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Effects of CO_2 and the harmful alga *Aureococcus anophagefferens* on growth and survival of oyster and scallop larvae

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ABSTRACT: Globally, the frequency of harmful algal blooms is increasing and CO_2 concentrations are rising. These factors represent serious challenges to a multitude of estuarine organisms as well as to efforts to restore depleted stocks of filter-feeding bivalves. In this study, we compared the responses of larval bivalves *Crassostrea virginica* and *Argopecten irradians* to the brown tide alga *Aureococcus anophagefferens* (250×10^6 cells l^{-1} and 1×10^9 cells l^{-1}, respectively) and a gradient of CO_2 concentrations (~240, ~390, and ~850 ppm). Results indicated that *A. anophagefferens* and higher levels of CO_2 significantly depressed rates of survival, development, growth, and lipid synthesis of *A. irradians* larvae with the combination of both factors having the largest effects. *C. virginica* larvae were also negatively impacted by the harmful alga and elevated CO_2, but displayed a higher overall survival rate when exposed to these combined stressors. For both species, high densities of *A. anophagefferens* (10^9 cells l^{-1}) elicited a stronger negative effect on larval survival than high levels of CO_2 concentrations (~850 ppm). Collectively, these results demonstrate that the concurrent occurrence of harmful algal blooms and high CO_2 concentrations will have negative consequences for bivalve populations and further demonstrate that some species of larval bivalves are more resistant to these stressors than others.

KEY WORDS: Bivalves · Carbon dioxide · Harmful algal bloom · Brown tide · Ocean acidification · Larvae · Shellfish · *Crassostrea virginica* · *Argopecten irradians*

INTRODUCTION

Bivalves are keystone organisms within estuarine ecosystems. The ecosystem services provided by bivalve filtration in marine habitats include control of natural and cultural eutrophication (Officer et al. 1982, Cerco & Noel 2007) as well as reduction of turbidity (Newell & Koch 2004). With increased water clarity caused by bivalve filtration, growth of submerged aquatic vegetation may be facilitated (Carroll et al. 2008, Wall et al. 2008). Beyond filtration, many bivalves also provide physical habitat structure (Jackson 2001). For all of these reasons, estuarine bivalves are often considered ecosystem engineers (Newell 2004). Bivalve populations throughout the world have declined significantly during the past century due loss of habitat, hypoxia, eutrophication, and overfishing (Jackson et al. 2001, Lotze et al. 2006, Beck et al. 2011). Two additional pressures on bivalve populations that have received relatively lesser attention but have progressively intensified in recent decades are harmful algal blooms (HABs) and coastal ocean acidification.

HABs have become increasingly common in recent decades (Hallegraeff 1993, Anderson et al. 2008, Heisler et al. 2008) and have had negative ecological and economical consequences on many marine ecosystems (Hoagland et al. 2002, Sunda et al. 2006, Jin et al. 2008). Many HABs can harm bivalve populations (Shumway 1990, Bricelj & Shumway 1998, Landsberg 2002). HABs can be particularly detrimental to bivalve larvae, and the success of larval

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bivalves can play an important role in sustaining adult populations (Caley et al. 1996, Gosselin & Qian 1997, Schneider et al. 2003, Arnold 2008). HAB-forming dinoflagellates have been shown to cause mortality in multiple species of bivalve larvae, including eastern oysters *Crassostrea virginica* (Springer et al. 2002, Leverone et al. 2006, Stoecker et al. 2008, Tang & Gobler 2009, 2012), northern quahogs *Mercenaria mercenaria* (Leverone et al. 2006, Tang & Gobler 2009, 2012), bay scallops *Argopecten irradians irradians* and *A. i. concentricus* (Springer et al. 2002, Yan et al. 2003, Leverone et al. 2006, Tang & Gobler 2009), and Japanese scallops *Chlamys farreri* (Yan et al. 2001). In addition, the brown tide-forming pelagophyte *Aureococcus anophagefferens* has been shown to reduce survival, growth, and lipid content of northern quahog larvae (Padilla et al. 2006, Bricelj & MacQuarrie 2007) and can slow the growth of larval bay scallops (Gallager et al. 1989).

Elevated concentrations of carbon dioxide (CO_2) are also a significant threat to coastal bivalve populations. CO_2 levels have risen by 40% since the industrial revolution (Caldeira & Wickett 2003), and the rate of increase has tripled since the mid-20th century (Fussel 2009, Friedlingstein et al. 2010). These increases may be even larger in coastal zones (Miller et al. 2009, Cai et al. 2011), which receive additional CO_2 inputs from upwelling (Feely et al. 2008), freshwater (Salisbury et al. 2008), and anthropogenic and terrestrial carbon sources (Gattuso et al. 1998, Paerl et al. 1998, Thomas et al. 2004, Koch & Gobler 2009). When the rise in CO_2 levels is not matched by the buffering capacity of terrestrial and/or marine minerals, levels of pH and carbonate ion concentration are depressed and 'ocean acidification' occurs (Cao et al. 2007). Ocean acidification can negatively affect a host of marine organisms and can be particularly harmful to animals with external, calcified shells such as bivalves (Doney et al. 2009). Marine bivalve larvae are particularly vulnerable to high CO_2 levels, displaying reduced rates of calcification, growth, and survival under levels of CO_2 found today (Talmage & Gobler 2010, Barton et al. 2012) or projected for later this century (Gazeau et al. 2007, Kurihara et al. 2007, Miller et al. 2009, Talmage & Gobler 2009, Gazeau et al. 2011). No study to date has investigated the concurrent effects of harmful algae and CO_2 levels on the growth and survival of bivalve larvae.

The goal of this research was to investigate the combined effects of the harmful alga *Aureococcus anophagefferens* (brown tide), and past (~240 ppm), present (~390 ppm), and future (~850 ppm) CO_2 levels on larvae of the bay scallop *Argopecten irradians* and eastern oyster *Crassostrea virginica*, a species whose vulnerability to *A. anophagefferens* has never been investigated. Larvae were exposed to bloom and moderate levels of *A. anophagefferens* or to a control alga and the survival and development of larvae through metamorphosis was monitored along with larval size and lipid content. Results provided important information regarding the vulnerability of these organisms to these stressors, as well as insight regarding prospects for future success of these populations.

MATERIALS AND METHODS

We examined the effects of multiple CO_2 and *Aureococcus anophagefferens* levels on the larval stages of 2 bivalves, *Argopecten irradians* and *Crassostrea virginica*. For all experiments, experimental vessels with bivalves (described below) were maintained in water baths set at 24°C using commercially available aquarium heaters (Aquatic Eco-systems). Temperatures were recorded every 6 min throughout experiments using *in situ* loggers (Onset), which demonstrated that temperatures varied within 2.5% of target values. The experimental temperature (24°C) is optimal for growth and survival of larvae of these 2 species (Kennedy 1996, Kraeuter & Castagna 2001, Cragg 2006). A gas proportionator system (Cole Parmer Flowmeter system, multitube frame) was used to deliver CO_2 gas to seawater within treatment vessels at multiple rates. The gas proportionator mixed appropriate flow rates of 5% CO_2 gas, low CO_2 gas, and pressurized air (~390 ppm CO_2) and distributed the gas equally and gently to all vessels to yield the desired CO_2 concentrations. Gas was supplied at a volume equal to >100× the volume of the experimental chamber per day. Plexiglass covers over the chambers sealed the vessels, ensuring that salinities did not change during experiments. We previously found that experiments performed with gases mixed via a proportionator as described here generate seawater chemistry and larval responses nearly identical to those obtained from tanked gases premixed at specific CO_2 levels (Talmage & Gobler 2010). For the experiments, the CO_2 gas mixtures from the proportionator system were continuously delivered to the bottom of replicated (n = 4) experimental vessels (detailed below). With continuous bubbling, all treatment vessels remained saturated with respect to oxygen (~8 mg l^{-1}).

To quantify precise CO_2 levels attained in experimental treatments at the start and end of each experiment, aliquots (~200 ml) were removed from each treatment vessel and analyzed during experiments

using an EGM-4 Environmental Gas Analyzer® system (PP Systems) 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 ±3.6% for replicated measurements of total dissolved inorganic carbon (DIC) and provided full recovery (102 ± 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 DIC kg^{-1} seawater). Levels of CO_2 were calculated based on measured levels of total inorganic carbon, pH (National Bureau of Standards [NBS]; mol kg^{-1} seawater), 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/). Once-daily measurements of pH made from the middle of each vessel with a high sensitivity microprocessor (Thermo Scientific Orion Star Series™ pH meter; ± 0.001; calibrated prior each use with NIST traceable standards) indicated that the experimental vessels maintained a constant pH level throughout the experiments (<0.5% relative standard deviation [RSD] 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 never significantly different than those obtained with the pH meter.

Larvae were grown at either 2 (for *Crassostrea virginica*) or 3 (for *Argopecten irradians*) levels of CO_2: an elevated level (~850 ppm CO_2), predicted for the year 2100 (Cao et al. 2007), an ambient level (~390 ppm CO_2), and a near pre-industrial level (~240 ppm CO_2) which provides maximal survival and growth rates for *A. irradians* (Talmage & Gobler 2010). Precise CO_2 levels and complete carbonate chemistry from experiments appear in Table 1. One liter high density polyethylene beakers were filled with 0.2 µm-filtered seawater from eastern Shinnecock Bay, NY, USA. Larvae from locally collected broodstock were obtained from Cornell Cooperative Extension, Southold, NY, and the East Hampton Shellfish Hatchery, East Hampton, NY. Within 6 to 12 h of fertilization, larvae were distributed to each treatment beaker at a concentration of ~ 400 l^{-1}, consistent with post-spawning densities in estuaries (Mackenzie 1996, Cragg 2006). Every 3 d, larvae from each beaker (n = 4 per treatment) were gently poured onto a 64 µm mesh and immediately transferred to a petri dish with filtered seawater. The condition (live or dead) and developmental stage (veliger, pediveliger,

Table 1. Mean ± SD of temperature, pH, carbonate chemistry, total alkalinity (TA), and salinity during the 2-level and 3-level CO_2 and *Aureococcus anophagefferens* experiments with *Crassostrea virginica* and *Argopectan irradians* larvae, respectively. Ambient CO_2: ambient, present day CO_2. pCO_2: partial pressure of CO_2. DIC: dissolved inorganic carbon

CO_2 condition	Temp. (°C)	pH	pCO_2 (ppm)	$\Omega_{calcite}$	$\Omega_{aragonite}$	Total DIC (µmol l^{-1})	CO_3^{2-} (µmol l^{-1})	TA	Salinity
With *Crassostrea virginica*									
Low density brown tide									
Ambient	24 ± 0.45	8.100 ± 0.010	381 ± 14.33	2.97 ± 0.03	1.92 ± 0.02	1466 ± 19.62	117.4 ± 1.18	1632.9 ± 17.37	28.0 ± 1.0
Elevated	24 ± 0.45	7.880 ± 0.010	833.4 ± 10.95	2.36 ± 0.07	1.52 ± 0.06	1880 ± 20.18	93.27 ± 3.10	1987.2 ± 24.35	28.0 ± 1.0
High density brown tide									
Ambient	24 ± 0.45	8.090 ± 0.010	398.2 ± 8.97	2.97 ± 0.08	1.91 ± 0.05	1493 ± 4.07	117.2 ± 2.81	1658.9 ± 7.96	28.0 ± 1.0
Elevated	24 ± 0.45	7.873 ± 0.006	863.5 ± 20.76	2.37 ± 0.06	1.53 ± 0.03	1916 ± 37.0	93.7 ± 2.17	2023.4 ± 38.74	28.0 ± 1.0
With *Argopectan irradians*									
Low density brown tide									
Near pre-industrial	24 ± 0.6	8.208 ± 0.015	233.18 ± 14.03	2.99 ± 0.04	1.92 ± 0.06	1171 ± 37.96	117.75 ± 5.24	1357 ± 38.74	28.0 ± 1.0
Ambient	24 ± 0.6	8.080 ± 0.001	368.3 ± 5.327	2.63 ± 0.04	1.69 ± 0.02	1348 ± 19.48	103.48 ± 1.49	1499 ± 20.80	28.0 ± 1.0
Elevated	24 ± 0.6	7.878 ± 0.005	870.1 ± 10.261	2.44 ± 0.06	1.58 ± 0.04	1951 ± 31.90	96.25 ± 2.46	2060 ± 34.31	28.0 ± 1.0
High density brown tide									
Near pre-industrial	24 ± 0.5	8.200 ± 0.005	230.05 ± 9.47	2.88 ± 0.08	2.47 ± 0.15	1142 ± 38.15	113.63 ± 3.32	1323 ± 40.78	28.0 ± 1.0
Ambient	24 ± 0.5	8.063 ± 0.033	377.13 ± 35.525	2.47 ± 0.15	1.60 ± 0.09	1318 ± 19.29	97.58 ± 5.66	1461 ± 11.15	28.0 ± 1.0
Elevated	24 ± 0.5	7.878 ± 0.010	856.68 ± 26.692	2.40 ± 0.06	1.55 ± 0.03	1920 ± 31.72	94.73 ± 2.19	2028 ± 32.20	28.0 ± 1.0

and metamorphosed) of every individual larva were determined under a dissecting microscope. Individuals were transferred into a new beaker with new filtered seawater, food, and antibiotics (details below) within a 15 min period. Experiments were terminated after all surviving larvae in all treatments had metamorphosed or when ~15 larvae per treatment remained for post-experiment analyses.

Brown tides caused by *Aureococcus anophagefferens* typically occur during the months of May, June, and July (Gobler et al. 2005, Gobler & Sunda 2012), a period co-incident with the spawning period of eastern oysters and bay scallops (Thompson et al. 1996, Cragg 2006). To simulate these post-spawning conditions, larvae of both bivalve species were fed 2 concentrations of brown tide (*A. anophagefferens* clone CCMP1850): (1) 2.5×10^8 cells l^{-1}, representing a low density bloom, and (2) 1.0×10^9 cells l^{-1}, representing a high density bloom (Gobler et al. 2005, Gobler & Sunda 2012). *A. anophagefferens* cultures were grown in GSe medium (Doblin et al. 1999) made with 0.2 µm filtered seawater collected from Shinnecock Inlet, Southampton, NY, during flood tide (salinity ~30) and maintained in an incubator at 21°C on a 14 h light:10 h dark cycle (Gobler et al. 1997). The strain of brown tide used for all experiments was Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP)1850, which was isolated from Great South Bay, NY, in 1998 and has been shown to be highly inhibitory to copepod nauplii (Smith et al. 2008) and adult bivalves (Harke et al. 2011). As a control treatment, 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 & Mann 1994, Cragg 2006, Talmage & Gobler 2009). Depending on the experimental treatment of either low or high levels of *A. anophagefferens*, control treatments were fed the bio-volume equivalent of *I. galbana* (3.125×10^7 and 1.25×10^8 cell l^{-1}, respectively; 1 *I. galbana* cell = ~8 *A. anophagefferens* cells; Harke et al. 2011). Cultures of *I. galbana* were grown as described for *A. anophagefferens*, and both cultures were maintained in exponential phase growth via daily supplementation with media. To assure that food was not a limiting factor, the lower food concentration was added to experimental vessels daily, whereas the higher density was added every third day during water changes. To promote high survivorship, all containers in contact with larvae were new and never exposed to chemicals or detergents (Talmage & Gobler 2009). 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% of its original concentration at the beginning of each experiment and at the time of each water change (~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 & Gobler 2009).

To meet the assumption of normality and heterogeneity, percent survival and metamorphosed data were arcsine square-root transformed, after which 2-way ANOVAs were performed where phytoplankton species and CO_2 were the treatment factors. Sizes of larvae were also statistically examined via 2-way ANOVAs. Post hoc Tukey multiple comparison tests were performed to examine the differences among survival rates, metamorphosis rates, and sizes for each algal food source and CO_2 level. The use of different batches of larvae from different broodstocks for each experiment prohibited statistical comparisons across experiments (different concentrations of *Aureococcus anophagefferens* or the 2 species of larvae). Statistical analyses were performed with SYSTAT 13® (Systat Software).

At the end of the 20 d experiments for *Crassostrea virginica* and *Argopecten irradians*, lipid content was analyzed. To estimate the relative lipid content of larvae, Nile Red stain was used to bind to neutral lipids and fluoresce under an a fluorescein isothiocyanate (FITC) filter on an epifluorescent microscope (Castell & 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 working solution made by a 1:9 dilution of the stock solution in 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 epifluorescent microscope. After exposure to the working dye solution, larvae stopped moving, which allowed them to be orientated, using a pipette tip, in the same plane of view. The total area and lipid accumulation area of each larva was determined from digital images using Image J® software. A lipid index was determined by dividing the area of the larva containing the fluorescing lipids by the total larval area thereby allowing for direct comparisons among treatments. Two-way ANOVAs and post hoc Tukey multiple comparison tests were performed to examine the differences among larval lipid indices, as well as shell length at each phytoplankton and CO_2 level.

RESULTS

Crassostrea virginica larvae exposed to low levels of *Aureococcus anophagefferens*

For *Crassostrea virginica* larvae exposed to the lower biomass level of *Aureococcus anophagefferens*, CO_2 concentrations, and food source both significantly affected metamorphosis ($p < 0.05$, see Table S1 in the supplement at www.int-res.com/articles/suppl/m464p121_supp.pdf), survival ($p < 0.05$, Table S2 in the supplement), and size ($p < 0.01$, Table S3 in the supplement). There was no interaction, between CO_2 concentrations and food type for metamorphosis, survival, or size, indicating that that increased CO_2 and *A. anophagefferens* had a predictable, additive effect on *C. virginica*. Metamorphosis and survival as well as larval size were greatest for *C. virginica* individuals grown under ambient CO_2 concentrations (~390 ppm) and with the control food source (*Isochrysis galbana*; Figs. 1 & 2a). At 20 d post-fertilization, 21 ± 4.9 (SD) and 14 ± 5.4% of larvae had metamorphosed at ~390 and ~850 ppm CO_2, respectively, when fed *I. galbana* compared to only 3.7 ± 0.31 and 2.4 ± 0.32% when fed *A. anophagefferens* and exposed to ~390 and ~850 ppm CO_2 (Fig. 1). After 20 d of development, 26 ± 7.8 and 17 ± 5.6% of *C. virginica* larvae fed *I. galbana* survived at ~390 and ~850 ppm CO_2, respectively, compared to 18 ± 0.32 and 14 ± 0.82% when fed *A. anophagefferens* and grown at ~390 and ~850 ppm CO_2, respectively (Fig. 1). Mean diameters for *C. virginica* larvae fed *I. galbana* were 363.9 ± 33.7 and 252.9 ± 6.7 µm at ~390 and ~850 ppm CO_2, respectively, but decreased to 328.7 ± 31.3 and 204.5 ± 11.5 µm at ~390 and ~850 ppm CO_2 on a diet of *A. anophagefferens* (Fig. 2a). Lipid indices for *C. virginica* larvae were significantly different among CO_2 concentrations and food sources ($p < 0.001$, Table S4 in the supplement) and there was a signifi-

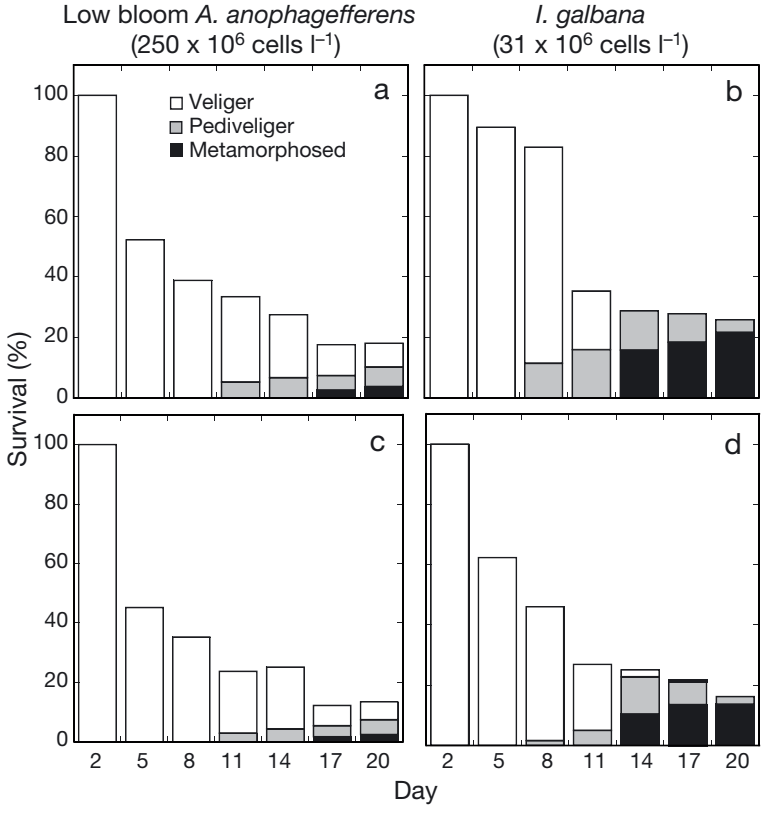

Fig. 1. *Crassostrea virginica.* Survival and development of larvae under 2 levels of CO_2, (a,b) ~390 and (c,d) ~850 ppm (see Table 1), and fed either (a,c) a low bloom density of *Aureococcus anophagefferens* or (b,d) the biovolume equivalent of *Isochrysis galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated (n = 4 per treatment)

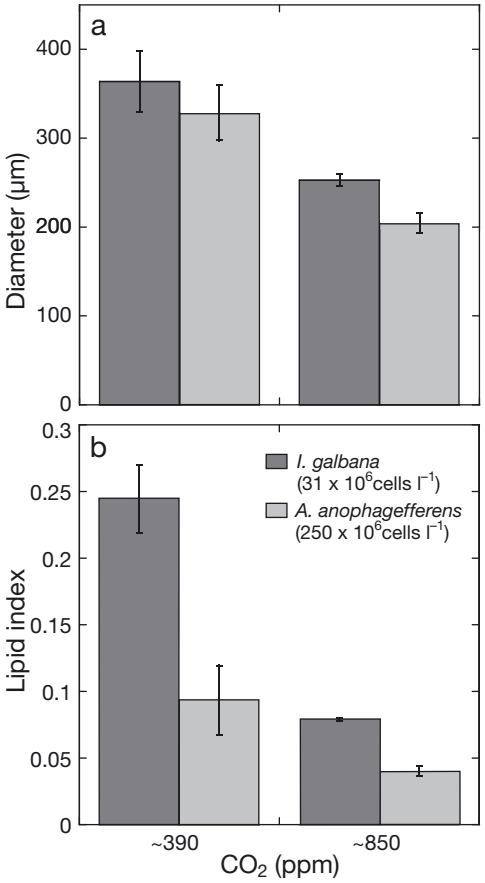

Fig. 2. *Crassostrea virginica.* Mean ± SD (a) diameter and (b) lipid indices of larvae under 2 levels of CO_2, ~390 and ~850 ppm (see Table 1), and fed either a low bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *Isochrysis galbana*

cant interaction between CO_2 concentrations and food source (Fig. 2b; $p < 0.001$). Lipid indices for *C. virginica* larvae decreased from 0.24 ± 0.03 and 0.08 ± 0.001 at ~390 and ~850 ppm CO_2, respectively, when fed *I. galbana* to 0.09 ± 0.03 and 0.04 ± 0.004 at ~390 and ~850 ppm CO_2 when fed *A. anophagefferens*, with that later index being slightly higher than predicted by the individual treatments, suggesting the statistical interaction was an antagonistic effect of increased CO_2 and *A. anophagefferens* on *C. virginica* lipids.

Crassostrea virginica larvae exposed to high levels of *Aureococcus anophagefferens*

With an increase to high concentrations of *Aureococcus anophagefferens* (10^9 cells l^{-1}), *Crassostrea virginica* larval performance worsened compared to the lower density of *A. anophagefferens*. After 20 d of exposure, CO_2 concentrations and food source both had a significant effect on larval metamorphosis ($p < 0.05$, Table S5 in the supplement), survival ($p < 0.01$, Table S6), and lipid content ($p < 0.001$, Table S7 in the supplement; Figs. 3 & 4). There was also a significant interaction between treatments for metamorphosis ($p < 0.05$), survival ($p < 0.01$), and lipids, with the treatments having an antagonistic effect on each of these response variables ($p < 0.001$; Figs. 3 & 4). While 24 ± 3.8 and 15 ± 5.2% of larvae had metamorphosed by Day 20 at ~390 and ~850 ppm CO_2, respectively, when fed *Isochrysis galbana*, almost none of the individuals fed *A. anophagefferens* metamorphosed (<0.2%; Fig. 3). On Day 20, survival of *C. virginica* larvae at ~390 ppm fed *I. galbana* and *A. anophagefferens* was 30 ± 5.6 and 4.6 ± 0.6%, respectively, whereas at ~850 ppm, survival was 19 ± 4.8 and 4.3 ± 0.5%, respectively (Fig. 3). At ~390 and

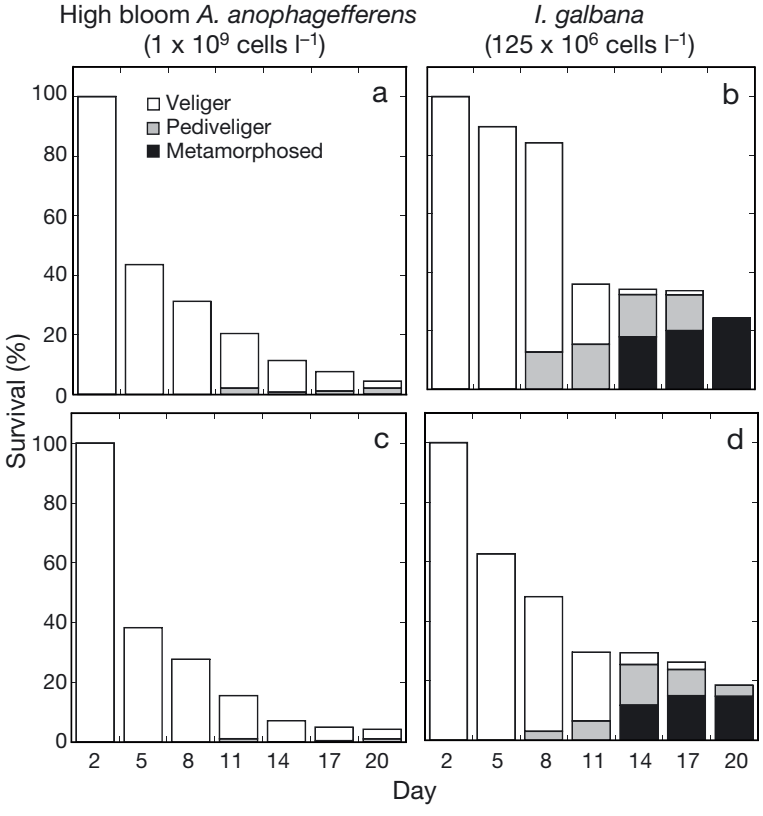

Fig. 3. *Crassostrea virginica.* Survival and development of larvae under 2 levels of CO_2, (a,b) ~390 and (c,d) ~850 ppm (see Table 1), and fed either (a,c) a high bloom density of *Aureococcus anophagefferens* or (b,d) the biovolume equivalent of *Isochrysis galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated (n = 4 per treatment)

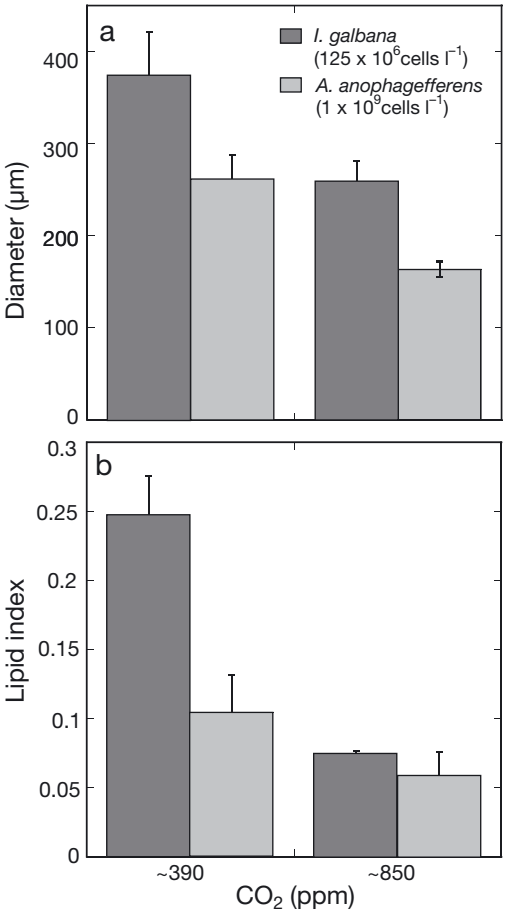

Fig. 4. *Crassostrea virginica.* Mean ± SD (a) diameter and (b) lipid indices of larvae under 2 levels of CO_2, ~390 and ~850 ppm (see Table 1), and fed either a high bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *Isochrysis galbana*

~850 ppm CO_2 with *I. galbana* as a food source, *C. virginica* lipid indices were 0.25 ± 0.03 and 0.07 ± 0.002, respectively, whereas individuals fed *A. anophagefferens* and exposed to the same CO_2 concentrations (~390 and ~850 ppm) had lipid indices of 0.1 ± 0.03 and 0.06 ± 0.02, respectively (Fig. 4b). CO_2 concentrations and *A. anophagefferens* also significantly affected larval diameters ($p < 0.001$, Table S8 in the supplement), although there was no significant interaction between these treatments. Diameters of *C. virginica* larvae fed *I. galbana* were 374.7 ± 46.8 and 259.5 ± 22.2 µm at ~390 and ~850 ppm CO_2, respectively, but decreased to 261.6 ± 25.7 and 163.6 ± 8.3 µm at ~390 and ~850 ppm CO_2 when fed *A. anophagefferens* (Fig. 4a).

Argopecten irradians larvae exposed to low levels of *Aureococcus anophagefferens*

Compared to *Crassostrea virginica*, *Argopecten irradians* larvae were more sensitive to both elevated CO_2 and *A. anophagefferens*. Both CO_2 concentrations and food source were significant treatments and had significant interactive effects on metamorphosis ($p < 0.001$, Table S9 in the supplement), survival ($p < 0.001$, Table S10 in the supplement), diameter ($p < 0.001$, Table S11 in the supplement), and lipid content ($p < 0.001$, Table S12 in the supplement; Fig. 5). The treatments interacted to have a synergistically negative impact on larval survival, size, and metamorphosis, but had an antagonistic effect on lipids. While 84.2 ± 0.24, 70.2 ± 0.69, and 51.5 ± 0.50% of larvae fed *Isochrysis galbana* metamorphosed after 20 d at ~240, ~390, and ~850 ppm CO_2, respectively, these percentages declined precipitously to 3.2 ± 0.24, 0.62 ± 0.14, and 0.50 ± 0.35% when their diet was comprised of *A. anophagefferens* (Fig. 5). Similarly, larval survival rates when fed *I. galbana* and exposed to ~240, ~390, and ~850 ppm CO_2 were 88.0 ± 0.20, 72.9 ± 0.72, and 44.8 ± 15.5%, respectively, but dropped to 14.5 ± 0.54, 7.2 ± 0.35, and 5.5 ± 0.24% when fed *A. anophagefferens* and exposed to ~240, ~390, and ~850 ppm CO_2 (Fig. 5). Diameters of individuals fed *I. galbana* decreased from 492.9 ± 4.7 to 389.3 ± 8.2 and 192.0 ± 3.7 µm with increasing CO_2 concentrations (~240, ~390, and ~850 ppm, respectively), and declined further when fed *A. anophagefferens* to 387.6 ± 4.5, 369.9 ± 18.2, and 126.1 ± 6.4 µm for ~240, ~390, and ~850 ppm, respectively (Fig. 6a). Lipid indices of larvae fed *I. galbana* and exposed to ~240, ~390, and

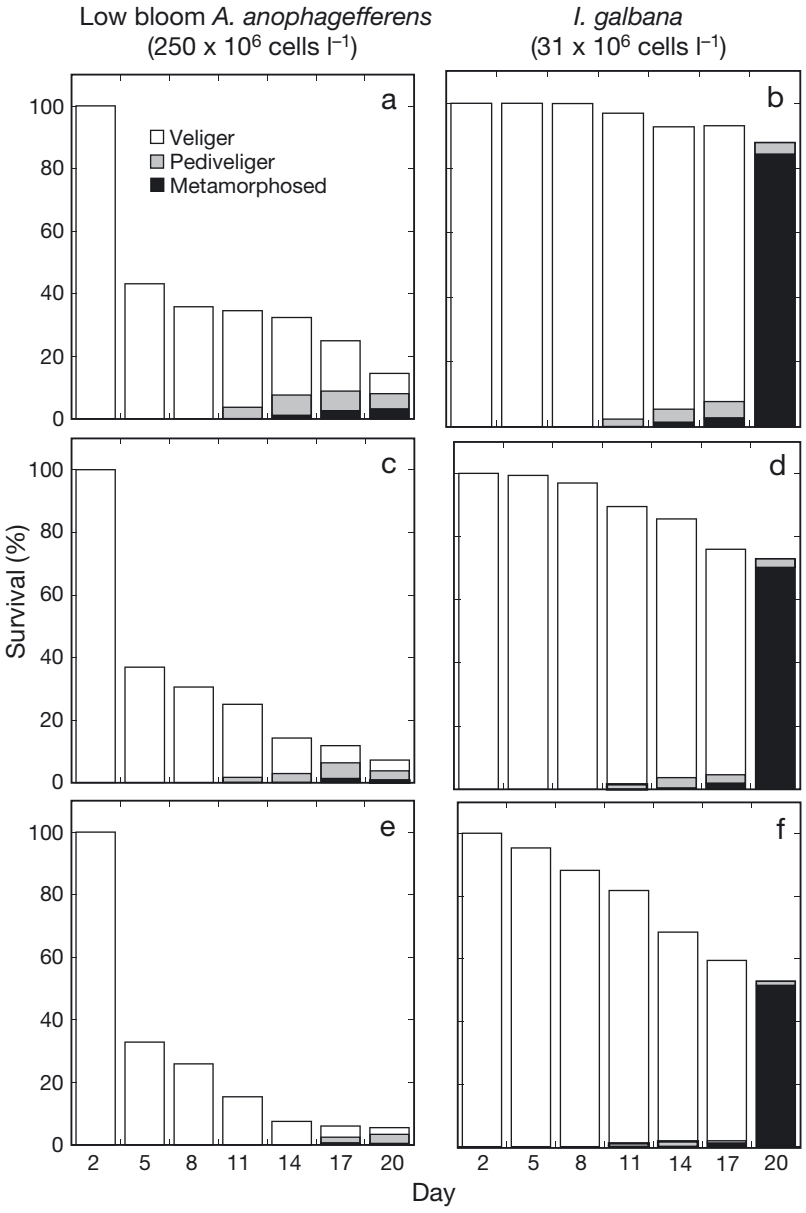

Fig. 5. *Argopecten irradians.* Survival and development of larvae under 3 levels of CO_2, (a,b) ~240, (c,d) ~390, and (e,f) ~850 ppm (see Table 1), and fed either (a,c,e) a low bloom density of *Aureococcus anophagefferens* or (b,d,f) the biovolume equivalent of *Isochrysis galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated (n = 4 per treatment)

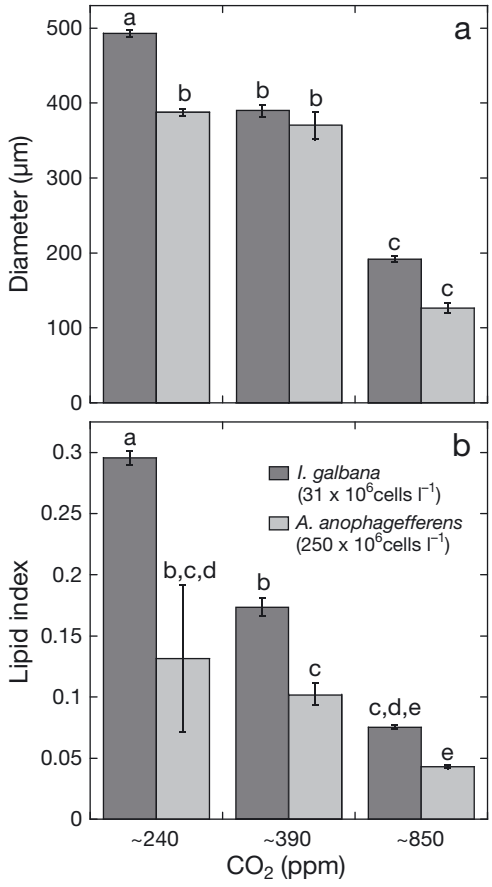

Fig. 6. *Argopecten irradians.* Mean ± SD (a) diameter and (b) lipid indices of larvae under 3 levels of CO_2, ~240, ~390, and ~850 ppm (see Table 1), and fed either a low bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *Isochrysis galbana*. Shared letters indicate treatments that are not significantly different (Tukey's multiple comparisons, $p > 0.05$)

~850 ppm CO_2 were 0.30 ± 0.006, 0.17 ± 0.007, and 0.08 ± 0.001, respectively, but were only 0.13 ± 0.06, 0.10 ± 0.009, 0.04 ± 0.001, respectively, when fed *A. anophagefferens* (Fig. 6b).

Argopecten irradians larvae exposed to high levels of *Aureococcus anophagefferens*

The strongest negative effects on bivalve larvae were observed during exposures of *Argopecten irradians* larvae to high concentrations of *Aureococcus anophagefferens* (10^9 cells l^{-1}) and high CO_2 concentrations. CO_2 concentrations and food source affected larval metamorphosis ($p < 0.001$, Table S13 in the supplement), survival ($p < 0.001$, Table S14 in the supplement), and lipid content ($p < 0.001$, Table S15 in the supplement; Fig. 7). These treatments also interacted to have significant ($p < 0.001$, Tables S13–S15) and a synergistically negative impact on larval survival, lipid content, and metamorphosis. None of the *A. irradians* larvae exposed to the high concentrations of *A. anophagefferens* metamorphosed or survived the 20 d experiment (Fig. 7). Maximal survival times for individuals exposed to high densities of *A. anophagefferens* and ~240, ~390, and ~850 ppm CO_2 were 17, 14, and 11 d, respectively (Fig. 7). On Day 11, larvae fed *Isochrysis galbana* had lipid indices of 0.30 ± 0.01, 0.18 ± 0.007, and 0.07 ± 0.002, respectively, while larvae exposed to high concentrations of *A. anophagefferens* and CO_2 concentrations of ~240, ~390, and ~850 ppm had lipid indices of 0.12 ± 0.004, 0.07 ± 0.008, and 0.03 ± 0.001 (Fig. 8b). Food source and CO_2 levels both significantly affected larval diameters ($p < 0.001$, Table S16 in the supplement), but did not have an interactive effect. When fed *I. galbana* and reared under CO_2 concentrations of ~240, ~390, and ~850 ppm, larval diameters were 494.33 ± 13.1, 404.10 ± 38.9, and 193.4 ± 14.6 µm, whereas individuals fed *A. anophagefferens* were 364.9 ± 9.8, 309.0 ± 10.4, and 93.8 ± 3.1 µm, respectively (Fig. 8a).

DISCUSSION

Many functions of coastal marine ecosystems have been negatively affected during the past century. Although issues such hypoxia and overfishing have received much attention, these waters have also been acidified by several mechanisms (Caldeira & Wickett 2003, Sabine et al. 2004, Fussel 2009) including anthropogenic carbon loading (Borges & Gypens 2010, Cai et al. 2011, Waldbusser et al. 2011), while anthropogenic nutrient loading has promoted more frequent HABs (Anderson et al. 2008, Heisler et al. 2008). Concurrent with these changing environmental conditions, populations of filter-feeding bivalves, including *Crassostrea virginica* and *Argopecten irradians*, have been depleted (Jackson 2001, Lotze et al. 2006, Beck et al. 2011). This study revealed the strong negative effects that ocean acidification and HABs can have on coastal bivalve larvae and further demonstrated that the effects of such stressors are synergistic for *A. irradians*. This study additionally demonstrated that organisms filling similar ecological niches (e.g. filter-feeding, resource bivalves) can vary substantially in their susceptibility to these stressors. Together, this research has important implications for the future success of bivalves as keystone species, trajectories of estuarine health and stability, and the management of estuarine ecosystems.

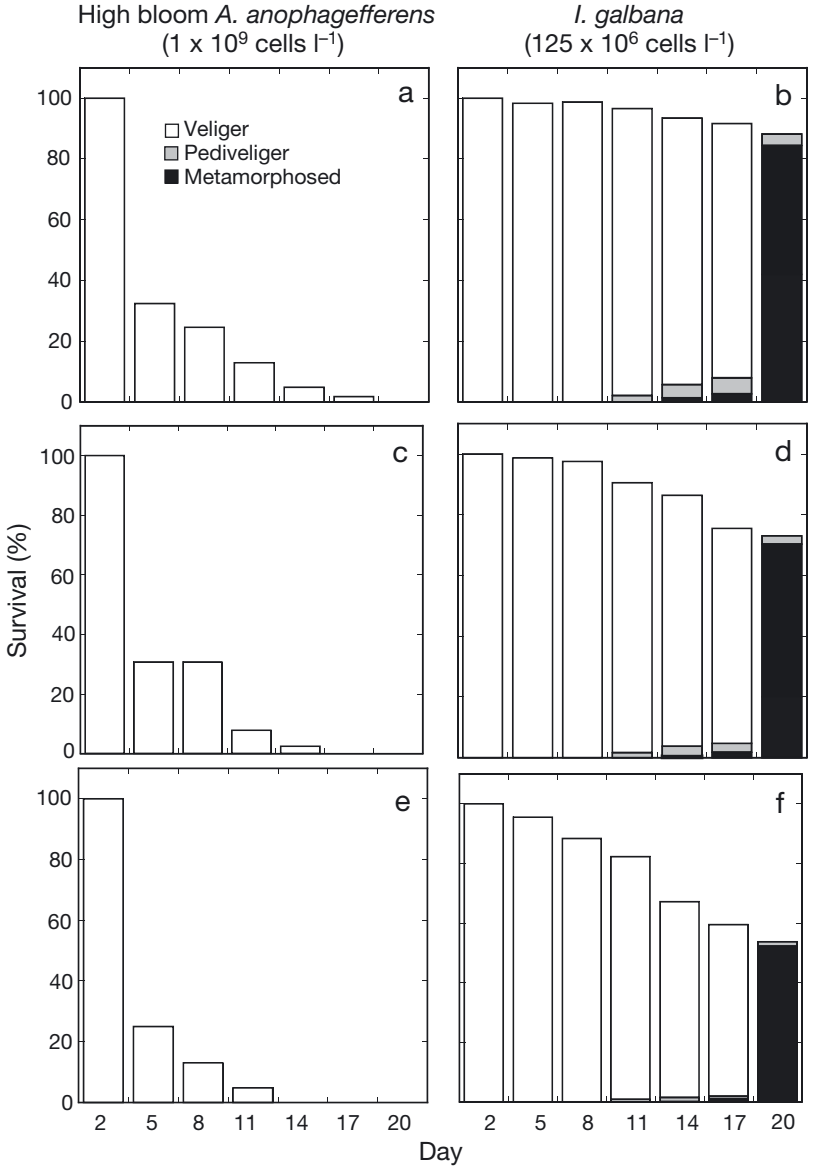

Fig. 7. *Argopecten irradians.* Survival and development of larvae under 3 levels of CO_2, (a,b) ~240, (c,d) ~390, and (e,f) ~850 ppm (see Table 1), and fed either (a,c,e) a high bloom density of *Aureococcus anophagefferens* or (b,d,f) the biovolume equivalent of *Isochrysis galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated (n = 4 per treatment)

During this study, the harmful alga *Aureococcus anophagefferens* had a more dramatic impact on larval performance than elevated CO_2. For example, while survival rates for *Crassostrea virginica* declined 10% when CO_2 concentrations increased from ~250 to ~850 ppm, they decreased 20% when their diet was switched from *Isochrysis galbana* to a high level (10^9 cells l^{-1}) of *A. anophagefferens*. Similarly, for *Argopecten irradians*, the higher level of CO_2 depressed survival rates by 40% while bloom densities of *A. anophagefferens* decreased survival rates by 90%. Similar declines in survival (60%) were observed in hard clam *Mercenaria mercenaria* larvae when fed bloom densities of *A. anophagefferens* (Bricelj & MacQuarrie 2007), although another study found that *A. anophagefferens* did not alter the survival of hard clam larvae (Padilla et al. 2006). All of the negative impacts on the performance of individuals during larval stages (reduced sizes, decreased lipid content, and delayed metamorphosis) would be likely to translate into elevated juvenile mortality rates in an ecosystem setting (Caley et al. 1996, Gosselin & Qian 1997, Munday et al. 2010).

Although the impacts of *Aureococcus anophagefferens* on oyster larvae have never been investigated, the negative responses of bay scallop larvae to *A. anophagefferens* shown here were stronger than those found by Gallager et al. (1989). This may be related to the strain of *A. anophagefferens* used in each study. *A. anophagefferens* strain CCMP1850 used in this study is generally more harmful than strains (CCMP 1784/1984, CCMP 1708, and CCMP 1794; Bricelj et al. 2001, Smith et al. 2008, Harke et al. 2011) used in prior studies of *A. anophagefferens* and bivalve larvae (Gallager et al. 1989, Padilla et al. 2006, Bricelj & MacQuarrie 2007). Strain CCMP1850 is the most recently isolated (1998) strain of *A. anophagefferens* and thus may be a more representative example of wild bloom populations than strains CCMP 1708 and 1794 isolated in 1995, or CCMP 1784/1984 isolated in 1986 (Martins et al. 2004).

Most ocean acidification experiments are bubbled with CO_2 to mimic the marine chemistry that will result from future atmospheric CO_2 scenarios (Riebesell et al. 2010). While this approach in the absence of biological activity should yield constant alkalinity, independent of CO_2 levels (Zeebe & Wolf-Gladrow 2001, Dickson et al. 2007), there are likely several biogeochemical processes that contributed to elevated alkalinity in our higher CO_2 treatments. First, since the goal of our experiments was to mimic dense

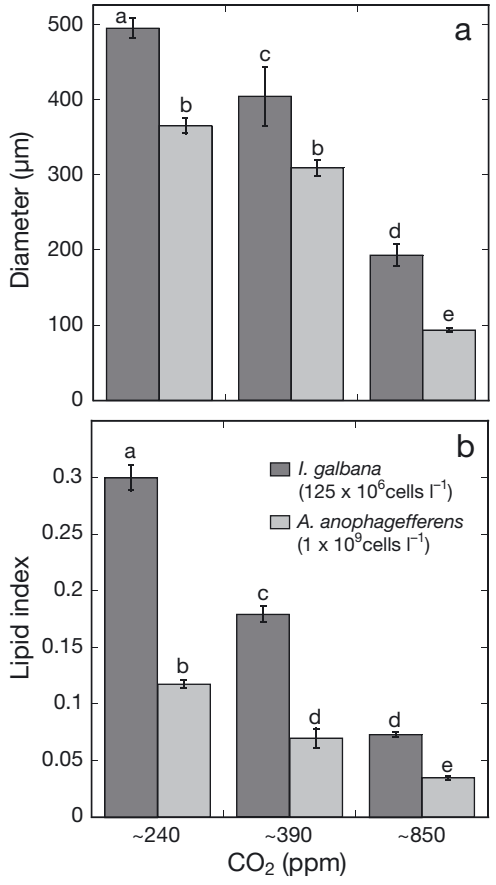

Fig. 8. *Argopecten irradians.* Mean ± SD (a) diameter and (b) lipid indices of larvae under 3 levels of CO_2, ~240, ~390 and ~850 ppm (see Table 1), and fed either a high bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *Isochrysis galbana*. Shared letters indicate treatments that are not significantly different (Tukey's multiple comparisons, $p > 0.05$)

algal blooms, we added high levels of algal biomass and nitrate (~1 mM in culture media) to our experimental vessels. The algal consumption of nitrate during experiments would cause an equimolar increase in alkalinity (Zeebe & Wolf-Gladrow 2001, Wolf-Gladrow et al. 2007). This effect should be exacerbated in high CO_2 vessels, where there were far fewer larvae to consume algae and thus many more phytoplankton to consume nitrate compared to low CO_2 treatments. Accordingly, alkalinity differences were larger in experiments with *Argopecten irradians*, the species that displayed a larger mortality gradient across CO_2 treatments, than in experiments with *Crassostrea virginica*. Also, the effect of CO_2-enhanced algal growth (Doney et al. 2009), and thus nitrate assimilation, under higher levels of CO_2 may also contribute toward higher alkalinity under higher CO_2 conditions. In addition, while larval survival rates and thus shell formation rates were high during the first week of our experiments, the second week saw high rates of larval mortality within high CO_2 treatments. Such disproportionately high rates of dissolution of calcium carbonate shells within high CO_2 treatments would increase alkalinity within these vessels. While we cannot constrain alkalinity changes to a single factor, we emphasize that these processes co-occur in the estuarine environments that our experiments sought to mimic and that the range of alkalinity variation in our experiments was smaller than the range found in the regions of estuaries where bivalves reside. Finally, we note that despite changes in alkalinity, all of our experiments achieved a desired gradient in CO_2, pH, and CO_3^{-2} and that Gazeau et al. (2011) demonstrated that it is variability in CO_3^{-2}, and not alkalinity, that controls the performance of larval bivalves during acidification.

Several ocean acidification studies have examined the effects of increased CO_2 and an additional stressor such as temperature. Sea urchins and barnacles have shown varied responses to simultaneous temperature and CO_2 increases (Brennand et al. 2010, Findlay et al. 2010). Bivalves including the Sydney rock oyster *Saccostrea glomerata*, the Pacific oyster *Crassostrea gigas* (Parker et al. 2010), *C. virginica*, and *Argopecten irradians* (Talmage & Gobler 2011) were negatively affected by elevated levels of temperature and CO_2. Prior to this work, no study has examined the effects of a poor food source such as *Aureococcus anophagefferens* coupled with increased CO_2 on the growth and survival of bivalves.

As coastal marine organisms adapt to anthropogenic stressors such as HABs and coastal ocean acidification, some species will outperform others in these rapidly changing environments. In this study, *Argopecten irradians* displayed higher survival rates than *Crassostrea virginica* under ideal conditions (ambient CO_2, ideal food), but the opposite was true in the presence of a harmful alga and coastal ocean acidification. At the end of 20 d, *C. virginica* larvae had twice the survival rates of *A. irradians* larvae at low density brown tide blooms despite increases in CO_2 concentrations (Figs. 1 & 5). At the highest densities of brown tide, some *C. virginica* survived and fully metamorphosed while all *A. irradians* individuals perished (Figs. 3 & 7). In addition, when *Aureococcus anophagefferens* and CO_2 interacted to affect the performance of these larvae, the effects on *A. irradians* were synergistically negative (performance worse than predicted by each individual treatment) but were often antagonistic (better than

predicted by each individual treatment) for *C. virginica* larvae. Collectively, all of these findings suggest that *C. virginica* larvae are more likely than *A. irradians* to persist and expand in the face of HABs and coastal ocean acidification. Bivalve restoration efforts are commonly implemented to enhance the densities of depleted wild populations (Arnold et al. 2002, Doall et al. 2008, Tettelbach & Smith 2008). In the future, such efforts will need to consider the differential vulnerabilities of these species to stressors such as acidification and HABs. Importantly, HABs and coastal ocean acidification are among a multitude of factors that must be considered in future coastal restoration efforts. It is possible that the differential vulnerability of bivalves to these stressors may be less important than other factors such as restoring degraded habitats. Regardless, our results demonstrate that, at least at the larval stage, *C. virginica* is a hardier bivalve and perhaps a more appropriate species for bivalve restoration in ecosystems which experience blooms of *A. anophagefferens* and acidification.

HABs have become an increasingly common phenomenon across coastal oceans and estuaries where many bivalves reside (Hallegraeff 1993, Smayda 1997, Sunda et al. 2006). These habitats have simultaneously been increasingly acidified via atmospheric CO_2 fluxes (Miller et al. 2009), the introduction of acidic river water (Salisbury et al. 2008), upwelling (Feely et al. 2008), and eutrophication-driven carbon loading (Borges & Gypens 2010, Cai et al. 2011, Hofmann et al. 2011), a process that may be directly and indirectly linked to HABs. For example, it has been well established that HABs are directly promoted by anthropogenic nutrient loading (Anderson et al. 2008, Heisler et al. 2008, Hattenrath et al. 2010). These blooms may promote coastal ocean acidification since many harmful algae rely on heterotrophic nutrition (Smayda 1997, Burkholder et al. 2008) and thus may be net producers, rather than consumers, of CO_2. Moreover, most HABs represent a large pool of organic carbon (Gobler et al. 2005, Heisler et al. 2008) that is ultimately degraded by bacteria and thus may create substantial increases in CO_2 (Borges & Gypens 2010, Cai et al. 2011). Moreover, as ocean acidification proceeds in coastal zones in the coming decades, it is expected that declines in pH will be greater than those expected from eutrophication and global ocean acidification alone due to a reduction in the ability of CO_2-rich coastal waters to buffer changes in pH (Cai et al. 2011). This is likely to have further negative impacts on bivalve populations.

Globally, declines in bivalve populations during the past century have been attributed to the loss of habitat, hypoxia, and overfishing (Jackson 2001, Lotze et al. 2006, Beck et al. 2011). However, HABs have negatively impacted important shellfisheries such as *Argopecten irradians* populations in NY and China due to *Aureococcus anophagefferens* blooms (Gobler et al. 2005, Gobler & Sunda 2006, 2012, Zhang et al. 2012). Furthermore, the increase in CO_2 that has occurred since the industrial revolution is capable of significantly reducing the survival of some bivalve larvae (Talmage & Gobler 2010, Barton et al. 2012) including *A. irradians*. In the future, HABs and coastal acidification together with other factors such as hypoxia and habitat loss are likely to be significant impediments to the restoration of depleted estuarine bivalve populations.

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