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Effects of increased seawater pCO_2 on early development of the oyster *Crassostrea gigas*

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ABSTRACT: This study demonstrated that the increased partial pressure of CO_2 (pCO_2) in seawater and the attendant acidification that are projected to occur by the year 2300 will severely impact the early development of the oyster *Crassostrea gigas*. Eggs of the oyster were artificially fertilized and incubated for 48 h in seawater acidified to pH 7.4 by equilibrating it with CO_2-enriched air (CO_2 group), and the larval morphology and degree of shell mineralization were compared with the control treatment (air-equilibrated seawater). Only 5% of the CO_2 group developed into normal 'D-shaped' veliger larvae as compared with 68% in the control group, although no difference was observed between the groups up to the trochophore stage. Thus, during embryogenesis, the calcification process appears to be particularly affected by low pH and/or the low $CaCO_3$ saturation state of high-CO_2 seawater. Veliger larvae with fully mineralized shells accounted for 30% of the CO_2-group larvae, compared with 72% in the control ($p < 0.005$). Shell mineralization was completely inhibited in 45% of the CO_2-group larvae, but only in 16% of the control ($p < 0.05$). Normal D-shaped veligers of the control group exhibited increased shell length and height between 24 and 48 h after fertilization, while the few D-shaped veligers of the CO_2 group showed no shell growth during the same period. Our results suggest that future ocean acidification will have deleterious impacts on the early development of marine benthic calcifying organisms.

KEY WORDS: Carbon dioxide · pH · *Crassostrea gigas* · Calcification · Larval development · Marine bivalves · Calcium saturation · Ocean acidification

INTRODUCTION

In addition to raising air and ocean temperatures, the increasing concentration of atmospheric CO_2 also has an acidifying effect on the oceans, which to date have absorbed about 40% of the anthropogenic CO_2 emissions (Royal Society 2005, GACGC 2006, Denman et al. 2007). Depending on partial pressure of CO_2 (pCO_2) gradients between air and surface waters, atmospheric CO_2 will dissolve in seawater to form carbonic acid, which then dissociates into protons and bicarbonate ions, thereby decreasing pH. Based on the IPCC IS92a emission scenario for the year 2000–2100 and a logistic function for the burning of the remaining fossil-fuel resources thereafter, Caldeira & Wickett (2003) projected that a maximum pH reduction of 0.77 units will occur around the year 2300, which is greater than any of the ocean acidifications that these authors inferred from a geochemical model and data from Crowley & Berner's (2001) geological record of atmospheric CO_2 levels over the past 300 million yr.

In addition to acidifying seawater, increases in pCO_2 also shift the equilibrium of oceanic carbonate; biologically, the most relevant consequence of this shift is a reduction of carbonate-ion (CO_3^{2-}) concentration. This is because a decrease in CO_3^{2-} concentration leads to a reduction in calcium carbonate saturation (Ω), which has significant impacts on calcifying marine organisms:

$$\Omega = [Ca^{2+}][CO_3^{2-}]/K^*_{sp} \quad (1)$$

where K^*_{sp} is the stoichiometric solubility product for $CaCO_3$, and $[Ca^{2+}]$ and $[CO_3^{2-}]$ are the *in situ* calcium and carbonate concentrations, respectively. Of the

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2 major biologically secreted forms of calcium carbonate, aragonite is more unstable and soluble than calcite, as described by the larger K^*_{sp} for aragonite than for calcite (Zeebe & Wolf-Gladrow 2001). The present ocean surface is supersaturated with respect to both calcite and aragonite, but recent models have predicted that the cold, high-latitude surface waters will become undersaturated ($\Omega < 1$) with respect to aragonite by the year 2050, due to the ocean acidification caused by the elevated atmospheric CO_2 (Feely et al. 2004, Orr et al. 2005).

Orr et al. (2005) demonstrated that the aragonite shells of live pteropods *Clio pyramidata* from the subarctic Pacific showed signs of shell surface dissolution when kept in seawater with $\Omega_{arag} < 1$ for 48 h. Moreover, it has been shown that the calcification rates of major groups such as coccolithophores, foraminiferans and corals decrease with reduced seawater CO_3^{2-} concentrations even when $\Omega > 1$ (Gattuso et al. 1998, Riebesell et al. 2000, Bijma et al. 2002).

As our knowledge on the effect of ocean acidification has begun to emerge, so has our understanding of the physiological impacts of acidification on marine organisms. Exposure to acidified seawater (pH 7.3) by elevated pCO_2 causes reductions in the growth rate of mussels *Mytilus galloprovincialis*, which is suggested to be due to a reduction in metabolic rate and the dissolution of shell as a result of extracellular fluid acidosis; at the same time, oxygen consumption decreases to 60% of the pre-exposure level (Michaelidis et al. 2005). Exposure to seawater pH 7.3 elicits complex changes in metabolic enzyme activities of the teleost *Sparus aurata*, which suggests a shift from aerobic to anaerobic pathways of substrate oxidation (Michaelidis et al. 2007).

The investigation of the biological impacts of future ocean acidification is still in its infancy. One potential impact that requires clarification is on the early development of calcifying marine organisms. From an ecological viewpoint, effects on early developmental stages are of pivotal importance for the following reasons: (1) early life stages are generally more sensitive to environmental disturbance than the adult stage, (2) most benthic calcifying organisms possess planktonic larval stages, and (3) fluctuations in these larval stages due to high mortality rates regulate population size (Green et al. 2004). In addition, $CaCO_3$ shells and skeletons of calcifying organisms usually begin to be synthesized during the larval stages. To our knowledge, Kurihara & Shirayama (2004a,b) and Kurihara et al. (2004) on the effects of high pCO_2 on spicule formation in sea urchin larvae are the only studies of effects of high pCO_2 on the early development of marine organisms.

In this study we evaluated the effects of increased seawater pCO_2 on the early life stages of the oyster *Crassostrea gigas*. Oysters are ecosystem engineers providing habitats for many rocky-shore benthic organisms, and also have a high commercial value as food. In addition, eggs, embryos and larvae of *C. gigas* are among the most commonly used organisms for assessing toxicity of pollutants and for evaluating water quality in coastal areas (Calabrese 1984, His et al. 1997). We incubated oyster eggs under an elevated pCO_2 condition (seawater pH 7.4) up to, and including, the veliger stage, and quantified the effects on early development including shell mineralization.

MATERIALS AND METHODS

Test animals. *Crassostrea gigas* were collected from subtidal shores near the Institute for East China Sea Research, Nagasaki University, Nagasaki, Japan. The oysters were kept for <1 wk in an indoor tank with continuous seawater supply or in an open-water cage of the Institute before the experiments (June through August 2006; water temperature: 23°C).

Seawater for culture. Filtered (GF/C 1.2 µm) and sterilized (121°C, 15 min) seawater (FSW) was placed in two 1 l beakers, and bubbled with air (control) or CO_2-enriched air (CO_2-FSW). The pH of the CO_2-FSW was adjusted to 7.4 by regulating the flow rates of air and CO_2 with flow meters (Kofloc 250). The pH and O_2 saturation of the FSW were measured before each experiment with a pH electrode (Mettler Toledo InLab413SG-IP67; pH meter Mettler Toledo MP125), and an O_2 electrode (OE-270AA, Toa; O_2 meter DO14-P, Toa) (Table 1). Salinity was measured with a refractometer (Atago 100-S), alkalinity was determined with a pH meter (PHM290, Radiometer) and an autoburette (ABU901, Radiometer), and $[Ca^{2+}]$ was analyzed with a calcium electrode (Thermo, Orion model 9720BN; ion analyzer Orion EA 920). pCO_2, dissolved carbon dioxide $[CO_2]$, biocarbonate $[HCO_3^-]$ and carbonate $[CO_3^{2-}]$ concentrations

Table 1. pH and O_2 saturation of seawater in control and CO_2 groups (mean ± SD); *T*: time after fertilization

T (h)	pH		O_2 saturation (%)	
	Control	CO_2	Control	CO_2
0	8.21 ± 0.09	7.42 ± 0.02	99 ± 1.3	97 ± 2.7
2	8.17 ± 0.08	7.46 ± 0.03	93 ± 1.7	93 ± 0.7
3	8.17 ± 0.07	7.45 ± 0.04	93 ± 2.9	93 ± 2.3
8	8.18 ± 0.07	7.46 ± 0.02	91 ± 2.7	90 ± 3.6
24	8.16 ± 0.07	7.44 ± 0.03	90 ± 1.9	90 ± 3.7
48	8.15 ± 0.07	7.42 ± 0.05	95 ± 1.9	94 ± 3.4

were calculated using the first and second dissociation constants of carbonic acid by Mehrbach et al. (1973), and calcite (Ω_{calc}) and aragonite (Ω_{arag}) saturation using stoichiometric solubility products for the respective crystalline forms by Mucci (1983) (Table 2).

Bioassay. Eggs were obtained by stripping the gonads of female oysters over a sieve (mesh size 80 mm) to remove tissue debris. The eggs were rinsed several times with FSW and transferred to a 500 ml beaker containing 300 ml of FSW. Then, 10 to 35 ml of 25% ammonia water (010-03166, Wako Pure Chemical Industries; $[NH_4^+]$ ca. 7 mol l^{-1}) was added to the beaker to induce germinal vesicle breakdown (GVBD), according to method suggested by Sagara (1958). Fifteen minutes after adding the ammonia water, the eggs were twice rinsed with FSW, and maintained at 23°C for about 1 h until GVBD had been completed. Sperm were obtained in the same way as the eggs, but using a 20 mm mesh sieve, and suspended in 300 ml of FSW. After completing GVBD, the eggs were placed in 2 Petri dishes (10 × 1.5 cm) filled with FSW or CO_2-FSW. After checking sperm mobility under a microscope, a few drops of sperm suspension were added to the Petri dishes; 15 min after fertilization, fertilized eggs were rinsed with FSW or CO_2-FSW to remove excess sperm. Thereafter, several hundred embryos were transferred from each dish to five 50 ml vials for each group (control or CO_2), keeping egg density at <10 eggs ml^{-1}, and the vials were completely filled with FSW or CO_2-FSW and sealed to prevent CO_2 exchange with the ambient air.

The developing embryos were fixed 2, 3, 8, 24 and 48 h after fertilization by adding 10% neutralized-formalin seawater solution to a final formalin concentration of 5%. Immediately before adding the formalin seawater solution, the pH and O_2 saturation of the FSW and CO_2-FSW were measured to verify that they had not changed significantly during the incubation period (Table 1).

Table 2. Carbon chemistry and calcite and aragonite saturation state of experimental seawater in control and CO_2 groups

Parameter	Control	CO_2
Temp. (°C)	23 ± 0.5	23 ± 0.4
pH (NBS scale)	8.21 ± 0.08	7.42 ± 0.02
Alkalinity (µmol kg^{-1})	1964 ± 0.003	
Salinity	33.7 ± 0.7	
pCO_2 (µatm)	348	2268
$[CO_2]$ (µmol kg^{-1})	10.4	67.8
$[HCO_3^-]$ (µmol kg^{-1})	1506	1825
$[CO_3^{2-}]$ (µmol kg^{-1})	161.4	36.4
Ca^{2+} (mmol l^{-1})	12	12
Ω_{calc}	4.54	1.02
Ω_{arag}	3.00	0.68

A light microscope was used to observe 100 oyster embryos or larvae from each 50 ml vial, and the numbers in each developmental stage were counted. Morphological criteria proposed by His et al. (1997) were adopted to differentiate 'normal' (D-shaped) from 'abnormal' larvae. To determine the effects of CO_2 on shell growth, larvae that were assessed to be normal were mounted on a glass slide, photographed, and measured for shell length (the anterior-posterior dimension of the shell parallel to the hinge line, Waller 1981) and height (the dorsal-ventral dimension perpendicular to the hinge) under a microscope with an ocular micrometer. Larvae incubated for 24 and 48 h were observed also with a polarized light microscope to evaluate the degree of shell mineralization. We estimated the degree of shell mineralization in our materials on the basis of the observed birefringence, which is due to the mineral phase (Weiss et al. 2002). The larval area exhibiting birefringence was interpreted to be covered by mineralized shell. Larvae were categorized into 3 types; fully mineralized, partially mineralized, and non-mineralized. Fully mineralized larvae are those individuals that exhibit birefringence over the entire surface of the larva, while non-mineralized larvae are the ones that exhibit no birefringence. Partially mineralized larvae are those individuals in which only part of the larval surface exhibits birefringence. We did not quantify the ratio of birefringent area to the entire larval area.

Statistics. Five replicates of the experiments, each consisting of one control and one CO_2 group originating from the same batch of eggs, were carried out, and the mean of each replicate was used to obtain grand means and SD values for statistical comparison. Paired *t*-tests were used to compare the percentage of each developmental stage at 2, 3, 8, 24 and 48 h after fertilization, as well as the percentage of fully, partially and non-mineralized larvae between the groups. Paired *t*-tests were also performed to compare shell length and height between the groups at 24 and 48 h, and between 24 and 48 h values within a group.

RESULTS

The percentage distribution of embryos in each stage did not differ between the control and the CO_2 groups for the samples obtained at 2, 3 and 8 h after fertilization (Fig. 1). The majority of larvae developed to the blastula and gastrula stages by 8 h in both groups (Fig. 1). However, effects of CO_2 became

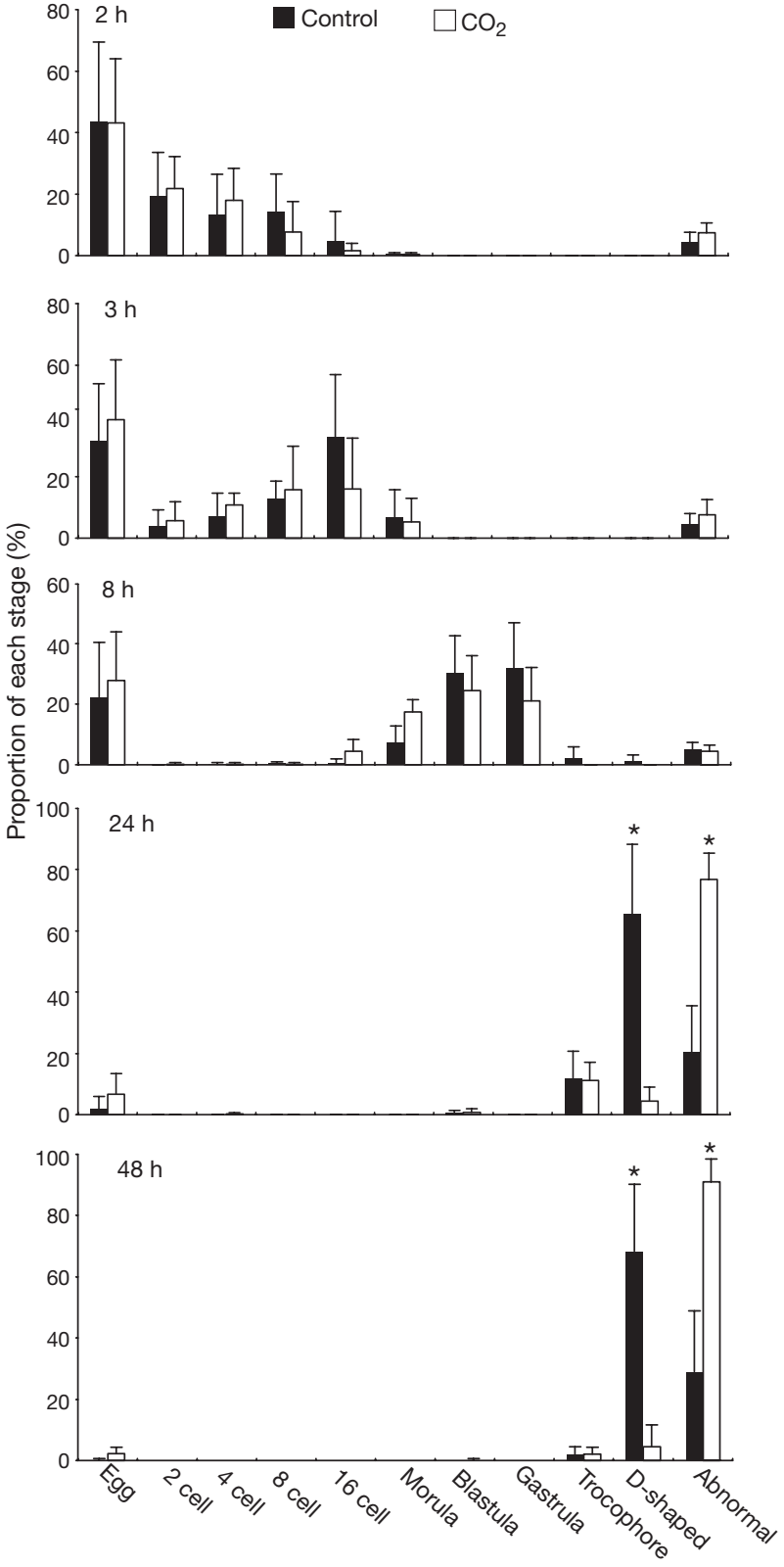

Fig. 1. *Crassostrea gigas*. Percentage distribution of each developmental stage of embryos/larvae incubated in control (black bars) or CO_2^- (white bars) seawater for 2, 3, 8, 24 and 48 h after fertilization (mean ± SD; n = 5). *Significant difference (paired *t*-test; $p < 0.005$)

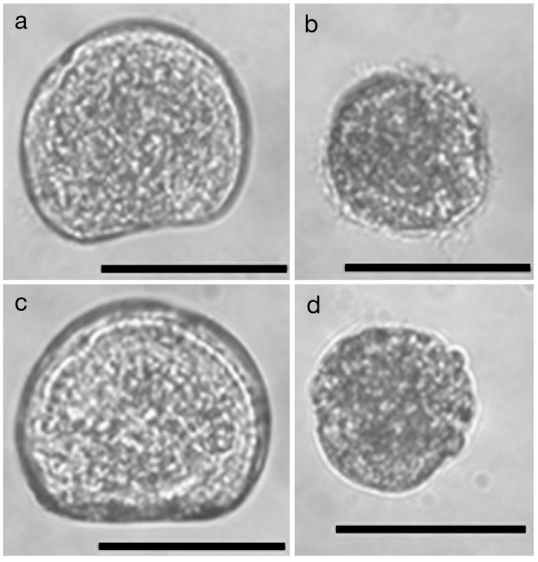

Fig. 2. *Crassostrea gigas*. Light micrographs of larvae incubated for (a,b) 24 h and (c,d) 48 h in (a,c) control or (b,d) CO_2-seawater. (a,c) Larval shells can be seen; (b,d) no larval shell formed. Scale bars = 50 mm

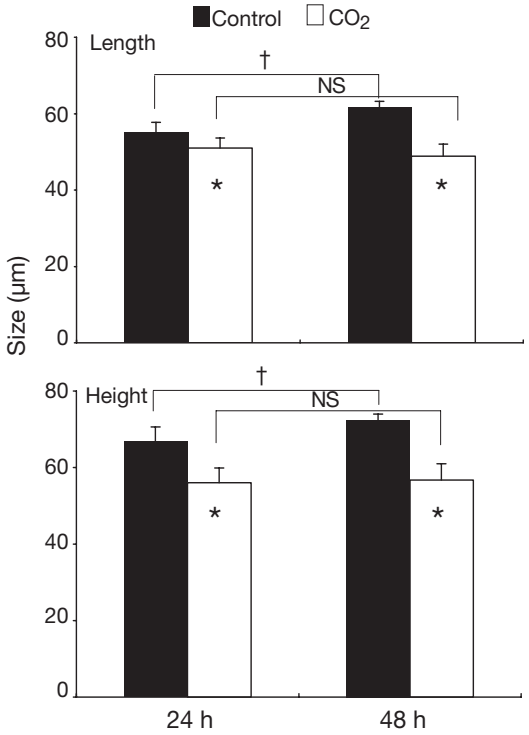

Fig. 3. *Crassostrea gigas*. Shell length and height of D-shaped larvae in control and CO_2 groups, determined 24 or 48 h after fertilization (mean ± SD; n = 5). *Significant difference between control and CO_2 groups at 24 h and 48 h (paired *t*-test, $p < 0.05$). †Significant difference between 24 and 48 h values within a group (paired *t*-test, $p < 0.05$). NS: not significant

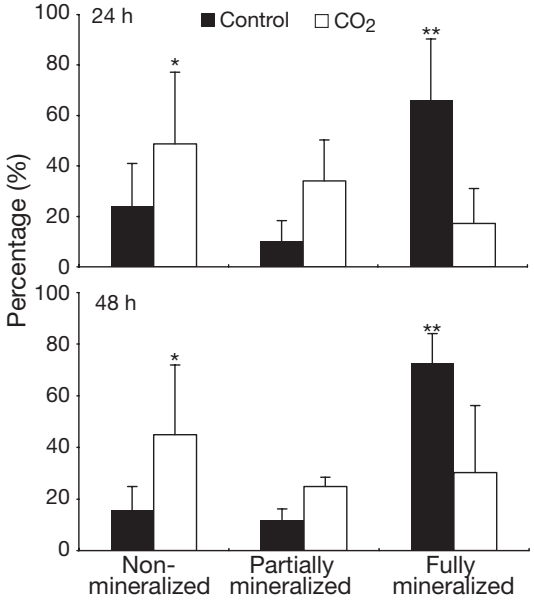

Fig. 4. *Crassostrea gigas*. Shell mineralization in embryos incubated for 24 or 48 h in control or CO_2 groups, determined by polarized light microscopy (mean ± SD; n = 5). Significant difference between control and CO_2 groups by paired *t*-test: $^{*}p < 0.05$, $^{**}p < 0.005$

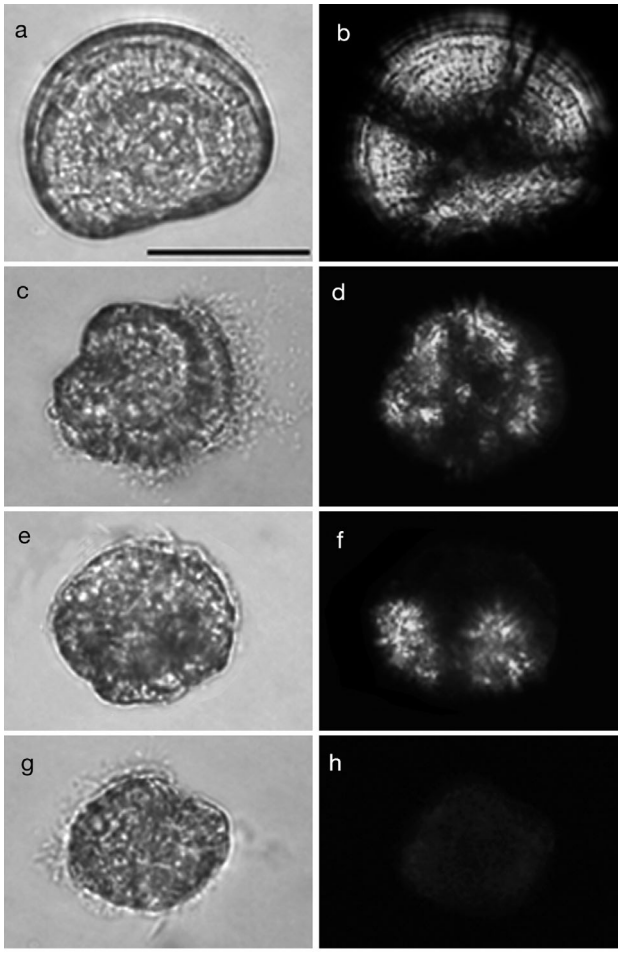

Fig. 5. *Crassostrea gigas*. Larval morphology after incubation for 48 h in (a,b) control or (c–h) CO_2-FSW. (a,c,e,g) Stereomicroscopic images of 4 different individuals; (b,d,f,h) polarized light microscopic images corresponding to (a,c,e,g). ~70% of control embryos developed into D-shaped larvae, which were fully covered by a $CaCO_3$ shell (a,b). Most CO_2 group embryos developed into abnormally shaped larvae either fully shelled (c, d), partially shelled (e, f), or not shelled (g, h). The CO_2 group larva in (c) was considerably smaller and had an irregular-shaped shell, although it was nearly fully mineralized. The CO_2 group larva in (e) showed limited mineralization in its shell, while the one in (g) showed no mineralization at all. Scale bar = 50 µm

manifest 24 h after fertilization. Only 4% of the embryos in the CO_2 group developed into D-shaped larvae by 24 h, in contrast to 65% of the control group (Figs. 1, 2a). The percentage of D-shaped larvae slightly increased to 68% in the control group by 48 h, whereas it remained unchanged in the CO_2 group (5%, Fig. 1). Nearly 80% of the embryos in the CO_2 group were abnormal in shape at 24 h (Figs. 1 & 2b), which increased to 91% by 48 h (Figs. 1 & 2d, see also Fig. 5).

Both shell length and height of the D-shaped CO_2-group larvae were significantly smaller than those of the control group at both 24 and 48 h (Fig. 3). Furthermore, no significant difference was detected in both shell length and height of the CO_2-group larvae between 24 and 48 h, in contrast to significant increases in both parameters for the control larvae (Fig. 3).

Fully mineralized individuals accounted for only 18 and 30% of the CO_2-group larvae at 24 and 48 h, respectively (Figs. 4 & 5c,d). These values are significantly lower than the respective 66 and 72% in the control group (Figs. 4 & 5a,b). In contrast, 48% (24 h) and 45% (48 h) of the CO_2-group larvae completely lacked mineralized shells (Figs. 4 & 5g,h); these values are significantly higher than those of the control group: 24% (24 h) and 16% (48 h) (Fig. 4). There was no significant difference in the number of partially shelled larvae (Fig. 5e,f) between the control and the CO_2 groups.

DISCUSSION

This study provides evidence that the increased seawater pCO_2 that is projected to occur by the year 2300 in the ocean surface is likely to seriously affect the early development of oyster embryos to the extent that possibly only 4 to 5% will develop into normal veliger larvae (this compares to ~70% in our control experiment) (Figs. 2 & 5). The percentage of normally developed embryos in our controls is lower than the value (91%) obtained by spontaneous spawning and

fertilization (His et al. 1997). However, Thain (1991) reported that gonadal stripping of adult bivalves could give lower percentages of normal embryos as compared with spontaneous production. Accordingly, we judged that our method for obtaining fertilized eggs was acceptable.

Embryonic development and larval morphology have been well documented for bivalves (see Waller 1981 for an early review), and the mechanism of larval shell formation has been a focus of recent investigations (Wilt 2005, Weiss & Schönitzer 2006). The larval shell is formed by a specialized group of ectodermal cells (called the 'shell gland') in the early embryos, the inner part of which is subsequently transformed into the larval mantle epithelium. Hayakaze & Tanabe (1999) studied the early larval shell development in the mussel *Mytilus galloprovincialis*, and showed that the organic matrix of the shell began to be secreted by the shell gland during the late trochophore larval stage, and calcification was indicated on the inner side of the organic matrix in the early veliger stage. Similarly, the calcification process in the early veliger stage has been described for the European oyster *Ostrea edulis* (Waller 1981). Since morphological effects of high pCO_2 were only apparent from the D-shaped veliger stage in our experiments, it appears that calcification during oyster embryogenesis is one of the processes most sensitive to CO_2 exposure, although other aspects such as protein synthesis and metabolism require further investigation. Similar adverse effects of high pCO_2 seawater on the synthesis of $CaCO_3$ spicules and morphology were reported in sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* pluteus larvae, even at a seawater pH of 7.8 (Kurihara & Shirayama 2004a,b).

Although the results of this study do not allow us to distinguish whether $CaCO_3$ synthesis was directly impaired or the synthesized shell was subsequently dissolved by the CO_2, we suspect that both phenomena occurred in the oyster larvae. We think that reduced $CaCO_3$ synthesis likely occurred in our experimental oysters, since many calcifying marine organisms reduce calcification rates under high CO_2 conditions (Kleypas et al. 2006). Dissolution of the larval shell is also likely for the following reasons: (1) The CO_2-FSW used in this study was undersaturated for aragonite and nearly at saturation for calcite (Table 2). (2) Oyster larvae initially deposit amorphous calcium carbonate (ACC) in the larval shell that is then partially transformed to aragonite (Carriker & Palmer 1979, Weiss et al. 2002), in contrast to adult oyster shells that are predominantly composed of calcite (Stenzel 1964). The K^*_{sp} of ACC is larger than that of aragonite and therefore more soluble (Brečević & Nielsen 1989). (3) Michaelidis et al. (2005) suggested that dissolution of $CaCO_3$ shells as a result of extracellular acidosis occurs in adult mussels *Mytilus galloprovincialis* reared in high pCO_2 seawater. Sea urchin larvae first deposit ACC that subsequently transforms to calcite with a high magnesium content in a few days during the larval stage (Politi et al. 2004). High magnesium calcite continues to be the main constituent of the exoskeleton in juvenile and adult sea urchins. Thus, we suspect that the $CaCO_3$ shells of bivalve larvae as well as spicules of sea urchin larvae are less tolerant to increased seawater pCO_2 than the corresponding structures in juveniles and adults.

We expect survivorship of bivalve larvae to decrease in high pCO_2 environments. Green et al. (2004) evaluated the effect of carbonate saturation state (with respect to aragonite) at the sediment– water interface (SWI) on the survivorship of juvenile bivalve *Mercenaria mercenaria*, and found significantly higher mortality rates and shell dissolution in undersaturated seawater than in supersaturated conditions. They also demonstrated that the mortality rates were higher for small size classes (0.2 and 0.3 mm) than for larger individuals (1 and 2.0 mm). Extremely high mortality rates (>98%) are a characteristic of settling marine clams (Thorson 1966), where an exponential loss is common within a few days following their recruitment from the pelagic to the benthic juvenile phase (Gosselin & Qian 1997). Green et al. (2004) postulated that dissolution-induced mortality by aragonite undersaturation at the SWI helps explain the exponential loss of juvenile bivalves. Relatively small perturbations in the initial population of settling marine bivalves translate to large alterations in adult populations (Gosselin & Qian 1997, Hunt & Scheibling 1997), and therefore the observed impacts of high pCO_2 seawater on the oyster larvae could have profound ecological implications to bivalve populations. Disruption of calcification by elevated pCO_2 may well diminish survivorship not only of bivalves, but of calcifying marine organisms in general, because $CaCO_3$ structures have vital functions such as protection, feeding, buoyancy regulation and pH regulation (Simkiss & Wilbur 1989).

In addition to the significant effects of CO_2 on *Crassostrea gigas*' developmental success into D-shaped larvae, CO_2 also inhibits shell growth (Fig. 3). Similarly, Kurihara & Shirayama (2004a) reported that size of sea urchin larvae at pluteus stage tended to be smaller with increased seawater CO_2. It has been postulated that smaller larvae encounter and clear less food than larger ones, and are therefore more prone to starvation (Anger 1987, Strathmann 1987, Hart & Strathmann 1995). Hence, CO_2 may reduce fitness of affected larvae through inefficient food intake capability, even when its concentration is sublethal.

In contrast to our results on the significant effects of seawater acidification (to pH 7.4) through increased pCO_2, Calabrese & Davis (1966) reported no signifi-

cant differences in embryonic development, larval growth, or survival of the American oyster *Crassostrea virginica*, when incubated in seawater of a pH between 6.75 and 8.75 (through the addition of hydrochloric acid or sodium hydroxide). Therefore, increased pCO_2 probably has more severe impacts than strong mineral acids on oyster early development, as observed for the early development of sea urchin embryos (Kurihara & Shirayama 2004a,b) and the mortality rate of embryos and larvae of the red seabream *Pagrus major* (Kikkawa et al. 2004). Kikkawa et al. (2004) speculated that the difference was due to the greater permeability of biological membranes to uncharged CO_2 molecules, compared with H^+ ions (Vandenberg et al. 1994). The alkalinity of our seawater (Table 2) was at the low end of alkalinity measurements in the ocean surface, which is typically ca. 2300 µmol kg^{-1} (Kleypas et al. 2006). Using the first and second dissociation constants of carbonic acid by Mehrbach et al. (1973), seawater with the typical alkalinity is expected to have pH 7.50 when equilibrated with pCO_2 of 2268 µatm, which is slightly higher than the measured seawater pH of 7.42 ± 0.02 SD. Thus, though we suspect it to be unlikely, if pH is responsible for the observed effects, the present results might have overestimated the effect of the pCO_2 used.

In conclusion, this study strongly indicates that the worst-case ocean acidification scenario, in which seawater pH is projected to decrease by 0.77 pH units by the year 2300, will bring about deleterious alterations in coastal ecosystems. It is possible that some marine organisms will adapt to the future acidified marine environments through genetic responses; however, we presently have no information on this possibility, except that the green alga *Clamydomonas reinhardtii* did not show genetic selection for growth even after exposure to 1050 µatm CO_2 for 1000 generations (Collins & Bell, 2004). Further studies are needed to evaluate adaptability of marine organisms to future acidified oceans. In addition, increased atmospheric CO_2 will not only acidify surface oceans but also simultaneously increase the seawater temperature and the sea level (Denman et al. 2007); their synergetic effects on the marine ecosystem require clarification.

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