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Elevated pCO_2 exposure during fertilization of the bay scallop *Argopecten irradians* reduces larval survival but not subsequent shell size

Meredith M. White[1,3,*], Lauren S. Mullineaux[1], Daniel C. McCorkle[2], Anne L. Cohen[2]

[1]Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA
[2]Department of Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

[3]*Present address:* Bigelow Laboratory for Ocean Sciences, East Boothbay, Maine 04544, USA

ABSTRACT: Ocean acidification, characterized by elevated partial pressure of CO_2 (pCO_2), generally has negative effects on early life stages of invertebrates. We tested the idea that fertilization is a critical CO_2 exposure stage for the bay scallop *Argopecten irradians* by determining the effects on bay scallops of exposure to high CO_2 (pCO_2 ~2600 ppm, pH ~7.30) from fertilization to 7 d old. To assess the possibility of persistent effects of exposure during fertilization, further treatments included switches from high CO_2 to ambient CO_2 (pCO_2 ~480 ppm, pH ~7.96) and from ambient CO_2 to high CO_2 at 2 h post-fertilization. Survival of larvae decreased significantly when they were fertilized in high CO_2. A switch in CO_2 conditions 2 h post-fertilization did not change this effect, suggesting that the critical exposure window for this survival effect is within the first 2 h. In contrast, CO_2 conditions during fertilization did not affect larval shell size, but the switch treatments showed that exposure to high CO_2 after 2 h post-fertilization decreased shell size, indicating that the exposure window for a size effect was later in development, possibly during shell calcification. Finally, a shell deformity was seen in scallops with continuous exposure to high CO_2 and those switched from ambient CO_2 to high CO_2 at 2 h post-fertilization. Decreased survival during fertilization and smaller larval shell size due to ocean acidification could ultimately reduce the population size of this commercially important bivalve, which has already seen dramatic population decline due to loss of juvenile habitat.

KEY WORDS: Ocean acidification · Bay scallop · Early development · Hypercapnia · Shell development · Fertilization

INTRODUCTION

The ocean and the atmosphere naturally exchange large amounts of carbon dioxide (CO_2), but as human activities, primarily fossil fuel burning, increase the atmospheric concentration of CO_2, the ocean is taking up an increasing amount of CO_2 (Le Quéré et al. 2009). Once in the ocean, dissolved CO_2 goes through a series of reactions that release H^+ ions, decreasing the pH of the water — a process known as ocean acidification (OA) (Feely et al. 2004, Orr et al. 2005, Doney et al. 2009). In addition to lowering seawater pH, OA also results in a decrease in the calcium carbonate saturation state (Ω), which has the potential to make calcification (shell building) more difficult or more energetically costly for calcifying organisms (Orr et al. 2005, Gazeau et al. 2007, Waldbusser et al. 2011).

While OA is affecting the surface ocean on a global scale, additional processes affect pH and Ω in coastal

*Corresponding author: meredith@whoi.edu

and estuarine environments, and coastal OA conditions are more extreme than open-ocean OA conditions (Feely et al. 2010, Cai et al. 2011, Howarth et al. 2011). Water chemistry in coastal and estuarine environments is influenced by many factors, including freshwater inputs, eutrophication, respiration, and stratification (Borges & Gypens 2010, Cai et al. 2011, Waldbusser et al. 2011, Melzner et al. 2013, Sunda & Cai 2012). For example, when algae in blooms resulting from eutrophication die and sink to the seafloor, they fuel microbial respiration, which releases CO_2 (Rabalais et al. 2002, Diaz & Rosenberg 2008). Stratification during summer months isolates the CO_2 released from benthic microbial respiration (Chou et al. 2009) and can result in bottom waters that are undersaturated with respect to calcium carbonate (Feely et al. 2010). Models have shown that the carbonate chemistry of coastal environments is strongly influenced by eutrophication (Borges & Gypens 2010) and that at current atmospheric CO_2 levels, eutrophication can cause bottom waters to become severely undersaturated (saturation state of aragonite, $\Omega_{aragonite}$, <0.3) (Melzner et al. 2013).

Marine invertebrates living in coastal and estuarine environments are exposed to dissolved CO_2 levels that fluctuate on time scales ranging from daily to seasonal as a result of both natural processes and human activities (Cai & Wang 1998, Hofmann et al. 2011, Howarth et al. 2011, Saderne et al. 2013). Many coastal bivalve species spawn during summer months, when O_2 and pH tend to be low as a result of stratified waters and increased microbial respiration. The coinciding timing of unfavorable conditions and spawning is a serious issue for marine invertebrates, because early life stages are key for successful recruitment and survival of the species (Cowen et al. 2000), and may be particularly vulnerable to both chemical and physical environmental stressors (Pechenik 1987).

Several studies have shown negative effects of high CO_2 conditions on early life stages of marine invertebrate species, especially bivalve species that produce calcareous shells. Some bivalve species appear to be more tolerant of high CO_2 conditions than others (Miller et al. 2009), but studies have shown negative effects of high CO_2 on all early life stages of bivalves, including fertilization, larval development, and juvenile development (Miller et al. 2009, Parker et al. 2009, Watson et al. 2009, Gazeau et al. 2010, Parker et al. 2010, Gazeau et al. 2011, Waldbusser et al. 2011, Van Colen et al. 2012). Talmage & Gobler (2010) showed that larvae of hard clams *Mercenaria mercenaria* and bay scallops *Argopecten irradians* experienced delayed metamorphosis at present-day CO_2 concentrations relative to lower pre-industrial CO_2 levels, suggesting that the pH decrease since pre-industrial times has already had an impact on larval development. Gazeau et al. (2010) demonstrated that blue mussels *Mytilus edulis* had significantly smaller shells at 2 d old when exposed to water with pH 7.6, relative to water with pH 8.1, and had significantly thinner shells at 15 d old when exposed to water with pH 7.8, relative to water with pH 8.0. In addition to developmental delays and stunted growth, decreases in survival of larval bivalves have also been observed; Watson et al. (2009) found that survival of oyster larvae *Saccostrea glomerata* raised in pH 7.6 was reduced by 72% relative to larvae raised in pH 8.1.

Most previous bivalve studies introduced larvae to high CO_2 conditions after embryonic development, or even after larval development had begun, leaving open the question of effects during fertilization. However, Byrne (2010, 2011) pointed out that if embryogenesis fails due to a stressor, then the stressor's impact on larval development becomes less relevant. The few studies that have examined high CO_2 effects during fertilization have produced mixed results. Both the Sydney rock oyster *Saccostrea glomerata* and the Pacific oyster *Crassostrea gigas* had reduced fertilization success and impaired larval development when exposed to pH 7.85 and suboptimal temperatures, relative to exposure to pH 8.2 at optimal temperature (Parker et al. 2009, 2010). Barros et al. (2013) confirmed reduced fertilization success and reduced larval viability in *C. gigas* exposed to low pH conditions, and further found reduced sperm motility and hatching success. Similarly, a decrease in fertilization success of the clam *Macoma balthica* was seen when gametes were fertilized at pH ~7.5 and ~7.8, compared with pH 8.1 (Van Colen et al. 2012). In contrast, Havenhand & Schlegel (2009) found no significant difference in fertilization success in *C. gigas* at pH 8.15 and 7.8, nor did they find a difference in sperm mobility or swimming speed. It is possible that the conflicting results for the same species arose due to experimental differences among the interstudy comparisons (Byrne 2011). The studies described here did not examine how exposure to elevated CO_2 during fertilization affects bivalve larval survival and development beyond 3 d of age, nor did they investigate whether negative effects differ based on the timing of early exposure.

The mechanism of and larval response to OA effects may differ depending on when during development the larvae are exposed, and such stage-

specific responses may in part be responsible for inconsistencies among previous studies in the literature. Previous work has shown a decrease in larval growth, first seen at 24 h and persisting throughout the first week of development in bay scallop *Argopecten irradians* larvae exposed to pH 7.4 (White et al. 2013), indicating that the first day is critical to overall larval growth of this commercially important species. However, it is unclear what period during the first 24 h of development creates this impact on larval growth. To further investigate the idea of stage-specific responses and to determine whether fertilization is a critical exposure stage, we designed a culturing experiment where adults were induced to spawn in either ambient or high CO_2 conditions and the resulting larvae were raised for 1 wk. Additionally, half of the embryos from each fertilization group were switched to the alternative CO_2 level at 2 h post-fertilization. This switch allowed us to determine whether any negative effects of high CO_2 exposure during fertilization could be reversed by subsequent ambient exposure, and to isolate the stage during early development (i.e. 0 to 2 h post-fertilization or after 2 h) when larvae exhibit particular responses. Furthermore, the timing of this switch in exposure conditions has ecological importance, as estuarine and near-shore communities have been shown to experience diurnal pH and partial pressure of CO_2 (pCO_2) cycles (Hofmann et al. 2011, Saderne et al. 2013). Embryos and larvae developing in such communities would therefore be subject to changing pCO_2 conditions as they develop.

MATERIALS AND METHODS

Adult collection

Adult *Argopecten irradians* individuals were collected during winter and spring months of 2012 from coastal waters around Martha's Vineyard and Woods Hole, Massachusetts, and were held in submerged cages in Little River, an estuarine river near Waquoit Bay, Massachusetts, until needed. The pH at this location ranges from ~8.2 during winter months to ~7.6 during summer months. For the spring months when adult scallops were held in cages prior to these experiments, monthly pH values ranged from 7.8 to 8.1, and monthly pCO_2 values ranged from 340 to 790 ppm (McCorkle et al. 2012). Scallops were collected under a research collection permit issued by the Commonwealth of Massachusetts Department of Fish and Game, Division of Marine Fisheries. Several days prior to spawning, the adults were brought to Woods Hole Oceanographic Institution, where they were maintained in 16°C flowing seawater and fed daily with Instant Algae Shellfish Diet (Reed Mariculture). During feedings, the flowing water was stopped for 2 h and adults were fed at a concentration of 200 000 cells ml^{-1}.

Manipulation of water chemistry

Water chemistry was manipulated using mass flow controllers (Aalborg) to precisely mix compressed air and pure CO_2, as described by White et al. (2013). Treatments were bubbled with either compressed air (ambient conditions) or a compressed air and CO_2 mixture (high CO_2 conditions). We chose both ambient and high CO_2 conditions that are represented in local coastal and estuarine waters during summer months when many bivalve species (including bay scallops) spawn. The ambient CO_2 treatment (target pCO_2 ~480 ppm, Table 1) is similar to carbonate chemistry conditions during the summer in Great Harbor, Woods Hole, Massachusetts (D. C. McCorkle unpubl.), where bay scallops can be found throughout the year. The high CO_2 treatment (target pCO_2 ~2600 ppm, Table 1) produced carbonate chemistry conditions comparable to those seen in Childs River, Waquoit Bay, Massachusetts, during summer months (McCorkle et al. 2012). Childs River is particularly isolated within Waquoit Bay and is heavily influenced by terrestrial water sources, leading to stratified waters (Thompson 2011) with extremely seasonally elevated pCO_2 and decreased pH and $\Omega_{aragonite}$ (McCorkle et al. 2012). While bivalve larvae have been found at Childs River during summer months (Thompson 2011), there are no longer adult bay scallop populations at that location, presumably due to loss of juvenile eel grass habitat (US EPA 2002). Therefore, the ambient treatment represents conditions in which adult bay scallops are known to spawn, while the high CO_2 treatment represents conditions that are more extreme, but to which bay scallop larvae may be exposed to at present through larval transport or in the future as coastal conditions deteriorate.

Characterization of water chemistry

To characterize the carbonate chemistry of the treatment water, pH measured on the total hydrogen ion concentration scale (pH_T), total alkalinity, salin-

Table 1. Mean (±SD) water chemistry for the ambient and high CO_2 conditions during Experiments 1 and 2. Values for the spawning baths reflect the conditions in which adult scallops were induced to spawn; values for buckets represent the equilibrated water used to replace old culture water at water changes; values for culture cups represent the water in culture cups just prior to each water change; values for blank cups represent the water in culture cups with no larvae or algae added, just prior to each water change. pH measured on the total hydrogen ion concentration scale (pH_T), total alkalinity (A_T), temperature, and salinity were measured; all other data were calculated from pH_T and alkalinity using CO2SYS software (Pierrot et al. 2006) using the first and second dissociation constants of carbonic acid in seawater from Mehrbach et al. (1973) refit by Dickson & Millero (1987), and $KHSO_4$ from Dickson (1990). DIC = dissolved inorganic carbon; $[HCO_3^-]$ = bicarbonate ion concentration; $[CO_3^{2-}]$ = carbonate ion concentration; $[CO_2]$ = dissolved carbon dioxide concentration; $\Omega_{aragonite}$ = aragonite saturation state

Parameter	n	pH_T	A_T (µEq kg^{-1})	Temperature (°C)	Salinity	pCO_2 (µatm)	DIC (µmol kg^{-1})	$[HCO_3^-]$ (µmol kg^{-1})	$[CO_3^{2-}]$ (µmol kg^{-1})	$[CO_2]$ (µmol kg^{-1})	$\Omega_{aragonite}$
Experiment 1											
Ambient spawning bath	1	7.93	2111	19.8	31.5	511	1942	1798	127	17	2
Ambient bucket	8	7.96 ± 0.01	2135 ± 17	23.6 ± 0.5	31.7 ± 0.3	474 ± 8	1924 ± 13	1756 ± 13	154 ± 4	14 ± 0.3	2.5 ± 0.1
Ambient culture cups	14	7.95 ± 0.01	2149 ± 22	23.8 ± 0.3	32.1 ± 0.3	490 ± 13	1938 ± 15.3	1770 ± 12	154 ± 5	15 ± 0.4	2.5 ± 0.1
Switch-to-ambient culture cups	15	7.95 ± 0.02	2157 ± 18	23.6 ± 0.2	32.1 ± 0.3	492 ± 21	1947 ± 11.2	1779 ± 10	154 ± 7	15 ± 1	2.5 ± 0.1
Ambient blank cups	2	7.96 ± 0.03	2182 ± 26	23.8 ± 0.3	32.5 ± 0.5	491 ± 30	1968 ± 17.2	1794 ± 6	159 ± 12	15 ± 1	2.5 ± 0.2
High CO_2 spawning bath	1	7.25	2120	20.2	31.4	2834	2169	2046	30	93	0.5
High CO_2 bucket	8	7.31 ± 0.03	2131 ± 12	23.5 ± 0.5	31.7 ± 0.2	2513 ± 160	2150 ± 13.3	2035 ± 12	39 ± 3	75 ± 5	0.6 ± 0.1
High CO_2 culture cups	15	7.29 ± 0.02	2158 ± 17	23.7 ± 0.3	32.0 ± 0.3	2657 ± 133	2181 ± 17.6	2063 ± 16	39 ± 2	79 ± 4	0.6 ± 0.03
Switch-to-high-CO_2 culture cups	14	7.28 ± 0.01	2175 ± 31	23.7 ± 0.3	32.3 ± 0.5	2714 ± 60	2200 ± 28.4	2081 ± 27	39 ± 2	81 ± 2	0.6 ± 0.03
High CO_2 blank cups	3	7.28 ± 0.01	2133 ± 7	23.7 ± 0.2	31.7 ± 0.2	2650 ± 51	2157 ± 4.6	2041 ± 5	38 ± 1	79 ± 1	0.6 ± 0.01
Experiment 2											
Ambient spawning bath	1	7.93	2114	21.4	31.9	522	1936	1786.5	133	17	2.1
Ambient bucket	5	7.95 ± 0.01	2133 ± 12	25.1 ± 0.1	32.0 ± 0.2	488 ± 19	1915 ± 13	1743 ± 15	159 ± 5	14 ± 1	2.6 ± 0.1
Ambient culture cups	15	7.97 ± 0.01	2215 ± 36	24.3 ± 0.1	33.2 ± 0.5	475 ± 8	1979 ± 26	1794 ± 20	171 ± 7	14 ± 0.3	2.7 ± 0.1
Switch-to-ambient culture cups	15	7.97 ± 0.01	2185 ± 30	24.3 ± 0.1	32.9 ± 0.5	481 ± 11	1958 ± 22	1779 ± 17	165 ± 6	14 ± 0.4	2.6 ± 0.1
Ambient blank cups	3	7.97 ± 0.01	2185 ± 19	24.3 ± 0.1	32.7 ± 0.2	472 ± 8	1955 ± 13	1774 ± 8	167 ± 5	14 ± 0.3	2.7 ± 0.1
High CO_2 spawning bath	1	7.25	2127	21.6	31.8	2826	2169	2047	32	89	0.5
High CO_2 bucket	5	7.31 ± 0.01	2122 ± 14	25.1 ± 0.0	31.9 ± 0.1	2500 ± 30	2134 ± 13	2020 ± 13	42 ± 1	72 ± 1	0.7 ± 0.01
High CO_2 culture cups	15	7.29 ± 0.01	2185 ± 23	24.3 ± 0.1	32.6 ± 0.2	2658 ± 38	2193 ± 21	2075 ± 20	41 ± 1	77 ± 1	0.7 ± 0.02
Switch-to-high-CO_2 culture cups	15	7.30 ± 0.02	2179 ± 34	24.3 ± 0.1	32.7 ± 0.5	2628 ± 92	2196 ± 33	2078 ± 32	41 ± 2	77 ± 3	0.7 ± 0.03
High CO_2 blank cups	3	7.29 ± 0.01	2145 ± 11	24.3 ± 0.1	32.2 ± 0.1	2650 ± 37	2166 ± 11	2049 ± 10	39 ± 1	77 ± 1	0.6 ± 0.01

ity, and temperature were measured following the procedures described by White et al. (2013). Briefly, pH_T was measured spectrophotometrically following the procedure described by Clayton & Byrne (1993) and Dickson et al. (2007), and using the refit equation of Liu et al. (2011). Alkalinity was measured by Gran titration with 0.01 M HCl. Based on the measured values of pH_T, total alkalinity, temperature, and salinity, CO2SYS software (Pierrot et al. 2006) was used to calculate pCO_2, $\Omega_{aragonite}$, and total dissolved inorganic carbon using the first and second dissociation constants of carbonic acid in seawater from Mehrbach et al. (1973), refit by Dickson & Millero (1987).

In addition to the replicate culture cups containing larvae for each treatment (see 'Larval culture in CO_2 treatments'), 2 additional cups, one for each CO_2 level, were maintained without larvae or algae added to them to serve as controls for the chemistry characterization. The filtered seawater (FSW) in these cups was analyzed for carbonate chemistry and changed every 2 d, as for the culture cups.

Control of spawning conditions

Spawning was induced by dividing the hermaphroditic adults between two 15 l, 20°C static seawater baths and gradually raising the temperature of each bath to a maximum of 25°C. The CO_2 level of each bath was controlled by bubbling the bath with a microbubbler air stone (RENA) at a rate of 1 l min^{-1} with the appropriate air-CO_2 mixture for 24 h prior to spawning. One bath was bubbled with high CO_2 and the other was bubbled with ambient CO_2. Both baths were covered with clear plastic wrap to help ensure CO_2 equilibration, while allowing observers to view the scallops in the baths. When an individual spawned, it was moved to a 1 l beaker of 20°C FSW and the spawned gametes were examined to distinguish eggs from sperm. These individual beakers continued to be bubbled with the appropriate (ambient or high) CO_2 level at a rate of 100 ml min^{-1} using a micropipette tip inserted into an airline and were covered with clear plastic wrap to ensure CO_2 equilibration. To prevent self-fertilization, the water in the beakers was frequently changed with pre-CO_2-equilibrated FSW. Eggs were rinsed through a 75 µm filter to remove debris, collected on a 20 µm filter, and subsequently pooled. Seawater with sperm was rinsed through a 20 µm filter to remove debris and the sperm was pooled. Separate sets of filters were used for gametes spawned in ambient and high CO_2 so there was no cross contamination. For gametes spawned in each CO_2 treatment, sperm and eggs were each pooled separately into about 1 l of seawater, which was bubbled at 100 ml min^{-1} with the appropriate CO_2 level. About 1 ml of sperm spawned in ambient CO_2 was added to the eggs spawned in ambient CO_2, and 1 ml of sperm spawned in high CO_2 was added to the eggs spawned in high CO_2; both sets of embryos were left to develop in their beakers for 45 min, bubbled with ambient or high CO_2 as appropriate, until the embryonic development was at the polar body stage or first cleavage (Belding 1910).

This induction of spawning was performed twice, once in late May 2012 and once in late June 2012, resulting in 2 separate experiments, henceforth referred to as Experiment 1 and Experiment 2. For Experiment 1, ambient CO_2-spawned eggs were collected from 2 individuals, although most of the eggs came from one of these individuals, and ambient CO_2-spawned sperm was collected from 2 individuals. High CO_2-spawned eggs were collected from 1 individual and high CO_2-spawned sperm was collected from 1 individual. For Experiment 2, ambient CO_2-spawned eggs were collected from 3 individuals and ambient CO_2-spawned sperm was collected from 3 individuals. High CO_2-spawned eggs were collected from 2 individuals and high CO_2-spawned sperm was collected from 5 individuals. Ideally, we would want at least 2 adults contributing each type of gamete to each treatment, to identify an overall effect of high CO_2 beyond any maternal effects, so the results of Experiment 1 are interpreted cautiously. For both experiments, if a scallop released both eggs and sperm, only the eggs were used.

Assessment of fertilization success and embryo density estimation

When the scallop embryos were 45 min post-fertilization, they were homogeneously suspended in the beaker by the up and down motion of a graduated cylinder (Helm & Bourne 2004) and 1 ml was removed to assess fertilization success and to estimate embryo density. Embryos were counted at 100× magnification on a gridded slide in which each grid square held 1 µl (Widman et al. 2001). An embryo was considered fertilized by evidence of a polar body or by evidence of first or second cleavage (Belding 1910). The total number of embryos seen and the total number of unfertilized eggs were counted. This

count was performed twice per experiment, once for embryos fertilized in ambient CO_2 conditions and once for embryos fertilized in high CO_2 conditions. These counts were used to calculate the percent of embryos from each CO_2 treatment that were successfully fertilized.

Larval culture in CO_2 treatments

When the embryos were about 2 h old, they were stocked at an initial density of 30 embryos ml^{-1} and were maintained in 800 ml of 0.35 µm filtered FSW in 1 l covered polyethylene cups that had previously been conditioned in running seawater for at least 4 wk. Filtered seawater was pre-CO_2 equilibrated by bubbling with the appropriate air-CO_2 mixture in covered 14 l buckets for 24 h prior to filling the 800 ml culture cups. Embryos that had been fertilized in ambient CO_2 were stocked into 5 culture cups containing ambient CO_2 water and 5 cultures cups containing high CO_2 water. Embryos that had been fertilized in high CO_2 were stocked into 5 cultures cups containing ambient CO_2 water and 5 culture cups containing high CO_2 water. This resulted in 4 experimental treatments: ambient CO_2 fertilized and ambient CO_2 grown (ambient treatment), high CO_2 fertilized and high CO_2 grown (high CO_2 treatment), ambient CO_2 fertilized and high CO_2 grown (switch-to-high-CO_2 treatment), and high CO_2 fertilized and ambient CO_2 grown (switch-to-ambient treatment). Each culture cup was bubbled with the appropriate air or CO_2-compressed air mixture for the 7 d duration of the experiment at a rate of approximately 100 ml min^{-1}.

Cultures were fed daily with laboratory-raised *Isochrysis galbana* (Tahitian strain, T-iso) in the exponential phase of growth at a density of 37 500 cell ml^{-1}. This ration has been shown to produce good growth rates and survivorship of bay scallop larvae (Widman et al. 2001). Culture water was changed every 2 d with pre-CO_2-equilibrated FSW from the 14 l buckets. Prior to water changes, the carbonate chemistry of the pre-equilibrated water in the 14 l buckets was measured as described above. During water changes, each culture was gently poured through a 20 µm sieve, which caught the larvae. The larvae were rinsed back into the cup and the cup was filled to 800 ml. To maintain a stable temperature, all culture cups were contained in a water bath controlled by an aquarium chiller and heater at a temperature of 23.7 ± 0.3°C (mean ±SD). This temperature was chosen because it has been shown to produce favorable growth rates in *Argopecten irradians* larvae (Widman et al. 2001). Because pH was measured every 2 d, but algae was added daily, we do not know the effect that algal photosynthesis had on the pH of the larval cultures.

Microscopic imaging and shell measurements

At 1, 3, and 7 d old, ~75 larvae from each culture were preserved in 95% ethanol for microscopic imaging and shell measurement. Preserved larvae were imaged at 200× magnification under both bright-field transmitted and cross-polarized light using a Nikon ECLIPSE 50i POL microscope and a SPOT Insight™ Camera controlled by SPOT Basic Software (Diagnostic Instruments). Using the built-in measurement capabilities of the software program, shell length (the longest dimension parallel to the hinge) was measured for at least 15 larvae from each replicate culture. When larvae exhibited unequal-sized valves, the shell length of the larger valve was measured. Preliminary experiments showed that 4 replicates gave sufficient statistical power to detect differences in mean larval size. A fifth culture cup was included in each treatment to ensure that if one culture crashed, 4 would remain available for size measurements. Mean growth rate (µm d^{-1}) for each replicate was calculated as the increase in mean shell length from 1 to 7 d old, divided by the number of days (6 d).

Scanning electron microscopy

Upon visualization of larvae through transmitted and polarized light microscopy, a shell abnormality near the hinge (appearing as a dark spot or line) was frequently observed in larvae in the high CO_2 and switch-to-high-CO_2 treatments. The nature of the shell abnormality was unclear, so subsets of larvae from one replicate of each treatment from Experiment 1 were mounted on stubs for scanning electron microscopy (SEM). Approximately 12 larvae from each treatment at 1, 3, and 7 d old were mounted on stubs using double-sided carbon tape and subsequently coated with 5 nm gold using a Leica EM MED020 vacuum coating system. Digital SEM images of the larvae were obtained with a Zeiss NTS Supra40VP electron microscope at 500× magnification using a voltage of 12 kV and a HKL Premium electron backscatter diffraction system.

Survival estimation

Percent survival was estimated at 1, 3, and 7 d old by removing a subsample of known volume from each culture in turn and counting all of the live larvae in this volume under a stereomicroscope at 10× magnification (White et al. 2013). The density of live larvae in this subsample was used to calculate the percent survival based on the initial stocking density. Due to an error in quantifying initial stocking densities during Experiment 2, survival estimates are calculated only for Experiment 1 and are based on an initial stocking density of ~30 embryos ml^{-1}. Survival estimates from all 5 replicate cultures were included in analyses to improve statistical power.

Statistical analysis

All statistical analyses were performed using Systat® 13 Software (Systat Software). Percent survival data were arcsine square-root transformed prior to statistical analyses. Repeated measures ANOVA tests were run to compare survival and shell length (separately) among the 4 treatments at 1, 3, and 7 d old. One-way ANOVAs followed by Tukey's honestly significant difference tests were run separately for each date to compare survival and shell length among the 4 treatments. One-way ANOVAs were run separately for each experiment to compare growth rates from Days 1 to 7 among the 4 treatments.

RESULTS

Fertilization success

The percent of eggs fertilized was reduced for gametes exposed to high CO_2 relative to gametes exposed to ambient CO_2 in both Experiments 1 and 2. In Experiment 1, the percent of eggs fertilized was 94.5 and 74.5% for gametes exposed to ambient CO_2 or high CO_2, respectively. In Experiment 2, the percent of eggs fertilized was 98.2 and 95.7% for gametes exposed to ambient CO_2 or high CO_2, respectively.

Shell development and length

Development through larval stages in all treatments progressed typically for this species (Fig. 1) (Belding 1910, Widman et al. 2001). At 1 d, most larvae were in the fully shelled veliger stage, while those larvae that were still in the trochophore stage (Fig. 2) exhibited birefringence under cross-polarized light, which indicated the presence of crystalline calcium carbonate. The shells of 1 d old larvae from the ambient and switch-to-ambient treatments showed stronger birefringence than those of 1 d old larvae from the switch-to-high-CO_2 and high CO_2 treatments (Fig. 1B, D). By Day 3, all larvae were post-D-stage veligers, although some larvae had unequal-sized valves (for example, the 3 d switch-to-high-CO_2 larva in Fig. 1A). This asymmetry in valve size was not related to CO_2 treatment; it was seen in a small number of larvae in all treatments. Nearly all larvae in the high CO_2 and switch-to-high-CO_2 treatments displayed a dark spot or line near the hinge under transmitted light (Fig. 1A,C) that was also evident under cross-polarized light (Fig. 1B,D). Based on subsequent SEM visualization, it was determined that this dark spot or line was an indicator of a dorsoventrally oriented abnormal indentation of the larval shell located near the hinge (Fig. 3). This abnormal indentation was rarely seen on larvae from the ambient and switch-to-ambient treatments, although the frequency of occurrence was not quantified.

The CO_2 conditions experienced during fertilization did not have an impact on larval growth or size (Fig. 4). However, exposure to high CO_2 conditions during subsequent larval development (starting ~2 h post-fertilization) caused a decrease in shell size relative to exposure to ambient CO_2 conditions, regardless of CO_2 exposure during fertilization (Experiment 1: repeated measures ANOVA, Wilk's Lambda = 0.0117, F = 14.187, df = 9, 24, $p < 0.00001$; Experiment 2: repeated measures ANOVA, Wilk's Lambda = 0.0052, F = 20.888, df = 9, 24, $p < 0.00001$). This decrease in shell size was seen consistently throughout the 7 d duration of both Experiments 1 and 2. For Experiment 1, the mean shell lengths of larvae from the ambient and switch-to-ambient treatments were significantly larger than the shell lengths of larvae from the switch-to-high CO_2 and high CO_2 treatments at 1 and 3 d old (Fig. 4A, Table 2, & Table A1 in Appendix 1). At 7 d old, the mean shell lengths of larvae from the ambient treatment were significantly larger than the shell lengths of larvae from the high CO_2 treatment. For Experiment 2, the mean shell lengths of larvae from the ambient and switch-to-ambient treatments remained significantly larger than the shell lengths of larvae from the switch-to-high-CO_2 and high CO_2 treatments throughout the

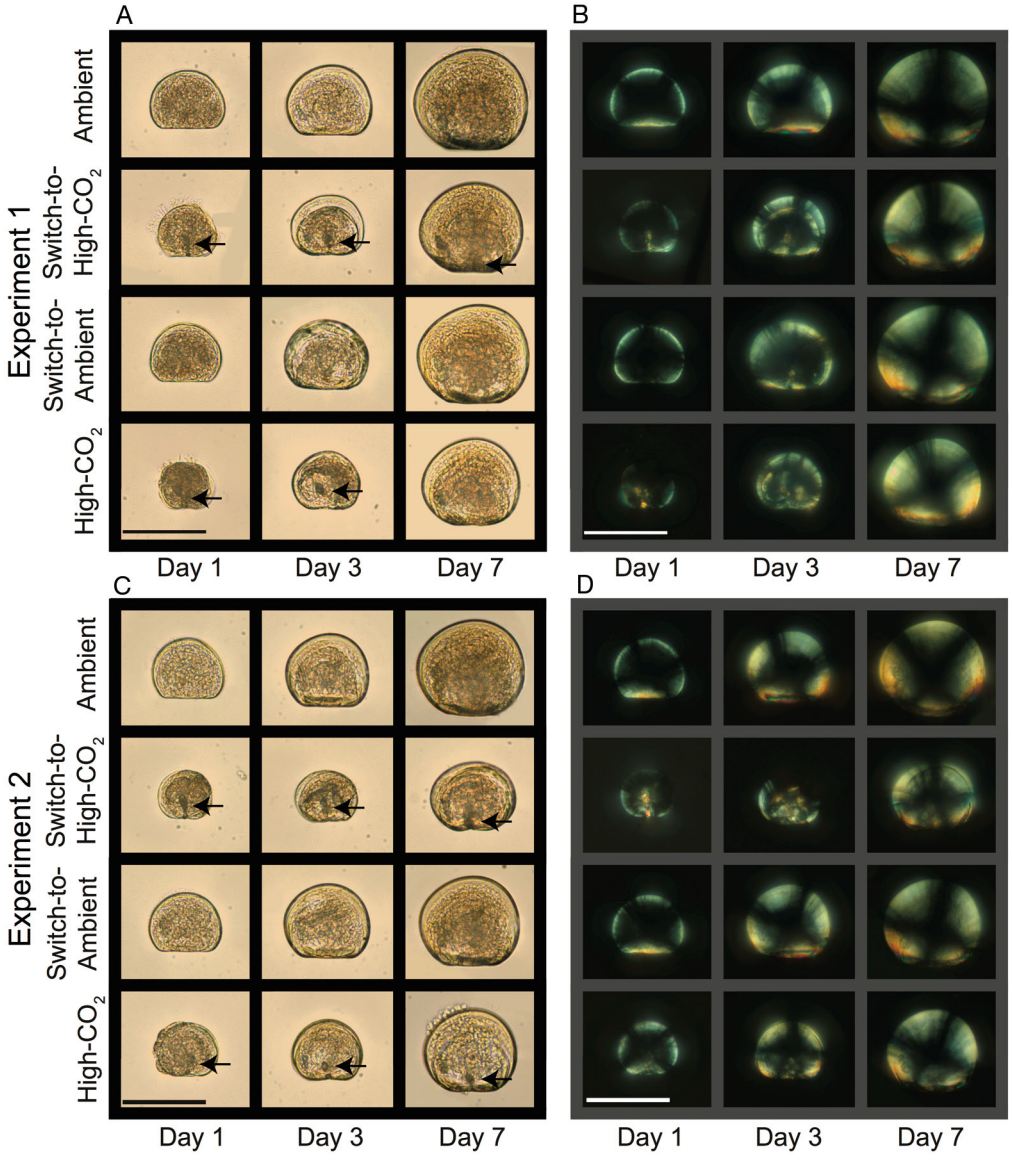

Fig. 1. *Argopecten irradians*. Larval morphology of bay scallops exposed to varied pCO_2 conditions from Experiment 1 (A,B) and Experiment 2 (C,D), viewed under both transmitted (A,C) and cross-polarized light (B,D). Larvae were preserved in 95% ethanol after incubation for 1, 3, or 7 d in one of the 4 CO_2 treatment regimes (ambient, switch-to-high-CO_2, switch-to-ambient, and high CO_2). Larvae shown represent the mean shell length for each treatment and age. Arrows indicate the dark spot on the larval shell that represents a shell abnormality. Images are all to the same scale; scale bar = 100 µm

entire experiment (Fig. 4B, Table 2, & Table A1 in Appendix 1). At 1 and 3 d old, larvae from the high CO_2 treatment were significantly larger than larvae from the switch-to-high-CO_2 treatment; this difference in size was no longer significant by Day 7 (Fig. 4B). No significant difference was seen in mean shell growth rates integrated over Days 1 to 7 for all 4 treatments (Experiment 1: 1-way ANOVA, $F = 0.97$, df = 3, p = 0.44; Experiment 2: 1-way ANOVA, $F = 1.97$, df = 3, p = 0.17).

Larval survival

Exposure to high CO_2 during fertilization resulted in consistently lower larval survival from Day 1 to 7, relative to exposure to ambient CO_2 at the same stage (Fig. 5, Table A2 in Appendix 1). This difference was significant on Days 1 and 3, and on Day 7, survival in the continuous ambient treatment was significantly higher than survival in all other treatments (Fig. 5). Additionally, within each fertilization

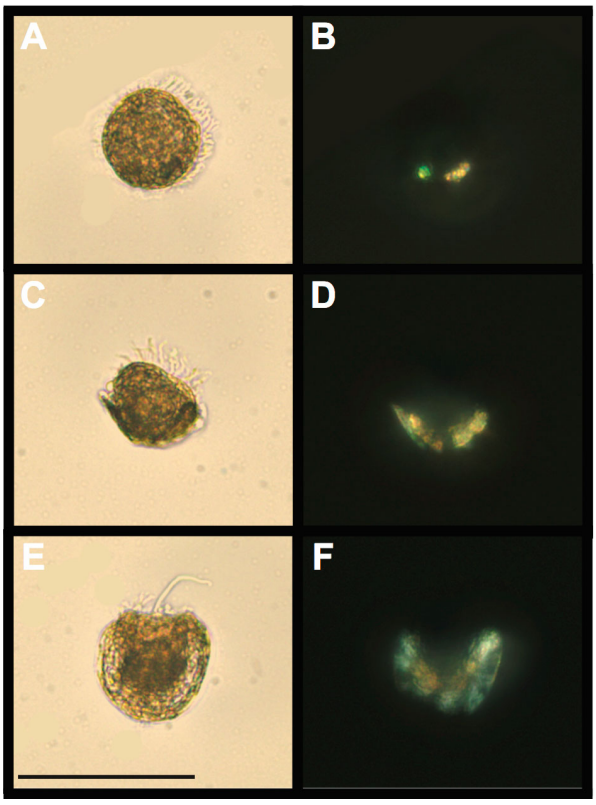

Fig. 2. *Argopecten irradians*. Three different 1 d old bay scallop trochophore larvae viewed under both transmitted (A,C,E) and cross-polarized light (B,D,F). Larvae shown are from the switch-to-high-CO_2 treatment and were selected because they represent progressive shell development, visualized in the cross-polarized images (B,D,F). Images are all to the same scale; scale bar = 100 µm

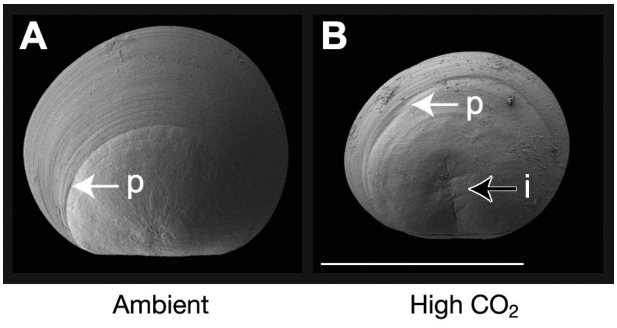

Fig. 3. *Argopecten irradians*. Scanning election microscopy images of larval shell morphology of 7 d old bay scallops exposed to ambient (A) or high (B) CO_2 conditions from Experiment 1. Larvae shown represent the typical shell appearance for each treatment and age, but do not represent mean shell size. The black arrow indicates the abnormal indentation (i) near the hinge of the larvae in the high CO_2 treatment. This abnormal indentation was also seen on larvae from the switch-to-high-CO_2 treatment. The white arrows indicate the demarcation line (p) between the prodissoconch I and the prodissoconch II, 2 distinct early stages of larval shell development. Images are to the same scale; scale bar = 100 µm

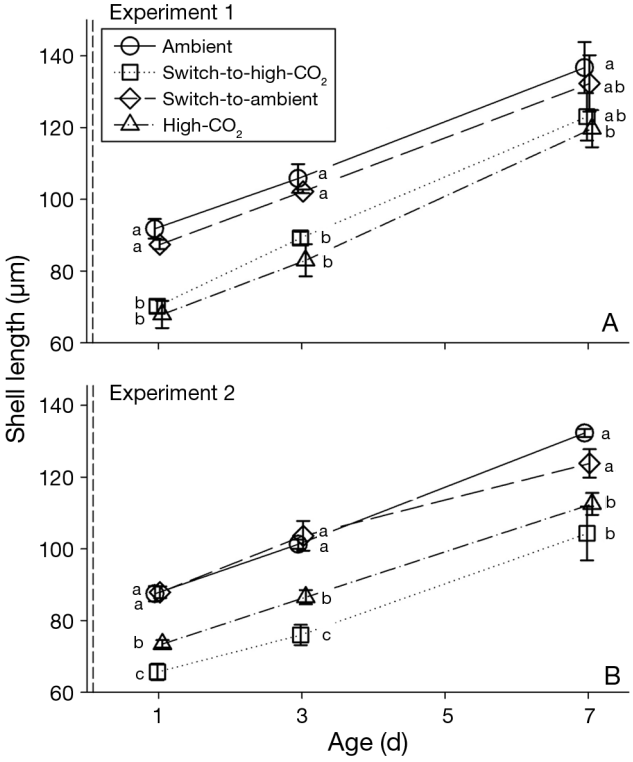

Fig. 4. *Argopecten irradians*. Shell length of larval bay scallops from Experiment 1 (A) and Experiment 2 (B) during the first week of larval development. Values are mean ± SD of 4 replicate culture containers. The dashed vertical line at 2 h indicates the age at the time of inoculation into culture cups, which was also the age at which CO_2 conditions were switched for the 2 switch treatments. Different letters denote significant differences ($p < 0.05$) between treatments at a given age, as determined in 1-way ANOVA (Table A1 in Appendix 1), followed by Tukey's honestly significant difference test

group (ambient or high CO_2), survival was lower in those treatments where larvae subsequently developed in high CO_2. On Day 1, survival was significantly lower in the high CO_2 treatment, relative to the switch-to-ambient treatment and in the switch-to-high-CO_2 treatment, relative to the ambient treatment. This significant decrease in survival persisted through to Days 3 and 7 with regard to the switch-to-high-CO_2 and ambient treatments, respectively.

DISCUSSION

The 2 fertilization experiments showed that the timing of exposure to high CO_2 has a clear influence on larval responses, and the design of the switch experiment allowed us to isolate the timing of these different effects. Exposure during fertilization reduced survival, but did not affect shell size or

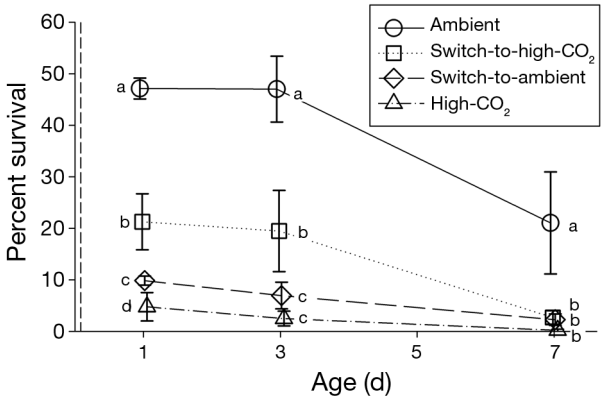

Fig. 5. *Argopecten irradians*. Survival of larval bay scallops from Experiment 1, expressed as the percent of larvae surviving from the time of inoculation (age = 2 h), during the first week of larval development. Values are mean ± SD of n = 5 replicate culture containers. All percent survival data are estimated from a single initial stocking density estimate of 30 embryos ml^{-1} in each culture cup for each treatment. The dashed vertical line at 2 h indicates the age at the time of inoculation into culture cups, which was also the age at which CO_2 conditions were switched for the 2 switch treatments. Letters denote significant differences ($p < 0.05$) between treatments at a given age, as determined in 1-way ANOVA (Table A2 in Appendix 1), followed by Tukey's honestly significant difference test

development. In contrast, exposure to high CO_2 2 h post-fertilization reduced shell size and caused a deformity in the larval shell.

The switch experiments suggest that larval survival is influenced by CO_2 exposure during both fertilization and larval development. We think this survival effect is due to CO_2 exposure, not a consequence of maternal effects, because while we were unable to estimate survival in Experiment 2, the good replication of size trends between the 2 experiments when different parent scallops were used is an indication that the observed effects were treatment effects, not maternal effects.

Exposure of gametes to high CO_2 conditions prior to and during fertilization did not negatively impact subsequent larval development and growth. Instead, larval development and growth were only influenced by CO_2 conditions experienced during larval development (e.g. after 2 h post-fertilization), with larvae in both the switch-to-high-CO_2 and high CO_2 treatments having significantly smaller shells than larvae in both the ambient and switch-to-ambient treatments (Fig. 4).

Impacts of exposure to high CO_2 during larval development (starting at 2 h post-fertilization) on larval shell size were evident starting when larvae were just 24 h old in both experiments. While the size differences persisted throughout the experiment, growth rates from Day 1 to 7 were not significantly different among any treatments in either experiment, indicating that the size differences at the end of the first week of larval development were a function of differences that appeared within the first day of larval development. Similar to results previously seen in *Argopecten irradians* larvae (White et al. 2013), it appears that growth during the first day of larval development, when the larvae are initiating calcification and building their first shell (Belding 1910, Widman et al. 2001), is critical in determining the size that larvae ultimately reach within the first week of development. Our results indicate that inhibition to growth observed in other studies of *A. irradians* larvae (e.g. Gobler & Talmage 2013) is probably triggered within the first day of development. During the first day of development, larvae rely on maternal energy reserves as they do not develop mouths until they are 24 h old (Belding 1910). Waldbusser et al. (2013) suggest, through stable isotope analysis, that disruption of bivalve initial shell formation during early development is probably due in part to energetics, i.e. limited supply of energy from maternal stores.

Calcium carbonate crystals cause cross-polarized light to exhibit birefringence (Weiss et al. 2002), which can be used as a qualitative indication of calcification. Thus, the stronger birefringence in the shells of 1 d old larvae from the ambient and switch-to-ambient treatments suggests that those larvae calcified more during the first day than the larvae exposed to high CO_2 during larval development (2 h post-fertilization).

In the wild, a decrease in the size of scallop larvae exposed to high CO_2 may have indirect effects on subsequent survival due to delayed metamorphosis and therefore, increased vulnerability to predation (Thorson 1950, Sastry 1965). The abnormal shell indentation seen near the hinge of shells of larvae reared in high CO_2 (Figs. 1A,C & 3) could further impact survival. The bivalve hinge allows the larval shell to open and close, facilitating the intake of food particles and release of waste (Cragg 2006), so that an abnormality in the hinge could prevent larvae from properly obtaining the food particles necessary for survival (Talmage & Gobler 2010). A difference in feeding rates between larvae with normal and abnormal shells would support the hypothesis that the abnormal shell indentation could impact feeding ability. Unfortunately, such data were not collected in this study as we did not anticipate finding this shell abnormality.

Kurihara et al. (2007) found that *Crassostrea gigas* larvae in the high CO_2 treatment had abnormally formed shells, similar to the abnormally indented *Argopecten irradians* shells described here. Barros et al. (2013) also documented increased frequency of prodissoconch abnormalities in *C. gigas* exposed to reduced pH conditions, although the types of abnormalities they found did not appear to be abnormal shell indentations. We hypothesize that, as observed in bay scallops, these shell abnormalities in oysters are a result of exposure to high CO_2 during early larval development, not of exposure during fertilization. Interestingly, this shell abnormality was not reported for *Mytilus galloprovincialis* larvae (Kurihara et al. 2008), indicating that mussel shell development may not be affected by early exposure to high CO_2 in the same way.

Our observation of a negative effect of high CO_2 during early larval development (>2 h post-fertilization) on shell size is consistent with other studies of bivalves, although the sensitivity of the response varied by species (Fig. 6). The magnitude of this CO_2 effect on early larvae of the Pacific oyster *Crassostrea gigas* (Kurihara et al. 2007) was similar to that observed in our experiments, while the effect on *Mytilus galloprovincialis* larvae was slightly greater (Kurihara et al. 2008) (Fig. 6). The difference in sensitivity among species was relatively small, and an overall negative size trend with increasing CO_2 was seen in all 3 of these species, each of which represents a different order of bivalve.

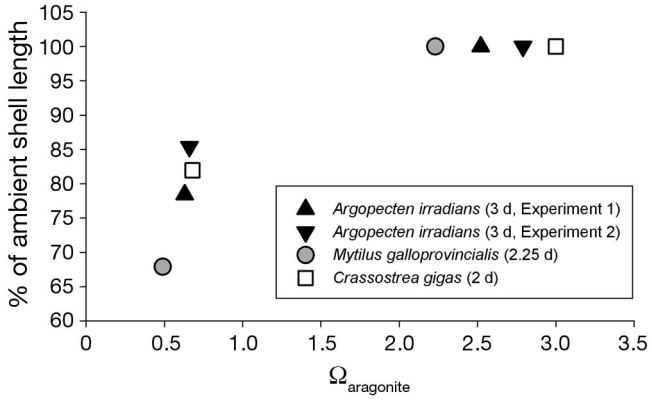

Fig. 6. *Argopecten irradians*, *Mytilus galloprovincialis*, and *Crassostrea gigas*. Shell length of early veliger larvae of 3 bivalve species, expressed as % of ambient shell length, where 'ambient' refers to the experimental treatment with water pCO_2 closest to the current atmospheric pCO_2 value. Values for bay scallops *Argopecten irradians* are calculated from the present study; data for the mussel *Mytilus galloprovincialis* are calculated from Kurihara et al. (2008); data for the oyster *Crassostrea gigas* are calculated from Kurihara et al. (2007). In all cases, gametes were exposed to treatment conditions (ambient or low aragonite saturation state, $\Omega_{aragonite}$) during fertilization and embryos and larvae were maintained in their respective $\Omega_{aragonite}$ conditions for the duration of the experiment with no switch in conditions (i.e. only the continuous ambient and high CO_2 treatments from the bay scallop fertilization experiments described in the present study are shown)

In both Experiments 1 and 2, fertilization success was reduced when gametes were exposed to high CO_2 conditions prior to and during fertilization. However, the extent to which fertilization success was reduced was not consistent between experiments. This variable fertilization success could be due to variable gamete quality (Havenhand & Schlegel 2009), but is also commonly a result of variable compatibilities between male and female gametes (Styan et al. 2008). A reduction in fertilization success as a result of exposure to high CO_2 has previously been documented in the clam *Macoma balthica* (Van Colen et al. 2012) and the oysters *Saccostrea glomerata* and *Crassostrea gigas* (Parker et al. 2009, 2010, Barros et al. 2013). In contrast, other groups have not observed a CO_2 exposure-induced reduction in fertilization success of *C. gigas* (Kurihara et al. 2007, Havenhand & Schlegel 2009), perhaps due to intraspecific differences among oyster populations. By comparison, fertilization success of echinoids appears to be robust at near-future levels of OA (Carr et al. 2006, Byrne et al. 2009, 2010, Ericson et al. 2010), but reduced at pH <7.4 (Bay et al. 1993, Kurihara & Shirayama 2004). Because the fertilization success measurements in the current experiments were not replicated, they cannot be definitively attributed to differences in CO_2 exposure.

OA may interact with other stressors, such as rising sea surface temperature. Parker et al. (2010) found that larvae of the oyster *Saccostrea glomerata* failed to develop to D-stage veligers when exposed to high CO_2 and increased temperature only when the larvae came from gametes fertilized in high CO_2, not when larvae came from gametes fertilized in ambient CO_2. This indicates that the high CO_2 fertilization had a lasting effect on larval development when combined with another stressor. Had we also included temperature as a synergistic stressor, we may have seen an impact of exposure to high CO_2 during fertilization on larval size and development of bay scallops.

Bay scallops living in coastal and estuarine environments will face increasingly unfavorable conditions in coming years as atmospheric CO_2-induced OA exacerbates already-high pCO_2 conditions in these locales. Survival through fertilization and successful larval development are critical to maintaining

or even increasing the bay scallop population for commercial consumption. While larval development appears to be affected most strongly by CO_2 conditions experienced after fertilization has taken place, larval survival is impacted when gametes are exposed to high CO_2 during fertilization. Larvae that were spawned in regions of high CO_2 may be at an ecological disadvantage to those spawned in regions with lower CO_2 conditions. If adult bay scallops were able to control the timing of their spawning, they might be able to ensure that embryos and larvae developed during the more favorable pH conditions during daylight hours (Hofmann et al. 2011). Hatchery managers may help ensure survival and successful development of larvae by raising the pH and/or lowering the pCO_2 of the water in which adult scallops spawn and larvae develop for the first day.

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Appendix 1

Table A1. *Argopecten irradians*. One-way ANOVAs of mean shell length (µm) of larvae from Experiments 1 and 2 raised in 4 CO_2 treatment regimes (ambient, switch-to-high-CO_2, switch-to-ambient, and high CO_2) at 1, 3, and 7 d old; n = 4

	Source of variation	Type III SS	df	MS	*F*-ratio	p-value
Experiment 1						
Day 1	Treatment	1734.69	3	578.23	85.97	<0.001
	Error	80.71	12	6.73		
Day 3	Treatment	1392.15	3	464.05	46.69	<0.001
	Error	119.27	12	9.94		
Day 7	Treatment	752.42	3	250.81	5.49	0.013
	Error	548.23	12	45.69		
Experiment 2						
Day 1	Treatment	1437.10	3	479.03	143.06	<0.001
	Error	40.18	12	3.35		
Day 3	Treatment	2035.41	3	678.47	88.87	<0.001
	Error	91.61	12	7.63		
Day 7	Treatment	1822.33	3	607.44	29.13	<0.001
	Error	250.25	12	20.85		

Table A2. *Argopecten irradians*. One-way ANOVAs of mean percent survival (arcsine square-root transformed) of larvae from Experiment 1 raised in 4 CO_2 treatment regimes (ambient, switch-to-high-CO_2, switch-to-ambient, and high CO_2) at 1, 3, and 7 d old; n = 5

	Source of variation	Type III SS	df	MS	*F*-ratio	p-value
Day 1	Treatment	0.84	3	0.28	122.78	<0.001
	Error	0.04	16	0.00		
Day 3	Treatment	1.04	3	0.35	73.31	<0.001
	Error	0.08	16	0.00		
Day 7	Treatment	0.50	3	0.17	33.74	<0.001
	Error	0.08	16	0.00		

Editorial responsibility: James McClintock, Birmingham, Alabama, USA

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