INTRODUCTION

Anthropogenic emissions of atmospheric carbon dioxide (CO₂) have increased and led to a rapid increase in atmospheric CO₂ concentration (Canadell et al. 2007). Atmospheric CO₂ concentration is expected to rise to 550–1000 ppm by the end of this century (scenarios B1-A1F1; IPCC 2007), and reach 2000 ppm (projected emission of 5000 pg C) by the year 2300 (Caldeira & Wickett 2003). By diffusion into ocean surface waters, the increasing atmospheric CO₂ increases seawater pCO₂ (partial pressure of CO₂), which leads to dissociation of carbonic acid, which decreases both seawater pH (termed ocean acidification) and carbonate ion concentration [CO₃²⁻] (Caldeira & Wickett 2003, Feely et al. 2008). The [CO₃²⁻] largely determines the calcium carbonate (CaCO₃) saturation state of seawater, since [Ca²⁺] varies little under given salinity conditions:

\[ \Omega = [\text{CO}_2^{2-}] [\text{Ca}^{2+}] / K_{sp} \]  

(1)

where [Ca²⁺] is the calcium ion concentration and K_{sp} is the stoichiometric solubility product of CaCO₃.

Models predict that the intensification of ocean acidification will make the entire ocean surface undersaturated with respect to aragonite (the more soluble crystalline form of common biogenic CaCO₃ minerals) by the year 2300 (Feely et al. 2004, Caldeira & Wickett 2005). Hence, the predicted increase in seawater pCO₂ may have catastrophic impacts on marine organisms, especially marine calcifiers (Orr et al. 2005, Raven et al. 2005, Fabry et al. 2008, Doney et al. 2009).

Research into the effects of elevated seawater pCO₂ on marine organisms has so far focused on corals, and
has demonstrated that high seawater pCO2 reduces calcification rates of coccolithophores (Riebesell et al. 2000) and corals (Kleypas et al. 1999, Leclercq et al. 2000, 2002, Langdon & Atkinson 2005, Guinotte et al. 2006, Kleypas et al. 2006, Hoegh-Guldberg et al. 2007, Cooper et al. 2008). Several studies have also shown reduced calcification under elevated CO2 conditions in other marine calcifiers such as molluscs (Orr et al. 2005, Gazeau et al. 2007, Kurihara et al. 2007), echinoderms (Miles et al. 2007) and crustaceans (Spicer et al. 2007), although 2 recent studies demonstrated the opposite, i.e. increased calcification under elevated CO2 conditions in the coccolithophore Emiliania huxleyi (Iglesias-Rodriguez et al. 2008) and the ophiuroid brittlestar Amphiura filiformis (Wood et al. 2008). Furthermore, increases in seawater CO2 disrupt physiological processes other than calcification: acid–base regulation, metabolism, growth, and immune responses in marine bivalves (Michaelidis et al. 2005, Berge et al. 2006, Bibby et al. 2008). Thus, high CO2 appears to interfere with a spectrum of homeostatic and developmental processes of affected animals, with both direction and intensity of a response varying from species to species.

Molluscs play an important role in coastal ecosystems as ecosystem engineers (Gutiérrez et al. 2003), food source for other marine organisms (Nagarajan et al. 2006), and carbonate producers. Marine benthic molluscs produce 50 to 1000 g CaCO3 m–2 yr–1, with the value for oyster as high as 90 000 g CaCO3 m–2 yr–1 (Gutiérrez et al. 2003). These facts make marine molluscs one of the key groups of animals in investigations on the biological impacts of ocean acidification. Investigation on early life stages is particularly of importance since mortality during early life stages is the decisive factor regulating subsequent adult population size of benthic marine invertebrates (Gosselin & Qian 1997). Most previous studies on the effect of ocean acidification have dealt with adult individuals; little is known about CO2 impacts on marine organisms during early development. In addition, several toxicity studies rank bivalve life stages in terms of relative sensitivity to sublethal and lethal effects as: embryos > veligers (D-larvae) > metamorphosing larvae > pediveligers > adults (Beiras & His 1994, His et al. 1999). Shell synthesis is significantly suppressed when larvae of the oyster Crassostrea gigas are exposed to seawater equilibrated with CO2-enriched air (2000 ppm) (pH 7.4; Kurihara et al. 2007). Green et al. (2004) indicated that aragonite undersaturation of seawater led to significant mortality of just-settled juveniles of Mercenaria mercenaria. These results suggest that embryos and larvae of bivalves are critically sensitive to elevations of seawater pCO2. In the present study, we aimed to evaluate the effect of high-CO2 seawater (2000 ppm) on the early development of the mussel Mytilus galloprovincialis, and compare CO2 sensitivity of mussels and oysters, to examine the generality of the previous findings among bivalves and to evaluate interspecific differences in CO2 vulnerability.

MATERIALS AND METHODS

Test animals. For each experimental run, 8 to 10 adult mussels Mytilus galloprovincialis (30 to 40 mm shell length) were collected by hand from buoys of aquaculture cages placed in the harbor fronting the Institute for East China Sea Research, Nagasaki University, Japan (32°48’34.21”N, 129°46’15.07”E). Seawater temperature and salinity at the time of collection ranged from 13 to 15°C and 33 to 34, respectively. The mussels were brought to the laboratory after collection and immediately used for the experiments, which were conducted 5 times from February through March 2007 at 14°C.

Seawater. Filtered (GF/C 1.2 µm) and sterilized (121°C, 15 min) seawater (FSW) was placed in two 1 liter beakers, and was bubbled with air (control, CO2 concentration: 380 ppm) or CO2-enriched air (CO2-seawater). By regulating flow rates of air and CO2 gas with flow meters (Kofloc 250) and monitoring with a CO2 gas analyzer (Telaire 7001), the CO2 concentration of the gas mixture was adjusted to 2000 ppm. The pH (NBS scale) and the O2 saturation (%) of the test seawater were measured before each experiment with a pH electrode (Mettler Toledo InLab413SG-IP67) connected to a pH meter (Mettler Toledo MP125) and with an O2 electrode (TOA OE-270AA) connected to an O2 meter (TOA DO14-P), respectively. Seawater salinity (35.5) was determined with a refractometer (Atago 100-S), alkalinity with a pH meter (PHM290, Radiometer) and an autoburette (ABU901, Radiometer), and [Ca2+] with a calcium electrode (Thermo, Orion model 9720BN) connected to an ion analyzer (Orion EA 920). Dissolved carbon dioxide [CO2], bicarbonate [HCO3–] and carbonate [CO32–] concentrations were calculated using the first and second dissociation constants of carbonic acid (Mehrbach et al. 1973), and calcite (Ωcalc) and aragonite (Ωarag) saturation using stoichiometric solubility products for respective crystalline forms (Mucci 1983) (Table 1).

Bioassay. Eggs and sperm were obtained as follows: collected individuals were kept individually in 200 ml beakers containing control FSW, the temperature of which was adjusted to be ~5° higher (18°C) than the natural seawater temperature (13–14°C) until the mussels released eggs or sperm into the FSW (~1 h). Each beaker with released eggs and sperm was inspected visually under a light microscope to check the number...
of eggs and sperm motility, and the batches with the largest quantity of eggs or the highest motility of sperm were used. Thus, sperm from 1 male and eggs from 1 female were used in each of 5 experimental runs. The eggs were rinsed several times with FSW, and transferred into Petri dishes containing control- or CO2-seawater. After rechecking sperm mobility under a microscope, a few drops of the sperm suspension in seawater. After rechecking sperm mobility under a microscope, a few drops of the sperm suspension in seawater was added to the Petri dishes containing eggs. After 15 min, the fertilized eggs were rinsed with control or CO2-seawater to remove excess sperm. A preliminary test confirmed the constancy of seawater pH during the 15 min fertilization period. Thereafter, ~400 embryos were transferred from each dish into six 50 ml vials for each experimental group (control or high-CO2 group) keeping the egg density at 10 eggs ml–1. The vials were completely filled with control or CO2-seawater to remove excess sperm. A preliminary test confirmed the constancy of seawater pH during the 15 min fertilization period. Thereafter, ~400 embryos were transferred from each dish into six 50 ml vials for each experimental group (control or high-CO2 group) keeping the egg density at <10 eggs ml–1. The vials were completely filled with control or CO2-seawater and sealed to prevent CO2 exchange with the ambient air. All vials were incubated at 13°C. The test seawater of the vials was renewed at 55, 96 and 121 h. At 2, 4, 24, 54, 120 and 144 h after fertilization, half of the seawater was removed from one vial of each group using a pipette with a mesh attached on its tip to prevent sucking embryos, then developing embryos and larvae were fixed by adding 10% neutralized formalin-seawater solution to attain the final formalin concentration of 5%, for ordinary light and polarized light microscopy. At 120 and 144 h, some larvae were transferred into 2% glutaraldehyde-paraformaldehyde in 0.2 M cacodylate buffer in seawater at pH 8.1, for scanning electron microscopy (SEM). Immediately before fixing, pH of the control and CO2-seawater was rechecked to ensure that no significant changes occurred during the incubation (Fig. 1). Oxygen saturation was determined at 55, 96 and 121 h, and remained above 90%. A total of 100 embryos or larvae from each 50 ml vial were observed under a light microscope, and the number of individuals in each developmental stage was counted. Morphological criteria proposed by His et al. (1997) were adopted to differentiate normal D-shaped larvae. To determine the effects of CO2 on shell growth, D-shaped larvae were mounted on a glass slide, photographed and measured for shell length (anterior to posterior dimension of the shell parallel to the hinge line) and height (dorsal to ventral dimension perpendicular to the hinge) under a microscope with an ocular micrometer. Larvae incubated for 54, 120 and 144 h were also observed with a polarized light microscope to evaluate the degree of shell mineralization, which we estimated based on the observed birefringence due to the mineral phase (Weiss et al. 2002; see Kurihara et al. 2007 for further information). For SEM, embryos were dehydrated through a series of ethanol, freeze-dried in t-butylalcohol and mounted onto SEM stubs. Embryos were coated with platinum and then examined using a scanning electron microscope (JEOL model JSM-6460).

**Statistics.** Statistical analyses were performed using JMP software package, version 7 (SAS Institute, Cary, NC). Data are based on 5 replicate experimental runs, each with eggs from 1 female and sperm from 1 male. Three-way ANOVA was used to compare the percentage of each developmental stage between treatments (control vs. CO2-seawater) and time (2 to 144 h after fertilization). Paired t-test was used to compare the percentage of (1) embryos at each developmental stage between control and CO2-seawater at 2, 4, 24, 54, 120 and 144 h after fertilization, and (2) normal and abnormal D-shaped larvae at 122 and 144 h. Repeated measures ANOVA and Tukey-Kramer tests were run to compare shell length and height of control and CO2-seawater individuals at 54, 120 and 144 h.

**RESULTS**

The pH of control seawater varied from 8.00 to 8.14 (mean 8.05 ± 0.05 SD) during the experiment, whereas that of CO2-seawater ranged from 7.41 to 7.46 (mean 7.43 ± 0.02, Fig. 1). There were significant interactions between Treatment (control vs. CO2-seawater), Developmental stage and Time (3-way ANOVA, F = 37.9, df = 35,385, p < 0.001). There was no significant difference in the percentage distribution of each stage between control and CO2-seawater embryos until the gastrula stage (Fig. 2). However, at 54 h after fertilization, all individuals developed into D-shaped larvae in the control, in contrast to only 20% in the high-CO2 group (paired t-test, p < 0.005; Fig. 2). Approximately 70% of the CO2-seawater larvae remained at the trophophore stage, demonstrating a development delay. At 120 and 144 h, all CO2-seawater individuals developed into D-shaped larvae, but nearly all (>99%) had
abnormal morphology compared to <1% of control larvae (paired t-test, p < 0.005; Figs. 2 & 3). The abnormalities in CO₂-seawater embryos included indentation of the shell margin (1 to 2%; Fig. 3b), protrusion of the mantle from the shell (23 to 24%; Fig. 3c), convexation of the hinge (8 to 11%; Fig. 3d) and a combination of the latter 2 conditions (64 to 65%; Fig. 3e).

Both shell length (repeated measures ANOVA, \( F = 988, \text{df} = 1,8, p < 0.001 \)) and height (repeated measures ANOVA, \( F = 326, \text{df} = 1,8, p < 0.001 \)) of the CO₂-seawater larvae were significantly smaller than those of control larvae at 54, 120 and 144 h (Tukey-Kramer test, p < 0.05; Fig. 4).

DISCUSSION

The present results demonstrate that exposure to seawater acidified by equilibrating with air containing 2000 ppm CO₂ (pH 7.4) leads to morphological abnormalities and a significant size reduction in D-shaped larvae of the mussel *Mytilus galloprovincialis*. The observations under polarized light and SEM provided evidences of disrupted shell formation and dissolution in the CO₂-seawater larvae: the rugged shell surface, and the absence of the prodissococonch I/II boundary and commarginal growth lines. Other morphological abnormalities include convex hinge and protrusion of
the mantle, both of which are typical criteria used to distinguish between normal and abnormal development of veliger larvae in embryotoxicology bioassay (His et al. 1997). Although no observations were made on larval behaviour in the present study, Beiras & His (1994) reported swimming inhibition in larvae with protruded mantle, which could reduce environmental fitness.

The present results are consistent with our earlier findings on CO₂ effects in the oyster Crassostrea gigas larvae (Kurihara et al. 2007). In both Mytilus galloprovincialis and C. gigas, morphological differ-
ences between control and experimental larvae became manifest at stages when the larval shell starts to form (trochophore stage), and only 0.2 and 4% of high-CO$_2$ exposed *Mytilus galloprovincialis* and *C. gigas* larvae, respectively, developed into normal D-shaped larvae. Since no effects were detected before the trochophore stage, the most vulnerable process during larval development appears to be shell synthesis. Shell formation in molluscs is a complex process, the mechanisms of which are still not well understood. The larval shell is thought to be formed in the early embryos by a specialized group of ectodermal cells called the shell gland, the inner part of which is subsequently transformed into the larval mantle epithelium (Wilt 2005, Weiss & Schönitzer 2006). Hayakaze & Tanabe (1999) showed that the organic shell in *M. galloprovincialis* larvae begins to be secreted by the shell gland at the late trochophore stage, and that calcification was initiated underneath the organic shell in the early veliger stage. Since the CO$_2$-seawater used in the present study was undersaturated for both aragonite and calcite (Table 1), we suspect that CaCO$_3$, even if normally deposited, incurred dissolution from the larval shell. We also speculate that elevated seawater CO$_2$ influenced biological processes responsible for larval shell calcification. In a study of molecular mechanisms of larval shell formation, Weiss & Schönitzer (2006) demonstrated the presence of chitinous material in the larval shell of *M. galloprovincialis*; in a subsequent study, Schönitzer & Weiss (2007) showed that larval shell formation was radically impaired by treatment of the *M. galloprovincialis* larvae with a chitin synthesis inhibitor, Nikkomycin Z. Observed types of larval shell malformation at the organism scale included asymmetry of the 2 shell valves, reduced size, shell undulation, and convex hinge, which are similar to our observations in high-CO$_2$ exposed embryos. At present, we have only started to list phenotypic alterations in high-CO$_2$ exposed marine bivalves; further research is needed to understand underlying mechanisms of the CO$_2$ effects.

Despite the overall similarity of CO$_2$ effects on the early development of the 2 bivalve species, the effect of CO$_2$ on larval shell mineralization is less severe in *Mytilus galloprovincialis* than in *Crassostrea gigas*, suggesting interspecific differences in CO$_2$ tolerance. All high-CO$_2$ exposed mussel larvae were fully mineralized in spite of the morphological abnormalities, while only 30% of high-CO$_2$ exposed oysters were...
fully mineralized, with the rest either completely lacking a shell or developing only a partially mineralized shell (Kurihara et al. 2007). In contrast to our findings on larval individuals, Gazeau et al. (2007) demonstrated that the net calcification of adult C. gigas was less sensitive to elevated seawater pCO₂ than that of adult M. edulis, suggesting ontogenetic changes in CO₂ vulnerability. Gazeau et al. (2007) ascribed the observed difference in calcification under elevated CO₂ conditions to differences in shell mineralogy between the species. Adult oyster shells are mainly composed of calcite (Stenzel 1964), while adult mussel shells contain up to 83% aragonite (M. edulis; Hubbard et al. 1981). Since the K*ₐ is larger for aragonite than for calcite, aragonite is less stable and therefore more soluble than calcite (Zeebe & Wolf-Gladrow 2001). On the other hand, aragonite has been identified as the CaCO₃ mineralization form in the larval shells of 2 bivalve species, the hard clam Mercenaria mercenaria and the Eastern oyster C. virginica (Stenzel 1964, Weiss et al. 2002), which raises the possibility that the larval shell of other bivalves including mussels may also consist of aragonite. If so, the observed difference in CO₂ sensitivity of calcification in mussel and oyster larvae cannot be explained by mineralogical considerations.

Similar species-specific responses to high pCO₂ seawater are found in scleractinian corals and calcifying algae. Marubini et al. (2003) reported that although 4 scleractinian corals (Acropora verweyi, Galaxea fascicularis, Pavona cactus and Turbinaria reniformis) cultured under 866 ppm CO₂ (pH 7.76) showed significantly suppressed calcification rates, the magnitude of microstructural crystallization responses was highly species-specific. They suggested that these differences are due to reaction differences of the organic matrix which produce differences in the size and shape of growing crystal fibres. On the other hand, in calcifying coccolithophores, the calcification rate of some species is sensitive to high CO₂, whereas that of other species is not (Riebesell et al. 2000, Langer et al. 2006, Iglesias-Rodriguez et al. 2008). These differences in CO₂ sensitivity could also be explained by genetic diversity between species (Fabry et al. 2008).

Another possible explanation for the difference in CO₂ sensitivity between Mytilus galloprovincialis and Crassostrea gigas is the difference in experimental temperature. Because C. gigas spawns in summer, the experiment for C. gigas was conducted at 23°C (Kurihara et al. 2007), which is 10°C higher than that used in the present study. A possible temperature dependence of CO₂ impact was also reported for 2 species of sea urchins by Kurihara & Shirayama (2004), who found a greater negative effect of high CO₂ on egg fertilization in Echinometra mathaei (experiments conducted in June to October at 24°C) than in Hemicentrotus pulcherrimus (January to March at 14°C). Thus, the negative effects of high CO₂ on marine animals may act synergistically with higher temperatures. If true, the ocean ecosystem may suffer from greater damages than have been predicted by experiments in the context of global warming or ocean acidification alone. In fact, Reynaud et al. (2003) demonstrated that elevations in both CO₂ and temperature synergistically reduced the calcification rate of scleractinian corals. Conversely, Zeebe & Wolf-Gladrow (2001) reported CaCO₂ to be more soluble at lower temperatures. Further comparison of CO₂ sensitivity is needed for marine organisms from different latitudes.

The present study revealed that the maximum CO₂ concentration predicted to occur in the next centuries would significantly affect the early development of mussels. As in our previous study on the oyster, we collected eggs and sperm of the ‘best’ quality for the present experiment (see ‘Material and methods – Bioassay’), which may have led to underestimation of the effects on natural populations of the 2 species. Michaelidis et al. (2005) reported that incubation of adult mussels for 3 mo under high CO₂ conditions (pH 7.3) caused a decrease in metabolic rate and shell size. Synergy effects may result in dwindling mussel populations, unless mussels acclimate to the predicted environmental alteration. Marine mussels are dominant competitors for space and can exclude other large benthic organisms such as kelps and barnacles that require primary space for attachment (Paine 1966). Mussel beds provide a biogenic habitat for algae and small-bodied invertebrates, thereby playing an important role in structuring intertidal communities (Suchanek 1992). Mussel beds also form an important trophic link in intertidal food webs, being consumed by whelks, crabs, lobsters, sea stars, fish, shore birds and sea otters (Seed & Suchanek 1992), and also have a high commercial value as food source for humans. Hence, ocean acidification due to increasing CO₂ may result in considerable ecological and economic losses through negative effects on mussel populations.

In conclusion, the results of our present and previous studies on mussel and oyster larvae demonstrate that high-pCO₂ seawater is harmful to the early development of marine bivalves as it affects shell synthesis. Considering the different taxonomic positions of the 2 species (Mytilus galloprovincialis: Order Mytiloida, Crassostrea gigas: Order Ostreoida), it is quite possible that early development of other bivalves is similarly affected by high-CO₂ seawater, although further verification is necessary for other bivalves.
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