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ORIGINAL ARTICLE



The effects of inbreeding on stress resistance of the Pacific oyster *Crassostrea gigas* at different temperatures and salinities

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ABSTRACT

The Pacific oyster (*Crassostrea gigas*) is a commercially important shellfish widely cultured worldwide. Understanding the effect of inbreeding on *C. gigas* is critical to the long-term feasibility of breeding programmes, especially when selected lines are developed in hatcheries with limited effective population sizes. The effect of inbreeding on stress resistance in *C. gigas* remains to be explored. The present study evaluated the physiological and immune responses to different temperatures (16–36°C) and salinities (20–40 psu) in an inbreeding line and a wild population of *C. gigas*. Two physiological parameters, including ammonia-N excretion rate (AER) and oxygen consumption rate (OCR), and three enzyme activities including superoxide dismutase activity (SOD), catalase activity (CAT), and contents of malondialdehyde (MDA) were measured on day 14 of the temperature and salinity exposure. Compared with the wild population, the physiological parameters (AER and OCR) were significantly lower, and the enzyme activities (SOD, CAT, and MDA) were significantly higher in the inbreeding line at suboptimal temperatures or salinities. These results showed that inbreeding has negative effects on stress resistance in *C. gigas*. In addition, multiple groups with different inbreeding levels would be needed to quantify the effects of inbreeding on stress resistance in *C. gigas*.

KEY POLICY HIGHLIGHTS

- This is the first study to examine the effect of inbreeding on the stress resistance of *C. gigas* under temperature and salinity challenges.
- Inbred oysters showed an equally good performance as wild oysters under benign conditions.
- The potential disadvantages of inbred oysters in adaptive capacity were shown at suboptimal conditions.

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Introduction

Inbreeding depression (reduced survival or fertility in a population) occurs when closely related individuals mate (Charlesworth and Willis 2009), which is due to increased homozygosity. It is well known that the extent of inbreeding depression is usually profoundly affected by environmental stress (Armbruster and Reed 2005; Liao and Reed 2009; Joubert and Bijlsma 2010; Fox and Reed 2011). Generally, the relationship between inbreeding depression and environmental stress is synergistic (Armbruster and Reed 2005), which can be explained by two hypotheses (Reed et al. 2012). The first theory is that inbreeding increases

the expression level of recessive deleterious alleles, which leads to intrinsic stress on basic cell function. Therefore, the individual's ability to respond to environmental stress is reduced. The second theory is that environmental stress may cause the expression of certain deleterious alleles that may not be expressed under benign conditions, which exacerbates inbreeding depression (Nadya 2018). However, some systems showed no relationship between inbreeding depression and environmental stress, while others suggested that environmental stress can decrease the magnitude of inbreeding depression (Dahlgard and Hoffmann 2000; Armbruster and Reed 2005). The

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variation in the relationship between inbreeding depression and environmental stress may be explained by differences in population history or other causes (Hedrick and Garcia-Dorado 2016; Sander and Matthies 2016), which suggests that the relationship between inbreeding and environment may not be easily generalized across species or populations. Therefore, understanding information regarding inbreeding-environmental interactions in a threatened population is important for implementing conservation policies or breeding schemes.

Temperature and salinity as environmental factors are crucial to immune enzyme activities and physiological metabolism in aquatic animals (Pourmozaffar et al. 2019; Wang and Li 2020). When seasonal or transient variations in temperature and salinity occur, aquatic animals can sense and respond to these variations through active regulation of physiological and biochemical activities. However, inbreeding may depress this adjustment ability. For example, it was observed that the mean value of salinity and temperature tolerance was significantly decreased in the inbred guppy (*Poecilia reticulata*) (Shikano et al. 2001; Shikano and Taniguchi 2003). In bivalves, it has been reported that the change in temperature and salinity was a crucial factor in large-scale mortality (Fuhrmann et al. 2016; Gajbhiye and Khandeparker 2017). Considering the reduced temperature and salinity tolerance could exacerbate the occurrence of large-scale mortality, it is necessary to explore whether adaptability will be affected by inbreeding in bivalves.

The Pacific oyster *Crassostrea gigas* is the most widely cultured shellfish species around the world due to its strong environmental adaptability, fast growth, and high economic value (Meng et al. 2021). Inbreeding depression of performance characters has become pervasive because of small effective population size, great fecundity, and very high larvae mortality (Launey and Hedgecock 2001; Langdon et al. 2003; Evans et al. 2004; Camara et al. 2007; Dégremont et al. 2007; Hedgecock and Davis 2007). Most researches exploring the effects of inbreeding reported significant reduction of growth and/or survival for inbreeding oysters during larval and adult stages (Evans et al. 2004). It was observed that inbreeding larvae can have a good performance like commercially cultured populations at optimal salinity and temperature but showed disadvantages in adaptive capacity under suboptimal conditions (Han and Li 2018). However, it has not been reported whether inbreeding will affect the temperature and salinity tolerance of adult oysters.

Four orange-shell variants were obtained in our artificial breeding practice of *C. gigas*. To obtain a genetically stable orange-shell line and further improve the growth performance, three successive generations of family selection and seven generations of consecutive mass selection were performed from 2011 to 2020 (Han et al. 2019). It was reported that the orange-shell line was a typically inbred line with significantly reduced allelic richness and expected heterozygosity (Han et al. 2019). Therefore, the orange shell line is suitable for exploring the effects of inbreeding on the temperature and salinity tolerance of adult oysters.

In this study, two physiological parameters (oxygen consumption rate and ammonia-N excretion rate) and activities of three immune-related enzymes (catalase, malondialdehyde content, and superoxide dismutase) of the orange-shell strain and a wild population were measured at different temperatures and salinities. This study is expected to explore whether inbred oysters have the worse adaptive capacity and help to formulate the breeding strategy of oysters and select appropriate aquaculture sea areas.

Materials and methods

Biological material

A rare orange-shell strain of *C. gigas* was obtained through three successive generations of family selection from 2011 to 2013 based on four orange-shell mutant individuals. To improve the growth performance of this strain, seven successive generations of improved mass selection were conducted from 2014 to 2020 (Han and Li 2020). The 10th generation of orange shell strain (OS) and the control group (WI) was produced on 28 May 2020 based on the 9th generation of orange shell strain and the wild population, respectively, which were collected from Sanggou Bay, Rongcheng, Shandong province, China. All spat were cultured in the same sea area of Sanggou Bay. In October 2021, oysters of two groups were collected from Sanggou Bay. Before the start of the experiment, all oysters were conditioned for 2 weeks in 2 aerated 1 m³ pools (temperature: 15°C, salinity: 30 psu). In addition, to assess the inbreeding level of OS oysters, 36 oysters were randomly selected from the 10th generation of the orange-shell strain, and the adductor muscles from each oyster were stored and used for microsatellite analysis. Six multiplex PCRs containing 18 microsatellite loci (Table I) were used to genotype according to Liu et al. (2017), and the inbreeding coefficient was

Table 1. Eighteen microsatellite loci of *C. gigas* used in this study.

Panel	Locus	Primer sequence (5' to 3')	Size (bp)
1	ucdCg-117F	CCAAGCTTGGCACTCACTCAA	290
	ucdCg-117R	GAGTGTCTGGTGTGCCAAAT	
	ucdCg-120F	GGGTGAGATTTAGGGGGAGA	152
	ucdCg-120R	CTCCATCAAACCTGCCAAAC	
	ucdCg-198F	GAAAGACACGACCGGAGAGA	
2	ucdCg-198R	CTGATGATGTCCACACCTG	230
	ucdCg-146F	CGCTCTGGTCTTTGTCCAT	
	ucdCg-146R	ACCCCAACAGATCACAATCC	218
	Crgi3F	TAGGATGAGGCTGGCACCTTGGGA	
	Crgi3R	GCCTGCCTTGCCCTTGAGGAATA	
3	uscCgi-210F	TTACAATGAAGATGACAGTGC	345–348
	uscCgi-210R	CCTCCTGCCTCCATATCA	
	ucdCg-170F	TGGTGGTCAGTGAATGTGAGA	276
	ucdCg-170R	CGGACAGTAGCCTTTAACACA	
	ucdCg-156F	AGCAGACCTTGGCAAATACG	
4	ucdCg-156R	CCGTCATCAGGTCCTGTTTT	325
	ucdCg-199F	GGGAAGAGTTGAATTCTGCAA	
	ucdCg-199R	AAACCGAGGCTCAGGAAAAT	270
	otgfa0_0007_B07F	TATCATCGCGCAATTCGTG	
	otgfa0_0007_B07R	GCAACTTAGCTGGTCTGTTCC	
5	otgfa0_0129_E11F	TGACTGTTCTTCGTACCCATCA	155–165
	otgfa0_0129_E11R	AGGTGGAACGAGATTGCCTTT	
	Crgi4F	CCAAAACACGATAAGATACACTTTC	235,238
	Crgi4R	GATCAGTCCCTCACATCTTTCCTC	
	ucdCg-152F	TGTTTTGGAGCTTGGCTTA	
6	ucdCg-152R	TCAAGCAAAGAAAGTCACCTCA	257
	Crgi39F	TTCCAAGTCCGTTTTGTCATCGT	
	Crgi39R	GTGCACAAACCACCATCAGCTC	190–214
	Crgi45F	GAGTCACCATGAAGATATCTGAA	
	Crgi45R	ATGATTACATAACTCTGACCCAAT	
6	ucdCg-200F	AAAGTTGCTTTGCTGTCGTC	254
	ucdCg-200R	CGCTAACGTGCTTCATTCAA	
	otgfa0_408293F	ACCCTGGTTTGATCTGAGAAATG	118–122
	otgfa0_408293R	TCTAAGGAGTGTGAGTGTAGTAG	
	otgfa0_0139_G12F	GTGCTTCAGGGTATCTCTTCC	
otgfa0_0139_G12R	AGCTACTGCATGGACACGATT	169–173	

calculated using GenAlEx 6.5 (Peakall and Smouse 2012).

Experimental design

The oysters of similar sizes from two groups were transferred into 30 small aquariums (350 mm × 250 mm × 300 mm) with 10 oysters per aquarium for each treatment. Five salinity treatments (15, 20, 25, 30, 35 psu) and five temperature treatments (16°C, 21°C, 26°C, 31°C, 36°C) were selected to evaluate the effects of inbreeding on stress resistance of *C. gigas* at different temperatures and salinities (Chu et al. 2005; Liu et al. 2008). The salinity was gradually adjusted from 30 psu to the test salinity at a rate of 2 psu/day by adding sea salt or diluting seawater with fresh water. The seawater in aquariums holding the oysters for the temperature was heated gradually from 15°C to the test temperature at a rate of 1°C/day by immersed heaters. When the targeted salinity and temperature were reached, it was kept for two weeks. The temperature was set at 26°C in the salinity treatments and the salinity was set at 30 psu in the temperature test. Exposure

to the test salinity or temperature lasted for 2 weeks.

Physiological measurement

The ammonia excretion rate (AER) and oxygen consumption rate (OCR) were determined by closed-chamber respiration methods (Meng et al. 2021). Before the measurement, oysters were kept unfed for 24 h to eliminate the effects of organics produced by fecal excretion and food decomposition. One oyster was placed in a 3-L plastic respiration chamber at the test salinity or temperature level, which had been pre-aerated for 24 h to reach oxygen saturation before the treatment. Each group had three replicates and one blank chamber with no oyster served as the control. Before the testing, dissolved oxygen (DO) and ammonia of water samples were measured using a DO meter (YSI, 600XL, USA) and the hypo-bromate oxidimetry method (Li 1995) respectively. Liquid paraffin was used to seal all chambers to ensure tightness. After 3 h of testing, water samples were collected by siphoning, and DO and ammonia at the end of the experiment were measured again. Each sample was

measured 3 times to minimize measurement errors. The OD and ammonia readings in the blank chamber were subtracted from the experimental units to correct for autogenic trends. After the physiological measurement, the mean growth traits of sampled oysters were measured (Table II). Soft tissues of the sampled oysters were dissected and dried at 80°C for 48 h, and then dry meat weight was determined. Condition index (CI) which reflects physiological and nutritional status (Rainer and Mann 1992) was calculated for both groups according to the following equation (Lawrence and Scott 1982):

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

AER and OCR were calculated according to following equations (Valverde et al. 2006):

$$AER = (N_t - N_0) \times V / (DW \cdot T)$$

$$OCR = (DO_0 - DO_t) \times V / (DW \cdot T)$$

N_0 and N_t are the initial and final concentrations of ammonia and DO_0 and DO_t are the initial and final concentrations of dissolved oxygen respectively. V is the volume of the respiration chamber (L), DW is the dry weight (g), and T is the time between the initial and final measurements (h).

The oxygen: nitrogen (O: N) atomic ratio, used to estimate the proportion of proteins in relation to carbohydrates or lipids for metabolism, was calculated based on the oxygen consumed and ammonium nitrogen excreted as the following equation:

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

In order to measure the rate of change of a chemical or biological system as a consequence of increasing the temperature by 10°C, Q_{10} was calculated as the following equation (Bayne and Newell 1983):

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

where t_1 and t_2 are the temperatures of two groups, and R_1 and R_2 are the corresponding OCR under each temperature group respectively.

Enzyme activity analysis

After exposure, the gill tissues of 3 oysters from each group were collected, cleaned thoroughly with phosphate-buffered saline (PBS, pH = 7.4), frozen with liquid nitrogen immediately, and stored at -80°C for the enzyme activity analysis. Before analysis, the frozen gill samples were thawed on ice. About 100 mg gill tissues were homogenized in nine volumes of 0.9% ice-cold saline using a homogenizer, and then immediately centrifuged at 3500 rpm for 20 min at 4°C. After centrifugation, the supernatants were used to measure enzyme activity. The total protein content was measured according to Bradford (1976) with Coomassie brilliant blue. The activities of superoxide dismutase (SOD) and catalase (CAT) and the content of malondialdehyde (MDA) were selected to evaluate the physiological response to different temperatures and salinities. These three indicators were determined by the corresponding commercial kits respectively (Nanjing Jiancheng, China). The activities of SOD and CAT were expressed as units per mg protein (U/mgprot) and MDA content was expressed as nanomole per mg protein (nmol/mgprot).

The superoxide anion (O_2^-) can reach with water-soluble tetrazolium salt (WST-1) to form blue formamide under the catalysis of xanthine oxidase. SOD inhibited the above reaction by catalyzing the disproportionation of O_2^- to generate hydrogen peroxide (H_2O_2) and elemental oxygen, so the optical density of formazan was inversely proportional to the activity of SOD.

CAT activity was determined according to Góth (1991). H_2O_2 (6.5×10^{-3} μmol) was decomposed by CAT of the supernatant (100 μL) in phosphate buffer (4.60 μmol, pH 7.4) at 37°C for 1 min. Then the reaction was immediately terminated by ammonium molybdate ($(NH_4)_2MoO_4$), and the residual H_2O_2 combined with $(NH_4)_2MoO_4$ to form a yellow compound. Its maximum absorbance was measured at 405 nm on a spectrophotometer in a 0.5 cm cuvette. One unit of CAT catalytic activity was defined as the decomposition of 1 μmol H_2O_2 per second per milligram protein.

The MDA content was measured according to Esterbauer and Cheeseman (1990). Briefly, the extract was mixed with an equal volume of 1% thiobarbituric acid and bathed in water at 95°C for 40 min. After cooling down on the ice, the mixture was centrifuged at 4000 rpm for 10 min. The maximum absorbance of

Table II. Biological characteristics of *C. gigas* used in this experiment.

Group	Shell height (mm)	Shell length (mm)	Shell width (mm)	Live weight (g)	Dry meat weight (g)	Shell wet weight (g)	Condition index
OS	93.67 ± 5.78	53.43 ± 6.13	30.91 ± 3.56	72.16 ± 8.98	0.79 ± 0.13	48.96 ± 6.67	3.41 ± 1.03
WI	92.89 ± 7.86	48.15 ± 5.12	28.21 ± 3.40	66.61 ± 7.97	0.84 ± 0.33	44.63 ± 5.73	3.82 ± 1.36

the supernatant was measured at 532 nm on a spectrophotometer against a blank control which consisted of anhydrous ethanol mixed with 1% thiobarbituric acid. Tetraethoxypropane was used as a standard.

Statistical analysis

All data were analyzed using SPSS 23.0. Firstly, the impacts of inbreeding status, environmental factors (temperature or salinity), and their interaction with the physiological parameters' levels and immune-related enzyme activities were analyzed using two-way ANOVA. Secondly, the physiological parameters' levels or immune-related enzyme activities between the two groups within a certain environmental factor were compared using one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

Physiological parameters

Two-way ANOVA analysis showed that inbreeding and environmental factors (salinity and temperature) had a significant effect on physiological parameters (Table III).

In the range of salinity, AER of both groups showed a trend of increasing first and then decreasing with the salinity increasing from 20 psu to 40 psu, which reached a maximum at 30 psu (Figure 1a). AER of WI was significantly higher than that of OS except the salinity was 30 psu. Dissimilar trends were observed in the temperature treatment (Figure 1b). AER of WI increased with the seawater warming up (16°C–36°C), however AER of OS reached the maximum at 31°C. AER of OS was significantly lower than that of WI except the temperature was 31°C.

At different temperatures, the trend of OCR in the two groups was not the same (Figure 1c). For WI, OCR increased with rising temperature, however, OCR of OS reached the highest level at optimal temperatures of 26°C. WI had significantly higher OCR than OS when the temperature was suboptimal. With increasing salinity from 20 psu to 40 psu, OCR of both groups firstly increased to the highest level at 30 psu and then decreased to a lower level (Figure 1d). Statistically, WI had significantly higher OCR than OS when the salinity was not 30 psu or 35 psu.

The O:N ratios at temperatures of 16–36°C ranged from 7.49 to 15.08 in OS and 8.30 to 17.36 in WI (Table IV). At salinities of 20–40 psu, the O:N ratios ranged from 7.56 to 16.99 in OS and 8.42 to 11.83 in WI. Inbreeding had no significant effect on O:N ratios

Table III. Two-way ANOVA results of effects of two environmental factors and inbreeding on physiological parameters and immune parameters of *C. gigas*.

Source of variation	OCR ($\text{mg g}^{-1} \text{h}^{-1}$)			AER ($\text{mg g}^{-1} \text{h}^{-1}$)			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	0.861	49.415	<0.001	0.020	482.349	<0.001	11354.048	24.738	<0.001	9529.452	58.097	<0.001	133.770	20.241	<0.001
Inbreeding	1.784	102.398	<0.001	0.012	287.624	<0.001	19509.638	42.507	<0.001	3108.186	18.949	<0.001	315.855	47.794	<0.001
Temperature × inbreeding	0.177	10.176	<0.001	0.001	19.780	<0.001	618.215	1.347	0.287	947.929	5.779	0.003	19.125	2.894	0.048
Salinity	1.734	111.149	<0.001	0.10	82.153	<0.001	27209.848	24.901	<0.001	2900.087	22.156	<0.001	42.900	12.315	<0.001
Inbreeding	0.574	36.807	<0.001	0.10	82.536	<0.001	40761.319	37.302	<0.001	699.803	5.346	0.032	149.561	42.934	<0.001
Salinity × inbreeding	0.017	1.102	0.383	0.01	4.693	0.008	3599.436	3.294	0.032	307.893	2.351	0.089	14.667	4.210	0.012

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

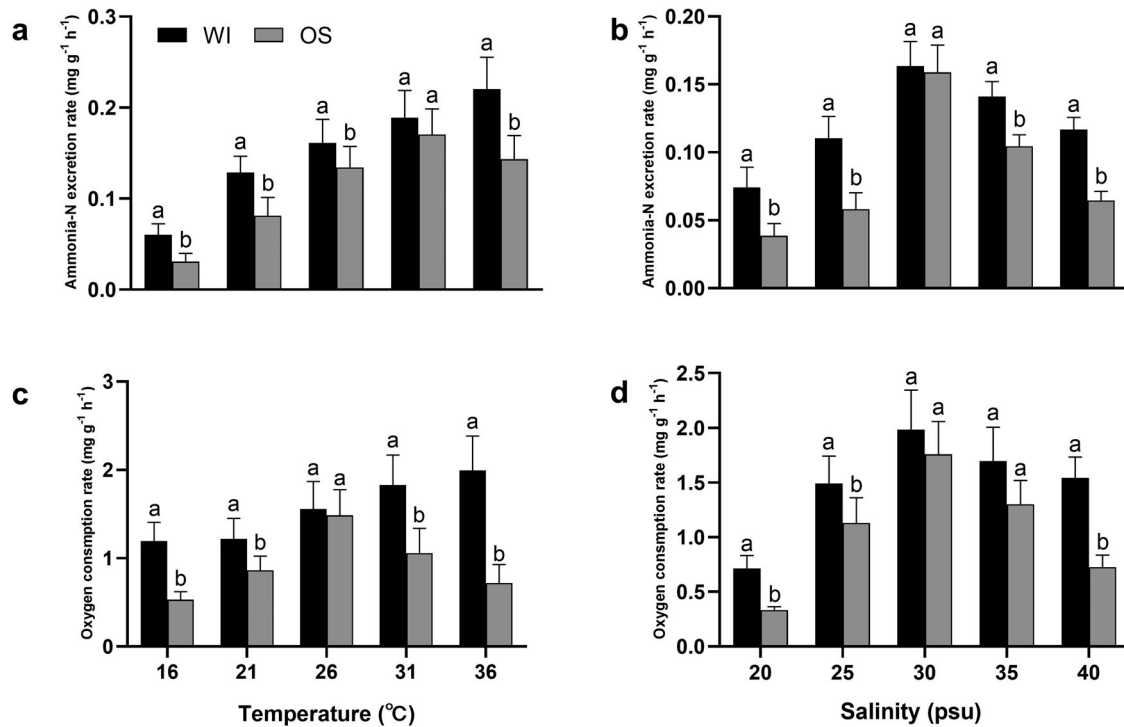


Figure 1. Ammonia-N excretion rate and oxygen consumption rate of two groups under different temperatures (a and c) and salinities (b and d). Different lowercase letters indicate significant differences ($P < 0.05$) in AER and OCR between two groups at a certain temperature or salinity.

Table IV. The O:N ratio of two groups of *C. gigas* at different temperatures and salinities.

Group	Temperature (°C)					Salinity (psu)				
	16	21	26	31	36	20	25	30	35	40
OS	15.08	9.27	9.67	7.49	7.44	7.56	16.99	9.69	10.89	9.85
WI	17.36	8.30	8.47	8.48	8.71	8.42	11.83	10.63	10.53	11.58

at different temperatures and salinities. Overall, there was no significant difference in O:N ratios between the two groups.

For WI, the highest value was recorded at 1.63 between 21°C and 26°C, followed by 1.44 between 31°C and 36°C, 1.38 between 26°C and 31°C and 1.04 between 16°C and 21°C (Table V). Similarly, the highest Q_{10} value in OS was recorded at 2.96 between 21°C and 26°C, followed by 2.63 between 16°C and 21°C, 0.96 between 16°C and 21°C and 0.70 between 31°C and 36°C.

Enzyme activity assays

Two-way ANOVA showed that inbreeding and temperature had a significant effect on SOD levels (Table III). With increasing temperature from 16°C to 36°C, SOD of both groups firstly decreased to the lowest level at 26°C and then increased to a higher level (Figure 2a). The SOD levels in WI were

Table V. Q_{10} coefficients in two groups of *C. gigas* calculated from different temperature ranges.

Temperature range	OS	WI
16–21	2.63	1.04
21–26	2.96	1.63
26–31	0.51	1.38
31–36	0.46	1.44

significantly higher than that in OS when the temperature was 16°C, 21°C, 31°C, 36°C. In the salinity experiment, the same trend was observed (Figure 2b).

Two-way ANOVA showed that temperature, inbreeding, and their interaction had significant effects on CAT levels (Table III). The CAT of both groups reached the highest level at 31°C (Figure 2c). When the temperature was 31°C and 36°C, the OS had significantly higher CAT compared with WI. Two-way ANOVA showed that salinity and inbreeding had significant effects on CAT levels (Table III). When in seawater with high salinity (35 and 40 psu), OS had significantly higher CAT levels than WI (Figure 2d).

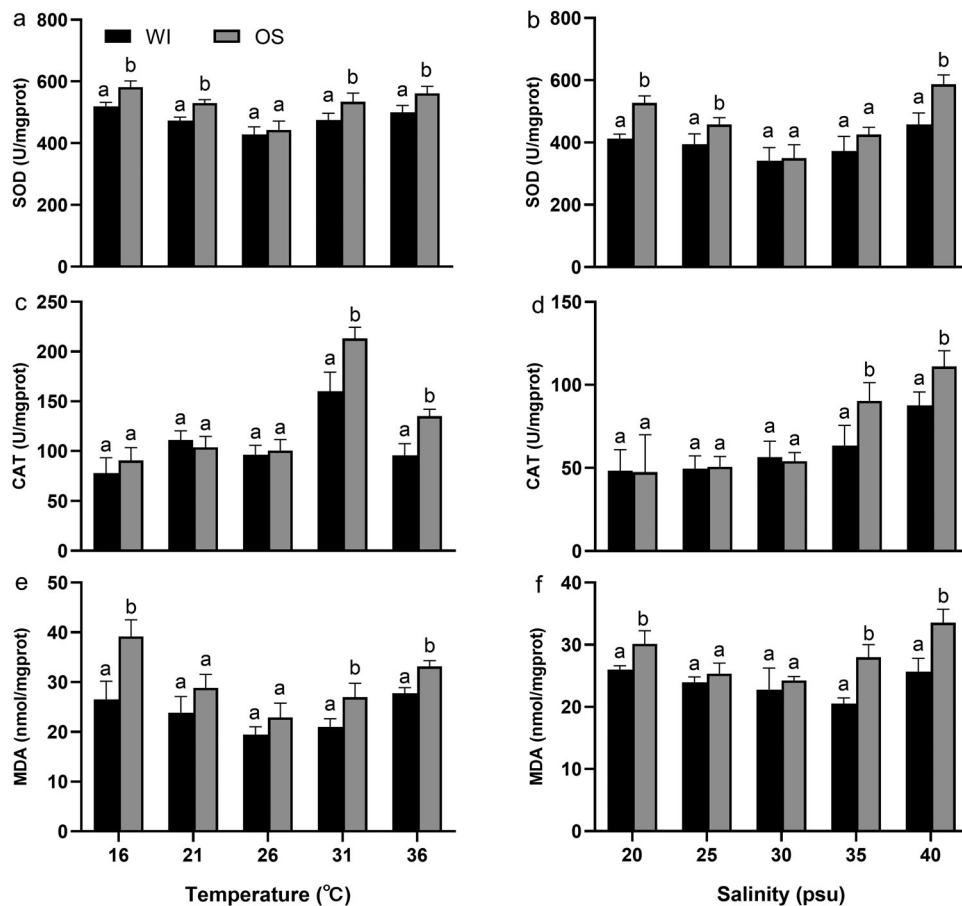


Figure 2. Effects of different temperatures (a, c and e) and salinities (b, d and f) on enzyme activity of two groups. Different lowercase letters indicate significant differences ($P < 0.05$) in the enzyme activity between two groups.

Two-way ANOVA showed significant effects of temperature, inbreeding, and their interaction on MDA contents (Table III). On the whole, WI got a lower MDA level than OS (Figure 2e). However, when the temperature was 21°C and 26°C, there was no significant difference between the two groups. In the salinity experiment, two-way ANOVA showed significant effects of salinity, inbreeding, and their interaction on MDA levels (Table III). Similarly, OS got higher MDA levels when the salinity was 20, 25, and 40 psu (Figure 2f). However, there was no significant difference between the two groups at 30 and 35 psu.

Discussion

Inbreeding usually causes offspring to show lower fitness and physiological efficiency (Crnokrak and Roff 1999; Keller and Waller 2002). Environmental stress may exacerbate inbreeding depression if genes that affect stress tolerance are affected by inbreeding (Kristensen et al. 2005). In the present study, the physiological and immune responses of inbred and wild oysters to temperature and salinity stress were

compared. The results suggested that stressed temperature and salinity can influence the physiological and immune responses of oysters and inbreeding weakened tolerance to environmental stress of oysters.

Physiological parameters

Oxygen consumption and ammonium excretion can usually reflect the biotic (food availability and body size) and abiotic (salinity and temperature) effects on the organism (Sarà et al. 2008). OCR is a crucial physiological parameter that reflects the energy metabolism level of aerobic respiration, which indicates the environmental and stress conditions accurately and sensitively (Guzman-Agüero et al. 2013). Ammonia is the end product of protein metabolism of aquatic invertebrates with high toxicity and reflects the energy lost as nitrogen, but it diffuses rapidly into seawater due to its small molecular size and high solubility in water (Pourmozaffar et al. 2019), so easy to measure to evaluate the tolerance of bivalves to environmental stress (Nie et al. 2017). Several studies have reported that OCR and AER are related to seawater temperature

and salinity in marine mollusks (Pourmozaffar et al. 2019; Chen et al. 2021). In this study, the OCR and AER of inbreeding oysters tended to increase with the temperature, up to a maximum beyond which they decrease, however, did not reach the maximum in temperature test in the wild oysters. In addition, when the temperature was not optimal, WI had significantly higher OCR and AER compared with OS. Therefore, OS had a weaker temperature tolerance limit due to inbreeding depression. Similarly, the responses of OCR and AER were observed to be significantly affected by salinity. The difference was that the OCR and AER of both groups increased with salinity, up to a maximum beyond which they decreased, which may be due to the length of the salinity acclimation period being higher than temperature because the bivalves needing more time to modulate their osmotic pressure (Gosling 2015). When bivalves are exposed to a change in salinity, they close the valve to avoid extremely different osmotic pressures of their tissues and open it periodically for a short time (Kurihara 2017). During valve closure, OCR is reduced to conserve energy by reducing energy expense on respiration after exposure to salinity fluctuation (Sarà et al. 2008; Peteiro et al. 2018). Meanwhile, under stressful (hypertonic and hypotonic) conditions, the level of ammonia excretion would be influenced by ingestion, leading to lower AER. In our study, the OCR and AER of WI were significantly higher than these of OS when environmental conditions were sub-optimal, which may indicate that inbreeding decrease the adaptability to salinity stress.

The O:N of an organism represents the ratio of protein to lipid and carbohydrate catabolism (Mayzaud 1976), which has proved useful in assessing the physiological response of bivalves to environmental stress (Bayne et al. 1976). The O:N value is 7 when the energy is completely supplied by protein, whereas the ratio is 24 if the energy is supplied by equal quantities of protein and lipid. The ratio will be infinite for the catabolism of lipids or carbohydrates (Zhang et al. 2021). In our study, the O:N for two groups in experimental temperature ranged from 7.44 to 17.36 and ranged from 7.56 to 16.99 in experimental salinity, which indicates utilization of protein was more than lipid and carbohydrate. Additionally, the effect of inbreeding on the O:N was not observed in the present study.

The value of Q_{10} is an index to reflect the adjustments related to the physiological and enzymatic requirements for energy when temperature increases within the natural range (Manush et al. 2004; Kim et al. 2005; Nie et al. 2017). In a certain temperature

range, higher Q_{10} values mean that the respiration of the organism increases rapidly to react sensitively at these temperatures (Rao and Bullock 1954). In this study, Q_{10} values were 0.51 for OS between 26 and 31°C, 0.46 between 31°C and 36°C, and for WI Q_{10} values were 1.38 between 26 and 31°C, 1.44 between 31°C and 36°C, which indicated that OS may have a lower tolerance to this temperature intervals compared to WI due to inbreeding.

Enzyme activity

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), singlet oxygen (O_2), and hydroxyl radicals (OH^\cdot), can be induced when organisms are exposed to environmental stress (Abele et al. 2002). However, the physiological antibody defense mechanisms can minimize the negative effect which is regulated by enzymes such as SOD and CAT (Rahman et al. 2019). Excess of these components can damage the cellular structure and protein function (Valko et al. 2006). SOD and CAT are essential in the ROS scavenging process. O_2^- can be catalytically decomposed into H_2O_2 and O_2 by SOD, and then H_2O_2 will be catalytically disproportionated by CAT into H_2O and O_2 . In addition, as a product of lipid peroxidation, the level of MDA is often regarded as a marker of oxidative stress (Zanette et al. 2011).

In this study, SOD activity was significantly affected by temperature. However, there were opposite results in *Mytilus galloprovincialis* and *Katelsia rhytiphora* (Rahman et al. 2019), which was considered to be the results that a high concentration of ROS could be neutralized by SOD in a short time (Rahman et al. 2019). This difference may be because inbreeding made oysters vulnerable to oxidative damage under temperature stress and led to large fluctuations in the SOD activity of OS at different temperatures. The CAT of both groups showed higher activity at higher temperatures (31°C and 36°C), consistent with the results in mussels *M. galloprovincialis* and *M. coruscus*, which suggested that oxidative stress is prone to produce peroxide radicals (Khessiba et al. 2005; Hu et al. 2015). The SOD and CAT activity of OS were significantly higher than these of WI at higher temperatures (31°C and 36°C), indicating that inbreeding weakened the antioxidant defense mechanism of oysters, resulting in cells more vulnerable to the adverse effects of oxidative stress (Meng et al. 2021). As well, the variation of salinity can cause oxidative stress in marine bivalves, which respond to oxidative damage by increasing antioxidant defense to purge ROS. The

changing trend of activity of SOD and CAT observed in the salinity experiment was similar to that in the temperature experiment. The overall levels of SOD and CAT in OS were significantly higher than those in WI, which once again provided evidence that inbreeding reduces the ability of oysters to deal with oxidative damage.

ROS produced by changes in temperature and salinity can also cause lipid membrane oxidation, which increases the risk of cell damage in marine invertebrates (Abele et al. 2002). It was reported that the MDA content of the pearl oyster *Pinctada maxima* increased significantly with increasing intensity and duration of marine heatwaves, indicating thermal stress response. When exposed to repeatedly-occurring marine heatwaves scenarios, the MDA content of the pearl oyster decreased compared with that during short-lasting marine heatwaves scenarios, which demonstrated the potential of the pearl oyster to acclimate rapidly to marine heatwaves (Xu et al. 2021). In another study, MDA levels in oysters exposed to diesel increased compared to control groups at 9, 15, and 35 ppt salinities, which suggested the occurrence of lipid peroxidation in those salinities, but not at 25 ppt salinity (Zanette et al. 2011). The result of the present study showed that when temperatures and salinities were not benign values, the oysters tended to produce more ROS and significantly increase lipid peroxidation. Meanwhile, the overall MDA content of OS was higher than that of WI, suggesting that the inbred oysters are susceptible to oxidative damage under environmental stress compared to the control oysters.

Inbreeding depression

For traits directly related to fitness, homozygotes are more responsive to environmental stresses than heterozygotes (Lerner 1954), which was consistent with the results of numerous subsequent researches. For example, the meta-analysis on model species and plants showed that inbreeding depression increased in 76% of these cases when changing from a benign to a stressful environment (Armbruster and Reed 2005). Inbreeding seems to negatively affect the capacity to maintain the main phenotype under various environments (Kristensen et al. 2010). Similarly, the adaptability significantly decreased under the stressful environment in this study, whereas there was no significant inbreeding depression under benign temperatures or salinities. This interaction may be the result of higher fitness costs for harmful alleles and increased expression of genetic load under the stressful environment, which leads to

increased phenotypic variation and ultimately an increased expression of inbreeding depression (Reed et al. 2012).

Conclusion

We analyzed and compared the physiological and immune responses of inbred and wild *C. gigas* to temperature and salinity stress to explore the effect of inbreeding on the stress resistance of *C. gigas*. OS showed an equally good performance like WI under the benign condition, but weak temperature and salinity tolerance and antioxidant defense mechanism of OS were observed under the stress condition, suggesting that the potential disadvantages in environmental stress resistance in inbred *C. gigas* need more attention and the genotype-environment interaction needs to be better incorporated in the breeding schemes of *C. gigas* (Reed et al. 2012), especially given the effect of environmental stress on production levels and economic viability (Mader et al. 2009). In this study, a single inbred strain was compared to one control group, which limits the scope of our findings. The study of multiple groups with different inbreeding levels would be needed to confirm and quantify the effect of inbreeding on reproductive capacity in *C. gigas* (Dégremont et al. 2022). There is also a strong need to explore more comprehensively the adaptive traits of *C. gigas* and the molecular mechanism of inbreeding-environment interaction.

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Author contributions

Jiafeng Fang performed the experiments and analyzed the data; Qi Li conceived and designed the experiments, authored and revised the paper.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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