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RESEARCH ARTICLE

Intertidal oysters reach their physiological limit in a future high-CO_2 world

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ABSTRACT

Sessile marine molluscs living in the intertidal zone experience periods of internal acidosis when exposed to air (emersion) during low tide. Relative to other marine organisms, molluscs have been identified as vulnerable to future ocean acidification; however, paradoxically it has also been shown that molluscs exposed to high CO_2 environments are more resilient compared with those molluscs naive to CO_2 exposure. Two competing hypotheses were tested using a novel experimental design incorporating tidal simulations to predict the future intertidal limit of oysters in a high-CO_2 world; either high-shore oysters will be more tolerant of elevated P_{CO_2} because of their regular acidosis, or elevated P_{CO_2} will cause high-shore oysters to reach their limit. Sydney rock oysters, *Saccostrea glomerata*, were collected from the high-intertidal and subtidal areas of the shore and exposed in an orthogonal design to either an intertidal or a subtidal treatment at ambient or elevated P_{CO_2}, and physiological variables were measured. The combined treatment of tidal emersion and elevated P_{CO_2} interacted synergistically to reduce the haemolymph pH (pH_e) of oysters, and increase the P_{CO_2} in the haemolymph (P_{e,CO_2}) and standard metabolic rate. Oysters in the intertidal treatment also had lower condition and growth. Oysters showed a high degree of plasticity, and little evidence was found that intertidal oysters were more resilient than subtidal oysters. It is concluded that in a high-CO_2 world the upper vertical limit of oyster distribution on the shore may be reduced. These results suggest that previous studies on intertidal organisms that lacked tidal simulations may have underestimated the effects of elevated P_{CO_2}.

KEY WORDS: Emersion, Ocean acidification, Hypercapnia, Multiple stressors, Mollusc

INTRODUCTION

Sessile marine molluscs living in the intertidal zone are periodically exposed to air (emersion) as the tide recedes. When emersed, marine bivalves close their shell, which restricts gas exchange and can result in internal hypercapnia and extracellular acidosis (Burnett, 1988; Truchot, 1990). When hypercapnic, normal physiological processes of bivalves are impeded, and metabolism is slowed until normocapnia returns (Burnett, 1988; Greenway and Storey, 1999; David et al., 2005). Intertidal emersion also reduces the time available to sessile organisms for feeding, waste excretion and other vital processes (Truchot, 1990).

Oysters are ubiquitous sessile molluscs found on many temperate shorelines, extending from the subtidal shore to the upper limits of the intertidal shore, where they provide ecosystem services ranging from complex habitat provision (Underwood and Barrett, 1990; Gutiérrez et al., 2003; Cole et al., 2007) to water quality improvement and benthic–pelagic coupling (Newell et al., 2005). The time oysters spend emersed is dependent on where they are located on the shore. Oysters in the subtidal zone are rarely, or never, emersed. Oysters in the intertidal zone, however, experience regular periods of emersion. Those oysters living at the upper level of the intertidal zone experience the longest periods of emersion and potentially greater respiratory and metabolic acidosis associated with hypercapnia. This emersion results in greater regular changes to the acid–base balance of intertidal oysters compared with subtidal oysters (Dugal, 1939; Burnett, 1988; Truchot, 1990).

Oysters and other molluscs have been identified as highly vulnerable to increasing CO_2 concentrations of the Earth's oceans in a process known as ocean acidification (Fabry et al., 2008; Gazeau et al., 2013). Ocean acidification occurs when CO_2 emissions are absorbed by the oceans, forming a weak acid and reducing oceanic pH (Caldeira and Wickett, 2003, 2005). Compared with pre-industrial levels, the mean pH of surface ocean waters has declined by more than 0.1 units (Caldeira and Wickett, 2005; Raven et al., 2005) and the Intergovernmental Panel on Climate Change (Houghton, 2001; Solomon, 2007; Collins et al., 2013) has predicted that the pH of ocean surface waters will fall a further 0.3–0.5 units (pH 7.8–7.6) by 2100 and 0.7–0.77 units (pH 7.4–7.43) by 2300, assuming median emission scenarios (Caldeira and Wickett, 2003, 2005; Solomon, 2007; Collins et al., 2013). Ocean acidification has been shown to alter the fundamental physiological functions of molluscs, especially acid–base regulation (Pörtner et al., 2004; Parker et al., 2013). This can then further impact a wide range of morphological and physiological characteristics of molluscs, including shell formation (Fabry, 2008; Fabry et al., 2008; Doney et al., 2009), and restrict the energy available for growth, reproduction, immune responses and homeostasis (Pörtner et al., 2004; Fabry et al., 2008; Doney et al., 2009; Parker et al., 2013). These negative effects extend to the larval stages of marine molluscs, which are known to be especially vulnerable to ocean acidification (Parker et al., 2010; Ross et al., 2011; Scanes et al., 2014). Larval growth, settlement and metamorphosis of molluscs has been shown to be negatively impacted by ocean acidification (Ross et al., 2011; Gazeau et al., 2013), and these effects are exacerbated in the presence of other environmental stressors (Ko et al., 2014; Cole et al., 2016). In all ontogenetic stages, molluscs are poor acid–base regulators compared with other taxa. They have a limited capacity to actively accumulate HCO_3^- in order to buffer their extracellular fluids against changes in pH (Melzner et al., 2009).

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Paradoxically, however, while the effects of ocean acidification are predicted to be most severe for molluscs living at the limits of their physiological stress tolerance (Sokolova, 2013; Gazeau et al., 2013), it has also been shown that molluscs living in environments naturally high in CO_2 are more resilient (Thomsen et al., 2010; Parker et al., 2012, 2015). The resilience of organisms to environmental stress can be mediated through plastic responses, genetic adaption and relocation (e.g. to refugia) (Williams et al., 2008; Dawson et al., 2011; Bellard et al., 2012). Pre-exposure to elevated P_{CO_2} changes the responses of some organisms including bivalves to elevated P_{CO_2} (Thomsen et al., 2010; Parker et al., 2012, 2015). When mussels (*Mytilus edulis*) were collected from a region of the Baltic Sea naturally high in CO_2, they were found to be more tolerant to laboratory exposure to elevated P_{CO_2} than *M. edulis* from control areas (Thomsen et al., 2010). Exposure of adult oysters to elevated CO_2 also has positive carry-over effects on offspring. Parker et al. (2012) exposed adult *Saccostrea glomerata* to elevated P_{CO_2} and found their larvae to be more resilient to elevated P_{CO_2}. When these larvae reached adult maturity, they retained those traits and were still more tolerant of elevated P_{CO_2} (Parker et al., 2015), perhaps because of transgenerational plasticity (Ross et al., 2016). Tolerance to ocean acidification has also been observed in organisms that experience greater levels of internal metabolic CO_2 (Melzner et al., 2009). Ectotherms capable of sustaining prolonged physical activity experience an accumulation of metabolic CO_2 as a result of muscle activity. Their ability to regulate this CO_2 and the associated acidosis is believed to explain the apparent tolerance of active organisms to ocean acidification (Melzner et al., 2009).

For oysters, the degree of stress experienced varies across their distribution. Individuals at the upper intertidal shore heights experience severe hypercapnia during emersion (Dugal, 1939; Burnett, 1988; Truchot, 1990), whereas oysters in the subtidal shore experience less hypercapnia. It is predicted that ocean acidification will make it more difficult for intertidal invertebrates to return to normocapnia during periods of immersion that follow emersion (Rastrick et al., 2014). This suggests that intertidal oysters, particularly those high on the shore, will experience greater stress from ocean acidification and have a lower capacity for resilience. Alternatively, because the physiological effects of emersion are similar to those experienced under ocean acidification, oysters living in a high-CO_2 environment may be more resilient to ocean acidification, as has been found in other studies (Thomsen et al., 2010; Parker et al., 2012, 2015; Ross et al., 2016).

The Sydney rock oyster, *Saccostrea glomerata* (Gould 1850), is found in south eastern Australia and is common from shallow subtidal to intertidal habitats in estuaries and protected embayments (Nell, 2001). To determine whether Sydney rock oysters will reach their intertidal limit in a future high-CO_2 world, two competing theories were tested: either oysters will reach their physiological and intertidal limit when exposed to elevated P_{CO_2} or, alternatively, oysters in the upper intertidal zone will be resilient to elevated P_{CO_2}. It was hypothesised that: (1) an intertidal environment would exacerbate the hypercapnic effects of elevated P_{CO_2} on oysters and (2) oysters from the high-intertidal zone will be more resilient to elevated P_{CO_2} and diurnal emersion compared with those from the subtidal zone.

MATERIALS AND METHODS

Location, collection and acclimation

Experimental *S. glomerata* individuals were collected from two locations and two different shore heights (high-intertidal and subtidal) on the shore within Port Stephens, NSW, Australia (location 1: 32°32′42.25″S, 152°03′42.69″E; location 2; 32°41′54.04″S, 152°03′27.71″E). Oysters from each location were pooled together according to their respective shore heights to better ensure genetic diversity. Each location was composed of sloping rocky shore, dominated by large flat surfaces of granite, giving a consistent gentle slope down to the water. At each location, two zones of shore height (high-intertidal and subtidal) were established by measuring the shore height on the rocky shore above Indian Spring Low Water (ISLW). The subtidal zone was identified as being 0.1–0.4 m above ISLW, and the high-intertidal zone as 1.1–1.5 m above ISLW. One hundred individuals were collected per zone and location by gently levering aggregations off the substratum.

Following collection, oysters were taken to the Port Stephens Fisheries Institute (PSFI, NSW, Australia), where they were cleaned of fouling organisms and separated into individual oysters. All experimental seawater (hereafter, FSW) used at PSFI was collected from Little Beach within Port Stephens (32°42′42.75″S, 152°9′26.48″E) and filtered to 1 µm. All experiments were conducted at a constant temperature of 22°C. The oysters from each zone were then transferred to separate 750 l header tanks filled with FSW. It was calculated from observations and measurements of diurnal tidal fluctuations that the high-intertidal zone received approximately 9 h emersion followed by 3 h immersion, and the subtidal zone received 12 h (constant) immersion on an average diurnal 12 h tidal cycle. The collected oysters remained in four 750 l tanks for 1 week to acclimate under a simulated tidal treatment of either 9 h emersion followed by 3 h immersion (for oysters collected from the high-intertidal shore) or constant submergence (for oysters collected from the subtidal shore).

Experimental treatments

A fully orthogonal design was used to test our hypotheses. Oysters were collected from both high-intertidal and subtidal zones, and were then exposed to all possible combinations of two tidal treatments and two P_{CO_2} scenarios. *Saccostrea glomerata* closes its valves when exposed to the air during emersion; this prevents any gaseous exchange with the air while emersed (Potter and Hill, 1982).

Twelve 750 l header tanks were divided among the two experimental tidal treatments: six intertidal and six subtidal. Within each tidal treatment, three header tanks were held at elevated P_{CO_2} (1000 µatm) and three at ambient P_{CO_2} (400 µatm). Each header tank circulated FSW through two 50 l tanks suspended above it, one of which held 16 oysters collected from the high-intertidal zone, while the other held 16 oysters from the subtidal zone. Therefore, each combination of tidal treatment×P_{CO_2}×intertidal/subtidal collection zone was replicated three times across three different header tanks (n=3). Experimental tidal treatments were the same as described previously for acclimation. The intertidal treatment consisted of 9 h emersion followed by 3 h immersion, and the subtidal treatment consisted of 12 h constant immersion. In all header tanks, FSW was pumped from the header tank into the replicate tanks via a spray bar, where it overflowed back to the header tank. Oysters were kept slightly elevated on a mesh stage in all tanks. To emulate tidal conditions, a small hole was drilled in the bottom of all 50 l tanks. When the allocated immersion time for the intertidal treatment (3 h) had been reached, pumps circulating water to the 50 l tank were switched off via an electronic timer (Hager electro Pty Ltd, Glendenning, NSW, Australia), and the FSW slowly drained out of the tank back into the header to simulate a naturally retreating tide. Following emersion (9 h),

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pumps automatically refilled the tank. Oysters in the subtidal treatment remained submerged under flowing FSW at all times. Oysters were allowed 10 days to acclimate to their tanks and new tidal treatments prior to the introduction of CO_2. Following the introduction of CO_2, oysters remained in experimental treatments for 3 weeks.

CO_2 monitoring

The two P_{CO_2} levels used in this study (400 µatm, 1000 µatm; based on the multi-model average projection by the IPCC for 2100; Collins et al., 2013) were a mean ambient pH_{NBS} of 8.19±0.02 and a mean pH_{NBS} at elevated CO_2 levels of 7.84±0.0035. The elevated CO_2 level was maintained using a pH negative feedback system (Aqua Medic, Aqacenta Pty Ltd, Kingsgrove, NSW, Australia; accuracy ±0.01 pH units). To determine the pH level corresponding to P_{CO_2} levels, total alkalinity (TA) was quantified at each water change using triplicate Gran-titration (Gran, 1952). Following the titration, the TA and chosen P_{CO_2} levels were entered into a CO_2 system calculation program (CO_2 SYS; Lewis et al., 1998), using the dissociation constants of Mehrbach et al. (1973), and the pH level corresponding with the desired P_{CO_2} level was calculated. Seawater physiochemical variables including pH_{NBS}, TA and salinity were measured at each water change (Table S1) – the desired pH values corresponding with P_{CO_2} levels were then recalculated accordingly. Food grade CO_2 was bubbled directly into the header tanks via a CO_2 reactor to ensure proper mixing and, in turn, reduce pH. A pH probe connected to a controlling computer was placed within each tank (probes were calibrated each water change using NBS buffers). When the desired pH level was reached, the delivery of CO_2 was automatically stopped by a computer signal to a solenoid valve. Each header tank set to elevated P_{CO_2} was controlled by its own independent pH controlling system. The pH values of each tank were monitored daily, and the pH electrode of each controlling system was checked daily against another calibrated pH probe (NBS buffers, WTW 3400*i*).

Animal husbandry

Every second day, each header tank received a complete water change. A second set of header tanks were filled and equilibrated to the temperature and, if appropriate, P_{CO_2} level of corresponding tanks already housing oysters. The 50 l tanks containing oysters were then transferred to a clean header tank (of the same treatment variables), ensuring they were out of the water for only a short amount of time. Tanks were completely drained and then scrubbed clean using Virkon S solution (Antec Corp, North Bend, WA, USA). Oysters were fed each day an algal mixture consisting of 50% *Chaetoceros muelleri* and 50% *Tisochrysis luteas* at a concentration equivalent to 2×10^9 *T. luteas* cells oyster^{-1} day^{-1} (Nell and O'Connor, 1991). Animal ethics approval was not required for this study.

Measurement of haemolymph variables

To determine the effects of experimental treatments on oyster haemolymph acid–base variables following 3 weeks of exposure, three oysters were randomly taken from each replicate tank. Oysters in the intertidal treatment were sampled at the end of both their allocated immersion and emersion times. Oysters were then immediately opened without rupturing the pericardial cavity. Haemolymph samples were drawn from the interstitial fluid filling the pericardial cavity chamber of an opened oyster using a sealed 1 ml needled syringe. A 0.2 ml sample was drawn carefully to avoid aeration of the haemolymph. Half of the sample was then immediately transferred to an Eppendorf tube where pH_e of the sample was measured at 20°C using a micro-pH probe (Metrohm 827 biotrode). The remaining haemolymph was transferred to a gas analyser (CIBA Corning 965) to determine total CO_2 concentration (C_{CO_2}). The micro-pH probe was calibrated prior to use with NBS standards at the acclimation temperature and the gas analyser was calibrated following the manufacturer's guidelines. Three oysters per replicate tank from the intertidal treatment were sampled after their entire allocated 3 h immersion cycle and another three oysters after their full 9 h emersion cycle. Three oysters were sampled per replicate tank in the subtidal treatment. Partial pressure of CO_2 in haemolymph (P_{e,CO_2}) and concentration of HCO_3^- in the haemolymph ($[HCO_3^-]_e$) were calculated from the C_{CO_2} using the modified Henderson–Hasselbalch equation (Eqns 1 and 2) according to Heisler (1984, 1986) as found in Riebesell et al. (2010), where the molarity of dissolved species was 1.033 mol l^{-1} (seawater; Hammer et al., 2011), $[Na^+]$ was 0.55 mol l^{-1} (measured previously) and protein concentration of *S. glomerata* was 0.05 g^{-1} l^{-1} (Peters and Raftos, 2003):

$$P_{e,CO_2} = C_{CO_2} \times (10^{pH_e - pK'''} \times \alpha + \alpha)^{-1}, \quad (1)$$

where P_{e,CO_2} is the calculated P_{CO_2} in haemolymph (mmHg), C_{CO_2} is the measured total CO_2 concentration in haemolymph (mmol l^{-1}), α is the physical solubility of CO_2 and pK''' is the apparent dissociation constant of carbonic acid in body fluids (after Heisler, 1986); and:

$$[HCO_3^-]_e = C_{CO_2} - (\alpha_{CO_2} \times P_{e,CO_2}), \quad (2)$$

where C_{CO_2} is the measured total CO_2 concentration in haemolymph (mmHg), α_{CO_2} is the solubility of CO_2 in haemolymph (calculated after Heisler, 1984, 1986) (0.0346 mmol l^{-1} mmHg^{-1}) and P_{e,CO_2} is the calculated P_{CO_2} in haemolymph (mmHg).

Standard metabolic rate and condition index

Standard metabolic rate (SMR) was determined using a closed respiratory system (Parker et al., 2012) when oysters were immersed. Following 3 weeks under experimental conditions, two individuals were randomly selected from each replicate tank for measurements. Oysters were placed in individual 500 ml airtight chambers filled with FSW set to the corresponding P_{CO_2} of that treatment. Each chamber was fitted with a fibre-optic O_2 probe (PreSens dipping probe DP-PSt3, AS1 Ltd, Regensburg, Germany). The probes were calibrated using a two-point calibration (0% and 100% air-saturated FSW) and all measurements were done at the experimental temperature of 22°C. The time taken to reduce the percentage oxygen saturation of seawater in the chamber from 100% to 80% was recorded. A 'blank' chamber containing only FSW was set up for each treatment to test for bacterial respiration. The change in this chamber over the duration of oyster measurements was negligible and therefore not included in the SMR calculation. Time was only recorded when oysters were actively respiring (time during which oxygen levels were decreasing). Prior to these SMR measurements, food was withheld for 24 h to remove any variability associated with digestive metabolism, and individuals were only measured following their allocated immersion time. Following the measurements, oysters were removed from the chambers, opened and tissue was separated from the shell. Both tissue and shells were dried in an oven at 70°C for 72 h then weighed using an electronic balance (±0.001 g). SMR was calculated for each individual using

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Eqn 3:

$$\mathrm{SMR} = \frac{[V_\mathrm{r} \times \Delta C_{\mathrm{O_2,w}}]}{\Delta t \times M_\mathrm{b}}, \quad (3)$$

where SMR is oxygen consumption normalised to 1 g of dry tissue mass (mg O_2 g^{-1} dry tissue mass h^{-1}), V_r is the volume of the respiratory chamber minus the volume of the oyster (l), $\Delta C_{\mathrm{O_2,w}}$ is the measured change in water oxygen concentration (mg O_2 l^{-1}), Δt is time (h) and M_b is the dry tissue mass (g) (Parker et al., 2012). Condition index was calculated as the ratio of shell mass (as a proxy for shell volume) to somatic mass using Eqn 4:

$$\mathrm{CI} = \left(\frac{M_\mathrm{b}}{M_\mathrm{s}}\right) \times 100, \quad (4)$$

where CI is condition index, M_b is dry tissue mass (g) and M_s is dry shell mass (g).

Shell growth

At the beginning of the experimental exposure, five oysters were randomly selected from each replicate tank and their shell length (antero-posterior measurement) was taken using digital Vernier callipers (±0.01 mm). This procedure was then repeated following 3 weeks of experimental exposure. For each replicate, the mean shell length of the five oysters at the beginning was subtracted from the mean shell length of the five oysters at the end of the exposure, to give mean shell growth (mm) per replicate tank.

Data analysis

To test for differences among haemolymph variables (pH_e, P_{e,CO_2}, $[HCO_3^-]_e$; n=3), condition index (n=3) and SMR (n=3) during immersion, data were analysed using an orthogonal nested 4-way ANOVA. P_{CO_2} treatment (ambient or elevated) was the first factor, tidal treatment (subtidal or intertidal) the second factor, shore collection height (subtidal or high-intertidal) the third factor and tank the fourth factor. Measurements taken during emersion were analysed using a 3-way orthogonal ANOVA where CO_2 (ambient or elevated) was the first factor, shore collection height (subtidal or high-intertidal) the second factor and tank the third factor. To compare emersion and immersion measurements taken from oysters in an intertidal cycle, a 4-way orthogonal nested ANOVA was used, where cycle (immersion versus emersion) was the first factor, CO_2 (ambient or elevated) was the second, shore collection height (subtidal or high-intertidal) was the third and tank was the fourth factor. Shell growth (n=3) was analysed using a 3-way ANOVA where CO_2 (ambient or elevated) was the first factor (fixed and orthogonal), tidal treatment (subtidal or intertidal) was the second (fixed and orthogonal) and shore collection height (subtidal or high-intertidal) was the third (fixed and orthogonal). In all analyses except shell growth, the first three (or two in the case of emersion measurements) factors were fixed and orthogonal, and the tank (TA) factor was random and nested in the three (or two) other factors. All data met Cochran's test for homogeneity of variance without transformation prior to analysis and were analysed using Gmav 5 software (Underwood et al., 2002). SNK tests were performed *post hoc* to determine the source of variation among means (Underwood, 1996). Mean results for measured variables were graphed using Microsoft Excel 2007, with error bars indicating s.e.m. Experimental factors that were not significant (α>0.1; Underwood, 1996) were pooled for some figures to give a mean of the combined non-significant factors.

RESULTS

Haemolymph variables

pH_e

Overall, the pH_e of oysters experiencing an intertidal cycle was higher when they were submerged in the water (immersed) rather than out of the water (emersed), i.e. following 3 h of immersion, the pH_e of oysters was significantly higher than it was following 9 h of emersion (Fig. 1A). Oysters that were emersed for 9 h had a similar pH_e in ambient and elevated P_{CO_2} treatments, which ranged between 6.8±0.04 and 6.79±0.05, respectively (ANOVA, P>0.5).

When oysters were immersed, pH_e was reduced at elevated (pH_e=7.4±0.04) compared with ambient (pH_e=7.58±0.02) P_{CO_2}, regardless of whether oysters were held in a subtidal or intertidal treatment (Fig. 1A). Further, when oysters were immersed, pH_e was lowest at elevated P_{CO_2} in the intertidal treatment (CO_2×tidal treatment interaction; ANOVA, $F_{1,16}$=5.52, P=0.032; Fig. 1A), i.e. the pH_e of oysters in the intertidal treatment remained lower than that in the subtidal treatment for oysters immersed and exposed to elevated P_{CO_2}. There was no significant difference in the pH_e of oysters collected from the subtidal or high intertidal shore (P>0.1).

P_{e,CO_2}

The mean P_{e,CO_2} of oysters was approximately three times greater when oysters were emersed (P_{e,CO_2}=0.67±0.06 kPa) compared with when they were immersed in water (P_{e,CO_2}=0.18±0.03 kPa; ANOVA, $F_{1,16}$=146.45, P<0.001). Further, when oysters were emersed, the P_{e,CO_2} in the haemolymph was greatest at elevated P_{CO_2} (P_{e,CO_2}=0.75±0.081 kPa) compared with the ambient P_{CO_2} treatment (P_{e,CO_2}=0.59±0.038 kPa; ANOVA, $F_{1,8}$=15.81, P=0.0041) (Fig. 1B). Oysters that were immersed and in an elevated P_{CO_2} treatment also had a significantly greater P_{e,CO_2} than those in the ambient treatment (ANOVA, $F_{1,16}$=12.82, P=0.0025).

Oysters collected from the high-intertidal zone had greater P_{e,CO_2} levels than those collected from the subtidal zone during emersion (cycle×shore height interaction; ANOVA, $F_{1,16}$=6.05, P=0.025).

$[HCO_3^-]_e$

There were no significant effects of any factors (ANOVA, P>0.07) on the $[HCO_3^-]_e$ of oysters except for among tanks ($F_{16,48}$=2.35, P=0.01), when measured during immersion. This suggests variability in $[HCO_3^-]_e$ among tanks. During immersion, there was a trend for $[HCO_3^-]_e$ of oysters to be greater in the subtidal compared with the intertidal treatment. Oysters collected from the high shore had a trend for greater $[HCO_3^-]_e$ under elevated P_{CO_2} (Fig. 1C).

Whole-organism measurements

SMR

The SMR of oysters was significantly increased by elevated compared with ambient P_{CO_2} (ANOVA, $F_{1,16}$=11.03, P=0.0043) and intertidal compared with subtidal treatment (ANOVA, $F_{1,16}$=8.97, P=0.0086). The greatest SMR was observed in the combined elevated P_{CO_2} and intertidal treatment (Fig. 2). The oysters collected from the subtidal shore increased their SMR when transferred to an intertidal treatment, whereas oysters from the high-intertidal shore did not change their metabolic rate (significant shore height×tidal treatment interaction; ANOVA, $F_{1,16}$=5.32, P=0.035; Fig. 2).

Condition index

Oysters in the subtidal treatment had significantly greater condition index (ANOVA, $F_{1,16}$=8.77, P=0.0092) compared with oysters in

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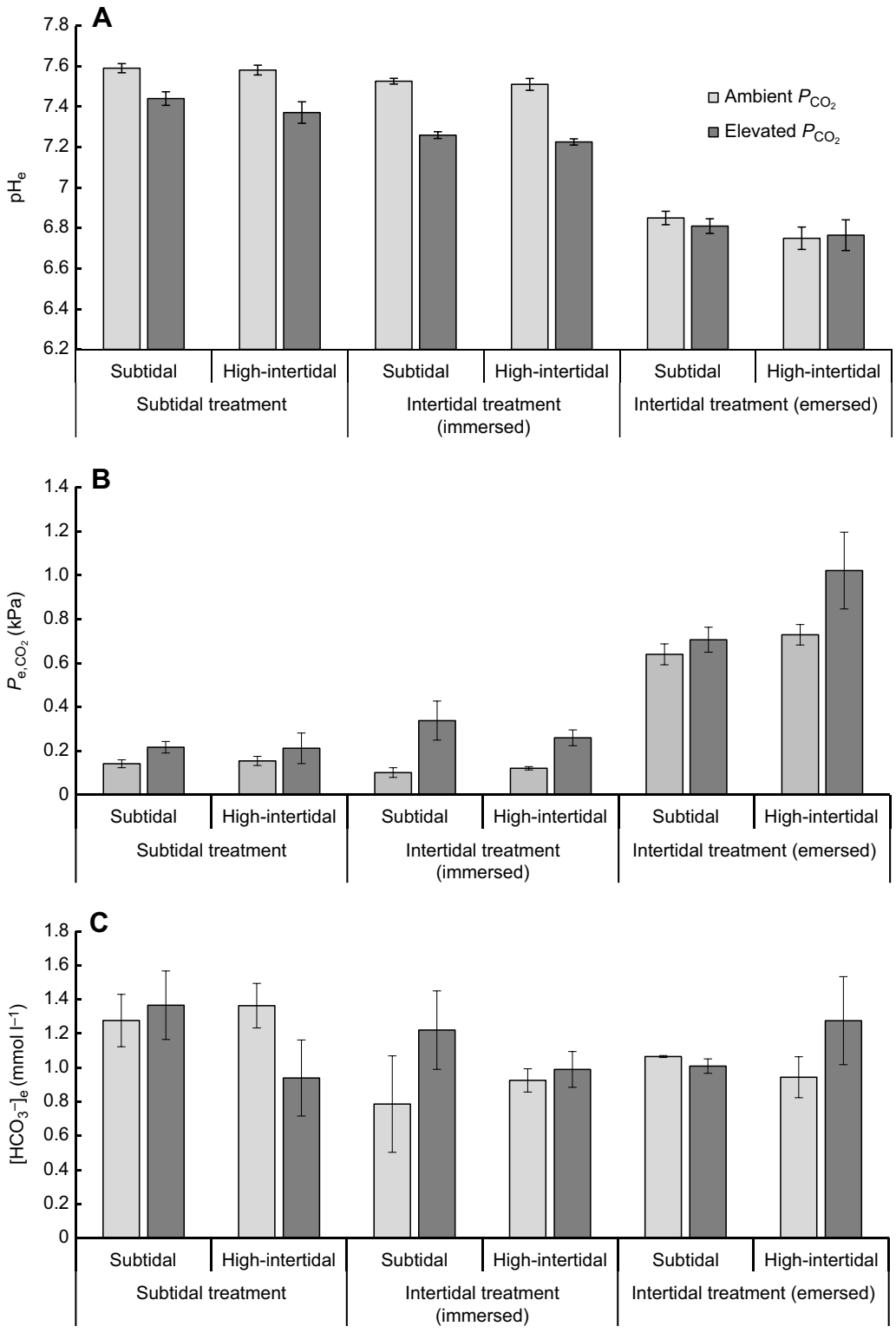

Fig. 1. Haemolymph variables of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. (A) Haemolymph pH (pH_e), (B) P_{CO_2} in the haemolymph (P_{e,CO_2}) and (C) the concentration of HCO_3^- in the haemolymph ($[HCO_3^-]_e$) were measured in the subtidal treatment and in the intertidal treatment during immersion and emersion at (400 µatm) and elevated (1000 µatm) P_{CO_2}. 'Subtidal' and 'High-intertidal' on the *x*-axis indicate the shore height at which oysters were collected; the tidal treatment that oysters were placed into is indicated below (subtidal treatment: constant immersion; intertidal treatment: 3 h immersion, 9 h emersion). Bars represent means±s.e.m. (*n*=9).

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Fig. 2. Standard metabolic rate (SMR) of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. SMR was measured at ambient (400 µatm) and elevated (1000 µatm) P_{CO_2}. Shore collection heights and tidal treatments as per Fig. 1. Measurements of SMR could not be taken during emersion as SMR can only be measured when immersed. Bars represent means±s.e.m. (n=6). Different letters represent significant differences (P<0.05, *post hoc* SNK) between subtidally collected oysters in different tidal treatments. Pairwise comparisons were conducted on significant factors following a 4-way ANOVA.

the intertidal treatment (Fig. 3). Condition index was not dependent on where oysters were collected on the shore or their P_{CO_2} exposure (Fig. 3).

Shell growth

Oysters in the subtidal treatment had significantly greater shell growth compared with oysters in the intertidal treatment (Riebesell et al., 2010; $F_{1,16}$=9.17, P=0.008; Fig. 4). Mean shell growth was lowest in the oysters in the intertidal treatment at ambient P_{CO_2} (Fig. 4). There was no significant effect of any other factor on shell growth.

DISCUSSION

This study found the acid–base balance of *S. glomerata* to be dependent on both tidal treatment (either intertidal or subtidal) and exposure to elevated P_{CO_2}. When oysters were kept in the intertidal treatment, they experienced a significantly greater reduction in pH_e at elevated P_{CO_2} compared with oysters that were kept in the subtidal treatment. These oysters also displayed a significantly greater increase in SMR and P_{e,CO_2}. These results support our first hypothesis: an intertidal environment exacerbates the hypercapnic effects of elevated seawater P_{CO_2}. The height on the shore where oysters were collected had no effect on pH_e. There was some effect of collection height on SMR and P_{e,CO_2}, although this was not sufficiently strong to provide support for our second hypothesis: oysters collected from the high-intertidal shore are more resilient than those from the subtidal shore to the effects of extracellular hypercapnia associated with emersion and elevated P_{CO_2}. We have shown that the impact of ocean acidification on the acid–base balance of oysters is greater when oysters are in an intertidal environment. For sessile organisms inhabiting a gradient of stress, such as oysters on the intertidal shore, future ocean acidification will

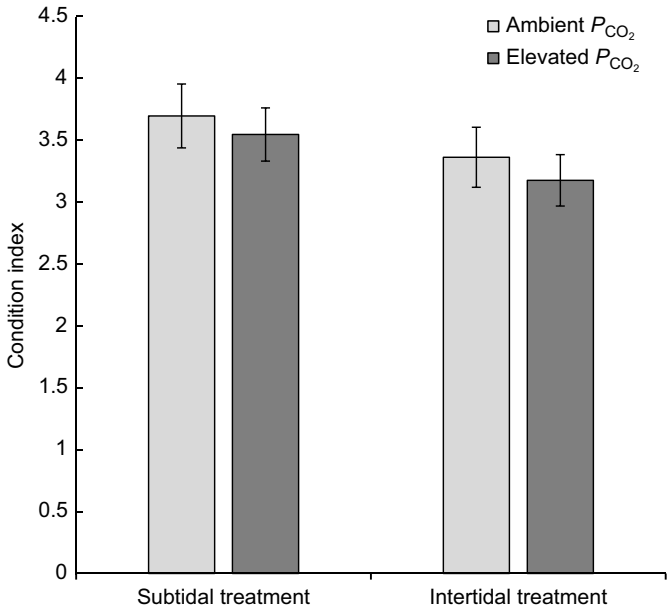

Fig. 3. Condition index of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. Condition index was measured at ambient (400 µatm) and elevated (1000 µatm) P_{CO_2} for subtidal and intertidal treatments (see Fig. 1). Bars represent means±s.e.m. (n=12). The shore height factor was pooled to give a combined mean.

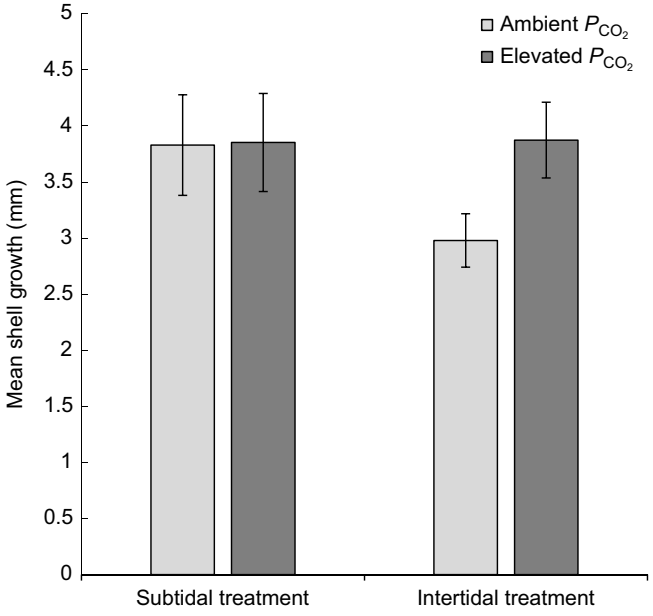

Fig. 4. Mean shell growth of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. Shell growth was measured at ambient (400 µatm) and elevated (1000 µatm) P_{CO_2} for subtidal and intertidal treatments (see Fig. 1). Bars represent means±s.e.m. (n=30). The shore height factor was pooled to give a combined mean.

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have a differential effect across the distribution. In a future acidified ocean, during emersion at low tide, crabs and other organisms capable of locomotion will be able to escape and seek refugia (Rastrick et al., 2014). Sessile organisms such as oysters, however, fixed to the rocky shore and unable to relocate may experience a contraction in their vertical range.

The impact of elevated CO_2 and intertidal air exposure on *S. glomerata*

Under ambient P_{CO_2}, adult *S. glomerata* experienced significant hypercapnia following emersion. When emersed for 9 h, P_{e,CO_2} rose to a level three times higher than that of oysters that were immersed. Further, oysters exposed to elevated P_{CO_2} experienced an even greater rise of P_{e,CO_2} while emersed compared with those in the ambient treatment. Bivalves such as oysters will close their valves during emersion, limiting respiration. As the organism excretes metabolic CO_2 via aerobic and then anaerobic mechanisms, hypercapnic conditions develop and extracellular acidosis occurs (Burnett, 1988; Truchot, 1990). While P_{e,CO_2} did not differ between the subtidal treatment and intertidal treatment during immersion, it is likely that the continual cost of 'defending' the increase in P_{e,CO_2} experienced in the intertidal treatment during emersion will have negative consequences for other fitness-sustaining processes (i.e. immune response, reproduction, shell and somatic growth; Michaelidis et al., 2005).

The hypercapnia experienced during emersion caused pH_e to fall by 0.6 units under both ambient and elevated P_{CO_2}. This fall was not completely compensated for once the oysters were immersed, with the oysters in the intertidal cycle maintaining a significantly lower pH_e than those in the subtidal cycle at ambient P_{CO_2}. Furthermore, these effects were exacerbated by elevated P_{CO_2}, as the lowest pH_e recorded during immersion was in the combined intertidal and elevated P_{CO_2} treatments. The multiple stressor effect of intertidal treatment and elevated P_{CO_2} was greater than what was predicted by a multiplicative model of the product of the individual effects of either intertidal or elevated P_{CO_2} treatment. Therefore, this interactive effect on pH_e of the two stressors could be considered a synergistic effect or as, Folt et al. (1999) describes, a 'multiplicative synergism'.

Decreasing pH_e in response to periods of emersion-induced hypercapnia is well established (e.g. Truchot and Duhamel-Jouve, 1980; Burnett, 1988). Further, a decrease of pH_e in response to ocean acidification in marine invertebrates is also a well-reported phenomenon (e.g. Lannig et al., 2010; Parker et al., 2013; Schalkhausser et al., 2013). In this study, the pH_e of oysters dropped approximately 0.2 units when they were kept subtidally and under elevated P_{CO_2}. This is comparable to other studies investigating the effects of ocean acidification on bivalve molluscs (Michaelidis et al., 2005; Lannig et al., 2010; Schalkhausser et al., 2013) including *S. glomerata* (Parker et al., 2013, 2015). However, we found that the addition of an intertidal treatment to the elevated P_{CO_2} treatment caused the pH_e of oysters to fall another 0.15 units during immersion. Rastrick et al. (2014) found that the physiological recovery from hypercapnia in the crab *Necora puba* associated with emersion was delayed by elevated P_{CO_2}. Extended periods of decreased pH_e are known to cause a significant reduction in protein synthesis (Kwast and Hand, 1996; Reid et al., 1997), which ultimately leads to decreased somatic growth (Michaelidis et al., 2005). The consistently lower pH_e in oysters in the intertidal and elevated P_{CO_2} treatments means those oysters are likely to have lower somatic growth because of the greater cost of homeostasis (Lannig et al., 2010; Parker et al., 2013).

Bivalves are known to have a limited capacity to compensate for extracellular acid–base disruptions (Schalkhausser et al., 2013). The buffering of extracellular fluids is mostly achieved in bivalves by dissolution of the shell to release HCO_3^- ions (Lindinger et al., 1984), although such shell dissolution is potentially unsustainable in the long term, especially when environmental conditions are unfavourable (Melzner et al., 2011). There were no significant effects of any treatments on the $[HCO_3^-]_e$ of oysters. The $[HCO_3^-]_e$ data were at times quite variable; however, there was a trend for greater $[HCO_3^-]_e$ during emersion and under elevated P_{CO_2}. Lannig et al. (2010) observed only a small increase in $[HCO_3^-]_e$ in the oyster *C. gigas* at a pH of 7.7 (comparable to this study), whereas Michaelidis et al. (2005) observed an increase in $[HCO_3^-]_e$ in the mussel *Mytilus galloprovincialis* when exposed to a pH of 7.3. It has been suggested that a high degree of acidosis is required for bivalves to undergo shell dissolution (Lannig et al., 2010).

An increase in SMR was observed under elevated P_{CO_2} and also when oysters were in an intertidal treatment. The oysters that were in the combined elevated P_{CO_2} and intertidal treatment experienced the greatest increase in P_{e,CO_2} and the lowest pH_e. Increased SMR under elevated P_{CO_2} is believed to occur in response to the increased cost of homeostasis (Fabry et al., 2008; Pörtner, 2008; Portner and Farrell, 2008). Consistently lower pH_e as experienced by oysters in the intertidal and elevated P_{CO_2} treatment may cause greater energy expenditure in homeostatic processes (i.e. defending intracellular pH; Fabry et al., 2008; Pörtner, 2008). Not only do water-breathing intertidal organisms need time immersed in water to excrete waste gases (Truchot, 1990) but also they use this time to feed. The lower condition and decreased growth of oysters in the intertidal treatment potentially reflects reduced opportunities for feeding and the additional metabolic cost which ultimately results in an imbalance of energy supply and demand (Pörtner et al., 2004).

Effect of shore collection height on the response of *S. glomerata* to elevated P_{CO_2}

Oysters were collected from two tidal heights (subtidal and intertidal) to determine whether oysters that had experienced a lifetime of acclimation to frequent emersion and internal acidosis were more resilient to elevated P_{CO_2}. The shore height where oysters were collected did not interact with tidal or elevated P_{CO_2} treatment on the pH_e and P_{e,CO_2} of oysters when measured during immersion. There were, however, some interactions of shore height with tidal treatment on the SMR of immersed oysters and P_{e,CO_2} of emersed oysters. When oysters collected from the subtidal shore were placed into an intertidal treatment, they increased their SMR to a level greater than that of oysters collected from the high-intertidal shore. Oysters collected from the high-intertidal shore did not adjust their SMR when under ambient P_{CO_2} and in either the subtidal or intertidal treatment. During emersion, oysters collected from the high-intertidal shore were shown to have a greater level of P_{e,CO_2} compared with subtidally collected oysters.

Other studies have found alterations in bivalve metabolism in response to tidal heights. Mussels (*M. edulis*) were shown to have differential metabolic indices such as glycogen stores and metabolic enzymes at different tidal heights on the shore (Lesser, 2016). These metabolic indices converged when mussels were transplanted to a common tidal cycle (Lesser, 2016). In another study on *M. edulis*, mussels transplanted from a subtidal to an intertidal treatment were shown to change their SMR within 14 days to suit their new environment (Widdows and Shick, 1985). When mussels acclimated to a subtidal environment were subjected to emersion, they were slower at repaying their accumulated 'oxygen debt'

compared with mussels acclimated to an intertidal environment (Widdows and Shick, 1985). Widdows and Shick (1985) also concluded that *M. edulis* that were intertidally acclimated were more efficient at metabolising food while emersed.

The greater SMR found in this study in subtidally collected oysters placed in the intertidal cycle suggests that these oysters are possibly trying to repay their oxygen debt accumulated during emersion, and metabolise food during their time immersed. The lower SMR while immersed and greater P_{e,CO_2} while emersed of oysters of high-intertidal origin may be the result of their lifetime acclimation to this environment. They are potentially more efficient at repaying an oxygen debt, and continue to metabolise ingested food while emersed.

The findings of this and previous studies (Widdows and Shick, 1985; Lesser, 2016) in relation to changing metabolisms of intertidal bivalves when transplanted, suggest that oysters are highly plastic (Collicutt and Hochachka, 1977; Greenway and Storey, 1999; Hamdoun et al., 2003; Ernande et al., 2004; David et al., 2005; Zhang et al., 2012). Plastic responses allow for an immediate response to cope with, and potentially overcome, a stressor (West-Eberhard, 1989). Although plastic responses are essential to coping with stress, they do come at an energetic cost (Koehn and Bayne, 1989; Van Buskirk and Steiner, 2009) and are not always sufficient to prevent death (Visser and Both, 2005). Despite the high plasticity of oysters (Zhang et al., 2012), they still have physiological limits that can be breached (Potter and Hill, 1982) and are especially vulnerable in early life stages (Dove and O'Connor, 2007; Parker et al., 2010). Here, it was found that any acclimation to a high-intertidal environment is likely to be due to a plastic acclimatory response, rather than adaption. In broadcast spawning organisms such as oysters, larval dispersal decreases the capacity to adapt to local conditions and increases the prevalence of phenotypic plasticity (Parsons, 1997; Kinlan and Gaines, 2003).

The common practice for ocean acidification experiments is to maintain organisms under a subtidal regime whilst exposing them to elevated P_{CO_2} (Riebesell and Gattuso, 2015). There is a paucity of studies that have measured the response of marine molluscs to simulated tidal scenarios (Gazeau et al., 2013; Rastrick et al., 2014). One major criticism of ocean acidification research is that the experimental environment is too static and not analogous to the 'real world' (Riebesell and Gattuso, 2015). Previous investigations into intertidal organisms neglecting tidal patterns may have underestimated the effects of ocean acidification on 'real world' populations.

Fate of high-shore oysters

Although oysters can be highly plastic, such plasticity in responses that allow them to withstand stress are energetically costly, reducing their condition and growth, and impacting their future fitness and capacity for resilience. The cost of homeostasis is likely to be greatest for those oysters on the high shore, where feeding time is also limited and emersion is most severe. This may result in energy trade-offs affecting gamete production (Rijnsdorp, 1990; Lester et al., 2004), somatic growth (Michaelidis et al., 2005) and immune responses (Bibby et al., 2008), which has implications for larval settlement and recruitment (Connell, 1985), and adult growth (Pörtner et al., 2004). When tidal emersion is coupled with other stressors such as increased temperature and disease, the resilience of oysters may be further reduced (Potter and Hill, 1982; Dwyer and Burnett, 1996; Willson and Burnett, 2000).

The combination of tidal emersion and elevated P_{CO_2} was found to be synergistic and sublethal rather than lethal. It is likely that under future levels of elevated P_{CO_2}, oysters may reach their physiological limit in the intertidal zone, and may not be able to exist as high on the shore as they currently do. Oysters are essential habitat-forming organisms in temperate intertidal systems, and provide a range of ecosystem services (Gutiérrez et al., 2003; Cole et al., 2007). Across the world, oyster reefs are already in decline. This experiment has shown that those oysters found in the high intertidal zone will be most vulnerable to ocean acidification. We conclude that in a high-CO_2 world, the upper vertical limit of oyster distribution on the shore may be reduced.

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Author contributions
E.S. was involved in the development of the concept, experimental design and setup, and data analysis. L.M.P. was also involved in the development of the concept, experimental design, running of experiments, measurement of organisms and collection of data. L.S.S. was involved in the collection of data. W.A.O. provided facilities and support, as well as contributing to experimental design. P.M.R. was responsible for supervision of the experiment, development of the concept and experimental design. All authors contributed to the writing of this manuscript.

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