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Short and long term consequences of larval stage exposure to constantly and ephemerally elevated carbon dioxide for marine bivalve populations

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Abstract

While larval bivalves are highly sensitive to ocean acidification, the basis for this sensitivity and the longer term implications of this sensitivity are unclear. Experiments were performed to assess the short term (days) and long term (months) consequences of larval stage exposure to varying CO_2 concentrations for calcifying bivalves. Higher CO_2 concentrations depressed both calcification rates assessed using ^{45}Ca uptake and RNA:DNA ratios in *Mercenaria mercenaria* and *Argopecten irradians* larvae with RNA:DNA ratios being highly correlated with larval growth rates ($r^2 > 0.9$). These findings suggested that high CO_2 has a cascading negative physiological impact on bivalve larvae stemming in part from lower calcification rates. Exposure to elevated CO_2 during the first four days of larval development significantly depressed *A. irradians* larval survival rates, while a 10 day exposure later in larval development did not, demonstrating the extreme CO_2-sensitivity of bivalve larvae during first days of development. Short- (weeks) and long-term (10 month) experiments revealed that individuals surviving exposure to high CO_2 during larval development grew faster when exposed to normal CO_2 as juveniles compared to individuals reared under ambient CO_2 as larvae. These increased growth rates could not, however, overcome size differences established during larval development, as size deficits of individuals exposed to even moderate levels of CO_2 as larvae were evident even after 10 months of growth under normal CO_2 concentrations. This 'legacy effect' emphasizes the central role larval stage CO_2 exposure can play in shaping the success of modern day bivalve populations.

1 Introduction

The partial pressure of CO_2 in the earth's atmosphere has risen by 40 % since the Industrial Revolution and concentrations are expected to double this century (IPCC, 2007). The ocean's ability to absorb CO_2 has resulted in 41 % of fossil fuel and cement manufacturing CO_2 emissions being stored in the world's oceans (Sabine et al., 2004).

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This flux of CO_2 has resulted in a reduction of ocean pH, a decrease in carbonate ion availability, and an environment that is challenging calcifying organisms in marine ecosystems around the globe (Orr et al., 2005; Doney et al., 2009). In addition, many coastal ecosystem have become increasingly acidified via atmospheric carbon dioxide fluxes (Miller et al., 2009), the introduction of acidic river water (Salisbury et al., 2008), upwelling (Feely et al., 2008), and/or eutrophication-driven carbon loading (Cai et al., 2011).

Ocean acidification can have a wide range of negative effects on marine organisms from minor to severe. The earliest stages of development for numerous marine species can be the most sensitive to decreased pH and carbonate ion availability. For example, larval stages of bivalves (Kurihara et al., 2007, 2008; Talmage and Gobler, 2009; 2010; 2011; 2012; Parker et al., 2010; Barton et al., 2012), corals (Albright et al., 2008), echinoderms (Dupont et al., 2010), pteropods (Comeau et al., 2010), and crustaceans (Walther et al., 2010) have all been shown to be negatively affected by the CO_2 concentrations expected later this century in the world's oceans. Despite these findings, the mechanisms by which CO_2 imparts negative effects on calcifying organisms are poorly understood. There have been few investigations of the biochemical effects of ocean acidification on marine organisms, and direct measurements of calcification rates, the process most likely to be altered by ocean acidification, have been made.

Most ocean acidification experiments conducted to date have administered a static exposure of specific CO_2 concentrations to organisms (Doney et al., 2009). In a coastal ecosystem setting, however, it is likely that marine organisms experience dynamic CO_2 concentrations. The effects of variable, compared to constant, exposure to high levels of CO_2 on larval stage marine bivalves has rarely been investigated. This exposure may be important since CO_2 concentrations in estuaries may vary tidally, diurnally, and with the succession of planktonic communities. Finally, longer term implications of larval stage exposure to high CO_2 for juvenile bivalves are poorly understood.

The objectives of this study were to examine the short term (days) and long term (months) implications of larval stage exposure to high CO_2 for calcifying bivalves.

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Mercenaria mercenaria (hard clam) and *Argopecten irradians* (bay scallop) larvae were grown under CO_2 concentrations of ~250, 390, 750, and 1500 µatm, their growth was estimated from their RNA : DNA ratio and their uptake of ^{45}Ca was quantified to estimate calcification rates. Additional experiments investigated the effects of variable high CO_2 exposure on development of *A. irradians* larvae. A final set of experiments investigated the growth of post-set juvenile bivalves exposed to different levels of CO_2 during larval development.

2 Methods

2.1 General methods

Five distinct experiments are presented in this manuscript: (1) measurements of calcium uptake rates in *M. mercenaria* and *A. irradians*, (2) measurements of RNA:DNA ratios and growth rates in *M. mercenaria* and *A. irradians*, (3) the effects differing durations of high CO_2 exposure on larval *A. irradians* survival, (4) the effects larval exposure to high CO_2 on larval and juvenile *M. mercenaria* survival, and (5) the effects larval exposure to high CO_2 on juvenile *A. irradians* growth. For all experiments, replicate ($n = 4$ except for calcium uptake experiments) experimental beakers with bivalve larvae (described below) were maintained in water baths maintained at 24 °C using commercially available aquarium heaters (Aquatic Eco-systems, Inc., Florida, USA). Temperature was recorded every 6 min throughout experiments using in situ data loggers (Onset©); temperatures varied within 2.5 % of target values. The experimental temperature (24 °C) is optimal for growth and survival of larvae from the two species used here (Kennedy, 1996; Kraeuter and Castagna, 2001; Cragg, 2006).

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2.2 Maintenance of CO_2 levels

A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used to deliver CO_2 gas to seawater treatments at different rates. The gas proportionator mixed appropriate flow rates of 5 % CO_2 gas, low CO_2 gas, and pressurized air (~ 390 µatm CO_2) to yield the concentrations of carbon dioxide desired for experiments at a net flow rate that turned over experimental vessels >100 times daily preventing equilibration with atmospheric CO_2. Experiments performed with gases mixed via a proportionator as described here generate nearly identical seawater chemistry and larval responses obtained from tanked gases premixed at specific CO_2 levels (Talmage and Gobler, 2010). For experiments, the CO_2 gas mixtures from the proportionator system were continuously delivered to the bottom of 1 L high density polyethylene beakers with polycarbonate lids. With continuous bubbling, all treatment vessels remained saturated with respect to oxygen (~ 7 mg L^{-1}). To quantify precise CO_2 levels attained in experimental treatments, aliquots were removed and analyzed during experiments with an EGM-4, Environmental Gas Analyzer® (PP Systems) system that quantified total dissolved inorganic carbon levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). This instrument provided a methodological precision ±4 % for replicated measurements of total dissolved inorganic carbon and provided full recovery (104 ± 5 %) of Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography) certified reference material for total inorganic carbon in seawater (Batch 102 = 2013 µmol dissolved inorganic carbon kg $seawater^{-1}$). Levels of CO_2 were calculated based on measured levels of total inorganic carbon, pH (mol kg $seawater^{-1}$; N.B.S.), temperature, salinity, phosphate, silicate, and first and second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/). Daily measurements of pH with a high sensitivity potentiometric electrode (Thermo Scientific Orion 3-Star™ Benchtop pH meter; ±0.002) calibrated prior each use with

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NIST/N.B.S. traceable standards indicated experimental vessels maintain a constant pH level throughout experiments (<0.5 % RSD within treatments). Spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson et al. (2007) and converted from total to N.B.S. scale (Dickson, 1993) were never significantly different from those obtained with the potentiometric electrode.

2.3 Effects of varying CO_2 exposure on calcification rates

M. mercenaria and *A. irradians* larvae were grown at three levels of CO_2: a high level ($\sim$ 750 µatm CO_2), predicted for the year 2100, a modern level ($\sim$ 390 µatm CO_2), and lower, near pre-industrial level ($\sim$ 250 µatm CO_2). Precise CO_2 levels and complete carbonate chemistry from this experiment appear Tables 1 and 2. One liter beakers were filled with 0.2 μm filtered seawater from eastern Shinnecock Bay, New York, USA, and within hours of fertilization, larvae were distributed to each treatment beaker at a concentration of $\sim 400\,L^{-1}$, consistent with post-spawning densities in estuaries (Carriker, 2001). Larvae were fed an ideal food source, *Isochrysis galbana*, (Tahitian strain, T-Iso) at a density known to maximize bivalve larval growth and survivorship through metamorphosis (Castell and Mann, 1994; Cragg, 2006; Talmage and Gobler, 2009). Cultures of *I. galbana* were maintained in exponential phase growth using standard culture conditions. To promote high survivorship, all containers in contact with larvae were never exposed to chemicals (Talmage and Gobler, 2009) and to discourage the growth of bacteria during experiments, an antibiotic solution (Sigma-Aldrich No. 4083, 5000 units of Penicillin, 5 mg of Streptomycin, and 10 mg of Neomycin per milliliter of solution) was added to each beaker at 1 % its original concentration at the beginning of each experiment and at the time of each water change (approximately 2 times weekly). This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae (Talmage and Gobler, 2009). Some experiments presented here (Fig. 1) were repeated without antibiotic treatments and yielded no difference in bivalve larval performance suggesting that neither the antibiotics nor the bacteria in seawater appreciable altered results. Every three days, larvae

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were gently poured onto a 64 µm mesh and all larvae from each beaker ($n = 8$, per treatment, 4 beakers for veligers, and 4 beakers for pediveligers at each CO_2 level) were removed and transferred into a new beaker with new filtered seawater, food, and antibiotics within a 15 min period for each beaker.

To measure calcification rates during the veliger and pediveliger stage for *Mercenaria mercenaria* larvae (3 and 10 d post-fertilization) and *Argopecten irradians* larvae (12 and 15 d post-fertilization), 250 larvae per treatment were removed and placed into 125 mL polyethylene bottles with 100 mL new filtered seawater; temperature was maintained at 24 °C and the bottles were continuously bubbled to achieve the same CO_2 levels the larvae had been grown under until that point. Differences in the rate of calcification of larvae exposed to differing levels of CO_2 was assessed using a ^{45}Ca isotope tracer method (Ho and Zubkoff, 1980). High specific activity, 9.25×10^6 Bq, ^{45}Ca was added to ~100 mL of filtered seawater with 250 larvae in 4 replicated, polypropylene 125 mL Nalgene bottles resulting in a final concentration of 3.7×10^3 Bq mL^{-1}. A killed-control bottle was established at each level of CO_2 via the addition of glutaraldehyde to a final concentration of 2 %. After 24 h, bottles were gently gravity filtered onto 20 µm polycarbonate membranes. For *A. irradians* larvae, time series uptake experiments were also made during the veliger and pediveliger stages when sub-samples of larvae were removed at 1, 2, 4, 6, and 12 h to obtain an estimate of calcium uptake over time. All larvae retained on filters were transferred to scintillation vials and digested with 1 mL of concentrated HNO_3 (15.8 N) for one hour after which 10 mL of UltimaGold™ scintillation cocktail was added. Beta activity of the samples was counted on a Perkin Elmer Tri-Carb 1600 liquid scintillation counter with the discriminator window optimized for ^{45}Ca detection. The weight-specific calcium uptake rate per individual was determined by the following equation:

$$\mathrm{CaU} = \frac{[(\mathrm{dpm})_\mathrm{l} - (\mathrm{dpm})_\mathrm{d}] \cdot \mathrm{Ca}_\mathrm{m}}{(\mathrm{dpm})_\mathrm{m} \cdot t \cdot L} \quad (1)$$

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where CaU is the calcium uptake rate (ng Ca larva^{-1} h^{-1}), $(dpm)_l$ is radioactivity of live larvae, $(dpm)_d$ is the radioactivity of killed larvae which represents non-biological factors including background, ion-exchange and isotope absorption, Ca_m is the calcium content of unit medium water in mg L^{-1}, $(dpm)_m$ is the radioactivity of unit medium water, t is the length of the incubation (days) and L is the number of larvae per beaker

This equation is based on two assumptions: (1) there is no significant discrimination by larvae for ^{45}Ca and ^{40}Ca uptake and (2) the shell dissolution was negligible compared to shell deposition during the incubation (Ho and Zubkoff, 1980). Calcium content of water was estimated by the ratio of its salinity to that of typical oceanic water of 35 which has 408 ppm of Ca^{2+} (Sverdrup, 1942).

2.4 Effects of varying CO_2 on RNA : DNA ratios and growth rates

To assess RNA : DNA ratios of *M. mercenaria* and *A. irradians* larvae, individuals were grown at four levels of CO_2 : a high level (~ 1500 µatm CO_2), predicted for the year 2200, a mid level (~ 750 µatm CO_2), predicted for the year 2100, a modern level (~ 390 µatm CO_2), and a near pre-industrial level (~ 250 µatm CO_2). The general experimental set-up followed the description above; precise CO_2 levels and complete carbonate chemistry from this experiment appear in Table 3. The size of individual larvae (n = 20 per beaker) at the end of the larval cycle was quantified microscopically using ImageJ software as described by Talmage and Gobler (2010). The ratio between ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content in larvae was assessed (Clemmesen, 1994). This approach provides an RNA : DNA ratio which has been used in other marine organisms, especially fish larvae, to estimate growth and nutritional condition, with elevated ratios being associated with fast growing individuals (Malzahn et al., 2003, 2007). Larvae (n = 15) were removed from each treatment beaker, poured onto a sieve, macerated using a Pellet Pestle® Motor, heated to 50 °C for 15 min, and then frozen in −80 °C for at least 90 min. Nucleic acids from groups of larvae were extracted using a modified CTAB technique and quantified using Quant-iT™ RiboGreen

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RNA® and Quant-iT™ PicoGreen® DNA assay kits (Invitrogen), according to manufacturer's protocol. RiboGreen® RNA and PicoGreen® DNA are ultra sensitive fluorescent nucleic acid stains for quantifying RNA and DNA, respectively, in solution. RiboGreen also binds DNA, therefore complete DNAse digestion of samples preceded analysis of RNA. RNA and DNA concentrations in the extracted samples were quantified by measuring fluorescence using an Applied Biosystems 7300 Real-Time PCR system-genetic analyzer and compared to a standard curve of nucleic acids.

2.5 Effects of varying CO_2 exposure on survival of larval bivalves

An experiment was conducted to investigate the effects of two levels of CO_2 (~390 and ~750 µatm) exposure on the growth and survival of *A. irradians* larvae over a 19 d period. The general experimental set-up followed the description above; precise CO_2 levels and complete carbonate chemistry from this experiment appear in Table 4. There experiment began with 15 treatment vessels at each CO_2 level (~390 and ~750µatm) and ~every three days, for the first 12 days of a 19 day experiment, three treatment vessels were switched from ~750 to ~390 µatm and three treatment vessels were switched from ~390 to ~750 µatm CO_2. Three vessels were maintained at 390 µatm and three were maintained at 750 µatm for the entire experiment so that *A. irradians* larvae experienced exposure time to each CO_2 level which ranged from 0–19 d. In the end, this experimental design exposed some larvae high CO_2 early in their development only while others were exposed later in their development.

To assess the implications of larval stage CO_2 exposure for juvenile stage *M. mercenaria*, larvae were grown at two CO_2 concentrations: ~390 and 1500 µatm. The general experimental set-up followed the description above; precise CO_2 levels and complete carbonate chemistry from this experiment appear in Table 5. After 24 d of development, 40 individuals that had metamorphosed into early juvenile stages were transferred from ~390 to ~1500 and from ~1500 to ~390 µatm CO_2; individuals were

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pooled from replicated treatment beakers. Early stage juveniles were cared for as described above for larvae and were monitored until day 36.

2.6 Long term growth experiment

To assess the longer term (months) implications of larval stage exposure to high CO_2, *Argopecten irradians* larvae (n = 1000) were grown at three CO_2 concentrations (~ 250, 390, and 750 µatm) for their entire larval cycle and early days as a juvenile. The general experimental set-up followed the description above; precise CO_2 levels and complete carbonate chemistry from this experiment appear in Table 6. After 35 d of development, all metamorphosed juveniles were quantified and placed on 64 µm mesh sieves submerged in seawater where they received a continuous flow of coarsely filtered (100 µm nylon) seawater from Shinnecock Bay, NY, USA which has been reported to have pH levels ranging from 7.66–8.01 (N.B.S. scale; Gobler and Talmage, 2009). At 47 d, individuals were moved into Three Mile Harbor, East Hampton, NY, USA, which is more oliogotrophic than Shinnecock Bay with 'near-normal' pH values (7.9–8.2 N.B.S. scale). At Three Mile Harbor, individuals were placed in mesh (500 µm) bags in a cage so as to receive ample water flow and food but to exclude predators. Bags were changed and the size of all individuals was recorded monthly over a ten month period and specific growth rates were estimated using the equation: [ln(final size) – ln(initial size)]/change in time.

2.7 Statistical analyses

Statistical analyses were performed with SYSTAT 13© Copyright, 2009, Systat Software, Inc. Percent survival values were arc-sin square root transformed before statistical analyses. Data sets not meeting the assumptions of normality and homogeneity were ranked prior to analyses. Two-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to assess differences among calcification rates at each CO_2 level and larval stage. Differences in RNA : DNA ratios, growth rates, and survival

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percentages among CO_2 levels were examined with one-way ANOVAs and post-hoc Tukey multiple comparison tests. Values reported in the results sections are means ± standard deviations.

3 Results

3.1 Calcium uptake rates by *M. mercenaria* and *A. irradians larvae*

For *Mercenaria mercenaria* larvae grown under three levels of CO_2 (~250, 390, and 750 µatm), there was a significant effect of CO_2 concentration on calcium uptake for the veliger stage and for the pediveliger stage ($p < 0.05$; Two-way ANOVA; Fig 1a). For *M. mercenaria* day 3, veliger larvae calcium uptake rates were 0.72 ± 0.001, 0.44 ± 0.005, and 0.35 ± 0.03 ng Ca larvae^{-1} h^{-1} under ~250, 390, and 750 µatm CO_2 respectively (Fig. 1a). *M. mercenaria* day 10, pediveligers under ~250, 390, and 750 µatm CO_2 had similarly decreasing calcium uptake rates of 0.72 ± 0.002, 0.53 ± 0.005, and 0.33 ± 0.002 ng Ca larvae^{-1} h^{-1} (Fig. 1a). Calcification rates did not differ between stages ($p > 0.5$; Two-way ANOVA; Fig. 1a).

Argopecten irradians larvae displayed a similar pattern of decreasing calcium uptake under increasing CO_2 concentrations. For *A. irradians* there was a significant effect of CO_2 on calcium uptake for day 12, veliger larvae as well as for day 15, pediveliger larvae ($p < 0.001$; two way ANOVA). There was also a significant effect of developmental stage and an interactive effect of CO_2 and developmental stage on *A. irradians* calcium uptake ($p < 0.05$ for both; two-way ANOVA; Fig. 1b). With an increase in CO_2 concentrations from ~250 to 390 to 750, *A. irradians* veliger larvae calcium uptake rates decreased from 0.61 ± 0.003, to 0.42 ± 0.001 and to 0.44 ± 0.002 ng Ca larvae^{-1} h^{-1} (Fig. 1b). *A. irradians* pediveliger larvae calcium uptake also decreased with increasing CO_2 concentrations from 0.76 ± 0.02, 0.53 ± 0.001, and 0.44 ± 0.004 ng Ca larvae^{-1} h^{-1} at ~250, 390, and 750 µatm CO_2 (Fig. 1b). A separate experiment found that *A. irradians* veliger larval uptake of calcium was linear over 12 h

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(Fig 2). Using this approach, *A. irradians* veliger larvae at 250 µatm had the greatest uptake rates (0.90 ng Ca larvae^{-1} h^{-1}; Fig. 2) while at ~390 and ~750 µatm CO_2, *A. irradians* larvae calcium uptake rates decreased to 0.52 and 0.29 ng Ca larvae^{-1} h^{-1}, respectively (Fig. 2).

3.2 *M. mercenaria* and *A. irradians* RNA : DNA ratios and growth rates

To understand the effects of CO_2 on larval development, RNA : DNA ratios and shell length-based growth rates were quantified. *Mercenaria mercenaria* larvae (day 24) and *Argopecten irradians* larvae (day 20) both displayed step-wise decreases in both measurements of growth with increasing CO_2 concentrations. CO_2 concentrations had a significant effect on RNA : DNA for *M. mercenaria* ($p < 0.001$; ANOVA). At ~250 µatm CO_2, the RNA : DNA ratio was 1.43 ± 0.07 for *M. mercenaria* larvae and progressively decreased to 0.21 ± 0.06 at ~1500 µatm CO_2 (Fig. 3). *A. irradians* also displayed decreasing RNA : DNA with increasing CO_2 (Fig. 3). When CO_2 concentrations increased from ~250 to 390 to 750 to 1500 µatm, RNA : DNA for *A. irradians* changed from 1.02 ± 0.07 to 0.49 ± 0.17 to 0.20 ± 0.03 to 0.25 ± 0.04 respectively (Fig. 3).

The second proxy for growth, changes in shell diameter followed a similar pattern as RNA : DNA. CO_2 concentrations had a significant effect on *M. mercenaria* larval shell growth ($p < 0.001$; ANOVA) and *A. irradians* larval shell growth ($p < 0.001$; ANOVA; Fig. 3). With increasing CO_2 from ~250, 390, 750 to 1500 µatm, *M. mercenaria* larvae growth decreased from 21.77 ± 1.58, 11.74 ± 0.20, 10.85 ± 0.28, to 8.76 ± 0.38 µm d^{-1}, respectively (Fig. 3). *A. irradians* larvae followed the same pattern of decreasing growth from 26.52 ± 2.53, 22.47 ± 1.75, 18.55 ± 2.02, and 15.56 ± 1.32 µm d^{-1} under CO_2 concentrations of ~250, 390, 750 and 1500 respectively (Fig. 3). Finally, there was the high degree of linear correlation between RNA : DNA ratios and shell-based growth rates of *M. mercenaria* and *A. irradians* larvae ($r^2 = 0.92$; $p = 0.08$ and $r^2 = 0.99$; $p < 0.01$, respectively).

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3.3 *A. irradians* survival under varying exposure to high CO_2

To assess how variable CO_2 exposure affected larval bivalve survival, *Argopecten irradians* larvae were exposed to normal CO_2 (~ 390 µatm) or high CO_2 (~ 750 µatm) and switched to either the higher (for vessels at 390 µatm CO_2) or normal (for vessels at 750 µatm CO_2) CO_2 concentration. The duration of exposure to high CO_2 significantly affected larval survival ($p < 0.001$; ANOVA; Fig. 4). *A. irradians* larvae which developed exclusively under 390 and 750 µatm CO_2 displayed survival rates of 78 ± 1.0 % and 56 ± 1.0 %, respectively (Fig. 4). In contrast, individuals which began their development at high CO_2 and were switched to lower CO_2 after 4, 7, 10, and 13 days displayed survival rates of 73 ± 3.0, 71.5 ± 2.3, 63.5 ± 3.1, and 61.7 ± 2.2 %, respectively (Fig. 4). Importantly, even three days of exposure to 750 µatm CO_2 significantly reduced larval survival rates compared to constant exposure to 390 µatm (Fig. 4). *A. irradians* larvae that began at ~ 390 µatm CO_2 and were switched to 750 µatm CO_2 after 7, 10, 13, and 16 d displayed survival rates of 76.8 ± 1.3, 75.3 ± 0.25, and 57.25 ± 0.9, and 56.7 ± 0.8 %, respectively (Fig. 4). Of this group, only individuals exposed for 13 and 16 d displayed survival that was significantly lower than the constant 390 µatm level (Fig. 4).

3.4 *M. mercenaria* survival under varying exposure to high CO_2

An experiment was conducted to investigate the effects of changing CO_2 exposure on survival by moving *Mercenaria mercenaria* from higher to lower concentrations of CO_2 and from lower to higher concentrations at the transition period from larvae to juvenile. For individuals exposed to ~ 390 and 1500 µatm CO_2 for the first 24 days of development there was a significant effect of CO_2 on total survival ($p < 0.001$; ANOVA) as survival at ~ 390 was 37 ± 3.3 % and at ~ 1500 was 23 ± 1.8 % (Fig. 5a). At day 24, a subset of individuals were moved from low CO_2 (~ 390 µatm) to high CO_2 (~ 1500 µatm) and from high CO_2 (~ 1500 µatm) to low CO_2 (~ 390 µatm). Survival from day 24–36 was also significantly affected by CO_2 ($p < 0.001$; ANOVA; Fig. 5b). Survival

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of *M. mercenaria* individuals at day 36 for ~390, ~390 switched to ~1500, ~1500 switched to ~390, and ~1500 µatm CO_2 were 70.31 ± 9.02, 62.5 ± 10.21, 97.5 ± 2.04, and 43.09 ± 4.72 %, respectively (Fig. 5b).

3.5 Juvenile *A. irradians* growth following larval stage exposed to high CO_2

To assess the longer term effects of elevated CO_2 exposure during larval development, the growth of post-set larvae was measured over a ten month period. For *A. irradians* growth during the first 12 weeks post-spawning, CO_2 had a significant effect on specific growth rates ($p < 0.001$; ANOVA; Fig. 6). With increasing CO_2 treatments during the larval stage development from ~250 to 390 to 750, specific growth rates for weeks 0–12 decreased from 0.25 ± 0.0008 to 0.23 ± 0.008 to 0.21 ± 0.0005 mm week^{-1} respectively (Fig. 6). For weeks 13–26, these trends reversed as individuals reared under ~250, 390, and 750 µatm as larvae displayed specific growth rates of 0.009 ± 0.001, 0.014 ± 0.001, and 0.02308 ± 0.001 mm week^{-1} respectively (Fig. 6). While shell diameters of individuals metamorphosed under ~390 and 750 µatm CO_2 were 21.19 ± 0.24 and 13.07 ± 0.08 mm in September 2010, they were similar in size by February 2011 (26.96 ± 0.79 mm and 23.59 ± 0.77; Fig. 7). However, at the end of the 10-month experiment, individuals reared at 250 µatm CO_2 as larvae and early stage juveniles were significantly larger than individuals exposed to 390 and 750 µatm ($p < 0.05$; ANOVA; Fig. 8).

4 Discussion

It has been well established that bivalve larvae experience reduced growth and survival when exposed to elevated concentrations of CO_2 (e.g. Miller et al., 2009; Talmage and Gobler, 2009, 2010; Barton et al., 2012). In this study, experiments examining calcification rates and RNA : DNA ratios provided insight regarding specific physiological processes that are impacted by elevated CO_2. Other experiments demonstrated that high

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CO_2 exposure during the first four days of development alone can inhibit the growth of larval growth while exposure during the final 10 days does not. Finally, experiments established the juvenile-stage implications of larval-stage exposure to elevated concentrations of CO_2 for the bivalves. Collectively, this data set provides novel insight regarding the short and long term implications of larval stage CO_2 exposure for calcifying bivalves.

At CO_2 concentrations exceeding ~250 µatm, both *M. mercenaria* (hard clam) and *A. irradians* (bay scallop) larvae displayed significant declines in calcium uptake and presumably rates of calcification. This observation is consistent with the thinner shells displayed by these bivalve larvae when exposed to higher CO_2 concentrations (Talmage and Gobler, 2010). The integrity of the bivalve shell may be one of the most important lines of defense for larval shellfish. Bivalve larvae depend on shells to provide physical support for soft and delicate internal organs, for protection from impact with suspended particles and physical stress (Carriker, 1986) and for protection from some benthic and pelagic predators (Purcell et al., 1991; Carriker, 1996). Therefore, the declines in calcification observed with increasing CO_2 levels likely contributes to larval bivalve shellfish mortality, and thus could be considered a primary impact of ocean acidification on these organisms.

Reductions in calcification under acidified ocean conditions have already been described for post-larval hard clam *Mercenaria* spp. that displayed decreased calcification rates with decreases in sea water pH (Waldbusser et al., 2010). Juvenile bivalves and larvae from the oyster species, *Saccostrea glomerata*, have displayed compromised shell integrity with decreasing calcium carbonate saturation states (Green et al., 2009; Watson et al., 2009). Juveniles spending only four days at undersaturated levels of calcium carbonate displayed signs of dissolution or shell pitting of the ostracum, with most dissolution of the surface shell in the umbonal region or the part of the shell that was deposited first (Green et al., 2009). These reductions in calcification by juvenile bivalves coupled with the current findings of reduced bivalve larvae calcification with increasing CO_2 collectively support the 'death by dissolution' hypothesis proposed as

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the underlying mechanism for how acidified waters and sediments lead to lower survival rates for early life stages of calcifying bivalves (Green et al., 2009). Different forms of calcium carbonate are secreted by bivalve larvae as they develop. Larval shells begin as amorphous calcium carbonate (ACC) which is significantly more soluble than aragonite and the combinations of aragonite and calcite synthesized by later stage bivalves (Weiss et al., 2002). Even the most resistant forms of calcite dissolve under high levels of CO_2 and leave organisms with shell loss (Harper, 2000). Hence, the dissolution and/or inhibition of calcification during larval stages contribute towards thin and frail shells. While thinner shells alone may leave larvae vulnerable to enhanced mortality rates, it is likely that the enhanced bioenergetic investment made to calcify under high levels of CO_2 also promotes mortality. Calcification is a significant metabolic cost for marine organisms, with other metabolic costs including, but not limited to the energy committed to the production of the organic matrix and somatic growth (Palmer, 1992). If the shells of bivalve larvae are more difficult to synthesize under increasing CO_2 levels and thus synthesized at a slower rates, this may make less energy available for growth and development for larvae and, thus, could contribute toward later stage mortality. The enhanced energetic investment has been evident from reduced lipid stores in larvae exposed to high CO_2 (Talmage and Gobler, 2010). This would represent a secondary effect of high CO_2 for surviving larvae: individuals that do not perish from high CO_2 are under enhanced physiological stress since more energy may be allocated to calcification and less is available for maintenance and growth. This is consistent with the observations of Melzner et al. (2011) who reported that mussels with access to excessive food were able to resist dissolution under high CO_2 while those on a restricted diet could not. This is also consistent with reported higher metabolic rates in juvenile oysters, *Crassostrea virginica*, exposed to elevated CO_2 indicating a higher energy demand of homeostasis under these conditions (Beniash et al., 2010). The additional stressors that coastal bivalve larvae encounter in parallel with acidification such as elevated temperatures and harmful algae are likely to exacerbate energy demands and further depress survival rates (Parker et al. 2010; Talmage and Gobler, 2011, 2012).

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The hypothesis that reduced calcification rates have "trickle down" effects on larval physiology and performance was supported by measurements of RNA : DNA ratios. RNA transcribes the genetic material stored in DNA and is subsequently translated by ribosomes to synthesize proteins. Hence, high levels of RNA compared to DNA are indicative of an organism in an active state of transcribing RNA, synthesizing proteins, and growth (Malzahn et al., 2003). Under high concentrations of CO_2, the ratio of RNA : DNA was reduced for both species of larvae, suggesting that rates of transcription and growth were compromised. This conclusion was supported by the high degree of correlation between RNA : DNA ratios and shell-based growth rates of *M. mercenaria* ($r^2 = 0.92$; $p = 0.08$) and *A. irradians* larve ($r^2 = 0.99$, $p < 0.01$). The reduced RNA : DNA ratios displayed by individuals developing under high levels of CO_2 indicates the systemic negative impact of ocean acidification on these organisms.

Most ocean acidification experiments conducted to date have administered a static exposure of specific CO_2 concentrations to organisms (Doney et al., 2009). In an ecosystem setting, however, it is likely that marine organisms, in general, and estuarine larvae, in particular, will experience varying CO_2 concentrations due to tidal effects and diurnal fluctuations in photosynthesis. By varying CO_2 levels, this study found that individuals grown at 'normal' CO_2 levels ($\sim$ 390 µatm) at the start of the larval cycle were able to withstand a longer exposure period at higher CO_2 (approximately 10 d) before significant declines in survival occurred. In contrast, individuals that began their development at higher CO_2 experienced significant declines in survival after only four days. This demonstrates that the first days of development are the most sensitive exposure period for *A. irradians* larvae, perhaps because more soluble forms of calcium carbonate are secreted by larvae during this period (Weiss et al., 2002) or because there is not internal compartmentalization for calcification at this time (Gilkin et al., 2007). Regardless of the mechanism, this result further suggests that shellfish hatcheries and restoration efforts should focus efforts on ensuring ideal chemical conditions for the first week of bivalve larval development. Importantly, however, the benefits of initial development under normal CO_2 levels are not enough to protect against extended, later

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exposure to high CO_2 as exposure to 13 days 750 µatm CO_2 after six days of optimal CO_2 caused a significant decline in larval survival. Given the in-situ variability of CO_2 concentrations, this experimental increase in CO_2 from ambient (~ 390 µatm) to elevated CO_2 concentrations (~ 750 µatm) mimics what these larvae may experience as they develop from pediveligers into juveniles and settle onto the seafloor. Future experiments should focus on the effects of diel changes of CO_2 concentrations on larval bivalves.

While larval exposure to high CO_2 can have strong negative impacts on growth and survival of many marine animals (Kurihara et al., 2008; Talmage and Gobler, 2009, 2010; Dupont et al., 2010; Barton et al., 2012), the post-larval stage implications of this exposure has not been fully established. Hettinger et al. (2012) reported that the Olympia oyster (*Ostrea lurida*) juveniles that had been reared as larvae under reduced pH experience significantly decreased shell growth rate regardless of the pH level the oysters experienced as juveniles, indicating a strong carry-over effect from the larval phase. During the current study, after 24 days of exposure to ambient and high CO_2 concentrations, *M. mercenaria* larvae displayed significantly higher survival under lower CO_2 concentrations (~ 390 µatm). When a subset of the surviving individuals were moved from high to low and low to high CO_2 concentrations, however, the highest survival rate over the next two weeks was found among individuals which developed under high CO_2 (~ 1500 µatm) as larvae and then were reared at ~ 390 µatm CO_2 as juveniles (Fig. 5b). As broadcast spawners, bivalves produce cohorts of larvae derived from multiple parents that are likely to display plasticity in their general fitness (Kraeuter and Castagna, 2001; Cragg, 2006). Although high CO_2 eliminated 80 % of the larval cohort, individuals that survived this treatment displayed superior survival rates as early stage juveniles suggesting high CO_2 may eliminate generally weaker individuals. Moreover, this finding indicates that individuals reared under high CO_2 as larvae can experience compensatory growth when the stress of elevated CO_2 levels is removed.

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The superior performance of individuals exposed to high CO_2 as larvae and reared under normal conditions as juveniles was also evident in *A. irradians* potentially evidencing a physiological plasticity. Although *A. irradians* bivalves had the greatest specific growth at the lowest CO_2 concentration during the larval stage, individuals surviving the highest CO_2 level experienced the highest specific growth rates as juveniles, although these rates were an order of magnitude lower than those displayed by larvae. This finding supports the hypothesis that individuals that survive high CO_2 as larvae are, on average, more fit as juveniles than individuals exposed to normal CO_2 levels as larvae. The compensatory, juvenile-stage growth displayed by individuals reared under high CO_2 as larvae resulted in their size differences being eliminated after two months of growth under normal CO_2 conditions. Interestingly, while the growth rate of the individuals reared at ambient and high CO_2 ($\sim$390 and 750 µatm) outpaced those of the lowest CO_2 treatment ($\sim$250 µatm) as early stage juveniles, they did not overcome the deficit in size established during the larval stage after ten months of growth under normal CO_2. Given that smaller juvenile bivalves are more susceptible to predators than larger individuals (Kraeuter, 2001), this finding suggests that the negative effects of larval stage exposure to even modern day levels of CO_2 represents a legacy that can persist for at least eight months in an ecosystem setting. This finding is similar to the 1.5 months carry-over effect of larval stage CO_2 exposure in juvenile Olympic oyster growth reported by Hettinger et al. (2012) and further emphasizes the critical role larval stage CO_2 exposure can play in influencing the success of modern day bivalve populations.

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Table 1. Mean temperature, pH_{NBS}, carbonate chemistry, total alkalinity, and salinity (±1 SD) during the three-level carbon dioxide experiments for calcium uptake with *Mercenaria mercenaria,* and *Argopecten irradians* larvae.

Parameter	Near pre-industrial CO_2	Ambient, present day CO_2	Elevated CO_2
Mercenaria mercenaria			
Temperature (°C)	24 ± 0.8	24 ± 0.8	24 ± 0.8
pH_{NBS}	8.2 ± 0.051	8.08 ± 0.076	7.800 ± 0.013
pCO_2 (µatm)	248.3 ± 21.03	372.6 ± 39.48	781.6 ± 29.99
$\Omega_{calcite}$	3.10 ± 0.13	2.59 ± 0.21	1.53 ± 0.13
$\Omega_{aragonite}$	1.98 ± 0.39	1.69 ± 0.21	0.99 ± 0.13
C_T (µmol L^{-1})	1117 ± 37.95	1365 ± 54.22	1458 ± 23.33
CO_3^{2-} (µmol L^{-1})	117.8 ± 3.96	104.8 ± 35.78	60.5 ± 15.632
Total alkalinity (A_T)	1412.7 ± 112.5	1516 ± 74.56	1528 ± 32.44
Salinity	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0
Argopecten irradians			
Temperature (°C)	24 ± 0.8	24 ± 0.8	24 ± 0.8
pH_{NBS}	8.2 ± 0.047	8.08 ± 0.061	7.810 ± 0.009
pCO_2 (µatm)	237.3 ± 5.32	396.5 ± 21.43	753.2 ± 19.04
$\Omega_{calcite}$	2.94 ± 0.25	2.60 ± 0.24	1.55 ± 0.11
$\Omega_{aragonite}$	1.89 ± 0.40	1.71 ± 0.18	1.01 ± 0.11
C_T (µmol L^{-1})	1220 ± 42.22	1327 ± 26.56	1438.6 ± 15.45
CO_3^{2-} (µmol L^{-1})	121.3 ± 5.98	101.9 ± 48.55	61.0 ± 17.843
Total alkalinity (A_T)	1352.7 ± 42.76	1518 ± 55.56	1511 ± 36.44
Salinity	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0

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Table 2. Mean temperature, pH_{NBS}, carbonate chemistry, total alkalinity, and salinity (±1 SD) during the three-level carbon dioxide for calcium uptake over time for *Argopecten irradians* larvae.

Parameter	Near pre-industrial CO_2	Ambient, present day CO_2	Elevated CO_2
Argopecten irradians			
Temperature (°C)	24 ± 0.8	24 ± 0.8	24 ± 0.8
pH_{NBS}	8.201 ± 0.022	8.041 ± 0.042	7.801 ± 0.013
pCO_2 (µatm)	242.4 ± 19.221	402 ± 21.268	764.7 ± 19.658
$\Omega_{calcite}$	3.01 ± 0.14	2.38 ± 0.32	1.51 ± 0.16
$\Omega_{aragonite}$	1.94 ± 0.46	1.54 ± 0.31	0.97 ± 0.26
C_T (µmol L^{-1})	1196 ± 45.76	1334 ± 35.62	1430 ± 28.45
CO_3^{2-} (µmol L^{-1})	118.4 ± 26.32	94.2 ± 28.65	59.5 ± 20.01
Total alkalinity (A_T)	1381.1 ± 30.05	1470.2 ± 43.02	1501 ± 43.88
Salinity	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0

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Table 3. Temperature, pH_{NBS}, carbonate chemistry, total alkalinity, and salinity (±1 SD) during the four-level carbon dioxide experiments with *Mercenaria mercenaria*, and *Argopecten irradians* larvae.

Parameter	Near preindustrial CO_2	Ambient, present day CO_2	Year 2100 CO_2	Year 2200 CO_2
Mercenaria mercenaria				
Temperature (°C)	24 ± 0.52	24 ± 0.52	24 ± 0.52	24 ± 0.52
pH_{NBS}	8.171 ± 0.022	8.052 ± 0.036	7.801 ± 0.004	7.532 ± 0.021
pCO_2 (µatm)	247.1 ± 6.231	380.0 ± 33.02	742.3 ± 9.111	1516 ± 31.21
$\Omega_{calcite}$	5.31 ± 0.47	4.53 ± 0.41	2.82 ± 0.05	1.67 ± 0.05
$\Omega_{aragonite}$	3.42 ± 0.30	2.92 ± 0.26	1.82 ± 0.03	1.08 ± 0.03
C_T (µmol L^{-1})	1646 ± 94.21	1831 ± 52.34	1947 ± 21.33	2108 ± 18.06
CO_3^{2-} (µmol L^{-1})	208.0 ± 20.22	178.0 ± 16.03	111.0 ± 1.806	66.0 ± 1.904
Total alkalinity (A_T)	1938 ± 117.3	2070 ± 66.42	2080 ± 22.63	2127 ± 49.71
Salinity	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0
Argopecten irradians				
Temperature (°C)	24 ± 0.51	24 ± 0.52	24 ± 0.52	24 ± 0.52
pH_{NBS}	8.170 ± 0.026	8.041 ± 0.044	7.801 ± 0.005	7.530 ± 0.011
pCO_2 (µatm)	244.1 ± 4.006	386.5 ± 40.04	738.9 ± 9.941	1529 ± 35.05
$\Omega_{calcite}$	5.18 ± 0.06	4.55 ± 0.47	2.81 ± 0.06	1.66 ± 0.05
$\Omega_{aragonite}$	3.34 ± 0.35	2.94 ± 0.30	1.81 ± 0.04	1.07 ± 0.03
C_T (µ L^{-1})	1613 ± 53.54	1850 ± 30.98	1941 ± 25.54	2101 ± 9.221
CO_3^{2-} (µmol L^{-1})	202.0 ± 23.42	180.0 ± 18.44	111.0 ± 2.341	66.02 ± 1.911
Total alkalinity (A_T)	1899 ± 35.24	2090 ± 50.01	2075 ± 26.84	2146 ± 11.21
Salinity	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0

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Table 4. Temperature, pH_{NBS}, carbonate chemistry, total alkalinity, and salinity (±1 SD) during the two-level carbon dioxide varying exposure experiment with *Argopecten irradians* larvae.

Parameter	Normal CO_2	ElevatedCO_2
Argopecten irradians		
Temperature (°C)	24 ± 0.6	24 ± 0.6
pH_{NBS}	8.08 ± 0.049	7.82 ± 0.015
pCO_2 (µatm)	372.6 ± 23.21	732.5 ± 29.02
$\Omega_{calcite}$	2.66 ± 0.47	1.58 ± 0.17
$\Omega_{aragonite}$	1.71 ± 0.28	1.02 ± 0.38
C_T (µmol L^{-1})	1364.1 ± 32.44	1432.6 ± 46.87
CO_3^{2-} (µmol L^{-1})	104.7 ± 23.24	62.2 ± 20.03
Total alkalinity (A_T)	1516.3 ± 66.54	1508 ± 52.56
Salinity	28.0 ± 1.0	28.0 ± 1.0

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Table 5. Temperature, pH_{NBS}, carbonate chemistry, total alkalinity, and salinity (±1 SD) during the four-level carbon dioxide experiments with *Mercenaria mercenaria* larvae for varying CO_2 exposure experiment.

Parameter	Normal CO_2	Elevated CO_2
Mercenaria mercenaria		
Temperature (°C)	24 ± 0.4	24 ± 0.4
pH_{NBS}	8.08 ± 0.037	7.58 ± 0.054
pCO_2 (µatm)	407.5 ± 27.832	± 17.996
$\Omega_{calcite}$	2.93 ± 0.32	1.02 ± 0.08
$\Omega_{aragonite}$	1.87 ± 0.39	0.65 ± 0.12
C_T (µmol L^{-1})	1492 ± 42.22	1592 ± 23.28
CO_3^{2-} (µmol L^{-1})	114.5 ± 24.3	40.0 ± 15.4
Total alkalinity (A_T)	1650 ± 36.56	1611 ± 37.81
Salinity	28.0 ± 1.0	28.0 ± 1.0

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Table 6. Temperature, pH_{NBS}, carbonate chemistry, total alkalinity, and salinity (±1 SD) during the three-level carbon dioxide experiments for *Argopecten irradians* larvae growth before deployment in the estuary for juvenile growth.

Parameter	Near pre-industrial CO_2	Ambient, present day CO_2	Elevated CO_2
Argopecten irradians			
Temperature (°C)	24 ± 0.7	24 ± 0.7	24 ± 0.7
pH_{NBS}	8.20 ± 0.067	8.08 ± 0.072	7.81 ± 0.009
pCO_2 (µatm)	231.3 ± 34.538	373.1 ± 29.78	755.9 ± 14.226
$\Omega_{calcite}$	2.86 ± 0.53	2.66 ± 0.55	1.55 ± 0.09
$\Omega_{aragonite}$	1.94 ± 0.37	1.71 ± 0.50	1.00 ± 0.43
C_T (µmol L^{-1})	1141 ± 98.33	1365.7 ± 56.79	1443.8 ± 38.92
CO_3^{2-} (µmol L^{-1})	112.9 ± 25.35	104.8 ± 37.37	61.3 ± 14.54
Total alkalinity (A_T)	1320.9 ± 119.3	1518.1 ± 43.36	1516.4 ± 47.54
Salinity	28.0 ± 1.0	28.0 ± 1.0	28.0 ± 1.0

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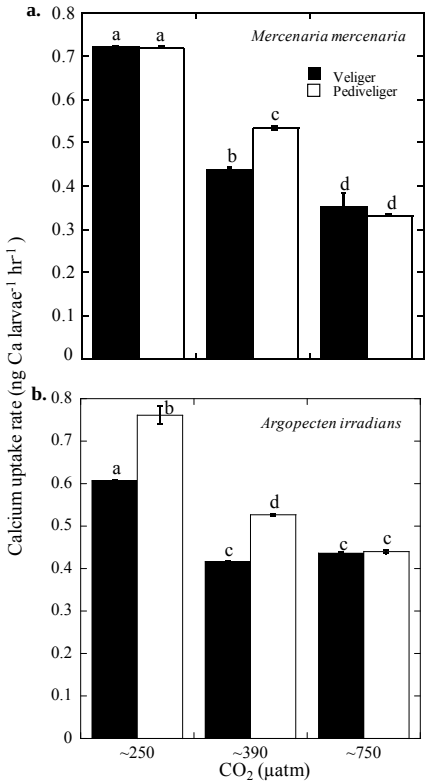

Fig. 1. Mean calcium uptake ±1 standard deviation for larvae from two developmental stages of *Mercenaria mercenaria* (veligers from day 3, and pediveligers from day 10) and *Argopecten irradians* larvae (veligers from day 12, and pediveligers from day 15). Larvae were grown under CO_2 concentrations of approximately 250, 390, and 750 µatm CO_2 (Table 1). Letters indicate significant differences revealed from Tukey post-hoc multiple comparisons, $p \leq 0.05$ for all.

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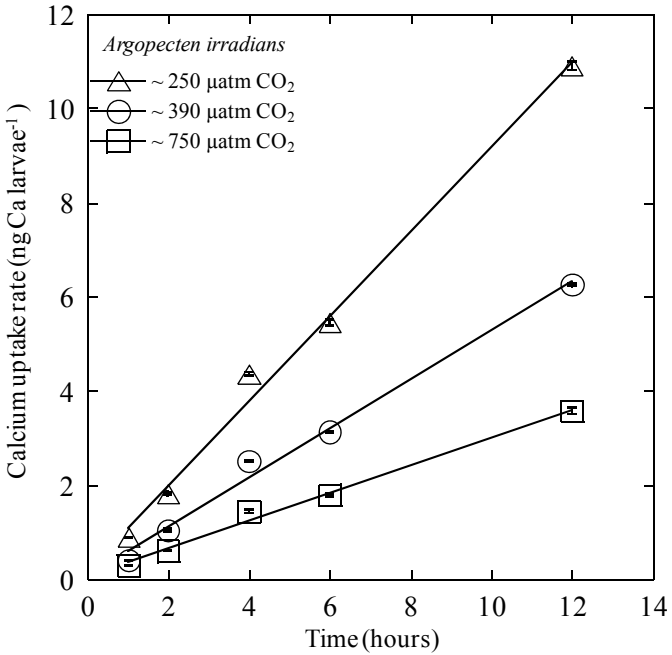

Fig. 2. Mean (±1 SD) calcium uptake rates over 12 h for *Argopecten irradians* larvae (veligers from day 12). Larvae were grown under CO_2 concentrations of approximately 250, 390, and 750 µatm CO_2 (Table 2), $n = 4$ per treatment. Regressions corresponding to each treatment are listed in legend. Regression are 250 µatm CO_2, $y = 0.19 + 0.90 \times$ ($R^2 = 0.99$), ~390 µatm CO_2, $y = 0.05 + 0.52 \times$ ($R^2 = 0.99$), and ~750 µatm CO_2, $y = 0.06 + 0.29 \times$ ($R^2 = 0.99$).

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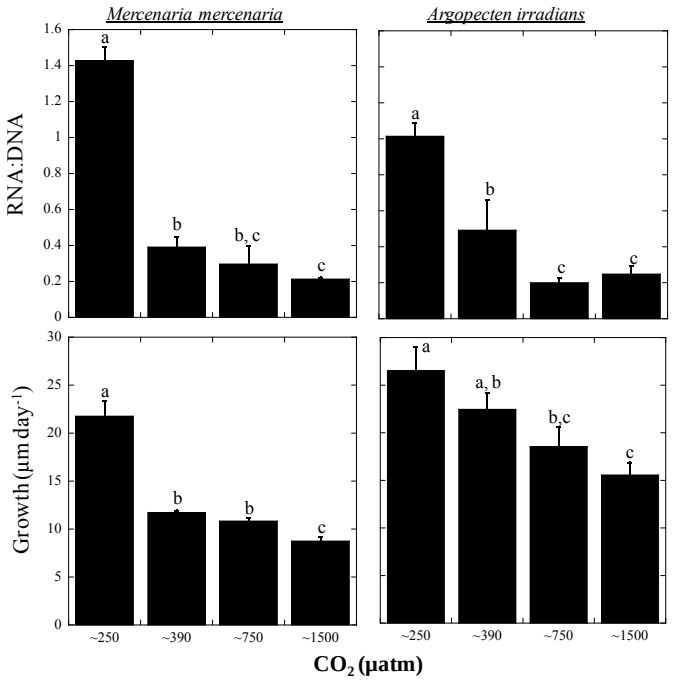

Fig. 3. Mean RNA : DNA (±1 SD) and mean, shell-based, specific growth rates (±1 SD) for *Mercenaria mercenaria* (day 24) and *Argopecten irradians* (day 20) larvae $n = 15$ larvae per treatment. Larvae were grown under CO_2 concentrations of approximately 250, 390, and 750 µatm CO_2 (Table 3). Letters indicate significant differences revealed from Tukey post-hoc multiple comparisons, $p \leq 0.05$ for all.

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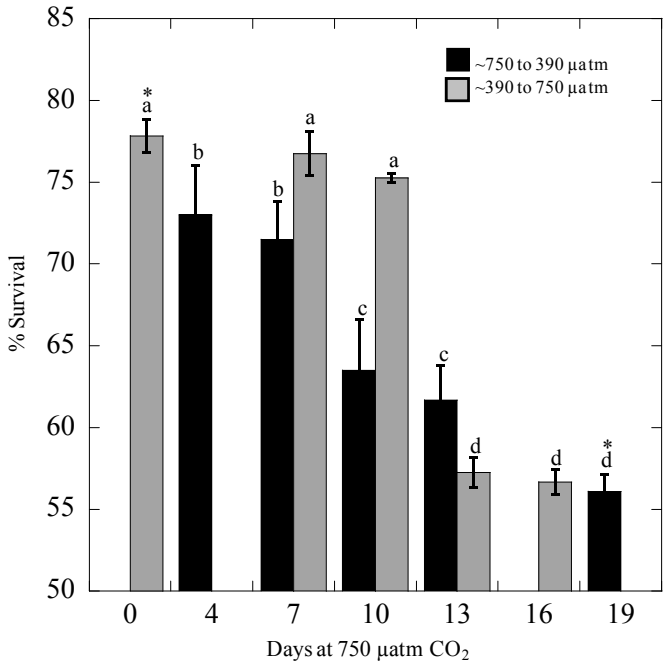

Fig. 4. Survival ± standard deviation of *Argopecten irradians* larvae under varying days of exposure to approximately 750 µatm CO_2 (Table 4). Different colors indicate the CO_2 exposure the larvae experienced first and then were switched to ($n = 4$ per treatment). Letters indicate significant differences revealed from Tukey post-hoc multiple comparisons, $p \leq 0.05$ for all, and * indicated these individuals only experienced either 390 µatm or 750 µatm and were never switched.

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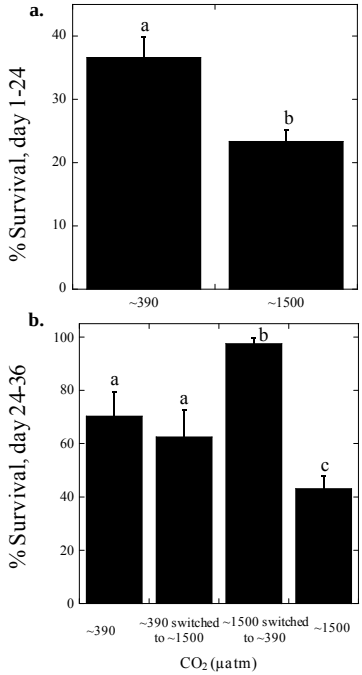

Fig. 5. (a) Percent survival of *Mercenaria mercenaria exposed to* 390, and 1500 µatm CO_2 during larval development, (Table 5) **(b)** percent survival of post-set, juvenile *M. mercenaria* under four treatments of CO_2: (1) individuals exposed to 390 µatm since fertilization, (2) individuals exposed to 390 µatm as larvae and increased to 1500 at day 24, (3) individuals exposed to 1500 µatm as larvae and decreased to 390 µatm at day 24, (4) individuals exposed to 1500 µatm since fertilization. Values are means ±1. SD; $n = 4$ per treatment. Letters indicate significant differences revealed from Tukey post-hoc multiple comparisons, $p \leq 0.05$ for all.

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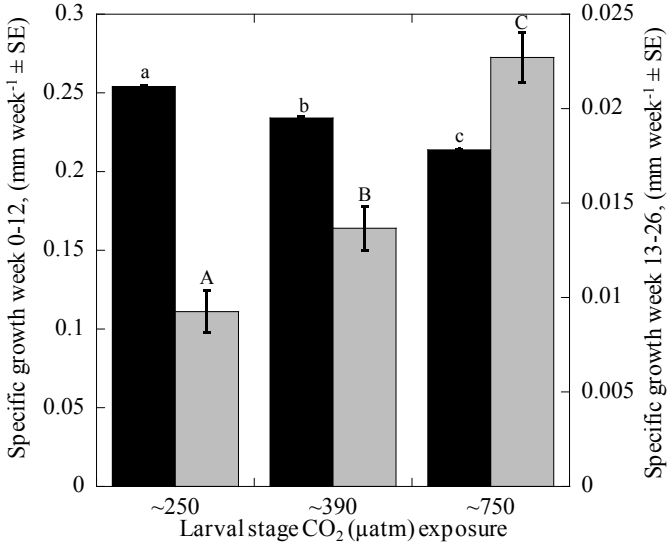

Fig. 6. Specific growth rates of *Argopecten irradians* for 0–12 weeks (black bars) and for 13–26 weeks (gray bars). Larvae (n = 1000) were grown under three CO_2 concentrations (250, 390, and 750 µatm CO_2; Table 6) during the larval stages before introduced into the field as juveniles. For Tukey multiple comparisons, $p \leq 0.05$ for all and capitalized letters indicate a separate analysis.

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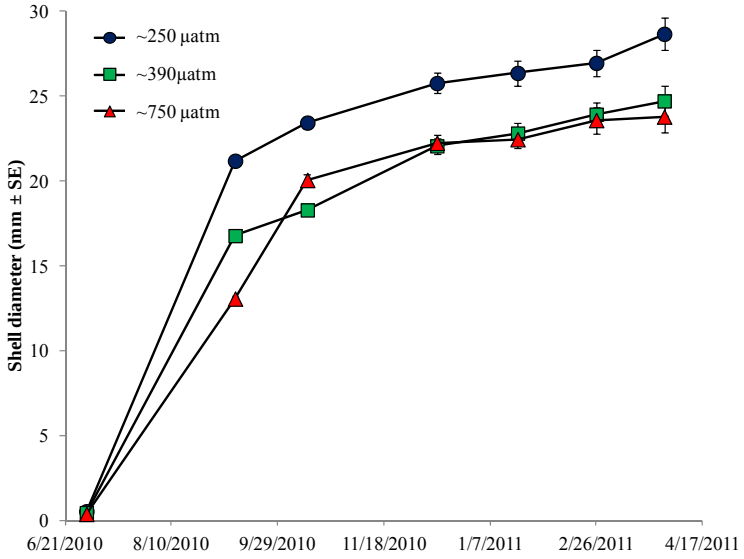

Fig. 7. Shell diameter for *Argopecten irradians* juveniles over a 26 week period in an estuary in East Hampton, NY. Larvae (n = 1000) were first grown under CO_2 concentrations of approximately 250, 390, and 750 µatm CO_2 (Table 6) during the larval stages before introduced into the field as juveniles. Estimates for first time point are from Sect. 3. Values are means ±1. SE.

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