



Effects of ocean acidification caused by rising CO₂ on the early development of three mollusks

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ABSTRACT: Increasing atmospheric CO₂ can decrease seawater pH and carbonate ions, which may adversely affect the larval survival of calcareous animals. In this study, we simulated future atmospheric CO₂ concentrations (800, 1500, 2000 and 3000 ppm) and examined the effects of ocean acidification on the early development of 3 mollusks (the abalones *Haliotis diversicolor* and *H. discus hannai* and the oyster *Crassostrea angulata*). We showed that fertilization rate, hatching rate, larval shell length, trochophore development, veliger survival and metamorphosis all decreased significantly at different pCO₂ levels (except oyster hatching). *H. discus hannai* were more tolerant of high CO₂ compared to *H. diversicolor*. At 2000 ppm CO₂, 79.2% of *H. discus hannai* veliger larvae developed normally, but only 13.3% of *H. diversicolor* veliger larvae. Tolerance of *C. angulata* to ocean acidification was greater than the 2 abalone species; 50.5% of its D-larvae developed normally at 3000 ppm CO₂. This apparent resistance of *C. angulata* to ocean acidification may be attributed to their adaptability to estuarine environments. Mechanisms underlying the resistance to ocean acidification of both abalones requires further investigation. Our results suggest that ocean acidification may decrease the yield of these 3 economically important shellfish if increasing CO₂ is a future trend.

KEY WORDS: Ocean acidification · Embryo · Larva · *Haliotis diversicolor* · *Haliotis discus hannai* · *Crassostrea angulata*

INTRODUCTION

Anthropogenic CO₂ emissions are the main cause of increasing atmospheric CO₂. Indeed, since the Industrial Age, atmospheric CO₂ had increased from 250 to 380 ppm by 2004 (Sabine et al. 2004). Due to its solubility, CO₂ is absorbed by the ocean, and subsequently decreases pH, carbonate ion concentration and calcium carbonate saturation, leading to ocean acidification (OA). By the middle of the 21st century, aragonite undersaturation is predicted to appear in northern subarctic surface waters (Feely et al. 2004), and by the end of the 21st century, atmospheric CO₂ may reach 800 ppm, with seawater pH 0.2 units lower than the present values (Gattuso & Lavigne 2009).

Early development of calcareous organisms has been a focus of OA research because invertebrate larvae are environmentally sensitive and their survival is important to community sustenance (Heath 1992, Gosselin & Qian 1997). In recent years, research has been conducted on the embryonic and larval development of marine organisms under simulated future atmospheric conditions. Although many organisms can endure OA to different degrees, fertilization and hatching rates, calcification and survival all decrease significantly for most species as levels of CO₂ increase (Byrne 2010, 2011, 2012, Dupont et al. 2010, Kroeker et al. 2013). Earlier studies have examined abalone responses, specifically *Haliotis discus hannai* (Kimura et al. 2011), *H. coccoradiata* (Byrne et al. 2011) and *H. kamschatkana* (Crim et al. 2011). In

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these earlier studies, fertilization and hatching rates decreased significantly when CO₂ exceeded 1000 ppm, and the abundance of calcified larvae was 30% lower than controls when CO₂ reached 700 to 1000 ppm for *H. discus hannai*. The number of calcified larvae also decreased significantly at pH 7.6 to 7.8, and most *H. coccoradiata* larvae were severely abnormal at pH 7.6. Only 45% of *H. kamtschatkana* larvae survived after an 8 d incubation at 800 ppm CO₂. These data suggest that abalones are sensitive to OA, and that even a slight increase in CO₂ may result in adverse effects.

As a taxon of calcareous organisms with low or no mobility (except the cephalopods), mollusks have the potential to be greatly affected by OA. Previous research on the effects of OA on mollusks indicated that rising CO₂ levels could negatively affect embryonic and larval development for most species (Gazeau et al. 2013). Previous studies have also found that tolerance to OA varies among taxonomically related species and even among different populations of the same species (Byrne 2010). Maintaining a normal larval developmental process is very important in aquaculture breeding. However, rising atmospheric CO₂ is a global problem, and the resulting OA could threaten the aquaculture industry and the economy of countries such as China, the world's largest mollusk producer. The abalones *H. diversicolor*, *H. discus hannai* and the oyster *Crassostrea angulata* are important aquaculture mollusks in China, and are extensively cultured. In 2011, the yield of abalone and oyster reached 76 786 and 3 756 310 t (output of 350 and 2.2 billion, respectively; www.fao.org). Compared to other abalones, *H. diversicolor* has a weaker resistance to environmental stress, and in recent years, industry in this abalone species has gradually decreased.

Understanding the effects of future climate change on these important culture species is essential for economic development. Thus, we chose to study these 3 representative mollusks to observe the effects of OA on early embryonic and larval development. We also compared differences in responses to OA among mollusks living in different habitats (subtidal abalone species and intertidal oyster species).

MATERIALS AND METHODS

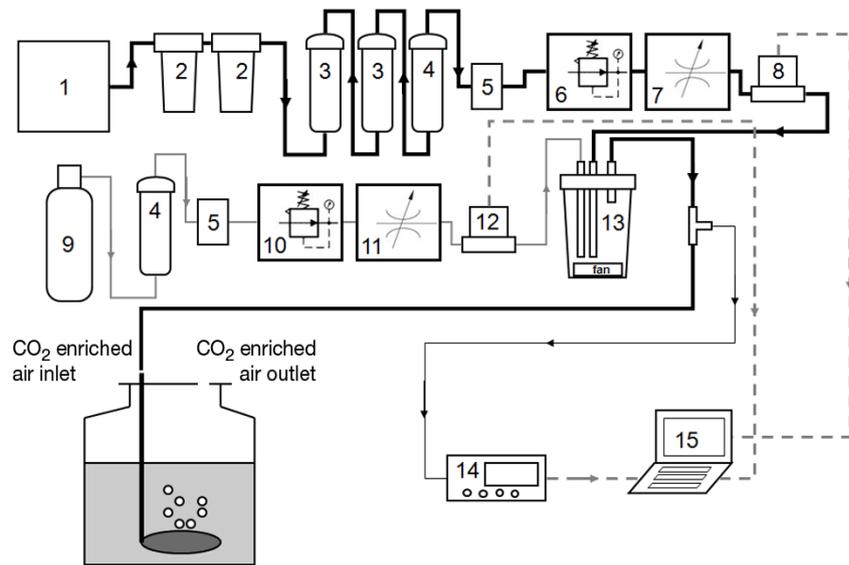
Construction of seawater CO₂ system

The principle of our experimental system was to mix CO₂-free, dry air and pure CO₂ (99.99% purity)

at different ratios of mass flow rates to generate CO₂-enriched air with different pCO₂ (Fangue et al. 2010). Atmospheric air was provided from an oil-free, medical air compressor (OLF-2524, Fengbao), and passed through 2 filters (GFR600-25, AIRTAC) to remove water and particles, and through 2 food-grade polypropylene (PP) columns (32 cm length, 6 cm diameter) filled with soda-lime to absorb the background CO₂, and then through a similar PP column filled with CaCl₂ anhydrate to further remove water, and finally through a 5 cm disc-type air filter before entering into regulating utilities. The treated air was then delivered into a pressure regulation valve (JLF-3, Trieder) and then a needle valve (ZTF-1, Trieder), such that a stable air flow could be maintained. CO₂ was supplied from a cylinder filled with pure CO₂ and was also delivered by a pressure regulating valve (JTF-1, Trieder) and a needle valve (TZF-1, Trieder) to produce a stable CO₂ flow. Mass flow of air and CO₂ were measured by 2 mass flow sensors (Siago MF5706-N-10 for air and 4100-30-CV-C for CO₂), and were then set to the desired level (for example: gas flow rates of 5 SLM (standard l min⁻¹) of air and 4 SCCM (standard cm³ min⁻¹) of CO₂ results in a CO₂ concentration of 800 ppm) by adjusting the needle valves. Both air and CO₂ were homogenized in a 2 l plastic jar equipped with a fan at the bottom to generate CO₂-enriched air with different CO₂ concentrations. A small proportion of gas mixture was directed through a bypass and monitored with a CO₂ detector (Li-7000, LI-COR). Further adjustment of the needle valve was carried out until the reading on the Li-7000 reached the set level (the needle valve in air route remained unaltered and the CO₂ route was adjusted). The mass flow sensors and CO₂ detector were connected to a computer, and measurements were recorded with the associated software.

The majority of CO₂-enriched air was mixed with filtered seawater (0.22 µm filtered) stored in a 60 l PP container. Two small holes were drilled in the lid for input and outlet of CO₂-enriched air. The bucket was kept semi-closed to ensure that the inner space of the bucket was filled with CO₂-enriched air and the treated seawater was separated from the ambient air. A brief diagram of the pCO₂ manipulation system is shown in Fig. 1. A total of 5 CO₂ concentration treatments were selected: 400 ppm, representing the current CO₂ concentration; 800 ppm, representing CO₂ by the end of the 21st century (Gattuso & Lavigne 2009); 1500 ppm (Caldeira & Wickett 2003), 2000 ppm (Caldeira & Wickett 2005) and 3000 ppm (Ries et al. 2009), representing the predicted CO₂ concentrations for the year 2300. Measurements of CO₂ con-

Fig. 1. The pCO₂ manipulation system, showing 1: air compressor, 2: filter, 3: soda-lime column, 4: CaCl₂ anhydrate column, 5: disc-type air filter, 6: pressure regulation valve, 7: needle valve, 8: mass flow sensor, 9: CO₂ cylinder, 10: pressure regulation valve, 11: needle valve, 12: mass flow sensor, 13: plastic jar, 14: CO₂ detector, 15: computer. Solid line: air or CO₂ flow; dashed line: digital signal transferring to computer



centrations and seawater pH among the 5 different treatments are provided in Supplement 1 at www.int-res.com/articles/suppl/b023p147_supp.xls (using 800 and 2000 ppm as examples).

Carbonate system parameters were calculated using CO2SYS software (version 2.1); pH, total alkalinity, temperature, and salinity data are listed in Table 1. Temperature and pH were measured with a pH meter (Thermo Scientific Orion Star Series™ Benchtop with a probe 8157BNUMD), which was calibrated with NBS buffer (4.00, 7.00 and 10.00, respectively). A seawater sample (500 ml) was taken from the tank and immediately fixed by 200 µl saturated mercuric chloride solution. Total alkalinity was determined by Gran acidimetric titration with an Apollo TA Analyzer (Cai & Wang 1998). Salinity was measured with an optical salinity meter gauge.

Collection and maintenance of the study organisms

Mature individuals of *H. diversicolor* and *H. discus hannai* were obtained from the Hongyun aquaculture

farm, Zhangzhou; parental oysters of *C. angulata* were obtained from the aquaculture area of Tong'an, Xiamen. The abalones and oysters were transferred to the laboratory where they were raised in aquaculture tanks with a flow-through seawater supply from a header tank. Seawater in the header tank was aerated with ambient air and the temperature was maintained at a similar level in all experiments. Abalones were fed ad libitum with *Gracilaria* spp.; oysters were fed with *Isochrysis* spp. When feeding oysters, seawater flow was stopped for 2 h so that the alga was sufficiently consumed by the oysters. The experiments were carried out after 1 wk of acclimation.

Continuous exposure experiments

Under continuous exposure, embryos were cultured at different CO₂ concentrations from fertilization until the end of the experiment. For abalone, fertilization, hatching and developmental conditions were measured at 16 and 24 h post-fertilization (hpf); time points corresponding to before and after larval

Table 1. Water chemistry and calcite (Ω_{cal}) and aragonite (Ω_{ara}) saturation state of seawater in control and high-CO₂ groups. Data are means \pm SD (n = 3). For all treatments, alkalinity = 228.1 \pm 4.6 $\mu\text{mol kg}^{-1}$, salinity = 31.3 \pm 0.6. Alkalinity was measured in triplicate

Treatment	Temp (°C)	pH (NBS scale)	pCO ₂ (μatm)	[CO ₂] ($\mu\text{mol kg}^{-1}$)	[HCO ₃ ³⁻] ($\mu\text{mol kg}^{-1}$)	[CO ₃ ²⁻] ($\mu\text{mol kg}^{-1}$)	Ω_{cal}	Ω_{ara}
Control	23.8 \pm 0.1	8.15 \pm 0.01	447.5	13.4	1844.3	179.9	4.45	2.90
800ppm	23.5 \pm 0.1	7.94 \pm 0.01	784.3	23.5	1993.0	118.8	2.94	1.91
1500ppm	23.2 \pm 0.2	7.71 \pm 0.02	1401.3	42.4	2103.7	73.1	1.81	1.17
2000ppm	23.3 \pm 0.0	7.61 \pm 0.02	1794.5	54.1	2137.5	59.2	1.46	0.95
3000ppm	23.5 \pm 0.0	7.43 \pm 0.02	2780.7	83.4	2183.6	40.2	0.99	0.65

torsion, respectively; and larval shell length was measured at 24 and 48 hpf. For oysters, fertilization, hatching and deformation rates were measured over the course of 24 h and larval shell length was measured at 24 and 48 hpf. Although previous studies have defined larval hatching based on straight hinge larvae (D-larvae), our study adopted the trochophore stage for oyster larvae measurements to be consistent with the abalone experiments.

A total of 2 males and 2 to 3 female abalones with fully developed gonads were selected as parents. Abalone gametes were obtained via stimulation with ultraviolet-irradiated seawater and gamete quality was examined before fertilization. Qualifying sperm were fast-swimming and mature eggs were round in shape with an intact membrane. In addition, 2 male and 3 female oysters with qualified gametes were selected. Oyster gametes were acquired using a stripping method. Selection of oyster sperm was similar to that of male abalone; selected oyster eggs were required to be globular shape after soaking in seawater for 30 min, with a transparent nucleus in the center surrounded by densely packed granules. Sperm and eggs were mixed, and fertilization was carried out in replicate 300 ml covered PP beakers in seawater under different pCO₂ conditions. The sperm suspension was gradually added to the eggs until a condition was achieved such that each egg had 8 to 10 sperm attached to the membrane for abalone, and 3 to 5 sperm for oyster. Thirty min after fertilization, fertilized eggs were washed with filtered seawater several times to remove excess sperm and then transferred into replicate 2 l PP buckets under each pCO₂ condition with seawater volume of 1.5 l at a density of 2 embryos ml⁻¹ for abalones and 10 embryos ml⁻¹ for oysters. Embryo densities applied in this experiment were sufficiently low to minimize any effect on seawater pH. After hatching, CO₂-enriched air was directed into the culture bucket to maintain seawater pH. In order to better sustain the pCO₂ condition in each culture bucket, CO₂-enriched air was divided into 2 parts: one part into seawater and the other part into the overlying space of the seawater. Seawater was refreshed every 24 h in the continuous experiment.

Samples for measuring fertilization were taken at 2 hpf and the fertilization rate (%) was calculated by checking the cleaved embryos in all eggs:

$$\text{Fertilization rate} = \frac{\text{no. cleaved embryos}}{\text{no. total eggs}} \times 100 \quad (1)$$

Samples for hatching rate (%) were taken at 8 hpf; hatching rate was quantified by observing the trocho-

phore larvae that swam out of the egg membrane in all eggs:

$$\text{Hatching rate} = \frac{\text{no. trochophores}}{(\text{total no. eggs} \times \text{fertilization rate})} \times 100 \quad (2)$$

For developmental measurement of abalone, the proportions of each developmental stage were calculated. The seawater was stirred gently and then 20 ml seawater was obtained using a syringe. Sampling of oyster deformation measurements was conducted in a similar manner. The sample was fixed with 2 ml 5% CaCO₃ buffered formalin and observed under a microscope (Olympus CX31). Approximately 50 embryos were observed in each sample. Classification of deformed and normal larvae was based on morphological criteria by His et al. (1997) for oysters and Hunt & Anderson (1990) for abalones.

Larval shell length was measured using Image-Pro Plus software. A total of 30 larvae were measured for each replicate in each pCO₂ treatment.

Periodic exposure experiments

Periodic exposure was only performed for abalone. In this experiment, embryos and larvae were cultured under ambient CO₂ and transferred to CO₂-acidified seawater at specific developmental stages. Hatching rates and metamorphosis of the trochophore were observed, as were veliger survival and metamorphosis.

Fifty fertilized embryos were carefully transferred into 300 ml PP beakers filled with different seawater treatments, and placed in 1.5 l PP boxes covered with lids containing aerated, CO₂-enriched air. Hatching rates was measured as in the continuous exposure experiment (Eq. 2).

Fifty trochophore larvae developed in ambient seawater were transferred and cultured under the same conditions as for hatching. Trochophore metamorphosis (%) was calculated at 24 hpf and defined as the ratio of normal veligers to total larvae:

$$\text{Trochophore metamorphosis} = \frac{\text{veliger number}}{\text{initial trochophore}} \times 100 \quad (3)$$

In addition, 50 veligers (24 hpf) developed in ambient seawater were transferred and cultured under the same conditions as for hatching for 48 h. Survival (%) was calculated as the ratio of swimming larvae to initial veligers. Immobile larvae on the beaker bottom were classified as dead.

$$\text{Survival} = \frac{\text{no. surviving larva}}{\text{no. initial larva}} \times 100 \quad (4)$$

Finally, 50 competent larvae developed in ambient seawater were transferred and cultured in the same conditions as for hatching, and a plastic biofilm membrane was placed in each beaker. After 3 d, metamorphosis was calculated as the ratio of veliger metamorphosed larvae on the membrane to initial veligers:

$$\text{Veliger metamorphosis} = (\text{no. metamorphosed larva} / \text{no. initial larva}) \times 100 \quad (5)$$

In all experiments, the seawater was refreshed every 12 h. Each group was studied in triplicate.

Statistical analysis

Percentage data were square-root and arcsine transformed before applying the Shapiro-Wilks normality test and Levene's test for variance homogeneity. Shell length data were not transformed. One-way ANOVA and Duncan's multiple comparisons were applied to compare differences among experimental groups when equal variances were assumed. A Kruskal-Wallis test was used when equal variances were not assumed. An independent samples *t*-test was performed to estimate shell increments between 24 and 48 hpf for each pCO₂ treatment. All tests were performed using SPSS v.17.0 statistical software. ANOVA results are provided in Supplement 2 at www.int-res.com/articles/suppl/b023p147_supp.pdf.

RESULTS AND DISCUSSION

Effects of OA on early development of mollusks

For both abalone species, significant effects of OA on fertilization appeared when CO₂ reached 3000 ppm ($F = 4.3$, $p = 0.028$ for *Haliotis diversicolor*; $F = 5.9$, $p = 0.01$ for *H. discus hannai*) and effects on hatching occurred at 1500 ppm ($F = 26.2$, $p < 0.001$ for *H. diversicolor*; $F = 33.1$, $p < 0.01$ for *H. discus hannai*) (Fig. 2).

Sixteen hours after fertilization, 89.8% of *H. diversicolor* larvae in the control treatment and 72.5% in the 800 ppm treatment had developed into veligers, but the veliger percentage was greatly reduced in the other 3 groups (0.68% for 1500 ppm, 0% for 2000 and 3000 ppm). With these high-CO₂ treatments, more than 60% of larvae remained as trochophore larvae, a sign of developmental delay. Deformations also increased in high pCO₂ treatments (1500, 2000 and 3000 ppm). At 24 h after fertilization, 50.1% larvae from the 1500 ppm group and 13.3%

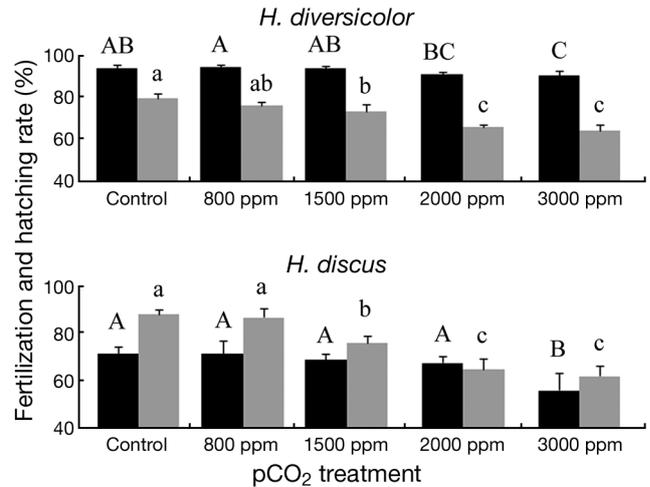


Fig. 2. Fertilization (black bar) and hatching (gray bar) rate of *Haliotis diversicolor* and *H. discus* in the continuous incubation experiment. Capital letters: significant differences of fertilization rate assessed by 1-way ANOVA and Duncan's multiple comparisons ($p < 0.05$); small letters: hatching rate. Data are mean + SD ($n = 3$)

from the 2000 ppm group had entered the veliger stage, but no veligers were found in the 3000 ppm group (Fig. 3).

Sixteen hours after fertilization, more than 70% of *H. discus hannai* larvae from the first 4 groups had developed into veligers, but fewer did so in the 1500 and 2000 ppm groups compared to the control and 800 ppm groups (77.4 and 75.8% vs. 90.9 and 87.7%, respectively). In the 3000 ppm group, the proportion of normal veligers was only 10.4% and deformation was as high as 75.7%. Veliger percentage decreased significantly with rising CO₂. Twenty-four hours after fertilization, larval development was similar to that at 16 h in the first 4 groups. Although the proportion of veligers was significantly reduced at 2000 ppm, the percentage (79.2%) was still high compared to that of *H. diversicolor*. However, no planktonic larva were found in the 3000 ppm group. Samples collected from the bottom of the culture bucket consisted of unfertilized eggs and deformed larvae (Fig. 4).

Deformed abalone larvae under the moderately high CO₂ (1500 ppm) treatment had smaller shells than normal (Fig. 5a,b). When CO₂ was increased further, no shells were observed on the larvae (Fig. 5c). A globular substance could be detected on the surface of these unshelled larvae, likely a residue of calcium carbonate precipitation (Fig. 5c).

For both abalones, shell length decreased significantly when pCO₂ increased to 800 ppm (at 24 hpf, $F = 144.9$, $p < 0.001$ for *H. diversicolor*, $F = 143.6$, $p < 0.001$ for *H. discus hannai*; at 48 hpf, $F = 106.9$,

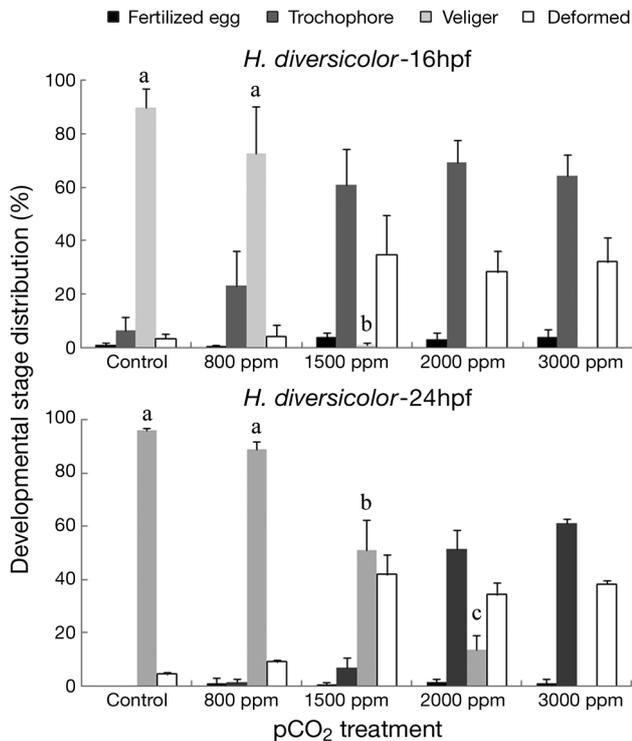


Fig. 3. Distribution (%) of each developmental stage of *Haliotis diversicolor* embryos cultured in each pCO₂ seawater treatment in the continuous incubation experiment at 16 and 24 hours post-fertilization (hpf). Small letters: significant differences of veliger % assessed by 1-way ANOVA and Duncan's multiple comparison ($p < 0.05$). Data are mean + SD ($n = 3$)

$p < 0.001$ for *H. diversicolor*, $F = 151.5$, $p < 0.001$ for *H. discus hannai*). Although significant differences were not observed in shell length between 24 and 48 hpf for any pCO₂ treatment (except for *H. discus* at 2000 ppm), a trend toward smaller shell length was detected at 48 hpf compared to 24 hpf with the 1500 and 2000 ppm treatments (Fig. 6).

Crassostrea angulata fertilization decreased with rising CO₂, and hatching did not vary significantly. Deformation of D-larvae (24 hpf) increased significantly with elevated CO₂. Unlike the 2 abalones studied, normal D-larvae (50.5%) were found in the 3000 ppm group (Fig. 7). Deformed oyster larvae had convex hinges, protruding mantles and smaller shells (Fig. 8) than normally developed larvae. Shell length was significantly reduced at 2000 and 3000 ppm, and shells were even smaller in the 3000 ppm treatment at 24 hpf. Significant reductions in length were also observed in all high CO₂ treatments, yet no differences were found between 2000 and 3000 ppm treatments at 48 hpf. Unlike abalone, significant shell increment from 24 to 48 hpf could be observed for all treatments (Fig. 9).

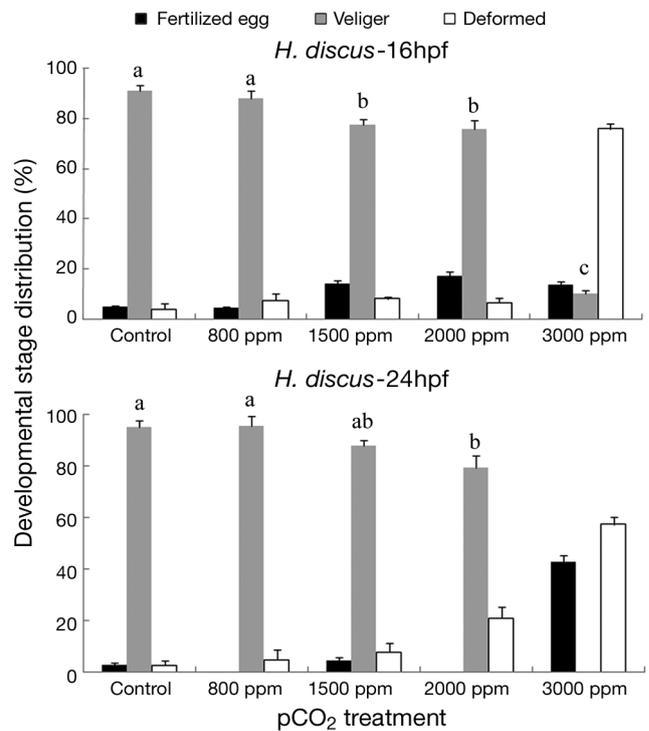


Fig. 4. Distribution (%) of each developmental stage of *Haliotis discus hannai* embryo cultured in each pCO₂ seawater treatment in the continuous incubation experiment at 16 and 24 hpf. Small letters: significant differences of veliger % assessed by 1-way ANOVA and Duncan's multiple comparison ($p < 0.05$). Data are mean + SD ($n = 3$)

With continuous exposure, fertilization of the 2 abalones decreased significantly when CO₂ reached 3000 ppm, and hatching was affected at 1500 ppm. Oyster fertilization also significantly decreased, but only at 3000 ppm (an extreme level), and hatching was unaffected in all treatments. Interestingly, shell formation was significantly affected when pCO₂ reached 800 ppm for both abalone and oyster species. CO₂, which is a non-polar molecule, may penetrate the cell membrane, lower intra-cellular pH, and affect various physiological processes. Thus, increasingly adverse

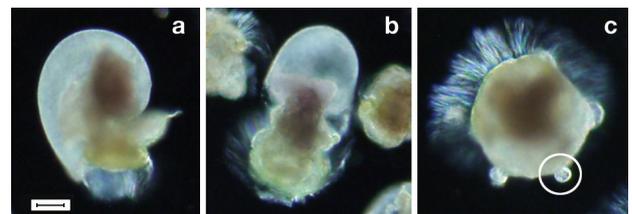


Fig. 5. Comparison of (a) normal and (b,c) deformed *Haliotis diversicolor* larvae at 24 hpf. White circle in (c): residue of calcareous precipitation. Scale bar: 25 μ m

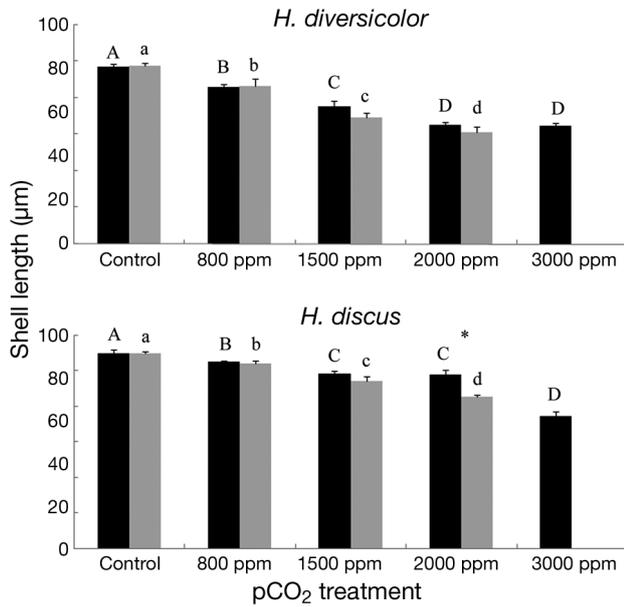


Fig. 6. Shell length of *Haliotis diversicolor* and *H. discus hannai* at 24 hours post-fertilization (hpf; black bars) and 48 hpf (grey bars). Capital letters: significant differences of 24 hpf assessed by 1-way ANOVA and Duncan's multiple comparison ($p < 0.05$), small letters: 48 hpf. No larva were found in the 3000 ppm treatment for either abalones at 48 hpf. Data are means + SD ($n = 3$); *significant difference between 24 and 48 hpf for each pCO₂ treatment

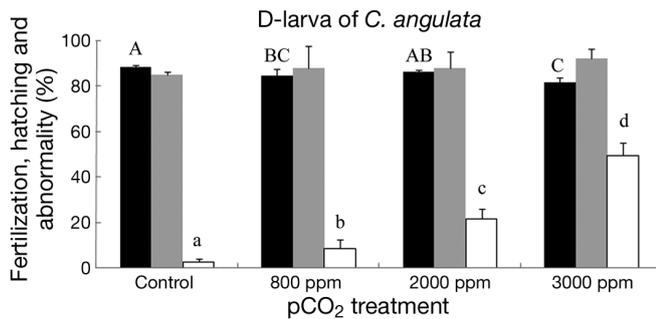


Fig. 7. Fertilization (black bars), hatching rate (grey bars) and abnormality (white bars) of D-larva of *Crassostrea angulata*. Capital letters: significant differences of fertilization rate assessed by 1-way ANOVA and Duncan's multiple comparison ($p < 0.05$); small letters: abnormality of D-larva. Data are means + SD ($n = 3$)

effects of CO₂ in embryonic development may be due to the gradual accumulation of CO₂ in mollusk embryos.

Kimura et al. (2011) reported that fertilization and hatching of *H. discus hannai* decreased significantly at a CO₂ concentration of 1650 ppm. Their hatching data were similar to our findings, but the results regarding fertilization were different. Differences in fertilization in identical species was also observed in

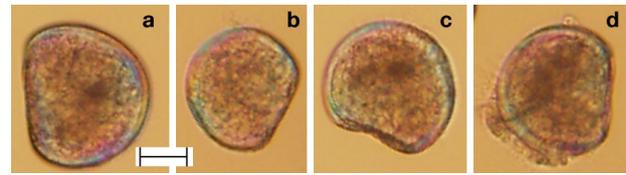


Fig. 8. Comparison of (a) normal and (b-d) deformed *Crassostrea angulata* larvae at 24 hours post-fertilization (hpf). Deformations show (b) smaller shell, (c) convex hinge, and (d) protruding mantle. Scale bar: 25 µm

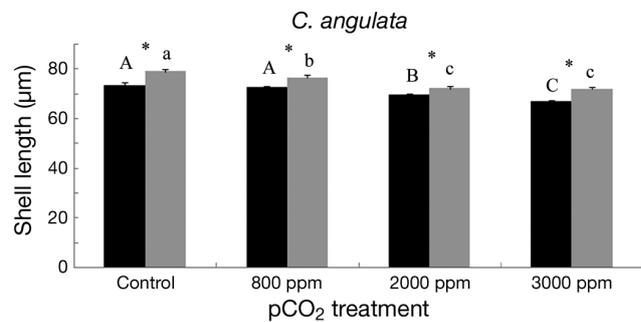


Fig. 9. Shell length of *Crassostrea angulata* at 24 hpf (black bars) and 48 hpf (grey bars). Capital letters: significant differences at 24 hpf assessed by 1-way ANOVA and Duncan's multiple comparison ($p < 0.05$); small letters: 48 hpf. Data are means + SD ($n = 3$); *significant difference between 24 and 48 hpf for each pCO₂ treatment

the oyster *Crassostrea gigas*, for which populations in Japan (Kurihara et al. 2007) and Sweden (Havenhand & Schlegel 2009) exhibited no sign of reduced fertilization under high CO₂, while a significant reduction in fertilization at elevated CO₂ was documented for the Australian population (Parker et al. 2010). These results indicate intraspecific variation in fertilization under OA stress due to geographic or genetic differences. Another possible explanation for fertilization differences between Kimura et al. (2011) and our study may have been due to the culture temperature used in the experiments (20°C for Kimura et al. 2011; 23°C for ours), and this further suggests that standardized experiment protocols are needed for OA research (Byrne 2010, Gazeau et al. 2013).

Fertilization rate and hatching of *C. angulata* was less affected by OA, which is in agreement with previous research on *C. gigas* (Kurihara et al. 2007). OA primarily affects the D-larvae. In Kurihara et al. (2007), only 4% of embryos developed into D-larvae, whereas 78.4% did so in our study. CaCO₃ saturation levels rather than pH or CO₂ may be the most important factor affecting mollusk embryos and larva (Gazeau et al. 2011). Seawater

alkalinity differed among laboratories (2247 $\mu\text{mol kg}^{-1}$ in our study vs. 1964 $\mu\text{mol kg}^{-1}$ in Kurihara et al. 2007), therefore the final CaCO_3 saturation state was different even though CO_2 concentrations were the same in CO_2 -enriched air. In addition, Kurihara et al. (2007) reduced the aragonite saturation (Ω_{ara}) to 0.68 at a CO_2 of 2000 ppm, which was equivalent to 3000 ppm in our study. This may explain different tolerances to OA between the 2 oysters in addition to the inter-species differences.

Increased CO_2 adversely affected shell formation for both abalone species, but tolerances exist with respect to OA between the 2 species. Embryonic development of *H. diversicolor* was significantly inhibited when CO_2 reached 1500 ppm, whereas *H. discus hannai* retained normal veliger larvae at 1500 and 2000 ppm, with development only inhibited at 3000 ppm. Shell formation in *H. discus hannai* was inhibited when CO_2 reached 3000 ppm ($\Omega_{\text{ara}} = 0.65$, highly unsaturated), but for *H. diversicolor*, shell formation was hindered at 1500 ppm ($\Omega_{\text{ara}} = 1.17$, still supersaturated). Shell formation of calcareous organisms is not simply chemical precipitation, but is a complicated biochemical process requiring a series of proteins. Protein synthesis for *H. diversicolor* may be more affected than *H. discus hannai* when CO_2 levels rise, and this may explain tolerance differences with respect to OA between the 2 abalones.

Rising CO_2 could cause larvae shell thinning and fragility. Talmage & Gobler (2010) found that in 2 bivalve larvae, increasing CO_2 loosened shell hinges and surface ribs became obscure while the surface became porous. The thickness of *Crassostrea virginica* juveniles increased with rising CO_2 , but their hardness decreased, indicating that the shell loosened (Beniash et al. 2010). Also, the shell aperture shape was altered under OA (Kimura et al. 2011). The shell of calcareous organisms is key for protecting the viscera from infection and resisting predators, so organismal survival is threatened when the shell is damaged. Another problem with a thinner shell is reduced larval density, which determines larval attachment. Therefore planktonic time could be prolonged for lighter larvae, and its life cycle would be disturbed, adversely affecting community recruitment.

In our study, shell length decreased under 1500 and 2000 ppm CO_2 treatments from 24 to 48 hpf, indicating a shell dissolution trend for both abalones. Considering the aragonite saturation state under these conditions (over-saturation or near saturation), the shell would be dissolved by larvae to buffer the

excess CO_2 absorbed into their bodies. In contrast, oyster larvae could grow shells at all treatments, yet their shell increment was still hindered at higher CO_2 concentrations. Shell length was unaffected at pCO_2 of 800 ppm at 24 hpf, but decreased significantly at 48 hpf, indicating a sign of a chronic effect of moderately high CO_2 concentration. Shell lengths in the 3000 ppm treatment were significantly smaller than those in the 2000 ppm treatment at 24 hpf, yet became no different from the 2000 ppm treatment at 48 hpf. This phenomenon could be attributed to a compensation effect, in which more energy was diverted to shell growth for oyster larvae. Because no alga was added during incubation, shell compensatory growth implied less energy allocation for somatic growth, which may be harmful for larval health and further development.

Periodic exposure experiment for abalone

Hatching of *H. diversicolor* decreased significantly at 2000 and 3000 ppm. For *H. discus hannai*, hatching decreased at 1500 ppm and at higher concentrations, with hatching in the latter 3 groups (1500, 2000 and 3000 ppm) being significantly less than the 2 lower concentrations (400 and 800 ppm). Trochophore metamorphosis in *H. diversicolor* decreased significantly at 3000 ppm, while for *H. discus hannai* it decreased significantly at 1500 ppm and decreased again at 3000 ppm compared with the 1500 and 2000 ppm treatments. Rising CO_2 significantly reduced survival and metamorphic rates of veligers (Fig. 10).

The periodic exposure experiment indicated that exposure to increased levels of CO_2 at many developmental stages could adversely affect embryonic development. When the CO_2 concentration was maintained at 800 ppm, no significant changes were detected; CO_2 at 1500 ppm appeared to be a threshold at which adverse effects began to appear. Although in a low proportion, larvae could still form complete shells at 3000 ppm if exposure began from the trochophore stage rather than before fertilization as in the continuous experiment. This may be due to a cumulative effect in which the shortened exposure time in the periodic experiment reduced CO_2 accumulated in embryos and hypercapnia was alleviated. The purpose of periodic exposure was to determine which larval stage is relatively sensitive to OA. However, alleviation of CO_2 toxicity indicated that the chronic exposure experiment better simulated natural conditions and had more ecological relevance.

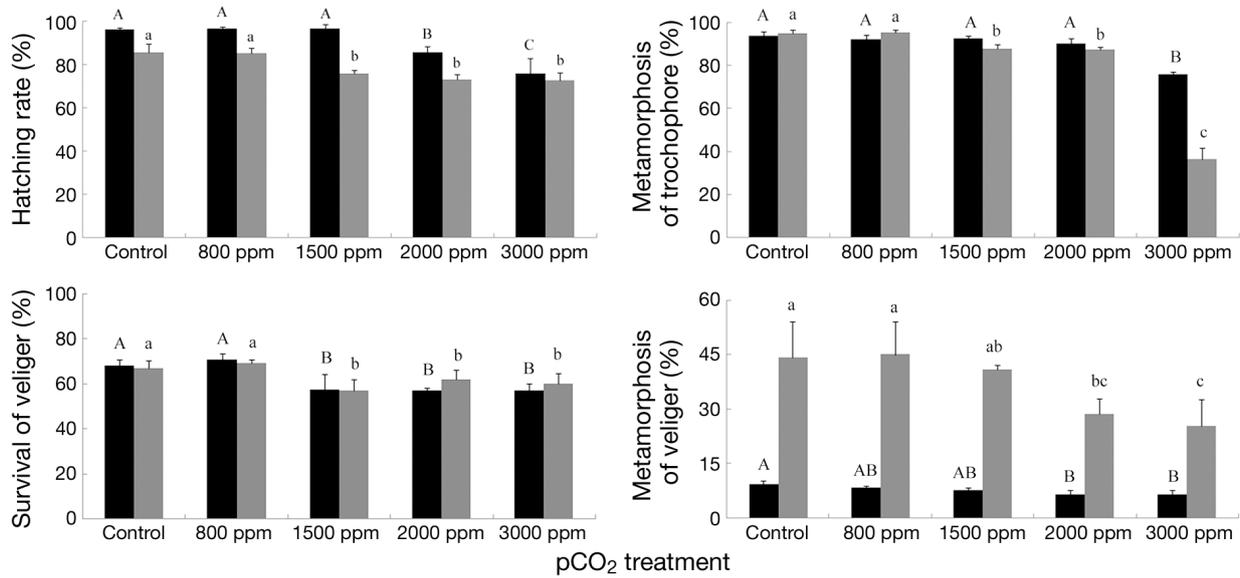


Fig. 10. Hatching rate, metamorphosis of trochophore, survival of veliger and metamorphosis of veliger of *Haliotis diversicolor* (black bars) and *H. discus hannai* (grey bars) across pCO₂ treatments. Capital letters denote significant differences of *H. diversicolor* assessed by 1-way ANOVA and Duncan's multiple comparison ($p < 0.05$); small letters indicate *H. discus hannai*. Data are means + SD ($n = 3$)

Comparison of OA tolerance in different mollusk species

Variation in tolerance to OA is evident among different species (Ries et al. 2009), and occasionally even among geographic populations of the same species. A slight increase in CO₂ could significantly reduce survival, normality, and shell length of the abalone *H. kamtschatica*, similar to *H. cocciradiata*, for which calcification decreased when pH levels fell to 7.8 (700 to 1000 ppm). Research on crab *Hyas araneus* communities from different latitudes indicated that crabs from low latitudes were more tolerant to OA than those from high latitudes (Walther et al. 2011). Clark et al. (2009) compared the response to OA of several urchins from tropical, temperate, and polar regions and reported a weaker tolerance to OA for polar urchins. Byrne et al. (2013) found that pCO₂ exerted the greatest influence on polar urchin larvae for arm growth, while for the tropical species a decreased carbonate mineral saturation was most important. *H. diversicolor* is a subtropical species whereas the original habitat of *H. discus hannai* is a temperate region. Seawater at a relatively high latitude tends to have a lower pH due to higher CO₂ solubility at lower temperatures; therefore, *H. discus hannai* could withstand a higher CO₂ perturbation, a sign of adaptation. The contrasting results between abalone and urchins with respect to the latitudinal difference in OA

response may be attributed to taxonomic variances in physiology and genetics. Moreover, the relationship between *H. diversicolor* and *H. discus hannai* is not close: *H. discus hannai* contains 18 chromosomes and *H. diversicolor* has 16. In aquaculture, *H. discus hannai* can be cultured and spread more easily. These differences may be due to divergence in genotypes and adaptation. A better understanding of different tolerances to OA requires further research using molecular biological, proteomic, physiologic and biochemical tools.

Compared with abalone, *C. angulata* had greater tolerance to OA, and normal larvae could still be observed at very high CO₂ concentrations. Both abalone species in this study inhabit environmentally stable, off-shore regions and are susceptible to reduced salinity. *C. angulata* is an estuarine intertidal species that lives in environments with strong fluctuations in pH and salinity, and is therefore more able to adapt to the changing environment during long-term acclimation. That may be the reason why *C. angulata* could tolerate a lower pH than the abalones.

Ion-regulating capacity is essential for dealing with hypercapnia, allowing organisms to excrete excess hydrogen ions out of the cell via ion channels. As an intertidal species that often experiences environmental changes, *C. angulata* is a better regulator than the 2 abalones studied here, and can withstand harsher OA conditions. Michaelidis et al.

(2005) found that the hemolymph pH of *C. gigas* exposed to air for 4 h decreased from 7.36 to 6.90. Because oysters experience air exposure at low tide in every tidal cycle, they may suffer acidosis frequently and to a degree that is more severe than that brought about by OA. Consequently, an adaptation mechanism may exist for oysters to cope with high CO₂ concentrations. However, to fully understand tolerance mechanisms, ion transportation, enzyme activity and genetic expression should be measured.

CONCLUSIONS

OA has adverse effects on embryonic development and larval shell formation, and threatens the viability of the mollusk aquaculture industry in the future. In this study, differences in OA tolerance among 3 mollusk species were observed. Organisms that inhabit frequently changing environments such as estuarine and intertidal regions were more resistant to OA than those from stable habitats, indicating that future research is warranted on more species to better estimate the effect of OA on the entire ecosystem.

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