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Elevated pCO_2 causes developmental delay in early larval Pacific oysters, *Crassostrea gigas*

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Abstract Increasing atmospheric CO_2 equilibrates with surface seawater, elevating the concentration of aqueous hydrogen ions. This process, ocean acidification, is a future and contemporary concern for aquatic organisms, causing failures in Pacific oyster (*Crassostrea gigas*) aquaculture. This experiment determines the effect of elevated pCO_2 on the early development of *C. gigas* larvae from a wild Pacific Northwest population. Adults were collected from Friday Harbor, Washington, USA (48°31.7′N, 12°1.1′W) and spawned in July 2011. Larvae were exposed to Ambient (400 µatm CO_2), MidCO_2 (700 µatm), or HighCO_2 (1,000 µatm). After 24 h, a greater proportion of larvae in the HighCO_2 treatment were calcified as compared to Ambient. This unexpected observation is attributed to increased metabolic rate coupled with sufficient energy resources. Oyster larvae raised at HighCO_2 showed evidence of a developmental delay by 3 days post-fertilization, which resulted in smaller larvae that were less calcified.

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Introduction

Ocean acidification is expected to affect ecosystems at an accelerating pace over the next century (Caldeira and Wickett 2003; IPCC 2007). Seawater pH declines (acidifies) in association with the uptake of anthropogenic CO_2 and resultant increased H^+ ion concentration. Projected changes in atmospheric pCO_2 may have significant consequences for natural populations ranging from physiological changes to broad-scale range shifts (Talmage and Gobler 2011; O'Donnell et al. 2009; Wong et al. 2011; Tomanek et al. 2011; Banks et al. 2010; Perry et al. 2005).

Acidification of nearshore waters can occur via a variety of processes, including equilibration with elevated pCO_2 in the atmosphere, upwelling events, terrestrial run-off, and respiration. The upper ocean acidification in the North Pacific is proportional to the anthropogenic increase in atmospheric CO_2, enforcing that the present-day pH changes are outside the range of natural variability (Byrne et al. 2010a, b). In addition to atmospheric sources of CO_2, oceanic upwelling and nearshore respiration further reduce the pH of water in which larvae develop (as low as pH 7.4 along the west coast of North America) and increasingly result in waters undersaturated with respect to aragonite (Feely et al. 2008, 2010). During the spring and summer off the US west coast, upwelling of waters rich in CO_2 and respiration from nearshore biological activity can cause under saturation of nearshore waters (Feely et al. 2008, 2010; Fassbender et al. 2011). These contemporary processes occur in the same area where planktonic invertebrate larvae congregate. As CO_2 emissions continue to equilibrate with ocean surface water, these habitats that already experience low pH could see further and more sustained increases in pCO_2.

Numerous studies have examined developmental consequences of ocean acidification on marine bivalve larvae.

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Exposure to low-pH water early in development caused decreased mid-stage growth and survival in *C. gigas* (Barton et al. 2012). *C. gigas*'s congener, *Crassostrea virginica*, grew more slowly and incorporated less $CaCO_3$ into their shells at elevated pCO_2 when compared to controls (Miller et al. 2009). Similarly, ocean acidification conditions decreased both shell integrity and tissue mass in larval mussels, *Mytilus californianus* (Gaylord et al. 2011). Larval Sydney rock oysters (*Saccostrea glomerata*) demonstrated reduced survival and slower growth and development when reared under conditions simulating future oceanic pCO_2 (Watson et al. 2009). Both clam (*Mercenaria mercenaria*) and scallop larvae (*Argopectens irrandians*) were impacted by elevated pCO_2 in their metamorphosis, growth, and lipid synthesis (Talmage and Gobler 2011). The effects of ocean acidification have been studied on populations of *C. gigas* from Japan (Kurihara et al. 2007), Australia (Parker et al. 2010, 2012), and Europe (Gazeau et al. 2011), but few studies to date look at these effects on populations of *C. gigas* from the United States. Due to differences in experimental design, it is difficult to directly compare the three aforementioned studies, but overall *C. gigas* larvae are smaller when raised at elevated pCO_2 (Kurihara et al. 2007; Parker et al. 2010; Gazeau et al. 2011), demonstrate a developmental delay (Kurihara et al. 2007), and have morphological and shell deformities (Kurihara et al. 2007; Parker et al. 2010; Gazeau et al. 2011).

Pacific oyster larvae are planktotrophs, spending an extended period of one to three weeks in the plankton, where they undergo a variety of important morphological and physiological changes (Strathmann 1985). These developmental changes are frequently associated with environmental cues (Bonar et al. 1990), and their successful completion is necessary for larval metamorphosis into a settled juvenile oyster. Organismal responses to ocean acidification vary among and within taxa suggesting that ecological and evolutionary history may influence responses to ocean acidification. Thus, empirical studies are needed to understand the mechanistic responses of species to a specific environmental stress and how the stress corresponds to the species' or population's original ecological niche.

One of the primary means by which marine organisms are directly influenced by ocean acidification is through relative concentrations of H^+ and associated decreased availability of CO_3^{2-}. These changes in water chemistry impact calcifying organisms as they rely on CO_3^{2-} to form and maintain carbonate-based structures (Beniash et al. 2010; Thomsen and Melzner 2010), while greater H^+ concentration can cause acidosis of body fluids. Acidosis can result in dissolution of calcium carbonate structures, reducing shell thickness and releasing ions into the hemolymph. Many adult aquatic invertebrates can make use of dissolved calcified structures or possibly actively dissolve their shell, to make HCO_3^- more available as a buffer against internal acidosis. Excess HCO_3^- for buffering can also be acquired from the aquatic environment. This phenomenon has been observed in Dungeness crabs, *Cancer magister* (Pane and Barry 2007); blue crabs, *Callinectes sapidus* (Henry et al. 1981); limpets, *Patella vulgata* (Marchant et al. 2010); and urchins, *Psammechinus miliaris* (Miles et al. 2007); however, internal acidosis was not successfully avoided in oysters, *C. gigas* (Lannig et al. 2010). It is not clear to what degree larvae can utilize this mechanism to maintain homeostasis under elevated pCO_2 conditions, but some invertebrates that inhabit naturally CO_2-rich environments are able to reproduce and the larvae settle without apparent adverse effects (Thomsen and Melzner 2010).

Sustained environmental change, such as ocean acidification, can negatively affect both the ecosystem and economy. Shellfish, including oysters, provide important ecosystem services such as improved water quality and benthic-pelagic coupling through the filtration of large volumes of water, release of feces to the benthos, and creation of habitat via reef formation (Coen and Luckenbach 2000). In addition to their ecological roles, mollusks are economically important to many coastal communities worldwide. In 2008, mollusks comprised 64.1 % (or 13.1 million tons) of worldwide aquaculture production, with oysters accounting for 31.8 % of the total production (FAO 2010). The global economic cost of ocean acidification to the mollusk fishery is unclear but has been estimated to increase with rising atmospheric CO_2 levels and terrestrial sources of acidification (Narita et al. 2012). Recently, in the Pacific Northwest of the US, concern has heightened over the already apparent effects of corrosive, acidified water on both natural and hatchery production of *C. gigas* larvae (Elston et al. 2008; Feely et al. 2010; Barton et al. 2012). Hatchery water supply comes from adjacent natural bays, and when upwelling events occur, the water that enters the hatchery can reach pCO_2 near 1,000 µatm (S. Alin, unpublished data; B. Eudeline, pers. comm.). These upwelling events have been linked to mortality episodes in the hatchery, perhaps due to a combination of acidic water and pathogens associated with the water masses (Elston et al. 2008). Acidification events are projected to become more frequent and sustained as atmospheric pCO_2 continues to rise.

This study characterized the effects of two elevated levels of pCO_2 on size, calcification, and development during early larval stages of the Pacific oyster, *Crassostrea gigas*. Oyster larvae were raised in two elevated levels of pCO_2 (700 and 1,000 µatm) and ambient (400 µatm) seawater through 72 h following fertilization. The chemistry scenarios simulated in this study are based on projections for the coming century, but these values of low pH and Ω are already occurring with increasing frequency in nearshore upwelling systems off the US West coast (Feely et al. 2010; Hauri et al. 2009).

Materials and methods

Seawater chemistry manipulation

Experimental conditions were maintained using a flow-through seawater system in Friday Harbor, Washington, USA. Water entering the system was filtered (to 0.2-μm), UV-sterilized, and CO_2-depleted using membrane contactors (Membrana, Charlotte, North Carolina, USA) under partial vacuum. Three experimental treatments were chosen to correspond with dissolved CO_2 levels of 400, 700, or 1,000 ppm in the atmosphere. These three treatments will be referred to throughout the manuscript as Ambient, MidCO_2, and HighCO_2. Set-point pH levels were determined with the program CO_2SYS (Robbins et al. 2010) using an average total alkalinity of 2,060 μmol kg^{-1} based on total alkalinity measurements taken the week prior to the experimental trial.

Larval *C. gigas* were held in 3-L microcosms within a large reservoir filled with the respective treatment water. Ambient air stripped of CO_2 by a CO_2 adsorption unit (Twin Tower Engineering, Broomfield, Colorado, USA) was used to aerate the seawater within the reservoirs through a Venturi injector into the larger reservoir of treatment water. This replaced oxygen lost through the degassing process. Reservoir pH was continuously monitored by a Durafet III pH probe (Honeywell, Morristown, New Jersey, USA). When the probe registered that the treatment's pH strayed from its set point, a solenoid would open or close to allow more or less pure CO_2 (Praxair, Danbury, Connecticut, USA) to be injected via the Venturi. The Durafet probe information was fed into a Honeywell UDA2182 pH controller, which also controlled the solenoids.

Seawater was pumped from the reservoir into larval microcosms through irrigation drippers (DIG Industries, Sun Valley, California, USA) at a rate of 1.9-L h^{-1}. An outflow tube at the top of the microcosms fitted with 35-μm mesh allowed water to exit the microcosms while retaining larvae. All systems were equilibrated to the correct treatment level 48 h prior to the start of the experiment. Water temperature was held at 20.4 ± 0.4 °C.

Oysters

Ten female and four male adult *C. gigas* were collected from Argyle Creek in Friday Harbor, Washington, in July 2011. Oysters were strip-spawned into Ambient seawater with eggs and sperm pooled separately (day 0). Pooled eggs (approximately 2 million) were divided equally into 18 7.5-cm diameter containers. Sperm was diluted (so as to approximate a 1:1 sperm/egg ratio) in Ambient seawater and added to each container of eggs. After the addition of sperm, the eggs were gently agitated and incubated for 15 min to allow for fertilization.

Six containers of fertilized eggs were transferred to microcosms containing one of three treatment conditions. Initial densities post-hatching were approximately 1 larva mL^{-1}. On days 1 and 3 post-fertilization, larvae were randomly sampled to determine survival, size, developmental stage, and presence or absence of calcification. For each microcosm sampled, larvae were filtered onto 35-μm mesh screens and washed with the appropriate seawater. Approximately 100 larvae were removed for each sample, relaxed with 7.5 % $MgCl_2$, and fixed in 4 % paraformaldehyde buffered in filtered seawater. The remaining larvae were returned to cleaned microcosms filled with new seawater. Larvae were fed *Dunaliellia* sp. and *Isochrysis* sp. at concentrations of 30,000 cells mL^{-1} each (concentrations for optimal larval growth) on day 2. During feeding, water flow was turned off in microcosms for 2 h. All microcosms were cleaned at each sampling event.

Larvae were examined using light microscopy to determine survival, size, developmental stage, and shell presence/absence. Survival was determined at 20–40×: larvae were counted as dead if there was a complete absence of ciliary movement. Larval hinge length and shell height were measured at 10× magnification with a Nikon Eclipse E600 and NIS Elements Basic Research software (Nikon, Tokyo, Japan). Larval developmental stage and shell presence were determined at 20× magnification using an inverted microscope and double polarized light. Larvae were scored as calcified on day 1 post-fertilization if calcified shell was observed at the hinge (Fig. 1a). On day 3 post-fertilization, larvae were classified as fully calcified if polarized light produced a "Maltese cross" in the larval shell (Fig. 1b; LaBarbera 1974).

Carbonate chemistry

Salinity was measured with a conductivity meter (Hach sensION5; Loveland, Colorado, USA), and temperature was measured using a Fluke 1523 thermometer (Fluke, Everett, Washington, USA). Seawater pH entering the microcosms was measured daily using the spectrophotometric (spec) technique outlined in SOP 6b by Dickson et al. (2007) to confirm pH measurements from the Durafet probe. When any discrepancies were observed, the Durafet probe was recalibrated. Seawater pH measurements were taken from two microcosms per treatment on days 0, 1, and 3. Final pH values reported here have been corrected for dye addition and temperature. Total alkalinity (A_T) was measured following the open cell titration of SOP 3b (Dickson et al. 2007). Samples for A_T were taken from incoming water and from two microcosms in each treatment on days 0, 2, and 3. CO_2SYS (Robbins et al. 2010)

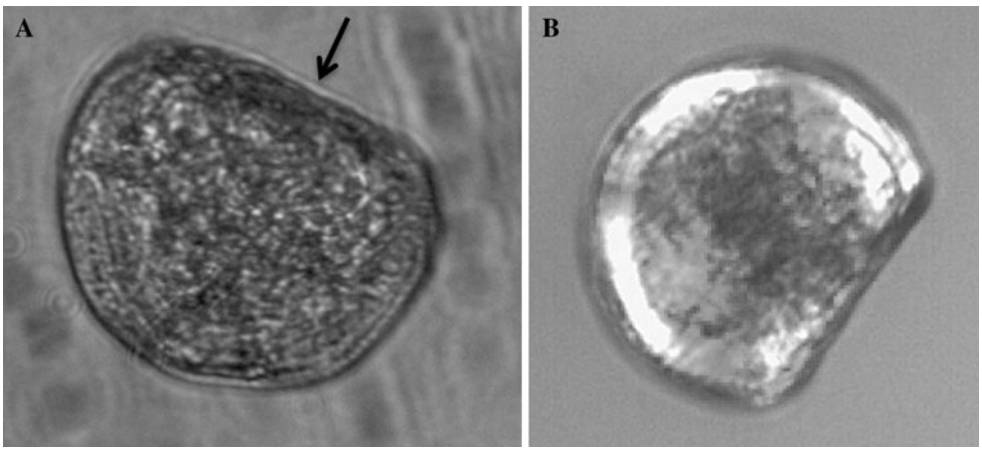

Fig. 1 D-hinge larvae under polarized light portraying calcification at the hinge without a Maltese cross in the shell (**a**) and full calcification as evidenced by the Maltese cross (**b**)

was used to calculate calcium carbonate saturation state (Ω) of aragonite and calcite, carbonate ion concentration, and pCO_2 with A_T and pH as inputs using the following constants: Lueker et al. (2000) for CO_2 constants; Dickson (1990) for $KHSO_4$, total scale (mol kg^{-1} SW) for pH scale; and Wanninkhof (1992) for air–sea flux.

Statistics

Differences in larval size and mortality across treatments were examined using a two-way ANOVA with fixed effects of treatment and day followed by Tukey's Honestly Significant Difference test (Tukey's HSD). A one-way ANOVA was also used to test for differences in larval size among treatments using the combined fixed factor of day-treatment. Larval calcification and developmental stage were compared among treatments using a generalized linear model (GLM). Binomial error distributions were used for GLM analyses. The occurrence of a developmental delay was assessed by fitting the regression of shell height on hinge length to a linear model and testing for differences in the slopes of these lines across treatments. Developmental delay would be demonstrated if the larvae maintained the same allometry across treatments (the slopes of the lines were the same) but were different in size. At least two replicates within treatments and time points were used for all statistical analyses. All analyses were performed in R (R Development Core Team 2011).

Results

Carbonate chemistry

Throughout the experiment, seawater pH differed across treatments and A_T varied slightly but to the same degree across treatments (Table 1). Mean seawater pH was consistent within but varied among treatments (Fig. 2). Mean pH (±standard deviation), as measured by the Durafet pH probes (Fig. 2), was 7.99 ± 0.04 in the Ambient treatment, 7.75 ± 0.06 in the $MidCO_2$ treatment, and 7.66 ± 0.09 in the $HighCO_2$ treatment. Aragonite and calcite saturation states were >1.0 for the duration of the experiment, except in the $HighCO_2$ treatment on days 1 and 2 (Table 1). Carbonate ion concentration was lowest in the $HighCO_2$ treatment (average ± SD of 61.15 ± 4.05 µmol kg^{-1} seawater, $N = 4$, Table 1), intermediate in $MidCO_2$ (74.05 ± 6.43 µmol kg^{-1}, $N = 4$), and highest in the Ambient treatment (120.24 ± 11.52 µmol kg^{-1}, $N = 4$). Partial pressure of CO_2 in the seawater averaged 468 ± 63 µatm in the Ambient treatment, 847 ± 67 µatm in the $MidCO_2$ treatment, and 1,065 ± 58 µatm in the $HighCO_2$ treatment.

Size, development, and calcification

Survival was near 100 % in all treatments on day 1 (Ambient = 99.0 %, $MidCO_2$ and $HighCO_2$ = 99.7 %). On day 3, survival was 92.9 % in the Ambient treatment and was approximately 88.6 % in the $MidCO_2$ and 85.6 % in the $HighCO_2$ treatment. Mortality was similar across treatments ($F = 0.59$, $P > 0.05$) but different across days ($F = 17.7$, $P < 0.05$).

On day 1, a slightly greater proportion (0.977) of larvae at $HighCO_2$ were at the D-hinge stage (compared with those that were still trocophores), but this difference was not significant (z value = 1.016, $P = 0.310$; data not shown). The proportion of larvae at the D-hinge stage on day 1 in the Ambient treatment was 0.875 and in $MidCO_2$ was 0.833. Amount of larvae with shell was significantly different among treatments for days 1 and 3 post-fertilization. Following 24 h of treatment (day 1), the proportion

Table 1 Water chemistry data for three experimental treatments—Ambient, $MidCO_2$, and $HighCO_2$: temperature and Durafet pH measurements are averages from each day based on the Honeywell controller logs

Treatment	Day	Temperature (°C)	Salinity (ppt)	Total alkalinity (nmol/kg)	pH (Durafet)	pH (spec)	pCO_2 (µatm)	Ω (calcite)	Ω (aragonite)	co_3^{2-} (µmol/kg)
Ambient	0	20.38	28.13	1,998.72	7.95	7.90	557.22	2.62	1.67	104.27
	1	20.50	27.97	2,005.28	7 99	7.97	466.27	2.14	1.36	120.26
	2	20.49	28.16	1,965.59	7.96	8.00	420.92	3.15	2.01	125.38
	3	20.46	28.91	2,021.96	8.00	8.00	428.43	3.27	2.10	131.03
	μ+SD	20.46±0.05	28.29±0.42	1,997.89±23.65	7.99±0.04		468±63			120.24±11.52
$MidCO_2$	0	20.00	28.13	2,003.25	775	7.73	860.92	1.83	1.17	72.79
	1	20.25	27.97	1,983.64	7.73	7.75	812.40	1.90	1.21	75.48
	2	20.18	28.16	1,969.06	7.78	7.69	935.39	1.66	1.06	66.21
	3	20.14	28.91	2,022.46	7.77	7.77	780.96	2.04	1.31	81.73
	μ±SD	20.14±0.11	28.29±0.42	1,994.60±23.26	7.75±0.06		847±67			74.05±6.43
$HighCO_2$	0	20.25	28.13	2,001.57	7.67	7.66	1,025.33	1.59	1.02	63.28
	1	20.47	27.97	1,979.61	7.64	7.61	1,149.99	1.42	0.91	56.46
	2	20.10	28.16	1,966.46	7 70	7.64	1,057.36	1.49	0.95	59.30
	3	20.41	28.91	2,023.45	7 64	7.66	1,030.70	1.64	1.05	65.54
	μ±SD	20.31±0.17	28.29±0.42	1,992.77±25.06	7.66±0.09		1,065±58			61.15±4.05

Salinity, total alkalinity (A_T), and spectrophotometric (spec) pH are point measurements taken each day. Partial pressure of CO_2, Ω, and CO_3^{2-} were calculated from spec pH and A_T. Mean and standard deviation ($\mu \pm$ SD) for the following parameters are given for all 3 days: temperature, salinity, A_T, pH, pCO_2, and CO_3^{2-}

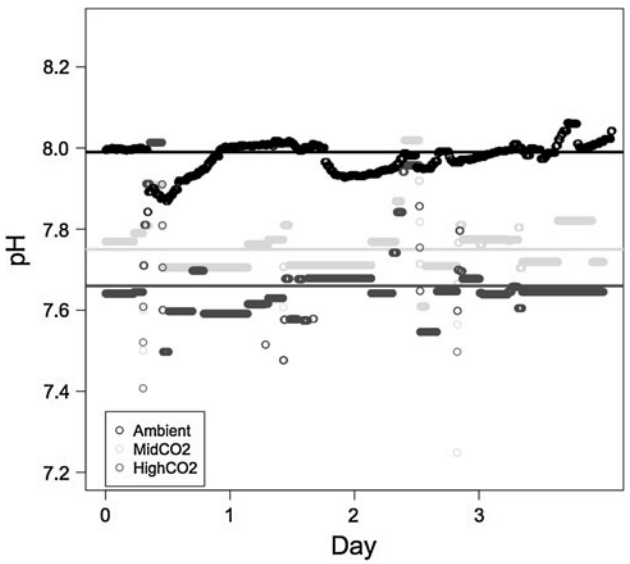

Fig. 2 Profiles of pH measurements in the three different treatments—Ambient (*black*), $MidCO_2$ (*light gray*), and $HighCO_2$ (*dark gray*). Average pH for the experiment for each treatment is represented by *solid lines*. The Durafet probes recorded pH measurements every minute

of larvae with shell present was inversely proportional to pCO_2 level with the greatest number of larvae with shell in the $HighCO_2$ treatment (z value = 2.084, $P = 0.0372$, Fig. 3). On day 3, fewer larvae at $HighCO_2$ conditions had full shell compared with the other two treatments (z value = −3.203, $P = 0.00136$).

Larval size (shell height and hinge length) was similar across experimental treatments after 24 h; however, by day 3, larvae grew significantly larger (height and length) in the Ambient and $MidCO_2$ as compared to the $HighCO_2$ treatment (Table 2, Figs. 4, 5). Between days 1 and 3, larvae increased in size under Ambient conditions (shell height, $P < 1e{-}7$) and $MidCO_2$ conditions (shell height and hinge length, $P < 1e{-}7$ and $P = 7.4e{-}6$, respectively; Figs. 4, 5) but did not significantly increase in size under $HighCO_2$ conditions. By day 3, all larvae observed across treatments were at the D-hinge stage. The slope of the linear regression through shell height versus hinge length for the larvae raised at Ambient pCO_2 was 0.6459 (Fig. 6), which was not significantly different from the slope of the regression line through the $MidCO_2$ data (0.8583, $P > 0.05$) or from the line through the $HighCO_2$ size data (0.3625, $P > 0.10$).

Discussion

Oyster larvae raised at $HighCO_2$ showed evidence of a developmental delay by 3 days post-fertilization, which caused them to be smaller and have less calcified material than controls. These results are consistent with other studies of *Crassostrea* spp. larvae in which elevated pCO_2 resulted in decreased growth and shell mineralization (Kurihara et al. 2007; Miller et al. 2009). Kurihara et al. (2007) raised *C. gigas* to 48 h post-fertilization at an elevated pCO_2 of about 2,268 µatm, much higher than pCO_2

Table 2 Results from post hoc Tukey's HSD following ANOVA for comparisons of hinge length and shell height among treatments

	Treatment	Hinge length		Shell height	
		$MidCO_2$	$HighCO_2$	$MidCO_2$	$HighCO_2$
2-way ANOVA	Ambient	0.250	0.0362	0.985	$\ll$0.001
	$MidCO_2$	–	$\ll$0.001	–	$\ll$0.001
1-way ANOVA day 1	Ambient	0.849	0.984	0.585	0.885
	$MidCO_2$	–	0.993	–	0.992
1-way ANOVA day 1	Ambient	0.565	0.0311	0.261	$\ll$0.001
	$MidCO_2$		$\ll$0.001		$\ll$0.001

The two-way ANOVA was performed with "treatment" and "day" as fixed effects and the one-way ANOVA was performed with the fixed effect of "day-treatment"

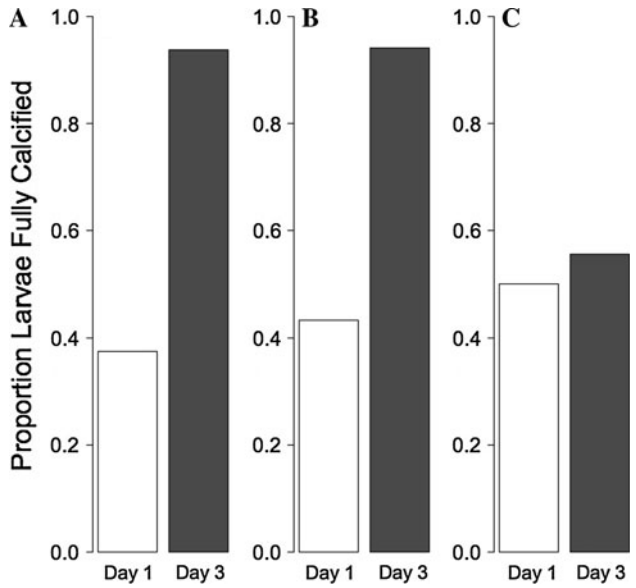

Fig. 3 Proportion of calcified larvae at different pCO_2 treatments. *Bars* represent calcification on day 1 (*white*) and day 3 (*gray*). Proportion of larvae calcified are provided from the Ambient treatment (panel A), $MidCO_2$ treatment (panel B), and $HighCO_2$ treatment (panel C). There is a significant difference in calcification among treatments, with the highest proportion of larvae calcified at $HighCO_2$ on day 1 and the fewest larvae calcified in $HighCO_2$ on day 3

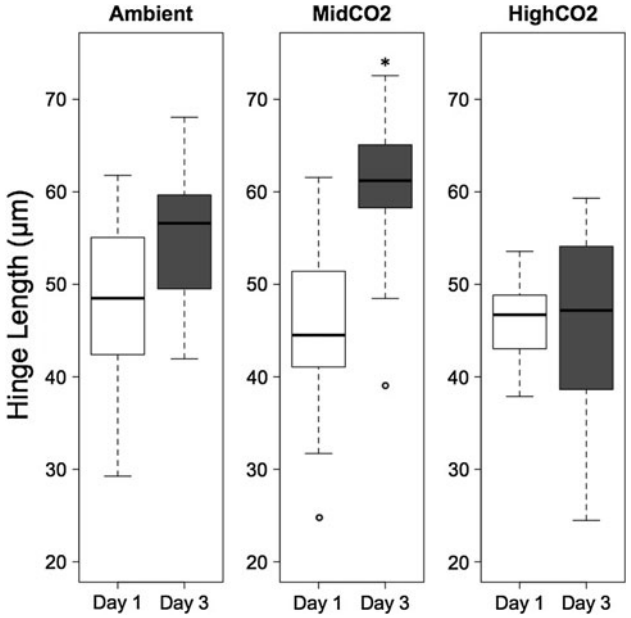

Fig. 4 Larval hinge length on day 1 (*white boxplots*) and day 3 (*gray boxblots*). Results are shown for the Ambient treatment, $MidCO_2$ treatment, and $HighCO_2$ treatment. Boxplots contain the middle 50 % of the data, and *dashed lines* encompass data within 1.5× the spread of the middle 50 %. Open circles represent outliers. *Horizontal black bars* indicate median values. An *asterisk* indicates significant differences within a treatment. On day 3, larvae in the $HighCO_2$ treatment were significantly smaller than those in the other two treatments ($P < 0.05$)

projected for the coming century, and observed a negative effect on calcification as early as 24 h post-fertilization. The authors also observed a developmental delay in reaching the D-hinge stage at 48 h post-fertilization (Kurihara et al. 2007). Since we did not measure growth or calcification in our larvae at 48 h post-fertilization, we are not able to draw direct comparisons with this time point, but we did observe a developmental delay by 72 h post-fertilization. Similarly, C. virginica larvae raised from 72 h post-fertilization through competency at different pCO_2 grew more slowly at elevated pCO_2 (560 and 800 µatm) and biomineralized less $CaCO_3$ than controls; however, *Crassostrea ariakensis* showed no effect of pCO_2 treatment (Miller et al. 2009). It is likely the observed differences between the studies are related to the much higher pCO_2 level used by Kurihara et al. (2007) and species- and population-specific differences in acclimation to ocean acidification.

The developmental delay is evidenced by similar growth trajectories across treatments (Figs. 3, 6) coupled with the smaller size of larvae in the $HighCO_2$ treatment. This suggests that change in size is not a direct effect of ocean acidification on shell growth and maintenance. In a study comparing faster growing hybrid *C. gigas* larvae to slower

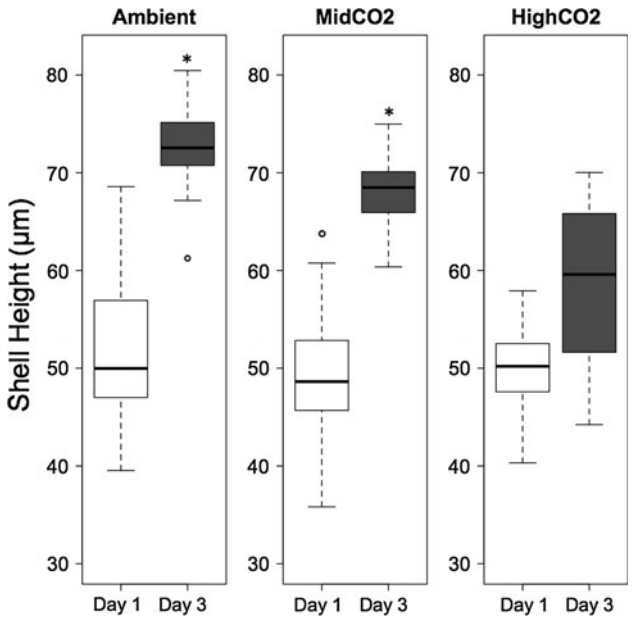

Fig. 5 Larval shell height on day 1 (*white boxplots*) and day 3 (*gray boxblots*). Results are shown for the Ambient treatment, $MidCO_2$ treatment, and $HighCO_2$ treatment. Boxplots contain the middle 50 % of the data, and *dashed lines* encompass data within 1.5× the spread of the middle 50 %. *Open circles* represent outliers. *Horizontal black bars* indicate median values. An *asterisk* indicates significant differences within a treatment. On day 3, shell height was reduced in larvae at $HighCO_2$ relative to those raised at Ambient and at $MidCO_2$ ($P < 0.01$)

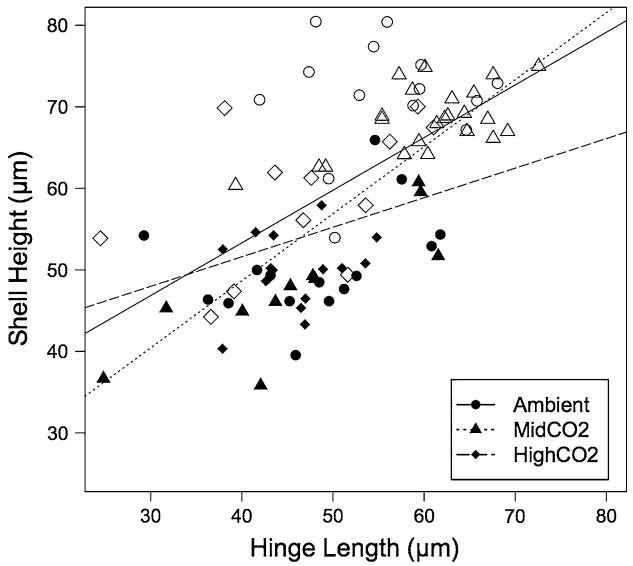

Fig. 6 Regression of larval shell height on hinge length by treatment and day. Data from larvae raised under Ambient pCO_2 conditions are represented by *circles*, $MidCO_2$ are *triangles*, and $HighCO_2$ are *diamonds*. Size data from day 1 are in *black* and day 3 are in *white*. The *solid line* is the regression line for the Ambient data (intercept = 27.47, slope = 0.65), *dotted* for $MidCO_2$ (intercept = 15.76, slope = 0.82), and *dashed* for $HighCO_2$ (intercept = 37.10, slope = 0.36). The slopes of all the lines are statistically the same ($P > 0.05$)

growing inbred larvae, slower growth was attributed to reduced feeding rate and differing allocation of internal energy reserves for metabolic processes (Pace et al. 2006). The stress of elevated pCO_2 can induce similar physiological changes via effects on metabolic demands, resulting in a developmentally delayed phenotype (Stumpp et al. 2011a). It is difficult to detect developmental delay with complete confidence in studies that do not follow larvae through to settlement. In one such study, larval *Strongylocentrotus purpuratus* were exposed to elevated pCO_2 throughout their larval period, and from this perspective, it was apparent that ocean acidification caused a delay in development, although at discrete time points, this delay could be interpreted as overall smaller size (Stumpp et al. 2011a). Developmental delay may give these species the energetic resources they need to survive stress and reach the later developmental stages of metamorphosis and settlement. However, a delay in development opens the possibility for a host of other complications for pelagic larvae, such as greater potential to be advected to unsuitable habitat (Strathmann 1985), greater chance of being exposed to predators (Underwood and Fairweather 1989), and an overall longer time in the water column where environmental conditions are variable and risky for a free-floating larva.

A greater percentage of the larvae in the $HighCO_2$ treatment had shell present by 24 h fertilization compared with both Ambient and $MidCO_2$. The impact of ocean acidification on larval invertebrates can change in direction and magnitude as the larvae switch from a non-feeding to a feeding stage. The larvae at $HighCO_2$ were most likely able to maintain a normal development rate and calcified structures early in developmental because an increased metabolic rate would have been supported by sufficient maternal energy resources. In early development, *C. gigas* depend on maternal lipid reserves, but after 24 h in the plankton, the larvae become dependent upon external resources (Gallager et al. 1986). Environmental stress frequently instigates an elevated metabolic rate (Lannig et al. 2010; Stumpp et al. 2011a). During the non-feeding stage, larvae may have enough maternal resources to support their increased metabolic rate and sustain normal or even accelerated growth and development. In the non-feeding lecithotrophic larvae of the common sun star (*Crossaster papposus*), larvae at low pH developed and grew faster than those in ambient conditions (Dupont et al. 2010). Once the metabolic switch to external resources occurs, the larvae may not be able to get enough resources to sustain the increased metabolic rate as well as normal development. A similar trend is seen in larval purple sea urchins, *S. purpuratus*. Ocean acidification had a larger impact on the feeding larval stage of *S. purpuratus* than it did on the non-feeding stage (Stumpp et al. 2011a). Similarly to *C. gigas*, *S. purpuratus* demonstrated a developmental

delay starting with the onset of feeding (Stumpp et al. 2011a). At the same time, routine metabolic rate increased in both elevated pCO_2 and ambient treatment, but increased more at low pH (Stumpp et al. 2011a). The results from these studies suggest that the maintenance of homeostasis becomes more difficult under the energetic demands of ocean acidification stress; however, the physiological stress is realized as developmental delay, with associated phenotypes of less shell and smaller size, only when larvae are in a feeding stage. Reallocation of resources associated with invertebrate responses to ocean acidification has been shown to affect several processes, including as soft tissue growth (Gaylord et al. 2011; Beniash et al. 2010), scope for growth (Stumpp et al. 2011a), and shell integrity (Gaylord et al. 2011; Melzner et al. 2011).

Larval shell formation is closely linked to development and begins by 24 h post-fertilization. Numerous species experience decreased calcification when water is undersaturated with respect to aragonite (Kurihara et al. 2007; Miller et al. 2009; Crim et al. 2011; Gazeau et al. 2011; Byrne et al. 2010a, b), although some species are still able to form apparently normal calcified structures in undersaturated conditions (Dupont et al. 2010; Catarino et al. 2011; Yu et al. 2011). Early *C. gigas* larval shells are made of amorphous calcium carbonate and aragonite (Weiss et al. 2002), two of the more soluble forms of $CaCO_3$ at low pH. Invertebrates are able to control calcification through amorphous mineral precursors and metabolites (Weiss 2011), thus decreasing the potential effects of a corrosive environment. On days 1 and 2, Ω_{Ar} was below 1.0, causing the seawater to be undersaturated with respect to aragonite. Calcification can become energetically costly due to scarcity of CO_3^{2-} ions in the environment and disruption of ionic gradients of the calcifying compartment from changes in H^+. If oyster larvae remove a fixed number of H^+ from their calcifying fluid versus maintaining a fixed ratio of extracellular/intracellular H^+, then their energy budget would be more taxed during environmental hypercapnia (Ries 2011). This added stress on the process of calcification could have contributed to the energy budget shifts that led to a developmental delay.

In this study, *C. gigas* tolerated the $MidCO_2$ treatment through 3 days post-fertilization. The lack of negative effects on shell formation and maintenance in the larvae from the $MidCO_2$ treatment suggests that a cut-off of $\Omega_{Ar} < 1.0$ is significant in terms of the ability of this population to biomineralize at this time point in development. It is also possible that the high level of food available to the larvae modulated the impact of ocean acidification and could have led to an underestimation its effect in this treatment (Melzner et al. 2011). An elevated pCO_2 of 750 ppm (Ω_{Ar} of about 1.0) had significant negative effects on hard clam (*Mercenaria mercenaria*) and bay scallop (*Argopecten irradians*) larvae after about 3 weeks of exposure as evidenced by decreased survival, development, growth, and lipid synthesis (Talmage and Gobler 2011). The comparable exposure conditions in our study ($MidCO_2$) did not have a negative impact over the time period observed. Due to the similarities of carbonate chemistry parameters with Talmage and Gobler (2011), the differential responses observed across species are likely indicative of variability in species, developmental stage tolerances, or length of exposure. Longer experiments in larvae have demonstrated that the negative effects of ocean acidification persist and sometimes worsen in mussels *M. californianus* (Gaylord et al. 2011), urchins *S. purpuratus* (Stumpp et al. 2011a, b), abalone *Haliotis kamtschatkana* (Crim et al. 2011), and oysters, *Crassostrea ariakensis* and *C. virginica* (Miller et al. 2009). The compounding negative effects of ocean acidification during an experiment may be due to a species' decreasing ability to tolerate a specific environmental stress as their metabolic needs change throughout development.

Conclusions

In this study, we observed that an acute, 72-h exposure to the end-of-century projections of ocean acidification ($HighCO_2$) has a negative impact on development in oyster larvae. Additionally, this study revealed that moderate changes in seawater chemistry ($MidCO_2$, about 800 µatm, mean $\Omega_{Ar} > 1.19 \pm 0.10$) did not have an observed significant impact on larvae through 3 days post-fertilization. It appears the effects of an environmental stress, such as ocean acidification, vary depending on developmental and metabolic stage of *C. gigas* larvae. This is most likely directly associated with a switch in larval energy metabolism as the oysters develop from a non-feeding stage to a feeding stage. In order to effectively evaluate the possibility of acclimation or adaptation, future research should focus on characterizing larvae from diverse genotypes and locations as well as assessing any influences that might be experienced later in development.

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References

Banks SC, Ling SD, Johnson CR, Piggott MP, Williamson JE, Behergegaray LB (2010) Genetic structure of a recent climate change-driven range extension. Mol Ecol 19:2011–2024

Barton A, Hales B, Waldbusser G, Langdon C, Feely RA (2012) The Pacific oyster, Crassostrea gigas, shows negative correlation to naturally elevated carbon dioxide levels: implications for near-term ocean acidification impacts. Limnol Oceanogr 57:698–710

Beniash E, Ivanina A, Lieb NS, Kurochkin Il, Sokolova IM (2010) Elevated levels of carbon dioxide affects metabolism and shell formation in oysters Crassostrea virginica. Mar Ecol Prog Ser 419:95–108

Bonar DB, Coon SL, Walch M, Weiner RM, Fitt W (1990) Control of oyster settlement and metamorphosis by endogenous and exogenous chemical cues. Bull Mar Sci 46:484–498

Byrne M, Ho M, Wong E, Soars NA, Selyakumaraswamy P, Shepard-Brennand H, Dworjanyn SA, David AR (2010a) Unshelled abalone and corrupted urchins: development of marine calcifiers in a changing ocean. Proc R Soc B. doi:10.1098/rspb.2010.2404

Byrne RH, Mecking S, Feely RA, Liu X (2010b) Direct observations of basin-wide acidification of the North Pacific Ocean. Geophys Res Lett. doi:10.1029/2009GL040999

Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. Nature 425:365

Catarino AI, De Ridder C, Gonzalez M, Gallardo P, Dubois P (2011) Sea urchin Arbacia dufresnei (Blainville 1825) larvae response to ocean acidification. Polar Biol. doi:10.1007/s00300-011-1074-2

Coen LD, Luckenbach MW (2000) Developing success criteria and goals for evaluating oyster reef restoration: ecological function or resource exploitation? Ecol Eng 15:323–343

Crim RN, Sunday JM, Harley CDG (2011) Elevated seawater CO2 concentrations impair larval development and reduce larval survival in endangered northern abalone (Haliotis kamtschatkana). J Exp Mar Biol Ecol 400:272–277

Dickson AG (1990) Thermodynamics of the dissociation of boric acid in synthetic seawater from 273.15 to 318.15 K. Deep Sea Res Part A, Oceanogr Res Pap 37(5):755–766

Dickson AG, Sabine CL, Christian JR (2007) Guide to best practices for ocean CO2 measurements. North Pacific Marine Science Organization, Sidney, British Columbia, p 176

Dupont S, Lundve B, Thorndyke M (2010) Near future ocean acidification increases growth rate of the lecithotrophic larvae and juveniles of the sea star Crossaster papposus. J Exp Zool (Mol Dev Evol) 314B:382–389

Elston RA, Hasegawa H, Humphrey KL, Polyak IK, Häse CC (2008) Re-emergence of Vibrio tubiashii in bivalve aquaculture: severity, environmental drivers, geographic extent, and management. Dis Aquat Org 82:119–134

Fassbender AJ, Sabine CL, Feely RA, Langdon C, Mordy CW (2011) Inorganic carbon dynamics during northern California coastal upwelling. Cont Shelf Res 31:1180–1192

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008) Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science 320:1490–1492

Feely RA, Alin SR, Newton J, Sabine CL, Warner M, Devol A, Krembs C, Maloy C (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

FAO Fisheries and Aquaculture Department (2010) World aquaculture 2010. FAO Fisheries and Aquaculture Department. Technical Paper. No. 500/1. Rome, FAO. 2011. 105 pp

Gallager SM, Mann R, Sasaki GC (1986) Lipid as an index of growth and viability in three species of bivalve larvae. Aquaculture 56:81–103

Gaylord B, Hill TM, Sanford E, Lenz EA, Jacobs LA, Sato KN, Russell AN, Hettinger A (2011) Functional impacts of ocean acidification in an ecologically critical foundation species. J Exp boil 214:2586–2594

Gazeau F, Gattuso J-P, Greaves M, Elderfield H, Peene J, Heip CHR, Middelburg JJ (2011) Effects of carbonate chemistry alteration on early embryonic development of the Pacific oyster (Crassostrea gigas). PLoS ONE. doi:10.1371/journal.pone.0023010

Hauri C, Gruber N, Plattner G-K, Alin S, Feely RA, Hales B, Wheeler PA (2009) Ocean acidification in the California current system. Oceanography 22:60–71

Henry RP, Kormanik GA, Smatresk NJ, Cameron JN (1981) The role of $CaCO_3$ dissolution as a source of HCO_3^- for the buffering of hypercapnic acidosis in aquatic and terrestrial decapod crustaceans. J Exp Biol 94:269–273

IPCC (2007) Climate change 2007: synthesis report. In: Core Writing Team, Pachauri RK, Reisinger A (eds) Contribution of working groups I, II and III to the fourth assessment report of the intergovernmental panel on climate change. IPCC, Geneva

Kurihara H, Kato S, Ishimatsu A (2007) Effects of increased seawater pCO_2 on early development of the oysters Crassostrea gigas. Aquatic Biology 1:91–98

LaBarbera M (1974) Calcification of the first larval shell of Tridacna squamosa (Tridacnidae: Bivalvia). Mar Biol 25:233–238

Lannig G, Eilers S, Pörtner HO, Sokolova IM, Bock C (2010) Impact of ocean acidification on energy metabolism of Oyster, Crassostrea gigas-changes in metabolic pathways and thermal response. Mar Drugs 8:2318–2339

Lueker TJ, Dickson AG, Keeling CD (2000) Ocean pCO_2 calculated from dissolved inorganic carbon, alkalinity, and equations for K_1 and K_2—validation based on laboratory measurements of CO_2 in gas and seawater at equilibrium. Mar Chem 70:105–119

Marchant HK, Calosi P, Spicer JI (2010) Short-term exposure to hypercapnia does not compromise feeding, acid-base balance or respiration of Patella vulgata but surprisingly is accompanied by radula damage. J Mar Biol Assoc UK 90:1379–1384

Melzner F, STange P, Trubenbach K, Thomsen J, Casties I, Panknin U, Gorb SN, Gutowska MA (2011) Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel Mytilus edulis. PLoS ONE 6:e24223. doi:10.1371/journal.pone.0024223

Miles H, Widdicombe S, Spicer JI, Hall-Spencer J (2007) Effects of anthropogenic seawater acidification on acid-base balance in the sea urchin Psammechinus miliaris. Mar Pollut Bull 54:89–96

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

Narita D, Rehdanz K, Tol RSJ (2012) Economic costs of ocean acidification: a look into the impacts on global shellfish production. Clim Change. doi:10.1007/s10584-011-0383-3

O'Donnell MJ, Hammond LM, Hofmann GE (2009) Predicted impact of ocean acidification on a marine invertebrate: elevated CO_2 alters response to thermal stress in sea urchin larvae. Mar Biol 156:439–446

Pace DA, Marsh AG, Leong PK, Green AJ, Hedgecock D, Manahan DT (2006) Physiological bases of energetically determined variation in growth of marine invertebrate larvae: a study of growth heterosis in the bivalve Crassostrea gigas. J Exp Mar Biol Ecol 335:188–209

Pane EF, Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Mar Ecol Prog Ser 334:1–9

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

Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Portner H-O (2012) Adult exposure influences offspring response to ocean acidification in oysters. Glob Change Biol 18:82–92

Perry A, Low PJ, Ellis JR, Reynolds JD (2005) Climate change and distribution shifts in marine fishes. Science 308:1912–1915

Ries JB (2011) A physicochemical framework for interpreting the biological calcification response to CO_2-induced ocean acidification. Geochim Cosmochim Acta 75:4053–4064

Robbins LL, Hansen ME, Kleypas JA, Meylan SC (2010) CO2calc: a user-friendly carbon calculator for windows, Mac OS X, and iOS (iPhone): U.S. geological survey open file report 2010–1280, 42 pp

Strathmann RR (1985) Feeding and nonfeeding larval development and life-history evolution in marine invertebrates. Ann Rev Ecol Syst 16:339–361

Stumpp M, Wren J, Melzner F, Thorndyke MC, Dupont ST (2011a) CO_2 induced seawater acidification impacts sea urchin larval development I: elevated metabolic rates decrease scope for growth and induce developmental delay comparative biochemistry and physiology—part a: molecular and integrative. Physiology 160:331–340

Stumpp M, Dupont ST, Thorndyke MC, Melzner F (2011b) CO_2 induced seawater acidification impacts sea urchin larval development II: gene expression patterns in pluteus larvae. Comparative biochemistry and physiology—part a: molecular and Integrative. Physiology 160:320–330

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. doi:10.1371/journal.pone.0026941

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel Mytilus edulis. Mar Biol 157:2667–2676

Tomanek L, Zuzow MJ, Ivanina AV, Beniash E, Sokolova IM (2011) Proteomic response to elevated pCO_2 level in eastern oysters, Crassostrea virginica: evidence for oxidative stress. J Exp Biol 214:1836–1844

Underwood AJ, Fairweather PG (1989) Supply-side ecology and benthic marine assemblages. Trends Ecol Evol 4:16–20

Wanninkhof R (1992) Relationship between wind speed and gas exchange over the ocean. J Geophys Res 97(5):7373–7382

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

Weiss IM (2011) Biomaterials: metabolites empowering minerals. Nat Chem Biol 7:192–193

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

Wong KKW, Lane AC, Leung PTY, Thiyagarajan V (2011) Response of larval barnacle proteome to CO_2-driven seawater acidification. Comp Biochem Physiol D Genomics Proteomics 6:310–321

Yu PC, Matson PG, Martz TR, Hofmann GE (2011) The ocean acidification seascape and its relationship to the performance of calcifying marine invertebrates: laboratory experiments on the development of urchin larvae framed by environmentally-relevant pCO_2/pH. J Exp Mar Biol Ecol 400:288–295