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 (1000 µatm). After 24 hours, 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.

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, RH *et al*. 2010). 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 U.S. west coast, upwelling of waters rich in CO_2 and respiration from nearshore biological activity can cause undersaturation 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. 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, *C. 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 *et al.* 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, molluscs are economically important to many coastal communities worldwide. In 2008, molluscs 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 mollusc 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 U.S., 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 1000 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 1000 µatm) and ambient (400 µatm) seawater through 72 hours 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 U.S. West coast (Feely *et al*. 2010; Hauri *et al*. 2009).

MATERIAL 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 1000 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 2060 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 adsorbtion 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,

196 USA). When the probe registered that the treatment's pH strayed from its set point, a
197 solenoid would open or close to allow more or less pure CO_2 (Praxair, Danbury,
198 Connecticut, USA) to be injected via the Venturi. The Durafet probe information was fed
199 into a Honeywell UDA2182 pH controller, which also controlled the solenoids.
200 Seawater was pumped from the reservoir into larval microcosms through
201 irrigation drippers (DIG Industries, Sun Valley, California, USA) at a rate of 1.9-L h^{-1}. An
202 outflow tube at the top of the microcosms fitted with 35-µm mesh allowed water to exit
203 the microcosms while retaining larvae. All systems were equilibrated to the correct
204 treatment level 48 hours prior to the start of the experiment. Water temperature was held
205 at 20.4°C ± 0.4°C
206
207 Oysters
208 Ten female and four male adult *C. gigas* were collected from Argyle Creek in
209 Friday Harbor, Washington in July 2011. Oysters were strip-spawned into Ambient
210 seawater with eggs and sperm pooled separately (day 0). Pooled eggs (approximately 2
211 million) were divided equally into 18 7.5-cm diameter containers. Sperm was diluted (so
212 as to approximate a 1:1 sperm:egg ratio) in Ambient seawater and added to each
213 container of eggs. After the addition of sperm, the eggs were gently agitated and
214 incubated for 15 minutes to allow for fertilization.
215 Six containers of fertilized eggs were transferred to microcosms containing one
216 of three treatment conditions. Initial densities post-hatching were approximately 1 larva
217 mL^{-1}. On days 1 and 3 post-fertilization, larvae were randomly sampled to determine
218 survival, size, developmental stage, and presence or absence of calcification. For each
219 microcosm sampled, larvae were filtered onto 35-µm mesh screens and washed with the
220 appropriate seawater. Approximately 100 larvae were removed for each sample,
221 relaxed with 7.5% $MgCl_2$ and fixed in 4% paraformaldehyde buffered in filtered seawater.
222 The remaining larvae were returned to cleaned microcosms filled with new seawater.
223 Larvae were fed *Dunaliellia* sp. and *Isochrysis* sp. at concentrations of 30,000 cells mL^{-1}
224 each (concentrations for optimal larval growth) on day 2. During feeding, water flow was
225 turned off in microcosms for two hours. All microcosms were cleaned at each sampling
226 event.
227 Larvae were examined using light microscopy to determine survival, size,
228 developmental stage and shell presence/absence. Survival was determined at 20-40x:
229 larvae were counted as dead if there was a complete absence of ciliary movement.
230 Larval hinge length and shell height were measured at 10x magnification with a Nikon
231 Eclipse E600 and NIS Elements Basic Research software (Nikon, Tokyo, Japan). Larval
232 developmental stage and shell presence were determined at 20x magnification using an
233 inverted microscope and double polarized light. Larvae were scored as calcified on day
234 1 post-fertilization if calcified shell was observed at the hinge (Figure 1A). On day 3

post-fertilization, larvae were classified as fully calcified if polarized light produced a "Maltese cross" in the larval shell (Figure 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) 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 (1990b) 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 (Figure 2). Mean pH (± standard deviation), as measured by the Durafet pH probes (Figure 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 greater than 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 1065 ± 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 to 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 hours of treatment (day 1) the proportion 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, Figure 6). On day 3, fewer larvae at HighCO_2 conditions had full shell compared to 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 hours, 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, Figures 3 and 4). Between days 1 and 3 larvae increased in size under Ambient conditions (shell height, $P < 1e\text{-}7$) and MidCO_2 conditions (shell height and hinge length, $P < 1e\text{-}7$ and $P = 7.4e\text{-}6$, respectively; Figures 3 and 4), 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 (Figure 5), 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, $P > 0.10$).

313
314 DISCUSSION
315 Oyster larvae raised at HighCO_2 showed evidence of a developmental delay by 3
316 days post-fertilization, which caused them to be smaller and have less calcified material
317 than controls. These results are consistent with other studies of *Crassostrea* spp. larvae
318 in which elevated pCO_2 resulted in decreased growth and shell mineralization (Kurihara
319 *et al*. 2007; Miller *et al.* 2009). Kurihara *et al*. (2007) raised *C. gigas* to 48 hours post-
320 fertilization at an elevated pCO_2 of about 2268 µatm, much higher than pCO_2 projected
321 for the coming century, and observed a negative effect on calcification as early as 24
322 hours post-fertilization. The authors also observed a developmental delay in reaching
323 the D-hinge stage at 48 hours post-fertilization (Kurihara *et al.* 2007). Since we did not
324 measure growth or calcification in our larvae at 48 hours post-fertilization, we are not
325 able to draw direct comparisons with this time point, but we did observe a developmental
326 delay by 72 hours post-fertilization. Similarly, *Crassostrea virginica* larvae raised from
327 72 hours post-fertilization through competency at different pCO_2 grew more slowly at
328 elevated pCO_2 (560 and 800 µatm) and biomineralized less $CaCO_3$ than controls;
329 however *Crassostrea ariakensis* showed no effect of pCO_2 treatment (Miller *et al.* 2009).
330 It is likely the observed differences between the studies are related to the much higher
331 pCO_2 level used by Kurihara *et al*. (2007) and species- and population-specific
332 differences in acclimation to ocean acidification
333 The developmental delay is evidenced by similar growth trajectories across
334 treatments (Figure 5) coupled with the smaller size of larvae in the HighCO_2 treatment.
335 This suggests that change in size is not a direct effect of ocean acidification on shell
336 growth and maintenance. In a study comparing faster growing hybrid *C. gigas* larvae to
337 slower growing inbred larvae, slower growth was attributed to reduced feeding rate and
338 differing allocation of internal energy reserves for metabolic processes (Pace *et al.*
339 2006). The stress of elevated pCO_2 can induce similar physiological changes via effects
340 on metabolic demands, resulting in a developmentally delayed phenotype (Stumpp *et al.*
341 2011a). It is difficult to detect developmental delay with complete confidence in studies
342 that do not follow larvae through to settlement. In one such study, larval
343 *Strongylocentrotus purpuratus* were exposed to elevated pCO_2 throughout their larval
344 period and from this perspective it was apparent that ocean acidification caused a delay
345 in development, although at discrete time points this delay could be interpreted as
346 overall smaller size (Stumpp *et al*. 2011a). Developmental delay may give these species
347 the energetic resources they need to survive stress and reach the later developmental
348 stages of metamorphosis and settlement. However, a delay in development opens the
349 possibility for a host of other complications for pelagic larvae, such as greater potential
350 to be advected to unsuitable habitat (Strathmann 1985), greater chance of being
351 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 hours fertilization compared to 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 hours 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, *Strongylocentrotus 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 hours 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, M. *et al.* 2010), 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

391 amorphous calcium carbonate and aragonite (Weiss *et al*. 2002), two of the more
392 soluble forms of $CaCO_3$ at low pH. Invertebrates are able to control calcification through
393 amorphous mineral precursors and metabolites (Weiss 2011), thus decreasing the
394 potential effects of a corrosive environment. On days 1 and 2, Ω_{Ar} was below 1.0,
395 causing the seawater to be undersaturated with respect to aragonite. Calcification can
396 become energetically costly due to scarcity of CO_3^{2-} ions in the environment and
397 disruption of ionic gradients of the calcifying compartment from changes in H^+. If oyster
398 larvae remove a fixed number of H^+ from their calcifying fluid versus maintaining a fixed
399 ratio of extracellular:intracellular H^+, then their energy budget would be more taxed
400 during environmental hypercapnia (Ries 2011). This added stress on the process of
401 calcification could have contributed to the energy budget shifts that led to a
402 developmental delay.
403 In this study, *C. gigas* tolerated the MidCO_2 treatment through 3 days post-
404 fertilization. The lack of negative effects on shell formation and maintenance in the
405 larvae from the MidCO_2 treatment suggests that a cut-off of $\Omega_{Ar} < 1.0$ is significant in
406 terms of the ability of this population to biomineralize at this time point in development. It
407 is also possible that the high level of food available to the larvae modulated the impact of
408 ocean acidification and could have led to an underestimation its effect in this treatment
409 (Melzner *et al.* 2011). An elevated pCO_2 of 750 ppm (Ω_{Ar} of about 1.0) had significant
410 negative effects on hard clam (*Mercenaria mercenaria*) and bay scallop (*Argopecten*
411 *irradians*) larvae after about 3 weeks of exposure as evidenced by decreased survival,
412 development, growth and lipid synthesis (Talmage and Gobler 2011). The comparable
413 exposure conditions in our study (MidCO_2) did not have a negative impact over the time
414 period observed. Due to the similarities of carbonate chemistry parameters with
415 Talmage and Gobler (2011), the differential responses observed across species are
416 likely indicative of variability in species, developmental stage tolerances, or length of
417 exposure. Longer experiments in larvae have demonstrated that the negative effects of
418 ocean acidification persist and sometimes worsen in mussels *Mytilus californianus*
419 (Gaylord *et al*. 2011), urchins *S. purpuratus* (Stumpp *et al*. 2011 a and b), abalone
420 *Haliotis kamtschatkana* (Crim *et al.* 2011), and oysters *Crassostrea ariakensis* and *C.*
421 *virginica* (Miller *et al.* 2009). The compounding negative effects of ocean acidification
422 during an experiment may be due to a species' decreasing ability to tolerate a specific
423 environmental stress as their metabolic needs change throughout development.
424
425 *Conclusions*
426 In this study we observed that an acute, 72 hour exposure to end-of-century
427 projections of ocean acidification (HighCO_2) has a negative impact on development in
428 oyster larvae. Additionally, this study revealed that moderate changes in seawater
429 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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FIGURE LEGENDS

Treatment	Day	Temperature (°C)	Salinity (ppt)	Total Alkalinity (µmol/kg)	pH (Durafet)	pH (spec)	pCO_2 (µatm)	Ω (calcite)	Ω (aragonite)	CO_3^{2-} (µmol/kg)
	0	20.38	28.13	1998.72	7.95	7.90	557.22	2.62	1.67	104.27
	1	20.50	27.97	2005.28	7.99	7.97	466.27	2.14	1.36	120.26
Ambient	2	20.49	28.16	1965.59	7.96	8.00	420.92	3.15	2.01	125.38
	3	20.46	28.91	2021.96	8.00	8.00	428.43	3.27	2.10	131.03
	µ±SD	*20.46±0.05*	*28.29±0.42*	*1997.89±23.65*	*7.99±0.04*		*468±63*			*120.24±11.52*
	0	20.00	28.13	2003.25	7.75	7.73	860.92	1.83	1.17	72.79
	1	20.25	27.97	1983.64	7.73	7.75	812.40	1.90	1.21	75.48
$MidCO_2$	2	20.18	28.16	1969.06	7.78	7.69	935.39	1.66	1.06	66.21
	3	20.14	28.91	2022.46	7.77	7.77	780.96	2.04	1.31	81.73
	µ±SD	*20.14±0.11*	*28.29±0.42*	*1994.60±23.26*	*7.75±0.06*		*847±67*			*74.05±6.43*
	0	20.25	28.13	2001.57	7.67	7.66	1025.33	1.59	1.02	63.28
	1	20.47	27.97	1979.61	7.64	7.61	1149.99	1.42	0.91	56.46
$HighCO_2$	2	20.10	28.16	1966.46	7.70	7.64	1057.36	1.49	0.95	59.30
	3	20.41	28.91	2023.45	7.64	7.66	1030.70	1.64	1.05	65.54
	µ±SD	*20.31±0.17*	*28.29±0.42*	*1992.77±25.06*	*7.66±0.09*		*1065±58*			*61.15±4.05*

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. 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 (µ±SD) for the following parameters are given for all 3 days: temperature, salinity, A_T, pH, pCO_2, and CO_3^{2-}.

		Hinge Length		Shell Height	
	Treatment	*$MidCO_2$*	*$HighCO_2$*	*$MidCO_2$*	*$HighCO_2$*
2-way ANOVA	*Ambient*	0.250	0.0362	0.985	<<0.001
	$MidCO_2$	-	<<0.001	-	<<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 3	*Ambient*	0.565	0.0311	0.261	<<0.001
	$MidCO_2$	-	<<0.001	-	<<0.001

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

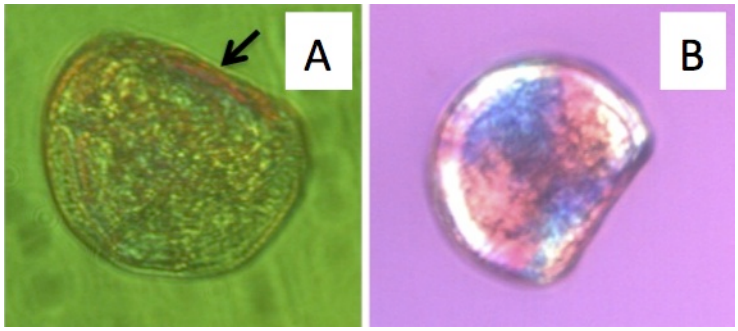

Fig. 1 D-hinge larvae under polarized light portraying calcification at the hinge without a Maltese cross in the shell (A) and full calcification as evidenced by the Maltese cross (B).

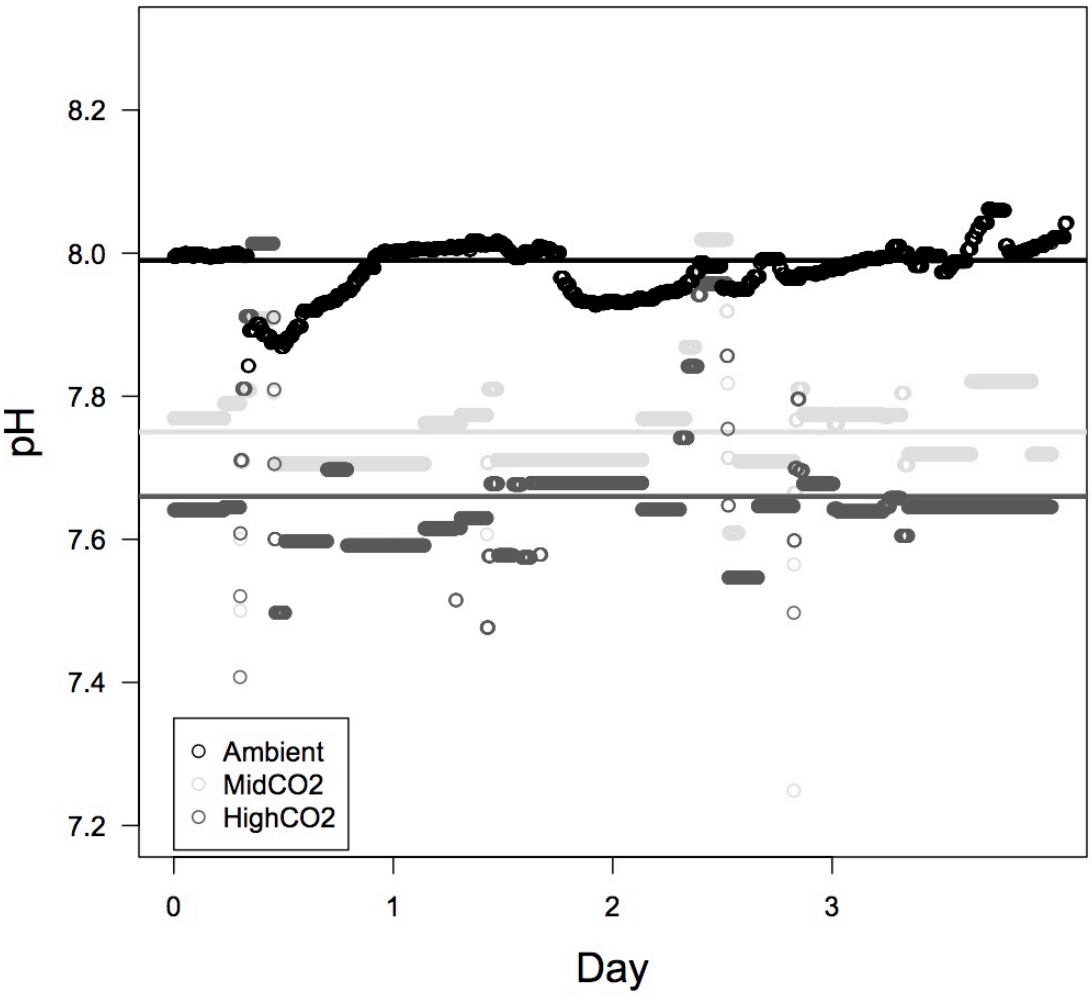

Fig. 2 Profiles of pH measurements in the three different treatments – Ambient (black), MidCO_2 (light gray), and HighCO_2 (dark gray). Average pH for the experiment for each

treatment is represented by solid lines. The Durafet probes recorded pH measurements every minute.

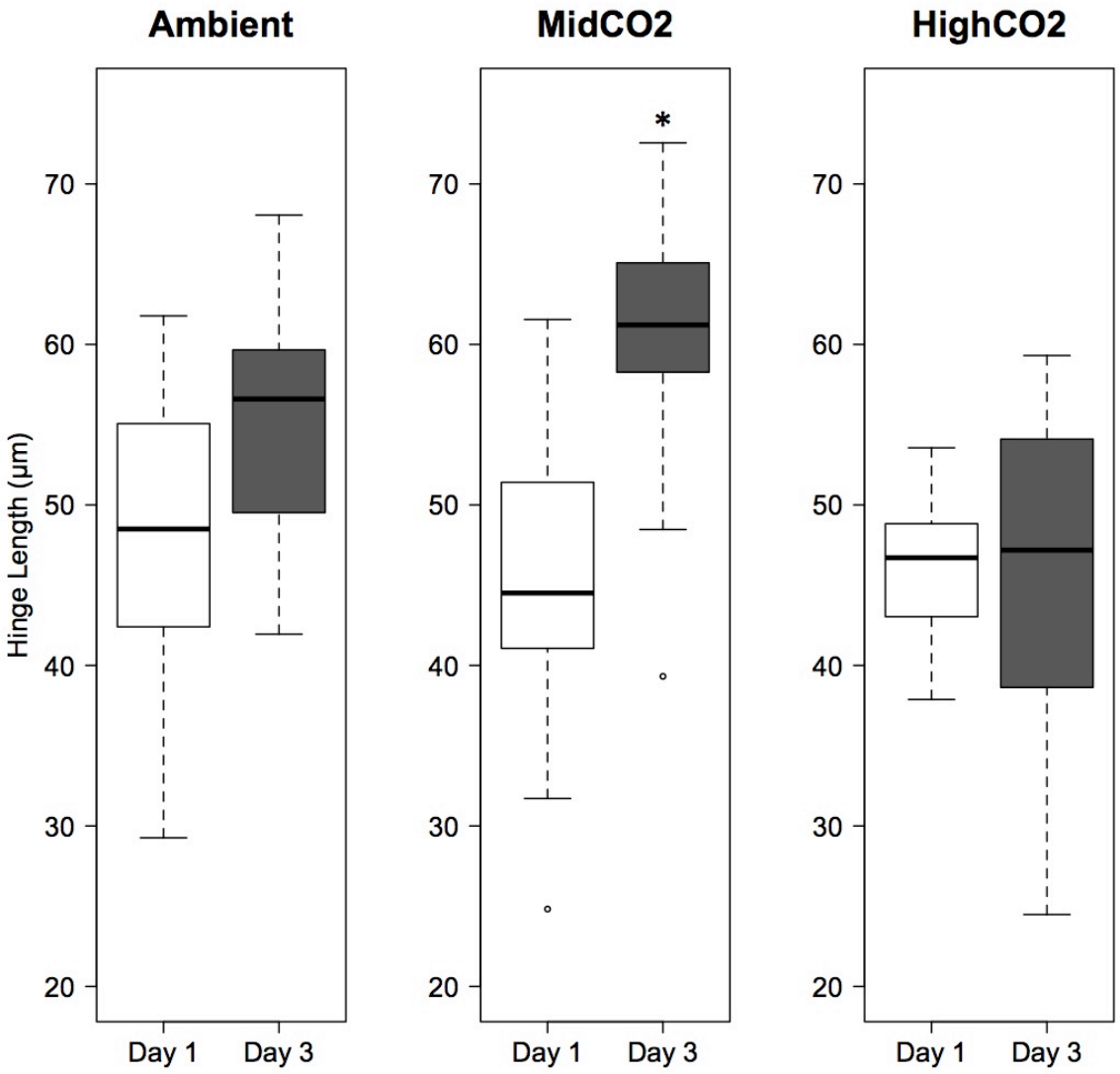

Fig. 3 Larval hinge length on day 1 (white boxplots) and day 3 (gray boxblots). Results are shown for the Ambient treatment, MidCO_2 treatment, and HighCO_2 treatment. Boxplots contain the middle 50% of the data and dashed lines encompass data within 1.5x the spread of the middle 50%. Open circles represent 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 ($P < 0.05$).

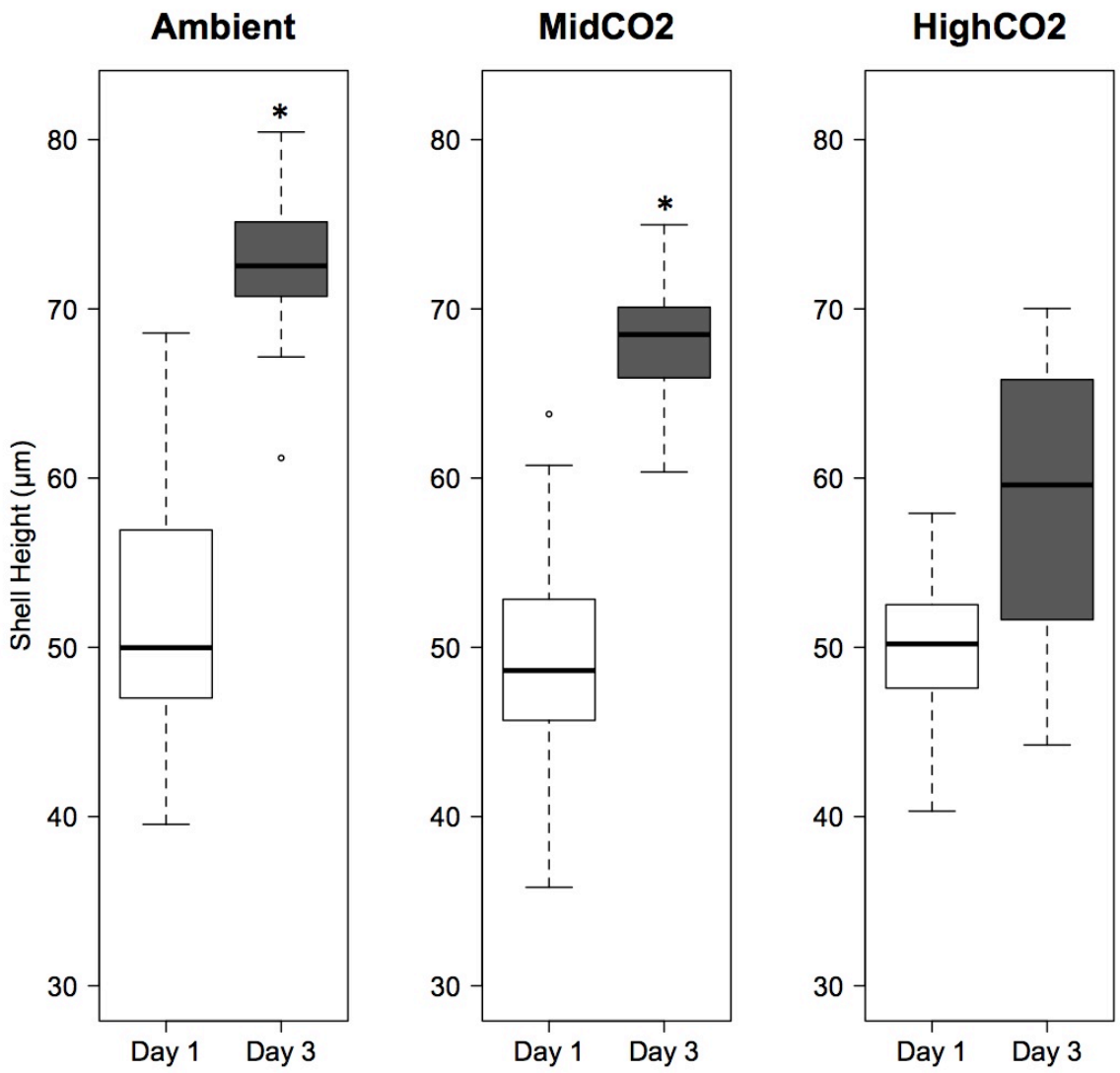

Fig. 4 Larval shell height on day 1 (white boxplots) and day 3 (gray boxblots). Results are shown for the Ambient treatment, MidCO_2 treatment, and HighCO_2 treatment. Boxplots contain the middle 50% of the data and dashed lines encompass data within 1.5x the spread of the middle 50%. Open circles represent 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 ($P < 0.01$).

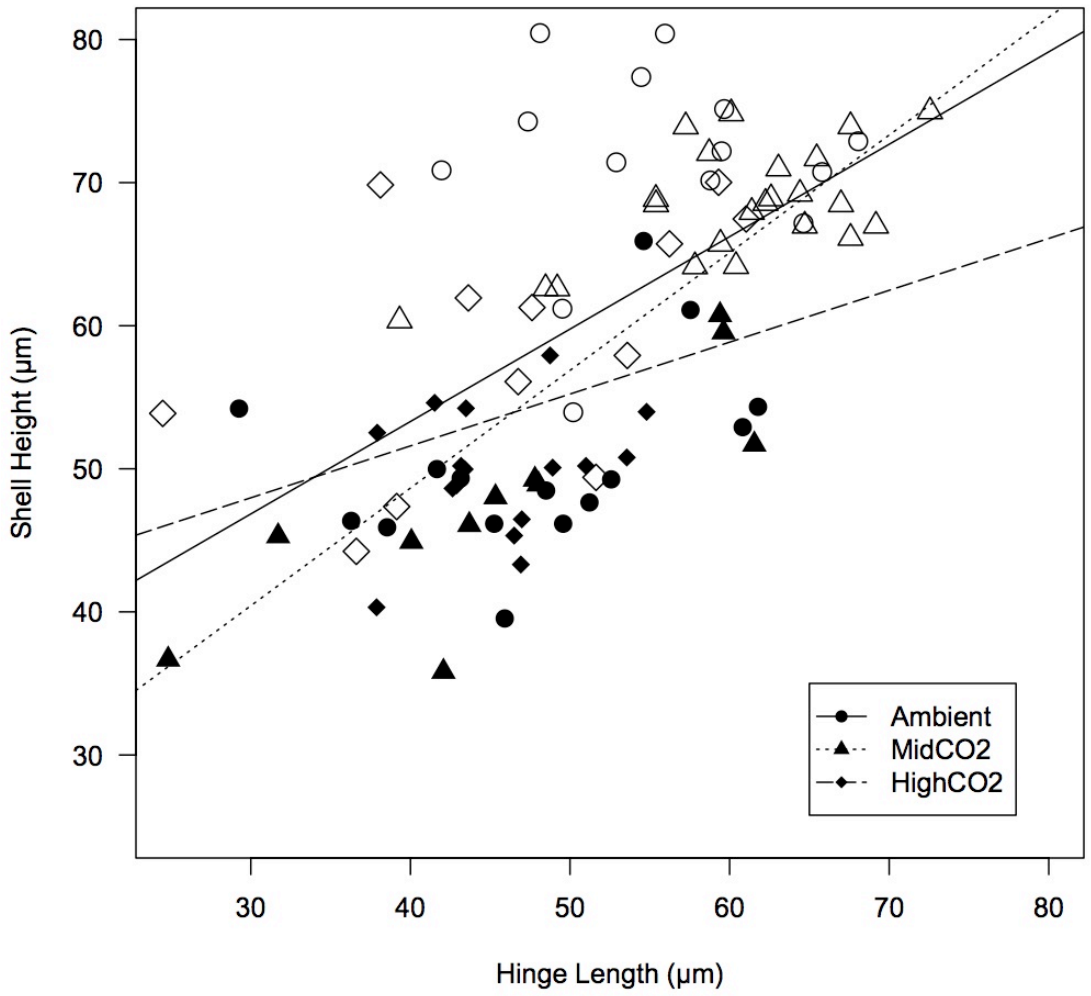

Fig. 5 Regression of larval shell height on hinge length by treatment and day. Data from larvae raised under Ambient pCO_2 conditions are represented by circles, MidCO_2 are triangles, and HighCO_2 are diamonds. Size data from day 1 are in black and day 3 are in white. The solid line is the regression 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 ($P > 0.05$).

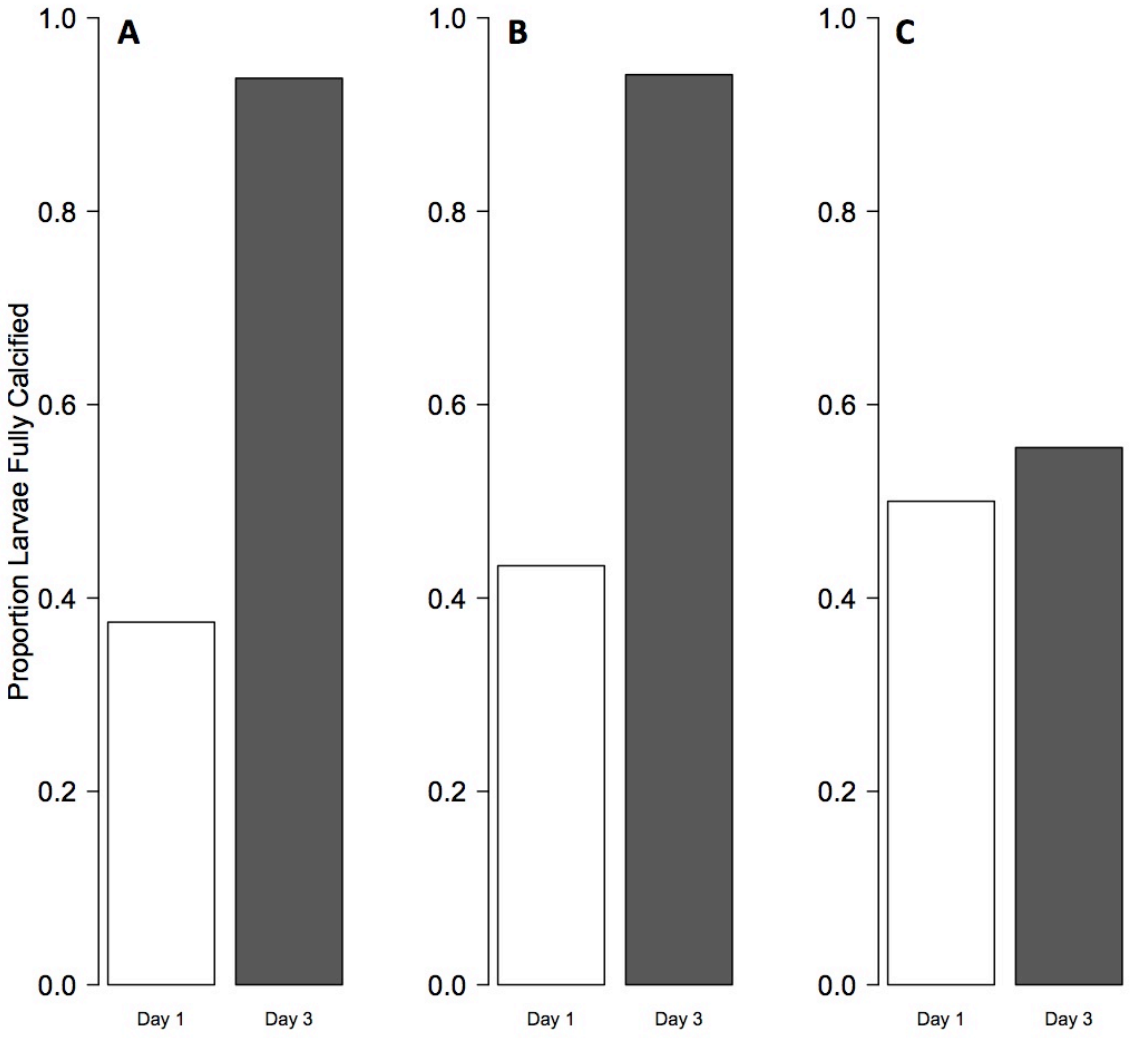

Fig. 6 Proportion of larvae calcified exposed to elevated pCO_2. Bars represent calcification on day 1 (white) and day 3 (gray). Proportion of larvae calcified are provided from the Ambient treatment (panel A), MidCO_2 treatment (panel B), and HighCO_2 treatment (panel C). There is a significant difference 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.