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Combined effects of ocean acidification and hypoxia on the early development of the thick shell mussel *Mytilus coruscus*

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Abstract

Ocean acidification has become serious, and seawater hypoxia has become evident in acidified waters. The combination of such stressors may have interactive effects on the fitness of marine organisms. In order to investigate the interactive effects of seawater acidification and hypoxia on the early development of marine bivalves, the eggs and sperm of the thick shell mussel *Mytilus coruscus* were exposed to combined treatments of pH (8.1, 7.7, 7.3) and dissolved oxygen (2, 6 mg/L) for 96 h culture observation to investigate the interactive effects of seawater acidification and hypoxia on the early development of marine bivalves. Results showed that acidification and hypoxia had significant negative effects on various parameters of the early development of the thick shell mussel. However, hypoxia had no effect on fertilization rate. Significant interactions between acidification and hypoxia were observed during the experiment. Short-term exposure negatively influenced the early development of the thick shell mussel but did not affect its survival. The effects of long-term exposure to these two environmental stresses need further study.

Keywords: Ocean acidification, Hypoxia, *Mytilus coruscus*, Early development

Background

Ocean Acidification (OA), i.e., a decrease in seawater pH (increased acidity) associated with a change in carbonate equilibrium system, is a consequence of the large amount of anthropogenic CO_2 absorbed by the ocean [1–3]. The current surface seawater pH is approximately 8.1–8.2 and is predicted to decrease to 7.7–7.8 by 2100 and 7.3–7.4 by 2300 [4, 5]. OA can affect the fertilization [6], embryonic development, metabolism [7], behavior [8–10], and immunity [11] of various marine organisms; threatens the survival and reproduction of marine animals; and damages global marine biodiversity and ecosystem stability [12–15]. Acidification negatively affects the early developmental stage of abalones *Haliotis diversicolor* and significantly reduces the survival rate of larvae [16]. In addition, low pH delays the development of mussel *Mytilus galloprovincialis* larvae [17].

Seawater acidification does not occur alone in the natural environment. Other stressors, such as hypoxia, also affect the marine environment [18–22]. Hypoxia are areas with hypoxic conditions that the dissolved oxygen (DO) concentration in the water is < 2.0 mg/L [23]. OA is likely to occur in areas where the seawater is deficient in oxygen [24, 25]. The decrease in seawater DO can enhance seawater acidification, and the seawater pH decreases with the value of DO [19]. Low DO in water negatively influences marine organisms and even threatens the survival of marine life [26–28]. Clark and Gobler [29] found that low DO inhibits the development and significantly reduces the survival rate of bay scallop *Argopecten irradians* larvae (2.33 mg/L).

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Marine mussels have become a popular model animal in marine environmental monitoring research owing to their relatively long lifespan, sessile benthic life style, filter feeding, bioaccumulation capability in body, and global distribution [30]. Embryonic and early larval developments are important stages of mollusk life cycle. Benthic animals experience stress, such as nutrition deficiency, pollution and infiltration, and growth inhibition during larval stage [31]. Early embryos and larvae of shellfish are sensitive to environmental disturbances. OA exposure of oysters during early development can potentially inhibit their subsequent growth and development [32]. Stevens and Gobler [33] studied the effects of ocean acidification and hypoxia on bay scallop *Argopecten irradians* and found that acidification significantly reduces their survival rate, and this effect is further aggravated by hypoxia. Gobler et al. [34] analyzed the effects of ocean acidification and hypoxia on the early development of hard clams *Mercenaria mercenaria* and found that acidification significantly inhibits larval growth, and hypoxia intensifies this influence. Considering that acidification and hypoxia exist simultaneously in actual marine ecosystems, the effects of complex ecological effects of acidification and hypoxia on marine organisms must be investigated. Exploring this aspect can further clarify the mechanism underlying acidification and hypoxia on marine organisms and help to assess the impact of environmental variability on coastal organisms.

Mussels are the key components of marine intertidal ecosystems and enhance the coastal diversity [35]. The thick shell mussel *Mytilus coruscus* is one of the most important aquaculture shellfish and possesses important economic value in China [36]. Heavy mortalities of mussels have occurred frequently during summer in the Shengsi island probably due to hypoxia and low pH conditions [37]. At present, the interactive effect of ocean acidification and hypoxia on the early development of *M. coruscus* is still unclear. We hypothesize that the early development of mussels is seriously affected by acidification and hypoxia, and these effects are aggravated when both stressors appear simultaneously. The eggs and sperm of the thick shell mussel were exposed to six treatments [three pH levels (8.1, 7.7, and 7.3) and two DO levels (2 and 6 mg O_2/L)] for 96 h culture observation to study the combined effects of ocean acidification and hypoxia on the early development of this species. Fertilization rate, cleavage rate, deformity rate, larval shell length, and shell height were also evaluated.

Materials and methods

Experimental animals

Mussels (shell length 8.0 ± 2.0 cm, dry weight 1.60 ± 0.90 mg) were collected from the Shengsi Island, Zhejiang Province, China. After cleaning, the mussels were acclimated in the aquarium for a week. Temperature, salinity, and DO were controlled at 20.0 ± 0.3 °C, 25.0 ± 0.5, and 6.0 ± 0.3 mg O_2/L, respectively. Water was completely changed once a day, and dead mussels were removed during acclimation. *Isochrysis galbana* (2.5×10^4 cells/mL) was fed to mussels daily. After 1-week acclimation, 60 parental mussels (45 females, 15 males) were stimulated with flowing filtered seawater for 10 min before spawning and then transferred to a 60 L spawning tank. Thermal shock method was applied by rapidly increasing the water temperature from 13 to 23 °C to achieve large-scale spawning [38, 39]. Male mussels that release sperm and female mussels that spawn were transferred to two 30 L spawning tanks for ejaculation and spawning, respectively. After ejaculation and spawning, 15 mL of seawater containing eggs (density of about 2×10^3/mL) and 15 mL of seawater containing sperm (density of about 1×10^6/mL) were sampled and transferred to aquariums (30 L) with different pH and DO levels for fertilization.

Experimental design

Six experimental treatments (① pH 8.1 × 6 mg O_2/L, ② pH 8.1 × 2 mg O_2/L, ③ pH 7.7 × 6 mg O_2/L, ④ pH 7.7 × 2 mg O_2/L, ⑤ pH 7.3 × 6 mg O_2/L, and ⑥ pH 7.3 × 2 mg O_2/L) and three replicates were set for each treatment. Exposure lasted for 4 days, and the development of fertilized eggs was investigated and recorded at 2, 4, 8, 24, 48, 72, and 96 h.

The pH in the experimental aquarium was controlled by using a pCO_2/pH system (DAQ-M) equipped with a WTW pH 3310 m and a SenTix 41 pH electrode (Loligo Systems Inc., Denmark) [40]. Low pH treatment was achieved by aerating CO_2 into the seawater by this pCO_2/pH system. Either N_2 or air was passed through the O_2 regulator (Loligo Systems Inc., Denmark) into the seawater to achieve the hypoxic conditions [41].

Seawater chemical parameters

Seawater parameters were monitored daily. Total alkalinity (AT) was determined by titration. Temperature, salinity, and DO were measured using a multi-parameter water quality instrument (5200A, YSI Inc., America). pH was detected by a pH meter (pH-201, MSITECH (Asia-Pacific) Pte. Ltd., Singapore). Other chemical parameters [dissolved inorganic carbon (DIC), pCO_2, calcite saturation state (Ωcal), and aragonite saturation state (Ωara)] were calculated by CO_2SYS [42].

Biological parameters

After 2 h of fertilization, the egg samples were observed by a microscope fitted with an ocular micrometer, and

fertilization was evaluated by the presence of polar body and embryonic cleavage [38, 39]. The egg samples were obtained 4 h after fertilization, and cleavage was assessed by observing embryonic morphology at 48, 72, and 96 h to assess embryo deformity. Malformations observed in the study of Kong et al. were used as evaluation criteria [15]. For the deformity, embryos were visually inspected and characterized as slightly deformed, broken, and deformed during embryonic division (Fig. 1). During sampling, 100 eggs were randomly selected from each aquarium, and fertilization rate, deformity rate, and cleavage rate were calculated after observation. At 48, 72, and 96 h, 50 D-shaped larvae were randomly obtained from each aquarium, and their shell length and height were measured by a microscope fitted with an ocular micrometer (Nikon Eclipse 55i, Japan).

Statistical analysis

Data analysis was performed by SPSS 19.0 software, and percentage data were arcsine square root transformed prior to analysis. Experimental data were tested for homogeneity of variance and normal distribution. Three-way ANOVA was used to assess whether the interaction among pH, DO, and time affects the deformation rate of the fertilized eggs of the thick shell mussel. Two-way ANOVA was employed to analyze whether the interaction between pH and DO influences the fertilization rate, cleavage rate, and shell length and height of the thick shell mussel. Tukey post hoc tests were performed for the effects of pH and DO, respectively, with $p < 0.05$ as a criterion for significant difference.

Results

The pH of each treatment was stably maintained within the required levels by using the pH/pCO_2 control system. Salinity and temperature were maintained at approximately 25 and 20 °C, respectively, during the whole experiment. The seawater chemical parameters of each experimental group are shown in Table 1.

The fertilization rate of mussel gametes was only significantly affected by pH ($P < 0.05$) (Table 2, Fig. 2). At DO of 6 mg/L, the fertilization rate under pH 7.3 was significantly lower (decreased by 14.7%) than that under pH 8.1 ($P < 0.05$). No significant difference was observed between pH 8.1 and pH 7.7 and between pH 7.7 and pH 7.3. At the DO of 2 mg/L, the fertilization rate under pH 7.3 was significantly lower than that under pH 8.1 and 7.7 ($P < 0.05$).

The cleavage rate was significantly affected by pH and DO ($P < 0.05$) (Fig. 3), and a significant interaction was

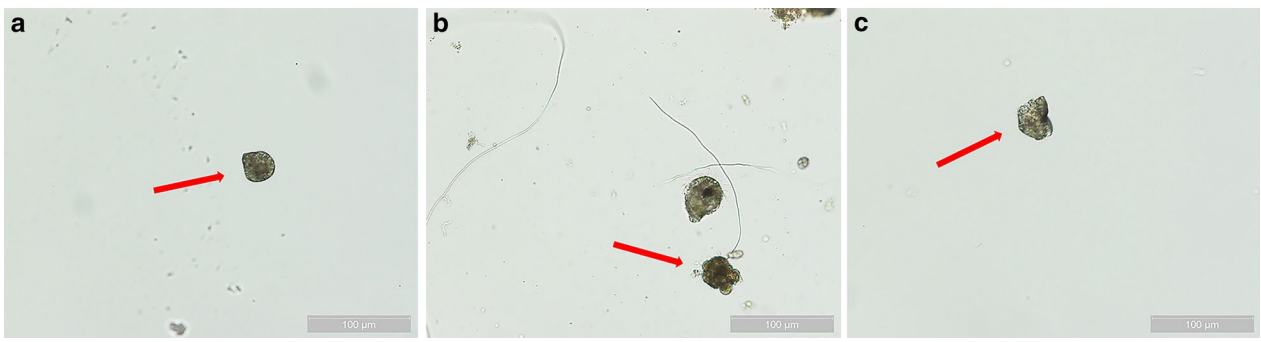

Fig. 1 The categorization of deformity of embryos. **a** Slightly deformed of the embryo; **b** deformity during embryonic division; **c** embryo breakage

Table 1 Seawater chemistry parameters during the experiment (mean ± SD)

DO (mg/L)	pH_{NBS}	Salinity	T (°C)	T_A (μmol/kg)	DIC (μmol/kg)	pco_2 (μatm)	Ωcal	Ωara
6.1 ± 0.1	7.33 ± 0.03	25.1 ± 0.1	20.0 ± 0.1	2316 ± 11	2343 ± 22	2715 ± 23	1.04 ± 0.08	1.19 ± 0.05
2.0 ± 0.2	7.30 ± 0.01	25.2 ± 0.1	20.1 ± 0.2	2345 ± 28	2379 ± 32	2939 ± 18	1.01 ± 0.03	1.22 ± 0.01
6.0 ± 0.1	7.69 ± 0.02	25.1 ± 0.3	20.0 ± 0.2	2376 ± 11	2224 ± 19	1015 ± 10	1.77 ± 0.04	1.24 ± 0.06
2.0 ± 0.2	7.70 ± 0.01	25.1 ± 0.2	20.0 ± 0.1	2295 ± 39	2114 ± 15	995 ± 11	1.84 ± 0.17	1.19 ± 0.03
6.1 ± 0.1	8.09 ± 0.02	25.0 ± 0.1	19.9 ± 0.2	2241 ± 41	1994 ± 19	351 ± 21	4.39 ± 0.07	2.76 ± 0.09
2.1 ± 0.1	8.10 ± 0.02	25.0 ± 0.1	20.0 ± 0.1	2208 ± 20	2059 ± 31	373 ± 12	4.40 ± 0.10	2.91 ± 0.07

pH was monitored by the pH/CO_2 system continuously during the experiment. Salinity, temperature and total alkalinity (T_A) were determined at each sampling time ($n = 5$). Dissolved inorganic carbon (DIC), partial pressure of CO_2 (pCO_2), saturation degrees for calcite (Ωcal) and aragonite (Ωara) were calculated based on the above parameters

Table 2 Summary of two-way ANOVA results on effects of pH and DO on fertilization rate and cleavage rate

		SS	df	MS	F	P
2 h fertilization rate	DO	80.222	1	80.222	2.911	0.114
	pH	837.444	2	418.722	15.196	0.001
	DO * pH	58.111	2	29.056	1.054	0.379
	Error	330.667	12	27.556		
	Total	96064.000	18			
4 h cleavage rate	DO	760.500	1	760.500	63.967	0.000
	pH	1477.000	2	738.500	62.117	0.000
	DO * pH	394.333	2	197.167	16.584	0.000
	Error	142.667	12	11.889		
	Total	8935.000	18			

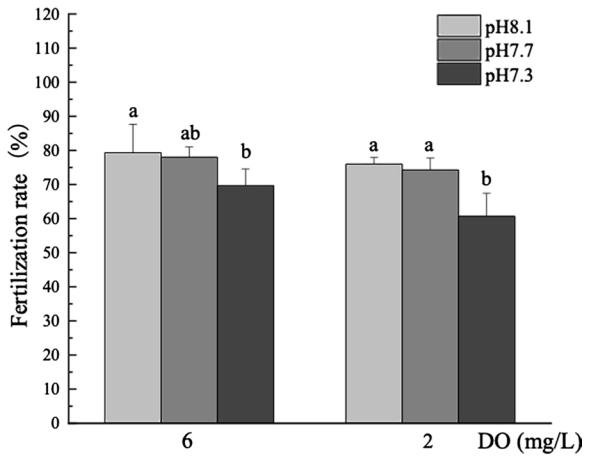

Fig. 2 The effects of different pH (8.1, 7.7, 7.3) and DO concentrations (6 mg/L, 2 mg/L) on the fertilization rate at 2 h. Different lowercase letters represent significant differences between different pH under the same DO ($P < 0.05$)

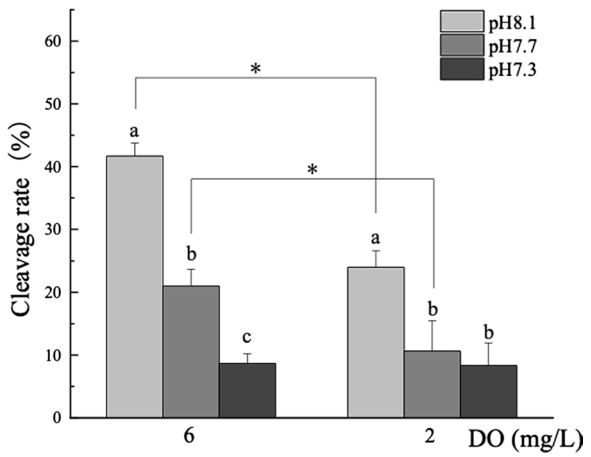

Fig. 3 The effects of different pH (8.1, 7.7, 7.3) and DO concentrations (6 mg/L, 2 mg/L) on the cleavage rate of mussels at 4 h. Different lowercase letters represent significant differences between different pH under the same DO ($P < 0.05$); * indicates significant difference between different DO under the same pH conditions ($P < 0.05$)

found between pH and DO ($P < 0.05$) (Table 2). At DO of 6 mg/L, the cleavage rate of mussel decreased significantly with the pH ($P < 0.05$). At DO of 2 mg/L, the cleavage rate of mussels under acidification (pH 7.7 and pH 7.3) was significantly reduced ($P < 0.05$). At low DO, the cleavage rate decreased significantly under pH 8.1 and 7.7 ($P < 0.05$).

The deformation rate of mussels was significantly affected by time, DO, and pH ($P < 0.05$) (Table 3), but these factors did not have significant interactions. When the fertilized eggs were exposed for 48, 72, and 96 h, the deformity rate increased significantly ($P < 0.05$) with the decrease in pH under the two DO conditions (Fig. 4). With prolonged exposure time, the deformation rate increased significantly at DO of 2 mg/L. At 96 h, hypoxia significantly increased the deformity rate of larvae under pH 7.3.

Table 3 Summary of three-way ANOVA results on effects of pH and DO on deformity rate

	Deformity rate				
	SS	df	MS	F	P
T	60.719	2	30.360	16.005	0.000
DO	20.488	1	20.488	10.801	0.002
pH	1063.576	2	531.788	280.347	0.000
T * DO	8.405	2	4.202	2.215	0.124
T * pH	12.157	4	3.039	1.602	0.195
DO * pH	8.687	2	4.343	2.290	0.116
T * DO * pH	0.421	4	0.105	0.056	0.994
Error	68.288	36	1.897		
Total	21588.839	54			

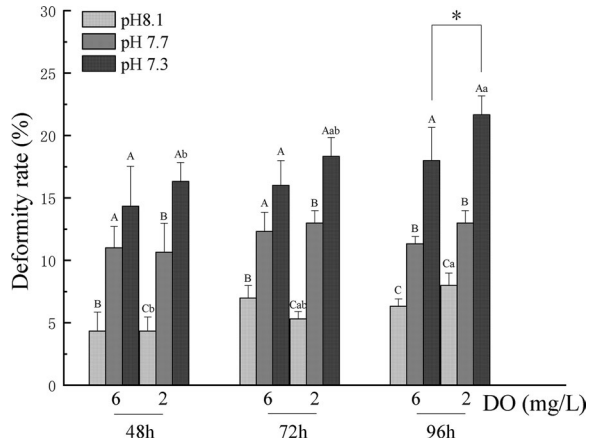

Fig. 4 The effect of different pH (8.1, 7.7, 7.3) and DO concentrations (6 mg/L, 2 mg/L) on the deformity rate of mussels during 96 h exposure. Different uppercase letters represent significant differences between different pH under the same DO ($P < 0.05$); different lowercase letters represent significant differences between different time points under the same DO and pH conditions ($P < 0.05$); * indicates significant difference between different DO under the same pH conditions ($P < 0.05$)

The shell length of D-shaped larvae was significantly affected ($P < 0.05$) by DO and pH throughout the experiment; however, DO and pH did not have significant interaction on the shell length of D-shaped larvae (Table 4, Fig. 5). At 24 h and 48 h, DO and pH did not significantly affect the shell length of D-shaped larvae. From 72 h, the growth of D-shaped larvae was significantly inhibited with the decrease in pH. Among the four time points observed in the experiment, hypoxia was observed to significantly inhibit the shell length growth of the D-shaped larvae of mussels only at 96 h and under pH 7.3 ($P < 0.05$).

The shell height of D-shaped larvae was significantly affected ($P < 0.05$) by DO and pH throughout the experiment, but no significant interaction was found between DO and pH (Table 4, Fig. 6). At 24, 48, and 72 h, no significant effect of acidification on the shell height of D-shaped larvae was observed under the two DO conditions. At 96 h, pH 7.3 significantly inhibited ($P < 0.05$) shell height growth under both DO conditions. At 72 h, hypoxia significantly inhibited ($P < 0.05$) the increase in the shell height of D-shaped larvae under pH 8.1 and pH 7.7. At 96 h, hypoxia significantly inhibited ($P < 0.05$) the increase in the shell height of D-shaped larvae under the three pH conditions.

Discussion

Ocean acidification and hypoxia phenomena have frequently occurred in recent years, and the affected areas in marine environments are still expanding [43, 44]. Although the effects of acidification or hypoxia on marine organisms have been studied, most works are based on single factor effects; only a few focused on the combined stress of acidification and hypoxia on marine organisms [34, 45]. In practice, acidification and hypoxia occur simultaneously, thus highlighting the need to investigate the synergic effects of these two stressors [18–20]. In the present experiment, the effects of ocean acidification and hypoxia on the early development of the thick shell mussel were studied. The results showed that ocean acidification and hypoxia negatively affected the early development of the thick shell mussel. In addition, the synergistic effects of acidification and hypoxia on the cleavage rate of fertilized eggs and the deformation rate of mussel larvae were observed during the experiment.

Chemical changes in seawater affects every developmental stage in the life cycle of various marine organisms, and early development is the most sensitive stage [46–48]. Shi et al. [6] suggested that OA may affect the fertilization success of broadcast spawning bivalves by (1) reducing sperm swimming speed and therefore lowering the probability for gamete collision in 3D water column, (2) hampering the gamete recognition that results in constrained gamete fusion, and (3) interrupting the generation of Ca^{2+} oscillation that triggers cortical reaction and embryonic development. Swiezak et al. [49] explored the effect of acidification on fertilization and found that the fertilization rate of clam *Limecola balthica* was significantly reduced at low pH. Similar to our experimental results, the fertilization rate of the thick shell mussel was significantly reduced at pH 7.3. We found that acidification and hypoxia significantly delayed the cleavage of fertilized eggs. Girard et al. [50] suggested that this phenomenon may be related to the interference of fertilized eggs on calcium homeostasis under ambient pressure. Moreover, acidification and hypoxia significantly increase the deformation rate when the fertilized eggs develop into larvae. When the pH of seawater is low, the deformation rate in early development of the undulated surf clam *Paphia undulate* significantly increases [51]. Possibly under seawater acidification, the early development stage of mussels is highly sensitive to elevated pCO_2, thereby leading to failed larval shell mineralization [46, 52–54]. Segerstråle [55] showed that hypoxic conditions could result in bivalve shell deformities. Low DO significant increases the deformation rate of the early development of mussel *Mytilus edulis*, and acidification exacerbates this effect [15]. This finding is consistent with the results of our experiments and may have occurred because under adverse environmental conditions, marine invertebrates spend much energy coping with stress, thereby resulting in shell malformations due to the less energy allocated to shell growth [56, 57].

Table 4 Summary of two-way ANOVA results on effects of pH and DO on larval shell length and shell height

		SS	df	MS	F	P
Shell length						
48 h	DO	150.222	1	150.222	1.777	0.207
	pH	248.778	2	124.389	1.471	0.268
	DO * pH	17.444	2	8.722	0.103	
	Error	1014.667	12	84.556		
	Total	164642.000	18			
72 h	DO	355.556	1	355.556	5.276	0.04
	pH	445.444	2	222.722	3.305	0.072
	DO * pH	15.444	2	7.722	0.115	0.893
	Error	808.667	12	67.389		
	Total	180826.000	18			
96 h	DO	138.889	1	138.889	4.762	0.050
	pH	1552.111	2	776.056	26.608	0.000
	DO * pH	22.111	2	11.056	0.379	0.692
	Error	350.000	12	29.167		
	Total	209002.000	18			
Shell height						
48 h	DO	88.889	1	88.889	1.752	0.210
	pH	125.778	2	62.889	1.240	0.324
	DO * pH	7.111	2	3.556	0.070	0.933
	Error	608.667	12	50.722		
	Total	92994.000	18			
72 h	DO	272.222	1	272.222	6.110	0.029
	pH	271.444	2	135.722	3.046	0.085
	DO * pH	15.444	2	7.722	0.173	0.843
	Error	534.667	12	44.556		
	Total	104758.000	18			
96 h	DO	213.556	1	213.556	24.484	0.000
	pH	688.000	2	344.000	39.439	0.000
	DO * pH	13.778	2	6.889	0.790	0.476
	Error	104.667	12	8.722		
	Total	119118.000	18			

During mussel larval growth, acidification and hypoxia significantly inhibited the growth of larval shells, and this effect was strengthened over time. Young and Gobler [58] studied the effects of ocean acidification on eastern oyster *Crassostrea virginica* and found that the growth rate of oyster shells is significantly reduced by low pH due to the dissolution of calcium carbonate, the main component of the shell, under acidification conditions [59, 60]. Under acidification conditions, the shellfish consumes much energy to fight against environmental stress (such as acid–base regulation), resulting in a decrease in energy allocated for growth [61–64]. Dorey et al. [64] studied the effects of low pH on the larvae of green sea urchin *Strongylocentrotus droebachiensis* and found that low pH significantly increases their respiration rate, indicating that a decrease in pH would increase the body's energy consumption. With regard to acidification, shellfish in the absence of oxygen also reduce their energy allocated for growth due to external pressure, resulting in growth inhibition [34, 56, 57]. In the present experiment, acidification additionally consumed a part of the energy used for body growth, and anti-hypoxia increased the energy consumption of this part. Therefore, acidification significantly inhibited the growth of mussel larvae, and hypoxia aggravated this effect. Shellfish exposed to hypoxia undergo anaerobic respiration and exert great resistance to external stress [65, 66], but this adaptive response is only short-term. Although the short-term acidification and hypoxia stress in this experiment only led to growth inhibition and did not cause death, a long-term exposure to such

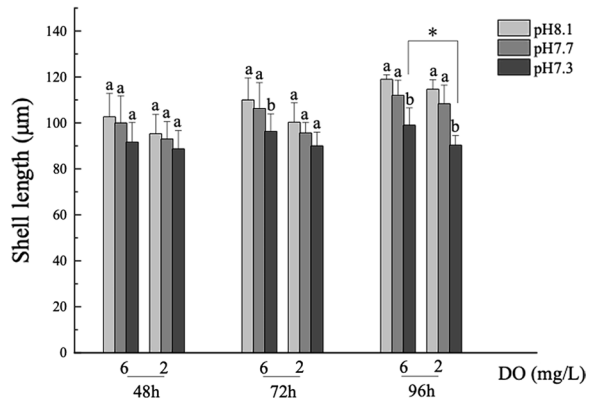

Fig. 5 The effect of different pH (8.1, 7.7, 7.3) and DO concentrations (6 mg/L, 2 mg/L) on the shell length of larvae of mussels during 96 h exposure. Different lowercase letters represent significant differences between different pH under the same DO ($P < 0.05$); * indicates significant difference between different DO under the same pH conditions ($P < 0.05$)

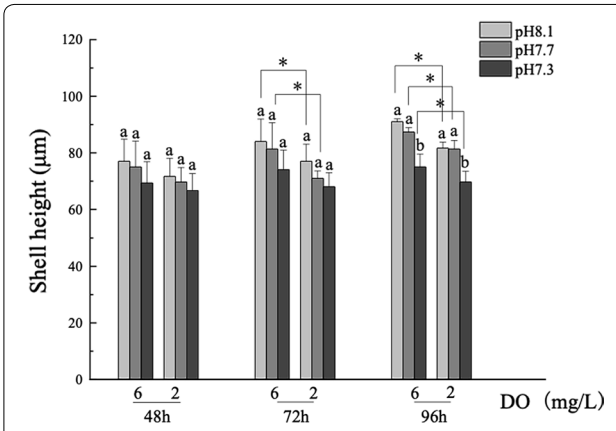

Fig. 6 The effect of different pH (8.1, 7.7, 7.3) and DO concentrations (6 mg/L, 2 mg/L) on the shell height of mussels during 96 h exposure. Different lowercase letters represent significant differences between different pH under the same DO ($P < 0.05$); * indicates significant difference between different DO under the same pH conditions ($P < 0.05$)

environmental stress may threaten the survival and even lead to death [57, 67]. Therefore, long exposures are necessary in studying the impact of acidification and hypoxia on marine mussels.

Conclusion

This experiment investigated the combined effects of acidification and hypoxia on the early development of the thick shell mussel *M. coruscus*. Results showed that hypoxia had no effect on fertilization rate. Acidification and hypoxia had significant negative effects on the various stages of early development of the thick shell mussel and showed significant interactive influences on the cleavage rate of fertilized eggs and the deformation rate of mussel larvae during the experiment. Although the short-term exposure test has not resulted in mussel death, the effects of long-term exposure to these two environmental stresses need further study.

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Authors' contributions
MH, JY, YW and YD conceived the study, XW and YS carried out the experiment, HK and YS analyzed the data, XW wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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