Global Change Biology

Global Change Biology (2013) **19**, 3317–3326, doi: 10.1111/gcb.12307

Larval carry-over effects from ocean acidification persist in the natural environment

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Abstract

An extensive body of work suggests that altered marine carbonate chemistry can negatively influence marine invertebrates, but few studies have examined how effects are moderated and persist in the natural environment. A particularly important question is whether impacts initiated in early life might be exacerbated or attenuated over time in the presence or absence of other stressors in the field. We reared Olympia oyster (*Ostrea lurida*) larvae in laboratory cultures under control and elevated seawater pCO_2 concentrations, quantified settlement success and size at metamorphosis, then outplanted juveniles to Tomales Bay, California, in the mid intertidal zone where emersion and temperature stress were higher, and in the low intertidal zone where conditions were more benign. We tracked survival and growth of outplanted juveniles for 4 months, halfway to reproductive age. Survival to metamorphosis in the laboratory was strongly affected by larval exposure to elevated pCO_2 conditions. Survival of juvenile outplants was reduced dramatically at mid shore compared to low shore levels regardless of the pCO_2 level that oysters experienced as larvae. However, juveniles that were exposed to elevated pCO_2 as larvae grew less than control individuals, representing a larval carry-over effect. Although juveniles grew less at mid shore than low shore levels, there was no evidence of an interaction between the larval carry-over effect and shore level, suggesting little modulation of acidification impacts by emersion or temperature stress. Importantly, the carry-over effects of larval exposure to ocean acidification remained unabated 4 months later with no evidence of compensatory growth, even under benign conditions. This latter result points to the potential for extended consequences of brief exposures to altered seawater chemistry with potential consequences for population dynamics.

Keywords: carbon dioxide, carry-over effect, climate change, field study, juvenile, larvae, ocean acidification, *Ostrea lurida*, oyster, pH

Received 1 March 2013; revised version received 13 June 2013 and accepted 17 June 2013

Introduction

Understanding the consequences of rising atmospheric carbon dioxide (CO_2) concentrations for natural populations is necessary to improve predictions of how ecosystems will respond under various drivers of global change. In the marine environment, a critical emerging concern is the 'acidification' of the oceans due to the absorption of atmospheric CO_2 into seawater, which drives a decline in pH and a reduction in the availability of carbonate ions used to synthesize shells and skeletons (Sabine *et al*., 2004). A growing body of laboratory studies documents impacts of ocean acidification, either through its action in isolation or in conjunction with other stressors (Doney *et al*., 2009; Kroeker *et al*., 2010, 2013). Impacts include reduced fertilization, metabolism, growth, and calcification, impaired immune responses, weakened shells, altered skeletal elemental composition, and decreased survival (Kurihara, 2008; Widdicombe & Spicer, 2008; Byrne, 2011; Gaylord *et al*., 2011; LaVigne *et al*., 2013). However, demonstrating how effects are transferred across life phase transitions, the degree to which responses are manifested in the natural environment, and for how long effects can persist, may provide a more complete understanding of potential impacts.

The transition from a pelagic larval stage to a benthic juvenile stage is a crucial phase in the life cycle of many marine invertebrates (Gosselin & Qian, 1997; Hunt & Scheibling, 1997). Mortality of benthic juveniles soon after settlement and metamorphosis is often high, due to a range of factors, both biotic (e.g., predation, competition for food and/or space) and abiotic (e.g., temperature, desiccation, wave dislodgement, UV radiation). The susceptibility of juveniles to elevated stress in the

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benthos can also be influenced by prior larval experience through so-called carry-over or latent effects (Pechenik, 2006). In particular, previous studies have indicated that a variety of factors (e.g., delayed metamorphosis, reduced nutrition, environmental pollutants, low pH / high pCO_2 conditions) experienced during the larval stage can reduce performance over at least the first weeks of juvenile life (Pechenik *et al*., 1998). For example, delayed metamorphosis induced carry-over effects in estuarine barnacles that lasted for 5 days postsettlement and did not abate even in surplus food environments (Thiyagarajan *et al*., 2007). Other carry-over effects of exposure to planktonic stresses include smaller size at settlement, slower growth, and decreased survival (e.g., Marshall *et al*., 2003; Emlet & Sadro, 2006; Kurihara & Ishimatsu, 2008; Hettinger *et al*., 2012; Gobler & Talmage, 2013).

Larval and juvenile life phases can also be influenced by maternal effects where offspring performance is shaped by the maternal environment or phenotype (Marshall, 2008). Maternal effects have been demonstrated in several ocean acidification studies (e.g., Miller *et al*., 2012; Parker *et al*., 2012; Dupont *et al*., 2013; Kelly *et al*., 2013), but the strength and sign of such effects have not been consistent. For example, Parker *et al.* (2012) found that larvae from adult oysters that had been preconditioned for 5 weeks in elevated pCO_2, grew larger and exhibited similar survival as larvae from adults preconditioned in ambient pCO_2, signifying a positive maternal effect. In contrast, Dupont *et al.* (2013) found that adult sea urchins preconditioned for 4 months in elevated pCO_2 produced fewer larvae with a lower rate of settlement success relative to controls. However, these negative maternal effects disappeared if adult urchins were preconditioned in elevated pCO_2 for 16 months.

While the importance of maternal effects and larval carry-over effects are increasingly evident, it is less clear whether environmental conditions during the juvenile phase might modulate the strength and persistence of these effects. Benign juvenile environments can sometimes allow organisms to compensate for stress experienced during the larval phase, leading to carry-over effects that weaken rapidly (e.g., Diederich *et al*., 2011; but see Phillips, 2002). Alternatively, exposure to benthic stressors (e.g., high temperatures) during juvenile life could exacerbate the magnitude of larval carry-over effects, and indeed, there is growing concern regarding the interactive effects of multiple environmental stressors in marine ecosystems (Crain *et al*., 2008; Boyd, 2011; Byrne, 2011). In some cases, the influence of two stresses can be synergistic, such that the combined effect is greater than the sum of the individual impacts (e.g., Parker *et al*., 2010). Alternatively, effects of multiple stressors can be additive, such that stressors operate independently of one another (e.g., Padilla-Gamiño *et al*., 2013). Furthermore, multiple stressors may be experienced sequentially during different life phases, although such effects have rarely been considered.

Prior work on Olympia oysters (*Ostrea lurida*), a native bivalve on the west coast of North America, demonstrated larval carry-over effects induced by ocean acidification (Hettinger *et al*., 2012). Specifically, juvenile Olympia oysters reared as larvae under low pH/high pCO_2 [7.73 pH; NBS scale (Zeebe & Wolf-Gladrow, 2001)] grew 41% less than oysters reared as larvae in control pH/pCO_2 (7.93 pH_{NBS}) conditions, regardless of the juvenile pH/pCO_2 environment. It remains unclear, however, how long larval carry-over effects induced by ocean acidification might persist in Olympia oysters and other species, and how such effects might be influenced by subsequent environmental conditions, especially when those conditions are varying realistically in the field. In this study, we reared Olympia oyster larvae through metamorphosis in both control and elevated pCO_2 concentrations in the laboratory, then outplanted the newly metamorphosed juveniles to field sites at two intertidal heights that differed in emersion time and temperature. We quantified subsequent juvenile growth and percent survival over 4 months, halfway to reproductive age. These efforts allowed us to address three questions: (i) Can larval carry-over effects of ocean acidification persist in *O. lurida* in their natural environment; (ii) in stressful habitats, are larval carry-over effects due to ocean acidification amplified by synergistic interactions with harsh environmental factors experienced during the juvenile phase; and (iii) in benign environments, do larval carry-over effects due to ocean acidification rapidly or strongly attenuate?

Materials and methods

Study system

Olympia oysters (*Ostrea lurida*) are native to bays and estuaries along the eastern Pacific coast from Alaska to Baja California, Mexico. Females brood developing larvae within their mantle cavity for approximately 10 days before releasing veliger larvae into the water column. Veliger larvae develop for 2–3 weeks before they settle onto hard substrate within low intertidal and shallow subtidal zones from +0.5 to −1.0 m mean lower low water (MLLW) (Baker, 1995; Deck, 2011).

Larval culturing

Adult Olympia oysters (n = 105) were collected in September 2010 from Tomales Bay, California (38°06′57.01″N, 122°51′14.39″W), a 20 km long and 1 km wide estuary. After transportation to Bodega Marine Laboratory (BML), adults were

cleaned of epiphytes and distributed evenly among three 100 l cylinders. Care was taken to maintain a similar average size of individuals among cylinders. Every day, adults in each cylinder were fed microalgal food (*Isochrysis galbana*) to encourage larval release. Seawater, filtered to 0.45 μm and held at 18–22 °C, was changed every other day. Seawater in the cylinders was not altered with respect to pCO_2 concentrations, and was bubbled with air. After 72 h, larvae were released in one of the cylinders, and subsets of these individuals were distributed by pipette among 4.5 l glass jars (n = 1000/jar) used for culturing (n = 18 jars / pCO_2 concentration). Each culture jar held 2 l filtered seawater (0.45 μm) pre-equilibrated to the target seawater pCO_2 concentration and temperature. Larvae were then reared through their entire planktonic duration in a culturing facility at BML that enabled continuous control of seawater pCO_2.

The target seawater pCO_2 concentration in larval treatment cultures was 1000 μatm (ca. 7.80 pH_{NBS}), roughly congruent with the year 2100 predictions from the (Intergovernmental Panel on Climate Change) IPCC (2007) emissions scenario A1FI ('fossil fuel intensive' case). The accompanying control seawater pCO_2 target was 400 μatm (ca. 8.10 pH_{NBS}), similar to the current global-mean atmospheric CO_2 concentration and within the range of seawater pCO_2 concentrations Olympia oysters currently experience in Tomales Bay (A. D. Russell, unpublished data). The diurnal variation in pH_{NBS} can be as large as 0.6 pH_{NBS} units in Tomales Bay during August–October (T. M. Hill, unpublished data). Jars were held in one of two seawater tables maintained at 20.0 °C (±0.2 °C), a temperature that is within the range of values encountered by Olympia oysters locally (A. D. Russell, unpublished data). Pre-equilibration of pCO_2 concentrations in seawater used during larval rearing was accomplished by bubbling 20 l carboys filled with filtered seawater for 2–3 days with NIST-traceable CO_2 gas mixtures (carboy water). After filling culture jars with carboy water, pCO_2 concentrations were maintained in the jar seawater by pumping the same CO_2 gas mixtures into sealed air spaces above the free surfaces of the seawater in the culture jars (hereafter 'headspaces') shared by 6 replicate jars per pCO_2 concentration. This latter procedure minimized net CO_2 exchange across the seawater surfaces in the jars. There were three headspaces for each pCO_2 concentration, and 6 jars associated with each headspace (n = 2 pCO_2 concentrations × 3 headspaces × 6 replicate jars = 36 jars). Further details of the larval culturing apparatus can be found in Hettinger *et al*. (2012).

Every other day, 90% of the seawater in each jar (jar water) was removed via reverse-filtration through 125 μm mesh and replaced with carboy water at the appropriate pCO_2 concentration. After each water change, larvae were fed *I. galbana* at a density of 100 000 cells per ml per jar. This food level is known to promote rapid growth of larval oysters (Strathmann, 1987), and falls within the range of phytoplankton cell densities in our study region during the time when Olympia oyster larvae are in the water column (Kimbro *et al*., 2009). High pCO_2 can induce shifts in phytoplankton C : N ratios over timescales of 10 days, with possible consequences for larval nutrition (Riebesell *et al*., 2007; van de Waal *et al*., 2010). However, algae in our experiment were cultured under control pCO_2 conditions and were added to larval cultures every 2 days. Presumably, the majority of algal cells were consumed before high pCO_2 substantially influenced their quality. Algae remaining in larval cultures were removed during water changes every other day.

On day 11 of the experiment, prior to the commencement of settlement and metamorphosis, larvae were transferred into new glass 'substrate' jars. Each of these jars was prepared in advance by removing the base of the jar, replacing it with a gray PVC plate (5 mm thick) attached with aquarium-safe silicone, and conditioning the new jar base in filtered seawater for 2 days. The surface of each new jar base was abraded with sandpaper to encourage larval settlement, and a 1 cm^2 grid was drawn onto each base to assist with subsequent field monitoring of juvenile growth and survival. The underside of each new jar base was scored to facilitate separation into four tiles after larvae had metamorphosed into benthic juveniles. These tiles were suitable for outplanting juvenile oysters to the field.

Seawater chemistry during larval culturing

The chemistry of jar water (water containing larvae and microalgae) and carboy water (no larvae or microalgae; water used to fill jars) was sampled every other day when a water change was performed. Seawater pH_{NBS} and temperature were quantified using a potentiometric pH/temperature meter (Accumet Excel XL60), salinity was determined using a YSI 6600V2 multi-parameter instrument, and total alkalinity (TA, μmol per kg of seawater) was measured using automated Gran titration with duplicates (Metrohm 809). A subset of samples was analyzed for dissolved inorganic carbon (DIC, μmol per kg of seawater) at the University of Georgia's infrared CO_2 analysis facility (Cai & Wang, 1998). Accepted methods (Dickson *et al*., 2007) were employed where possible throughout the study, and both TA and DIC measurements were standardized using certified reference material from A. Dickson at Scripps Institute of Oceanography (La Jolla, California). Calcite and aragonite saturation states ($\Omega_{calcite}$, $\Omega_{aragonite}$) and seawater pCO_2 were calculated using the carbonate system analysis software, CO2SYS (Lewis & Wallace, 1998) with TA, DIC, temperature, and salinity defined as input variables, and employing equilibrium constants K_1 and K_2 from Mehrbach *et al*. (1973), and K_{SO4} from Dickson (1990).

Metamorphosis and field outplants

Settlement of larvae and metamorphosis into benthic juveniles was assessed daily starting at day 11 when larvae were transferred into substrate jars. Although some settlement could have occurred over the course of several days, we defined settlement as the day when <5% of larvae were still visibly swimming (day 14). At this point, the bases of each substrate jar were removed, and the proportion of metamorphosed individuals was determined by subtracting dead and non-metamorphosed larvae (e.g., pediveligers) from the total initial number of larvae, divided by the total initial number of larvae. Each jar base was then divided into four 50 cm^2 tiles for outplanting.

To obtain tiles for outplanting with similar initial densities of juveniles, tiles from a given pCO_2 concentration with similar juvenile densities were arranged into four groups, each composed of six replicate tiles. The four groups were then randomly assigned to one of the two field sites and one of the two shore levels. Initial density of juveniles on the tiles did not vary with larval pCO_2 concentration, site, or shore level (ANOVA, larval pCO_2, $F_{1,\ 44} = 0.19$, $P = 0.667$; site, $F_{1,\ 44} = 0.3$, $P = 0.59$; shore level, $F_{1,\ 44} = 0.004$, $P = 0.95$). Tiles from the second pCO_2 concentration were arranged similarly and paired with those from the first pCO_2 concentration resulting in six pairs of tiles for each outplant location. Each tile was then secured to a stake. The overall design was: $n = 2$ larval pCO_2 concentrations × 2 sites × 2 shore levels × 6 tiles = 48 tiles. Tiles were outplanted to the field on the same day as settlement (day 14 post larval release).

A shoreline region half-way along the Tomales Bay estuary was selected for the two replicate field sites (38°09′01.01″N, 122°53′19.19″W). The replicate sites were 40 m apart in the alongshore direction, and were similar in substrate type, solar exposure, and bottom slope. At each replicate site, six PVC 'T' stakes, placed 0.5 m apart and each holding two tiles, were driven into the substrate such that the tiles were situated at either 0 or 0.3 m above MLLW (i.e., 'low' and 'mid' shore levels, respectively). Tiles deployed on four of the six stakes per site and shore level were outfitted with temperature loggers (iButton, Maxim, Sunnyvale, CA, USA) that sampled every 30 min (i.e., water temperature when submerged or aerial temperature when emersed) ($n = 4$ iButtons × 2 sites × 2 shore levels = 16 iButtons). Each iButton was coated in marine epoxy for waterproofing and was then secured to the respective tile in a style to mimic the orientation of attached juveniles. Temperature data were summarized using two temperature metrics: acute temperature stress, defined as the 99th percentile of maximum temperatures recorded at each site and shore level, and chronic high temperature exposure, defined as the average daily maximum temperatures recorded at each site and shore level (Helmuth *et al.*, 2002).

A YSI 6600V2 multi-parameter instrument was used to measure temperature, salinity, and pH_{NBS} at each of the outplant sites approximately weekly up to day 52 postsettlement, and then monthly up to day 127 postsettlement. The YSI 6600V2 conductivity probe was calibrated using a conductivity calibration standard (Ricca Chemical Company, Arlington, TX, USA) and low-ionic strength buffer solutions (pH_{NBS} 4.0, 7.0, 10.0) (Fisher Scientific, Pittsburg, PA, USA) held at 25 °C. Along with simultaneous field measurements of seawater properties, discrete water samples were collected for TA (500 ml sample) and DIC (20 ml sample). These samples were collected below the outplanted tiles at the low shore level, typically in the early morning or late afternoon during low tide. Water samples for DIC were immediately poisoned for a final 0.1% mercuric chloride concentration, and samples were analyzed as described for larval culturing.

Juvenile survival on each tile was calculated as the percentage of initial juveniles that were alive on each sampling date (day 6, 13, 27, 127 postsettlement). Tiles were retrieved from the field on each designated sampling day, placed in individual seawater-filled containers, and transported in coolers to BML. Each tile was examined for live juvenile oysters under a dissecting microscope (Leica M125 with DC290 camera). The edges, back, and tops of each tile were also inspected visually for pediveligers and new recruits each time the tiles were brought into the laboratory, to verify that minimal natural settlement occurred on the outplanted tiles. Only one new recruit was seen on the top of one tile at 6 days postsettlement; this low field recruitment was expected given that tiles were outplanted at the end of September, shortly after the typical recruitment peak in Tomales Bay (Deck, 2011).

Juvenile growth rates were estimated from photographs of juveniles randomly sampled on each tile using a random number table to select squares on the gridded tile. The total projected area of each shell was measured from these photographs using ImageJ software (ver. 1.37, National Institutes of Health). The area of the larval shell at metamorphosis, which remains visually distinct, was also determined from these photographs (Hettinger *et al.*, 2012). Juvenile growth rates were calculated on days 6, 13, and 27 postsettlement as the change in the total projected area of the shell between the sample date and when the larvae metamorphosed, divided by the intervening number of days (mm^2 per day). Thus, these growth rates represent the average growth rate over the full benthic life stage to the age examined, not an age-specific growth rate characteristic of the period between assay dates. On day 127 postsettlement, the larval shell at metamorphosis made up <1% of the total shell area and only the total projected area of the shell could be determined reliably from photographs. In this case, growth rate was estimated as the total projected area divided by the intervening number of days (mm^2 per day). After tiles were processed, they were transported back to the field during the next low tide, within 12–24 h of collection.

Statistical analyses

For the laboratory portion of the study, variation in percent metamorphosis and shell area at metamorphosis in the two pCO_2 concentrations were analyzed in separate, partly nested ANOVAS with jar means as replicates. In these analyses, larval pCO_2 concentration was a fixed factor, and headspaces were nested within pCO_2. In assessing jar chemistry, all measurements of jar water and carboy water were averaged across the experiment, and separate, partly nested ANOVAS were conducted on estimates of average conditions per jar.

In the field component of the study, juvenile survival and growth rates at days 6 and 13 postsettlement were analyzed using an ANOVA with outplant tiles as replicates, and larval pCO_2 concentration, site, shore level, and stake (site, shore level) as main effects. All interaction terms were included in the model. Juvenile survival and growth rates at days 27 and 127 postsettlement were each analyzed using an ANOVA with outplant tiles as replicates and larval pCO_2 concentration, site, and stake (site) as main effects. Shore level was not included in these analyses due to 100% mortality at the mid shore level by day 27 postsettlement. In all analyses, data fulfilled assumptions of normality and homogeneity of variance, tested using Shapiro-Wilks' and Bartlett's test, respectively, and thus untransformed data were used. The statistical software JMP

(ver. 8.0.1, Statistical Analysis Software) was used for all analyses.

Results

Seawater chemistry during larval culturing

As expected, pH_{NBS} of seawater differed between the two pCO_2 concentrations (ANOVA, $F_{1,\ 30} = 52628.8$, $P < 0.0001$; Table 1). In some cases, pH_{NBS} of seawater in the jars associated with headspaces in the same pCO_2 concentration differed (ANOVA, $F_{4,\ 30} = 26.12$, $P < 0.0001$); pH_{NBS} in seawater from 400 μatm headspace 2 differed by 0.02 from headspaces 1 and 3 (Tukey HSD, $P < 0.05$). pH_{NBS} of seawater from the three 1000 μatm headspaces did not differ (Tukey HSD, $P > 0.05$). TA of seawater did not differ between pCO_2 treatments (ANOVA, $F_{1,\ 30} = 1.86$, $P = 0.183$; Table 1) or among headspaces assigned to the same pCO_2 treatments (ANOVA, $F_{4,\ 30} = 0.96$, $P = 0.446$). DIC of seawater differed between pCO_2 treatments (ANOVA, $F_{1,\ 30} = 2102.84$, $P < 0.0001$; Table 1), and among headspaces assigned within the same pCO_2 concentration (ANOVA, $F_{4,\ 30} = 3.48$, $P = 0.019$). Mean DIC differed by 117 μmol kg_{sw}^{-1} in 400 μatm vs. 1000 μatm (Table 1).

Laboratory metamorphosis

Percent metamorphosis in the laboratory was 80% higher in control compared to high pCO_2 conditions (ANOVA, $F_{1,\ 30} = 19.72$, $P < 0.0001$; Fig. 1a), and did not vary among headspaces assigned to the same treatment (ANOVA, $F_{4,\ 30} = 0.16$, $P = 0.956$). Size at metamorphosis was 11% lower for individuals reared as larvae in high pCO_2 than those in control pCO_2 conditions (ANOVA, $F_{1,\ 28} = 19.44$, $P < 0.0001$; Fig. 1b), and also did not vary among headspaces assigned to the same treatment (ANOVA, $F_{4,\ 28} = 2.08$, $P = 0.11$).

Field site physical characteristics

pH_{NBS}, TA, and DIC values did not differ between the two sites (Student's *t*-test, pH_{NBS}, $t_{16} = 0.53$, $P = 0.603$; TA, $t_{16} = -0.12$, $P = 0.904$; DIC, $t_{16} = -0.13$, $P = 0.899$; Table 1). Mean pCO_2 values calculated from TA and DIC sampled at the field sites corresponded to control values in the laboratory culture conditions (Table 1). Average daily maximum temperatures were 2 °C warmer in the mid zone than in the low zone (black bars, Fig. 2a). The 99th percentile of maximum temperatures recorded were 1 °C warmer in the mid zone than in the low zone (white bars, Fig. 2a). Based on estimates from tide tables (http://tbone.biol.sc.edu/tide), juveniles in the mid zone were emersed for 12% more time than juveniles in the low zone (Fig. 2b).

Juvenile survival in the field

Juvenile survival in the field at day 6 postsettlement was not affected by the pCO_2 concentration in which larvae were reared (ANOVA, $P = 0.701$; Table S1, Fig. 3a and b). Survival was an average of 90% higher for juveniles in the low zone compared to the mid zone (ANOVA, $P < 0.0001$), regardless of larval pCO_2 exposure treatment, (larval pCO_2 x shore level, ANOVA, $P = 0.942$). Survival did not differ among stakes (ANOVA, $P = 0.363$) or between sites (ANOVA, $P = 0.971$; Table S1, Fig. 3a and b).

There were similar results for survival on day 13 postsettlement. Juvenile survival did not differ as a function of the larval pCO_2 concentration (ANOVA,

Table 1 A table of seawater properties during the larval culturing and field outplant portions of the experiment (±standard deviation)

	Larval culturing		Field outplant	
	400 μatm	1000 μatm	Site 1	Site 2
Temperature (°C)	19 ± 0.03	19 ± 0.04	17 ± 1.24	17 ± 1.32
Salinity	34.1 ± 0.07	34.1 ± 0.10	34.1 ± 0.48	34.2 ± 0.56
TA (μmol kg_{sw}^{-1})	2258 ± 6 (18)	^{NS}2259 ± 7 (18)	2321 ± 79 (9)	^{NS}2326 ± 70 (9)
DIC (μmol kg_{sw}^{-1})	2043 ± 8 (18)	*2160 ± 10 (18)	2125 ± 69 (9)	^{NS}2119 ± 62 (9)
pH_{NBS}	8.11 ± 0.011	*7.80 ± 0.003	8.20 ± 0.141	^{NS}8.16 ± 0.161
pCO_{2calc}	485 ± 54	1060 ± 189	531 ± 205	516 ± 230
$\Omega_{calcite}$	3.7 ± 0.30	2.0 ± 0.27	3.55 ± 1.04	3.70 ± 1.10
$\Omega_{aragonite}$	2.4 ± 0.19	1.3 ± 0.18	2.3 ± 0.68	2.4 ± 0.72

pCO_{2calc}, $\Omega_{calcite}$ and $\Omega_{aragonite}$ calculated from the measured values of total alkalinity (TA), dissolved inorganic carbon (DIC), temperature, and salinity.

The number of samples for TA and DIC are indicated in parentheses.

Significant or non-significant differences at the $\alpha = 0.05$ level are indicated with an * or NS, respectively (Student's *t*-test)

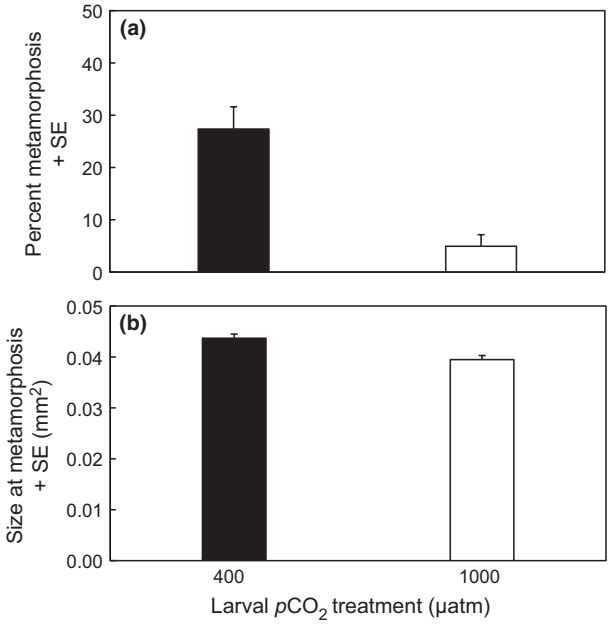

Fig. 1 Effect of elevated pCO_2 on *Ostrea lurida* (a) percent metamorphosis and (b) size at metamorphosis during the laboratory portion of the study. (a) The percent of metamorphosed individuals averaged across jar replicates in each larval pCO_2 treatment (+SE) (n = 18 jars per pCO_2 concentration). (b) The mean size of individuals at the time of metamorphosis averaged across tile replicates in each larval pCO_2 treatment (+SE). Sizes at metamorphosis were measured as the total area of the larval shell, which was visible on the shells of juveniles at day 6 postsettlement (n = 18 tiles per pCO_2 concentration).

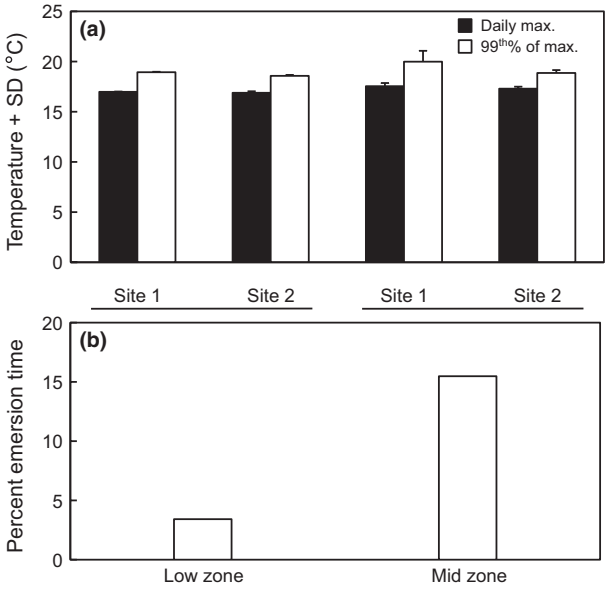

Fig. 2 Temperature data recorded during the field portion of our experiment in Tomales Bay, California. (a) The average daily maximum temperatures (+SD) (black bars) and the 99th percentile of maximum temperatures (+SD) (white bars) calculated using iButton data recorded by each of 4 iButtons deployed at each site per shore level over the 127 days of the experiment. (b) Percent emersion time of juveniles outplanted on tiles at each shore level over the 127 day period of the field portion of the experiment. Values were estimated using tide tables.

P = 0.737), but differed strongly between shore levels (ANOVA, $P < 0.0001$; Table S2, Fig. 3a and b). The effect of shore level on juvenile survival was consistent between oysters raised under the two larval pCO_2 concentrations (ANOVA, P = 0.972; Table S2).

By day 27 postsettlement, there was 100% juvenile mortality in the mid zone. In the low zone, juvenile survival again did not differ as a function of larval pCO_2 (ANOVA, P = 0.368; Table S3, Fig. 3a and b).

On day 127 (ca. 4 months) postsettlement, although there was not a significant effect of larval pCO_2 concentration on juvenile survival in the low zone, survival for individuals reared as larvae in control pCO_2 conditions was an average of 20% higher at both sites than survival for individuals reared as larvae in elevated pCO_2 conditions (ANOVA, P = 0.328; Table S4; Fig. 3a and b).

Juvenile growth rates in the field

At day 6 postsettlement, juvenile growth rates were 15–50% higher for individuals reared as larvae in control pCO_2 compared to individuals that spent the larval stage in elevated pCO_2 conditions (ANOVA, P = 0.026; Table S5, Fig. 4a). Juvenile growth rates were also higher for individuals outplanted to the low zone compared to the mid zone (ANOVA, P = 0.031; Table S5), with low zone juveniles growing 50% faster than those in the mid zone (Fig. 4a). This effect of shore level on juvenile growth rates was consistent between the two larval pCO_2 levels (ANOVA, P = 0.839; Table S5).

Juvenile growth rates at day 13 postsettlement did not differ as a function of larval pCO_2 concentration (ANOVA, P = 0.21; Table S6, Fig. 4b), but the trend of higher growth rates for juveniles reared as larvae in control compared to elevated pCO_2 conditions remained consistent. A reduction in statistical power due to low sample sizes might have caused trends to be non-significant. Growth rates differed between shore levels (ANOVA, P = 0.015), and this effect did not depend on larval pCO_2 concentration (ANOVA, P = 0.948; Table S6).

As noted previously, there was 100% juvenile mortality in the mid zone by day 27 postsettlement. Juvenile growth rates in the low zone at this time point did not differ between the two larval pCO_2 concentrations (ANOVA, P = 0.118; Table S7, Fig. 5a). As noted above for

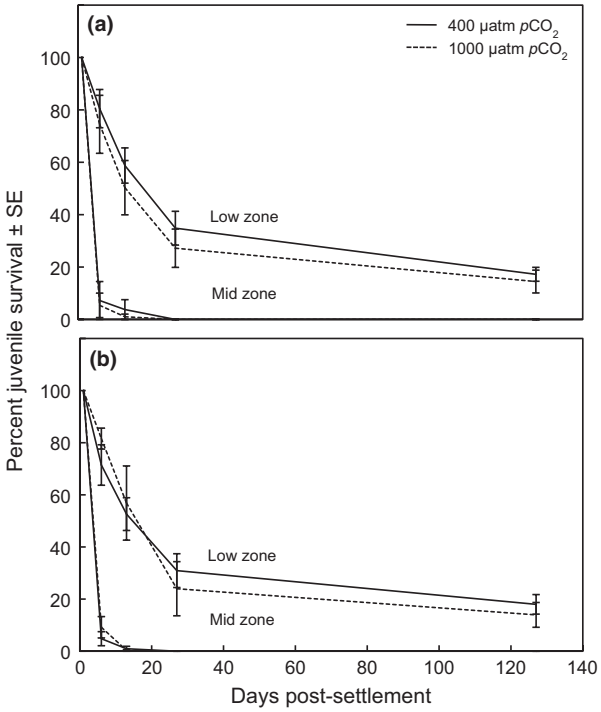

Fig. 3 Percent juvenile survival of *Ostrea lurida* as a function larval exposure to elevated pCO_2 and shore level while juveniles were outplanted in Tomales Bay, California. Values represent percent survival averaged across all tile replicates of a given treatment (±SE) at (a) site 1 and (b) site 2 on days 1, 6, 13, 27, and 127 postsettlement (n = 6 tiles per site, shore level, and larval pCO_2 combination). Solid lines and dashed lines represent the control (400 μatm) and elevated (1000 μatm) larval pCO_2 concentrations, respectively.

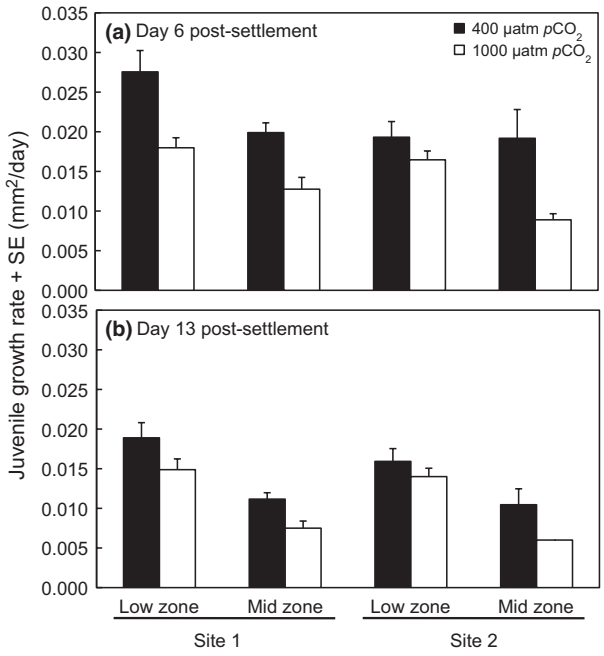

Fig. 4 Effect of larval exposure to elevated pCO_2 and shore level on juvenile growth rates of *Ostrea lurida* at (a) day 6 and (b) day 13 postsettlement, when juveniles were outplanted to two sites and shore levels in Tomales Bay, California. Growth rates were calculated as the change in the total projected area of the shell between the sample date (day 6 or 13) and when the larvae metamorphosed, divided by the intervening number of days (mm^2 per day). Values are means across all tile replicates of a given treatment (+SE) (n = 6 tiles per site, shore level, and larval pCO_2 combination).

growth rates at day 13 postsettlement, the trend of higher growth for juveniles reared as larvae in control compared to elevated pCO_2 conditions remained; again however, low sample sizes may have resulted in insufficient power to statistically resolve the differences in growth.

In the low zone, juvenile growth rates at 4 months (day 127) postsettlement were 20–40% greater for individuals reared as larval in control compared to high pCO_2 conditions (ANOVA, P = 0.003; Table S8, Fig. 5b).

Discussion

Complex life cycles and sequential stressors

Although there is growing concern about the impact of multiple environmental stressors on marine ecosystems, the extent of these interactive effects is unknown (Crain *et al*., 2008; Pörtner, 2008; Boyd, 2011). An increasing number of studies have considered the interactive effects of ocean acidification and temperature on marine invertebrates (reviewed by Byrne, 2011). In most studies, these stresses have been applied simultaneously, for example in testing the effects of increased temperature and acidification on early life stages (e.g., Parker *et al*., 2010). However, for marine organisms with complex life cycles, there is a strong possibility that stressors may be experienced sequentially during different life phases. Thus, while the influence of a single environmental stressor can certainly carry over to impact the performance of subsequent life stages (e.g., Pechenik *et al*., 1998; Thiyagarajan *et al*., 2007), there is also a possibility that the strength of these carry-over effects can be modified by exposure to a subsequent stress.

In our study, there was no evidence that larval carry-over effects were exacerbated through synergistic interactions with subsequent stressors in the juvenile environment (i.e., temperature extremes and emersion time in the mid zone). Instead, the larval and juvenile classes of stress appeared to operate additively. However, it is possible that environmental stress in the mid zone was severe enough that we were unable to detect a synergistic effect of ocean acidification and emersion

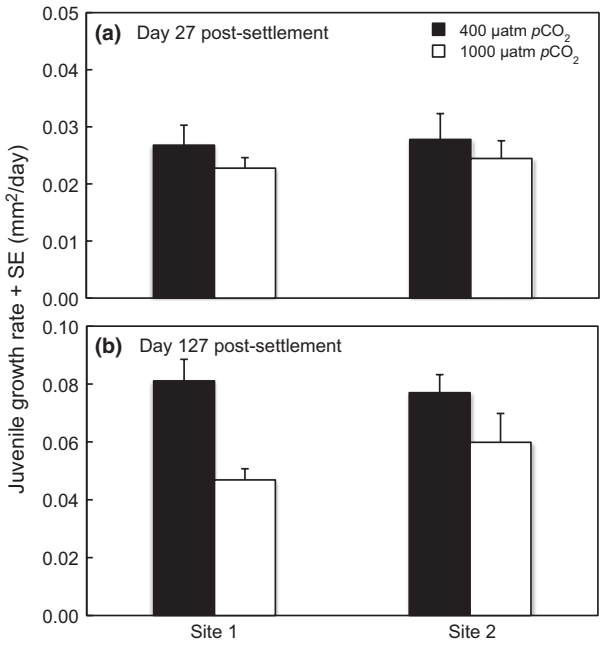

Fig. 5 Effect of larval exposure to elevated pCO_2 on juvenile growth rates at (a) day 27 and (b) day 127 postsettlement of *Ostrea lurida* in the low zone when juveniles were outplanted to Tomales Bay, California. Growth rates for day 27 postsettlement were calculated as the change in the total projected area of the shell between the sample date and when the larvae metamorphosed, divided by the intervening number of days (mm^2 per day). Growth rates for day 127 postsettlement were calculated as the total projected area of the shell divided by the intervening number of days (mm^2 per day). Values are means across all tile replicates of a given treatment (+SE) (n = 6 tiles per site, shore level, and larval pCO_2 combination).

stress. If oysters had been exposed to a more moderate level of stress (e.g., outplanted to a slightly lower tidal height), synergistic interactions might have been more apparent.

Persistence of carry-over effects and lack of compensatory growth

In our study, there was little indication that carry-over effects from larval exposure to high pCO_2 rapidly or strongly attenuated in a physically benign habitat. Even in the low zone where oysters were submerged nearly continuously with access to naturally high levels of food, the larval carry-over effect on juvenile growth was still evident after 4 months. This suggests two possibilities that are not mutually exclusive: (i) juvenile oysters continued to suffer energetic deficits or other abnormalities following larval exposure to acidified conditions; and (ii) Olympia oysters may have little or no capacity for compensatory growth.

Carry-over effects may often arise through energetic deficits accumulated during an earlier life history stage. By the completion of the larval phase, oysters exposed to acidified conditions may have reached metamorphosis in an energy-depleted state. Processes requiring substantial energy during the larval phase include calcification, maintenance of internal acid-base balance, somatic growth, swimming, and feeding, and each incur metabolic costs (Pörtner *et al.*, 2004). Calcification can be demanding energetically (Palmer, 1992), and costs of mineral accretion may increase as carbonate ion concentrations decline under ocean acidification scenarios. This appears to be especially true in larval oysters (Waldbusser *et al.*, 2013). Moreover, because metamorphosis is also an energetically demanding process in oysters (Videla *et al.*, 1998), any energetic deficits accrued during the larval period could be magnified during early juvenile life, resulting in further decreases in growth and survival (Hettinger *et al.*, 2012).

Even if such energetic deficits fade with increasing time after metamorphosis, juvenile oysters appear unable to later compensate for the negative carry-over effects of larval exposure to ocean acidification. Many organisms experience temporary periods of reduced nutrition or energetic stress during their early development. These periods of stress often shape the subsequent performance and life history trajectory of the organism (Metcalfe & Monaghan, 2001). However, in some cases, after stressful conditions subside, organisms exhibit increased growth to compensate for the initial setback and return to a normal growth trajectory (Metcalfe & Monaghan, 2001; Jespersen & Toft, 2003). Although compensatory growth has been documented in a few bivalve species (e.g., Eldridge & Eversole, 1982), the early growth rate of the Pacific oyster (*Crassostrea gigas*) was found to predict later growth with no evidence of compensation (Boudry *et al.*, 2003). Thomsen *et al.* (2013) reported that juvenile mussels were able to overcompensate for exposure to high pCO_2 during the juvenile phase if there was sufficient food available in the environment. It is unknown whether juvenile responses would differ if these mussels had been challenged energetically by carry-over effects induced during the larval stage.

Juvenile Olympia oysters exposed to high pCO_2 as larvae did not exhibit signs of compensatory growth, even in physically benign environments (Fig. S1). Four months after settlement, juvenile oysters exposed as larvae to high pCO_2 continued to lag behind control oysters in terms of their size and growth rates. Compensatory growth is typically achieved through a period of increased feeding activity, especially in mobile consumers (Jespersen & Toft, 2003). However, it is

possible that all oysters of a given size have a similar rate of suspension feeding that cannot be increased substantially. There are also often costs of compensatory growth (e.g., decreased lifespan; Metcalfe & Monaghan, 2001), and it is possible that unknown costs of accelerated feeding and growth might outweigh the potential benefits. The lack of compensatory growth was not likely a result of food limitation. Juveniles in the low zone were submerged nearly continuously, and thus had substantial access to food. During the season of our experiment, phytoplankton is abundant in the portion of Tomales Bay where our field sites were located (Kimbro *et al*., 2009). Rather, the lower growth rates exhibited for a given size oyster were likely due to persistent carry-over effects from ocean acidification.

The mechanisms for such effects are not completely understood (Pechenik, 2006). Perhaps stress in the larval stage leads to a reduction in the juvenile feeding apparatus, digestive capability, or assimilation efficiency, leading to slower growth postmetamorphosis (Pechenik *et al*., 2002). Stress during early life stages might also interfere with transcriptional or translational processes that have far-reaching effects across life history stages (e.g., Todgham & Hofmann, 2009). Finally, some stressors are known to alter proteins directly (e.g., Tomanek *et al*., 2011), potentially causing effects that persist long after the stress is removed. More research is necessary to better understand what mechanisms drive carry-over effects, and to identify which species are most likely to be sensitive to such effects.

Overall, our results indicate that larval carry-over effects from ocean acidification in Olympia oysters are substantial and produce persistent, negative effects on juvenile performance long after stresses during the larval stage have been removed. These consequences also appear to operate mostly independently of whether additional stresses in the juvenile stage are active, or whether benign conditions prevail. Because the success of early life history stages can influence the dynamics of marine populations, future studies are needed to consider the conditions that various life history stages experience and to tease apart the mechanisms driving the interactive effects of multiple stressors.

Acknowledgements

We thank K. N. S. Sato, K. Meyer, J. Newman, and R. Pennington for assistance with larval culturing and fieldwork, J. D. Hosfelt for help with seawater carbonate chemistry, and N. Willits for statistical advice. The Tomales Bay Oyster Company provided access to adult oysters. This work was funded by NSF grants OCE-0927255 and OCE-1041089. A. H. is grateful for a GK-12 graduate fellowship (NSF grant 0841297), a BML graduate fellowship, and a UC Davis Graduate Group in Ecology grant. This manuscript was greatly improved by comments from G. G. Waldbusser and two anonymous reviewers.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mean size of juvenile *Ostrea lurida* on each sampling date (days 6, 13, 27, and 127 postsettlement).
Table S1. Analysis of variance for *Ostrea lurida* juvenile survival at day 6 postsettlement.
Table S2. Analysis of variance for *Ostrea lurida* juvenile survival at day 13 postsettlement.
Table S3. Analysis of variance for *Ostrea lurida* juvenile survival at day 27 postsettlement.
Table S4. Analysis of variance for *Ostrea lurida* juvenile survival at day 127 postsettlement.
Table S5. Analysis of variance for *Ostrea lurida* juvenile growth at day 6 postsettlement.
Table S6. Analysis of variance for *Ostrea lurida* juvenile growth at day 13 postsettlement.
Table S7. Analysis of variance for *Ostrea lurida* juvenile growth at day 27 postsettlement.
Table S8. Analysis of variance for *Ostrea lurida* juvenile growth at day 127 postsettlement.