Early Exposure of Bay Scallops (*Argopecten irradians*) to High CO$_2$ Causes a Decrease in Larval Shell Growth

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Abstract

Ocean acidification, characterized by elevated pCO$_2$ and the associated decreases in seawater pH and calcium carbonate saturation state (Ω), has a variable impact on the growth and survival of marine invertebrates. Larval stages are thought to be particularly vulnerable to environmental stressors, and negative impacts of ocean acidification have been seen on fertilization as well as on embryonic, larval, and juvenile development and growth of bivalve molluscs. We investigated the effects of high CO$_2$ exposure (resulting in pH = 7.39, Ω_{ar} = 0.74) on the larvae of the bay scallop *Argopecten irradians* from 12 h to 7 d old, including a switch from high CO$_2$ to ambient CO$_2$ conditions (pH = 7.93, Ω_{ar} = 2.26) after 3 d, to assess the possibility of persistent effects of early exposure. The survival of larvae in the high CO$_2$ treatment was consistently lower than the survival of larvae in ambient conditions, and was already significantly lower at 1 d. Likewise, the shell length of larvae in the high CO$_2$ treatment was significantly smaller than larvae in the ambient conditions throughout the experiment and by 7 d, was reduced by 11.5%. This study also demonstrates that the size effects of short-term exposure to high CO$_2$ are still detectable after 7 d of larval development; the shells of larvae exposed to high CO$_2$ for the first 3 d of development and subsequently exposed to ambient CO$_2$ were not significantly different in size at 3 and 7 d than the shells of larvae exposed to high CO$_2$ throughout the experiment.

Introduction

Coastal marine invertebrates are exposed to dissolved carbon dioxide levels that fluctuate on time scales ranging from daily to seasonal as a result of both natural processes and human activities [1–3]. These aqueous CO$_2$ levels are likely to increase (and pH to drop) in the decades ahead as a consequence of ocean acidification (OA), the uptake of anthropogenic CO$_2$ by the ocean [4–6]. Since pre-industrial times, increases in anthropogenic emissions of CO$_2$ have caused a decrease in surface ocean water pH of 0.1 units, and a decrease of another 0.2–0.3 units is projected by the end of this century [6,7]. As seawater pH decreases, its calcium carbonate saturation state (Ω) also decreases, and drops in Ω have the potential to make calcification (or shell-building) more difficult, or more energetically costly for the organism [6,8,9].

While changes in surface ocean pH are happening on a global scale, the processes affecting coastal and estuarine ocean pH are different and result in more seasonally variable conditions than in the open ocean [2,10,11]. Anthropogenic eutrophication is a major factor influencing acidification of coastal and estuarine regions [8,12,13]. Eutrophication results in algal blooms, which eventually die, sink to the seafloor, and fuel microbial respiration [14,15]. This process is exacerbated during summer stratification events

[16] and can produce low-pH seasonal bottom waters that are undersaturated with respect to aragonite [11]. Because many coastal bivalve species spawn during summer months, the larvae are exposed to such conditions. Furthermore, because the buffering capacity of seawater is reduced as dissolved inorganic carbon (DIC) increases, it has been suggested that eutrophication could increase the susceptibility of coastal waters to ocean acidification [10]. As atmospheric CO$_2$-driven OA and anthropogenic eutrophication increase, the conditions experienced by bivalve larvae will become increasingly unfavorable for shell growth.

There is mounting evidence for negative effects of OA on marine organisms that produce calcareous skeletons, shells, or tests [6,8,9,17,18]. In particular, marine invertebrate larvae, including bivalves, are vulnerable to a variety of both chemical and physical environmental conditions including decreased pH [19]. For example, mussel and oyster larvae raised in water with pH ~7.4 were shown to have delayed development to D-stage veligers compared to larvae raised in ambient pH ~8.1 [20,21]. Talmage and Gobler [22] showed that hard clams (*Mercenaria mercenaria*) and, to a lesser extent, bay scallops (*Argopecten irradians*) experienced delayed metamorphosis at present-day conditions compared to pre-industrial conditions, suggesting that the 0.1 pH decrease in

the last 150 years has already had an impact on bivalve species. OA has been shown to have a negative impact on multiple early life stages of bivalves, including fertilization, D-stage (early) development, later larval development, and juvenile development [8,23–29]. Some species appear to be more tolerant of OA conditions than others. For example, two species of oysters displayed different trends when exposed to a range of pCO$_2$ values resulting in $\Omega_{ar} = 1.3$–0.6 for ~30 days; *Crassostrea virginica* had decreased shell area and decreased calcification with decreasing Ω_{ar}, but *Crassostrea ariakensis* showed no change in shell area or calcification [28]. Nonetheless, the majority of work has demonstrated that young bivalves are negatively impacted by the high CO$_2$/low pH conditions resulting from ocean acidification. There is evidence that the early larvae of some species are susceptible to OA [20,21,30]. Additionally, OA conditions have been shown to negatively affect the survival of bay scallop larvae and the size of competent and post-metamorphic bay scallops [22,31,32].

Here, we address the impact of early exposure to elevated CO$_2$ on the survival and growth of bay scallop larvae. Bay scallops are ideal as a model organism for this study because of their economic importance as a commercially harvested shellfish and because of their relatively short larval duration (~2–3 weeks). We also investigate whether transferring the larvae to ambient conditions can reverse the effects of early high CO$_2$ exposure. Such a scenario is ecologically relevant in a situation where larvae are spawned in an estuary with relatively high pCO$_2$ and are subsequently transported by currents or tides out of the estuary to sites with a lower pCO$_2$. We exposed larvae to ambient (nominally 390 ppm CO$_2$, pH = 7.93) and high (nominally 2200 ppm CO$_2$, pH = 7.39) CO$_2$ conditions for a total of 18 days. The high CO$_2$ treatment produced a calcium carbonate saturation state that was undersaturated with respect to aragonite ($\Omega_{aragonite}$). Such pCO$_2$ values and associated saturation states have been observed in summer months in a local estuary (Childs River, Falmouth, MA, USA) where bivalve larvae are found [33]. In addition, we exposed a third group of larvae to high CO$_2$ conditions for three days (through the larval D-stage), followed by exposure to ambient CO$_2$ conditions for 15 days. We hypothesize that larvae exposed continuously to elevated CO$_2$ will grow and develop more slowly throughout the larval period than those exposed to ambient CO$_2$ and that survival will also be negatively affected throughout the larval period. We explore whether effects of exposure to elevated CO$_2$ conditions during critical initial shell-formation (1–3 days post fertilization) can be altered by a return to ambient CO$_2$ conditions.

Methods

Adult Collection and Spawning

Adult *A. irradians* (subspecies *irradians*) individuals were collected during spring and summer months from coastal waters around Mashpee, 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 about 8.2 during winter months to about 7.6 during summer months [33]. From monthly water samples, pH (total scale) and pCO$_2$ were calculated from measured alkalinity and total DIC. For the summer months when adult scallops were held in cages, monthly pH values ranged from 7.6–7.9. During the same time period, calculated monthly pCO$_2$ values ranged from 578–986 ppm. All necessary permits were obtained for the described field studies; a research collection permit was 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, Campbell, CA, USA). During feedings, the flowing water was stopped for 2 h and adults were fed at a concentration of 200,000 cells ml^{-1}.

Spawning was induced by placing the hermaphroditic adults in a 20°C flowing seawater bath and gradually raising the temperature to a maximum of 25°C, over a period of 3 h. When an individual spawned, it was moved to a beaker of 20°C, 0.35 μm filtered seawater (FSW) and the spawned gametes were examined to distinguish eggs from sperm. To prevent self-fertilization, the water in the beakers was frequently changed. Eggs were rinsed through a 75 μm filter to collect debris, collected on a 20 μm filter, and subsequently pooled. Seawater with sperm was rinsed through a 20 μm filter to collect debris and the sperm was pooled. Eggs were collected from four individuals and sperm was collected from ten individuals. If a scallop released both eggs and sperm, only the eggs were used. Sperm and eggs were each pooled separately into about 1 L of seawater. About 1 ml of sperm was added to the eggs and the embryos were left to develop in the beaker for 11 h, until the larvae were at the swimming gastrula stage [34].

Larval Culture

When the scallop larvae were 11 h post-fertilization, they were homogeneously suspended in the beaker by gentle plunging with a graduated cylinder [35] and 1 ml was removed to estimate their density. Live larvae were counted at 100X magnification on a gridded slide in which each grid square held 1 μl [36].

Larvae were stocked at an initial density of 30 larvae ml^{-1} and were maintained in 800 ml of 0.35 μm FSW in six 1–l polyethylene cups per treatment, which were previously conditioned in running seawater for at least four weeks. Cultures were fed a daily pulse of 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 [36]. Culture water was changed every three days with pre-CO$_2$ equilibrated FSW. 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/heater (T = 22.5±0.3°C).

Manipulation of Water Chemistry

Water chemistry was manipulated by bubbling cultures with either compressed air or a mixture of compressed air and pure CO$_2$. The Ambient CO$_2$ treatment was bubbled with compressed air produced by an oil-free, portable air compressor (Porter Cable, Jackson, TN, USA). The High CO$_2$ treatment was bubbled with a compressed air/pure CO$_2$ mixture precisely controlled using two mass flow controllers (Aalborg, Orangeburg, NY, USA). To create the high CO$_2$ treatment, 8.1 ml min^{-1} CO$_2$ was mixed with 4.5 l min^{-1} compressed air. Each 800 ml culture cup was bubbled at a rate of approximately 100 ml min^{-1}.

Filtered seawater was pre-CO$_2$ equilibrated by bubbling with the appropriate air-CO$_2$ mixture in 14–l buckets for 24 h prior to filling the 1 l culture cups. Each of the Ambient CO$_2$ and High CO$_2$ treatment replicate cups was bubbled with ambient CO$_2$ compressed air or high CO$_2$-compressed air mixture, respectively, for the 18-d duration of the experiment. The High CO$_2$ to ambient switch treatment (hereafter referred to as the High CO$_2$/Ambient treatment) was bubbled with the high CO$_2$-compressed air mixture for the first three days of the experiment, followed by

ambient CO$_2$ compressed air for the remaining 15 d of the experiment.

Characterization of Water Chemistry

Prior to water changes, the carbonate chemistry of the pre-equilibrated water in the 14–1 buckets was measured. To characterize the carbonate chemistry of the water, pH, total alkalinity, salinity, and temperature were measured. Spectrophotometric pH measurements were made with 2 mM m-Cresol purple indicator dye to ensure high accuracy and precision using an Ocean Optics USB4000 Spectrometer with an LS-1 light source and a FIA-Z-SMA-PEEK 100 mm flow cell (Ocean Optics, Dunedin, FL, USA), following the procedure described by Clayton and Byrne [37] and Dickson et al. [38], and using the refit equation of Liu et al. [39]. This method proved to have a precision of ±0.002 pH units.

Samples for total alkalinity analysis were filtered to 0.45 μm, poisoned with saturated mercuric chloride, and stored in sealed glass vials until analysis. Total alkalinities were measured in duplicate via titration with 0.01 N HCl using a Metrohm Titrando 808 and 730 Sample Changer controlled by Tiamo software to perform automated Gran titrations of 1 ml samples. Internal seawater standards were standardized against seawater certified reference materials (supplied by the laboratory of Andrew Dickson, Scripps Institution of Oceanography) and were included in each run.

Salinity was determined using a Guildline model 8400B "Autosal" laboratory salinometer (Guildline Instruments, Smith Falls, Ontario, Canada). The temperature of the water bath was recorded every 10 min by a TidbiT v2 data logger (Onset Computer Corporation, Pocasset, MA, USA) and was also recorded for each replicate culture cup at the time of pH measurements.

Based on the measured values of pH (seawater scale), total alkalinity, temperature, and salinity, we used CO2SYS Software [40] to calculate pCO$_2$, $\Omega_{aragonite}$, and total DIC using the first and second dissociation constants (K$_1$ and K$_2$) of carbonic acid in seawater from Mehrbach et al. [41], refit by Dickson and Millero [42].

The High CO$_2$ treatment produced carbonate chemistry conditions (Table 1) comparable to those seen in Waquoit Bay during summer months [33] when many bivalve species, including bay scallops, spawn. Bay scallops live in coastal areas and estuaries, which experience seasonal fluctuations in pH and Ω_{ar} influenced by factors other than atmospheric CO$_2$. Consequently, the pH and Ω_{ar} currently experienced by coastal bivalve larvae [33] are lower than the values predicted for the open ocean at the end of this century [7,43].

Microscopic Imaging and Shell Measurements

At 1, 3, and 7 d, approximately 50 larvae from each culture were preserved in 95% ethanol for subsequent microscopic imaging and shell measurement. At the time of imaging, the preserved larvae were transferred to FSW and viewed at 200X magnification under bright field transmitted light using a Nikon ECLIPSE 50 i POL microscope. Images were captured using a SPOT Insight^{TM} Camera controlled by SPOT Basic Software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). 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. Mean growth rate (μm d^{-1}) for each replicate was calculated as the increase in mean shell length from 1 d to 7 d, divided by the number of days (6 d).

Table 1. Environmental parameters and carbonate chemistry for the Ambient CO$_2$ and High CO$_2$ treatments during the experiment (mean ± SD).

	Ambient CO$_2$	High CO$_2$
Measured parameters		
Temperature (°C)	22.4±0.3	22.5±0.2
Salinity	32.2±1.1	31.8±0.7
pH	7.93±0.01	7.39±0.03
A_T (μEq kg^{-1})	2130±68	2114±44
Calculated parameters		
pCO$_2$ (μatm)	509±13	1987±140
[HCO$_3^\times$] (μmol kg^{-1})	1779±46	2000±44
[CO$_3^{2\times}$] (μmol kg^{-1})	142±10	46±3
[CO$_2$] (μmol kg^{-1})	16±1	61±4
DIC (μmol kg^{-1})	1937±55	2107±47
$\Omega_{aragonite}$	2.26±0.14	0.74±0.04

Calculated parameters were calculated from pH and total alkalinity using CO2SYS software [40]. A_T = total alkalinity; DIC = dissolved inorganic carbon; $\Omega_{aragonite}$ = aragonite saturation state.
doi:10.1371/journal.pone.0061065.t001

Survival Estimation

Percent survival was estimated at 1, 3, 7, and 18 d. By 18 d, less than 0.5% of larvae remained alive in any treatment, so both survival and shell size analyses focused on 1, 3, and 7 d. On these days each culture in turn was homogeneously suspended by gently plunging with a graduated cylinder and a known volume (13, 25, and 40 ml for 1, 3, and 7 d, respectively) was removed and concentrated by gently pouring it through a 20 μm sieve. The volumes were chosen to yield a number of larvae (100–200) that could be counted in approximately 30–45 min. All of the live larvae in this volume were counted under a stereomicroscope at 10X magnification. The density of larvae in the removed volume was used to calculate the percent survival based on the initial stocking density of 30 larvae ml^{-1}. One replicate culture in the Ambient CO$_2$ treatment was discovered to have unreliable survival counts, as a result of a mistake by the counter, so only data from the other five replicate cultures were analyzed.

Statistical Analysis

All statistical analyses were performed using Systat$^{®}$ 13 Software (Systat Software, Inc., Chicago, IL, USA). Percent survival data were arcsine-square root-transformed prior to statistical analyses. Repeated measures ANOVA tests were run to compare survival among the three treatments at 1, 3, and 7 d and to compare shell length among the three treatments at 1, 3, and 7 d. One-way ANOVAs followed by Tukey's Honestly Significant Difference tests were run to compare survival and shell length among the three treatments at each time-point separately.

Results

Shell Length

Larval development (Fig. 1) in all treatments progressed in a sequence typical for this species as described by Belding [34] and Widman et al. [36]. All larvae were fully shelled at 1 d and were post-D-stage by 3 d. While fully shelled, some 1 d larvae
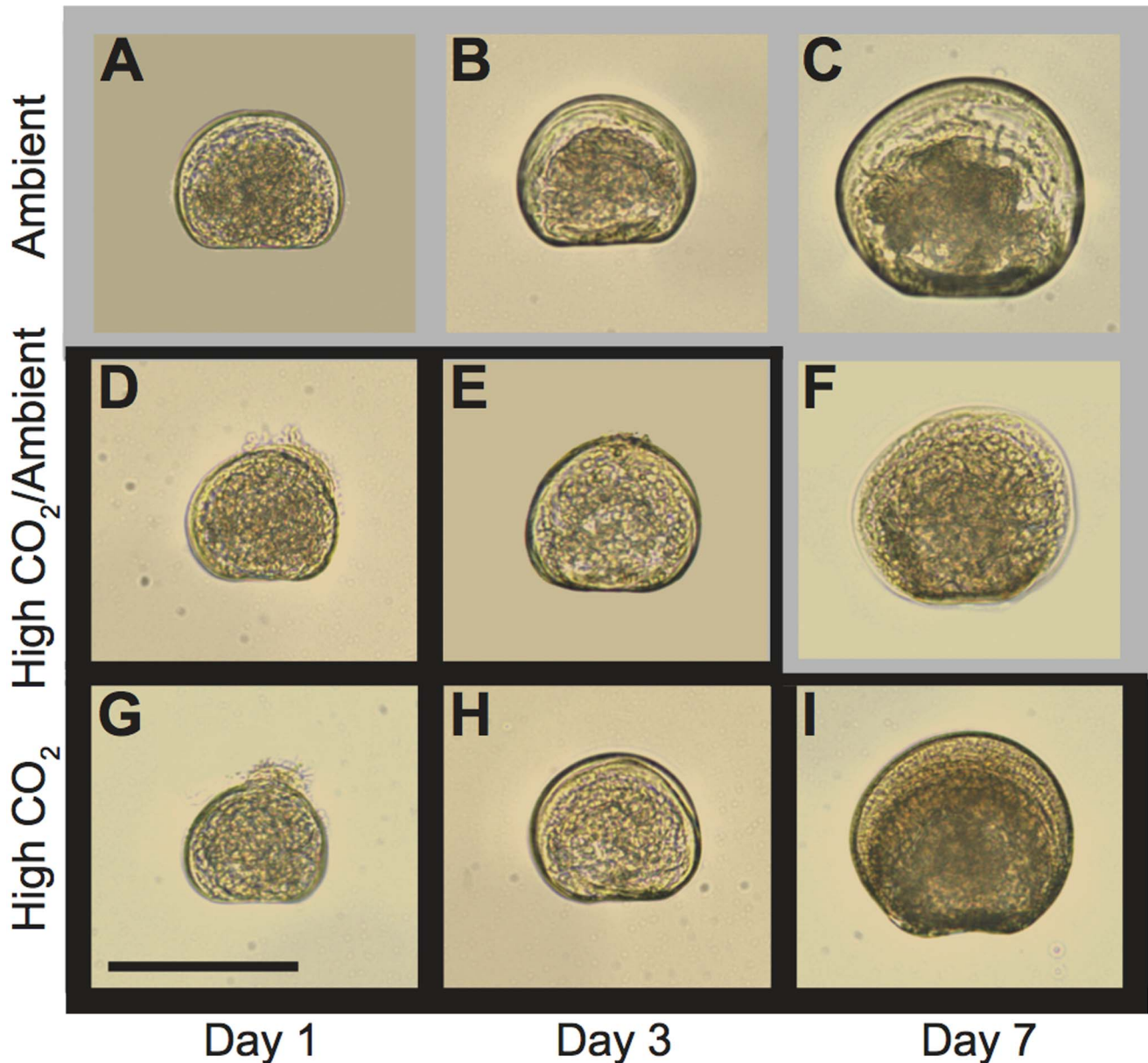
Figure 1. Larval morphology of bay scallops (*Argopecten irradians*) exposed to varied pCO$_2$ conditions. Larvae were preserved in 95% ethanol after incubation for 1 d, 3 d, and 7 d in (A–C) ambient CO$_2$, (D–F) high CO$_2$ for 3 d followed by ambient CO$_2$ for 4 d, and (G–I) high CO$_2$. Larvae shown represent the mean shell length for each treatment and age. The outline of each image corresponds to the CO$_2$ treatment the larvae were experiencing at the time of preservation. Gray = ambient CO$_2$, black = high CO$_2$. Images are all to the same scale; scale bar = 100 µm. doi:10.1371/journal.pone.0061065.g001

had velums that protruded from the shell (Fig. 1d, g); this occurred more frequently in larvae from the High CO$_2$/ Ambient and High CO$_2$ treatments than in larvae from the Ambient CO$_2$ treatment.

Exposure to high CO$_2$ (Table 1) caused a significant reduction in shell length (Fig. 2) during the first week of development when compared to exposure to ambient CO$_2$ (repeated measures ANOVA, Wilk's Lambda = 0.033; F = 19.47; df = 6, 26; p<0.00001). This pattern was significant for each of days 1, 3, and 7 (One-way ANOVA; Table 2). On day 1, after 12 h of exposure, the mean shell lengths of all three treatments were significantly different from each other (Fig. 2). The difference on day 1 between the High CO$_2$/Ambient and High CO$_2$ treatments was unexpected, as the larvae were in similar

conditions prior to the switch on day 3. On days 3 and 7, the mean shell lengths of larvae from the High CO$_2$ and High CO$_2$/Ambient treatments were no longer significantly different from each other, despite having been in different conditions since day 3. The mean lengths of shells from the High CO$_2$ treatment were 84.1%, 92.5%, and 88.5% of the mean lengths of shells from the Ambient CO$_2$ treatment on days 1, 3, and 7, respectively. After day 1, mean (±1 standard deviation) shell growth rates integrated over following 6 days were 7.4±1.5 µm d^{-1} for the Ambient CO$_2$ treatment, 6.7±1.2 µm d^{-1} for High CO$_2$/Ambient, and 7.2±1.0 µm d^{-1} for High CO$_2$; these differences were not significant (one-way ANOVA, F = 0.50, df = 2, p = 0.62).
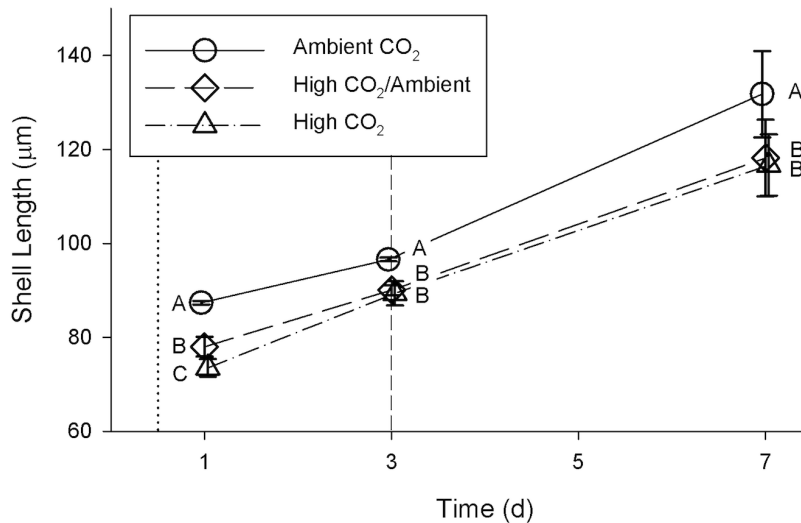
Figure 2. Shell length of larval bay scallops (*Argopecten irradians*) during the first week of larval development. Values are mean ± SD of 6 replicate culture containers. The dotted vertical line indicates the age at the time of inoculation (exposure to CO_2 treatments); the dashed vertical line indicates the age at which CO_2 conditions were switched for the High CO_2/Ambient treatment. Different letters (A, B, C) denote significant differences ($p<0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 2), followed by Tukey's HSD test.
doi:10.1371/journal.pone.0061065.g002

Larval Survival

Survival of scallop larvae in the Ambient CO_2 treatment was consistently higher than survival of scallop larvae in either the High/Ambient or High CO_2 treatments (Fig. 3) during the first week of development. This overall effect on larval survival was significant in a repeated measures ANOVA (Wilk's Lambda = 0.38; $F = 2.50$; df = 6, 24; $p = 0.05$). However, when the effect was examined for individual days, survival in the Ambient CO_2 treatment was significantly higher only on day 1 (Table 3, Fig. 3). Survival of larvae in the High/Ambient CO_2 treatment was not significantly different than in the other treatments at any time during the first week of development.

Discussion

Early exposure (12–24 hours post-fertilization) to high CO_2 significantly reduced larval shell size (Fig. 2) and survival (Fig. 3) relative to ambient CO_2 by the time the larvae were 1 d old. The initial reduction in size relative to the Ambient CO_2 treatment was still evident after the first week of larval development. This suggests

Table 2. One-way ANOVAs of mean shell length (μm) of *Argopecten irradians* larvae raised in three CO_2 treatment regimes (Ambient CO_2, High CO_2/Ambient, and High CO_2) at 1, 3, and 7 d; $n = 6$ replicate culture containers per treatment.

Day	Source of Variation	Type III SS	df	Mean Squares	F-Ratio	p-value
Day 1	Treatment	610.774	2	305.387	114.244	<0.001
	Error	40.097	15	2.673		
Day 3	Treatment	187.631	2	93.816	35.058	<0.001
	Error	40.140	15	2.676		
Day 7	Treatment	823.314	2	411.657	6.382	0.01
	Error	967.488	15	64.499		

doi:10.1371/journal.pone.0061065.t002

that CO_2 exposure during the first day is critical to shell development. Growth rate from 1–7 d was not significantly affected by CO_2 exposure, further indicating that growth during the first 24 h post-fertilization determines shell size later in development – the larvae did not increase their growth rate to compensate for initial slow growth. There is evidence that some bivalve larvae (Pacific oysters, *Crassostrea gigas*, and hard clams, *M. mercenaria*) use amorphous calcium carbonate (ACC) to produce the earliest stages of their shell [44,45]. If this is also true for bay scallops, then it may be a factor in explaining the sensitivity of bay scallop larvae to high CO_2 conditions during the first day of development, as ACC is more soluble than aragonite and its formation would therefore be less thermodynamically favorable. This demonstration of a significant and lasting CO_2 effect on shell size within the first day of larval development suggests that other studies on bivalve larval development in which CO_2 exposure was initiated after the first day of larval development and initial calcification [22,25,28–31] may have underestimated the magnitude of the effects of high CO_2 throughout larval development.
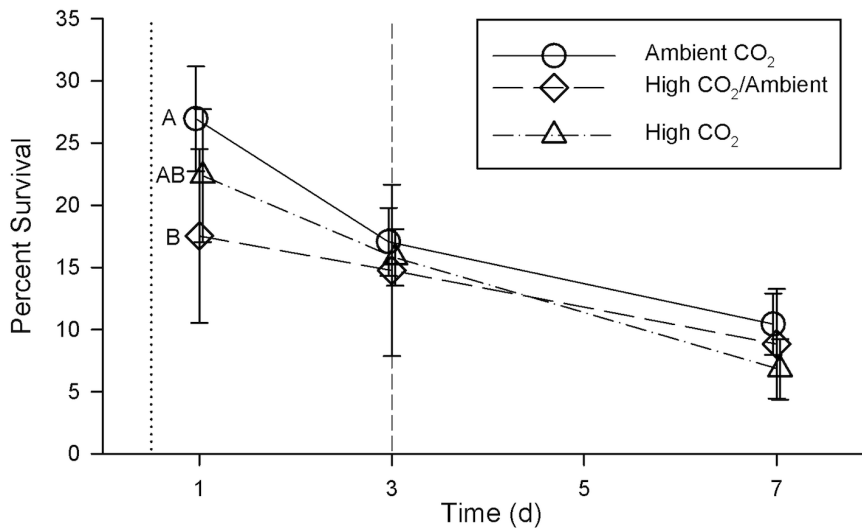
Exposure to high CO_2 caused a significant decrease, relative to ambient conditions, in larval survival at 1 d post fertilization, and a consistent, but not significant, decrease at 3 and 7 d post fertilization. Survival of <20% of individuals on day 7 is low compared to other studies of this species [22,31], probably due to a combination of not using antibiotics, and calculating survival from initial counts at 12 h post-fertilization rather than 3 d. Mortality during the first 3 d of larval development typically is high and can be variable between culture vessels even when conditions are held constant (Widman, J.C. Jr., personal communication). In our study, survival was highly variable among replicates within treatments and was likely influenced by factors other than carbonate chemistry. This variability may contribute to unexplainable patterns in survival such as the differences on day 1 between High CO_2 and High CO_2/Ambient treatments, despite the similar conditions.

A size reduction in scallop larvae exposed to high CO_2 may have indirect effects on subsequent survival in the field. The age at which a scallop larva is competent to metamorphose is often

Figure 3. Survival of larval bay scallops (*Argopecten irradians*). Survival is expressed as the percent of larvae surviving from the time of inoculation (age = 0.5 d), during the first week of larval development. Values are mean ± SD of $n = 5$ replicate culture containers for the Ambient CO_2 treatment and $n = 6$ replicate culture containers for the High CO_2/Ambient and High CO_2 treatments. The dotted vertical line indicates the age at the time of inoculation (exposure to CO_2 treatments); the dashed vertical line indicates the age at which CO_2 conditions were switched for the High CO_2/Ambient treatment. Letters denote significant differences ($p < 0.05$) between treatments at a given age, as determined in one-way ANOVA (Table 3), followed by Tukey's HSD test; only the Ambient and High CO_2 treatments on day 1 are significantly different.
doi:10.1371/journal.pone.0061065.g003

affected by size [46]. Small scallop larvae may delay metamorphosis, increasing their time in the plankton and risk of mortality from planktonic predators [47]. Previous work has found that exposure of bivalve larvae to high CO_2 treatments cause delayed metamorphosis, although it is not clear if small size was the cause of the delay [30–32]. In addition, if the smaller size of bay scallop larvae exposed to high CO_2 persisted through metamorphosis into adulthood, the reproductive output of those individuals could be stunted simply because the size of the gonad is proportional to the size of the scallop [48].

The negative effect of high CO_2 exposure on bay scallop larval size is consistent with negative effects of high CO_2 exposure on the size of other larval bivalves. The clam *Macoma balthica* has been shown to produce significantly smaller larval shells at 3 d when exposed to seawater with a pH of 7.5 or 7.8 directly after

fertilization, compared to a control treatment with pH 8.1 [30]. The same group also reported a similar trend in which *M. balthica* larvae produced (non-significantly) smaller shells from day 5 to 19 when exposed to lowered pH [30]. Similarly, larvae of the blue mussel, *Mytilus edulis*, had shells that, at 2 d, were 12.7% smaller when raised in water with pH 7.6 compared to larvae grown in water with pH 8.1 [25]. Larvae of the oyster *Saccostrea glomerata* showed at 8 d a decrease in shell size of 6.3% at pH 7.8 and 8.7% at pH 7.6 relative to pH 8.1 [29]. By documenting a size effect in the early larval stage of bay scallops, we can better understand the Talmage and Gobler observations of negative effects of high CO_2 exposure on shell size of 19–20 d old competent scallops [22,31,32]. Our results suggest that a decrease in size of competent larvae and set juveniles exposed to high CO_2 may be a lingering effect of compromised growth in early stages.

Recent work has suggested that marine organisms vary in their potential for evolutionary change in response to high CO2 conditions. For example, when exposed to high CO_2 conditions (2200 ppm), coccolithophore (*Emiliania huxleyi*) clones that had been raised in high CO_2 conditions for 500 generations showed a reduced negative calcification response when compared to clones that had been raised in ambient CO_2 conditions for 500 generations [49]. This indicates that the clones exposed to high CO_2 conditions for many generations adapted to the unfavorable conditions. Similarly, Sunday et al. [50] showed that larvae of the sea urchin *Strongylocentrotus franciscanus* may have faster evolutionary responses to high CO_2 than larvae of the mussel *Mytilus trossulus*, despite the fact that *M. trossulus* has a faster generation time and breeder replacement time. Studies of the evolutionary potential of marine invertebrates to adapt to high CO_2 conditions have been initiated only recently, and bay scallops may be a useful model organism for trans-generational laboratory culturing experiments, as they reach sexual maturity in just one year.

Acidification of coastal waters is affected by atmospheric CO_2 levels, but it is also strongly impacted by eutrophication [2,10–12], mixing and circulation [11,51], and input of fresh water [10,12]. We showed a CO_2 exposure effect on larval bay scallop growth,

Table 3. One-way ANOVAs of mean percent survival (arcsine-square root-transformed) of *Argopecten irradians* larvae raised in three CO_2 treatment regimes (Ambient CO_2, High CO_2/Ambient, and High CO_2) at 1, 3, and 7 d; $n = 5$ replicate culture containers for Ambient CO_2 treatment, $n = 6$ replicate culture containers each for High CO_2/Ambient and High CO_2 treatments.

Day	Source of Variation	Type III SS	df	Mean Squares	F-Ratio	p-value
Day 1	Treatment	0.040	2	0.020	3.731	0.050
	Error	0.074	14	0.005		
Day 3	Treatment	0.004	2	0.002	0.502	0.616
	Error	0.056	14	0.004		
Day 7	Treatment	0.012	2	0.006	1.694	0.219
	Error	0.051	14	0.004		

doi:10.1371/journal.pone.0061065.t003

but we did not include hypoxia as a treatment. Hypoxia typically co-occurs with high CO$_2$ (hypercapnia) as a result of eutrophication in coastal and estuarine waters [2,12,52]. Because hypoxia and hypercapnia act synergistically on the responses of marine invertebrates found in such environments [53], it is possible that the interaction of low oxygen and high CO$_2$ will affect larval bay scallop development even more strongly than high CO$_2$ alone.

The larvae in both High CO$_2$ and Ambient CO$_2$ treatments maintained similar growth rates from 1–7 d, but the negative effects on shell length of exposure to high CO$_2$ during the first 3 d of development were still present after a week of larval development. This result suggests that there is a critical initial window in which CO$_2$ exposure is particularly damaging to scallop larvae. Larvae that spawn in coastal and estuarine environments when pCO$_2$ is high may have smaller shells, even if they are transported within a few days to waters with lower CO$_2$. Aquaculture facilities may need to monitor pCO$_2$ in the water used for rearing early larvae to ensure that conditions do not impair growth.

Author Contributions

Conceived and designed the experiments: MMW DCM LSM ALC. Performed the experiments: MMW DCM LSM. Analyzed the data: MMW DCM LSM. Contributed reagents/materials/analysis tools: MMW DCM LSM ALC. Wrote the paper: MMW DCM LSM.

References

1. Cai W-J, Wang Y (1998) The chemistry, fluxes, and sources of carbon dioxide in the estuarine waters of the Satilla and Altamaha Rivers, Georgia. Limnol Oceanogr 43: 657–668.

2. Howarth R, Chan F, Conley DJ, Garnier J, Doney SC, et al. (2011) Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. Front Ecol Environ 9: 18–26. doi:10.1890/100008.

3. Hofmann GE, Smith JE, Johnson KS, Send U, Levin LA, et al. (2011) High-frequency dynamics of ocean pH: A multi-ecosystem comparison. PLoS ONE 6: e28983. doi:10.1371/journal.pone.0028983.t002.

4. Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: The other CO$_2$ problem. Ann Rev Mar Sci 1: 169–192.

5. Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, et al. (2004) Impact of anthropogenic CO$_2$ on the CaCO$_3$ system in the oceans. Science 305: 362–366. doi:10.1126/science.1097329.

6. Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, et al. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437: 681–686.

7. Feely RA, Doney SC, Cooley SR (2009) Ocean acidification: Present conditions and future changes in a high-CO$_2$ world. Oceanography 22: 36–47.

8. Waldbusser GG, Voigt EP, Bergschneider H, Green MA, Newell RIE (2011) Biocalcification in the Eastern Oyster (*Crassostrea virginica*) in relation to long-term trends in Chesapeake Bay pH. Estuaries Coast 34: 221–231. doi:10.1007/s12237-010-9307-0.

9. Gazeau F, Quiblier C, Jansen JM, Gattuso J-P, Middelburg JJ, et al. (2007) Impact of elevated CO$_2$ on shellfish calcification. Geophys Res Lett 34: L07603.

10. Cai W-J, Hu X, Huang W-J, Murrell MC, Lehrter JC, et al. (2011) Acidification of subsurface coastal waters enhanced by eutrophication. Nat Geosci 4: 766–770. doi:10.1038/ngeo1297.

11. Feely RA, Alin SR, Newton J, Sabine CL, Warner M, et al. (2010) The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. Estuar Coast Shelf Sci 88: 442–449. doi:10.1016/j.ecss.2010.05.004.

12. Sunda WG, Cai W-J (2012) Eutrophication Induced CO$_2$-acidification of subsurface coastal waters: interactive effects of temperature, salinity, and atmospheric pCO$_2$. Envir Sci Tech. doi:10.1021/es300626f.

13. Borges AV, Gypens N (2010) Carbonate chemistry in the coastal zone responds more strongly to eutrophication than ocean acidification. Limnol Oceanogr 55: 346–353.

14. Diaz RJ, Rosenberg R (2008) Spreading dead zones and consequences for marine ecosystems. Science 321: 926–929. doi:10.1126/science.1156401.

15. Rabalais NN, Turner RE, Wiseman WJ Jr (2002) Gulf of Mexico hypoxia, A.K.A. "The Dead Zone." Annu Rev Ecol Syst 33: 235–263. doi:10.1146/annurev.ecolsys.33.010802.150513.

16. Chou W-C, Gong G-C, Sheu DD, Jan S, Hung C-C, et al. (2009) Reconciling the paradox that the heterotrophic waters of the East China Sea shelf act as a significant CO$_2$ sink during the summertime: Evidence and implications. Geophys Res Lett 36: L15607. doi:10.1029/2009GL038475.

17. Green MA, Aller RC, Aller JY (1998) Influence of carbonate dissolution on survival of shell-bearing meiobenthos in nearshore sediments. Limnol Oceanogr 43: 18–28.

18. Green MA, Aller RC, Aller JY (1993) Carbonate dissolution and temporal abundances of foraminifera in Long Island Sound sediments. Limnol Oceanogr 38: 331–345.

19. Pechenik JA (1987) Environmental influences on larval survival and development. In: Giese AC, Pearse JS, Pearse VB, editors. Reproduction of Marine Invertebrates: General Aspects: Seeking Unity in Diversity. Palo Alto, CA: Blackwell Scientific Publications, Vol. 9. 551–607.

20. Kurihara H, Asai T, Kato S, Ishimatsu A (2008) Effects of elevated pCO$_2$ on early development in the mussel *Mytilus galloprovincialis*. Aquat Biol 4: 225–233.

21. Kurihara H, Kato S, Ishimatsu A (2007) Effects of increased seawater pCO$_2$ on early development of the oyster *Crassostrea gigas*. Aquat Biol 1: 91–98.

22. Talmage SC, Gobler CJ (2010) Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. Proc Nat Acad Sci USA 107: 17246–17251. doi:10.1073/pnas.0913804107.

23. Parker LM, Ross PM, O'Connor WA (2009) The effect of ocean acidification and temperature on the fertilization and embryonic development of the Sydney rock oyster *Saccostea glomerata* (Gould 1850). Glob Chang Biol 15: 2123–2136.

24. Van Colen C, Montserrat F, Vincx M, Herman PMJ, Ysebaert T, et al. (2010) Long-term divergent tidal flat benthic community recovery following hypoxia-induced mortality. Mar Pollut Bull 60: 178–186. doi:10.1016/j.marpolbul.2009.09.028.

25. Gazeau F, Gattuso JP, Dawber C, Pronker AE, Peene F, et al. (2010) Effect of ocean acidification on the early life stages of the blue mussel *Mytilus edulis*. Biogeosciences 7: 2051–2060. doi:10.5194/bg-7-2051-2010.

26. Gazeau F, Gattuso J-P, Greaves M, Elderfield H, Peene J, et al. (2011) Effect of carbonate chemistry alteration on the early embryonic development of the Pacific oyster (*Crassostrea gigas*). PLoS ONE 6: e23010. doi:10.1371/journal.pone.0023010.t001.

27. Parker LM, Ross PM, O'Connor WA (2010) Comparing the effect of elevated pCO$_2$ and temperature on the fertilization and early development of two species of oysters. Mar Biol 157: 2435–2452. doi:10.1007/s00227-010-1508-3.

28. Miller AW, Reynolds AC, Sobrino C, Riedel GF (2009) Shellfish face uncertain future in high CO$_2$ world: influence of acidification on oyster larvae calcification and growth in estuaries. PLoS ONE 4: e5661. doi:10.1371/journal.pone.0005661.t001.

29. Watson S-A, Southgate PC, Tyler PA, Peck LS (2009) Early larval development of the Sydney rock oyster *Saccostrea glomerata* under near-future predictions of CO$_2$-driven ocean acidification. J Shellfish Res 28: 431–437. doi:10.2983/035.028.0302.

30. Van Colen C, Debusschere E, Braeckman U, Van Gansbeke D, Vincx M (2012) The early life history of the clam *Macoma balthica* in a high CO$_2$ world. PLoS ONE 7: e44655. doi:10.1371/journal.pone.0044655.t001.

31. Talmage SC, Gobler CJ (2009) The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*). Limnol Oceanogr 54: 2072–2080.

32. Talmage SC, Gobler CJ (2011) Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juveniles of three species of Northwest Atlantic bivalves. PLoS ONE 6: e26941. doi:10.1371/journal.pone.0026941.t002.

33. McCorkle DC, Weidman C, Cohen AL (2012) Time series of pCO$_2$, pH, and aragonite saturation state in Waquoit Bay National Estuarine Research Reserve - "estuarine acidification" and shellfish. Ocean Sciences Meeting. Salt Lake City, UT, USA.

34. Belding DL (1910) A report upon the scallop fishery of Massachusetts, including the habits, life history of *Pecten irradians*, its rate of growth, and other facts of economic value. Boston, MA: Wright & Potter Printing Co., State Printers. 238 pp.

35. Helm MM, Bourne N (2004) Hatchery culture of bivalves: A practical manual. Lovatelli A, editor Rome: Food and Agricultural Organization of the United Nations. 177 pp.

36. Widman JC Jr, Choromanski J, Robohm RA, Stiles S, Wikfors GH, et al. (2001) Manual for Hatchery Culture of the Bay Scallop, *Argopecten irradians irradians*. Milford, CT: Connecticut Sea Grant College Program & NOAA National Marine Fisheries Service. 50 pp.

37. Clayton TD, Byrne RH (1993) Spectrophotometric seawater pH measurements: total hydrogen ion concentration scale calibration of *m*-cresol purple and at-sea results. Deep Sea Res Part I Oceanogr Res Pap 40: 2115–2129.

38. Dickson AG, Sabine CL, Christian JR (2007) Determination of the pH of sea water using the indicator dye *m*-cresol purple. In: Dickson AG, Sabine CL, Christian JR, editors. Guide to Best Practices for Ocean CO₂ Measurements. PICES Special Publication 3. p.191.

39. Liu X, Patsavas MC, Byrne RH (2011) Purification and characterization of meta-cresol purple for spectrophotometric seawater pH measurements. Envir Sci Tech 45: 4862–4868. doi:10.1021/es200665d.

40. Pierrot DW, Lewis E, Wallace D (2006) MS Excel program developed for CO₂ system calculations. doi:10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a.

41. Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol Oceanogr 18: 897–907.

42. Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res Part I Oceanogr Res Pap 34: 1733–1743.

43. Denman KL, Brasseur G, Chidthaisong A, Ciais P, Cox PM, et al. (2007) Couplings between changes in the climate system and biogeochemistry. In: Solomon S, Quin D, Manning M, Chen Z, Marquis M, et al., editors. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, United Kingdom and New York, NY, USA.: Cambridge University Press, Vol. 4. 499–587.

44. Weiss IM, Tuross N, Addadi L, Weiner S (2002) Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite. J Exp Zool 293: 478–491. doi:10.1002/jez.90004.

45. Lee SW, Hong SM, Choi CS (2006) Characteristics of calcification processes in embryos and larvae of the Pacific oyster, *Crassostrea gigas*. Bull Mar Sci 78: 309–317.

46. Sastry AN (1965) The development and external morphology of pelagic larval and post-larval stages of the bay scallop, *Aequipecten irradians concentricus* Say, reared in the laboratory. Bull Mar Sci 15: 417–465.

47. Thorson G (1950) Reproductive and larval ecology of marine bottom invertebrates. Biol Rev 25: 1–45.

48. Barber BJ, Blake NJ (2006) Reproductive Physiology. In: Shumway SE, Parsons GJ, editors. Scallops: Biology, Ecology, and Aquaculture. Elsevier. 357–416.

49. Lohbeck KT, Riebesell U, Reusch TBH (2012) Adaptive evolution of a key phytoplankton species to ocean acidification. Nat Geosci 5: 346–351. doi:10.1038/ngeo1441.

50. Sunday JM, Crim RN, Harley CDG, Hart MW (2011) Quantifying rates of evolutionary adaptation in response to ocean acidification. PLoS ONE 6: e22881. doi:10.1371/journal.pone.0022881.g003.

51. Gilbert D, Rabalais NN, Diaz RJ, Zhang J (2010) Evidence for greater oxygen decline rates in the coastal ocean than in the open ocean. Biogeosciences 7: 2283–2296. doi:10.5194/bg-7-2283-2010.

52. Dai M, Guo X, Zhai W, Yuan L, Wang B, et al. (2006) Oxygen depletion in the upper reach of the Pearl River estuary during a winter drought. Mar Chem 102: 159–169. doi:10.1016/j.marchem.2005.09.020.

53. Widdicombe S, Spicer JI (2008) Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? J Exp Mar Bio Ecol 366: 187–197. doi:10.1016/j.jembe.2008.07.024.