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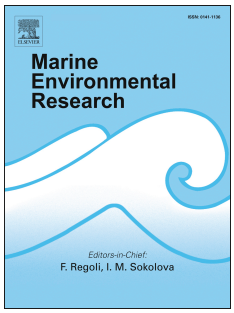

Transgenerational effects of short-term exposure to acidification and hypoxia on early developmental traits of the mussel *Mytilus edulis*

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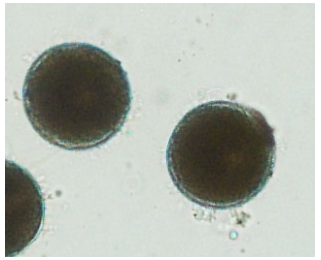

Low pH

Low pH
Low DO

Experiment #1

pH	DO
8.1	6mg O_2*L^{-1}
7.7	2mg O_2*L^{-1}
7.3	

Six treatments

Negative effects

Embryo deformity rate
D-stage shell length
Fertilization rate

Experiment #2

pH 8.1
DO 6mg O_2*L^{-1}

Six treatments

Positive transgenerational effect

Transgenerational effects of short-term exposure to acidification and hypoxia on early developmental traits of the mussel *Mytilus edulis*

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Abstract

Transgenerational effects of multiple stressors on marine organisms are emerging environmental themes. We thus experimentally tested for transgenerational effects of seawater acidification and hypoxia on the early development traits of the mussel *Mytilus edulis*. Fertilization rate, embryo deformity rate, and larval shell length were negatively impacted by acidification, while hypoxia had little effect except for increasing deformity rates under control pH conditions. Offspring from low pH/O_2 parents were less negatively affected by low pH/O_2 conditions than offspring from control parents; however, low pH/O_2 conditions still negatively affected developmental traits in offspring from acclimated parents compared to control seawater conditions. Our results demonstrate that experimental seawater acidification and hypoxia can adversely affect early developmental traits of *M. edulis* and that parental exposure can only partially alleviate these impacts. If experimental observations hold true in nature, it is unlikely that parental exposure will confer larval tolerance to ocean acidification for *M. edulis*.

Keywords: Carbon dioxide; Environmental stress; Hypoxia; Global change biology; Multiple stressors; Transgenerational plasticity

1. Introduction

Marine global change is anticipated to impact ocean life in the near-future. Two co-occurring stressors that have received relatively little combinatory attention are ocean acidification and deoxygenation (see Gobler & Baumann, 2016 for review).

Generally speaking, ocean acidification describes a decrease in oceanic pH, while deoxygenation refers to a global decrease in oceanic oxygen. In the open ocean, acidification is predominantly driven by the uptake of excess anthropogenic CO_2 from the atmosphere (Hoegh-Guldberg et al., 2014), while deoxygenation is primarily driven by global warming (Breitburg et al., 2018). By 2100, it is projected that open-ocean pH will decrease by 0.3–0.4 units (Feely et al., 2004; Orr et al., 2005), and oxygen will reduce 1–7% (Keeling et al., 2010; Schmidtko & Visbeck, 2017).

In contrast to the open ocean, coastal acidification can be affected by myriad processes such as coastal upwelling, ecosystem metabolism and watershed dynamics, and freshwater runoff (Duarte et al., 2013). Similarly, coastal deoxygenation is primarily caused by increased nutrient and organic loads that increase oxygen consumption through microbial decomposition (typically defined as dissolved O_2 below 2 mg O_2 L^{-1}; Vaquer-Sunyer & Duarte, 2008; Breitburg et al., 2018). Acidification and hypoxia are known to co-occur, and recent studies highlight tight linkages between acidification and hypoxia in coastal ecosystems, with acidification being more severe under hypoxic conditions (compared to normoxia; Feely et al., 2010; Cai et al., 2011; Paulmier et al., 2011; Melzner et al., 2013). Consequently, coastal organisms can already experience low pH and oxygen conditions that exceed near-future open ocean projections (Wallace et al., 2014; Baumann et al., 2015; Gobler & Baumann, 2016). Nonetheless, global climate change can exacerbate pH and oxygen declines in coastal regions, and coastal organisms are not, by default,

immune to such change (Waldbusser & Salisbury, 2014; Breitburg et al. 2018). It is thus necessary to understand the combined effects of short-term acidification and hypoxia on marine life.

Globally, marine bivalves are of ecological (Costanza et al., 1997; Dame 2011) and economic (Cooley & Doney, 2009; FAO, 2018) importance. It is well documented, however, that marine bivalves are sensitive to multiple global change stressors. With respect to ocean acidification and hypoxia (see Gobler & Baumann, 2016 for review), a limited number of studies suggest largely negative combined effects (Gobler et al., 2014; Clark & Gobler, 2016; Stevens & Gobler, 2018), but positive and null effects have also been reported (Jakubowska & Normant, 2014; Jansson et al., 2015). Given the contrasting effects across relatively few studies, more research testing the combined effects of acidification and hypoxia on marine bivalves is warranted.

The role of transgenerational effects (i.e., the effect caused by the parental environment on the offspring; Munday, 2014; Ross et al., 2016) in shaping offspring responses to environmental stress has recently drawn substantial attention. These transgenerational effects can be acclamatory (non-genetic; referred to as transgenerational acclimation or transgenerational plasticity) or adaptive (genetic; referred to as transgenerational adaptation), and can allow some organisms to adjust to projected environmental change (Munday, 2014). Recent studies have indicated that the potential for transgenerational acclimation to global change stressors is not

universal and varies across species (Munday, 2014; Munday et al., 2014; Sunday et al., 2014; Ross et al., 2016). With respect to marine bivalves, a limited number of transgenerational studies in the context of ocean acidification exist and report variable effects. For instance, larval clams (*Ruditapes philippinarum*) showed better growth performance under low pH when parents experienced similar low pH conditions (Zhao et al., 2018). Positive transgenerational effects under experimental ocean acidification have also been reported for larval oysters (*Saccostrea glomerata*; Parker et al., 2012) and juvenile mussels (*M. edulis*; Fitzer et al., 2014a). In contrast, Griffith & Gobler (2017) reported negative transgenerational effects associated with transgenerational exposure to ocean acidification in larval scallops (*Argopecten irradians*) and clams (*Mercenaria mercenaria*).

While transgenerational studies on ocean acidification exist for marine bivalves, to our knowledge there have been no studies testing for transgenerational acclimation to combined acidification and hypoxia. Consequently, the predictions for how these animals will respond to ocean and coastal acidification and hypoxia are, at present, unattainable. To explore this knowledge gap, we tested for transgenerational effects on early larval developmental traits of mussels (*M. edulis*) exposed to experimental acidification and hypoxia.

2. Materials and Methods

2.1 Animal collection and husbandry

Wild adult mussels (*M. edulis*; 75 ± 5 mm shell length) were collected from Gouqi Island, East China Sea (30°43′1.64″N, 122°46′3.25″E) in October 2017. Mussels were immediately transported to experimental facilities at Shanghai Ocean University (Shanghai, China), gently scrubbed clean of epibionts, and transferred to 30 L acclimation tanks (recirculating aquarium system with filtered seawater; density = 15 mussels $tank^{-1}$; flow rate ~10 L min^{-1}). The mussels were acclimated to laboratory conditions for two weeks at 13 ± 0.5 °C, salinity 28 ± 0.5 psu, dissolved oxygen (DO) concentration of 6.0 ± 0.3 mg O_2 L^{-1} and pH 8.1 ± 0.1 (simulated natural environment of mussels at collection site). During acclimation, the mussels were fed twice daily with 10 ml of the microalgae *Isochrysis galbana* (25,000 cells ml^{-1}). Animal condition did not change during the acclimation phase and adult mortality was minimal; only visually healthy mussels were selected for the experiment.

2.2 Seawater chemistry

Low pH was achieved by using a pCO_2/pH system (DAQ-M) equipped with WTW pH 3310m and SenTix 41 pH electrode (Loligo Systems Inc., Denmark). The pH level was maintained by bubbling pure CO_2 which was real-time connected with feedback STAT systems (DAQ-M). Dissolved oxygen was manipulated by bubbling a mixture of N_2 and air directly into the water via an O_2 regulator (Loligo Systems Inc., Denmark). The gas flow was maintained by a solenoid valve controlled by a computer connected to an O_2 regulator to achieve stable DO levels in each tank.

Abiotic seawater parameters including temperature, pH, DO and salinity were monitored twice a day for each tank and total alkalinity (A_T) was measured every two days. Temperature, salinity and DO were observed by a multi-parameter water quality instrument (5200A, YSI Inc., America). Total alkalinity (A_T) was determined by manual 2-point acid-base titration using a manual burette and applicable reagents (Phenolphthalein indicator, Methyl red indicator, and 0.025mol L^{-1} Hydrochloric Acid Standard Solution). Additional carbonate system parameters including pCO_2, dissolved inorganic carbon (DIC), calcite saturation state (Ω_{ca}) and aragonite (Ω_{ar}) were estimated from temperature, salinity, A_T, and pH_{NBS} measurements in CO2SYS (Pierrot et al., 2006) with dissociation constants from Mehrbach et al. (1973) refit by Dickson & Millero (1987). Summaries of seawater carbonate chemistry parameters are listed in Table 1 and Table 2 for the two experiments. Abiotic conditions were generally stable and representative of the targeted conditions.

2.3 Experimental design

Due to logistical constraints with experimental space, we conducted two separate experiments (hereafter Experiment 1 and Experiment 2) to test for transgenerational effects. In Experiment 1, parental mussels were acclimated in a fully-factorial manner to three pH treatments (8.1 [control], 7.7, and 7.3) and two DO treatments (6 mg O_2 L^{-1} [control] and 2 mg O_2 L^{-1} [hypoxia]) for four weeks and respective embryos (with a density of approximately 25 embryos ml^{-1}) from each parental treatment were reared

143 under the same conditions as their parents. The embryos were maintained in triplicates
144 in culture flasks (5L; n = 3 flasks) filled with filtered seawater under the same
145 conditions as the respective parental exposure (pH and O_2 conditions maintained as
146 previously described) and reared through to the D-stage of larval development.
147 Seawater was half-renewed every two days in each tank. Larvae were fed daily with
148 10ml of the microalgae *I. galbana* (25,000 cells ml^{-1}) 48 h post-fertilization.
149 Fertilization rate was observed at 8 h and embryo deformity rates were observed at 2 h,
150 4 h, and 8 h after fertilization. The shell length of the D-shaped larvae was observed at
151 48h, 72h, 96h, and 120h after euthanizing the larvae with paraformaldehyde solution
152 (4% PFA).

153 In Experiment 2, all parental mussels were acclimated under control conditions
154 (pH 8.1, 6mg O_2 L^{-1}) and respective offspring were reared under all experimental
155 treatment combinations as above. As with Experiment 1, embryos were maintained in
156 triplicate flasks (5L) filled with filtered seawater under the six pH × DO treatments as
157 Experiment 1. Subsequent experimental procedures were the same as Experiment 1.

158 For each treatment in each experiment, a total of 45 adult mussels were split
159 evenly among 3 tanks (n = 15 mussel per tank). The control pH level was chosen
160 based on ambient seawater pH at the collection site (pH 8.1; Li et al., 2014), while pH
161 7.7 mimicked the predicted average level by 2100 (Hoegh-Guldberg et al., 2014) as
162 well as the extreme of present natural variability at the sampling site (Li et al., 2014);
163 pH 7.3 represented the predicted extreme pH level relevant for hypoxic zones by 2100

(Cai et al., 2011). For DO levels, 6 mg O_2 L^{-1} was chosen based on normoxic conditions at the collection site, and 2 mg O_2 L^{-1} was chosen based on the typical defined threshold for seawater hypoxia (Zhang et al., 2010).

For artificial reproduction in each experiment, 45 parental mussels from each treatment combination were induced to spawn in three spawning tanks using the temperature shock method (Pronker et al., 2008). Prior to spawning, the mussels were cleaned with filtered seawater and stimulated with flowing filtered seawater for 10 min, then the mussels were transferred to a 60 L spawning tank. Massive spawning was achieved by rapidly raising the seawater temperature from 13 °C to 23 °C. Three spawning tanks per treatment and 15 mussels per spawning tank were used to spawned. Freshly filtered seawater was replaced every 30 minutes after fertilization (remove the upper sperm suspension and add the same amount of seawater).

2.4 Developmental bioassays

For embryonic development, 5 ml seawater (with a density of approximately 25 embryos ml^{-1}) was randomly sampled from each flask at 2, 4, and 8 h after fertilization. Fertilization rate and deformity rate were subsequently examined under a microscope. Fertilization was assessed by observing the release of polar bodies (Ventura et al., 2016) and embryo deformity was assessed by the observation of embryo morphology. For the latter, embryos were visually inspected and characterized as slightly deformed, irregular, lysed, broken and/or defective embryos

(Fig. 2); embryos falling into any of these categories were considered deformed. The number of fertilized eggs and deformed embryos in 100 randomly selected eggs from each flask were counted and fertilization and deformity rates were calculated as the percentage of fertilized and deformed eggs ([*n*/100] × 100). For larval development, seawater was randomly sampled as above at 48, 72, 96 and 120 h after fertilization. A random sample of 50 D-shaped larvae were isolated from each flask and the shell length of the D-shaped larvae (anterior to posterior dimension of the shell parallel to the hinge) was measured under a microscope fitted with an ocular micrometer.

2.5 Statistical analysis

Data analyses were performed using SPSS 24 software and the values of all parameters were expressed as the means ± S.D. Prior to analysis, data were tested for normality using the Shapiro-Wilk's test and homogeneity of variance using the Levene's test. Percentage data were arcsin-square root transformed prior to analyses. The independent and interactive effects of three fixed factors (DO, pH, and parental exposure) were analyzed by three-way analysis of variance (ANOVA). If an interaction existed, the significant effects were analyzed by a one-way ANOVA at each fixed DO value and parental exposure condition, followed by a Tukey's HSD test ($\alpha = 0.05$). Significant effects of DO and parental exposure were analyzed at fixed other two parameters respectively using Student's t-test ($\alpha = 0.05$).

3. Results

3.1 Fertilization and deformity rate

Fertilization rates ranged from 63% to 100%, and were significantly reduced by low pH in a stepwise fashion; low DO had no effect (Table 3). Significant interactions occurred between pH and parental exposure on the fertilization rates (Table 3; Fig. 3). Parental exposure significantly affected the fertilization rates under low pH conditions (7.7 and 7.3), with fertilization rates under low pH conditions being partially enhanced when parents were reared under low pH (Fig. 3). Regardless of parental exposure, low pH negatively affected fertilization rates compared to control conditions (Fig. 3).

Deformity rates at 2h, 4h, and 8h were significantly affected by low pH in a stepwise fashion, with severe deformity rates at pH 7.3 (Table 3, Fig. 4). Low DO significantly increased deformity rates at 2h, 4h, and 8h under control pH (pH 8.1) in both Experiment 1 and Experiment 2. Significant interactions occurred between pH and parental exposure, and pH and DO, at different times (Table 3; Fig. 4). More specifically, parental exposure significantly decreased the embryo deformity rates under pH 7.7 at DO 6mg O_2 L^{-1} at all three time points, and under all pH levels at DO 2mg O_2 L^{-1} for all three time points, with the exception of pH 8.1 × DO 2mg O_2 L^{-1} at 2h.

3.2 Shell length of D-shaped larvae

227 Shell length of D-shaped larvae ranged from 60 μm to 125 μm during the
228 observation period. A significant decrease in larval shell growth occurred at 48 h
229 under pH 7.3 in Experiment 1. In Experiment 2, low pH significantly decreased larval
230 shell growth in a stepwise fashion under 6 mg O_2 L^{-1}; at 2 mg O_2 L^{-1} larvae reared
231 under pH 7.3 had a significantly smaller shell length than control larvae (Table 3; Fig.
232 5). Larval shell growth at 48h were not significantly affected by low DO. Moreover,
233 parental exposure did not show a significant difference in the D-shaped larval shell
234 growth except in the condition of pH 8.1 × DO 2mg O_2 L^{-1} at 72h (Table 3; Fig.5). At
235 72, 96 and 120 h, larval shell length was significantly smaller under low pH; low DO
236 larvae showed significantly smaller shell lengths under control pH (pH 8.1).
237 Significant interactions did not occur on the D-shaped larval shell length (Table 3).
238

239 **4. Discussion**

240 In this study, we tested for transgenerational effects of exposure to combined
241 ocean acidification and hypoxia on the early development of mussels *M. edulis*. We
242 found that parental exposure to acidification and hypoxia could only partially alleviate
243 the negative effects of these stressors on embryonic and larval developmental traits, as
244 negative effects on developmental traits were still observed when parents were reared
245 under low pH and low O_2. As such, our results suggest that parental exposure may not
246 confer offspring tolerance to short-term ocean acidification and hypoxia in mussels *M.*
247 *edulis*.

248

249 *4.1 Effects of ocean acidification and hypoxia on larval development*

250 Considering the increased occurrences of hypoxia (Vaquer-Sunyer & Duarte,
251 2008) and the continuous decrease of pH levels (Hoegh-Guldberg et al., 2014)
252 globally, it is critical to evaluate the combined impacts on marine species and
253 ecosystems. However, the combined effect of low pH and oxygen on marine species
254 has not been widely studied (Gobler & Baumann, 2016). Our results indicated that
255 low pH conditions had negative effects on fertilization rates, larval deformity rates,
256 and larval shell growth, Furthermore, while positive transgenerational effects were
257 observed, they only partially alleviated the effects of acidification on the
258 aforementioned early developmental traits.

259 While we did not measure survival, our results showed that short-term exposure
260 to experimental ocean acidification negatively affected fertilization rate, embryo
261 deformity rate, and larval shell growth, while hypoxia had relatively little effect and
262 did not influence the effect of acidification. The reduced fertilization rates under
263 acidification may be due to the negative effect of acidification on sperm fitness such
264 as the percentage of motile sperm and the sperm swimming speed (Vihtakari et al.,
265 2013) and/or the process of sperm-egg collisions and gamete fusion (Shi et al., 2017).
266 Negative effects on larval shell growth may be due to the decreasing calcification
267 (Berge et al., 2006) and shell dissolution (Ramesh et al., 2017), or perhaps increases
268 in larval deformities (Talmage & Gobler, 2009). Regardless of mechanism, such

269 effects in nature could potentially increase juvenile mortality, particularly when food
270 shortages occur during the accumulation of energy reserves (Phillips, 2002).
271 Our findings indicated relatively little effect of hypoxia on early development.
272 While some comparatively small effects of hypoxia were observed at control pH
273 conditions, DO did not affect fitness under any of the low pH conditions, suggesting
274 that pH has a stronger influence on early development in mussels *M. edulis*. Similar
275 results have been observed for *M. edulis* from other locations (e.g. Frieder et al., 2014)
276 as well as other mussel species such as *Mytilus californianus* (Frieder, 2013), even at
277 extremely low DO concentrations (0.5 mg O_2 L^{-1}; Eerkes-Medrano et al., 2013). With
278 respect to calcification, mineralogical plasticity (e.g. increased calcite to aragonite
279 ratio and magnesium to calcium ratio) is thought to be one way in which calcifying
280 marine organisms can withstand low DO effects on calcification (e.g. polychaete
281 *Hydroides diramphus;* Leung & Cheung, 2018). Metabolic alterations have also been
282 reported to support organismal tolerance to hypoxia. For example, Pörtner et al. (2005)
283 reported that marine animals switch to an anaerobic metabolism and undergo
284 metabolic depression which contributes to energy savings during low DO. The
285 utilization of metabolic pathways that are less energetically demanding may also
286 support calcification and survival under hypoxic conditions (Risgaard-Petersen et al.,
287 2006; Nardelli et al., 2014). While we did not test for physiological underpinnings of
288 observed responses in this study, such mechanisms may explain the lack of DO effect
289 on deformation rates and shell growth observed herein. Alterations in metabolic

activity that result in increased energy availability under hypoxia could have also been responsible for the lack of low DO effect on fertilization rates as well. Collectively, these findings suggest that low DO has relatively little effect on the early development of mussels.

4.2 Transgenerational effects of combined ocean acidification and hypoxia

The role of parental exposure in shaping offspring responses to global change stressors has been observed in numerous marine species including fishes, copepods, and bivalves (Vehmaa et al., 2012; Parker et al., 2012; Munday, 2014). Despite numerous transgenerational studies for acidification and warming, this is, to our knowledge, the first study to test for transgenerational acclimation in response to combined acidification and hypoxia. While parental exposure to low pH and DO partially reduced negative effects on offspring compared to when parents were exposed to control conditions, the positive parental effects were weak at best. Our results thus suggest a limited capacity for parental exposure to alleviate the negative effects of low pH on early development in mussels. This is in contrast to studies documenting largely positive effects of parental exposure on offspring responses, particularly to low pH conditions, in bivalves (Parker et al., 2012; Fitzer et al., 2014; Zhao et al., 2016) and others reporting negative effects of parental exposure (Griffith & Gobler, 2017). Thus, there remains a high degree of uncertainty regarding the

ability of parental exposure to alleviate the effects of marine global change stressors on their offspring and more research is warranted.

While limited, the increased resistance to ocean acidification of *M. edulis* larvae from parents exposed to low pH and DO conditions may be the result of a higher concentration or activity of the enzyme carbonic anhydrase (CA) catalyzing the reversible hydration of CO_2 and accelerating the formation of bicarbonate (HCO_3^-) (Lionetto et al., 2012). Some studies have also found a correlation between CA activity and shell formation (Fitzer et al., 2014b; Medaković & Lucu., 1994), and enzyme activity increases linearly with shell formation (Medaković, 2000). Nonetheless, the mechanisms at play only conferred a small benefit of parental exposure. It is important to note here, however, that although our parental exposure time (4 weeks) was similar to other transgenerational studies on bivalves (e.g. Griffith & Gobler, 2017), a longer exposure may have yielded different results. For example, our exposure time may not have been enough for parental mussels to produce adequate proteins, hormones, or other somatic traits that would provide offspring with the ability to strongly resist more acidified, hypoxic conditions (Munday, 2014). Thus, while our results provide the first documentation of transgenerational effects to combined acidification and hypoxia, future studies with longer parental exposure times are warranted.

5. Conclusions

This study represents the first of its kind to assess the potential for transgenerational acclimation to combined acidification and hypoxia in marine bivalves. Our results suggest that ocean acidification has a comparatively stronger effect on the early development of mussels *M. edulis*. Although we did not directly measure survival, the observed effects of acidification represent a strong decline in function, as reduced fertilization rates, increased deformity rates, and decreased growth all represent negative functional consequences for larval bivalves. Furthermore, while transgenerational effects were positive, they were not sufficient to completely alleviate the negative effects of ocean acidification. Thus, if our experimental results hold true in nature, it appears the ocean acidification may have negative effects on *M. edulis* populations since the success of the early developmental stage of shellfish can affect population and community dynamics. Nonetheless, more research on the combined effects on ocean acidification and hypoxia are required before general conclusions can be drawn with respect to marine bivalves, and longer-term parental exposures are required before predicting whether or not the effects observed herein apply in nature.

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Table 1 A summary of seawater carbonate chemistry parameters in experiment 1. Seawater pH (pH_{NBS}), temperature of embryo and larvae period (T,℃), salinity (psu), total alkalinity (A_T, μmol kg^{-1}), dissolved inorganic carbon (DIC), the partial pressure of CO_2 (pCO_2, μatm) as well as aragonite (Ωar) and calcite (Ωca) saturation states were listed.

Treatments		salinity	T	pH_{NBS}	A_T	DIC	pCO_2	Ωca	Ωar
pH	DO (mg O_2 L^{-1})	(pus)	(℃)		(μmol*kg^{-1})	(μmol*kg^{-1})	(μatm)		
8.1	6.0 ± 0.2	28.0 ± 0.3	16.1 ± 0.3	8.11 ± 0.02	2236 ± 20	2027 ± 13	348 ± 13	4.31 ± 0.12	2.63 ± 0.11
7.7	6.1 ± 0.1	28.1 ± 0.2	16.2 ± 0.1	7.70 ± 0.02	2189 ± 29	2130 ± 23	1118 ± 22	2.03 ± 0.09	1.31 ± 0.05
7.3	6.0 ± 0.2	27.9 ± 0.2	16.0 ± 0.3	7.31 ± 0.03	2218 ± 12	2273 ± 20	2328 ± 34	0.81 ± 0.07	0.59 ± 0.03
8.1	2.1 ± 0.1	28.1 ± 0.2	15.9 ± 0.2	8.10 ± 0.03	2301 ± 21	2089 ± 12	356 ± 12	4.28 ± 0.08	2.59 ± 0.16
7.7	2.1 ± 0.1	28.1 ± 0.2	16.0 ± 0.2	7.73 ± 0.01	2257 ± 27	2159 ± 19	1089 ± 29	1.96 ± 0.18	1.29 ± 0.05
7.3	2.0 ± 0.2	28.0 ± 0.3	16.2 ± 0.3	7.29 ± 0.03	2261 ± 13	2318 ± 11	2401 ± 31	0.86 ± 0.05	0.63 ± 0.04

Table 2 A summary of seawater carbonate chemistry parameters in experiment 2. Seawater pH (pH_{NBS}), temperature of embryo and larvae period (T,℃), salinity (psu), total alkalinity (A_T, μmol kg^{-1}), dissolved inorganic carbon (DIC), the partial pressure of CO_2 (pCO_2, μatm) as well as aragonite (Ωar) and calcite (Ωca) saturation states were listed.

Treatments		salinity	T	pH_{NBS}	A_T	DIC	pCO_2	Ωca	Ωar
pH	DO (mg O_2 L^{-1})	(pus)	(℃)		(μmol*kg^{-1})	(μmol*kg^{-1})	(μatm)		
8.1	6.1 ± 0.1	28.1 ± 0.1	15.8 ± 0.3	8.09 ± 0.02	2228 ± 27	2021 ± 27	352 ± 11	4.29 ± 0.07	2.57 ± 0.10
7.7	6.0 ± 0.1	28.1 ± 0.3	16.1 ± 0.2	7.71 ± 0.03	2169 ± 17	2165 ± 21	1107 ± 17	2.09 ±	1.29 ±

								0.08	0.05
7.3	6.1 ± 0.2	28.0 ± 0.3	16.1 ± 0.2	7.32 ± 0.03	2231 ± 19	2284 ± 13	2427 ± 23	0.85 ± 0.03	0.62 ± 0.01
8.1	2.1 ± 0.1	27.9 ± 0.2	15.9 ± 0.3	8.10 ± 0.03	2311 ± 19	2098 ± 18	343 ± 10	4.28 ± 0.05	2.66 ± 0.13
7.7	2.2 ± 0.1	28.0 ± 0.2	16.0 ± 0.3	7.70 ± 0.01	2217 ± 13	2248 ± 15	1098 ± 16	2.06 ± 0.18	1.22 ± 0.07
7.3	2.0 ± 0.2	28.1 ± 0.3	16.0 ± 0.1	7.32 ± 0.03	2211 ± 17	2339 ± 20	2418 ± 33	0.86 ± 0.03	0.61 ± 0.04

Table 3 Summary of three-way ANOVA results on effects of pH, DO and parental exposure (PE) on the fertilization rate (FR), the deformity rate at 2h (DR2), 4h (DR4), 8h (DR8) and the shell length of D-shaped larvae at 48h (SL48), 72h (SL72), 96h (SL96) 120h (SL120) in experiment #1 and experiment #2. Significantly different values are represented in bold.

	FR				DR2				DR4			
	df	MS	F	P	df	MS	F	P	df	MS	F	P
PE	1	406.694	34.531	**<0.001**	1	448.028	16.836	**<0.001**	1	529.000	31.271	**<0.001**
pH	2	1656.694	140.663	**<0.001**	2	11858.778	445.633	**<0.001**	2	13307.194	786.632	**<0.001**
DO	1	30.25	2.568	0.122	1	272.250	10.231	**0.004**	1	484.000	28.611	**<0.001**
PE*pH	2	61.361	5.21	**0.013**	2	112.111	4.213	**0.027**	2	54.250	3.207	0.058
PE*DO	1	1.361	0.116	0.737	1	0.694	0.026	0.873	1	4.000	0.236	0.631
pH*DO	2	6.25	0.531	0.595	2	206.333	7.754	**0.003**	2	99.750	5.897	**0.008**
PE*pH*DO	2	0.694	0.059	0.943	2	0.444	0.017	0.983	2	33.583	1.985	0.159

	DR8				SL48				SL72			
	df	MS	F	P	df	MS	F	P	df	MS	F	P
PE	1	633.361	36.423	**<0.001**	1	352.667	9.197	**0.003**	1	273.375	8.480	**0.005**
pH	2	12572.583	723.024	**<0.001**	2	1371.885	35.777	**<0.001**	2	3146.281	97.595	**<0.001**

DO	1	521.361	29.982	**<0.001**	1	165.375	4.313	**0.041**	1	864.000	26.801	**<0.001**
PE*pH	2	67.861	3.903	**0.034**	2	44.135	1.151	0.321	2	2.844	0.088	0.916
PE*DO	1	14.694	0.845	0.367	1	.667	0.017	0.895	1	2.042	0.063	0.802
pH*DO	2	89.194	5.129	**0.014**	2	49.594	1.293	0.280	2	70.969	2.201	0.117
PE*pH*DO	2	17.361	0.998	0.383	2	3.510	0.092	0.913	2	24.448	0.758	0.472

	SL96				SL120			
	df	MS	F	P	df	MS	F	P
PE	1	137.760	2.893	0.093	1	433.500	12.113	**0.001**
pH	2	3806.542	79.950	**<0.001**	2	4585.073	128.115	**<0.001**
DO	1	1239.844	26.041	**<0.001**	1	1162.042	32.470	**<0.001**
PE*pH	2	19.542	0.410	0.665	2	13.031	0.364	0.696
PE*DO	1	1.760	0.037	0.848	1	16.667	0.466	0.497
pH*DO	2	76.625	1.609	0.206	2	103.323	2.887	0.061
PE*pH*DO	2	47.792	1.004	0.371	2	35.323	0.987	0.377

Figure legends

Fig. 1 Embyros observed at 2 to 8h after fertilization in all treatments. A: pH*DO condition of 8.1*6mg O_2 L^{-1} in experiment 1; B: 7.7*6mg O_2 L^{-1} in experiment 1; C: 7.3*6mg O_2 L^{-1} in experiment 1; D: 8.1*2mg O_2 L^{-1} in experiment 1; E: 7.7*2mg O_2 L^{-1} in experiment 1; F: 7.3*2mg O_2 L^{-1} in experiment 1; a: 8.1*6mg O_2 L^{-1} in experiment 2; b: 7.7*6mg O_2 L^{-1} in experiment 2; c: 7.3*6mg O_2 L^{-1} in experiment 2; d: 8.1*2mg O_2 L^{-1} in experiment 2; e: 7.7*2mg O_2 L^{-1} in experiment 2; f: 7.3*2mg O_2 L^{-1} in experiment 2;

Fig. 2 The categorization of deformity of embryos. A: Initial embryo deformity; B: Irregular deformation of the embryo; C: Slightly deformed of the embryo; D: Embryo rupture; E: Embryo breakage and incomplete; F: Deformity during embryonic division.

Fig. 3 The fertilization rate (FR) at 8h of *M. edulis* exposed to different combinations of pH (8.1, 7.7 and 7.3) and DO (6mg O_2 L^{-1} and 2mg O_2 L^{-1}) (N=100). The means denoted by different superscripts (A, B, C) at each fixed DO are significantly different among three pH levels ($P < 0.05$). The means denoted by red superscripts (+, -) at each fixed DO and pH are significantly affected by parental exposure ($P < 0.05$).

Fig. 4 The embryos deformity rate (DR) at 2h, 4h, and 8h of the *M. edulis* exposed to different combinations of pH (8.1, 7.7 and 7.3) and DO (6mg O_2 L^{-1} and 2mg O_2 L^{-1}) (N=100). The means denoted by different superscripts (A, B, C) at each fixed DO are significantly different among three pH levels ($P < 0.05$). The means sharing the different superscripts (a, b) between two DO levels at each fixed pH are significantly different ($P < 0.05$). The means denoted by asterisk (*) at each fixed DO and pH are significantly affected by parental exposure ($P < 0.05$).

Fig. 5 A, B, C, D respectively means the D-shaped larval shell length of the *M. edulis* at 48h, 72h, 96h, and 120h exposed to different combinations of pH (8.1, 7.7 and 7.3) and DO (6mg O_2 L^{-1} and 2mg O_2 L^{-1}) (N=50). The means denoted by different superscripts (A, B, C) at each fixed DO are significantly different among three pH levels ($P < 0.05$). The means sharing the different superscripts (a, b) between two DO levels at each fixed pH are significantly different ($P < 0.05$). The means denoted by red superscripts (+, -) at each fixed DO and pH are significantly affected by parental exposure ($P < 0.05$).

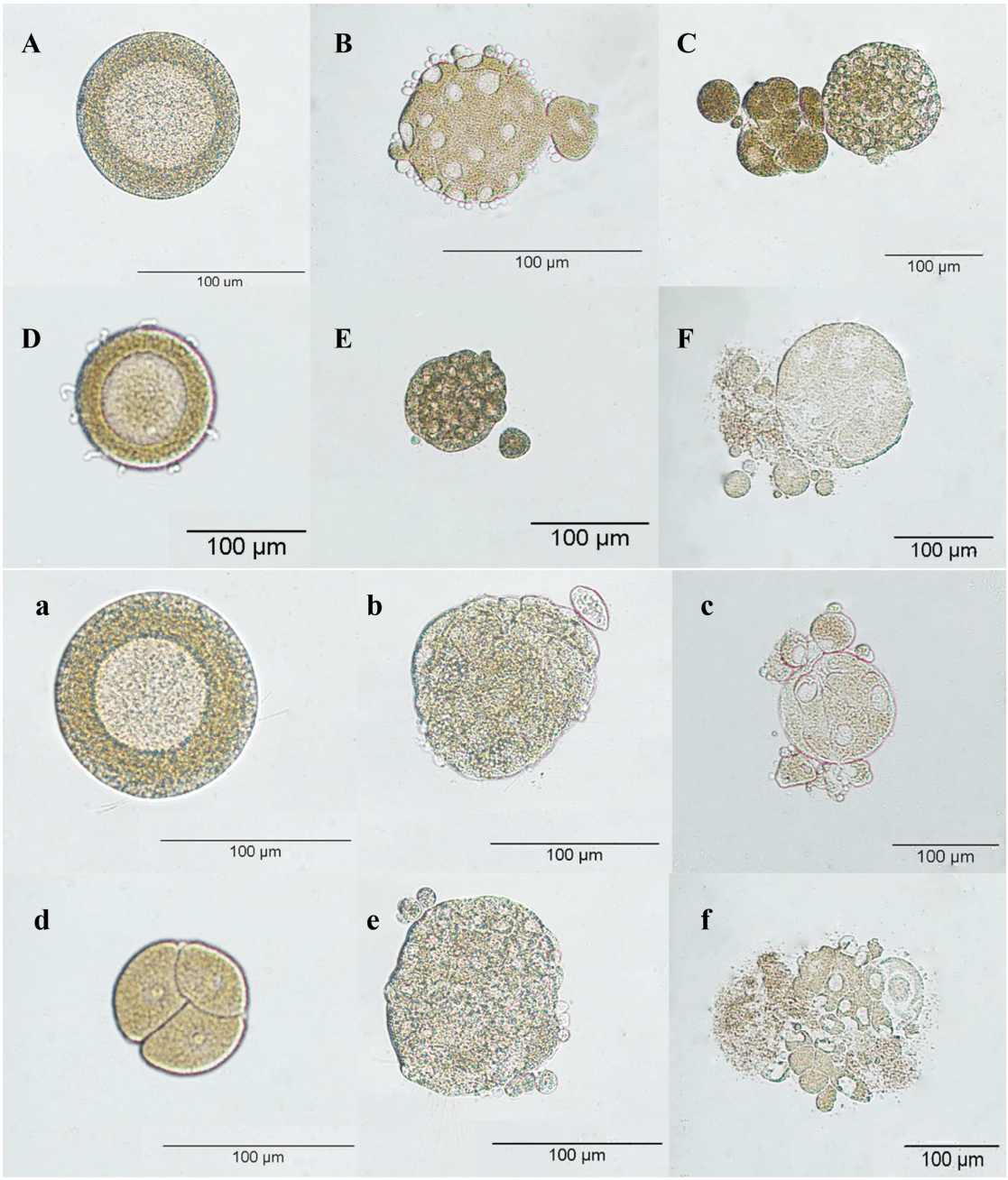
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Fig. 1

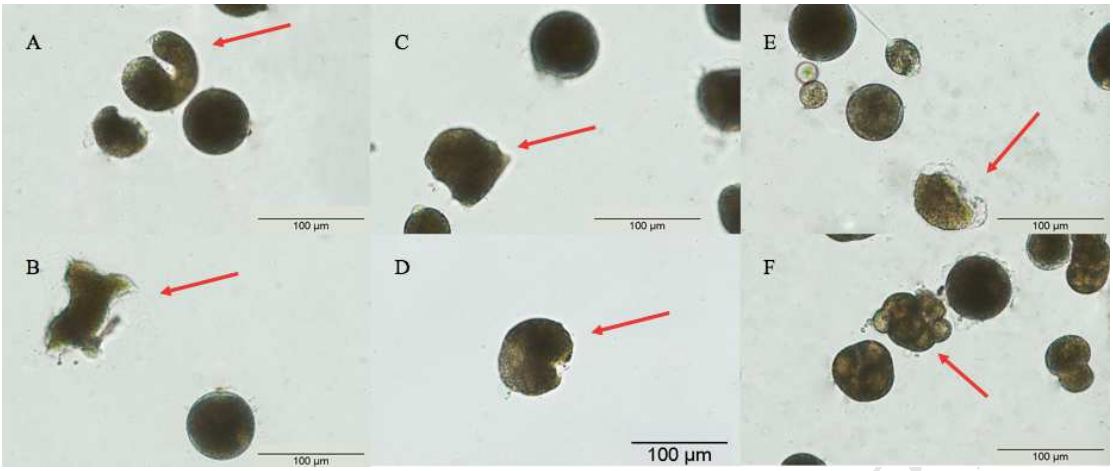


Fig. 2

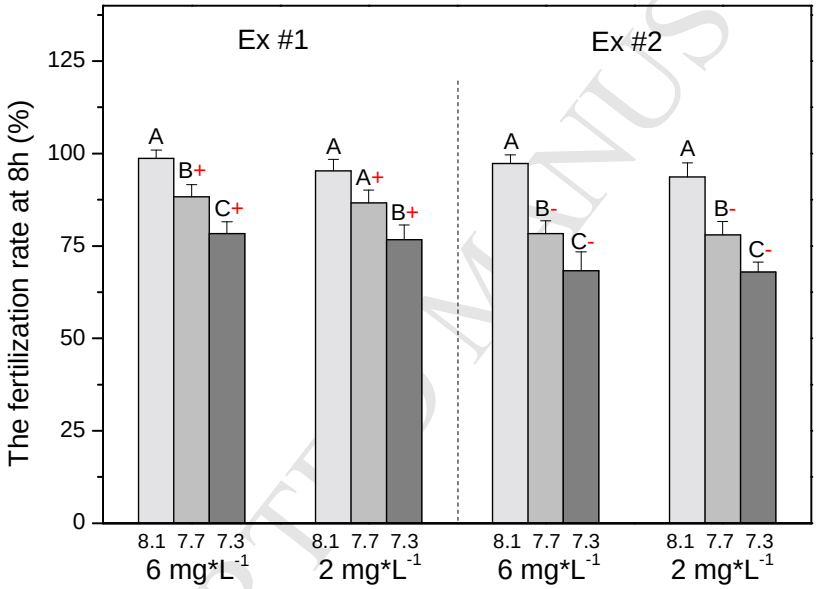


Fig. 3

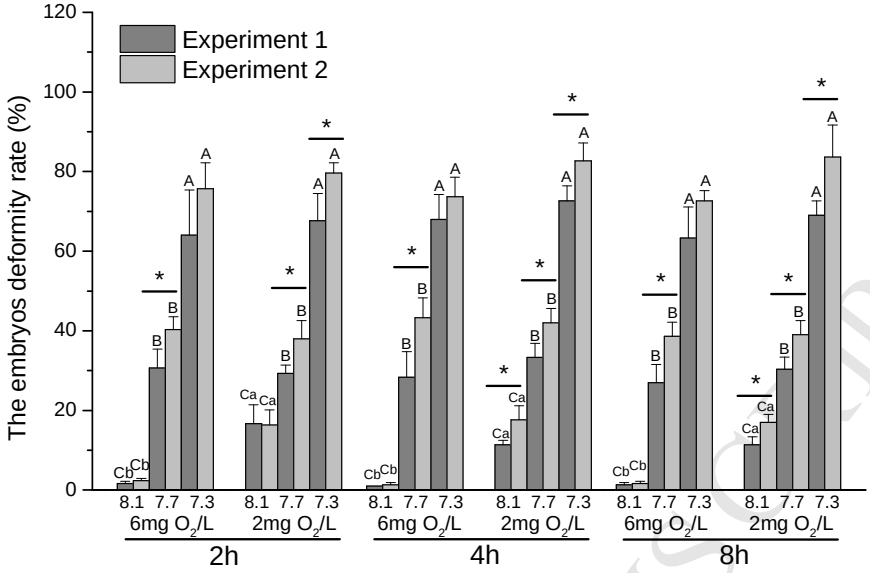
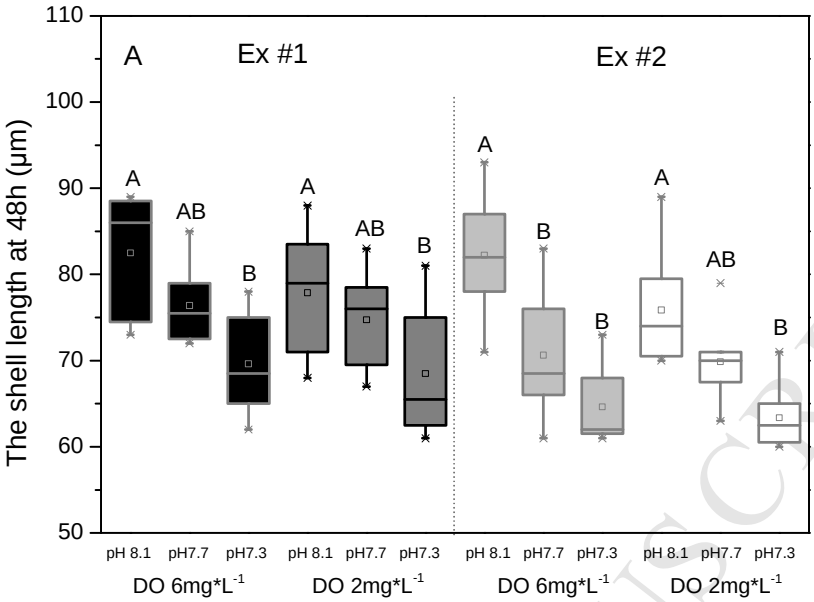
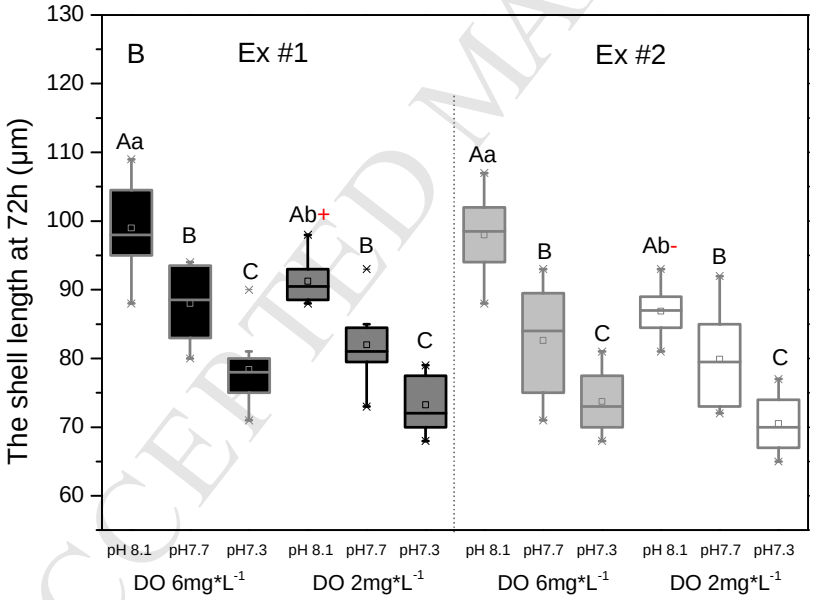
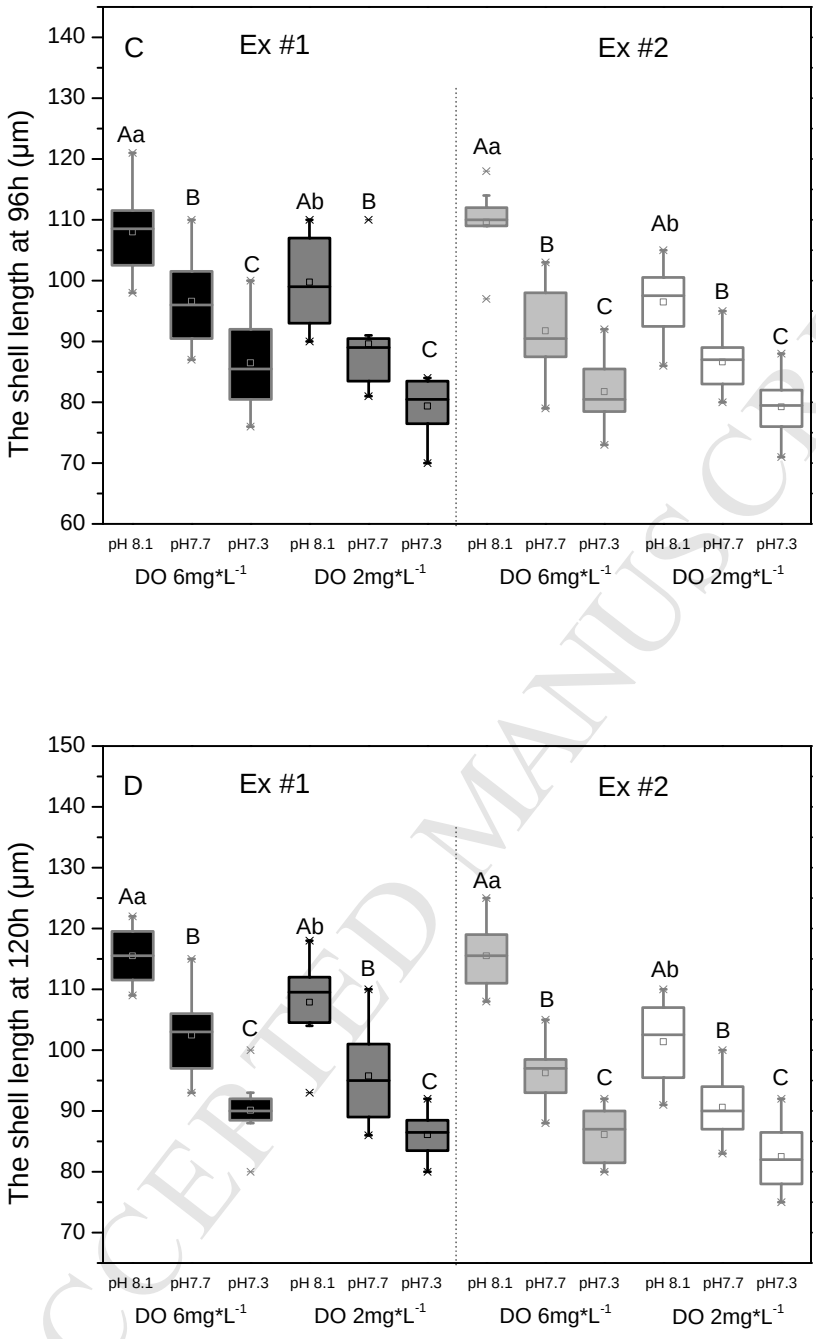


Fig. 4



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The shell length at 96h (μm)
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pH7.7
pH7.3
DO 6mg*L-1
DO 2mg*L-1
D
Ex #1
Ex #2
The shell length at 120h (μm)
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130
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Fig. 5

Transgenerational effects of short-term exposure to acidification and hypoxia on early developmental traits of the mussel *Mytilus edulis*

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Abstract

Transgenerational effects of multiple stressors on marine organisms are emerging environmental themes. We thus experimentally tested for transgenerational effects of seawater acidification and hypoxia on the early development traits of the mussel *Mytilus edulis*. Fertilization rate, embryo deformity rate, and larval shell length were negatively impacted by acidification, while hypoxia had little effect except for increasing deformity rates under control pH conditions. Offspring from low pH/O_2 parents were less negatively affected by low pH/O_2 conditions than offspring from control parents; however, low pH/O_2 conditions still negatively affected developmental traits in offspring from acclimated parents compared to control seawater conditions. Our results demonstrate that experimental seawater acidification and hypoxia can adversely affect early developmental traits of *M. edulis* and that parental exposure can only partially alleviate these impacts. If experimental observations hold true in nature, it is unlikely that parental exposure will confer larval tolerance to ocean acidification for *M. edulis*.

Keywords: Carbon dioxide; Environmental stress; Hypoxia; Global change biology; Multiple stressors; Transgenerational plasticity

1. Introduction

Marine global change is anticipated to impact ocean life in the near-future. Two co-occurring stressors that have received relatively little combinatory attention are ocean acidification and deoxygenation (see Gobler & Baumann, 2016 for review).

Generally speaking, ocean acidification describes a decrease in oceanic pH, while deoxygenation refers to a global decrease in oceanic oxygen. In the open ocean, acidification is predominantly driven by the uptake of excess anthropogenic CO_2 from the atmosphere (Hoegh-Guldberg et al., 2014), while deoxygenation is primarily driven by global warming (Breitburg et al., 2018). By 2100, it is projected that open-ocean pH will decrease by 0.3–0.4 units (Feely et al., 2004; Orr et al., 2005), and oxygen will reduce 1–7% (Keeling et al., 2010; Schmidtko & Visbeck, 2017).

In contrast to the open ocean, coastal acidification can be affected by myriad processes such as coastal upwelling, ecosystem metabolism and watershed dynamics, and freshwater runoff (Duarte et al., 2013). Similarly, coastal deoxygenation is primarily caused by increased nutrient and organic loads that increase oxygen consumption through microbial decomposition (typically defined as dissolved O_2 below 2 mg O_2 L^{-1}; Vaquer-Sunyer & Duarte, 2008; Breitburg et al., 2018). Acidification and hypoxia are known to co-occur, and recent studies highlight tight linkages between acidification and hypoxia in coastal ecosystems, with acidification being more severe under hypoxic conditions (compared to normoxia; Feely et al., 2010; Cai et al., 2011; Paulmier et al., 2011; Melzner et al., 2013). Consequently, coastal organisms can already experience low pH and oxygen conditions that exceed near-future open ocean projections (Wallace et al., 2014; Baumann et al., 2015; Gobler & Baumann, 2016). Nonetheless, global climate change can exacerbate pH and oxygen declines in coastal regions, and coastal organisms are not, by default,

immune to such change (Waldbusser & Salisbury, 2014; Breitburg et al. 2018). It is thus necessary to understand the combined effects of short-term acidification and hypoxia on marine life.

Globally, marine bivalves are of ecological (Costanza et al., 1997; Dame 2011) and economic (Cooley & Doney, 2009; FAO, 2018) importance. It is well documented, however, that marine bivalves are sensitive to multiple global change stressors. With respect to ocean acidification and hypoxia (see Gobler & Baumann, 2016 for review), a limited number of studies suggest largely negative combined effects (Gobler et al., 2014; Clark & Gobler, 2016; Stevens & Gobler, 2018), but positive and null effects have also been reported (Jakubowska & Normant, 2014; Jansson et al., 2015). Given the contrasting effects across relatively few studies, more research testing the combined effects of acidification and hypoxia on marine bivalves is warranted.

The role of transgenerational effects (i.e., the effect caused by the parental environment on the offspring; Munday, 2014; Ross et al., 2016) in shaping offspring responses to environmental stress has recently drawn substantial attention. These transgenerational effects can be acclamatory (non-genetic; referred to as transgenerational acclimation or transgenerational plasticity) or adaptive (genetic; referred to as transgenerational adaptation), and can allow some organisms to adjust to projected environmental change (Munday, 2014). Recent studies have indicated that the potential for transgenerational acclimation to global change stressors is not

universal and varies across species (Munday, 2014; Munday et al., 2014; Sunday et al., 2014; Ross et al., 2016). With respect to marine bivalves, a limited number of transgenerational studies in the context of ocean acidification exist and report variable effects. For instance, larval clams (*Ruditapes philippinarum*) showed better growth performance under low pH when parents experienced similar low pH conditions (Zhao et al., 2018). Positive transgenerational effects under experimental ocean acidification have also been reported for larval oysters (*Saccostrea glomerata*; Parker et al., 2012) and juvenile mussels (*M. edulis*; Fitzer et al., 2014a). In contrast, Griffith & Gobler (2017) reported negative transgenerational effects associated with transgenerational exposure to ocean acidification in larval scallops (*Argopecten irradians*) and clams (*Mercenaria mercenaria*).

While transgenerational studies on ocean acidification exist for marine bivalves, to our knowledge there have been no studies testing for transgenerational acclimation to combined acidification and hypoxia. Consequently, the predictions for how these animals will respond to ocean and coastal acidification and hypoxia are, at present, unattainable. To explore this knowledge gap, we tested for transgenerational effects on early larval developmental traits of mussels (*M. edulis*) exposed to experimental acidification and hypoxia.

2. Materials and Methods

2.1 Animal collection and husbandry

Wild adult mussels (*M. edulis*; 75 ± 5 mm shell length) were collected from Gouqi Island, East China Sea (30°43′1.64″N, 122°46′3.25″E) in October 2017. Mussels were immediately transported to experimental facilities at Shanghai Ocean University (Shanghai, China), gently scrubbed clean of epibionts, and transferred to 30 L acclimation tanks (recirculating aquarium system with filtered seawater; density = 15 mussels $tank^{-1}$; flow rate ~10 L min^{-1}). The mussels were acclimated to laboratory conditions for two weeks at 13 ± 0.5 °C, salinity 28 ± 0.5 psu, dissolved oxygen (DO) concentration of 6.0 ± 0.3 mg O_2 L^{-1} and pH 8.1 ± 0.1 (simulated natural environment of mussels at collection site). During acclimation, the mussels were fed twice daily with 10 ml of the microalgae *Isochrysis galbana* (25,000 cells ml^{-1}). Animal condition did not change during the acclimation phase and adult mortality was minimal; only visually healthy mussels were selected for the experiment.

2.2 Seawater chemistry

Low pH was achieved by using a *p*CO_2/pH system (DAQ-M) equipped with WTW pH 3310m and SenTix 41 pH electrode (Loligo Systems Inc., Denmark). The pH level was maintained by bubbling pure CO_2 which was real-time connected with feedback STAT systems (DAQ-M). Dissolved oxygen was manipulated by bubbling a mixture of N_2 and air directly into the water via an O_2 regulator (Loligo Systems Inc., Denmark). The gas flow was maintained by a solenoid valve controlled by a computer connected to an O_2 regulator to achieve stable DO levels in each tank.

Abiotic seawater parameters including temperature, pH, DO and salinity were monitored twice a day for each tank and total alkalinity (A_T) was measured every two days. Temperature, salinity and DO were observed by a multi-parameter water quality instrument (5200A, YSI Inc., America). Total alkalinity (A_T) was determined by manual 2-point acid-base titration using a manual burette and applicable reagents (Phenolphthalein indicator, Methyl red indicator, and 0.025mol L^{-1} Hydrochloric Acid Standard Solution). Additional carbonate system parameters including pCO_2, dissolved inorganic carbon (DIC), calcite saturation state (Ω_{ca}) and aragonite (Ω_{ar}) were estimated from temperature, salinity, A_T, and pH_{NBS} measurements in CO2SYS (Pierrot et al., 2006) with dissociation constants from Mehrbach et al. (1973) refit by Dickson & Millero (1987). Summaries of seawater carbonate chemistry parameters are listed in Table 1 and Table 2 for the two experiments. Abiotic conditions were generally stable and representative of the targeted conditions.

2.3 Experimental design

Due to logistical constraints with experimental space, we conducted two separate experiments (hereafter Experiment 1 and Experiment 2) to test for transgenerational effects. In Experiment 1, parental mussels were acclimated in a fully-factorial manner to three pH treatments (8.1 [control], 7.7, and 7.3) and two DO treatments (6 mg O_2 L^{-1} [control] and 2 mg O_2 L^{-1} [hypoxia]) for four weeks and respective embryos (with a density of approximately 25 embryos ml^{-1}) from each parental treatment were reared

143 under the same conditions as their parents. The embryos were maintained in triplicates
144 in culture flasks (5L; n = 3 flasks) filled with filtered seawater under the same
145 conditions as the respective parental exposure (pH and O_2 conditions maintained as
146 previously described) and reared through to the D-stage of larval development.
147 Seawater was half-renewed every two days in each tank. Larvae were fed daily with
148 10ml of the microalgae *I. galbana* (25,000 cells ml^{-1}) 48 h post-fertilization.
149 Fertilization rate was observed at 8 h and embryo deformity rates were observed at 2 h,
150 4 h, and 8 h after fertilization. The shell length of the D-shaped larvae was observed at
151 48h, 72h, 96h, and 120h after euthanizing the larvae with paraformaldehyde solution
152 (4% PFA).

153 In Experiment 2, all parental mussels were acclimated under control conditions
154 (pH 8.1, 6mg O_2 L^{-1}) and respective offspring were reared under all experimental
155 treatment combinations as above. As with Experiment 1, embryos were maintained in
156 triplicate flasks (5L) filled with filtered seawater under the six pH × DO treatments as
157 Experiment 1. Subsequent experimental procedures were the same as Experiment 1.

158 For each treatment in each experiment, a total of 45 adult mussels were split
159 evenly among 3 tanks (n = 15 mussel per tank). The control pH level was chosen
160 based on ambient seawater pH at the collection site (pH 8.1; Li et al., 2014), while pH
161 7.7 mimicked the predicted average level by 2100 (Hoegh-Guldberg et al., 2014) as
162 well as the extreme of present natural variability at the sampling site (Li et al., 2014);
163 pH 7.3 represented the predicted extreme pH level relevant for hypoxic zones by 2100

(Cai et al., 2011). For DO levels, 6 mg O_2 L^{-1} was chosen based on normoxic conditions at the collection site, and 2 mg O_2 L^{-1} was chosen based on the typical defined threshold for seawater hypoxia (Zhang et al., 2010).

For artificial reproduction in each experiment, 45 parental mussels from each treatment combination were induced to spawn in three spawning tanks using the temperature shock method (Pronker et al., 2008). Prior to spawning, the mussels were cleaned with filtered seawater and stimulated with flowing filtered seawater for 10 min, then the mussels were transferred to a 60 L spawning tank. Massive spawning was achieved by rapidly raising the seawater temperature from 13 °C to 23 °C. Three spawning tanks per treatment and 15 mussels per spawning tank were used to spawned. Freshly filtered seawater was replaced every 30 minutes after fertilization (remove the upper sperm suspension and add the same amount of seawater).

2.4 Developmental bioassays

For embryonic development, 5 ml seawater (with a density of approximately 25 embryos ml^{-1}) was randomly sampled from each flask at 2, 4, and 8 h after fertilization. Fertilization rate and deformity rate were subsequently examined under a microscope. Fertilization was assessed by observing the release of polar bodies (Ventura et al., 2016) and embryo deformity was assessed by the observation of embryo morphology. For the latter, embryos were visually inspected and characterized as slightly deformed, irregular, lysed, broken and/or defective embryos

(Fig. 2); embryos falling into any of these categories were considered deformed. The number of fertilized eggs and deformed embryos in 100 randomly selected eggs from each flask were counted and fertilization and deformity rates were calculated as the percentage of fertilized and deformed eggs ([*n*/100] × 100). For larval development, seawater was randomly sampled as above at 48, 72, 96 and 120 h after fertilization. A random sample of 50 D-shaped larvae were isolated from each flask and the shell length of the D-shaped larvae (anterior to posterior dimension of the shell parallel to the hinge) was measured under a microscope fitted with an ocular micrometer.

2.5 Statistical analysis

Data analyses were performed using SPSS 24 software and the values of all parameters were expressed as the means ± S.D. Prior to analysis, data were tested for normality using the Shapiro-Wilk's test and homogeneity of variance using the Levene's test. Percentage data were arcsin-square root transformed prior to analyses. The independent and interactive effects of three fixed factors (DO, pH, and parental exposure) were analyzed by three-way analysis of variance (ANOVA). If an interaction existed, the significant effects were analyzed by a one-way ANOVA at each fixed DO value and parental exposure condition, followed by a Tukey's HSD test ($\alpha = 0.05$). Significant effects of DO and parental exposure were analyzed at fixed other two parameters respectively using Student's t-test ($\alpha = 0.05$).

3. Results

3.1 Fertilization and deformity rate

Fertilization rates ranged from 63% to 100%, and were significantly reduced by low pH in a stepwise fashion; low DO had no effect (Table 3). Significant interactions occurred between pH and parental exposure on the fertilization rates (Table 3; Fig. 3). Parental exposure significantly affected the fertilization rates under low pH conditions (7.7 and 7.3), with fertilization rates under low pH conditions being partially enhanced when parents were reared under low pH (Fig. 3). Regardless of parental exposure, low pH negatively affected fertilization rates compared to control conditions (Fig. 3).

Deformity rates at 2h, 4h, and 8h were significantly affected by low pH in a stepwise fashion, with severe deformity rates at pH 7.3 (Table 3, Fig. 4). Low DO significantly increased deformity rates at 2h, 4h, and 8h under control pH (pH 8.1) in both Experiment 1 and Experiment 2. Significant interactions occurred between pH and parental exposure, and pH and DO, at different times (Table 3; Fig. 4). More specifically, parental exposure significantly decreased the embryo deformity rates under pH 7.7 at DO 6mg O_2 L^{-1} at all three time points, and under all pH levels at DO 2mg O_2 L^{-1} for all three time points, with the exception of pH 8.1 × DO 2mg O_2 L^{-1} at 2h.

3.2 Shell length of D-shaped larvae

227 Shell length of D-shaped larvae ranged from 60 μm to 125 μm during the
228 observation period. A significant decrease in larval shell growth occurred at 48 h
229 under pH 7.3 in Experiment 1. In Experiment 2, low pH significantly decreased larval
230 shell growth in a stepwise fashion under 6 mg O_2 L^{-1}; at 2 mg O_2 L^{-1} larvae reared
231 under pH 7.3 had a significantly smaller shell length than control larvae (Table 3; Fig.
232 5). Larval shell growth at 48h were not significantly affected by low DO. Moreover,
233 parental exposure did not show a significant difference in the D-shaped larval shell
234 growth except in the condition of pH 8.1 × DO 2mg O_2 L^{-1} at 72h (Table 3; Fig.5). At
235 72, 96 and 120 h, larval shell length was significantly smaller under low pH; low DO
236 larvae showed significantly smaller shell lengths under control pH (pH 8.1).
237 Significant interactions did not occur on the D-shaped larval shell length (Table 3).
238

239 **4. Discussion**

240 In this study, we tested for transgenerational effects of exposure to combined
241 ocean acidification and hypoxia on the early development of mussels *M. edulis*. We
242 found that parental exposure to acidification and hypoxia could only partially alleviate
243 the negative effects of these stressors on embryonic and larval developmental traits, as
244 negative effects on developmental traits were still observed when parents were reared
245 under low pH and low O_2. As such, our results suggest that parental exposure may not
246 confer offspring tolerance to short-term ocean acidification and hypoxia in mussels *M.*
247 *edulis*.

248

249 *4.1 Effects of ocean acidification and hypoxia on larval development*

250 Considering the increased occurrences of hypoxia (Vaquer-Sunyer & Duarte,
251 2008) and the continuous decrease of pH levels (Hoegh-Guldberg et al., 2014)
252 globally, it is critical to evaluate the combined impacts on marine species and
253 ecosystems. However, the combined effect of low pH and oxygen on marine species
254 has not been widely studied (Gobler & Baumann, 2016). Our results indicated that
255 low pH conditions had negative effects on fertilization rates, larval deformity rates,
256 and larval shell growth, Furthermore, while positive transgenerational effects were
257 observed, they only partially alleviated the effects of acidification on the
258 aforementioned early developmental traits.

259 While we did not measure survival, our results showed that short-term exposure
260 to experimental ocean acidification negatively affected fertilization rate, embryo
261 deformity rate, and larval shell growth, while hypoxia had relatively little effect and
262 did not influence the effect of acidification. The reduced fertilization rates under
263 acidification may be due to the negative effect of acidification on sperm fitness such
264 as the percentage of motile sperm and the sperm swimming speed (Vihtakari et al.,
265 2013) and/or the process of sperm-egg collisions and gamete fusion (Shi et al., 2017).
266 Negative effects on larval shell growth may be due to the decreasing calcification
267 (Berge et al., 2006) and shell dissolution (Ramesh et al., 2017), or perhaps increases
268 in larval deformities (Talmage & Gobler, 2009). Regardless of mechanism, such

effects in nature could potentially increase juvenile mortality, particularly when food shortages occur during the accumulation of energy reserves (Phillips, 2002).

Our findings indicated relatively little effect of hypoxia on early development. While some comparatively small effects of hypoxia were observed at control pH conditions, DO did not affect fitness under any of the low pH conditions, suggesting that pH has a stronger influence on early development in mussels *M. edulis*. Similar results have been observed for *M. edulis* from other locations (e.g. Frieder et al., 2014) as well as other mussel species such as *Mytilus californianus* (Frieder, 2013), even at extremely low DO concentrations (0.5 mg O_2 L^{-1}; Eerkes-Medrano et al., 2013). With respect to calcification, mineralogical plasticity (e.g. increased calcite to aragonite ratio and magnesium to calcium ratio) is thought to be one way in which calcifying marine organisms can withstand low DO effects on calcification (e.g. polychaete *Hydroides diramphus;* Leung & Cheung, 2018). Metabolic alterations have also been reported to support organismal tolerance to hypoxia. For example, Pörtner et al. (2005) reported that marine animals switch to an anaerobic metabolism and undergo metabolic depression which contributes to energy savings during low DO. The utilization of metabolic pathways that are less energetically demanding may also support calcification and survival under hypoxic conditions (Risgaard-Petersen et al., 2006; Nardelli et al., 2014). While we did not test for physiological underpinnings of observed responses in this study, such mechanisms may explain the lack of DO effect on deformation rates and shell growth observed herein. Alterations in metabolic

activity that result in increased energy availability under hypoxia could have also been responsible for the lack of low DO effect on fertilization rates as well. Collectively, these findings suggest that low DO has relatively little effect on the early development of mussels.

4.2 Transgenerational effects of combined ocean acidification and hypoxia

The role of parental exposure in shaping offspring responses to global change stressors has been observed in numerous marine species including fishes, copepods, and bivalves (Vehmaa et al., 2012; Parker et al., 2012; Munday, 2014). Despite numerous transgenerational studies for acidification and warming, this is, to our knowledge, the first study to test for transgenerational acclimation in response to combined acidification and hypoxia. While parental exposure to low pH and DO partially reduced negative effects on offspring compared to when parents were exposed to control conditions, the positive parental effects were weak at best. Our results thus suggest a limited capacity for parental exposure to alleviate the negative effects of low pH on early development in mussels. This is in contrast to studies documenting largely positive effects of parental exposure on offspring responses, particularly to low pH conditions, in bivalves (Parker et al., 2012; Fitzer et al., 2014; Zhao et al., 2016) and others reporting negative effects of parental exposure (Griffith & Gobler, 2017). Thus, there remains a high degree of uncertainty regarding the

ability of parental exposure to alleviate the effects of marine global change stressors on their offspring and more research is warranted.

While limited, the increased resistance to ocean acidification of *M. edulis* larvae from parents exposed to low pH and DO conditions may be the result of a higher concentration or activity of the enzyme carbonic anhydrase (CA) catalyzing the reversible hydration of CO_2 and accelerating the formation of bicarbonate (HCO_3^-) (Lionetto et al., 2012). Some studies have also found a correlation between CA activity and shell formation (Fitzer et al., 2014b; Medaković & Lucu., 1994), and enzyme activity increases linearly with shell formation (Medaković, 2000). Nonetheless, the mechanisms at play only conferred a small benefit of parental exposure. It is important to note here, however, that although our parental exposure time (4 weeks) was similar to other transgenerational studies on bivalves (e.g. Griffith & Gobler, 2017), a longer exposure may have yielded different results. For example, our exposure time may not have been enough for parental mussels to produce adequate proteins, hormones, or other somatic traits that would provide offspring with the ability to strongly resist more acidified, hypoxic conditions (Munday, 2014). Thus, while our results provide the first documentation of transgenerational effects to combined acidification and hypoxia, future studies with longer parental exposure times are warranted.

5. Conclusions

This study represents the first of its kind to assess the potential for transgenerational acclimation to combined acidification and hypoxia in marine bivalves. Our results suggest that ocean acidification has a comparatively stronger effect on the early development of mussels *M. edulis*. Although we did not directly measure survival, the observed effects of acidification represent a strong decline in function, as reduced fertilization rates, increased deformity rates, and decreased growth all represent negative functional consequences for larval bivalves. Furthermore, while transgenerational effects were positive, they were not sufficient to completely alleviate the negative effects of ocean acidification. Thus, if our experimental results hold true in nature, it appears the ocean acidification may have negative effects on *M. edulis* populations since the success of the early developmental stage of shellfish can affect population and community dynamics. Nonetheless, more research on the combined effects on ocean acidification and hypoxia are required before general conclusions can be drawn with respect to marine bivalves, and longer-term parental exposures are required before predicting whether or not the effects observed herein apply in nature.

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Table 1 A summary of seawater carbonate chemistry parameters in experiment 1. Seawater pH (pH_{NBS}), temperature of embryo and larvae period (T,℃), salinity (psu), total alkalinity (A_T, μmol kg^{-1}), dissolved inorganic carbon (DIC), the partial pressure of CO_2 (pCO_2, μatm) as well as aragonite (Ωar) and calcite (Ωca) saturation states were listed.

Treatments		salinity	T	pH_{NBS}	A_T	DIC	pCO_2	Ωca	Ωar
pH	DO (mg O_2 L^{-1})	(pus)	(℃)		(μmol*kg^{-1})	(μmol*kg^{-1})	(μatm)		
8.1	6.0 ± 0.2	28.0 ± 0.3	16.1 ± 0.3	8.11 ± 0.02	2236 ± 20	2027 ± 13	348 ± 13	4.31 ± 0.12	2.63 ± 0.11
7.7	6.1 ± 0.1	28.1 ± 0.2	16.2 ± 0.1	7.70 ± 0.02	2189 ± 29	2130 ± 23	1118 ± 22	2.03 ± 0.09	1.31 ± 0.05
7.3	6.0 ± 0.2	27.9 ± 0.2	16.0 ± 0.3	7.31 ± 0.03	2218 ± 12	2273 ± 20	2328 ± 34	0.81 ± 0.07	0.59 ± 0.03
8.1	2.1 ± 0.1	28.1 ± 0.2	15.9 ± 0.2	8.10 ± 0.03	2301 ± 21	2089 ± 12	356 ± 12	4.28 ± 0.08	2.59 ± 0.16
7.7	2.1 ± 0.1	28.1 ± 0.2	16.0 ± 0.2	7.73 ± 0.01	2257 ± 27	2159 ± 19	1089 ± 29	1.96 ± 0.18	1.29 ± 0.05
7.3	2.0 ± 0.2	28.0 ± 0.3	16.2 ± 0.3	7.29 ± 0.03	2261 ± 13	2318 ± 11	2401 ± 31	0.86 ± 0.05	0.63 ± 0.04

Table 2 A summary of seawater carbonate chemistry parameters in experiment 2. Seawater pH (pH_{NBS}), temperature of embryo and larvae period (T,℃), salinity (psu), total alkalinity (A_T, μmol kg^{-1}), dissolved inorganic carbon (DIC), the partial pressure of CO_2 (pCO_2, μatm) as well as aragonite (Ωar) and calcite (Ωca) saturation states were listed.

Treatments		salinity	T	pH_{NBS}	A_T	DIC	pCO_2	Ωca	Ωar
pH	DO (mg O_2 L^{-1})	(pus)	(℃)		(μmol*kg^{-1})	(μmol*kg^{-1})	(μatm)		
8.1	6.1 ± 0.1	28.1 ± 0.1	15.8 ± 0.3	8.09 ± 0.02	2228 ± 27	2021 ± 27	352 ± 11	4.29 ± 0.07	2.57 ± 0.10
7.7	6.0 ± 0.1	28.1 ± 0.3	16.1 ± 0.2	7.71 ± 0.03	2169 ± 17	2165 ± 21	1107 ± 17	2.09 ±	1.29 ±

								0.08 ± 0.03	0.05 ± 0.01
7.3	6.1 ± 0.2	28.0 ± 0.3	16.1 ± 0.2	7.32 ± 0.03	2231 ± 19	2284 ± 13	2427 ± 23	0.85 ± 0.05	0.62 ± 0.13
8.1	2.1 ± 0.1	27.9 ± 0.2	15.9 ± 0.3	8.10 ± 0.03	2311 ± 19	2098 ± 18	343 ± 10	4.28 ± 0.18	2.66 ± 0.07
7.7	2.2 ± 0.1	28.0 ± 0.2	16.0 ± 0.3	7.70 ± 0.01	2217 ± 13	2248 ± 15	1098 ± 16	2.06 ± 0.03	1.22 ± 0.04
7.3	2.0 ± 0.2	28.1 ± 0.3	16.0 ± 0.1	7.32 ± 0.03	2211 ± 17	2339 ± 20	2418 ± 33	0.86 ± 0.03	0.61 ± 0.04

Table 3 Summary of three-way ANOVA results on effects of pH, DO and parental exposure (PE) on the fertilization rate (FR), the deformity rate at 2h (DR2), 4h (DR4), 8h (DR8) and the shell length of D-shaped larvae at 48h (SL48), 72h (SL72), 96h (SL96) 120h (SL120) in experiment #1 and experiment #2. Significantly different values are represented in bold.

	FR				DR2				DR4			
	df	MS	F	P	df	MS	F	P	df	MS	F	P
PE	1	406.694	34.531	**<0.001**	1	448.028	16.836	**<0.001**	1	529.000	31.271	**<0.001**
pH	2	1656.694	140.663	**<0.001**	2	11858.778	445.633	**<0.001**	2	13307.194	786.632	**<0.001**
DO	1	30.25	2.568	0.122	1	272.250	10.231	**0.004**	1	484.000	28.611	**<0.001**
PE*pH	2	61.361	5.21	**0.013**	2	112.111	4.213	**0.027**	2	54.250	3.207	0.058
PE*DO	1	1.361	0.116	0.737	1	0.694	0.026	0.873	1	4.000	0.236	0.631
pH*DO	2	6.25	0.531	0.595	2	206.333	7.754	**0.003**	2	99.750	5.897	**0.008**
PE*pH*DO	2	0.694	0.059	0.943	2	0.444	0.017	0.983	2	33.583	1.985	0.159

	DR8				SL48				SL72			
	df	MS	F	P	df	MS	F	P	df	MS	F	P
PE	1	633.361	36.423	**<0.001**	1	352.667	9.197	**0.003**	1	273.375	8.480	**0.005**
pH	2	12572.583	723.024	**<0.001**	2	1371.885	35.777	**<0.001**	2	3146.281	97.595	**<0.001**

DO	1	521.361	29.982	**<0.001**	1	165.375	4.313	**0.041**	1	864.000	26.801	**<0.001**
PE*pH	2	67.861	3.903	**0.034**	2	44.135	1.151	0.321	2	2.844	0.088	0.916
PE*DO	1	14.694	0.845	0.367	1	.667	0.017	0.895	1	2.042	0.063	0.802
pH*DO	2	89.194	5.129	**0.014**	2	49.594	1.293	0.280	2	70.969	2.201	0.117
PE*pH*DO	2	17.361	0.998	0.383	2	3.510	0.092	0.913	2	24.448	0.758	0.472

	SL96				SL120			
	df	MS	F	P	df	MS	F	P
PE	1	137.760	2.893	0.093	1	433.500	12.113	**0.001**
pH	2	3806.542	79.950	**<0.001**	2	4585.073	128.115	**<0.001**
DO	1	1239.844	26.041	**<0.001**	1	1162.042	32.470	**<0.001**
PE*pH	2	19.542	0.410	0.665	2	13.031	0.364	0.696
PE*DO	1	1.760	0.037	0.848	1	16.667	0.466	0.497
pH*DO	2	76.625	1.609	0.206	2	103.323	2.887	0.061
PE*pH*DO	2	47.792	1.004	0.371	2	35.323	0.987	0.377

Figure legends

Fig. 1 Embyros observed at 2 to 8h after fertilization in all treatments. A: pH*DO condition of 8.1*6mg O_2 L^{-1} in experiment 1; B: 7.7*6mg O_2 L^{-1} in experiment 1; C: 7.3*6mg O_2 L^{-1} in experiment 1; D: 8.1*2mg O_2 L^{-1} in experiment 1; E: 7.7*2mg O_2 L^{-1} in experiment 1; F: 7.3*2mg O_2 L^{-1} in experiment 1; a: 8.1*6mg O_2 L^{-1} in experiment 2; b: 7.7*6mg O_2 L^{-1} in experiment 2; c: 7.3*6mg O_2 L^{-1} in experiment 2; d: 8.1*2mg O_2 L^{-1} in experiment 2; e: 7.7*2mg O_2 L^{-1} in experiment 2; f: 7.3*2mg O_2 L^{-1} in experiment 2;

Fig. 2 The categorization of deformity of embryos. A: Initial embryo deformity; B: Irregular deformation of the embryo; C: Slightly deformed of the embryo; D: Embryo rupture; E: Embryo breakage and incomplete; F: Deformity during embryonic division.

Fig. 3 The fertilization rate (FR) at 8h of *M. edulis* exposed to different combinations of pH (8.1, 7.7 and 7.3) and DO (6mg O_2 L^{-1} and 2mg O_2 L^{-1}) (N=100). The means denoted by different superscripts (A, B, C) at each fixed DO are significantly different among three pH levels ($P < 0.05$). The means denoted by red superscripts (+, -) at each fixed DO and pH are significantly affected by parental exposure ($P < 0.05$).

Fig. 4 The embryos deformity rate (DR) at 2h, 4h, and 8h of the *M. edulis* exposed to different combinations of pH (8.1, 7.7 and 7.3) and DO (6mg O_2 L^{-1} and 2mg O_2 L^{-1}) (N=100). The means denoted by different superscripts (A, B, C) at each fixed DO are significantly different among three pH levels ($P < 0.05$). The means sharing the different superscripts (a, b) between two DO levels at each fixed pH are significantly different ($P < 0.05$). The means denoted by asterisk (*) at each fixed DO and pH are significantly affected by parental exposure ($P < 0.05$).

Fig. 5 A, B, C, D respectively means the D-shaped larval shell length of the *M. edulis* at 48h, 72h, 96h, and 120h exposed to different combinations of pH (8.1, 7.7 and 7.3) and DO (6mg O_2 L^{-1} and 2mg O_2 L^{-1}) (N=50). The means denoted by different superscripts (A, B, C) at each fixed DO are significantly different among three pH levels ($P < 0.05$). The means sharing the different superscripts (a, b) between two DO levels at each fixed pH are significantly different ($P < 0.05$). The means denoted by red superscripts (+, -) at each fixed DO and pH are significantly affected by parental exposure ($P < 0.05$).

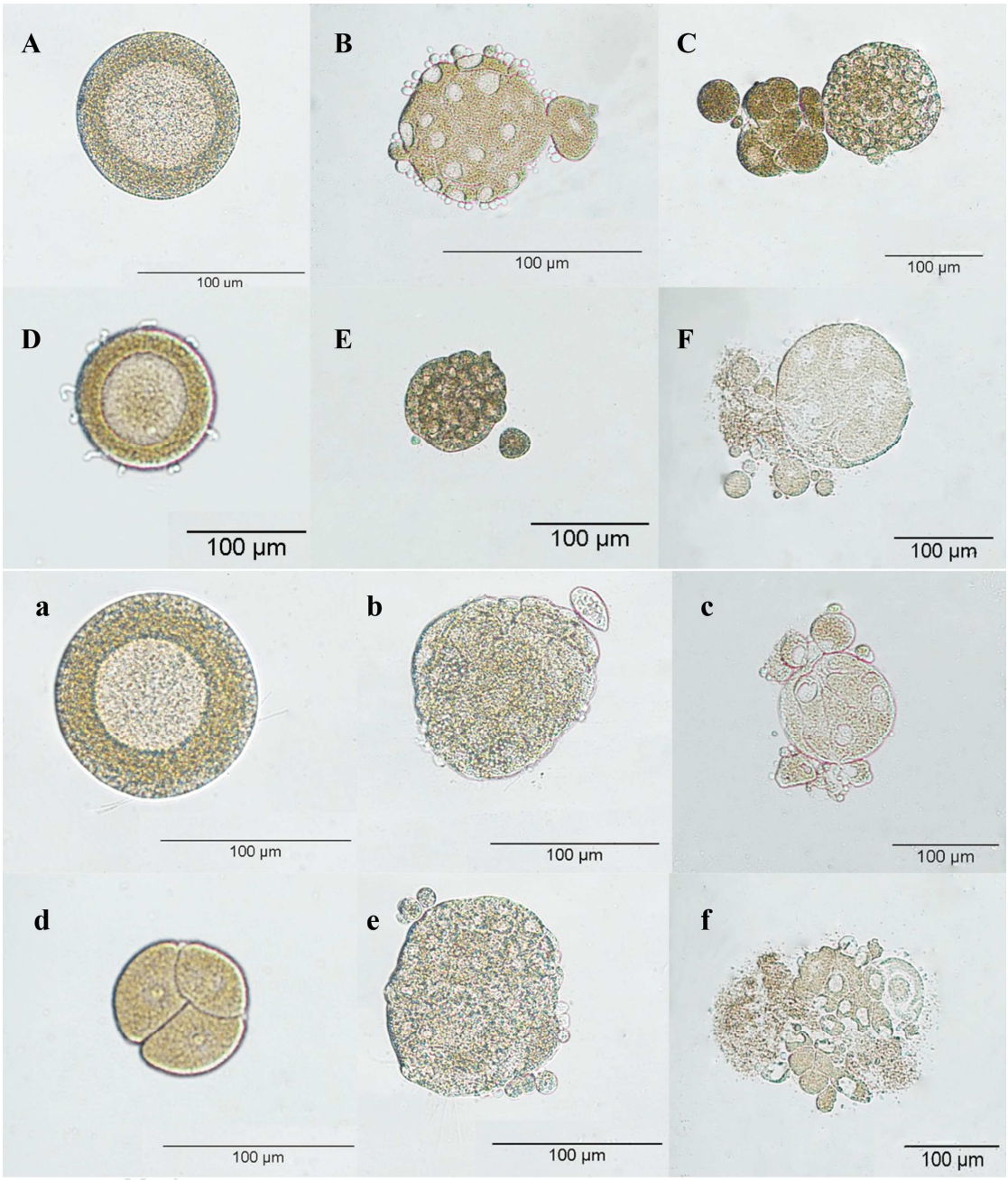

A
100 µm
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F
100 µm
a
100 µm
b
100 µm
c
100 µm
d
100 µm
e
100 µm
f
100 µm

Fig. 1

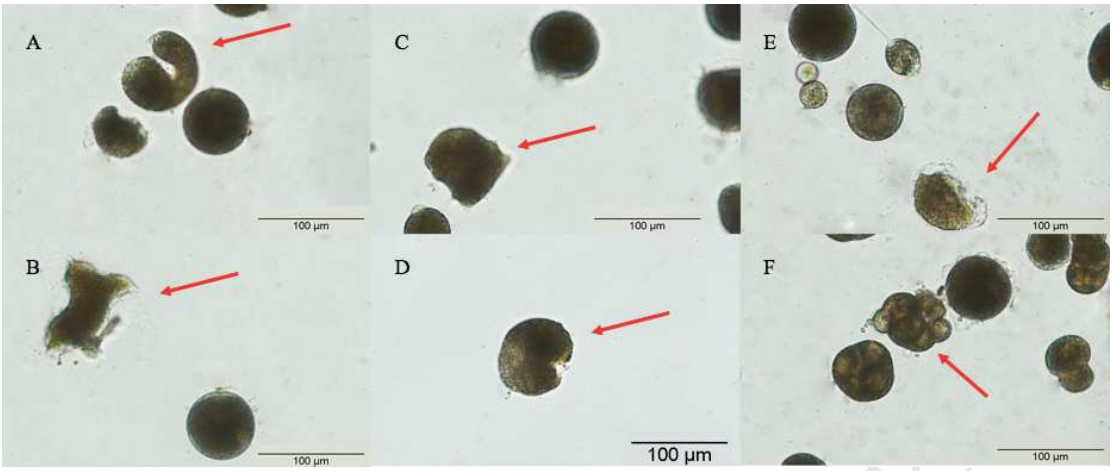

A
C
E
B
D
F
100 µm

Fig. 2

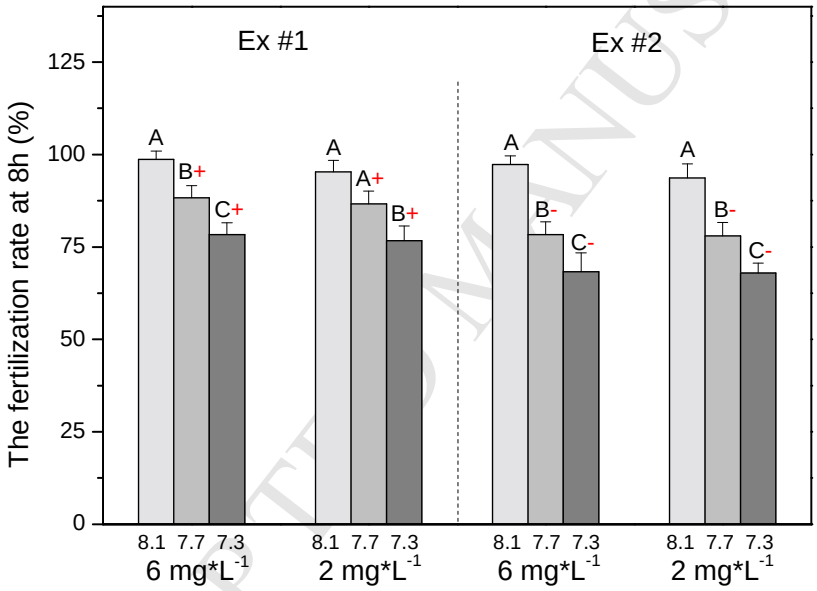

Ex #1
Ex #2
The fertilization rate at 8h (%)
0
25
50
75
100
125
A
B+
C+
A
A+
B+
A
B-
C-
A
B-
C-
8.1 7.7 7.3
6 mg*L-1
8.1 7.7 7.3
2 mg*L-1
8.1 7.7 7.3
6 mg*L-1
8.1 7.7 7.3
2 mg*L-1

Fig. 3

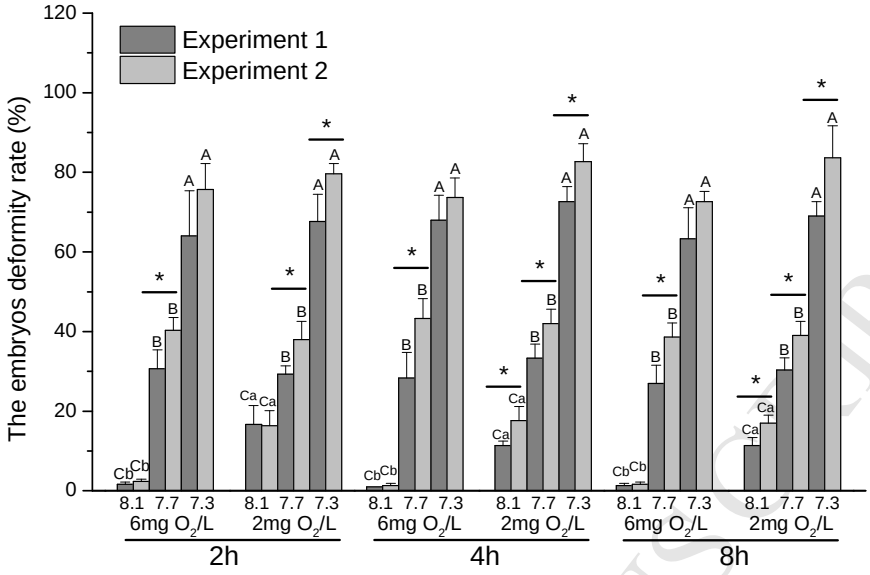
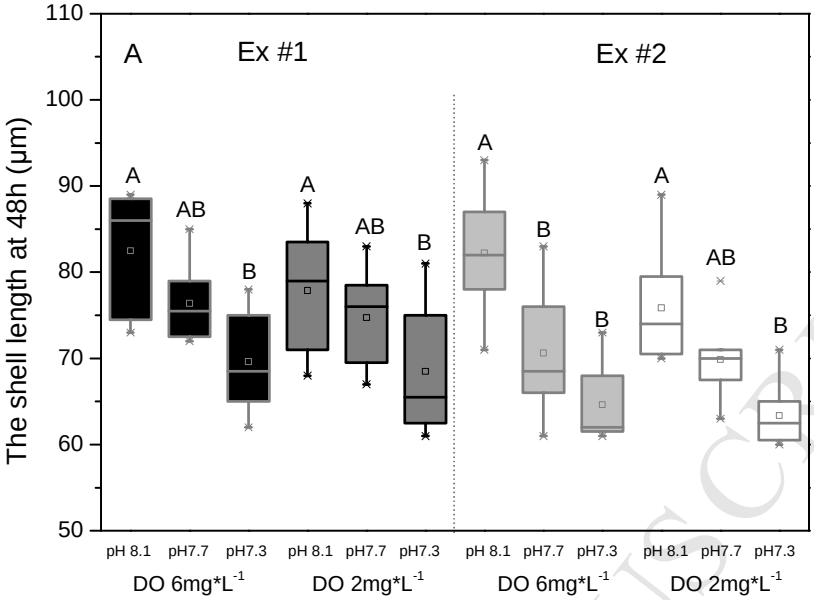
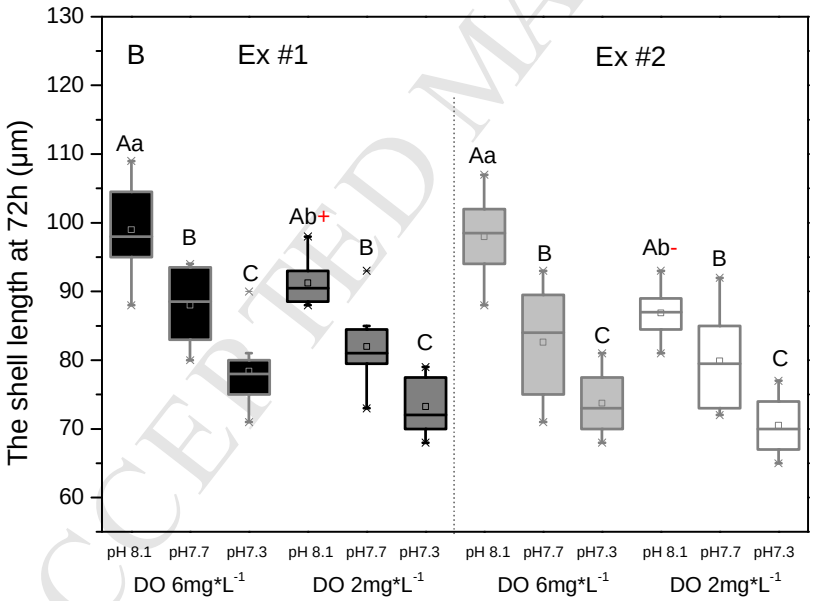
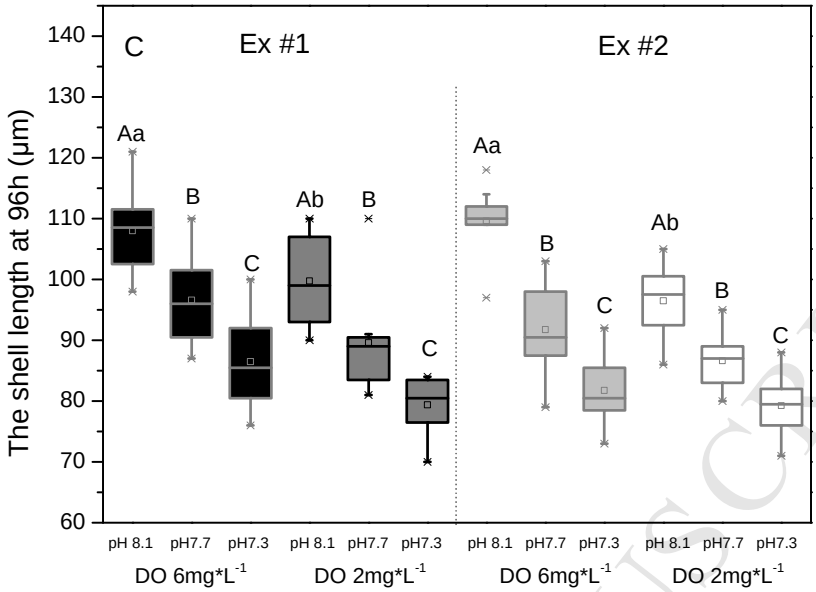


Fig. 4






C
Ex #1
Ex #2
The shell length at 96h (µm)
140
130
120
110
100
90
80
70
60
Aa
B
C
Ab
B
C
Aa
B
C
Ab
B
C
pH 8.1
pH7.7
pH7.3
DO 6mg*L-1
DO 2mg*L-1

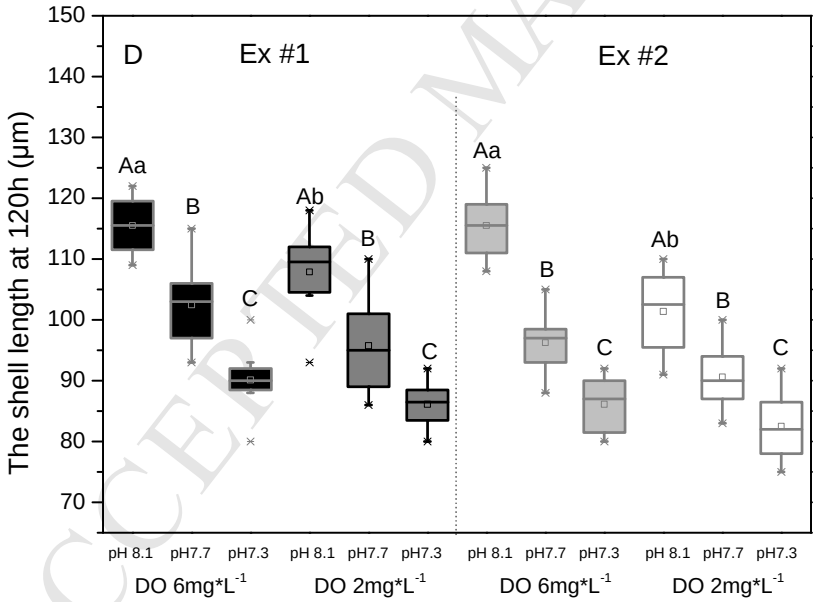

D
Ex #1
Ex #2
The shell length at 120h (µm)
150
140
130
120
110
100
90
80
70
Aa
B
C
Ab
B
C
Aa
B
C
Ab
B
C
pH 8.1
pH7.7
pH7.3
DO 6mg*L-1
DO 2mg*L-1

Fig. 5

Highlights

- Effects of ocean acidification and hypoxia on the early development of the mussel *M. edulis* were investigated.
- Positive carry-over effects of adult mussels exposed to low pH and hypoxia were observed on larvae performance.
- Low pH showed key negative effects on the early development of the mussel *M. edulis*.