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Saturation-state sensitivity of marine bivalve larvae to ocean acidification

George G. Waldbusser[1]*, Burke Hales[1], Chris J. Langdon[2], Brian A. Haley[1], Paul Schrader[2], Elizabeth L. Brunner[1], Matthew W. Gray[2], Cale A. Miller[3] and Iria Gimenez[1]

Ocean acidification results in co-varying inorganic carbon system variables. Of these, an explicit focus on pH and organismal acid–base regulation has failed to distinguish the mechanism of failure in highly sensitive bivalve larvae. With unique chemical manipulations of seawater we show definitively that larval shell development and growth are dependent on seawater saturation state, and not on carbon dioxide partial pressure or pH. Although other physiological processes are affected by pH, mineral saturation state thresholds will be crossed decades to centuries ahead of pH thresholds owing to nonlinear changes in the carbonate system variables as carbon dioxide is added. Our findings were repeatable for two species of bivalve larvae could resolve discrepancies in experimental results, are consistent with a previous model of ocean acidification impacts due to rapid calcification in bivalve larvae, and suggest a fundamental ocean acidification bottleneck at early life-history for some marine keystone species.

Ocean acidification (OA) is described as an imbalance between the acidic influence of rapidly accelerating anthropogenic CO_2 emissions and the slow buffering response due to weathering of continental rock and carbonate marine sediment, causing increased acidity of marine waters[1,2]. The release of CO_2 from fossil fuel emissions and cement production, and decreasing uptake efficiency of CO_2 by land and sea has resulted in the fastest increase in p_{CO_2} (partial pressure of carbon dioxide) in the past 800,000 years[3]. Conversely the natural mechanisms that buffer acidic perturbations from increasing p_{CO_2} occur over timescales of hundreds of thousands to millions of years[1,2]. Modern anthropogenic changes in the open ocean have tightly coupled aqueous p_{CO_2}, pH and mineral solubility responses, but it was not always thus. Previous instances of elevated p_{CO_2} in the geologic record, such as the Cretaceous, seem to coincide with significantly elevated alkalinity[4], and were fairly benign with respect to OA, with elevated p_{CO_2} not indicative of low pH or mineral corrosivity. Throughout the geologic record and in many coastal habitats the marine carbonate system decouples, resulting in changes in pH, p_{CO_2} and saturation state that do not follow the co-variance assumed for modern open-ocean average surface waters[5].

Effects of ocean acidification on a suite of marine organisms have been the subject of significant recent work. Although many experimental results have shown equivocal impacts when taken in composite, the process of calcification has mostly exhibited negative sensitivity to OA (ref. 6). Physiological processes that may experience OA sensitivity occur across all taxa in nearly all natural waters; however, persistent calcified structures can elevate species that precipitate calcium carbonate to keystone status in marine waters. Bivalves, which provide a number of critical ecosystem services, have been noted as particularly sensitive to OA (refs 7–10). Some experiments have even found OA impacts at present-day, compared with pre-industrial, p_{CO_2} levels[11]. Marine bivalves seem to be sensitive to OA owing to the limited degree to which they regulate the ionic balance and pH of their haemolymph (blood)[12–15], and acute sensitivities at specific, short-lived, life-history stages that may result in carryover effects later in life[16–20]. Bivalve larvae are particularly sensitive to OA during the hours- to days-long bottleneck when initial shell (called prodissoconch I or PDI) is formed during embryogenesis[17]. Before PDI shell formation, larvae lack robust feeding and swimming appendages and must rely almost exclusively on maternal energy from eggs; and during calcification of PDI the calcification surfaces are in greater contact with ambient seawater than during following shell stages[17]. Failure of larvae to complete shell formation before exhausting maternal energy reserves leads to eventual mortality, as seen in well-documented oyster hatchery failures[18]. So far, the prevailing physiological mechanism identified for OA effects on organisms has been in their ability to regulate internal acid–base status; however, short-term exposure impacts and carryover effects documented in bivalve larvae[18–21] and greater exposure of PDI calcification to ambient seawater[17] points to another mechanism for the early larval sensitivity not captured by regulation of internal acid–base chemistry[22].

In most natural waters the dissolved inorganic carbon (DIC) system controls both pH and the thermodynamic mineral solubility (saturation state), but in different ways. pH is determined by the ratio of dissolved concentrations of CO_2 to carbonate ions, whereas saturation state is predominantly controlled by absolute carbonate ion concentration. The potential that organisms will respond differently to pH (ratio) or mineral saturation state (abundance), highlights how the decoupling of carbonate system variables in coastal zones[5] or geologic time[1,2] provides a formidable challenge in interpreting and predicting organismal responses to OA. The seemingly simple experimental perturbation of CO_2 bubbling results in the

[1]College of Earth, Ocean, and Atmospheric Sciences, Oregon State University, 104 COAS Admin. Bldg., Corvallis, Oregon 97331, USA. [2]Coastal Oregon Marine Experimental Station and Department of Fisheries and Wildlife, Hatfield Marine Science Center, Oregon State University, 2030 SE Marine Science Drive, Newport, Oregon 97365, USA. [3]Department of Fisheries and Wildlife, Oregon State University, 104 Nash Hall Oregon State University, Corvallis, Oregon 97331, USA. *e-mail: waldbuss@coas.oregonstate.edu

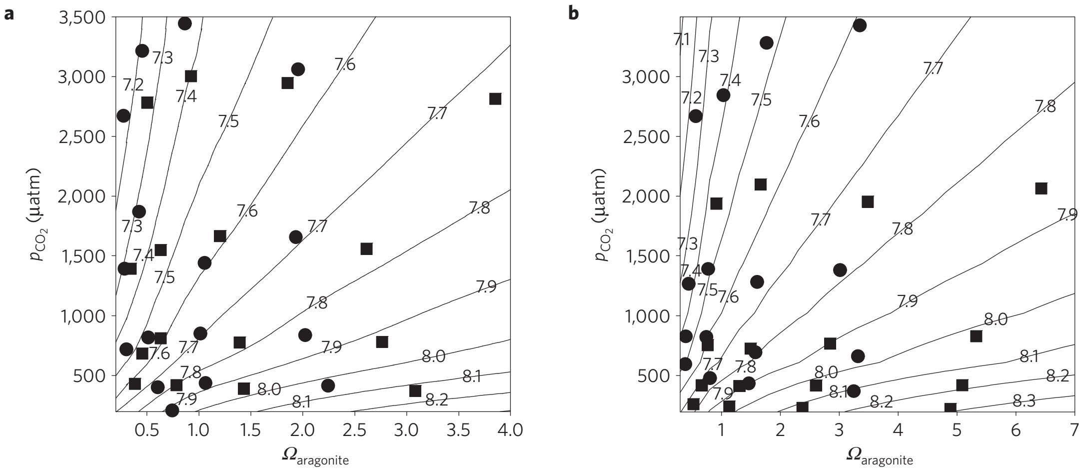

Figure 1 | Carbonate chemistry values for the 16 experimental treatments for each of the four experiments grouped by species, plotted against p_{CO_2} and saturation state, with isopleths of pH plotted in p_{CO_2}/saturation state space. a, Values for the two experiments on *Mytilus galloprovincialis*. **b**, Values for the two experiments on *Crassostrea gigas*. Circle and square symbols represent chemistry for the first and second experiments, respectively.

equilibrium redistribution of the acid–base species with pH, saturation state, p_{CO_2} and dissolved inorganic carbon (DIC) all changing simultaneously. The co-variance of carbonate parameters leaves interpretation of experimental responses unclear if organismal sensitivity to each parameter is physiologically distinct, particularly if the importance of each process varies across ontology (for example, respiration, shell formation, feeding rate). The underlying mechanisms of organismal sensitivity to OA may therefore not be constrainable without special experimental techniques.

We conducted series of experiments in which we applied a unique chemical manipulation approach to decouple the carbonate system parameter-covariance and evaluated larval growth and development of two bivalve species: the Pacific oyster, *Crassostrea gigas*, and the Mediterranean mussel, *Mytilus galloprovincialis*. Through simultaneous manipulation of DIC and alkalinity, we generated a 4×4 factorial design with aragonite saturation state (Ω_{ar}) and p_{CO_2}. Our experimental design separated Ω_{ar} and p_{CO_2} effects on larval responses; and responses to pH were evaluated by examining responses to pH within a p_{CO_2} and Ω_{ar} treatment level. Using this approach, we assessed which carbonate system parameter is most important to early larval shell development and growth: pH, p_{CO_2}, or Ω_{ar}.

Results

We successfully decoupled pH, p_{CO_2}, and Ω_{ar} experimentally to find Ω_{ar} as the primary variable affecting early larval shell development and growth in these two bivalve species. Below we describe why this direct sensitivity to Ω_{ar} demands a refinement of our current model of OA responses of calcifying organisms, and the environmental relevance of these results.

Chemistry manipulations. We simultaneously altered abundance and ratio of DIC and alkalinity to provide three orthogonal experimental axes in pH, p_{CO_2} and saturation state of the calcium carbonate mineral aragonite (Ω_{ar}) (Fig. 1 and Supplementary Table 1). The sensitivity of these parameters to DIC:alkalinity means that there is some variability within treatment suites, but that variability was far less than the differences among treatments. We were able to replicate treatment conditions via DIC and alkalinity, as evidenced by the concordance between expected versus measured values (Supplementary Fig. 1). At the termination of the 48 h incubation period we found p_{CO_2} generally increased by approximately 10–30% relative to initial conditions. The greatest p_{CO_2} increases were in treatments with the poorest larval development, probably due to elevated microbial respiration associated with larval mortality in these treatments.

Prodissoconch I shell development. The dominant effect of Ω_{ar} on proportion normal shell development (PNS) is immediately apparent in Fig. 2. The Ω_{ar} effect is clear for both species, with highly significant effects (Mussels $F_{3,15} = 105.53$, $p < 0.0001$, Oysters $F_{3,15} = 76.79$, $p < 0.0001$, Supplementary Table 2); Ω_{ar} explained 88% and 86% of the variance in proportion normal for the mussels and oysters, respectively. p_{CO_2} and the interaction between Ω_{ar} and p_{CO_2} were not significant (Supplementary Table 2). Experiment number was found to be statistically significant, but only explained 3% and 6% of the variance for the mussels and oysters, respectively (Supplementary Table 2). We fit a three-parameter logistic equation to the untransformed treatment means of PNS (Fig. 2) to determine the functional response of both species to saturation state. The fit was found to be highly significant for mussel ($F_{2,29} = 223.01$, $R^2 = 0.93$, $p < 0.0001$) and oyster larvae ($F_{2,29} = 72.61$, $R^2 = 0.83$, $p < 0.0001$).

Our results unequivocally show that saturation state is the primary carbonate system variable of importance for normal shell development for these two bivalve species; we will, however, further explore possible pH effects in our experiments, given its importance to physiological acidosis and the historical emphasis on pH in OA experiments. Because pH covaries with the primary factors in the analysis of variance (ANOVA), and a slight visual pattern is apparent (Fig. 2), we ran a series of regression analyses of PNS versus pH, within a saturation state treatment. Although we found some statistically significant slopes, generally in the low-Ω_{ar} treatments (Supplementary Table 3), the effect is equivocal and its magnitude markedly smaller than the Ω_{ar} effect. The largest effect we found was in the lowest saturation state treatment for oysters, with a 0.1 increase in PNS per 0.1 pH units from pH 7.27 to 7.51. Other significant slopes were less than half of this, 0.02 to 0.04 PNS per 0.1 pH unit within a Ω_{ar} treatment. The pH effect across the entire experimental range seen in Fig. 2 is, therefore, primarily an artefact of pH covariance with Ω_{ar}. Furthermore, at pH values of <7.6 and <7.4 in the mussel and oyster experiments respectively, we still see excellent PNS of >80% if Ω_{ar} is high. We therefore reiterate that Ω_{ar} is the primary carbonate system variable driving successful shell development of early larvae in these two species.

Shell growth. Even among larvae that seemed to develop normal shell morphology, Ω_{ar} was still the primary factor influencing growth (Fig. 3 and Supplementary Table 4). Ω_{ar} had statistically

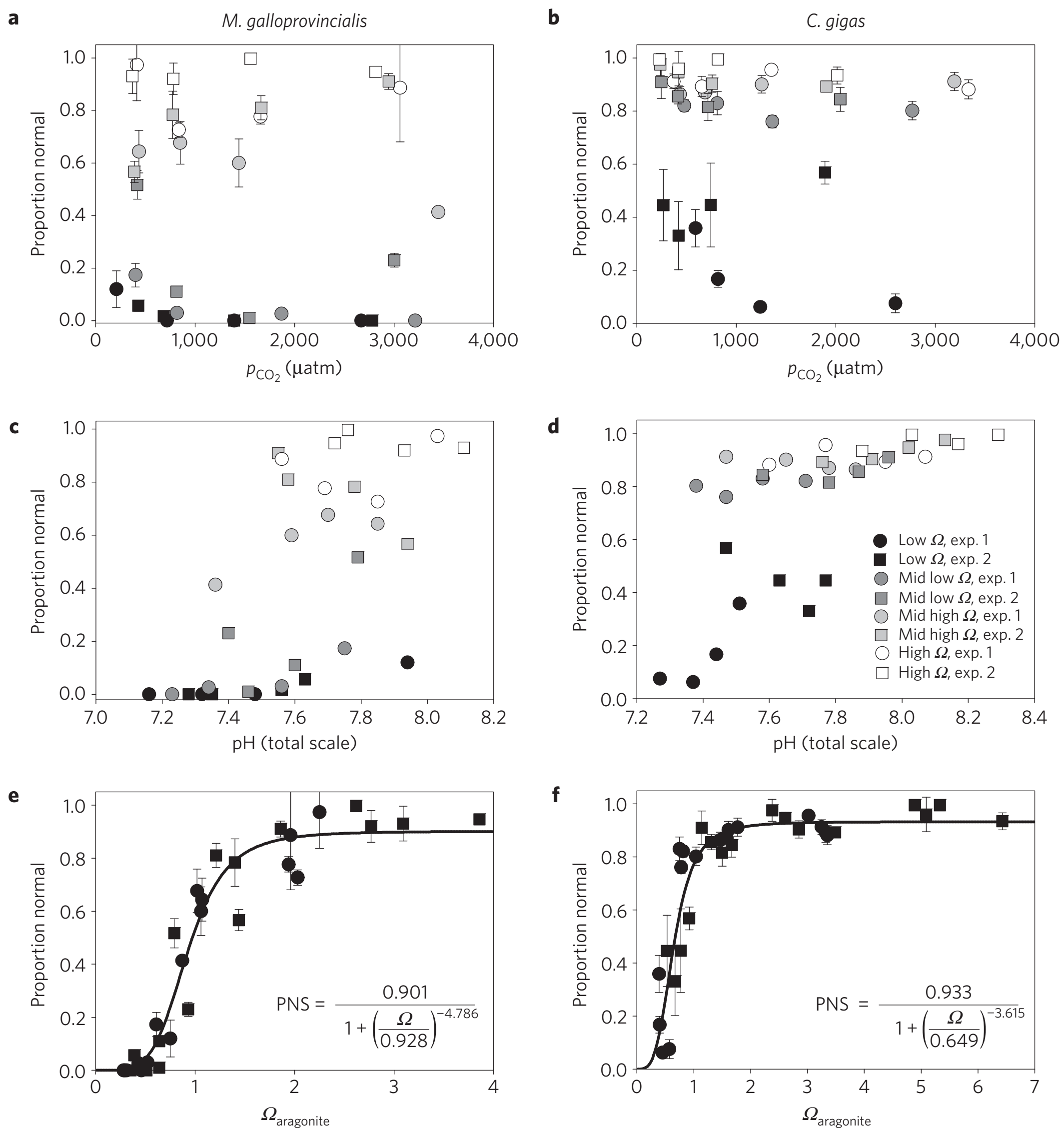

Figure 2 | Shell development in response to carbonate system variables for both species. Mean proportion of normal shell development (PNS) of D-hinge larvae for mussel (**a**,**c**,**e**) and oyster (**b**,**d**,**f**) experiments in response to p_{CO_2} (**a**,**b**), pH (**c**,**d**), and saturation state (**e**,**f**). Circles and squares are the first and second experiments, respectively. Fills from black to white represent increasing saturation state. Symbols are the mean values of the three replicate containers, each of which was sub-sampled three times (approximately 100–200 larvae per sub-sample). Error bars are standard deviations of mean replicate values per treatment. Error bars were excluded from the pH plot to allow easier visual interpretation.

significant effects on the mussels ($F_{2,24} = 707.63$, $p < 0.0001$) and oysters ($F_{3,9} = 219.29$, $p < 0.0001$), explaining 93% and 81% of the variance in normal shell length for each species. The lack of normally developed mussel larvae in the low-Ω_{ar} treatments prevented size estimates. Shell length decreased by nearly 25% and 10% with decreasing Ω_{ar} across our experimental range in the mussel and oyster larvae, respectively. p_{CO_2} had minor significant positive effects on mussel ($F_{3,24} = 5.83$, $p = 0.0039$) and oyster larvae shell length ($F_{3,32} = 27.64$, $p < 0.0001$), (Fig. 3 and Supplementary Table 4), explaining 1% and 10% of the variance in shell length. The interaction between Ω_{ar} and p_{CO_2} was also statistically significant for both species, but only explaining 5% of the shell growth variance for both species (Supplementary Table 4 and Fig. 2). The positive response to p_{CO_2} may seem counter-intuitive at first; however, within an Ω_{ar} treatment level, DIC concentrations are proportional to p_{CO_2} (Supplementary Table 1) and inversely proportional to pH. We did not evaluate pH effects on shell growth, given what seems to be a positive response to decreasing pH, and thus a probable response to increasing DIC concentrations (Fig. 3). We will argue below that shell growth is responding to DIC within an Ω_{ar} treatment level, but saturation state is again the dominant parameter affecting shell growth of these early larvae. Shell length continues to increase with increasing saturation state even at the highest values in our treatments, $\Omega_{ar} \sim 4$ and $\Omega_{ar} \sim 6.5$ for the mussel and oyster larvae, respectively. We therefore fitted a power function to the response of shell length to saturation state (Fig. 3). The fit of the model for both species was highly significant: mussel larvae ($F_{2,10} = 81.36$, $R^2 = 0.89$, $p < 0.0001$) and oyster larvae ($F_{2,14} = 103.22$, $R^2 = 0.88$, $p < 0.0001$).

Why saturation state matters to bivalve larvae

Our results initially seems contradictory to the physiological basis for understanding ocean acidification impacts on organisms; particularly the overarching role of seawater pH, acid–base regulation, and extracellular acidosis in marine organisms[12,13,22–24]. Specifically, we found that seawater pH seems to have little

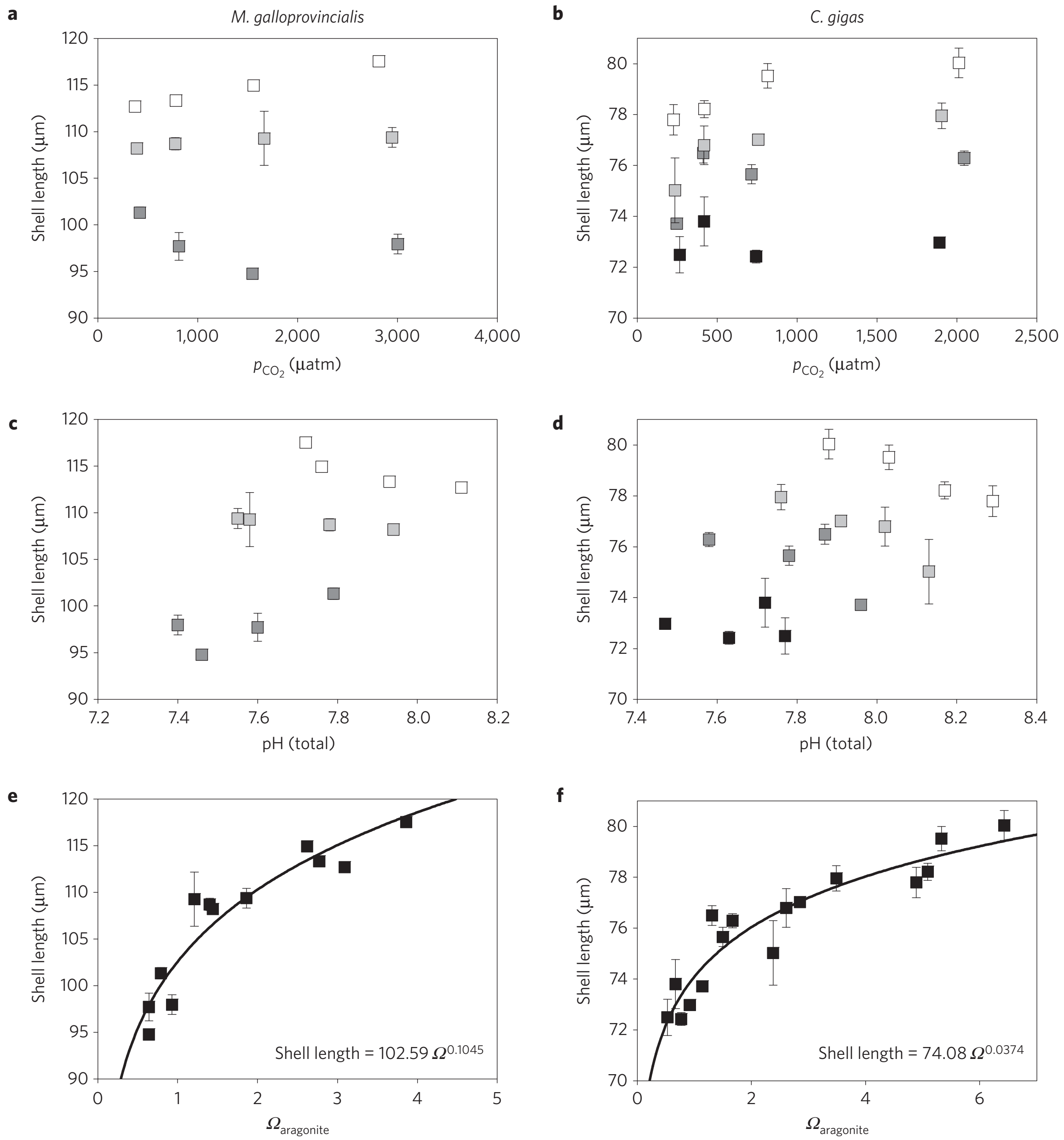

Figure 3 | Shell growth in response to carbonate system variables for both species. Mean shell length of normally developed larvae in response to p_{CO_2} (**a**,**b**), pH (**c**,**d**), and saturation state (**e**,**f**). The grey scale symbols are the same as used previously. Means and standard deviations are of replicate containers per treatment, as above. We lack larvae from low-Ω treatments owing to very poor development in the mussel experiments. The total number of normal larvae measured for shell length was 3,132 and 7,106 for the mussel and oyster experiments, respectively. Control shell lengths were 108.44 ± 2.57 μm and 78.78 ± 2.06 μm for the mussels and oysters, respectively.

to no measurable effect on early larval shell development and growth, except for the case where pH and Ω_{ar} are both very low (Figs 2 and 3). At these low levels, seawater pH probably becomes very important (particularly to bivalves, which show limited ability to regulate extracellular pH), as eukaryote intracellular pH typically ranges from 7.0 to 7.4 (ref. 25) and additional energy is needed to maintain physiochemical gradients crucial for passive and active cross-membrane ion transport[26] if extracellular pH approaches these values. Some species seem to be able to mitigate acidosis via bicarbonate accumulation; however, ability to do so is variable across taxa, and bicarbonate accumulation often requires several days to months[14,15,22]. During the transient (days) early larval stage it is unlikely that bivalve larvae have the time or physiological capacity to compensate for acidosis[22], with their limited energy budget and the embryological development taking place during this time period. Therefore, although seawater pH effects on organismal acidosis may also be at work during this early larval stage, we have experimentally shown that any pH effect is overwhelmed by the impact of saturation state during initial shell formation. The likelihood of organisms experiencing such low pH conditions without coinciding low-Ω conditions is also very unlikely (Fig. 1 and Supplementary Table 1). Therefore, the conclusions from this study do not contradict the importance of pH on marine bivalve larvae, but rather highlight the overwhelming significance of saturation state at this critical bottleneck for bivalve larvae.

We have previously argued[17] that during PDI shell formation in bivalve larvae the rapid rate of calcification (as shown in Fig. 4)

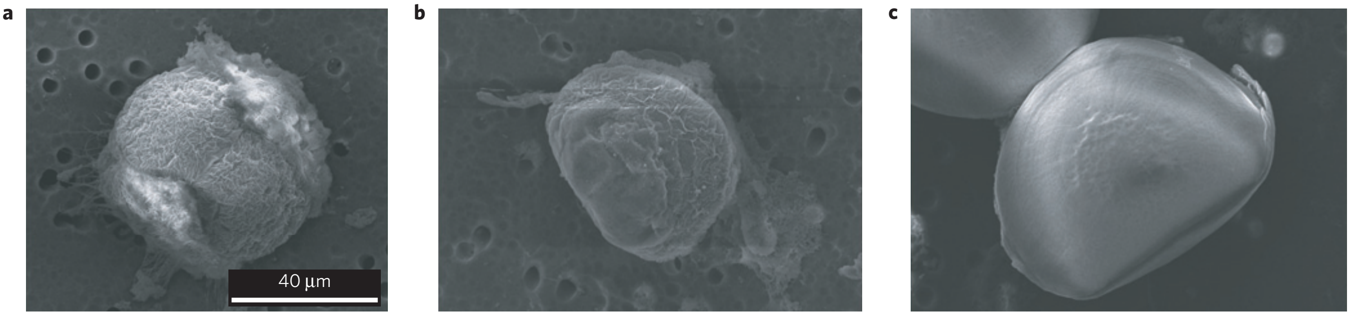

Figure 4 | Development of prodissoconch I shell in Pacific oyster larvae. Representative scanning electron micrographs of Pacific oyster larvae at 10 h (**a**), 14 h (**b**) and 16 h (**c**) post fertilization. Over the course of development from **a** to **c** the formation of the periostracum (wrinkled) is seen, followed by increasing amounts of hardening by calcium carbonate until, by 16 h, the prodissoconch I shell is formed and fully calcified and the periostracum is taut over the shell surface. Larvae were reared at 23 °C and salinity = 34, under atmospheric CO_2.

and increased exposure of crystal nucleation sites to seawater puts an important kinetic-energetic constraint on the larvae; thereby mandating an Ω_{ar} sensitivity (as in equation (1)). The classical representation of the calcification rate (r) following the standard empirical formulation is:

$$r = k(\Omega - 1) \quad (1)$$

The apparently predetermined amount of rapid calcification required to form the PDI shell and begin feeding requires the biocalcification rate constant (k) to be several orders of magnitude higher than inorganic precipitation[17]. This constraint also demands a rapidly accelerating biocalcification rate constant (k) as Ω approaches saturation (and thus $\Omega - 1$ approaches 0) to maintain the calcification rate necessary to complete the PDI shell without depleting maternal energy reserves. However, the logical extension of this argument that biocalcification is not possible below saturation is erroneous. Bivalve larvae clearly precipitate mineral when ambient conditions are undersaturated and must, therefore, create some level of supersaturation at crystal nucleation sites which are semi-exposed to the external environment. We suggest that larvae are both elevating Ω at the site of calcification and elevating k through physico-chemical changes at the organic–inorganic nucleation interface[17]. That is, the dependency on seawater Ω_{ar} in our experimental results (Fig. 2) supports the importance of this kinetic-energetic constraint; increasing seawater supersaturation lowers the energetic cost of shell building, increasing the scope for growth, as seen in the shell length response to Ω_{ar} (Fig. 3).

A curious pattern is observed in our shell length data. Figure 3 seems to indicate a minor (positive/negative) effect of p_{CO_2}/pH on shell growth. Within a Ω_{ar} treatment group, our experimental manipulations result in decreasing DIC with increasing pH (Supplementary Table 1). Previous studies in corals have suggested that total DIC (driven mostly by bicarbonate ion) is an important factor for calcification[27–29]. Alternatively, the ratio of DIC/[H^+] (which is in fact a proxy for carbonate ion and thus saturation state) due to the proton flux model[30] may be the controlling parameter for coral calcification. Given the differences in calcification mechanisms and shell morphology between larval prodissoconch I (PDI) and prodissoconch II (PDII; ref. 31), we postulate that any minor, secondary DIC effect may be acting during the latter PDII shell formation. In fact, a previous study[32] found that larval *C. gigas* shell size was not affected by elevated CO_2 at day one (PDI), but by three days post fertilization shells were significantly smaller in the high-CO_2 treatment (PDII). Larvae from our two-day experiments had already begun PDII shell formation; therefore, it seems plausible the impacts of Ω_{ar} (negative) and DIC (slightly positive) on shell length were acting on PDII. Conversely, the range of Ω_{ar} tested in this previous study[32] was roughly between 1 and 2 (p_{CO_2} ~400–1,100 µatm), and may have resulted in undetectable PDI size differences over the smaller experimental range (highlighting the value of experimental treatments extending beyond open-ocean projections). The minor secondary positive effect of DIC (at supersaturation) seems consistent with previous studies. However, saturation state was still the dominant factor impacting the shell length of normally developed larvae. Although we cannot determine whether compensatory growth is possible if saturation state is improved later in larval life, the carry-over effects found on US West Coast oyster larvae indicate there is limited capacity to recover from OA exposure during the sensitive early larval stages[18–20].

Even the most critical of OA meta-analyses on organismal responses[6] note calcification as being the 'most sensitive' of responses to ocean acidification. For developing embryos of bivalve larvae, calcification is a process that determines whether larvae will survive or perish; without the development and calcification of the PDI shell, larvae probably lack a functional velum to support swimming and feeding owing to lack of muscular–skeletal attachment. Without an effective feeding mechanism, larvae will eventually exhaust endogenous energy reserves[17]. Although larvae may be able to support basal metabolism using dissolved organic matter (DOM; ref. 33), the velum is also responsible for DOM uptake[34]. Our results show that seawater Ω_{ar} directly affects shell development and growth, and this effect is not an indirect pH impact on internal acid–base status. Without shell development, or if it is too energetically expensive, there seems little opportunity for larvae to overcome OA during this early stage[17]. A previous study[35] suggested carbonate ion concentration, rather than saturation state, matters to larvae. Calcium addition was used to manipulate mineral solubility without a control for excess calcium at already supersaturated mineral solubility[35]. The addition of calcium to roughly twice that of seawater, as in ref. 35, at Ω_{ar} ~2.0 (increasing Ω_{ar} to ~3.64) resulted in very poor shell development (PNS = 0.39 ± 0.8) and much smaller normal larvae (S.L. = 68.63 ± 3.22 µm) in *C. gigas*. This result is not surprising, given the role of calcium in cellular ion transport and immune response, and the lack of osmo-regulation in marine bivalves[14,36]. This is a minor point ultimately, because carbonate ion concentration usually controls saturation state in marine waters. Importantly, however, the carbonate in marine bivalve shell is derived from all forms of DIC, including respiratory carbon (ref. 17 and references therein); increased seawater saturation state seems to make the kinetics of shell formation less energetically expensive.

Environmental context

In marine waters, the increase of p_{CO_2} decreases saturation state and pH, but their declines approach potential thresholds differentially.

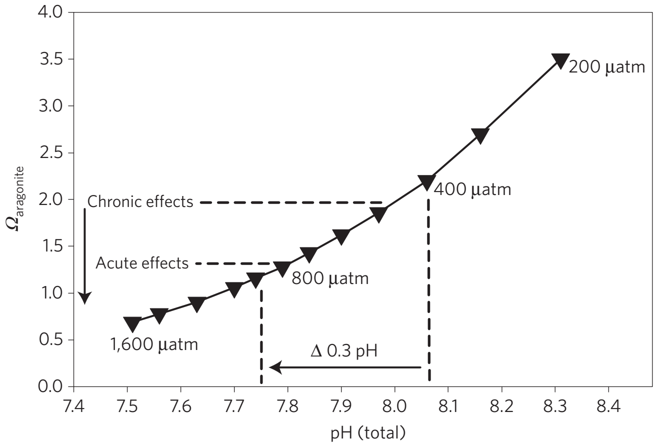

Figure 5 | Calculated response of pH and aragonite saturation state to increasing p_{CO_2} from 200 to 1,600 µatm (triangles) at typical upwelling conditions along the Oregon coast. Conditions calculated for total alkalinity = 2,300 µmol kg^{-1}, temperature = 13 °C, and salinity = 33. Symbols are values of p_{CO_2}. Chronic and acute effects due to saturation state decreases from experiments have been noted for bivalve larvae. The Δ 0.3 pH was previously noted as significant to many physiological processes in molluscs[8].

We have plotted the change in Ω_{ar} and pH as p_{CO_2} increases for typical upwelling conditions in Oregon's coastal waters (Fig. 5). We found acute responses of bivalve larvae begin to manifest at saturation states (Ω_{ar}) of ~1.2–1.5 (Figs 2 and 3). Other studies have documented sub-lethal chronic exposure effects in Pacific oyster larvae (~2.0: refs 18,35), Olympia oyster larvae (~1.4, ref. 19), Eastern oyster larvae (~1.9, ref. 11), and California mussel larvae (~1.8, ref. 37). Although far from an exhaustive list of experimental studies, placing these Ω_{ar} values in context of the present conditions in the California Current ecosystem illustrate two key points. First, there is limited remaining capacity for Oregon's coastal waters to absorb more CO_2 before sub-lethal Ω_{ar} thresholds are crossed for bivalve larvae. Increasing atmospheric CO_2 pushes saturation state across these thresholds more frequently and with greater magnitude in the California Current[38,39]. Second, these saturation state thresholds will be crossed long before recently documented pH changes found to be physiologically important in molluscs[8] (often >0.3 pH units). If transient conditions during spawning are unfavourable for bivalve larvae, in hatcheries or in the wild, then these impacts would result in diminished larval supply and recruitment to adult populations.

Larval supply and recruitment are vital to maintaining many benthic marine invertebrate populations[40]. Survival to metamorphosis requires normal development and rapid growth to limit larval predation[40]. Larger larval size indicates greater scope for growth and the repletion of energy stores needed to complete metamorphosis[41]; energy stores that are diminished under acidification stress[11]. Recruitment to adult populations can be highly variable year to year and often related to regional climatology[42,43]. The coastal zones where many ecologically and economically important marine calcifiers are found will not experience acidification gradually, as seen in the oligotrophic open ocean, but rather as increases in frequency, duration and magnitude of events that are unfavourable for specific life-history stages[5,38,44]. Our experimental work has shown that successful larval development and growth during rapid shell formation is dependent on seawater saturation state in temporal windows lasting two days or less (Fig. 4); thus providing increasing evidence for a mechanism by which transient, moderate acidification impacts[18] nearly resulted in collapse of the Pacific Northwest oyster industry[45]. These impacts occur on timescales relevant to changes already observed in coastal zones[5,18,46], regardless of future changes or direct cause, and thus decreases in saturation state can limit recruitment to present bivalve populations. Our experimental approach and findings shed new light on the organismal responses to OA, while indicating the importance of monitoring the complete carbonate chemistry system; without which successfully linking biological responses and chemical observations will prove exceptionally challenging.

Methods

Water collection and stripping dissolved inorganic carbon. For each experiment, 1 µm filtered seawater was collected from Yaquina Bay. The alkalinity was reduced by the addition of trace metal grade HCl in near-alkalinity equivalence, followed by bubbling with ambient air for 48 h to strip (DIC) as CO_2. The acidified, stripped seawater was then 0.22 µm-filtered, pasteurized, and stored at 2–5 °C. Before treatment manipulation, the seawater was bubbled with 0.2 µm-filtered outside air until atmospheric conditions were achieved, then carbonate DIC and alkalinity values were determined for manipulations.

Experimental manipulation. A 4×4 factorial experimental design was developed to target 16 total treatment combinations of p_{CO_2} and Ω_{ar} (saturation state with respect to aragonite; Fig. 1 and Supplementary Table 1), with triplicate 500 ml biological oxygen demand (BOD) bottles per treatment. Two separate experiments were conducted with each species. DIC and alkalinity concentrations were calculated for each of the 16 target treatment combinations (p_{CO_2} and Ω_{ar}). Experimental treatments were created by gravimetric addition of mineral acids and bases to the decarbonated seawater in gas-impermeable bags customized with Luer lock fittings. Aliquots of a concentrated, ambient-p_{CO_2}, solution of Na_2CO_3 and and $NaHCO_3$ were added to adjust DIC to target treatment levels followed by 0.1N HCl to adjust alkalinity. Immediately following chemical manipulation, the bags with treatment water were stored without head-space at 2–5 °C for up to several weeks before spawning broodstock. Antibiotics were added to BOD bottles (2 ppm chloramphenicol and 10 ppm ampicillin), which we found to have no negative effects on larvae or carbonate chemistry in previous trials. Controls were included to evaluate experimental manipulations and incubation conditions by hatching eggs in open culture containers, as well as by using stored seawater collected before decarbonation and not subjected to the chemical manipulations described in this study.

Carbonate chemistry measurements. Carbonate chemistry samples were collected from the treatment water bags just before stocking larvae in BOD bottles, and also from each BOD bottle at the end of the incubation period. Carbonate chemistry samples were collected in 350 ml amber glass bottles with polyurethane-lined crimp-sealed metal caps and preserved by the addition of 30 µl of saturated $HgCl_2$. Analyses of p_{CO_2} and DIC were carried out following the procedure of Bandstra *et al.*[47], modified for discrete samples as in Hales and colleagues[48]. Gas and liquid standards that bracketed the experimental range (Supplementary Table 1) were employed to ensure accuracy.

Larval rearing. Broodstock for mussel (*Mytilus galloprovincialis*) and oyster (*Crassostrea gigas*) experiments were obtained from Carlsbad Aquafarm, or from selected stocks of the Molluscan Broodstock Program (MBP; ref. 49), Yaquina Bay, respectively. Broodstock spawning was stimulated by a rapid increase of 10 °C in ambient seawater temperature. Gametes were collected from at least two male and two female parents, and the eggs fertilized in ambient seawater. Developing embryos were added at a density of 10 larvae ml^{-1} to triplicate BOD bottles per treatment after visual verification of successful fertilization. Sealed BOD bottles were oriented on their side and incubated for 48 h at culture temperature (18 °C for mussels and 22 °C and 25 °C for oyster trials 1 and 2, respectively). Larvae from each BOD bottle were concentrated after a filtered chemistry sample was collected, sampled in triplicate, and preserved in 10% formalin buffered to ~8.1–8.2.

Larval shell development and size. Larvae were examined microscopically to determine the proportion of normally and abnormally developed D-hinge (prodissoconch I) larvae as well as larval shell lengths. Normally developed larvae were characterized by a straight hinge, smooth curvature along the edge of the valve, and the appearance of tissue within the translucent shells[50]. Digital images were used to determine the shell length (longest axis perpendicular to the hinge) of normally developed larvae only. Images were analysed using ImageJ (V1.42).

Data analyses. Proportion normal data were scaled to the unmanipulated, seawater control for each experiment by dividing treatment values by control values. We used a two-way ANOVA, with p_{CO_2} and Ω_{ar} as the primary factors, with experiment number as a blocking factor. Proportion normal data were square-root arcsine transformed. Assumptions of normality and homoscedasticity were checked, and any violations were managed as noted. Initial data analyses found unequal variance across treatment groups in the transformed proportion normal data, and mean values per treatment were used to improve heteroscedasticity as well as blocking by experiment. To evaluate pH effects on shell development we ran a series of regression analyses of transformed proportion normal regressed on pH, within each Ω_{ar} treatment and experiment. We then used a Bonferroni correction for multiple tests of significance to reduce Type 1 error. Analyses were conducted with the SAS software suite (v9.3). Nonlinear least-squares regression in Sigma-Plot (v12.5) was used to fit functional responses of development (logistic) and shell length (power).

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Author contributions
G.G.W., B.H., C.J.L. and B.A.H. conceived and planned the research. G.G.W. designed and supervised experiments. G.G.W. and B.H. analysed data. P.S. organized study components and P.S., M.W.G., E.L.B., I.G. and C.A.M. developed and carried out the experiments. M.W.G., E.L.B., I.G. and C.A.M. analysed organism and chemistry samples. All authors contributed to writing the manuscript.

Additional information
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Competing financial interests
The authors declare no competing financial interests.