The Journal of Experimental Biology 214, 2586-2594

doi:10.1242/jeb.055939

RESEARCH ARTICLE

Functional impacts of ocean acidification in an ecologically critical foundation species

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Accepted 9 May 2011

SUMMARY

Anthropogenic CO_2 is reducing the pH and altering the carbonate chemistry of seawater, with repercussions for marine organisms and ecosystems. Current research suggests that calcification will decrease in many species, but compelling evidence of impaired functional performance of calcium carbonate structures is sparse, particularly in key species. Here we demonstrate that ocean acidification markedly degrades the mechanical integrity of larval shells in the mussel *Mytilus californianus*, a critical community member on rocky shores throughout the northeastern Pacific. Larvae cultured in seawater containing CO_2 concentrations expected by the year 2100 (540 or 970 ppm) precipitated weaker, thinner and smaller shells than individuals raised under present-day seawater conditions (380 ppm), and also exhibited lower tissue mass. Under a scenario where mussel larvae exposed to different CO_2 levels develop at similar rates, these trends suggest a suite of potential consequences, including an exacerbated vulnerability of new settlers to crushing and drilling attacks by predators; poorer larval condition, causing increased energetic stress during metamorphosis; and greater risks from desiccation at low tide due to shifts in shell area to body mass ratios. Under an alternative scenario where responses derive exclusively from slowed development, with impacted individuals reaching identical milestones in shell strength and size by settlement, a lengthened larval phase could increase exposure to high planktonic mortality rates. In either case, because early life stages operate as population bottlenecks, driving general patterns of distribution and abundance, the ecological success of this vital species may be tied to how ocean acidification proceeds in coming decades.

Key words: biomineralization, early survivorship, environmental change, form and function, shell properties.

INTRODUCTION

There is increasing urgency in organismal biology and ecology to understand the consequences of human-induced changes to the physical environment. Among the more effective approaches for pursuing this issue include those of ecophysiology and ecomechanics, which directly link functional performance of individuals to population-level factors (Helmuth et al., 2006; Denny and Gaylord, 2010; Kearney et al., 2010). Most studies attempting such integration have examined implications of thermal shifts tied to elevated atmospheric CO_2 concentrations and global warming. However, there is growing awareness that equivalently dramatic perturbations may ensue from the absorption of CO_2 into the world's oceans.

Between 25 and 40% of anthropogenic carbon emissions have entered the marine realm since the advent of the industrial age (Sabine et al., 2004; Zeebe et al., 2008). This process is decreasing seawater pH and carbonate ion concentration in a process termed 'ocean acidification' (OA) (Caldeira and Wickett, 2003). Global mean pH levels have declined 0.1 units since the year 1750, representing a 30% increase in acidity, and another 0.3–0.4 unit reduction is expected by 2100 (Orr et al., 2005). Mean carbonate ion concentrations in surface waters have dropped concomitantly, by 20–30% (Feely et al., 2004). Although the full breadth of impacts is still emerging (Hofmann et al., 2010), particular concern revolves around the capacity for reduced pH and carbonate ion to impair the ability of marine organisms to construct calcium carbonate ($CaCO_3$) shells and skeletons (Fabry et al., 2008; Doney et al., 2009).

Recent studies have documented important decreases in calcification in a number of taxa (e.g. Gattuso et al., 1998; Langdon et al., 2000; Riebesell et al., 2000; Gazeau et al., 2007; Hoegh-Guldberg et al., 2007; Kuffner et al., 2008; Miller et al., 2009; Ries et al., 2009; Kroeker et al., 2010). Resultant impacts on the mechanical function of affected carbonate structures, however, have received little attention – despite the connection of function to performance and, ultimately, fitness. Even in relatively well-studied organisms like corals it is uncertain whether OA-induced declines in skeletal growth and density increase vulnerability to storm breakage or damage from grazers like parrotfish (Hoegh-Guldberg et al., 2007). Similarly, although many temperate invertebrates rely on shells for protection, and although those shells could be weakened under OA, explorations of the effects of acidified seawater on shell strength have been limited (McDonald et al., 2009; Beniash et al., 2010; Welladsen et al., 2010).

Potential tradeoffs among calcification and other physiological responses are likewise poorly understood (Hofmann and Todgham, 2010). Most marine calcifiers can increase fluid pH and carbonate ion concentration at the site of crystal nucleation, which enables synthesis of shells and/or skeletons even when external seawater parameters are thermodynamically unfavorable for the formation of $CaCO_3$ (Cohen and Holcomb, 2009). Maintenance of local conditions that differ from those of surrounding waters, however, often depends on active ion transport that is energetically costly (Palmer, 1992; Cohen and Holcomb, 2009). Whether OA-induced energetic expenditures require that organisms differentially prioritize certain physiological processes is largely unknown (Widdicomb and Spicer, 2008). In shell-forming species, for instance, it is unclear whether decreased growth arises from somatic resources being redirected to fortify the shell or as a direct consequence of acidified seawater. If OA reduces both growth and shell integrity, it is uncertain which might be degraded more strongly.

Such questions of mechanical function and physiological prioritization apply with particular force to organisms that play key ecological roles. The California mussel, *Mytilus californianus*, operates as a foundation species, creating extensive beds that provide habitat, food and refuge for hundreds of organisms that live on exposed rocky shores throughout the northeastern Pacific (Suchanek, 1992). *Mytilus californianus* is also competitively dominant over much of its range, preventing other taxa from accessing primary substrate. The biological diversity of many intertidal communities is therefore linked tightly to the ecological performance of this bivalve (Paine, 1966; Dayton, 1971). Within this context, the performance of larvae and newly settled juveniles – as well as adults – are relevant, because early life stages often operate as demographic bottlenecks driving overall population abundance (Thorson, 1950; Gosselin and Qian, 1997).

Despite its community importance, impacts of ocean acidification on *M. californianus* have not been explored in any detail. Field transition data and modeling suggest a vulnerability of this species to decreased pH (Wootton et al., 2008), and effects of acidification have been observed in its congeners *M. edulis* and *M. galloprovincialis* (Bamber, 1990; Michaelidis et al., 2005; Berge et al., 2006; Gazeau et al., 2007; Beesley et al., 2008; Bibby et al., 2008; Kurihara et al., 2008; Ries et al., 2009; Gazeau et al., 2010). Direct OA tests on *M. californianus*, however, are lacking. It therefore remains difficult to assess the degree of impact, as well as the relative susceptibility to OA of various life stages in this species. It might be expected that larval responses would be especially pronounced because larvae are affected strongly by environmental stressors (Pechenik, 1999). Larval bivalves also employ amorphous $CaCO_3$ in their shells, a form of $CaCO_3$ that is particularly vulnerable to dissolution under low carbonate ion concentration (Weiss et al., 2002; Addadi et al., 2003).

In the present study, we tested effects of OA on *M. californianus*, focusing on the larval stage (Fig. 1). We raised larvae over their pelagic duration in controlled, elevated-CO_2 seawater cultures. CO_2 levels were selected to match predictions for the end of this century. Shell strength and area were measured at the middle of the larval period and immediately preceding the initiation of settlement (days 5 and 8 following fertilization, respectively), and shell thickness and larval tissue mass were assayed at day 8. Magnitudes of response of these performance indices were quantified to assess tradeoffs among competing demands and determine implications for early-life survivorship.

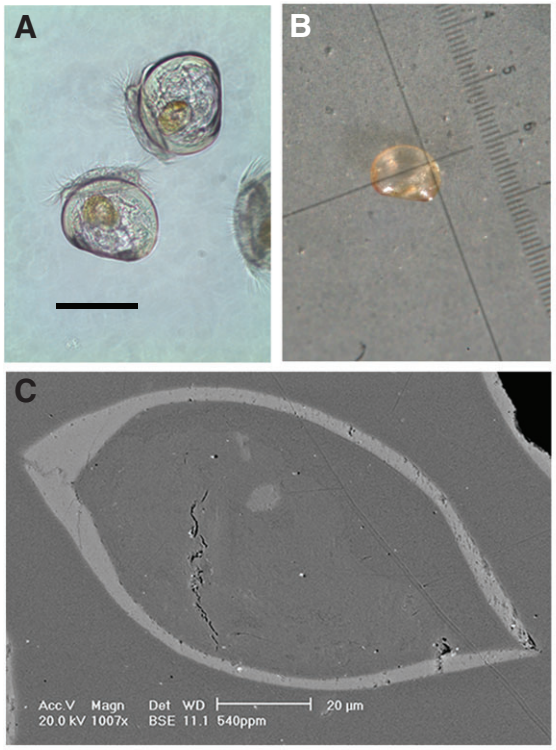

Fig. 1. Larvae of *Mytilus californianus* at various stages during the experiment. (A) Swimming veligers at day 5 post-fertilization. Scale bar, 100 µm. (B) Dried day-8 larva placed on a microscope slide for tests of shell strength. Smallest scale division equals 12.5 µm. (C) Representative scanning electromicrograph of a transverse section through both valves of a day-8 larva. Mean shell thickness was computed as the mean of four measurements made at equidistant locations around the shell, avoiding the thicker region near the hinge.

MATERIALS AND METHODS

Larval culturing

Thirty adult *Mytilus californianus* Conrad 1837 were collected from the mid-intertidal zone in the Bodega Marine Reserve, Bodega Bay, CA, USA (38°19.110′N, 123°04.294′W), on 6 January 2010 and placed immediately into running seawater. Spawning was induced the next day by scrubbing the mussels with a brush and immersing them for 1.5 h in 0.45 µm filtered seawater (FSW) with 30 mmol l^{-1} hydrogen peroxide buffered with 17 mmol l^{-1} TRIS (Trevelyan and Chang, 1983). The mussels were then rinsed in FSW and transferred to individual 0.5 l vessels, which were monitored until eggs or sperm were released. Eggs from a single female were removed to a clean 1 l beaker where they were fertilized with four drops of dilute sperm suspension from each of three males, followed by two rinses with FSW to minimize exposure to any residual TRIS or peroxide. The embryos were stirred briskly to establish a uniform suspension, and the concentration of embryos in the beaker was estimated by averaging the number of embryos in each of five 1 ml aliquots examined under a dissecting microscope (Leica M125). The beaker was again stirred and a volume sufficient to contain 1000 embryos was transferred to 2 l of FSW in each of eighteen 4.5 l glass culture jars.

Embryos and ensuing larvae in the culture jars were held at 15°C in two paired seawater tables, where they were subjected to controlled seawater CO_2 conditions throughout their pelagic period. CO_2 concentrations were matched to climate model projections for

the year 2100 (IPCC, 2007), with levels associated with a present-day global-mean atmospheric CO_2 concentration (~380 ppm) contrasted against a 'fossil-fuel intensive' projection (970 ppm; A1F1 emissions scenario) and a more optimistic prediction (540 ppm; B1 scenario). The CO_2 treatments were established by bubbling FSW for 2–3 days, prior to addition of larvae, with NIST-traceable gas mixtures containing the appropriate CO_2 concentrations. This adjustment was the dominant agent of CO_2 modification. CO_2 off-gassing during the culturing was prevented subsequently by pumping the same gas mixtures through the headspaces above the jars. Note that in this latter step it was necessary to supply headspaces above triplicate sets of jars on each seawater table by means of a single mixed-gas airstream. In theory, this feature could have reduced the degree of independence among jars. It is worth emphasizing, however, that biological processes likely dominated the minor fluctuations in chemistry of the already-CO_2-equilibrated seawater. These latter processes operated fully independently in each jar. The experimental design thus incorporated three jars placed in each of two tables, for each of three different CO_2 levels (three jars × two tables × three CO_2 levels=18 jars). The positions of CO_2 treatments within a table were determined randomly.

Preliminary experiments indicated that *M. californianus* from the Bodega area typically initiate settlement after a 9 day larval duration when cultured with full food rations at 15°C. Larvae were therefore allowed to develop through this period, during which the seawater within jars was stirred using oscillating paddles driven at 0.2 Hz using low-rpm electric motors. Every 2 days, 90% of the water in each jar was reverse-filtered through 37 μm mesh and exchanged with new FSW again pre-equilibrated with air at the appropriate CO_2 concentration. Immediately following each water change, 50,000 cells ml^{-1} of the microalga *Isochrysis galbana* were added for food (Strathmann, 1987).

Seawater chemistry

Total alkalinity (TA) of the seawater entering and exiting the culture jars was determined at each water change by automated Gran titration (Metrohm 809), and pH was measured on the National Bureau of Standards (NBS) scale (Zeebe and Wolf-Gladrow, 2001) using an Acumet Excel XL60D benchtop meter equipped with glass double-junction electrodes calibrated in low-ionic strength certified buffers. A subset of samples was analyzed for dissolved inorganic carbon (DIC) at the University of Georgia's infrared CO_2 analysis facility (Cai and Wang, 1998). Accepted methods (Dickson et al., 2007) were employed 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, CA, USA). Because three carbonate system parameters were measured and a fourth (CO_2) was prescribed, it was also possible to check for internal consistency among the full suite of parameters (including CO_2, HCO_3^-, CO_3^{2-}, H^+, DIC and TA), using the calculation software CO_2SYS (Lewis and Wallace, 1998). TA and DIC were employed as input variables because of their greater accuracy compared with pH determined using potentiometric methods, and recommended values for the equilibrium constants K_1, K_2 and K_{SO4} were used (Mehrback et al., 1973; Dickson and Millero, 1987; Dickson, 1990).

Shell strength

Shell strengths of the cultured larvae were measured individually (N=720) using a materials testing apparatus (Instron 3345, Norwood, MA, USA) as follows. Twenty veliger larvae were sampled haphazardly by pipette from each culture jar at both day 5 and day 8 post-fertilization, after which they were placed on 37 μm Nitex plankton filters and air-dried for 1 week. Although moisture content can influence mechanical properties in some biomaterials, the use of dried shells ensured uniformity across samples and also increased the abruptness with which they broke, facilitating identification of failure events. Following drying, each larva was transferred using an artist brush to a ground microscope slide, which was then positioned under a dissecting microscope (Leica S8APO) mounted beneath the crosshead of the materials testing apparatus. The position of the larva was adjusted with the fine tip of the artist brush until it was oriented flat on the slide, with one valve facing the microscope lens. The larva was then photographed at 80× magnification, with the scale of an ocular micrometer in the field of view to enable later determination of shell area (Fig. 1B). A 60 μm diameter, horizontally oriented stainless steel rod with its distal tip bent to the vertical was fixed in a 5 N load cell so that it extended into the field of view of the microscope. The crosshead was jogged downward to position the vertically oriented tip of the rod just above the shell, and the microscope slide was adjusted to center the larval shell under the rod tip. The crosshead was then activated to move at an extension rate of 1000 μm min^{-1}, crushing the larval mussel shell against the microscope slide. Force and position were recorded at 10 Hz by manufacturer software (Bluehill® version 2.21, Instron). Compressive extension continued until the shell broke, visible in 95% of cases as an abrupt step in the force record. Cases where an abrupt step was not visible were discarded as ambiguous and an additional sample was analyzed. Follow-up experiments indicated that ambiguous cases were associated with shells that had existing imperfections (e.g. small cracks or irregularities), presumably from damage that had occurred during pipetting.

Shell area

The sizes of unbroken shells from larvae sampled at day 5 and day 8 post-fertilization (N=20 for each of 18 jars per day) were determined from image analysis of the photographs taken at 80×. The projected area of a single valve was measured by tracing its outline using standard software tools (ImageJ, version 1.37, National Institutes of Health). Note that the measurements did not determine surface area proper because a projected area measurement does not follow the curved contour of the shell. All measurements were made by a single researcher, with the origin of each larval sample kept blind to eliminate the potential for subjective bias.

Shell thickness

Unbroken and dried day-8 mussel larvae (N=72) were analyzed by scanning electron microscopy to determine the influence of OA on shell thickness (Fig. 1C). The larvae were drawn evenly from across the 18 jars and three CO_2 treatments. They were encased in 0.25% Formvar film (Ted Pella, Inc., Redding, CA, USA) within single-hole copper grids, and were dehydrated in ascending concentrations of ethyl alcohol (30–100%; 10 min each step). Pure epoxy resin was infiltrated to replace the ethyl alcohol overnight. The grids with larvae were then placed in the bottom of a flat embedding mold, which was subsequently filled with additional epoxy resin and polymerized overnight in a 70°C oven. After extraction from the mold and removal of the copper grid, the resulting epoxy blocks were trimmed and faced on an ultra-microtome (Leica Ultracut UCT, Vienna, Austria) using a diamond knife. Effort was made to ensure that final cuts passed through the midlines of the shells, although due to their small size slight deviations could not always be prevented. For imaging, the epoxy blocks were mounted on

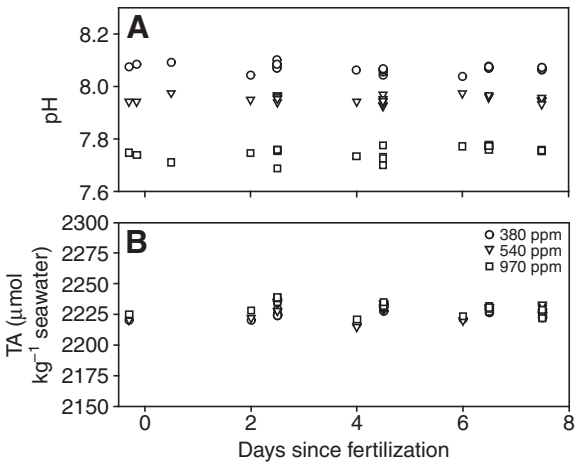

Fig. 2. Seawater properties in the culture jars used to raise mussel larvae. (A) pH. (B) Total alkalinity (TA). Mean pH and TA values differed significantly among the three treatments (Tukey's HSD, $P<0.05$). Distinct symbols indicate each of the three CO_2 levels: 380, 540 and 970 ppm. Note that some points on a given day within a CO_2 treatment cannot be distinguished because of overlap.

aluminum stubs using double-stick carbon tape, and were gold-coated using a PELCO SC-7 auto-sputter coater (Ted Pella Inc.). The blocks with larval samples were then viewed on a Philips XL30 TMP scanning electron microscope (FEI Company, Hillsboro, OR, USA) using a solid-state backscatter detector. Digital photographs were stored on computer and analyzed using ImageJ, calibrated with scale indicators embedded in the photographs.

Larval tissue mass

Masses of day-8 larvae were determined using standard methods. After placement on a 37 µm Nitex plankton filter, approximately 100 larvae from each culture jar were rinsed twice with distilled water, with all excess distilled water wicked immediately through the back of the filter. The larvae were transferred individually with an artist brush to small aluminum vessels (pre-ashed at 500°C for 3 h) and were dried at 50°C for >1 day, after which they were weighed on a microbalance (Sartorius Ultramicro, Goettingen, Germany). The larvae were dried in their vessels again for 1 day, weighed a second time to verify negligible weight loss (<0.01%) and then combusted at 460°C for 4 h in a muffle furnace (Thermo Scientific FB1415M) to volatilize the organic matter. Ash-free dry tissue weights were determined from the difference in weights before and after combusting, and total dry weights (i.e. weights including the shell) were determined by subtracting ashed weights of the empty aluminum vessels. A standard condition index was computed by converting weights to masses and taking the ratio of dry tissue mass to total dry mass.

Statistical analyses

Impacts of CO_2 concentration on seawater chemistry, shell properties and tissue mass were tested using ANOVA and JMP software (version 8.0, SAS Institute, Inc., Cary, NC, USA). Potential effects of CO_2 and exposure to a particular headspace airstream were assessed using a mixed-model ANOVA with CO_2 the main, fixed factor and airstream (nested within CO_2) as the subsidiary factor, and with the mean response of a jar employed as the replicate. If an airstream effect was detected, the pattern of response was inspected further, because of the potential for airstream and table effects to be confounded (because a given airstream supplied jars on only one table). The latter scenario was evaluated under the additional assumption that jars with a given airstream functioned independently, using two-way ANOVA with CO_2 and table as fixed factors, and with the mean response of a jar again employed as the replicate. In the absence of an airstream or table effect, or a significant interaction term, jar means were pooled across airstreams and tables, and a simple one-way ANOVA was run with CO_2 as the sole factor. When a significant difference among means was detected, a Tukey's honestly significant difference (HSD) multiple comparisons test was used to determine which means differed. Mean values are presented ±s.e.m.

RESULTS

Seawater chemistry

Mussel larvae cultured in jars equilibrated with the three different atmospheric CO_2 concentrations experienced significantly different seawater chemistries during their pelagic phase (ANOVA, $F_{2,15}=34015$, $P<0.0001$; Fig. 2A). Mean pH across all 380 ppm control jars was 8.06±0.001, whereas the 540 and 970 ppm jars had mean pH values of 7.96±0.001 and 7.75±0.001, respectively. TA differed only slightly among CO_2 levels, as would be expected, with the 380 and 970 ppm treatments exhibiting values of 2223±0.15 and 2228±0.29 µmol kg^{-1} seawater, respectively (ANOVA, $F_{2,15}=102.34$, $P<0.0001$; Fig. 2B).

Checks on internal consistency of the carbonate system demonstrated that pH_{NBS} and seawater P_{CO_2} calculated from TA and DIC were within 1.2 and 8.6% of measured values (means within 1.0 and 6.6%; Table 1), respectively. Calculated pH_{NBS} values were consistently 0.08 units higher than those measured, corresponding to a ~17% discrepancy in $[H^+]$ due to the logarithmic nature of the pH scale. Such offsets are not unusual for potentiometric pH

Table 1. Mean (±s.d.) carbonate system parameters during the larval culturing

	Supplied or measured parameters			Calculated parameters based on TA/DIC		
Treatment gas P_{CO_2} (ppm)	pH_{NBS}	TA (µmol kg^{-1} seawater)	DIC (µmol kg^{-1} seawater)	Carbonate saturation state ($\Omega_{aragonite}$)	Seawater P_{CO_2} (ppm)	pH_{NBS}
380±8	8.07±0.01 (28)	2226±4 (27)	2019±6 (3)	2.28±0.02	405±10	8.15±0.01
540±11	7.95±0.01 (28)	2229±5 (27)	2071±2 (3)	1.82±0.03	545±7	8.03±0.01
970±19	7.75±0.02 (28)	2230±5 (27)	2144±2 (3)	1.19±0.02	928±21	7.83±0.01

Precision of CO_2 concentrations of NIST-certified, pre-mixed treatment gases are as stated by the supplier (Airgas, Inc.).
The saturation state for the aragonite polymorph of calcium carbonate was calculated as $\Omega_{aragonite}=[Ca^{2+}][CO_3^{2-}]/K_{sp}$, where K_{sp} is the solubility product (Zeebe and Wolf-Gladrow, 2001).
The number of samples is indicated in parentheses.
Salinity and temperature (mean ± s.d.) were 15.37±0.15°C and 34.00±0.11 psu, respectively.
DIC, dissolved inorganic carbon; TA, total alkalinity.

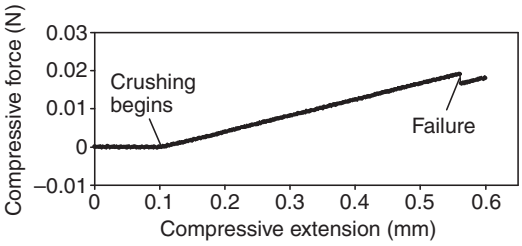

Fig. 3. Representative strength test of a larval mussel shell, showing how force increased linearly with shell compression. Compressive forces were intended as a mimic to crushing attacks by predators such as crabs. However, because failure in biomaterials often varies with the speed and geometry of force application, and with hydration state, results are unlikely to duplicate natural predatory interactions in all respects. The slope of the trace does not provide a measure of shell stiffness because of compliance in the testing apparatus.

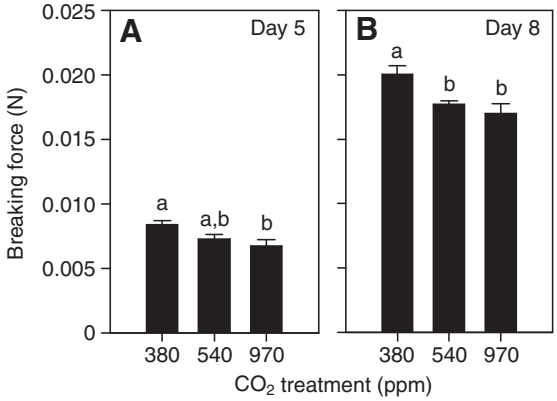

Fig. 4. Shell breaking force as a function of CO_2 treatment at (A) day 5 and (B) day 8 post-fertilization in mussel larvae. Shared letters above bars in each panel indicate shell breaking forces that did not differ (Tukey's HSD, $P<0.05$). Error bars denote +s.e.m.

measurements given that pH_{NBS} values can displaced from absolute levels by magnitudes approaching 0.1 units, mostly from large inter-electrode differences in liquid junction potential (Dickson, 1984). The offsets do not influence the validity of relative comparisons among pH data acquired, as in the present study, by means of a single electrode.

Effects on shell strength

Cultured larvae placed in the materials testing apparatus sustained linearly increasing compressive forces until a point of catastrophic shell failure (Fig. 3). As is often seen in mechanical tests, the force required to induce breakage varied appreciably among individuals. Coefficients of variation for breaking force ranged from 18 to 43% in a given culture jar. Mean strength of the larval shells also increased over the course of development, with breaking forces from day 8 exceeding those from day 5 by over a factor of two (Fig. 4). Note that one 380 ppm control culture developed a filamentous algal bloom and the mean shell strength of this jar failed outlier tests (Dixon's $r_{10}=5.58$, $N=6$, $P<0.05$; Grubb's $G=1.83$, $N=6$, $P<0.05$). This jar was therefore discarded from subsequent analyses.

The degree of seawater acidification strongly impacted shell strength on both day 5 and day 8 after fertilization (ANOVA, day 5, $F_{2,15}=4.47$, $P=0.03$; day 8, $F_{2,14}=6.48$, $P=0.01$; Fig. 4). At day 5, larvae reared in the 540 ppm CO_2 treatment had shells that were 13% weaker than those of control individuals, and individuals reared in the 970 ppm CO_2 treatment had shells that were 20% weaker. At day 8, the corresponding reductions in shell strength were 12 and 15%.

Impacts on shell area

Ocean acidification had a much smaller effect on larval shell growth (Fig. 5) compared with its impact on strength. At day 5, larvae reared in the 970 ppm CO_2 treatment had shells that were 7% smaller in area than those of control individuals. At day 8, the corresponding reductions in shell area were 5%. The decreases in growth were not the principal cause of the reductions in shell strength, as there was no relationship between shell area and strength on a given day. In addition to the main treatment effect, the pattern of variation in shell area among jars also implied a significant effect of the nested factor airstream [CO_2] in the mixed-model ANOVA (day 5 CO_2, $F_{2,12}=37.94$, $P<0.0001$; day 5 airstream [CO_2], $F_{3,12}=3.94$, $P=0.04$; day 8 CO_2, $F_{2,11}=16.59$, $P<0.0005$; day 8 airstream [CO_2], $F_{3,11}=13.55$, $P<0.0005$). However, further examination suggested that the nested effect actually resulted from jars of a given airstream being located in a specific table. In particular, shell areas of larvae reared in the second of the two tables were uniformly smaller, regardless of CO_2 level (Fig. 5), and no airstream-associated differences in water chemistry were evident. Consistent with the interpretation of a table effect, a two-way ANOVA with CO_2 and table as fixed factors indicated a significant effect of both factors on shell area, with no interaction between them, for both day-5 and day-8 larvae (day 5 CO_2, $F_{2,14}=40.26$, $P<0.0001$; day 5 table, $F_{1,14}=11.28$, $P<0.005$; day 8 CO_2, $F_{2,13}=19.28$, $P=0.001$; day 8 table, $F_{1,13}=46.17$, $P<0.0001$; Fig. 5). These results suggest a subtle impact of CO_2 on growth rivaled by minor experimental effects associated with use of a particular seawater table. Although possible causes of a table effect are uncertain, mussel growth varies with seawater temperature (Widdows, 1991), and the two tables differed in mean temperature by ~0.2°C. Such apparent sensitivity to experimental conditions indicates a place for substantial care in the design of OA culturing studies.

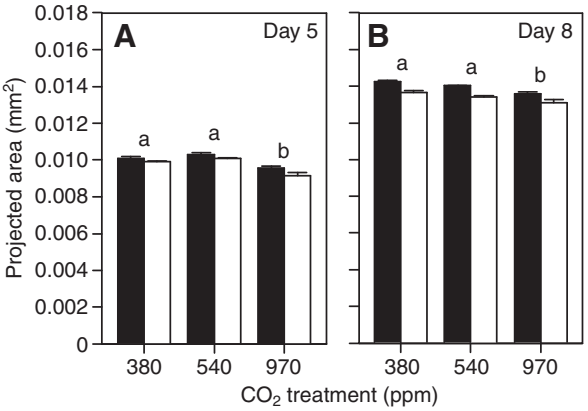

Fig. 5. Shell area as a function of CO_2 treatment at (A) day 5 and (B) day 8 post-fertilization for mussel larvae cultured in each of two seawater tables (black and white bars for each CO_2 level). Shared letters above paired bars in each panel indicate mean shell areas that did not differ among CO_2 treatments (Tukey's HSD, $P<0.05$). Note that shell areas differed subtly but significantly between larvae raised in the two seawater tables ($P<0.05$). Error bars denote +s.e.m.

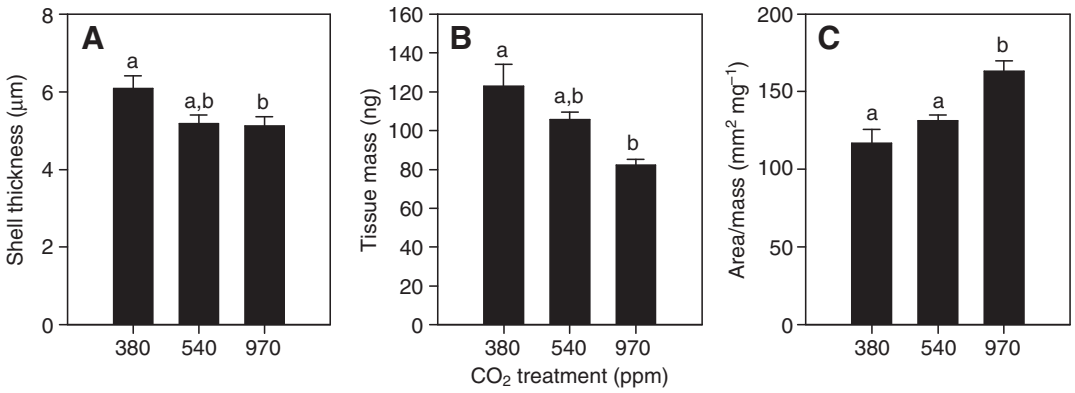

Fig. 6. Effects of acidified seawater on additional day-8 larval parameters potentially influencing early-life survivorship of mussels. (A) Mean larval shell thickness. (B) Mean dry tissue mass. (C) Mean ratio of surface area to dry tissue mass. Shared letters above bars in each panel indicate parameters that did not differ among CO_2 treatments (Tukey's HSD, $P<0.05$). Error bars denote +s.e.m.

Effects on shell thickness and tissue mass

Other factors tied to larval shell strength and growth were also impacted by acidification. Shell thickness was significantly altered under OA (ANOVA, $F_{2,15}=4.29$, $P=0.03$; Fig. 6A), leading to larval shells at day 8 that were approximately 15% thinner in elevated CO_2 treatments compared with the control. The condition index (ratio of ash-free dry tissue mass to total dry mass) of larvae varied significantly across treatments (ANOVA, $F_{2,13}=8.49$, $P=0.004$), with larvae from the 970 ppm treatment exhibiting values that were 17% lower than those of larvae from 540 ppm and control cultures. Note, however, that because shell mass enters into the calculation of the condition index and was reduced in the elevated-CO_2 treatments, dry tissue mass may provide a more direct measure of the energetic status of larvae. Dry tissue mass varied across CO_2 treatments (ANOVA, $F_{2,13}=9.93$, $P=0.002$; Fig. 6B), with values for larvae from the 540 and 970 ppm treatments reduced by 14 and 33%, respectively, relative to that of the control. The relationship between shell area and dry tissue mass differed substantially across treatments (ANOVA, $F_{2,13}=12.88$, $P=0.0008$; Fig. 6C), with larvae cultured in 970 ppm CO_2 seawater exhibiting an area/mass ratio that was 40% larger than that of control larvae.

DISCUSSION

Functional costs of ocean acidification

The role of $CaCO_3$ as a key building block for support structures such as shells, skeletons and spines is well recognized. Although decreased calcification under OA is not universal (Gutowska et al., 2008; Iglesias-Rodriguez et al., 2008; Wood et al., 2008; Checkley et al., 2009; Ries et al., 2009; Dupont et al., 2010; Hofmann et al., 2010; Kroeker et al., 2010), much of the concern regarding OA derives from the widespread assumption that acidified seawater will impair function in affected structures. Studies explicitly testing for declines in the performance of calcified hard parts – as opposed to just changes in growth rates or sizes – however, are rare and limited to a subset of life stages (i.e. primarily only juveniles and adults) (McDonald et al., 2009; Beniash et al., 2010; Welladsen et al., 2010). It therefore remains difficult to anticipate whether OA will generally compromise the ability of these protective and support structures to serve their purpose. This gap in understanding degrades the ability of scientists to effectively forecast ocean acidification's full consequences.

The observed OA-induced decrease in shell integrity in *M. californianus* represents a clear functional decline. It must be recognized that this decline in strength need not proceed inevitably from reductions in calcification and/or shell thickness. On the contrary, there can be considerable complexity in the relationship between shell properties and breaking force. Consider, for example, the morphological plasticity in shell architecture common to many invertebrates, which arises both at micro-crystalline scales and at the scale of whole-shell shape (Bibby et al., 2007; Bourdeau, 2010). If a valve of a larval shell is approximated as a hemispherical dome, peak stresses (σ) induced by a compressive force (F) applied at the shell's apex can be quantified as:

$$\sigma = C\frac{F}{t^2}\ , \qquad (1)$$

where t is the thickness of the shell and C is a coefficient that varies with shell geometry, material properties and other factors (Timoshenko and Woinowsky-Krieger, 1959; Young, 1989). The presence of t^2 in the denominator indicates that a thinner shell will experience greater stresses and an elevated risk of breakage, all else held equal. However, C declines rapidly as the shell's radius of curvature decreases. According to this relationship, morphologically plastic larvae could produce equivalently strong, but thinner, shells simply by forming more highly domed structures. Along similar lines, many invertebrates have the capacity to alter the crystalline structure of their shells. Bivalves employ different polymorphs of $CaCO_3$ during various developmental stages (including amorphous $CaCO_3$, aragonite and calcite) and incorporate different combinations of polymorphs as a function of environmental conditions (Hubbard et al., 1981; Al-Dabbas et al., 1984; Falini et al., 1996; Weiss et al., 2002; Addadi et al., 2003). Each of these changes has the potential to influence shell integrity. It is therefore notable that *M. californianus* larvae, even in the face of multiple factors under biological control, still experience appreciable reductions in shell strength when exposed to OA. Such reductions may in fact be common in bivalves given that similar effects on shell size and thickness have been observed in other species (Gazeau et al., 2010; Talmage and Gobler, 2010).

Results of the present study are insufficient to isolate the precise causes of decreased shell strength. Magnitudes of observed shell thinning are large enough to account for the pattern, with Eqn 1 predicting a 28% decline in sustainable stress (or equivalently, breaking force) due to a 15% decrease in thickness. The somewhat lower reduction in measured strength (Fig. 4B) compared with the predicted decrease could reflect subtle shifts in shell architecture or

material properties. However, no obvious changes to shell characteristics other than thickness were apparent. Whether shell thinning itself resulted from bulk declines in accretion, reductions in specific crystalline layers or external dissolution is also unclear. Although the fact that aragonite saturation states in the cultures did not drop below a threshold of 1.0 (Table 1) suggests a limited role for passive dissolution (but see Nienhuis et al., 2010), bivalves subjected to pH<7.5 actively reabsorb shell material to maintain an internal acid–base balance (Lindinger et al., 1984; Michaelidis et al., 2005).

Neither shell production nor somatic growth was obviously prioritized. Shell strength, shell area, shell thickness and ash-free dry tissue mass all decreased in the high-CO_2 treatments. However, there is some indication that shell area was influenced to a lesser degree than shell strength or thickness, and that tissue mass was more heavily affected than other factors. Note that although the quantity or quality of food may have differed among treatments because of CO_2 effects on phytoplankton added to the cultures, such an effect would have likely increased food levels in the acidified treatments, thereby tending to offset observed impacts.

Potential ecological repercussions

An evaluation of the population-level implications of OA for *M. californianus* requires considering certain nuances in how ocean acidification might influence larval development in this species. Two general projections are possible. Under the assumption that mussel larvae complete development at approximately the same rate regardless of CO_2 level, the full suite of impacts observed on day 8 would likely apply to settling individuals, given a 9 day pelagic period. Alternatively, if the primary consequence of elevated CO_2 is to slow development, observed effects on larval size and shell mechanics could be transient. In this latter scenario, all larvae, regardless of CO_2 exposure, could eventually reach the same size and produce shells of equivalent strength prior to the initiation of settlement. It would simply take individuals exposed to elevated CO_2 longer to reach this point. Developmental delays of this nature have been observed in a number of taxa as a consequence of low food availability (e.g. Strathmann et al., 1992; Strathmann et al., 2008). A combination of both persistent impacts and delayed development is, of course, also possible (e.g. Talmage and Gobler, 2010).

Because all larvae in the current experiment were sampled by day 8, none of the above alternatives can be immediately discounted. Nonetheless, an initial assessment of potential ramifications of the two outcomes is possible. In the case of unaltered larval durations where observed impacts likely hold at settlement (including reduced shell strength, thickness and size, together with decreased tissue mass; Figs 4–6), there are at least three critically important implications for the ecology of *M. californianus*. Consider first the role of shell strength (Fig. 4) in predation resistance. Although it is uncertain how much protection shells afford larvae in the plankton (probably little against planktivorous fish; perhaps some against larval crustaceans that employ raptorial or crushing attacks) (Young and Chia, 1987; Rumrill, 1990), mussels retain their larval shells upon settlement. Mortality rates in newly settled invertebrates can exceed 90% in early juvenile life, with up to 30% of individuals dying within the first 1–2 days following entry into benthic habitat (Gosselin and Qian, 1997). A substantial component of this mortality is thought to accrue from predation (Gosselin and Qian, 1997; Hunt and Scheibling, 1997), in the case of mussels often due to juvenile crabs that feed by crushing their prey. An additional class of predation-induced mortality arises from carnivorous snails that drill through shells of bivalves. Whelks of the genus *Nucella* operate as major consumers of *M. californianus* in southern portions of this mussel's range (Sanford and Worth, 2009), and it is likely that thinner-shelled mussels (Fig. 6A) experience elevated vulnerability to this feeding strategy.

Observed reductions in larval condition create a second class of potential impacts on the success of new settlers (Fig. 6B). Juvenile survivorship and growth are often depressed in individuals that settle at smaller size or have smaller lipid stores (Phillips, 2002; Phillips, 2004; Pechenik, 2006). The acidification-induced decline in condition index and >30% decrease in dry tissue mass (Fig. 6B) implies that OA may contribute to the suite of environmental factors that induce energetic stresses around the critical time of metamorphosis.

A third consideration emerges from the differential influence of acidification on shell area and body mass. Desiccation during low tide joins predation as one of two dominant agents of mortality in newly settled juveniles (Gosselin and Qian, 1997; Bownes and McQuaid, 2009). Although drying rates of emergent organisms depend on multiple factors tied to evaporative water loss and heat gain, they are strongly dependent on the ratio of surface area to mass. Projected areas determined in the present study are not precisely coincident with surface areas, but provide a relevant index for scaling rates of thermal input. Likewise, dry tissue mass correlates strongly with wet tissue mass, and thus provides a measure of how much dehydration could take place before an individual dries out during low tide. Area/mass ratios in day-8 larvae therefore yield a first-order estimate of susceptibility to desiccation at the larval–juvenile transition. The dramatic 40% increase in this ratio for late-stage larvae reared in elevated-CO_2 conditions suggests the potential for increased desiccation-induced death rates in new recruits.

Potential consequences of a lengthened larval duration are also substantial – even if impacts on shell and tissue properties are completely attenuated by the time of settlement. In such a situation, the major cost of OA may be the extended exposure to high mortality rates believed to be characteristic of the planktonic environment. Such rates can exceed several percent per day (Rumrill, 1990), so even a modestly longer pelagic phase could appreciably elevate accumulated mortality over the course of the larval period.

Regardless of whether mussel larvae or newly settled juveniles are ultimately affected more strongly by OA, reduced survivorship during early life can have repercussions for ensuing population structure and dynamics. Initial age classes often operate as demographic bottlenecks constraining abundances of individuals in subsequent stages (Thorson, 1950; Gosselin and Qian, 1997; Hunt and Scheibling, 1997; Bownes and McQuaid, 2009). Given the foundational role of *M. californianus* in rocky shore communities, a decrease in later-stage mussel densities could alter occupancy rates in what are frequently space-limited ecosystems (Wootton et al., 2008). Such effects could, in turn, influence the dynamics of patch formation and loss, known to play important roles in controlling species diversity (Dayton, 1971; Paine and Levin, 1981). Although the degree to which such cascading effects might arise requires further study, research in other systems has revealed OA impacts on species interactions and trophic dynamics (Hall-Spencer et al., 2008). Indeed, the possibility that OA might increase feeding rates in the keystone seastar *Pisaster ochraceus* (Gooding et al., 2009) introduces a scenario whereby enhanced predation by a major consumer of *M. californianus* could combine with multiple additional factors, including greater consumption by other predators (crabs and whelks), elevated energetic stresses and exacerbated

desiccation, to alter mussel survivorship and thus overall community dynamics.

Interestingly, *M. californianus* would not have been an immediately obvious candidate to exhibit vulnerability to OA. California mussels in the northeastern Pacific have a long history of exposure to waters of low pH and low carbonate saturation state. Even without OA, pH values in the California Current system drop below 7.6 during episodes of upwelling of high-CO_2 waters (Hauri et al., 2009). One might therefore have expected that *M. californianus* would be well adapted to, and minimally affected by, acidified seawater. The opposing outcome seen in the present study reiterates the potential seriousness of OA; clearly many and strong effects can arise even where one might expect them to be few and weak. Further work using mechanistic approaches that reveal functional, ecologically relevant costs of OA are necessary to improve understanding of this major anthropogenic alteration to the world's oceans.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for valuable feedback. P. Kysar and G. Abramson helped with electron microscopy; W. J. Cai and Y. Wang assisted with chemical measurements; and E. Chang and J. Clegg loaned equipment and provided insightful discussion. This work was funded by NSF grants OCE-0927255 and OCE-1041089, support from the University of California Multicampus Research Programs and Initiatives, and a grant from the UC Davis Academic Senate Committee on Research.

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