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RESEARCH REPORT

Combined effects of temperature and salinity on growth, survival, gill morphology, and antioxidant capabilities in the horse mussel *Modiolus modiolus*

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Abstract

Temperature and salinity are critical to the reproduction, growth, and survival of shellfish. We investigated the combined effects of temperature (15 °C, 20 °C, and 25 °C) and salinity [20, 30, and 40 practical salinity units (PSUs)] on the horse mussel *Modiolus modiolus,* which inhabits the northern Chinese coast. The specific growth rate of *M. modiolus* increased with salinity at a low temperature (15 °C). The interaction between temperature and salinity with respect to *M. modiolus* survival was significant (Two-Way ANOVA; $p < 0.01$). Across all temperatures tested, *M. modiolus* specimens kept at low salinity (20 PSU) had narrower gill filament lumens than those kept at moderate salinity (30 PSU); the gill filament lumens of specimens kept at high salinity (40 PSU) were wider than either of these. The combined effects of temperature and salinity on superoxide dismutase (SOD) activity, total antioxidant capacity (T-AOC), and glutathione peroxidase (GPx) activity in the gills of *M. modiolus* were significant (Two-Way ANOVA; $p < 0.05$). Our results suggested that temperature and salinity significantly affect the survival, gill morphology, and antioxidant capabilities of *M. modiolus*. Our findings not only enrich our knowledge of the interactions between temperature and salinity with respect to shellfish but also provide a framework for *M. modiolus* aquaculture development.

Key Words: horse mussel; gill lumen; *SOD*; *T-AOC*; *GPx;* superoxide dismutase; total antioxidant capacity; glutathione peroxidase; SGR

Introduction

The horse mussel, *Modiolus modiolus* (Mollusca: Bivalvia), is a benthic, sessile species distributed worldwide in coastal areas (Hutchison *et al.*, 2016). In China, *M. modiolus* is mainly found in the subtidal zones of the Yellow and Bohai Seas (Ning *et al.*, 2015). *M. modiolus* modifies its habitat via bio-deposition, forming beds on various substrata (Fariñas-Franco *et al*., 2016). It is therefore believed that *M. modiolus* has an important impact on the ecological equilibrium of the sedimentary environments of the shallow sublittoral (Ragnarsson and Burgos, 1984). *M. modiolus* is highly edible and grows rapidly to a large size (Ning *et al*., 2016). This species has thus recently been proposed as a potential new aquaculture species in China (Ning *et al.*, 2015).

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Temperature and salinity are the two primary environmental factors affecting the reproduction, growth, and survival of shellfish (Meryem *et al.,* 2015). For example, increased water temperature and water quality were thought to contribute to mortality in the oyster *Crassostrea gigas* (Shelaghk *et al*., 2009), while high temperature significantly affected survival, oxygen consumption, behavior, ammonia-N excretion, and related immune indicators in the Japanese scallop *Mizuhopecten yessoensis* (Hao *et al*., 2014). In addition, the restoration of normal antioxidant enzyme activity in the clam *Cyclina sinensis* was increased after exposure to abnormal salinities; *C. sinensis* had a greater tolerance for low salinity than for high salinity (Li *et al.*, 2012). Recently, the combined effects of temperature and salinity on shellfish have received more attention (Samuel *et al*, 2015). For example, Wang *et al.* (2017) showed that a temperature of 26 °C and a salinity of 30 practical salinity units (PSU) were optimal for the culture of juvenile ark shells (*Anadara broughtonii*). Unfortunately, studies of the combined influence of temperature and salinity on shellfish remain scarce.

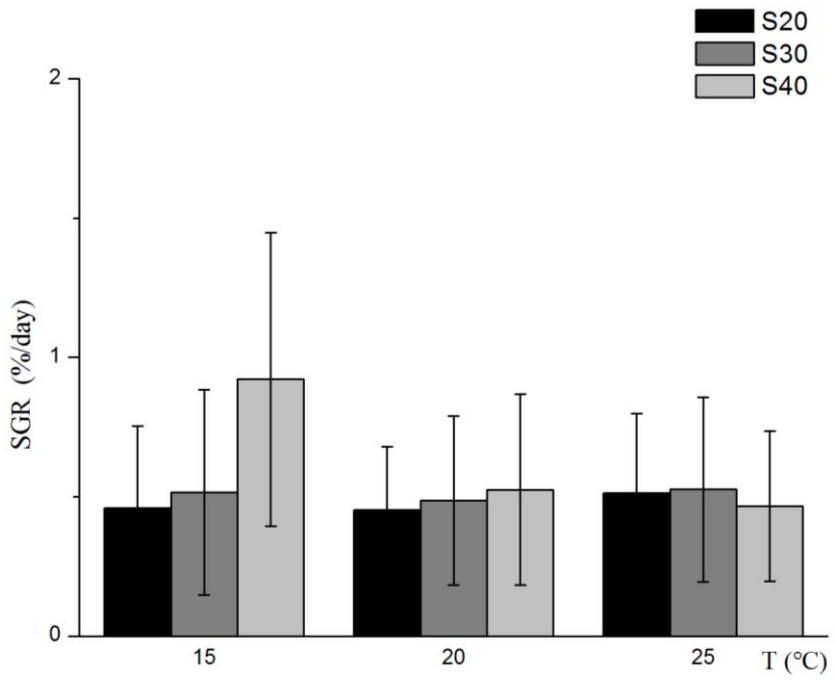

Fig. 1 Effects of temperature and salinity on SGR in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU. Bars show the means of 3 replicates. Error bars show SD.

Table 1 One-way and two-way ANOVA of the effects of different temperatures and salinities on SGR in *M. modiolus.* Values are means ± SD (n = 3).

	Temperature (°C)	Salinity (PSU)	p-value			Duncan test
			Temperature	Salinity	Interaction	
One-way ANOVA (factor: Temperature)	15, 20, 25	20	0.85^{ns}	--	--	15 °C = 20 °C = 25 °C
		30	0.96^{ns}			15 °C = 20 °C = 25 °C
		40	0.04*			15 °C > 20 °C = 25 °C
One-way ANOVA (factor: Salinity)	15	20, 30, 40	--	0.02*	--	40 PSU > 30 PSU = 20 PSU
	20			0.84^{ns}		20 PSU = 30 PSU = 40 PSU
	25			0.91^{ns}		20 PSU = 30 PSU = 40 PSU
Two-way ANOVA (Fixed factors: Temperature & Salinity)	15, 20, 25	20, 30, 40	0.15^{ns}	0.16^{ns}	0.11^{ns}	15 °C = 20 °C = 25 °C 20 PSU = 30 PSU = 40 PSU

*, value is significant based on the Duncan tests ($p < 0.05$); ns, value is not significant based on the Duncan test.

Although *M. modiolus* tolerates a wide range of environmental conditions (Lesser and Kruse, 2004), it has previously been shown that alterations in a single environmental factor (such as temperature, salinity, or pH) affect growth, survival, and feeding (Lesser and Kruse, 2004; Ning *et al.*, 2012). Before large-scale farming of *M. modiolus* begins, it is critical to identify the optimal conditions for such farms.

We therefore explored the combined impact of temperature and salinity on *M. modiolus*, specifically aiming to 1) identify the effects of temperature and salinity on growth and survival in *M. modiolus*; 2) identify morphological changes in the gills of *M. modiolus* in response to temperature and salinity; 3) identify alterations in superoxide dismutase (SOD) levels, total antioxidant capacity (T-AOC), and glutathione peroxidase (GPx) levels in the gills of *M. modiolus* in response to changes in temperature and salinity. Our results will help to determine the ideal nursery conditions and farming locations for *M. modiolus*.

Materials and Methods

Animal collection and treatments

In November 2015, we collected 220 healthy adult *Modiolus modiolus* specimens (mean shell height = 42.71 ± 3.08 mm; mean shell length = 96.09 ± 4.65 mm; mean shell width = 49.82 ± 3.21 mm; and mean mass = 13.39 ± 2.32 g) from the near-shore sublittoral zone of the North China Sea near the Ministry of Agriculture Key Laboratory of Mariculture & Stock Enhancement at Dalian Ocean University, Dalian, China (121°33′47″ E, 38°51′55″ N). All specimens were kept in ~60 L recirculating seawater tanks; each tank was fitted with an automatic temperature control and monitoring system (Dalian Huixin Titanium Equipment Development Co., Ltd, Liaoning, China). Seawater was sand filtered and continuously aerated. Specimens were kept in natural light. All specimens were supplied with 0.25 g spirulina powder (Dalian Golden Bay Technology Co., Ltd, Liaoning, China) daily. The amount of spirulina powder supplied exceeded the daily requirements of the specimens (Ning *et al.*, 2015) to ensure that our results were not biased by insufficient nutrition. We also judged that the spirulina was supplied in excess because extra powder remained in the water after a day of circulation.

All specimens were acclimated to default laboratory conditions (13.95 ± 1.32 °C and 31.69 ± 0.35 PSU) for one week before experiments began. Experiments were conducted between November 2015 and January 2016.

After acclimation, all specimens were dried with a paper towel and weighed with digital balance (0.01 g sensitivity; AL204; Mettler Toledo, Shanghai, China) to obtain initial mass (W_1). We then divided mussels into 27 groups of three specimens each (three temperatures × three salinities with three replicates of each combination; Table S1). Each group was housed in a separate tank. To reach the desired temperature/salinity combinations, we removed half of the seawater from each tank every day, and replaced it with seawater at a different temperature/salinity. We changed the temperature of the new seawater such that the temperature of the entire tank did not increase by more than 1 °C per day; this was based on previous study on *M. modiolus* (Ning *et al.*, 2015) and on field survey data of the coastal waters of the Yellow Sea (Zhang *et al.*, 2014).

We altered the salinity of the replacement seawater either by adding fresh filtered water or by adding synthetic sea salt (Aqua Salt; Dalian Salt Industry Co., Ltd, Liaoning, China). We altered the salinity of the new seawater such that salinity in the entire tank did not change by more than 2 PSU per day (Ning *et al.*, 2015, Hamer *et al.,* 2008).

We monitored the temperature and salinity in each tank both using the automatic temperature control, and with a water quality monitor (A329 Portable Meter; Thermo Scientific Orion Star, Beijing, China).

Growth and survival in each treatment

During the 30-day experimental period, dead *M. modiolus* [defined as loss of motility and lack of external stimulus response (Ning *et al.*, 2015)], were noted and removed immediately. At the end of the experiment, all living specimens were again dried and weighed to obtain a final mass (W_2). We then calculated the specific growth rate (SGR; defined as the growth rate per day) and survival rate (SR) for each group as follows:

$$SGR\ (\% \times day^{-1}) = 100 \times (\ln W_2 - \ln W_1)/t$$
$$SR\ (\%) = 100 \times (N_2/N_1)$$

where N_1 is the initial number of live specimens; N_2 is the final number of live specimens; and t is the duration of the experiment in days.

Gill filament morphology and antioxidant analyses

The gills of all living *M. modiolus* specimens were carefully removed at the end of the experimental period. Gills were divided into two parts: one for morphological analysis, and the other for detection of SOD, T-AOC, and GPx.

To example gill filament morphology, we fixed gill tissue samples for 24 h in Bonn Fluid (75 ml saturated picric acid, 25 ml 40 % formaldehyde, and 5 ml acetic acid), following the procedures of Wu *et al*. (2007). We then transferred the fixed samples into different concentrations of ethanol for gradient dehydration (Han *et al.,*2014). Dehydrated fixed tissues were embedded in paraffin wax, and 5 μm sections were stained with hematoxylin and eosin (Qu *et al.,* 2013). We then examined gill filament morphology and photographed the gill filament lumens using an optical microscope with attached camera (50i; Nikon, Japan). Using DN-2 (Dalian Zhonghe Company, Liaoning, China) with our gill photographs, we measured the width of each gill filament lumen for each specimen, as it has been shown that lumen width is affected by changes in temperature and salinity (Wang *et al.,* 2016).

To determine SOD, T-AOC, and GPx, gill samples were dried with paper towels and homogenized immediately in an ice bath. Gill homogenates were centrifuged at 3,500 rpm for 10 min at 4 °C. Supernatants were stored at -80 ºC. We used commercial kits to determine SOD, T-AOC, and GPx (Nanjing Jiancheng Company, China; kit model used for SOD: A001-1; for T-AOC: A015-1; for GPx: A005), following the manufacturer's instructions. SOD activity, T-AOC level, and GPx activity were calculated in units per mg protein (U × mg protein^{-1}) as follows:

$$SOD = (A_2 - A_3)/0.5 \times (V_1/V_0)/C_0$$

$$T\text{-}AOC = (A_3 - A_2)/0.01/30 \times (V_1/V_0)/C_0$$

$$GPx = (A_2 - A_3)/(A_1 - A_0) \times C_1 \times 5/5/(V_0 \times C_0)$$

where A_0 is the light absorption (O.D.) of the blank group; A_1 is the light absorption (O.D.) of the standard group; A_2 is the light absorption (O.D.) of the control group; A_3 is the light absorption (O.D.) of the given experimental group; C_0 is the protein concentration of the homogenate; C_1 is the protein concentration of the standard (20 μg/L); V_0 is the volume of the sample; and V_1 is the volume of the total reaction.

The protein concentration of each gill homogenate was determined with the Bradford method, using bovine serum albumin as the standard, on a multimode reader (SpectraMax i3x; Molecular Devices, Shanghai, China).

Data analysis

All statistical analyses were performed with SPSS 16.0 (IBM, Shanghai, China). We first confirmed that our data were normally distributed and homogeneous with the Shapiro-Wilk test and with Levene's test. We then compared differences in SGR, survival rate, gill filament lumen size, SOD activity, T-AOC level, and GPx activity among treatments with one-way ANOVA (factor: temperature or salinity) and with two-way ANOVAs (fixed factors: temperature and salinity). We considered $p < 0.05$ statistically significant. Significant differences between pairs of treatments were identified with Duncan's multiple range test.

Results

Effects of temperature and salinity on growth and survival

Only *M. modiolus* specimens cultured at 15 °C in high salinity (40 PSU) had an increased SGR (Fig. 1; Table 1, S2).

We found that *M. modiolus* cultured in moderate salinity (30 PSU) had relatively higher survival rates as compared to other salinity treatments at the same temperature (Fig. 2). High survival rates (> 90%) were also observed at temperatures below 20 °C and salinities between 30 and 40 PSU. *M. modiolus* specimens kept at 20 °C in low salinity (20 PSU) had lower survival rates (68.75 ± 0.08 %). For *M. modiolus* specimens maintained at 25 °C, survival rate decreased as salinity increased (Fig. 2). The interaction between temperature and salinity was significant with respect to survival (Two-way ANOVA, $p < 0.01$), and salinity had a more pronounced effect

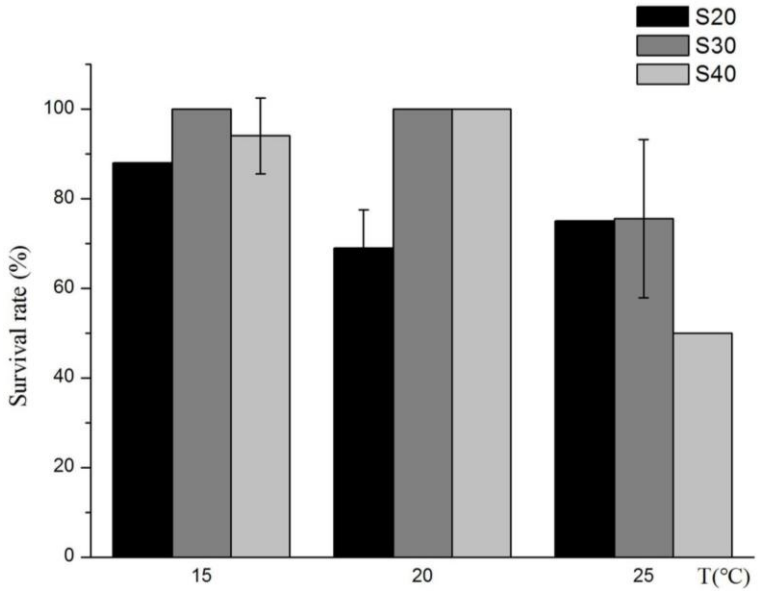

Fig. 2 Effects of temperature and salinity on survival in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU. Bars show the means of 3 replicates. Error bars show SD.

Table 2. One-way and two-way ANOVA of the effects of different temperatures and salinities on survival rate in *M. modiolus.* Values are means ± SD (n = 3).

	Temperature (°C)	Salinity (PSU)	p-value			Duncan test
			Temperature	Salinity	Interaction	
One-way ANOVA (factor: Temperature)	15, 20, 25	20	0.06^{ns}	--	--	15 °C > 25 °C > 20 °C
		30	0.15^{ns}			15 °C = 20 °C = 25 °C
		40	0.004**			20 °C = 15 °C > 25 °C
One-way ANOVA (factor: Salinity)	15	20, 30, 40	--	0.19^{ns}	--	20 PSU = 30 PSU = 40 PSU
	20			0.01*		40 PSU = 30 PSU > 20 PSU
	25			0.14^{ns}		20 PSU = 30 PSU = 40 PSU
Two-way ANOVA (Fixed factors: Temperature & Salinity)	15, 20, 25	20, 30, 40	0.02*	2.05×10^{-4}****	0.50×10^{-2}*	15 °C = 20 °C > 25 °C 30 PSU > 20 PSU = 40 PSU

, value is significant based on the Duncan tests ($p < 0.05$, **$p < 0.01$, ****$p < 0.0001$); ns, value is not significant based on the Duncan test.

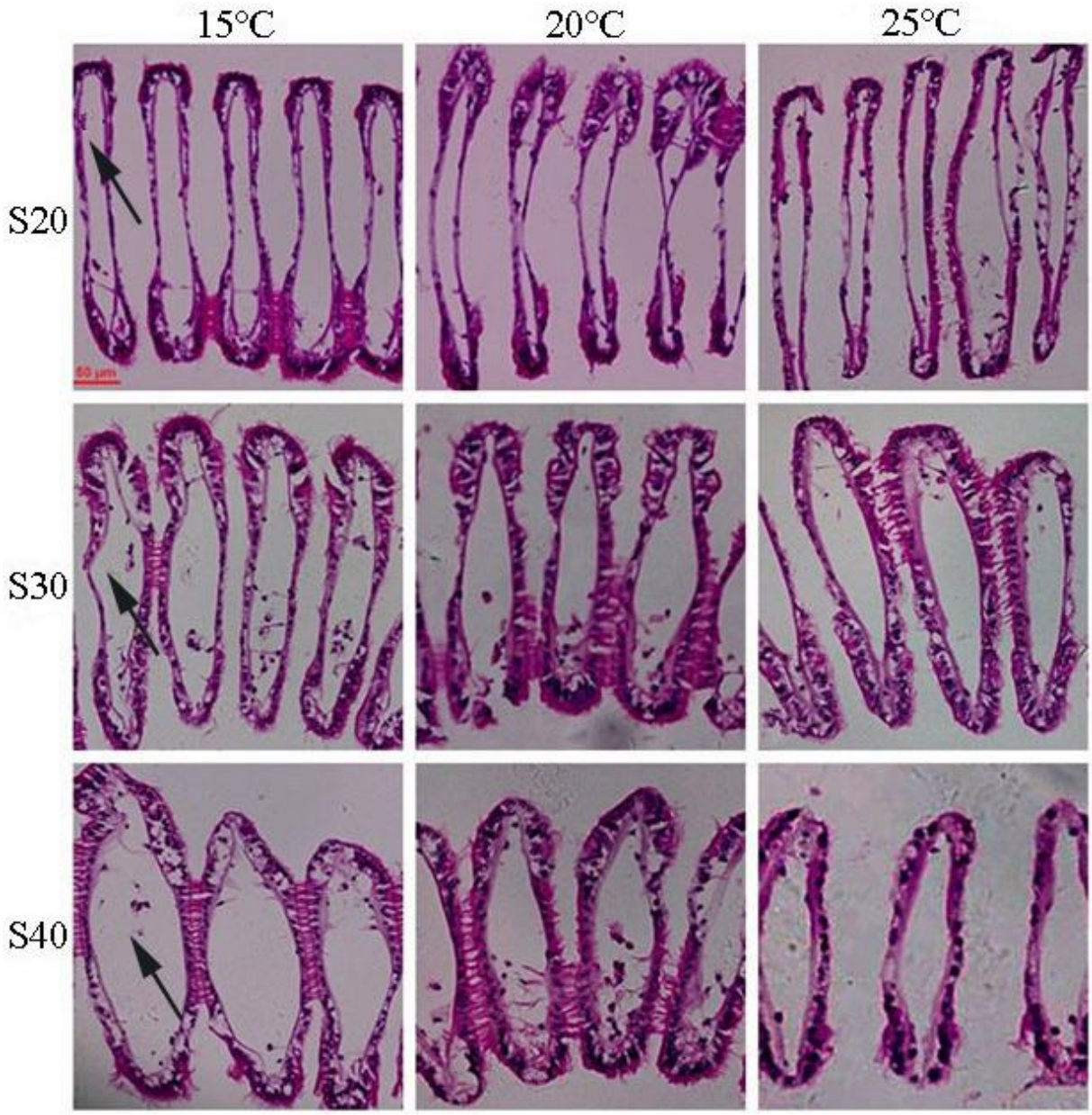

Fig. 3 Effects of temperature and salinity on gill filament morphology in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU.

on *M. modiolus* survival than the temperature (Two-way ANOVA, $p < 0.01$). It is interesting to note that the negative effects of low salinity (20 PSU) on *M. modiolus* were somewhat alleviated at the low temperature (*i.e.,* 15 °C) and the high temperature (25 °C) (One-way ANOVA, $p > 0.05$; Table 2).

Effects of temperature and salinity on gill filament morphology

As salinity and/or temperature increased, the gill filament lumens of *M. modiolus* specimens widened (Fig. 3). Relatively narrow gill filament lumens were observed in *M. modiolus* specimens cultured at 20 °C in low salinity (20 PSU). At moderate salinity (30 PSU), gill filament lumen size did not vary obviously with incubation temperature. Wider gill filament lumens were observed in *M. modiolus* specimens cultured at 15 °C in high salinity (40 PSU). At 25 °C, gill filament lumens were significantly smaller in low salinity (20 PSU) than in high salinity (40 PSU; Fig. 4; Table 3). The interaction between temperature and salinity with respect to the width of gill lumen was significant (Two-way ANOVA, $p < 0.01$), and salinity had a more pronounced effect on gill filament morphology than did temperature (Two-way ANOVA, $p < 0.01$). High temperatures (25 °C) partially alleviated the negative effects of high salinity on the gill filament morphology of *M. modiolus* (One-way ANOVA, $p < 0.05$; Table 3).

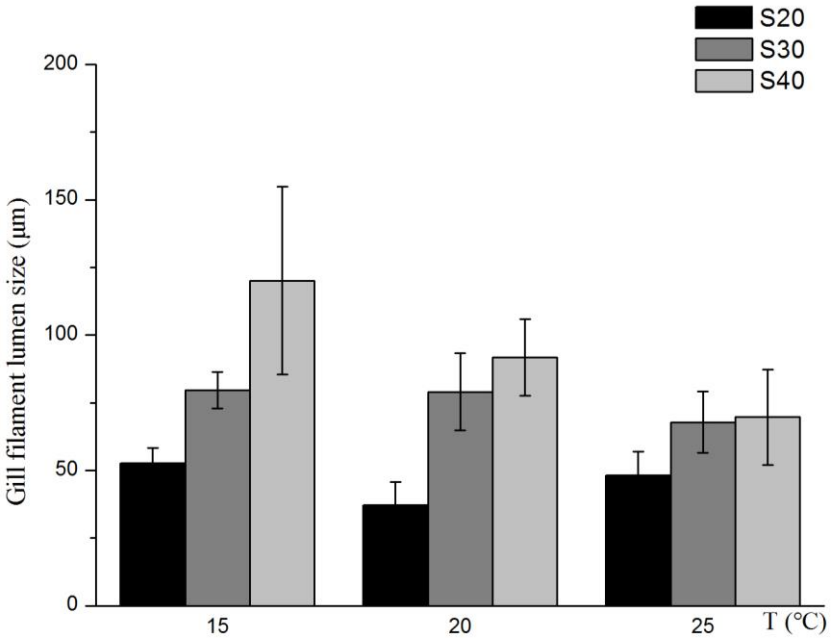

Fig. 4 Effects of temperature and salinity on gill filament lumen width in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU. Bars show the means of 3 replicates. Error bars show SD.

Table 3 One-way and two-way ANOVA of the effects of different temperatures and salinities on gill filament lumen size in *M. modiolus.* Values are means ± SD (n = 3).

	Temperature (°C)	Salinity (PSU)	p-value			Duncan test
			Temperature	Salinity	Interaction	
One-way ANOVA (factor: Temperature)	15, 20, 25	20	3.54×10^{-11}****	--	--	15 °C = 25 °C > 20 °C
		30	1.51×10^{-4}****			15 °C = 20 °C > 25 °C
		40	4.60×10^{-11}****			15 °C > 20 °C > 25 °C
One-way ANOVA (factor: Salinity)	15	20, 30, 40	--	1.93×10^{-9}****	--	40 PSU > 30 PSU > 20 PSU
	20			4.43×10^{-31}****		40 PSU > 30 PSU > 20 PSU
	25			0.10×10^{-2}**		40 PSU = 30 PSU > 20 PSU
Two-way ANOVA (Fixed factors: Temperature & Salinity)	15, 20, 25	20, 30, 40	3.27×10^{-13}****	2.09×10^{-40}****	1.57×10^{-10}****	15 °C > 20 °C = 25 °C 40 PSU > 30 PSU > 20 PSU

*, value is significant based on the Duncan tests (**p < 0.01, ****p < 0.0001); ns, value is not significant based on the Duncan test.

Effects of temperature and salinity on SOD activity, T-AOC level, and GPx activity

SOD activity in the gills of *M. modiolus* specimens kept at 15°C declined significantly with increased salinity (p < 0.001); in specimens kept at 25°C, SOD activity increased with increased salinity (Fig. 5; Table 4). At 20 °C, SOD activity was significantly higher in specimens cultured in low salinity (20 PSU) and in those cultured in high salinity (40 PSU), as compared to those cultured in moderate salinity (30 PSU). The interaction between temperature and salinity with respect to SOD activity was significant (Two-way ANOVA, p < 0.001).

The T-AOC of the *M. modiolus* gill was significantly affected by both temperature (One-way ANOVA, p < 0.05) and salinity (One-way ANOVA, p < 0.05; Fig. 6; Table 5). At each of the tested temperatures, the gills of *M. modiolus* specimens cultured in moderate salinity (30 PSU) had lower T-AOC than those cultured in low (20 PSU) and high salinities (40 PSU; Fig. 6). At each of the tested salinities, the gills of *M. modiolus* specimens kept at

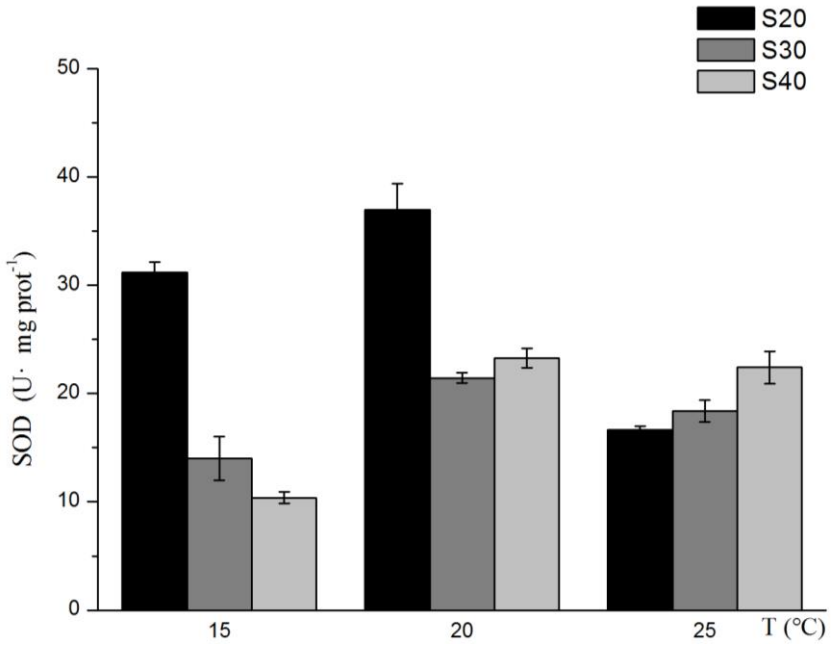

Fig. 5 Effects of temperature and salinity on gill SOD level in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU. Bars show the means of 3 replicates. Error bars show SD.

Table 4 One-way and two-way ANOVA of the effects of different temperatures and salinities on SOD levels in *M. modiolus.* Values are means ± SD (n = 3).

	Temperature (°C)	Salinity (PSU)	p-value			Duncan test
			Temperature	Salinity	Interaction	
One-way ANOVA (factor: Temperature)	15, 20, 25	20	8.49×10^{-6}****	--	--	15 °C = 25 °C > 20 °C
		30	0.10×10^{-2}**			20 °C > 25 °C > 15 °C
		40	0.30×10^{-2}**			20 °C = 25 °C > 15 °C
One-way ANOVA (factor: Salinity)	15	20, 30, 40	--	2.53×10^{-6}****	--	20 PSU > 30 PSU > 40 PSU
	20			3.03×10^{-5}****		20 PSU > 30 PSU = 40 PSU
	25			0.10×10^{-2}**		40 PSU > 30 PSU = 20 PSU
Two-way ANOVA (Fixed factors: Temperature & Salinity)	15, 20, 25	20, 30, 40	1.59×10^{-12}****	3.00×10^{-11}****	9.96×10^{-12}****	15 °C > 20 °C = 25 °C 40 PSU > 30 PSU > 20 PSU

*, value is significant based on the Duncan tests (**$p < 0.01$, ****$p < 0.0001$); ns, value is not significant based on the Duncan test.

a moderate temperature (20 °C) had higher T-AOC than those maintained at low (15 °C) or high temperatures (25 °C; Fig. 6). The interaction between temperature and salinity with respect to T-AOC in the *M. modiolus* gill was significant (Two-way ANOVA; $p < 0.01$).

GPx activity in the gills of *M. modiolus* was significantly affected by both temperature (One-way ANOVA; $p < 0.0001$) and salinity (One-way ANOVA; $p < 0.0001$; Fig. 7; Table 6). At each of the tested temperatures, specimens kept at both low (20 PSU) and high salinities (40 PSU) had significantly less GPx activity than those kept at a moderate salinity (30 PSU). At each of the tested salinities, the gills of *M. modiolus* specimens cultured at a moderate temperature (20 °C) had relatively higher GPx activity than those cultured at low (15 °C) and high (25 °C) temperatures (Fig. 7). The interaction between temperature and salinity with respect to GPx activity in the *M. modiolus* gill was significant (Two-way ANOVA; $p < 0.0001$).

Discussion

Previous studies have indicated that shellfish growth is affected by both temperature and salinity (Hao *et al.*, 2014; Çelik *et al.*, 2015). Hao *et al.* (2014) showed that exposing *M. yessoensis* to a low temperature (15 °C) for 30 days increased SGR significantly, while Bashevkin and Pechenik (2015)

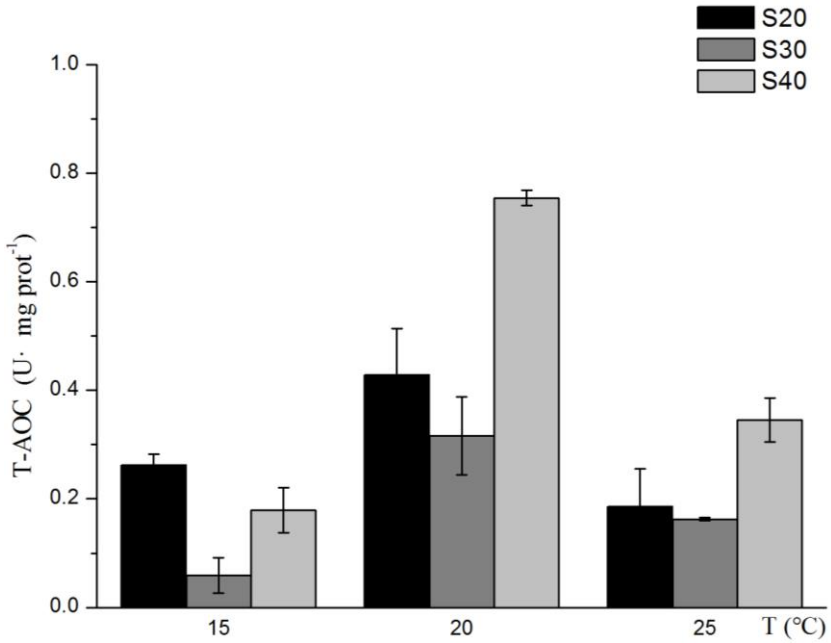

Fig. 6 Effects of temperature and salinity on gill T-AOC in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU. Bars show the means of 3 replicates. Error bars show SD.

Table 5 One-way and two-way ANOVA of the effects of different temperatures and salinities on T-AOC in *M. modiolus.* Values are means ± SD (n = 3).

	Temperature (°C)	Salinity (PSU)	p-value			Duncan test
			Temperature	Salinity	Interaction	
One-way ANOVA (factor: Temperature)	15, 20, 25	20	0.1×10^{-1}*	--		20 °C > 15 °C = 25 °C
		30	0.1×10^{-2}**			20 °C >25 °C > 15 °C
		40	2.32×10^{-6}****			20 °C > 25 °C > 15 °C
One-way ANOVA (factor: Salinity)	15	20, 30, 40	--	0.1×10^{-2}**	--	20 PSU > 40 PSU > 30 PSU
	20			4.26×10^{-4}****		40 PSU > 20 PSU = 30 PSU
	25			0.60×10^{-2}**		40 PSU > 20 PSU = 30 PSU
Two-way ANOVA (Fixed factors: Temperature & Salinity)	15, 20, 25	20, 30, 40	1.95×10^{-8}****	6.24×10^{-11}****	9.71×10^{-6}****	20 °C > 15 °C = 25 °C 40 PSU > 20 PSU > 30 PSU

, value is significant based on the Duncan tests ($p < 0.05$, **$p < 0.01$, ****$p < 0.0001$); ns, value is not significant based on the Duncan test.

found that exposure to low temperature and low salinity significantly reduced both the larval and juvenile growth rates of the gastropod, *Crepidula fornicata*. It has also been shown that salinity had the greatest effect on the growth rate of *A. broughtonii* (Wang *et al.*, 2017). Here, we found that the SGR of adult *M. modiolus* specimens increased with salinity at a low temperature (15 °C). We therefore hypothesize that response to temperature and salinity in marine mollusks varies with species.

Adult *M. modiolus* specimens survived ten days at salinities of 20 and 40 PSU and temperatures between 10 °C and 12 °C; at moderate salinity (~31 PSU) and temperatures above 25 °C, survival decreased (Ning *et al.*, 2015). Our results were consistent with this previous study. We also found that salinity alone, especially low salinity, was the main cause of reduced survival in *M. modiolus.* Therefore, our data support the hypothesis that salinity maintenance should be the first concern of shellfish aquaculture (Wang *et al.*, 2017). As low salinity significantly reduced the feeding rate of *C. fornicata* juveniles (Samuel and Pechenik, 2015), we postulate that the reduced survival rate of *M. modiolus* might be due to a dramatic decrease in feeding rate. With respect to aquaculture management, it was worth noting that the negative effects of low salinity were somewhat alleviated by decreased or increased temperatures.

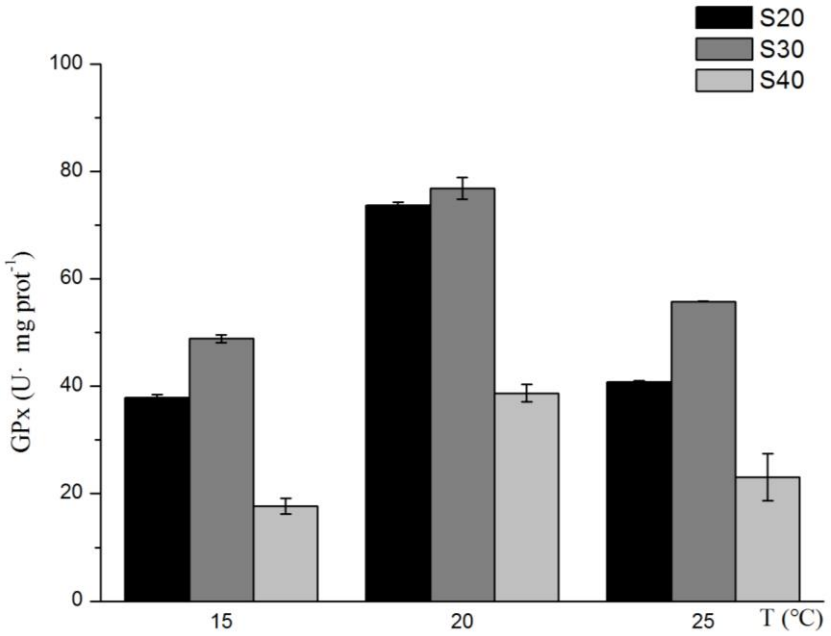

Fig. 7 Effects of temperature and salinity on gill GPx levels in *M. modiolus*. S20, salinity of 20 PSU; S30, salinity of 30 PSU; S40, salinity of 40 PSU. Bars show the means of 3 replicates. Error bars show SD.

Table 6 One-way and two-way ANOVA of the effects of different temperatures and salinities on GPx levels in *M. modiolus.* Values are means ± SD (n = 3).

	Temperature (°C)	Salinity (PSU)	p-value			Duncan test
			Temperature	Salinity	Interaction	
One-way ANOVA (factor: Temperature)	15, 20, 25	20	1.31×10^{-10}****			20 °C > 25 °C > 15 °C
		30	3.79×10^{-7}****	--	--	20 °C > 25 °C > 15 °C
		40	2.33×10^{-4}****			20 °C > 25 °C = 15 °C
One-way ANOVA (factor: Salinity)	15	20, 30, 40	--	5.59×10^{-8}****	--	30 PSU > 20 PSU > 40 PSU
	20			1.40×10^{-7}****		30 PSU > 20 PSU > 40 PSU
	25			1.20×10^{-5}****		30 PSU > 20 PSU > 40 PSU
Two-way ANOVA (Fixed factors: Temperature & Salinity)	15, 20, 25	20, 30, 40	1.89×10^{-17}****	1.30×10^{-18}****	1.17×10^{-6}****	20 °C > 25 °C > 15 °C; 30 PSU > 20 PSU > 40 PSU

*, value is significant based on the Duncan tests (****$p < 0.0001$); ns, value is not significant based on the Duncan test.

The adverse effects of temperature and salinity on *M. modiolus* were also clear in our examination of gill morphology. Gills are the primary organs of both respiration and defense in bivalves (Fiddy *et al.*, 2016). Here, abnormal temperatures and salinities damaged gill filaments and altered gill filament lumen width. Salinity affects the respiration and growth of larval and juvenile *C. fornicata* (Samuel *et al.,* 2015); thus, it is possible that the reduction in *M. modiolus* survival observed here might have been due to the effects of temperature and salinity on respiration, instead of feeding rate. Alternatively, it has been shown that changes in gill filament lumen size indirectly demonstrate an imbalance in osmotic pressure (Han *et al.,* 2014). Therefore, it might be that temperature and salinity affect the survival of *M. modiolus* by disrupting endogenous osmotic pressure. Further work is necessary to assess the combined impact of temperature and salinity on respiration and osmotic pressure in *M. modiolus*.

The generation of reactive oxygen species (ROS) is a typical physiological phenomenon in aerobic organisms. It is clear that excessive accumulation of ROS (such as O_2, H_2O_2, and OH^-) can cause oxidative damage to lipids, proteins, carbohydrates, and nucleic acids (Khazri *et al.*, 2016; Shenaitirodkar *et al.*, 2017). The maintenance of optimal ROS levels is thus vital for endo-redox homeostasis and for the survival of aerobic species.

SOD and GPx, two important enzymes in the antioxidant defense system, also serve as ROS scavengers (Levent *et al.,* 2005); T-AOC typically reflects the balance of oxidants and antioxidants in aerobic organisms (Fan *et al.,* 2004). Decreases in temperature (from 26 ºC to 15 ºC) significantly decreased SOD activity in *M. yessoensis* at normal salinity (~31 PSU; Hao *et al.*, 2014). In adult *C. gigas*, SOD activity decreased and then increased as temperature increased from 24 ºC to 32 ºC, but in adult *C. brasiliana,* SOD activity decreased steadily with the same temperature increase (Moreira *et al.,* 2017). Here, we found that SOD activity increased and then decreased as temperature rose from 15 ºC to 25 ºC. These results suggested that the effects of temperature on SOD activity vary among bivalves; there may be species-specific mechanisms that manage temperature variations.

At 15 °C, SOD activity decreased significantly as salinity increased, but at 25 °C, SOD activity increased significantly as salinity increased. This interesting result indicated that salinity might have a more profound impact on SOD activity in the gill of *M. modiolus* than temperature.

It was reported that elevated temperature increased the activity of GPx in the gills of the brown mussel *Perna perna* (Almeida *et al.,*2007). In contrast, GPx activity in the gills of *M. modiolus* at both low and high temperatures (15 °C and 25 °C) was lower at all tested salinities than GPx activity at 20 °C. This discrepancy might be due to differences in GPx function among species. In addition, the decreased gill GPx activity at both low and high salinities (20 and 40 PSU) observed in *M. modiolus* might indicate that increased salinity interrupts the antioxidant system of this species by reducing the catalytic capacity of GPx.

T-AOC levels in *M. modiolus* increased at both low and high salinities, suggesting that *M. modiolus* might compensate for the increased numbers of ROS induced by abnormal temperatures and salinities by increasing T-AOC. Further research is necessary to clarify the mechanism by which *M. modiolus* increases T-AOC.

Our results suggested that *M. modiolus* might be suitably cultured between 15 °C and 20 °C, and between 30 and 40 PSU. Our study provides a framework for the selection of suitable locations for *M. modiolus* aquaculture, as well as for the development of sustainable aquaculture methods. Our data also increase our understanding of the impact of temperature and salinity on molluscan growth and survival. Future studies should focus on identifying and testing additional ROS markers to further clarify the effects of temperature and salinity on endo-redox homeostasis in *M. modiolus*. In addition, molecular studies (including differential gene expression, transcriptome comparison, and microRNA analysis) would help to further clarify the mechanisms affected by temperature and salinity in adult *M. modiolus*.

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