

Physiological response and resilience of early life-stage Eastern oysters (*Crassostrea virginica*) to past, present and future ocean acidification

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The Eastern oyster, *Crassostrea virginica* (Gmelin, 1791), is the second most valuable bivalve fishery in the USA and is sensitive to high levels of partial pressure of CO₂ (pCO₂). Here we present experiments that comprehensively examined how the ocean's past, present and projected (21st and 22nd centuries) CO₂ concentrations impact the growth and physiology of larval stages of *C. virginica*. *Crassostrea virginica* larvae grown in present-day pCO₂ concentrations (380 μatm) displayed higher growth and survival than individuals grown at both lower (250 μatm) and higher pCO₂ levels (750 and 1500 μatm). *Crassostrea virginica* larvae manifested calcification rates, sizes, shell thicknesses, metamorphosis, RNA:DNA ratios and lipid contents that paralleled trends in survival, with maximal values for larvae grown at 380 μatm pCO₂ and reduced performance in higher and lower pCO₂ levels. While some physiological differences among oysters could be attributed to CO₂-induced changes in size or calcification rates, the RNA:DNA ratios at ambient pCO₂ levels were elevated, independent of these factors. Likewise, the lipid contents of individuals exposed to high pCO₂ levels were depressed even when differences in calcification rates were considered. These findings reveal the cascading, interdependent impact that high CO₂ can have on oyster physiology. *Crassostrea virginica* larvae are significantly more resistant to elevated pCO₂ than other North Atlantic bivalves, such as *Mercenaria mercenaria* and *Argopecten irradians*, a finding that may be related to the biogeography and/or evolutionary history of these species and may have important implications for future bivalve restoration and aquaculture efforts.

Key words: Bivalve, ocean acidification, oyster, restoration

Editor: Steven Cooke

Received 11 December 2013; Revised 14 January 2014; Accepted 16 January 2014

Cite as: Gobler CJ, Talmage SC (2014) Physiological response and resilience of early life-stage Eastern oysters (*Crassostrea virginica*) to past, present and future ocean acidification. *Conserv Physiol* 2: doi:10.1093/conphys/cou004.

Introduction

Rising levels of atmospheric CO₂ have led to world's oceans acidifying since the dawn of the industrial revolution, a process expected to continue for at least two centuries (Caldeira and Wickert, 2003). This ocean acidification depresses the availability of carbonate ions (CO₃²⁻) and thus is expected to have adverse effects on a diverse assemblage of marine animals that incorporate calcium carbonate into their hard parts

or shells (Orr *et al.*, 2005; Doney *et al.*, 2009). The shallow nature of coastal zones makes them more vulnerable to external fluxes of CO₂ than open ocean regions (Duarte *et al.*, 2013). Many coastal ecosystems already experience elevated levels of CO₂ (Salisbury *et al.*, 2008; Cai *et al.*, 2011; Hu and Cai, 2013), in part due to decomposition of the large amount of terrestrial, algal and anthropogenic organic carbon delivered to estuaries (Gattuso *et al.*, 1998; Paerl *et al.*, 1998; Thomas *et al.*, 2004; Gupta *et al.*, 2009). Furthermore,

coastal zones may be impacted by discharge of acidic river water (Salisbury *et al.*, 2008; Hu and Cai, 2013) or upwelling of acidified deep waters (Feely *et al.*, 2008). Hence, animals in coastal zones are, in some cases, already subjected to acidification.

Oysters are a keystone species in coastal zones due to their economic value and the array of ecosystem services they provide, including robust water filtration and the creation of reefs that represent important benthic habitat (Newell, 2004). Many species of oyster are vulnerable to high CO₂ levels. Larvae of the Pacific oyster (*Crassostrea gigas*) exposed to experimentally elevated CO₂ levels experienced slowed developmental rates, slowed shell mineralization rates and malformations (Kurihara *et al.*, 2007), while juveniles have displayed significant declines in calcification with increases in partial pressure of CO₂ (pCO₂) to levels expected at the end of the century (Gazeau *et al.*, 2007). In contrast, decreases (−0.35 pH units) in pH did not affect the fertilization rate or sperm motility of *C. gigas* (Havenhand and Schlegel, 2009). The survival of *C. gigas* larvae at a hatchery was shown to be strongly dependent on the aragonite saturation state of waters in which larval oysters were spawned and reared for the first 48 h of life (Barton *et al.*, 2012), probably due to the strong dependence of these larvae on external, rather than internal, sources of inorganic carbon for shell synthesis during this developmental period (Waldbusser *et al.*, 2013). Exposure of *C. gigas* and a second species of oyster (*Saccostrea glomerata*) to high CO₂ and increased temperature caused declines in fertilization success, development of embryos and the size of larvae, as well as abnormal larval morphology (Parker *et al.*, 2010). Estuarine acidification has been shown to cause mortality of rock oysters (*Saccostrea glomerata*; Dove and Sammut, 2007). Pearl oysters (*Pinctada fucata*) grown in acidic seawater have weaker, malformed shells compared with normal oysters (Welladsen *et al.*, 2010). In contrast, growth and calcification of the larvae from the Suminoe oyster (*Crassostrea ariakensis*) were unaffected by pCO₂ levels ranging from 280 to 800 μatm (Miller *et al.*, 2009). However, high levels of CO₂ have been shown to have deleterious consequences for the Eastern oyster (*Crassostrea virginica*, Gmelin, 1791; see below).

The Eastern oyster (*Crassostrea virginica*) has a geographical range of 8000 km from Brazil to Canada in the Western Atlantic Ocean, where it inhabits a wide range of salinities (5–30) in coastal zones (Carriker and Gaffney, 1996). In the USA, *C. virginica* is the second most valuable bivalve fishery (NMFS, 2010). Waldbusser *et al.* (2011) reported that calcification rates of juvenile *C. virginica* declined significantly during experimental reductions in pH that matched those observed in Chesapeake Bay in recent decades, while Talmage and Gobler (2011) found that juvenile Eastern oysters exposed to high CO₂ levels (~1700 μatm) grew 40% slower than those exposed to normal CO₂ levels. With increasing CO₂ levels, *C. virginica* larvae experience a reduction in calcium content (Miller *et al.*, 2009) as well as decreased size and survivorship (Talmage and Gobler, 2009). Moreover,

Miller *et al.* (2009) reported that *C. virginica* larvae grown with low CO₂ levels (280 μatm) yielded slight increases in shell area and calcium carbonate (CaCO₃) composition, suggesting that, as was the case for other bivalve species (*Mercenaria mercenaria* and *Argopecten irradians*; Talmage and Gobler, 2010), *C. virginica* larvae may experience optimal growth and performance at the CO₂ levels that were present during the pre-industrial era.

The goal of this study was to provide a comprehensive assessment of how past, present and projected CO₂ levels (~250, 380, 750 and 1500 μatm pCO₂) affect the physiology of larval Eastern oysters, *C. virginica*. Reductions in the growth and survival at this stage can cause substantial declines in adult populations (Caley *et al.*, 1996; Schneider *et al.*, 2003). Oyster larvae were exposed to each pCO₂ level for the entirety of the larval stage, and the calcification rate, RNA:DNA ratio, shell length, shell thickness, lipid content and shell morphology were quantified. Next, we compared differences in these physiological parameters as a function of size and calcification rates of larval oysters. A final goal was to compare the vulnerabilities of *C. virginica* to elevated pCO₂ levels with those of other North American resource bivalves. This multi-faceted approach provided a comprehensive assessment of the biological response of early life stages of this species to varying levels of pCO₂.

Materials and methods

Carbon dioxide treatments and measurements

This study examined the effects of multiple pCO₂ levels on larval stages of *C. virginica*. For all experiments, experimental vessels with bivalves (described below) were maintained in water baths at 24°C using commercially available aquarium heaters (Aquatic Eco-systems, Inc., Apopka, FL, USA), a temperature ideal for the growth and survival of *C. virginica* larvae (Matthiessen, 2001). Temperatures were recorded every 6 min throughout experiments using *in situ* data loggers (Onset®, Bourne, MA, USA) and remained within ±0.5°C of target values. A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame, Cole Parmer, Court Vernon Hills, IL, USA) was used to deliver CO₂ gas to seawater treatments at multiple rates. The gas proportionator delivered pre-mixed gases (250, 380, 750 or 1500 μatm; Praxair®, Danbury, CT, USA) at flow rates which turned over the volume of experimental vessels several times an hour, ensuring that vessels did not equilibrate with the atmosphere. For experiments, the CO₂ gas mixtures were continuously delivered to the bottom of replicated (*n* = 4) experimental vessels (detailed below). With continuous bubbling, all treatment carboys remained saturated with respect to dissolved oxygen (~8 mg l⁻¹). To quantify precise CO₂ levels attained in experimental treatments, aliquots were removed immediately before commencing experiments as well as immediately after the conclusion of experiments and analysed immediately using an

EGM-4 Environmental Gas Analyzer® (PP Systems) system that quantified total dissolved inorganic carbon (DIC) levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). These analyses provided a methodological precision of $\pm 2.6\%$ for replicated measurements of total DIC and provided full recovery ($102 \pm 3\%$) of Dr 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 total DIC (kg seawater) $^{-1}$]. Levels of CO_2 were calculated based on measured levels of total inorganic carbon, pH [mol (kg seawater) $^{-1}$; NBS scale], temperature, salinity, 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 (Thermo Scientific Orion Star Series™ Benchtop pH meter; ± 0.001 ; calibrated prior to each use with NIST traceable standards) indicated that experimental vessels maintained a constant pH level throughout experiments ($< 0.5\%$ relative standard deviation within treatments). Spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson *et al.* (2007) and corrected for scale (Dickson, 1993) were highly similar to and never significantly different from those obtained with the pH electrode. The levels of precision for measurements of pH and DIC adequately resolved the hundreds of microatmosphere differences among our CO_2 treatments (~ 250 , 380, 750 and 1500 μatm).

Larvae and treatments

Crassostrea virginica larvae were obtained from the East Hampton Shellfish Hatchery (East Hampton, NY, USA) within hours of fertilization. Wild broodstock were obtained from the eastern Peconic Estuary (NY, USA), which experiences normoxic and normocapnic levels of oxygen and pCO_2 (Gobler *et al.*, 2014). Broodstock consisted of at least five individuals of both sexes to ensure genetic diversity among larvae. Broodstock were conditioned for > 2 months using standard methods (Loosanoff and Davis, 1963) in seawater with a salinity of ~ 28 and a pH of ~ 8 through the entire gametogenic phase prior to spawning. For experiments, the following four levels of pCO_2 were administered: a pre-industrial level ($\sim 250 \mu\text{atm}$ pCO_2), a modern level ($\sim 380 \mu\text{atm}$ pCO_2), an elevated level ($\sim 750 \mu\text{atm}$ pCO_2) and a high level ($\sim 1500 \mu\text{atm}$ pCO_2). These concentrations span the levels of pCO_2 observed and expected in open ocean waters from the year 1750 to 2200 (Caldeira and Wickett, 2003) and can be observed in estuaries during summer (Talmage and Gobler, 2009; Cai *et al.*, 2011), when *C. virginica* spawn (Kennedy *et al.*, 1996). We note that the static pCO_2 levels used in these experiments are likely to deviate from the diurnal, tidal and seasonal fluctuations in pCO_2 that *C. virginica* larvae are likely to experience in estuaries (Duarte *et al.*, 2013). All treatment vessels were filled from a single reservoir containing 0.2- μm -filtered seawater obtained from eastern Shinnecock Bay to ensure equivalent seawater chemistry. Larvae were fed *Isochrysis galbana* (Tahitian strain,

T-Iso), an ideal food source, at a density of $4 \times 10^4 \text{ ml}^{-1}$ daily to maximize bivalve larval growth and survivorship through metamorphosis (Cragg, 2006; Padilla *et al.*, 2006). To promote high survivorship, all containers in contact with larvae were never exposed to chemicals or detergents. 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 millilitre of solution) was added to each treatment vessel at 1% of its original concentration at the beginning of each experiment and at the time of each water change (approximately two times weekly). While this is not a standard practice in shellfish hatcheries, it was pursued due to the length of the experiments. This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae (Padilla *et al.*, 2006).

For each experiment, larvae were distributed to each 1 l experimental vessel at a concentration of $\sim 400 \text{ l}^{-1}$ in quadruplicate ($n = 4$) ~ 18 h post-fertilization. Twice weekly during experiments, larvae were gently poured onto a 64 μm mesh, and the condition (live or dead) and developmental stage of each larva (veligers, pediveligers and metamorphosed) was determined visually under a dissecting microscope; every individual live larva was counted at every water change. Larvae from each vessel ($n = 4$ per treatment) were removed, counted, observed, and transferred into a new vessel with new filtered seawater, food and antibiotics within a 15 min period. Experiments were terminated after all surviving larvae in all treatments metamorphosed, after ~ 3 weeks.

Size and lipid analysis

At the end of experiments, the relative lipid content of larvae was measured as described by Talmage and Gobler (2010). Briefly, Nile Red stain was used to bind to neutral lipids and fluoresce under a fluorescein isothiocyanate filter on a fluorescence microscope (Castell and Mann, 1994; Phillips, 2002). A Nile Red stock solution was made of 1.25 mg of Nile Red crystals in 100 ml of acetone. Randomly selected larvae ($n = 15$) from each treatment were stained with a 1:9 dilution of the stock solution and 0.2- μm -filtered seawater. Larvae were exposed to the stain for ~ 1.5 h, rinsed with filtered seawater, and digitally photographed with a Roper Scientific Photometrics CoolSNAP ES camera under an epifluorescence microscope to observe lipids. Photographs of each larva were taken and analysed using ImageJ® software for the area of lipid accumulation, the diameter and the area of individuals. Diameters were based on shell lengths. The proportional area of lipid content was estimated as the ratio of lipid area to larval area.

Calcification rates

In order to measure calcification rates during the veliger (day 7) and pediveliger stage (day 16), a separate set of larvae were grown at 250, 380 and 750 μatm pCO_2 as described above (in the 'Larvae and treatments' subsection). These larvae were bubbled with the same tanked gases delivered at the

same rates, held in the same vessels within the same water baths, fed the same food, with water changes on the same dates, and displayed survival rates that were similar to and not significantly different from those in the other experimental vessels at the same pCO₂ levels. For experiments, ~200 larvae per treatment were 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 deliver the same CO₂ levels that the larvae had been grown in until that point.

Differences in the rate of calcification of larvae exposed to different levels of CO₂ were assessed using a ⁴⁵Ca isotope tracer method (Ho and Zubkoff, 1980). High specific activity, 9.25 × 10⁶ Bq, ⁴⁵Ca was added to ~100 ml of filtered seawater with larvae in four replicated, polypropylene 125 ml Nalgene bottles, resulting in a final concentration of 3.7 × 10³ Bq ml⁻¹. A killed-control bottle was established at each level of CO₂ via the addition of glutaraldehyde to a final concentration of 2%. After 24 h, bottles were gently gravity filtered onto 20 µm polycarbonate membranes. Time series uptake experiments were also made during the veliger stage, 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 rinsed with filtered seawater to remove surface adsorbed ⁴⁵Ca, transferred to scintillation vials, and digested with 1 ml of concentrated HNO₃ (15.8 N) for 1 h, 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 ⁴⁵Ca detection. The weight-specific calcium uptake rate per individual was determined by the following equation:

$$\text{CaU} = \frac{[(\text{dpm})_l - (\text{dpm})_d] \times \text{Ca}_m}{(\text{dpm})_m \times t \times L}$$

where CaU is the calcium uptake rate (in nanograms of calcium per larva per hour), (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 milligrams per litre), (dpm)_m is the radioactivity of unit medium water, *t* is the length of the incubation (in days) and *L* is the number of larvae per beaker. This equation is based on the assumption that there is no significant discrimination by larvae for ⁴⁵Ca and ⁴⁰Ca uptake (Ho and Zubkoff, 1980). Given that bivalves exposed to high levels of CO₂ and low levels of pH can dissolve (Green *et al.*, 2009; Talmage and Gobler, 2010), these should be considered net calcification rates. The calcium content of water was estimated by the ratio of its salinity to that of typical oceanic water of 35, which has 408 p.p.m. of Ca²⁺ (Sverdrup *et al.*, 1942).

RNA:DNA ratios

In order to assess RNA:DNA ratios of *C. virginica* larvae, individuals were grown at the four levels of CO₂ used in the experiments. The general experimental set-up followed the description above (see *Larvae and treatments*); precise CO₂ levels and complete carbonate chemistry from this experiment appear in Table 1. The overall growth and RNA synthesis rate of larval shellfish was assessed by analysis of the ratio between RNA and DNA content in the organisms (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 (*n* = 4), poured onto a sieve, and were then macerated using a Pellet Pestle® Motor, heated to 50°C for 15 min, and then frozen to -80°C for at least 90 min. Nucleic acids from pooled groups of larvae per treatment (*n* = 15) were extracted using a modified cetyl trimethylammonium bromide technique, and quantified using Quant-iT™ RiboGreen RNA® and Quant-iT™ PicoGreen® DNA assay kits (Invitrogen), according to the manufacturer's protocol. RiboGreen® RNA and PicoGreen® DNA are ultra-sensitive fluorescent nucleic acid stains for quantifying RNA and DNA, respectively, in solution.

Table 1: Temperature, pH, carbonate chemistry, alkalinity and salinity (±1 SD) during the four-level carbon dioxide experiment

Parameter	Pre-industrial CO ₂	Ambient CO ₂	Elevated CO ₂	Elevated CO ₂
Temperature (°C)	24 ± 1.0	24 ± 1.0	24 ± 1.0	24 ± 1.0
pH	8.32 ± 0.01	8.16 ± 0.02	7.93 ± 0.001	7.68 ± 0.05
Partial pressure of CO ₂ (µatm)	249 ± 7.15	394 ± 26.7	745 ± 23.0	1430 ± 162
Saturation state of calcite	5.67 ± 0.06	4.27 ± 0.20	2.822 ± 0.06	1.69 ± 0.19
Saturation state of aragonite	3.68 ± 0.04	2.77 ± 0.13	1.83 ± 0.04	1.10 ± 0.12
Total dissolved inorganic carbon (µmol l ⁻¹)	1710 ± 11.8	1790 ± 31.3	1932 ± 14.2	2040 ± 11.5
CO ₃ ²⁻ (µmol l ⁻¹)	227 ± 2.45	171 ± 8.33	113 ± 2.14	67.8 ± 7.43
Total alkalinity (µmol l ⁻¹)	2020 ± 8.41	2040 ± 35.3	2070 ± 12.6	2090 ± 11.8
Salinity	30.0 ± 1.0	30.0 ± 1.0	30.0 ± 1.0	30.0 ± 1.0

RiboGreen® also binds DNA; therefore, complete DNase digestion of the sample preceded analysis of RNA. The RNA and DNA concentrations in the extracted samples were determined by measuring fluorescence using an Applied Biosystems 7300 Real-Time PCR system-genetic analyser and compared with a standard curve of nucleic acids.

Scanning electron microscopy

In order to document differences in the size and structure of larval and early oysters exposed to differing levels of CO₂, randomly chosen individuals ($n = 6$ per vessel) were mounted for scanning electron microscopic imaging as described by Talmage and Gobler (2010). Firstly, to image the outside of shells, individuals were attached at 45° relative to a level surface to a conductive substrate using carbon double-sided tape and were subsequently coated with ~12 nm of gold using an Edwards® 150B rotary pump.

In order to image the thickness and internal dimensions, cross-sections of shellfish were prepared. Individuals were mounted on glass microscope slides using ultraviolet-curing adhesive coating (Locite® 4304) and were impregnated with low-viscosity epoxy (Stuers® Specifix-20) under vacuum outgassing, a step which did not alter the original shape or size of individuals. After curing, the epoxy mount was progressively ground and polished to the centreline (hinge to shell edge) of the shellfish using silicon carbide sandpapers, followed by successively finer diamond polishing grits (15, 6 and 3 μ), 0.05 μm aluminum oxide suspension and, finally, with colloidal silica. All individuals were cross-sectioned at the same location (hinge to shell edge) across the shell. Measurements of shell thicknesses were made at the precise mid-point between the hinge and valve opening on the upper and lower shell for all individuals. This mount was then attached to a conductive substrate using carbon double-sided tape and coated with ~4 nm of gold.

Scanning electron microscopic images were collected on both types of samples with a Leo (Zeiss) Model # 1550 electron microscope using a high voltage of 20 kV and a Robinson back-scatter detector. All components of individual bivalve shells displayed in Fig. 6 were probed using an advanced energy dispersive x-ray analyzer microanalysis in the LEO (Zeiss) Model # 1550 electron microscope and were confirmed to contain almost exclusively carbon, oxygen and calcium.

Statistical analyses

Percentage data (survival, metamorphosis) were arcsine square-root transformed prior to statistical analyses. Differences in survival, size, shell thickness, metamorphosis, RNA:DNA ratios, calcification rates and lipid indexes were assessed using one-way ANOVAs and Tukey's *post hoc* multiple comparison tests where CO₂ levels were the treatment effects. Data that did not meet assumptions of normality and homogeneity were tested using Kruskal–Wallis tests. Statistical analyses were performed with SYSTAT 13® Copyright, 2009, Systat Software, Inc.

Allometric- and calcification-based analyses

Larvae exposed to elevated pCO₂ levels have been shown to grow more slowly than conspecifics grown at lower pCO₂ levels (Talmage and Gobler, 2010; Waldbusser *et al.*, 2013). As a consequence, comparing individuals in different pCO₂ conditions at a single sampling time may make it difficult to discriminate between the direct impact of pCO₂ on physiological performance and the indirect effects on physiology via slowed growth and development, because physiological performance is often stage and size specific. To explore this hypothesis, an allometric approach was taken whereby the length of individuals at the end of the larval stage was used as a proxy for size and age, and the shell thickness, lipid index, RNA:DNA ratio and calcification rates of individuals were divided by their length. Differences in length-based physiological performance were compared by means of a one-way ANOVA and Tukey's *post hoc* multiple comparison tests.

Given that ocean acidification compromises the ability of bivalves to calcify, it is possible that the primary impact of acidification on larvae may be compromised calcification, which has a cascading effect on the physiology of individuals. To explore this hypothesis, the length, shell thickness, lipid index and RNA:DNA ratio of individuals at the end of the larval stage were divided by their calcification rate. Differences in calcification-based physiological performance were compared by means of a one-way ANOVA and Tukey's *post hoc* multiple comparison tests.

Results

Crassostrea virginica larvae grown at present-day CO₂ levels (380 μatm) generally performed better than those reared at higher and lower CO₂ levels (250, 750 and 1500 μatm). More *C. virginica* larvae survived at present-day CO₂ levels (29 ± 8.1%) compared with the survival at 250 (6.0 ± 3.8%), 750 (19 ± 5.5%) and 1500 μatm CO₂ (21 ± 1.7%; Fig. 1). While survival at 250 μatm CO₂ was significantly lower than at all other levels ($P < 0.05$; Tukey's test), the differences among the other levels were not statistically significant ($P > 0.05$).

Developmental rates were most rapid for *C. virginica* larvae grown at present-day CO₂ levels ($P < 0.001$; Fig. 1). After 16 days of development at present-day CO₂ levels (~380 μatm), 51 ± 15% of surviving larvae had metamorphosed, while at ~250, 750 and 1500 μatm, 22 ± 18, 41 ± 12 and 39 ± 0.4% had done so (Fig. 1).

Shell lengths of *C. virginica* larvae at day 21 were significantly larger (392 ± 9.8 μm) when grown in present-day CO₂ conditions (380 μatm), compared with 250 μatm pCO₂ (317 ± 4.7 μm), 750 μatm CO₂ (208 ± 7.5 μm) or 1500 μatm pCO₂ (127 ± 4.6 μm; $P < 0.001$; Fig. 2A). Shells of *C. virginica* larvae were also significantly thicker at present-day CO₂ levels (8.1 ± 1.1 μm) compared with 250 (6.4 ± 0.76 μm), 750 (5.1 ± 0.53 μm) or 1500 μatm pCO₂ (5.1 ± 0.62 μm;

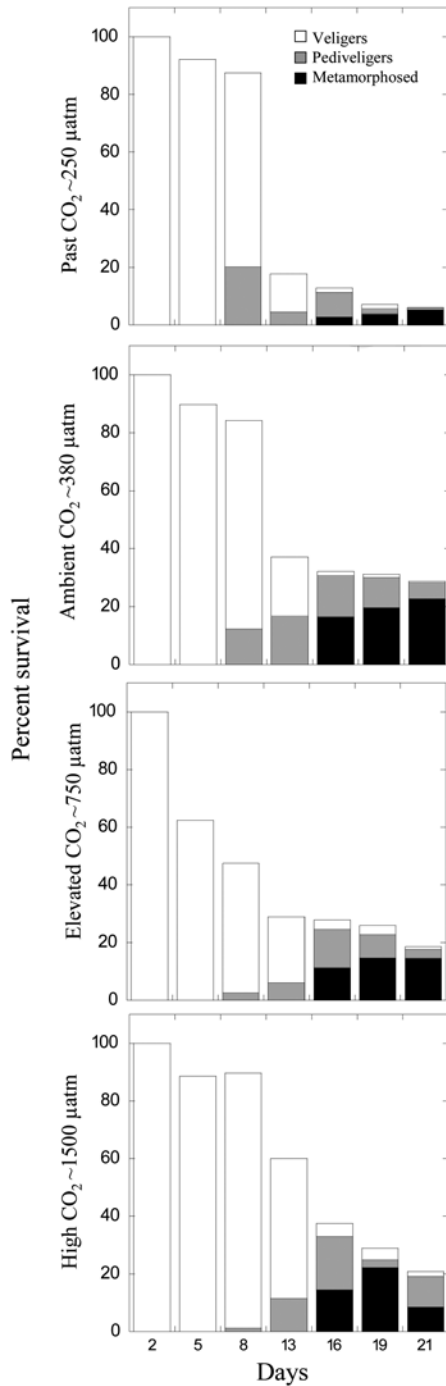


Figure 1: Development and survival of *Crassostrea virginica* larvae. Percentage survival and developmental stage (veliger, pediveliger and metamorphosed) of larvae grown in the presence of four levels of pCO₂, i.e. ~250, 380, 750 and 1500 µatm (Table 1). The relative standard deviation of larval survival among replicated vessels per treatment for all times points and experiments was 15% ($n = 4$ per treatment).

$P < 0.001$; Fig. 2B). The lipid content (as estimated by an index) of larvae was highest in larvae grown in 380 µatm pCO₂ (0.40 ± 0.05) compared with the lower (0.3 ± 0.1) and the two higher levels of pCO₂ (0.12 ± 0.01 and 0.11 ± 0.01 ; $P < 0.001$; Fig. 2C). The RNA:DNA ratios of larvae differed across pCO₂ treatments. Larvae grown at 380 µatm displayed a high ratio of 1.75 ± 0.42 , while larvae within other treatments had significantly lower ratios of 0.32 ± 0.02 , 0.30 ± 0.03 and 0.23 ± 0.04 at 250, 750 and 1500 µatm pCO₂, respectively ($P < 0.001$; Fig. 2D).

Calcification rates of oyster larvae varied as a function of the ambient pCO₂ levels. During 24 h measurements, calcification rates of 7-day-old veligers and 16-day-old pediveligers were significantly faster at 380 µatm CO₂ (0.78 ± 0.003 ng calcium per larva h⁻¹) compared with higher (750 µatm; 0.45 ± 0.002 ng calcium per larva h⁻¹) and lower levels of pCO₂ (250 µatm; 0.58 ± 0.003 ng calcium per larva h⁻¹; $P < 0.001$; Fig. 3A). Calcification rates were highly linear during 12 h experiments with veligers, and followed trends observed during 24 h exposures, with rates being 0.77, 0.67 and 0.44 ng calcium per larva h⁻¹ at 380, 250 and 750 µatm pCO₂ (Fig. 3B).

Scaling the oyster larval performance metrics to larval lengths indicated that some differences in larval performance may have been driven by larval size, while other attributes still differed by pCO₂ exposure level, even when size was considered (Fig. 4). For example, there were no significant differences in the thickness or lipid index of individuals when scaled to larval length (Fig. 4A and B). Interestingly, calcification rates were slightly but significantly higher (8%) at 750 compared with 380 µatm pCO₂ when corrected for size ($P < 0.05$; Fig. 4C). In contrast, RNA:DNA ratios of individuals grown at 380 µatm pCO₂ remained significantly higher than those grown at higher and lower pCO₂ levels even when corrected for the length of the larvae ($P < 0.05$; Fig. 4D).

Scaling the larval oyster performance metrics to calcification rates suggested that some patterns in larval performance depended on these rates, while others were still affected by pCO₂ exposure, even when differences in calcification rates were considered (Fig. 5). For example, once calcification rates were considered, the lengths of oyster larvae were proportional to pCO₂ levels, with lengths decreasing as pCO₂ levels increased ($P < 0.05$; Fig. 5A). This trend was not found with regard to shell thickness ($P > 0.05$; Fig. 5B), suggesting that this parameter was more directly affected by calcification rate. While the calcification rate-corrected lipid contents of individuals grown at 250 and 380 µatm pCO₂ were nearly identical, the lipid content was still lower at elevated pCO₂ even after correcting for calcification rates ($P < 0.05$; Fig. 5C). Consistent with the size-corrected RNA:DNA ratios, ratios of individuals grown at 380 µatm pCO₂ remained significantly higher than those grown at higher and lower pCO₂ levels, even when corrected for calcification rates ($P < 0.05$; Fig. 5D).

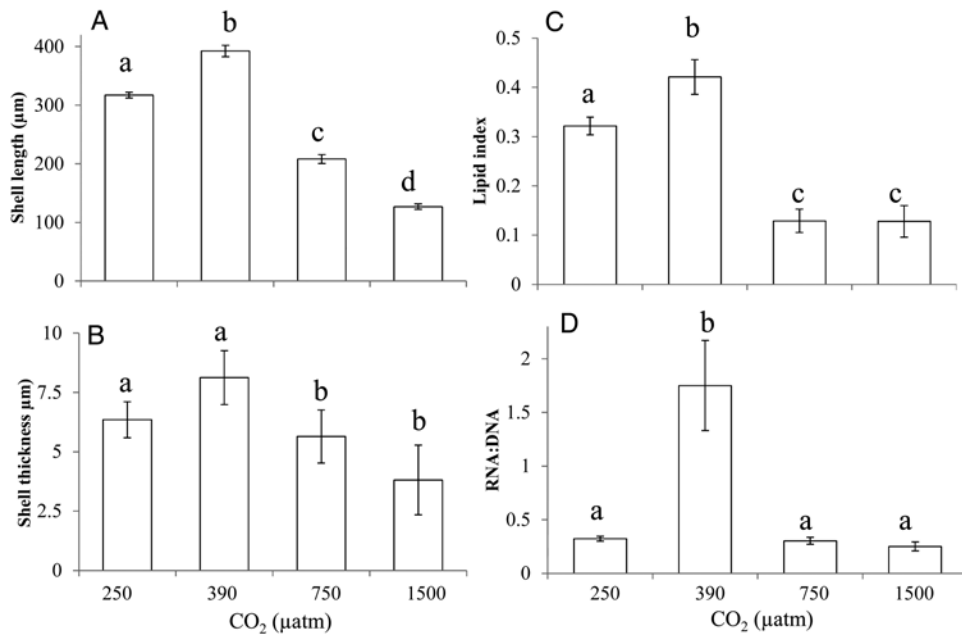


Figure 2: Shell length and thickness, lipid index and RNA:DNA ratio of *C. virginica* larvae in the presence of pCO₂ levels of ~250, 380, 750 and 1500 µatm. (A) Shell length of *C. virginica*. (B) Shell thickness of *C. virginica* shells at mid-point between the hinge and valve edge of the upper and lower shell of cross-sectioned individuals. (C) Lipid index (lipid area/total area) of *C. virginica* individuals (day 21). (D) RNA:DNA ratio of *C. virginica* individuals. Error bars represent standard deviation of replicated vessels per treatment ($n = 4$ per treatment). Lower case letters signify treatments that are statistically equal (same letter) or different (different letters) as revealed via a Tukey test ($P < 0.05$).

Scanning electron microscopic images illustrated the effects of higher and lower CO₂ levels on shell diameter and thickness, as well as hinge structure of early life-stage oysters (Fig. 6). While there was a clear development of the metamorphic boundary between the prodissoconch and dissoconch shell parts for larvae grown in 380 µatm pCO₂, this feature was not apparent at high and lower levels (Fig. 6). At the highest CO₂ level in this experiment, larval shells were the smallest and thinnest and often displayed minor shell fractures (Fig. 6). Cross-sectional magnification of the *C. virginica* hinge indicated that this structure was the largest and most resembled a tongue-and-groove structure at 380 µatm pCO₂ but appeared smaller, degraded and more disconnected at higher CO₂ levels (Fig. 6).

Discussion

This study demonstrated that, like many calcifying ocean animals, early life-history stages of the Eastern oysters, *C. virginica*, are negatively affected by high levels of pCO₂. Multiple aspects of oyster physiology were strongly altered by pCO₂, including their size, metamorphosis, lipid content, calcification rate, RNA:DNA ratios and morphology. Scaling the oyster performance to their size and calcification rates revealed that some physiological parameters may have been compromised directly by elevated pCO₂, while others may have been controlled by slower growth or calcification rates. Unlike some other larval bivalves from North America, such as

M. mercenaria and *A. irradians*, *C. virginica* larvae performed best at the levels of CO₂ found in today's oceans, not lower levels (Talmage and Gobler, 2010). As discussed below, these differences may be caused by evolutionary differences among these species as well as extant physiologically driven differences in biogeography. Regardless of the causes, these findings have important implications for understanding the current and future fate of these bivalves in coastal ecosystems.

During this study, we carefully assessed the physiological condition of *C. virginica* larvae exposed to four levels of pCO₂ by measuring metamorphosis, size, shell thickness, lipid content, RNA:DNA ratios and calcification rates, as well as the physical structure of the larval hinge. High levels of pCO₂ (750 and 1500 µatm) were found to have a consistent, cascading negative impact on early life-stage oysters during this study. Individuals experiencing higher (750 or 1500 µatm) levels of pCO₂ were smaller, slower to metamorphose and less calcified and had thinner shells in comparison to those at lower pCO₂ levels. In an ecosystem setting, these smaller individuals would be more vulnerable to predation (Carriker, 1996; Tamburri and Zimmer-Faust, 1996; Marshall *et al.*, 2003). Oyster larvae grown at higher pCO₂ (750 or 1500 µatm) also accumulated fewer lipids compared with individuals grown at lower pCO₂ levels. Lipids are a key energy store in larvae that are used for growth during early larval stages and are accumulated in later larval stages as an energy store that is used by early juvenile stages (Waldbusser *et al.*, 2013). *Crassostrea virginica* larval growth and survival

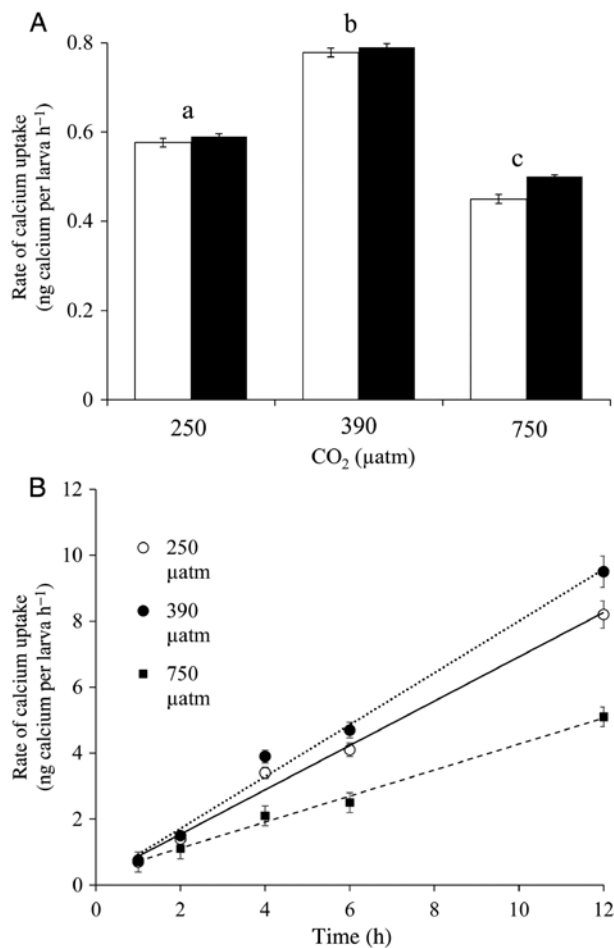


Figure 3: Calcification (calcium uptake) rates of *C. virginica* larvae grown under three levels of pCO₂, ~250, 380 and 750 µatm. **(A)** Calcification rates of day 7 veligers (filled bars) and day 16 pediveligers (open bars) during 24 h, single point measurements. **(B)** Time series measurements during a 12 h incubation with day 7 veligers with best-fit linear regressions at each CO₂ level shown. Error bars represent standard deviation of replicated vessels per treatment ($n = 4$ per treatment). Equations and correlation coefficients of lines were as follows: 250 µatm, $y = 0.67x + 0.20$, $r^2 = 0.99$; 380 µatm, $y = 0.78x + 0.14$, $r^2 = 0.99$; and 750 µatm, $y = 0.39x + 0.33$; $r^2 = 0.99$. Lower case letters in **(A)** signify treatments that are statistically equal (same letter) or different (different letters) as revealed via a Tukey test ($P < 0.05$).

rates have been shown to parallel lipid levels (Gallager and Mann, 1986). Moreover, larvae with lower lipid contents are more likely to perish as settled juveniles (Wikfors *et al.*, 1992; Phillips, 2002; Wacker and von Elert, 2002). Given that the lipid content, size, thickness and rates of metamorphosis were all lower in individuals grown at higher CO₂ levels, their post-metamorphosis survival rates in an ecosystem setting would probably be even lower than those found during this study. Finally, while the rates of metamorphosis of individuals reared at 380 µatm were consistent with our prior studies at this pCO₂ level (Talmage and Gobler, 2009), the slower rates of metamorphosis at high pCO₂ levels would leave larvae

vulnerable to predation for a longer period of time in the plankton (André and Rosenberg, 1991).

Scanning electron micrographs of oysters reared at higher levels of pCO₂ displayed a series of attributes associated with compromised calcification, including declines in the size, integrity and connectedness of their hinge, a structure that facilitates opening and closing of shells, allowing for intake of food and the excretion of waste (Eble, 2001). These compromised hinges have been observed in other bivalves exposed to high pCO₂ (Talmage and Gobler, 2010) and may hinder the ability of individuals to obtain and process suspended particles for nutrition and thus contribute to declines in other physiological parameters, including growth and lipid content. Larvae stages of bivalves rely almost exclusively on external food sources for growth as they develop and metamorphose (Waldbusser *et al.*, 2013). Individuals grown at the highest pCO₂ levels also displayed extremely thin and, in many cases, fractured shells that could leave individuals more vulnerable to predation (Gaylord *et al.*, 2011). Interestingly, at the lowest pCO₂ levels, the hinge and hinge teeth were more fused than at 380 µatm CO₂, a potential indication of reduced functionality at this pCO₂ level that may partly account for compromised performance with this treatment. While there was a clear development of the metamorphic boundary between the prodissoconch and dissoconch shell parts for larvae grown in 380 µatm CO₂, this feature was not apparent at high and lower levels, a difference probably associated with the slower growth and delayed development of individuals exposed to higher and lower pCO₂ levels.

At high and low pCO₂ concentrations, oyster larvae displayed significant declines in calcium uptake and, presumably, rates of net calcification, an observation consistent with the thinner shells displayed by these individuals. Reductions in calcification are often associated with compromised shell integrity and morphology and lower growth rates of bivalves (Green *et al.*, 2009; Waldbusser *et al.*, 2010), symptoms also seen in individuals exposed to higher pCO₂ during this study. Calcification is a significant metabolic cost for marine organisms (Palmer, 1992). If the shells of bivalve larvae are more difficult to synthesize with increasing CO₂ levels, this would represent an additional physiological stress that may leave less energy available for maintenance and growth. For example, juvenile oysters (*C. virginica*) exposed to elevated CO₂ display higher metabolic rates, indicating a higher energy demand for homeostasis in these conditions (Beniash *et al.*, 2010). A similar occurrence in larvae would result in less energy being available for growth and development and, thus, could contribute to smaller sizes and other negative impacts, such as reduced lipid stores, observed during this study.

The hypothesis that reduced calcification rates have ‘trickle-down’ effects on larval physiology and performance was further supported by measurements of RNA:DNA ratios. RNA transcribes the genetic material stored in DNA and is subsequently translated by ribosomes into proteins. Hence, high levels of RNA compared with DNA (ratios >1)

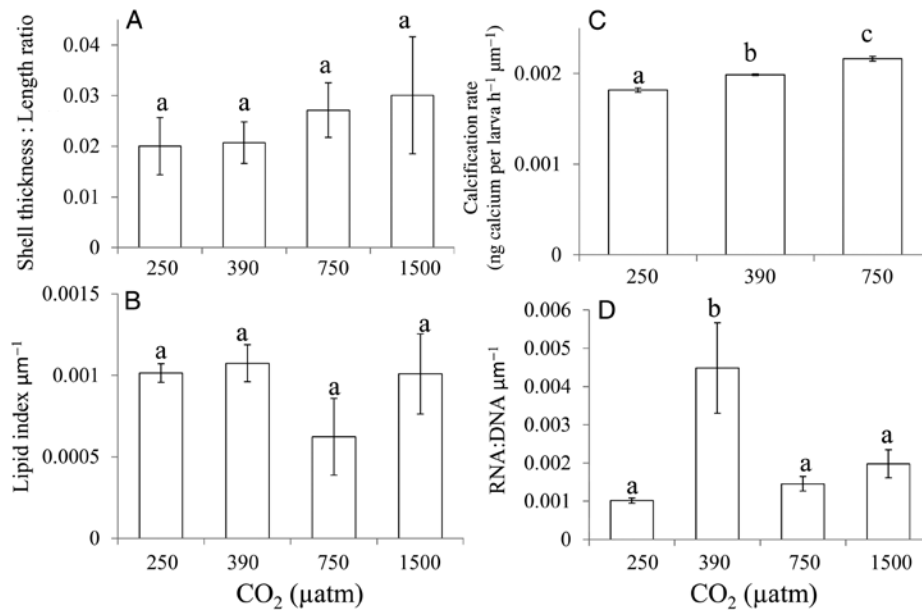


Figure 4: Shell length-corrected thickness, lipid index, calcification rate and RNA:DNA ratio of *C. virginica* larvae grown in pCO₂ concentrations of ~250, 380, 750 and 1500 μatm. (A) Shell thickness to shell length ratio (B) Lipid index per micrometre of shell length. (C) Calcification rate per micrometre of shell length. (D) RNA:DNA ratio per micrometre of shell length. Error bars represent standard deviation of replicated vessels per treatment (*n* = 4 per treatment). Lower case letters signify treatments that are statistically equal (same letter) or different (different letters) as revealed via a Tukey test (*P* < 0.05).

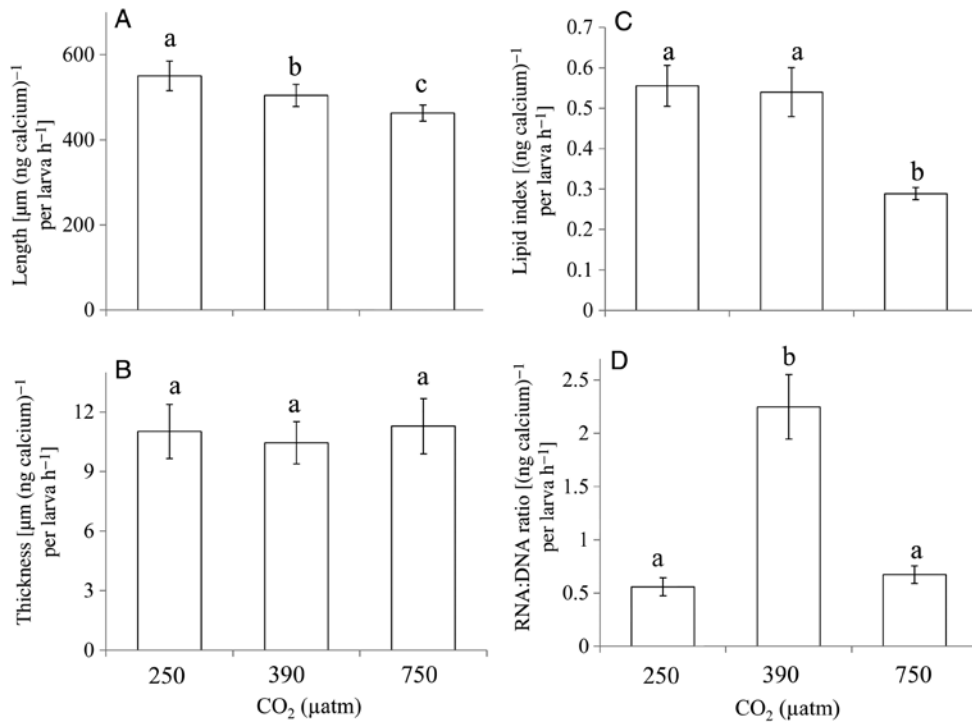


Figure 5: Calcification rate-corrected shell length, shell thickness, lipid index and RNA:DNA ratio of *C. virginica* larvae grown in pCO₂ concentrations of ~250, 380, 750 and 1500 μatm. (A) Micrometres of shell length per nanogram of calcium per larva per hour. (B) μm of shell thickness per nanogram of calcium per larva per hour. (C) Lipid index per nanogram of calcium per larva per hour. (D) RNA:DNA ratio per nanogram of calcium per larva per hour. Error bars represent standard deviation of replicated vessels per treatment (*n* = 4 per treatment). Lower case letters signify treatments that are statistically equal (same letter) or different (different letters) as revealed via a Tukey test (*P* < 0.05).

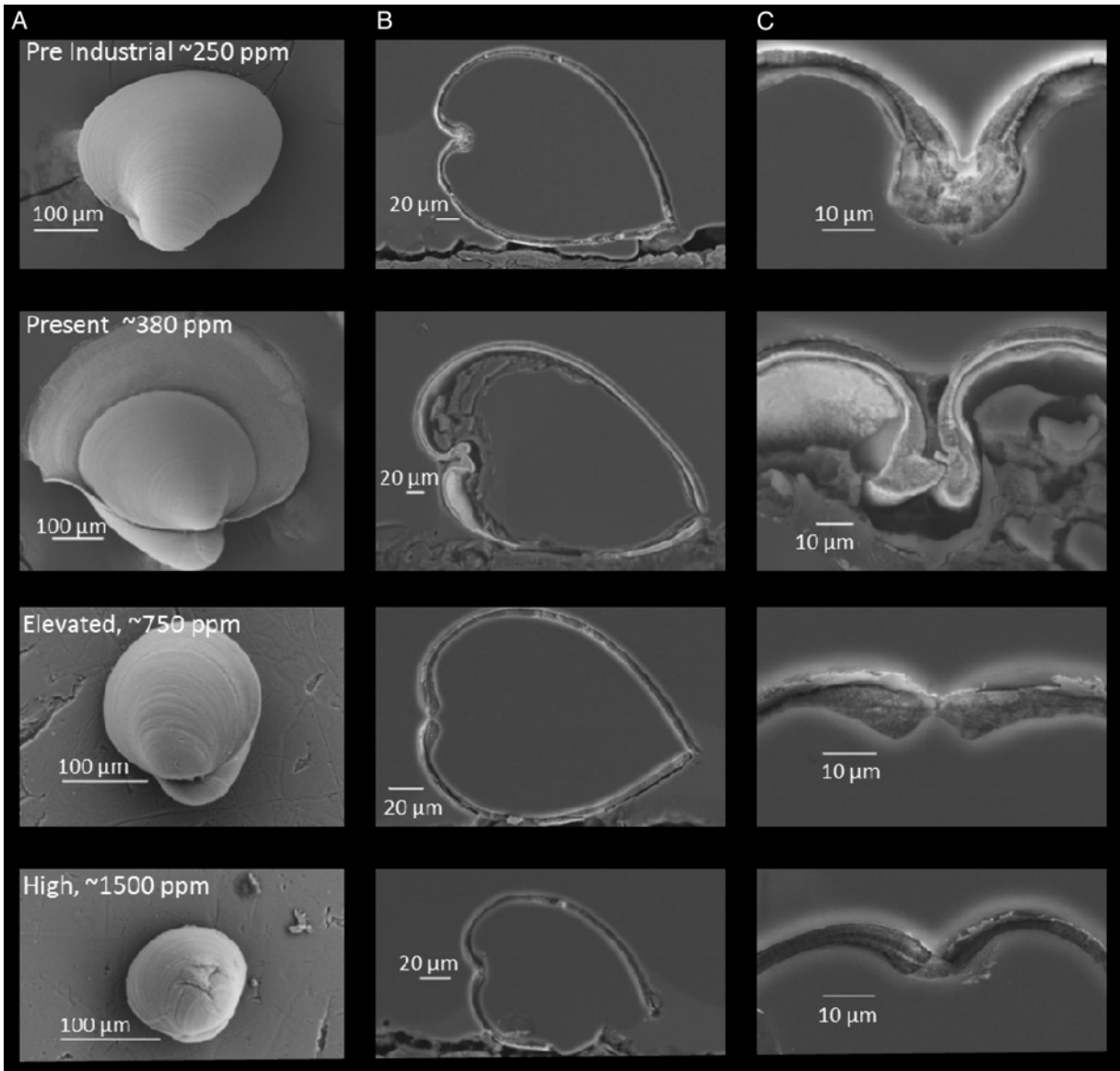


Figure 6: Scanning electron microscopic images of 21-day-old *Crassostrea virginica* grown in the presence of different levels of $p\text{CO}_2$, i.e. ~250, 380, 750 and 1500 μatm (Table 1). (A) Images of individual larvae grown at each $p\text{CO}_2$ level. (B) Hinge-to-valve cross-sections of individuals grown at each CO_2 level. (C) The hinge of individuals grown at each CO_2 level. Note that shells of individuals at 380 μatm $p\text{CO}_2$ display both the prodissoconch and dissoconch shell growth.

are indicative of an organism in an active state of transcribing RNA, synthesizing proteins and growth, while lower values are indicative of physiological impairment (Chícharo *et al.*, 1998; Malzahn *et al.*, 2003; Gobler and Talmage, 2013). While oyster larvae grown at 380 μatm had RNA:DNA ratios exceeding 1.5, those grown at higher $p\text{CO}_2$ levels had values <0.5 . In high concentrations of CO_2 , individuals are likely to have invested more energy in overcoming a kinetically less favourable environment for calcification. With more physiological investment in calcification, other processes, such as lipid accumulation and growth

rates, were reduced, a trend also reflected in the ratios of RNA:DNA of individuals, which were also reduced at high $p\text{CO}_2$, indicating that rates of transcription and growth were compromised.

Given the strong effects of $p\text{CO}_2$ on calcification and, in turn, on growth of early life-stage oysters, we attempted to resolve the interacting effects of $p\text{CO}_2$, calcification and size by correcting several physiological measures for size and calcification rates. This approach provided evidence of the direct effects of calcification on some attributes of oysters,

the size-dependent effect on other attributes, and the overarching effect of pCO₂ on other aspects of physiology. For example, both shell thickness and lipid content scaled with shell length, demonstrating the allometric, size-dependent nature of these attributes; pCO₂ slowed the growth of individuals and, in turn, the smaller individuals were thinner and accumulated fewer lipids because they were less developed (Waldbusser *et al.*, 2013). Neither length nor calcification rate, however, could account for the overwhelmingly elevated RNA:DNA ratios in larvae grown at ambient pCO₂, suggesting that these ratios are indicative of a more universally positive physiological state of early life-stage bivalves grown in the presence of ideal levels of pCO₂ (Gobler and Talmage, 2013).

Calcification rates seemed strongly to influence some, but not all, aspects of larval oyster performance. For example, while shell thickness corrected for calcification rate did not differ among pCO₂ levels, calcification-corrected lengths were a direct function of pCO₂ levels, suggesting that in the most favourable conditions for calcification (lowest pCO₂ level) the largest amounts of calcium carbonate were likely to accrete on the outer shell of larvae. The significantly lower lipid content of individuals grown at higher pCO₂, even after calcification rates were accounted for, further affirmed that these individuals were physiologically challenged and thus unable to devote energy needed for the formation and storage of lipids compared with individuals growing in the presence of lower pCO₂ levels (Waldbusser *et al.*, 2013).

During this study, most physiological parameters paralleled trends observed in survival, being maximal for individuals grown at ~380 µatm, a trend not observed by Miller *et al.* (2009), who found no significant difference in the calcium content and shell size of individuals grown at 280 and 380 µatm pCO₂. The experiments of Miller *et al.* (2009), however, began with 4-day-old larvae, while the present study exposed 18-h-old larvae to CO₂ treatments through metamorphosis. It is during the first 48 h of oyster larval existence that calcium carbonate shells of oyster larvae are deposited for the first time (Waldbusser *et al.*, 2013), and exposure to different levels of pCO₂ at this time can have strong effects that may be manifested days or weeks later (Barton *et al.*, 2012; Gobler and Talmage, 2013; Waldbusser *et al.*, 2013). As such, exposing 4-day-old bivalve larvae to different levels of pCO₂ (Miller *et al.*, 2009) may mute the impacts compared with exposure during the first 2 days of life.

Ocean acidification research to date has repeatedly demonstrated that there exists substantial variability in the vulnerabilities of ocean animals to high CO₂ (Doney *et al.*, 2009; Ries *et al.*, 2009), and bivalve larvae are not an exception (Gazeau *et al.*, 2013). A comparison of the results presented here with prior studies that have used identical methods to those employed here (Talmage and Gobler, 2009, 2010, 2011, 2012) indicate that Eastern oyster larvae are substantially less sensitive to high CO₂ than other US east coast bivalves, such as hard clams (*M. mercenaria*) and bay scallops (*A. irradians*).

For example, in the presence of moderate pCO₂ concentrations (650 µatm), *M. mercenaria* and *A. irradians* larvae exhibited large declines (>50%) in survivorship, while *C. virginica* did not (Talmage and Gobler, 2009). Moreover, while *C. virginica* can experience depressed survival at higher levels of pCO₂ (>1500 µatm), the relative decline in their survival is smaller than that of *M. mercenaria* and *A. irradians* (Talmage and Gobler, 2009, 2010, present study). Finally, while *M. mercenaria* and *A. irradians* experience maximal survival at low pCO₂ concentrations (250 µatm; Talmage and Gobler, 2010), the present study has shown that the growth and survival of *C. virginica* is maximal at slightly higher pCO₂ levels (380 µatm). Collectively, these findings demonstrate that *C. virginica* is less sensitive to acidification than some other North Atlantic bivalves and, in fact, performs best at current levels of pCO₂ (380 µatm).

Estuaries are the principal habitat for many bivalves and are dynamic ecosystems where organisms may be exposed to a wide range of temperature, dissolved oxygen, pH and, of course, salinity. In many estuaries, and particularly in the eastern USA where *C. virginica* is abundant, freshwater tributaries are acidic and, thus, the low-salinity regions of estuaries that naturally have a lower buffering because of low alkalinity also harbour low pH and low calcium carbonate saturation states (Frankignoulle *et al.*, 1996; Salisbury *et al.*, 2008; Miller *et al.*, 2009; Hu and Cai, 2013). Bivalves are differentially suited to subsisting at low salinities, with *C. virginica* being tolerant of, and found within, lower salinities (down to 5; Kennedy *et al.*, 1996) compared with other North American bivalves, such as *M. mercenaria* (down to 18; Kraeuter and Castagna, 2001) and *A. irradians* (down to 20; Shumway and Parsons, 2006). As such, the greater resistance of *C. virginica* to acidification compared with *M. mercenaria* and *A. irradians* may partly be related to its adaptation to lower salinities and, thus, lower pH and calcium carbonate saturation states. This may also partly explain why *M. mercenaria* and *A. irradians* benefit from low CO₂ levels (250 µatm; Talmage and Gobler, 2010) and *C. virginica* does not. The adaptation of this species to waters of lower salinity and pH makes it less likely that it would experience, and thus be adapted to, low CO₂ levels in an ecosystem setting. Lower-salinity regions of estuaries are also likely to be associated with greater fluctuations in pH, which may foster a greater inherent genetic variability with respect to pH tolerance in these oyster populations (Curole *et al.*, 2010; Meyer and Manahan, 2010) and, thus, may also account for the greater resistance to low pH in *C. virginica*.

Molluscs first appeared during the Cambrian period ~500 million years ago, while prehistoric oysters are likely to have evolved during the Mesozoic era (Haglund, 1998). *Crassostrea virginica* emerged as a species ~80 million years ago (Ren *et al.*, 2010), during a period of higher levels of CO₂ compared with the present day (Falkowski and Raven, 1997; Royer, 2006). In contrast, *M. mercenaria* and *A. irradians* have fossil records that began in the mid- to late Cenozoic (Jablonski *et al.*, 2003), which were periods of lower CO₂ levels compared with the present day and compared with

when *C. virginica* evolved (Pearson and Palmer, 2000; Pagani *et al.*, 2005; Royer, 2006; Ren *et al.*, 2010). Hence, differences in the evolution of these species may partly account for the differences in the vulnerabilities of these species to elevated CO₂ levels. Specifically, species that evolved during periods of higher CO₂ (i.e. *C. virginica*) may be more tolerant of higher CO₂ levels than species that evolved during eras of lower CO₂ levels (i.e. *M. mercenaria* and *A. irradians*) that seem to perform best at the levels of CO₂ that were found prior to the industrial revolution. Still, open questions regarding these species, including the evolution of modern strains, the evolution of their calcification pathways and their ability to adapt to rapidly rising levels of CO₂, remain unanswered.

Yet another reason why oyster larvae might be more tolerant to acidification could be their general population structure. Oyster reefs typically host hundreds to thousands of oysters per square metre (Kennedy *et al.*, 1996), and the microclimate around the oyster reef would be expected to be high in pCO₂ and low in pH due to metabolic processes. In contrast, other bivalves more sensitive to acidification, such as hard clams and bay scallops, do not approach these densities in the wild. Another bivalve that also exists in high-density beds, the blue mussel (*Mytilus edulis*), has also shown tolerance to acidification (Gazeau *et al.*, 2010; Thomsen *et al.*, 2010). As such, the adaptation to living in dense reef communities may also contribute towards the Eastern oyster's tolerance of ocean acidification.

Regarding the optimal performance of *C. virginica* larvae in the presence of modern levels of pCO₂, it has been postulated previously that the tolerance limits and optimal conditions of temperature and salinity for development of larvae are determined by the conditions in which gonadal development occurred prior to spawning (Davis, 1958; Newkirk *et al.*, 1977). As such, the short-term history of the parent stock could have an effect on the tolerances of the larvae. The conditioning of broodstock used during the present study was in 'normal' salinities and pH values, which may, therefore, have contributed to the preference of their larvae for 380 µatm pCO₂ rather than higher or lower levels. This hypothesis should be explored in future studies.

Be it due to its evolutionary history, its biogeography, gametic conditioning, a combination of these factors or factors not considered here, *C. virginica* larvae are more tolerant of moderate pCO₂ levels than some other bivalves. As such, this bivalve may be better suited for aquaculture and/or restoration purposes within eutrophic, acidified estuaries than bivalves common to US east coast estuaries that are more sensitive to acidification, such as *M. mercenaria* and *A. irradians*. This will also be the case in the future as atmospheric pCO₂ levels continue to rise. Importantly, however, given that *C. virginica* can be highly susceptible to diseases and the larvae require hard substrates (e.g. oyster shell) to settle on, oyster restoration can be a challenge (Mann and Powell, 2007). Moreover, as an estuarine species *C. virginica* faces additional stressors, such as elevated temperatures and

sub-optimal food, both of which have been shown additively to reduce the survival rates of bivalve larvae, including those of *C. virginica* (Talmage and Gobler, 2011, 2012). Hypoxia may also combine with acidification to threaten these bivalves (Gobler *et al.*, 2014). Lastly, all estuarine bivalves will cope with multiple acidification threats in the coming decades, namely rising levels of atmospheric CO₂ (i.e. ocean acidification) coupled with coastal ocean acidification driven by anthropogenically enhanced organic carbon loading (Borges and Gypens, 2010; Cai *et al.*, 2011). These processes have probably contributed to the recent acidification of some estuaries (Waldbusser *et al.*, 2011) and the occurrence of pCO₂ levels in some estuaries that are not predicted to occur in the open ocean until the end of this century but that yield lower survival rates of larvae (e.g. ≥1000 µatm; Talmage and Gobler, 2009; Cai *et al.*, 2011). As such, it seems plausible that acidification in estuaries may be contributing, at least in part, to the 'functional extinction' that has been observed for many oyster populations around the globe during the past century (Beck *et al.*, 2011).

Acknowledgements

We are grateful for our supply of *C. virginica* larvae obtained from the East Hampton Shellfish Hatchery, East Hampton, NY. We thank Jim Quinn for scanning electron microscope assistance and James Waldvogel for cross-sectioning assistance during this project. We also thank Gordon Taylor and his laboratory for the use of his liquid scintillation counter. This research was supported by the National Science Foundation (NSF # 1129622), NOAA's Ocean Acidification Program through award #NA12NOS4780148 from the National Centers for Coastal Ocean Science, the Laurie Landeau Foundation, and the Chicago Community Trust.

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