



Physiological effects of environmental acidification in the deep-sea urchin *Strongylocentrotus fragilis*

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Abstract. Anthropogenic CO₂ is now reaching depths over 1000 m in the Eastern Pacific, overlapping the Oxygen Minimum Zone (OMZ). Deep-sea animals are suspected to be especially sensitive to environmental acidification associated with global climate change. We have investigated the effects of elevated pCO₂ and variable O₂ on the deep-sea urchin *Strongylocentrotus fragilis*, a species whose range of 200–1200 m depth includes the OMZ and spans a pCO₂ range of approx. 600–1200 μatm (approx. pH 7.6 to 7.8). Individuals were evaluated during two exposure experiments (1-month and 4 month) at control and three levels of elevated pCO₂ at in situ O₂ levels of approx. 10 % air saturation. A treatment of control pCO₂ at 100 % air saturation was also included in experiment two. During the first experiment, perivisceral coelomic fluid (PCF) acid-base balance was investigated during a one-month exposure; results show *S. fragilis* has limited ability to compensate for the respiratory acidosis brought on by elevated pCO₂, due in part to low non-bicarbonate PCF buffering capacity. During the second experiment, individuals were separated into fed and fasted experimental groups, and longer-term effects of elevated pCO₂ and variable O₂ on righting time, feeding, growth, and gonadosomatic index (GSI) were investigated for both groups. Results suggest that the acidosis found during experiment one does not directly correlate with adverse effects during exposure to realistic future pCO₂ levels.

in the Eastern Pacific (Gruber and Sarmiento, 2002; Sabine et al., 2004), overlapping the Oxygen Minimum Zone (OMZ) and driving ocean acidification (OA). Increased pCO₂ raises even further the already elevated pCO₂ of OMZs, which are known to be stressful environments for many taxa. As OA and climate-related deoxygenation (Doney et al., 2009b; Keeling and Garcia, 2002; Stramma et al., 2011) intensifies in the future, animals in OMZs are expected to experience increased physiological stress (Dupont et al., 2010; Melzner et al., 2012; Pörtner et al., 2011). Deep-sea animals living in this dark, energy-poor environment have evolved with moderate to mild temporal variation in biochemical conditions, but very large spatial variation. In particular, taxa spanning a range of depths within and beyond an OMZ experience very large ranges of temperature, pH, pCO₂, oxygen, and food; not as individuals, but as populations. The high amount of genetic diversity and/or plasticity presumably required for a population to persist throughout a bathymetric range including an OMZ suggests we should expect fairly high tolerance to at least the extent of conditions seen within its bathymetric range. We expect the apparent limitation in tolerance of OMZ taxa to global climate change is not a product of evolutionary constancy, but rather is a product of the limited energy (food and oxygen) available to cope with the stress of environmental change (Barry et al., 2011; Pane and Barry, 2007; Seibel and Walsh, 2003). Taking these considerations into account, we aim to investigate the physiological effects of elevated pCO₂ on a calcifying OMZ taxon, the deep-sea fragile urchin *Strongylocentrotus fragilis*.

S. fragilis is a member of the Strongylocentrotidae, a worldwide, and largely shallow water family, that inhabits the upper continental slope along the eastern North Pacific, ranging in depth from 200–1200 m off central California. Its

1 Introduction

Anthropogenic CO₂ is penetrating to the deep waters of the oceans through thermohaline circulation and other vertical mixing processes, and has now reached depths over 1000 m

relatively large bathymetric range indicates that individuals from the *S. fragilis* population used in this study may experience temperatures from approx. 3 to 9 °C, oxygen levels of approx. 10 to 100 μM , pH of 7.6 to 7.8 (approx. 600–1200 $\mu\text{atm } p\text{CO}_2$), calcite saturation near $\Omega = 1$, and aragonite saturation of $\Omega < 1$. *S. fragilis* is a key member of the benthic megafaunal community on the upper slope, where it is an important detritivore. As described, the bathymetric range of *S. fragilis* spans a fairly large gradient in temperature, oxygen, and pH, and presumably also food supply; each of which may affect individual energy budget as well as population distribution.

Environmental acidification can affect an individual marine animal's energy budget by disrupting homeostatic processes; parameters including growth rates, reproduction, and nutritional status may consequently be reduced (Dupont et al., 2012; Pörtner, 2012a; Uthicke and Fabricius, 2012). The extent to which a marine animal can regulate extracellular fluid acid-base balance thus offers a reasonable predictor of the extent to which behavior, growth, and reproduction may be affected by increases in environmental acidity (Seibel and Walsh, 2003; Spicer et al., 2007). Proper function of enzymes, including those necessary for homeostatic processes, demands precise intra- and extracellular pH conditions (pH_i and pH_e , respectively). Precise regulation of acid-base balance is typically far better honed in more advanced animals (e.g., bony fishes as compared to aquatic invertebrates). Studies suggest that some echinoderms simply accumulate bicarbonate in the perivisceral coelomic fluid (PCF) as a byproduct of stereom (carbonate test) dissolution, rather than (or in addition to) actively accumulating bicarbonate for acid-base balance (Holtmann et al., 2013; Spicer et al., 1988). Furthermore, species-dependence has been shown in capacity of sea urchins to regulate acid-base balance (Calosi et al., 2013). Shallow-living sea urchins *Psammechinus miliaris* and *Echinus esculentus* were shown to perfectly compensate the respiratory acidosis induced by emersion (Spicer et al., 1988). Similarly, *Strongylocentrotus droebachiensis*, a taxa experiencing considerable environmental variability, is able to fully or partially compensate extracellular pH changes by accumulation of bicarbonate (Stumpp et al., 2012). However, taxa distributed around a CO_2 vent revealed very different capacities for accumulation of extracellular bicarbonate between sea urchins *Arbacia lixula* and *Paracentrotus lividus* (Calosi et al., 2013). The ability of deep-sea urchin taxa to regulate extracellular acid-base balance is yet unknown. Interestingly, seastars that are unable to compensate for increased seawater $p\text{CO}_2$ have been shown to perform with uncompensated acidosis for up to 6 months (Dupont and Thorndyke, 2012; Hernroth et al., 2011), suggesting extracellular acid-base balance may not always have direct implications for fitness.

Ocean acidification typically does not happen as a solitary environmental change; it is often accompanied by warming temperatures and less frequently by hypoxia. When $p\text{CO}_2$, temperature, and O_2 act simultaneously on individual phys-

iology, negative effects of one stressor may be offset, or additive (or even synergistic) negative effects may be observed. For example, concomitant low O_2 and high $p\text{CO}_2$ interfere with aquatic respiration as O_2 becomes more difficult to obtain from the hypoxic water and CO_2 becomes more difficult to excrete to the water along a weaker concentration gradient. Despite a recent surge in studies investigating interactive effects of ocean acidification and other climate stressors, the consequences of these scenarios are still largely undetermined (Crain et al., 2008; Pörtner, 2012b, 2010; Wernberg et al., 2012). Calcification is a more clearly visible processes adversely affected by rising ocean acidity (Orr et al., 2005). Larval exposure to elevated $p\text{CO}_2$ has been shown to delay development in some echinoderms (Byrne et al., 2013; Dupont et al., 2010; Hofmann et al., 2010; Kurihara et al., 2012), and adults of some taxa have been inferred to suffer dissolution of the stereom on exposure to elevated $p\text{CO}_2$ (Holtmann et al., 2013; Miles et al., 2007; Orr et al., 2005; Ries et al., 2009); however, bicarbonate liberated into the PCF during stereom dissolution may in fact be useful in buffering PCF acidification (Holtmann et al., 2013).

Regulation of acid-base balance can be particularly energetically costly (Holtmann et al., 2013; Stumpp et al., 2012), and is vital not only for maintaining extracellular fluid chemistry but also intracellular chemistry including processes linked to calcification (Beniash et al., 1997, 1999). Since acid-base balance regulation is an energetically costly process associated with oxygen demand, it follows that the physiological performance of *S. fragilis* will be most vulnerable to ocean acidification and hypoxia in the core of the OMZ. Notably, this and other oxygen-deplete zones are expected to expand in future oceans (Brewer and Peltzer, 2009). Two experiments have been used to test the effects of elevated $p\text{CO}_2$ and variable O_2 on *S. fragilis*; the first addresses the ability of *S. fragilis* to regulate perivisceral coelomic fluid (PCF) acid-base balance at near- and far-future levels of environmental $p\text{CO}_2$, over the course of one month. The second set of experiments addresses the behavioral, nutritional, and growth consequences of similar levels of $p\text{CO}_2$ on *S. fragilis* over more than four months, and includes an additional experimental variable of fed vs. fasted groups. This set of experiments also examines whether *S. fragilis* is able to improve upon baseline righting time, feeding, and growth, on exposure to surface levels of O_2 rather than typical OMZ levels. Both experiments include an “extreme $p\text{CO}_2$ ” scenario in which we hope not only to add an additional data point, but also to perhaps shed light on processes that may be impacted more subtly in other treatments yet represent important mechanisms by which physiological disruption may occur on exposure to elevated $p\text{CO}_2$. However, we also recognize the possibility that different physiological mechanisms may be used for a mild vs. extreme acidosis, and have taken this caveat into account in our interpretation of the data.

2 Methods

Urchin collection and maintenance

S. fragilis were captured by suction sampler from the Remotely Operated Vehicle (ROV) *Ventana*, operated from the R/V *Point Lobos*, from depths of approx. 500 to 1000 m in Monterey Bay, CA (Fig. 1), during late 2010 and early 2011. Once aboard the R/V *Point Lobos*, individuals were kept in 5 °C seawater for 3–5 h until arrival at the Monterey Bay Aquarium Research Institute (MBARI). Urchins were then held in climate-controlled environmental chambers in the laboratory, in an approx. 50 L acrylic tank receiving flow-through chilled seawater pumped in from Monterey Bay (5.5 ± 0.5 °C, 220 ± 20 μ M O₂, pH 7.9 ± 0.1 , p CO₂ 500 ± 100 μ atm, and salinity 34 ± 1). While it is a caveat worth mentioning, the transfer of *S. fragilis* to lab conditions (i.e. higher oxygen and pH than in situ) has been shown to have negligible effects on metabolic rates, as oxygen consumption appears to saturate around 40 μ M O₂ (J. R. Taylor et al., unpublished data, 2012).

Experimental conditions, setup, and procedure

Experiments one and two are described below and use similar ranges of experimental conditions. Both experiments include a “control p CO₂” treatment of surface Monterey Bay seawater p CO₂ levels (pH 7.9 to 8.0); a “moderate p CO₂” treatment of expected near-future (approx. 2100) p CO₂ at the relevant depth range of 200–1200 m (pH 7.5 to 7.7); a “high p CO₂” treatment of far-future (approx. 2300) p CO₂ at the relevant depth range (pH 7.1 to 7.3); and an “extreme p CO₂” treatment with little relevance to foreseeable ocean p CO₂ (pH 6.6 to 6.7).

During both experiments, individual urchins were held in 1 L glass jars overflowing with seawater of the desired chemistry at a rate of approx. 30 mL min⁻¹, for the duration of the experiment. Target conditions for seawater O₂ and p CO₂ (as pH) were maintained throughout the study period by a PC-manipulated, Gas-Controlled Aquarium (GCA) system supplying seawater to the laboratory’s climate-controlled environmental chamber. In brief, oxygen and pH for each seawater treatment holding tank were controlled using a combination of sensors (Aanderra oxygen optodes and Honeywell pH probes) and membrane contactors connected to recirculation pumps and gas sources. A LabVIEW software system integrated with mass flow controllers for oxygen, carbon dioxide, and nitrogen sources allowed real-time, automated regulation of gas concentrations in each tank (Barry et al., 2008).

Experimental seawater conditions were monitored throughout using a logging system for pH, oxygen, and temperature sensors in the seawater head tanks, and from samples collected directly from jars containing urchins for pH and DIC measurements. During weekly spot-sampling of three randomly selected jars from each treatment, exper-

imental conditions were checked using a Thermo Scientific Orion 5-Star handheld meter with an optode and temperature compensated pH probe. The Orion optode was calibrated using a two-point method at temperature (approx. 6 °C). The probe comes with a sleeve to provide a 100 % air saturation calibration point; a sodium dithionate solution was used to generate a 0 % air saturation point. An Aanderra optode was also used to crosscheck the oxygen (% air saturation, μ M) measurements determined by the GCA system. During jar sampling, the water flow delivery line was removed and the probes and cables (bound together) were lowered mid-way into the 1 L volume of seawater in the jars, easily avoiding the animal contained therein. Output was given five min to stabilize, after which values from each probe were recorded.

During urchin sampling events, jar seawater and tap samples were also collected in glass, gas-tight Scintillation vials with negligible headspace and analyzed within one hour, using the same protocols by which concurrently sampled animal fluids were analyzed. For these samples, DIC was measured by non-dispersive infrared analysis (LI-COR model 6262), as detailed by Friederich et al. (2002) In brief, samples were acidified (5 % phosphoric acid) and the stripped gas introduced into an infrared analyzer. A standard curve was created using sodium carbonate (dried for 4 h at 250 °C before making carbonate standards), and resultant values standardized to certified reference material (A. Dickson CRM, Scripps Institute of Oceanography, La Jolla, CA). Seawater pH was measured by spectrophotometry using the indicator dye m-cresol purple (Clayton and Byrne, 1993; SOP 6b of Dickson et al., 2007; Part 1 of Riebesell et al., 2010). The program CO2Calc (Robbins et al., 2010) was used to calculate p CO₂ from seawater pH and DIC measurements, using CO₂ constants from Millero et al. (2006)

2.1 Experiment one: perivisceral coelomic fluid (PCF) acid-base balance

Experiment one was used to measure the ability of *S. fragilis* to regulate PCF acid-base balance under a range of environmental conditions, and evaluate the hypothesis that its acid-base regulatory capacity is weak. The impact of elevated p CO₂ on urchin acid-base balance was examined over the course of 31 days. Four experimental treatments (30 individuals per treatment) with chemistry defined in Table 1 were applied at the in situ temperature of 5 °C. Individuals were fed kelp (*Macrocystis pyrifera*) weekly to satiation during the experimental period, but food was withheld for > 72 h prior to PCF sampling.

PCF buffering capacity

The non-bicarbonate buffer value (β) of PCF was determined for urchins collected at the same time, but not undergoing experimentation. Samples of PCF were drawn anaerobically

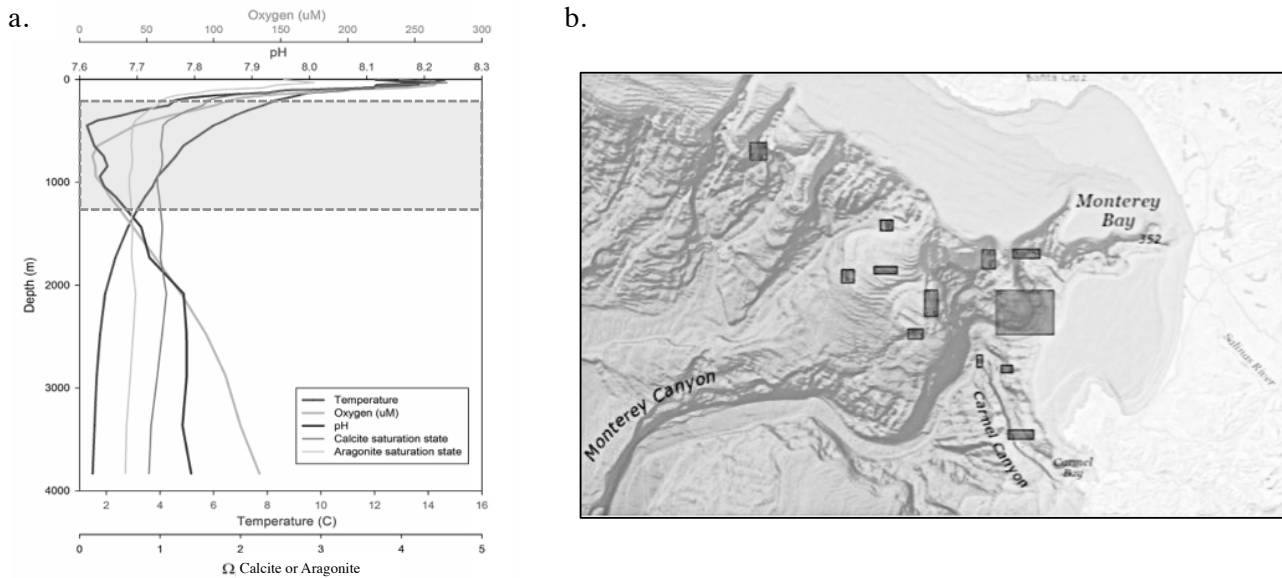


Fig. 1. *S. fragilis* lives at depths of 200–1200 m, in seawater with chemistry approximating that described in the shaded area of (a). Urchins were collected within the boxed regions of Monterey Bay, CA (b), at depths of 500–1000 m, during ROV dives in late 2010 and early 2011. Data shown in (a) acquired from the World Ocean Circulation Experiment (WOCE) station P17C (central CA coast).

through the oral membrane into ice-cold gas-tight Hamilton syringes rinsed with a modified Crab Ringer comprised of NaCl (460 mM), KCl (10 mM), CaCl₂ (20 mM), MgCl₂ (9.5 mM), adjusted to pH 7.8 (Lang and Gainer, 1969; Pane and Barry, 2007). Fluid samples were then centrifuged aerobically at 4000 × g at 5 °C for 1 min to remove debris, added (200 µL) to round-bottom flasks for equilibration with humidified gas mixtures of CO₂ and nitrogen from pre-analyzed cylinders (Airgas), and allowed to equilibrate for 90 min in a shaking cooler at 5 °C. Samples were then drawn into gas-tight syringes and pH_(total scale) was measured using a microelectrode and in-line reference electrode (Microelectrodes) thermostatted to 5 °C and coupled to an Accumet (Fisher Scientific) pH meter. pH was standardized with Radiometer Analytical precision buffers adjusted to the ionic strength of *S. fragilis* PCF (~1050 mOsm). Total CO₂ (*C*_{CO₂}) was measured as the physiological equivalent to seawater DIC, by non-dispersive infrared analysis (LI-COR). PCF *p*CO₂ and [HCO₃⁻], based on measured pH and *C*_{CO₂}, were calculated by rearrangement of the Henderson-Hasselbalch equation:

$$p\text{CO}_2 = C_{\text{CO}_2} / (10^{\text{pH} - \text{pK}'} \times \alpha_{\text{CO}_2} + \alpha_{\text{CO}_2}) \quad (1)$$

Values for CO₂ solubility (α_{CO_2}) and apparent dissociation constant (pK') at the appropriate temperature were taken from Boutilier et al. (1984) and Truchot (1976). The PCF [HCO₃⁻] was calculated from *p*CO₂ and *C*_{CO₂} according to the equation:

$$[\text{HCO}_3^-] = C_{\text{CO}_2} - (\alpha_{\text{CO}_2} \times p\text{CO}_2) \quad (2)$$

β s were derived from linear regression of pH vs. HCO₃⁻ plots.

Blood chemistry

Five individuals were terminally sampled from each treatment five days before treatment conditions were achieved; sampling proceeded on days 0 (when treatment conditions were achieved after a 5-day ramp to conditions), 1, 3, 7, and 31 of treatment. PCF was attained while individuals were gently held by hand at the top of their respective jars in the environmental chamber, inverted with oral surface just below the water's surface. Approx. 2 mL fluid was drawn anaerobically through the oral membrane into ice-cold gas-tight Hamilton syringes rinsed with the modified Crab Ringer described above. PCF samples were held on ice in their respective syringes until measurement of pH by the spectrophotometric method (Clayton and Byrne, 1993; Dickson et al., 2007; Riebesell et al., 2010). Samples were then prepared in triplicate for *C*_{CO₂} measurements by non-dispersive infrared analysis (LI-COR, as above) by diluting 300 µL PCF in 3300 µL Nanopure water in glass scintillation vials (by Hamilton syringe through a septum with negligible headspace). One 16-gauge needle was used to vent the septa, and another delivered sample to the LI-COR. A standard curve was created using three sets of four standards prepared as above, in parallel, and measured before, during, and after PCF samples. Resultant values were standardized to certified reference material (A. Dickson CRM, Scripps Institute of Oceanography, La Jolla, CA).

Table 1. Seawater chemistry for experiments one and two, as measured by GCA logging and spot-sampling (pH, DIC, O₂) and calculated (pCO₂) using CO2SYS (Robbins et al., 2010).

Treatment	pH (total scale)	DIC (μM)	pCO ₂ (μatm)	O ₂ (μM)
Experiment One				
1	7.98 ± 0.030	2142.0 ± 27.26	447.5	25 ± 2.5
2	7.51 ± 0.035	2264.7 ± 38.20	1404.8	25 ± 2.5
3	7.11 ± 0.012	2343.7 ± 26.50	3538.6	25 ± 2.5
4	6.65 ± 0.029	2497.7 ± 162.90	9455.3	25 ± 2.5
Experiment Two				
1	7.92 ± 0.014	2388.6 ± 289.78	585.0	220 ± 22
2	7.92 ± 0.024	2387.1 ± 306.80	585.1	25 ± 2.5
3	7.64 ± 0.014	2482.8 ± 329.00	1144.9	25 ± 2.5
4	7.23 ± 0.011	2710.4 ± 357.40	3191.8	25 ± 2.5
5	6.61 ± 0.054	3413.6 ± 530.03	14053.2	25 ± 2.5

2.2 Experiment two: behavior and nutritional status

The second of our two experimental groups of *S. fragilis* was used to investigate the impacts of pCO₂ and O₂ on righting time, feeding, growth, and reproductive capacity during approx. four months (140 days) exposure. Urchins collected from a depth of 660 m at Sponge Ridge (Monterey Bay, CA) were allowed two days to acclimate en masse in aquaria as described above. The health of 150 urchins (< 48 mm diameter) was evaluated prior to their inclusion in the experiment using a test of ‘righting time’ in which urchins were turned by hand aboral side down. Individuals were monitored for the time required to “right” themselves (oral side down). All 150 urchins showed a righting time less than 10 min and were segregated haphazardly into five groups (30 individuals per group) and each individual placed in a 1 L jar. The jars (A–E; 1–30) were then connected to flow-through seawater supply lines of the Gas Controlled Aquaria (GCA) as described above. The five experimental treatments defined in Table 1 were applied at the in situ temperature of 5 °C. In each treatment, two feeding regimes were applied; odd numbered urchins were fed kelp to satiation, while even numbered urchins were fasted during the experimental period.

2.2.1 Righting time

Video analysis of righting time

The day following urchin segregation into jars, video recordings were made of a righting time test for each individual. Down-looking video through the open-top jar was collected using simple web cameras in waterproof housings connected to a PC. With this setup, 8–10 individuals were monitored simultaneously. The time taken for each individual to right itself was noted, with recording stopped if the individual failed to turn over within two hours. At a number of time points before (5 and 2 days prior to commencement of ex-

perimental conditions) and during (after 6, 16, 23, 44, 99, and 130 days of experimental conditions) the experiment, one righting time was evaluated for each individual from all treatments.

2.2.2 Feeding

Urchins in the fed treatment groups (odd numbered jars in treatments A–E) were offered weighed stipes of kelp (*Macrocystis pyrifera*) to satiation; kelp in jars was replaced every 4–12 days and weighed for calculation of feeding rates. Visual evidence of kelp consumption was also recorded to document feeding frequency; this was used as a Y/N measure of feeding during the feeding period in question. The weight change of kelp upon cutting and exposure to seawater was quantified in parallel for each feeding period, and found to be negligible. A “blotting” technique was validated in our laboratory and used to ensure consistency in attaining kelp weights on removal from treatment jars.

2.2.3 Growth

Photo sizing methods

All urchins in this experiment were digitally photographed in anticipation of sizing individuals from images. Once urchins had been initially evaluated for righting time and placed in assigned jars, a digital 35 mm camera mounted on a tripod and operated via infrared remote was used to take photographs of each animal against a solid black background. Two internal metric scales (one on the background and one on a wand allowing its placement along the major axis midline of the individual) were included in each image. Midway through the experiment this process was repeated. At the termination of the experiment (half of each treatment group was sacrificed at 30 and the other half at 140 days), digital images were again collected. As before, overhead shots were taken of the aboral surface of the individual; additionally, images of the oral side were taken at this time. Individuals were then removed from jars and measured to the nearest 0.1 mm using calipers; these measurements were used to validate measurements made from the digital images.

2.2.4 Reproductive capacity

Gonadosomatic index (GSI) was measured as a proxy for reproductive capacity. Urchin whole body wet weight was measured in the urchins sacrificed after 140 days of treatment exposure; gonads were also extracted and weighed separately in order to calculate the terminal GSI (percentage of gonad to whole body wet weight) for each animal at this time.

2.3 Statistical analyses

All data were analyzed using StatPlus (AnalystSoft) software. Mean values are reported with their standard error of

mean (mean \pm SEM). Statistical evaluation of pH data was done using H^+ values, rather than pH, to achieve a normal distribution (Boutilier and Shelton, 1980). Repeated measures (RM) analysis of variance (ANOVA) was used when applicable to test for differences between timepoints among treatment groups (i.e. pre-treatment measurements for the pH 7.6 group were used as a “control” for later measurements of individuals in the pH 7.6 group). One-way ANOVA, followed by the Tukey HSD test for differences between means, was used to test the impact of treatment conditions between groups. In experiment two, most data did not meet assumptions of normality; Kruskal-Wallis (or Friedman, as appropriate) ANOVAs followed by pairwise Mann-Whitney U tests (or Wilcoxon pairs, as appropriate) were thus employed, using Holm’s sequential Bonferroni correction (Holm, 1979) to determine the reported statistical significance.

3 Results

3.1 Experiment one: perivisceral coelomic fluid (PCF) acid-base balance

During the experimental period, *S. fragilis* PCF underwent significant changes in acid-base balance as compared with pre-treatment (day “-5”) values, evaluated by ANOVA followed by Tukey’s HSD test (Fig. 2). In all treatments, *S. fragilis* PCF sustained a pCO_2 gradient with higher concentration inside the individual ($pCO_{2(in)}$) than in the treatment water outside ($pCO_{2(out)}$) (Table 1 and Fig. 2); however, the average ratio of $pCO_{2(in)} : pCO_{2(out)}$ dropped from 3.5 in the control group to 1.7 in the low pCO_2 group, 1.3 in the high pCO_2 group, and 1.4 in the extreme pCO_2 group. Within the first 24 h of all three elevated pCO_2 exposures, PCF chemistry was titrated in an acidic direction, roughly along the non-bicarbonate buffering (β) line (data not shown). The PCF pCO_2 of high (one-way ANOVA, $F_{5,24} = 28.70$, $P < 0.001$) and extreme (one-way ANOVA, $F_{5,24} = 7.98$, $P < 0.001$) pCO_2 treatment groups did not recover by the end of the experiment; nor did the pH of high (one-way ANOVA, $F_{5,24} = 21.46$, $P < 0.001$) and extreme (one-way ANOVA, $F_{5,24} = 4.80$, $P = 0.004$) pCO_2 treatment groups. Moderate pCO_2 exposed individuals (pCO_2 one-way ANOVA, $F_{5,22} = 7.98$, $P = 0.017$ and pH one-way ANOVA, $F_{5,22} = 7.82$, $P < 0.001$) recovered these parameters at the final (31 days) time point to levels of pCO_2 (Tukey HSD; $P = 0.271$) and pH (Tukey HSD; $P = 0.503$) not significantly different from pre-treatment (Fig. 2). HCO_3^- levels were elevated only towards the end of the experimental period, in the high (one-way ANOVA, $F_{5,24} = 3.16$, $P = 0.025$) and extreme (one-way ANOVA, $F_{5,24} = 6.33$, $P < 0.001$) pCO_2 treatment groups. The high pCO_2 group had significantly elevated HCO_3^- at 31 days (Tukey HSD; $P = 0.017$) and the extreme pCO_2 group at 7 days (Tukey

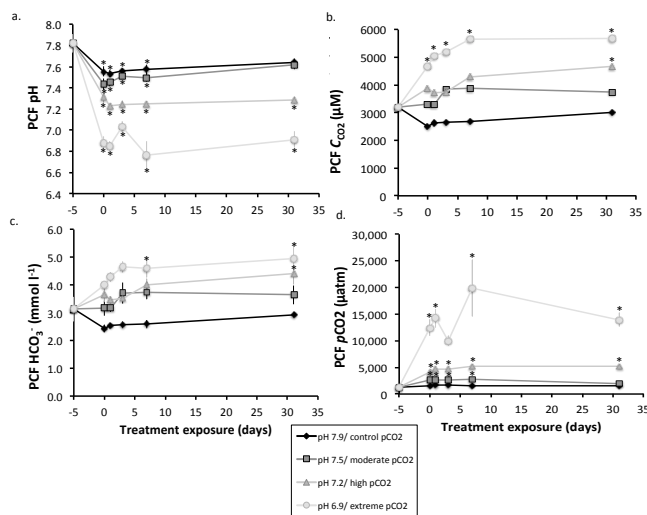


Fig. 2. During experiment one, *S. fragilis* perivisceral coelomic fluid (PCF) pH and C_{CO_2} were measured (a and b, respectively), and HCO_3^- and pCO_2 were calculated (c and d, respectively) as described in the text. Statistically significant differences from pre-treatment (day “-5”) values were evaluated by ANOVA followed by Tukey’s HSD test, and are shown with * denoting $P < 0.05$.

HSD; $P = 0.007$) and 31 days (Tukey HSD; $P = 0.010$) of exposure (Fig. 2). No mortality occurred in experiment one.

3.2 Experiment two: behavior and nutritional status

3.2.1 Mortality

In the pH 6.6 group, there was one mortality (3.3 %) before the first terminal samples were taken (day 30) and four before the final terminal samples were taken on day 140. In the pH 7.2 group, three mortalities occurred before the final sampling day. In the pH 7.6 group, there was one mortality before day 140. In the pH 7.9 group, no mortality occurred. In the pH 7.9/ high O_2 group, there were two mortalities before the final sampling day.

3.2.2 Righting time

No significant correlation was seen between righting time and feeding, so data for fed and fasted animals were pooled for further analyses. Righting time (Fig. 3) increased over time for the extreme pCO_2 (pH 6.6) treatment (RM ANOVA, $F_{(7,56)} = 7.44$, $P < 0.001$), compared to pre-treatment righting times. Transiently increased righting time was seen in *S. fragilis* exposed to pH 7.2 (RM ANOVA, $F_{(7,63)} = 2.63$, $P = 0.019$), solely after 16 days of exposure (Tukey HSD; $P = 0.028$).

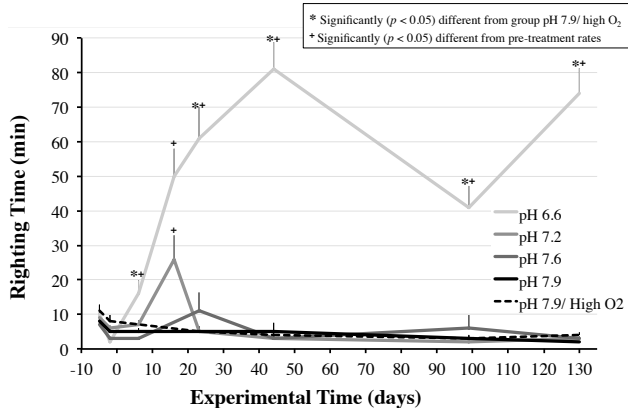


Fig. 3. Righting time for *S. fragilis* is shown before and during exposure to extreme (pH 6.6), high (pH 7.2), moderate (pH 7.6) and control (pH 7.9) $p\text{CO}_2$ at in situ O_2 levels, and to control $p\text{CO}_2$ (pH 7.9) at 100 % air saturation. Times are shown as mean \pm SEM.

3.2.3 Feeding

During the entire experimental period, urchins fed significantly less (Fig. 4) on exposure to extreme $p\text{CO}_2$ (pH 6.6) as compared with their pre-treatment rates (RM ANOVA, $F_{(17,68)} = 2.23$, $P = 0.010$). Exposure to high $p\text{CO}_2$ (pH 7.2) yielded less dramatic results over time (RM ANOVA, $F_{(7,63)} = 1.07$, $P = 0.385$) but showed treatment effects at several timepoints when compared with the control $p\text{CO}_2$ /high O_2 group (Fig. 4). Feeding rates of urchins at pH 6.6 were also significantly less than those of urchins at pH 7.9/high O_2 at most timepoints after 10 days of exposure (Fig. 4). The F Test for equal variances revealed significantly greater variation in feeding rates of individuals at moderate $p\text{CO}_2$ (pH 7.6) during the first week of exposure ($F = 40$, $P < 0.001$), as compared with pre-treatment variance (Fig. 4).

3.2.4 Growth

After 30 days of exposure to treatment conditions, changes in body width were significantly correlated (negatively) only with pH 6.6 fed individuals (Fig. 5a; Mann-Whitney U test, $U = 52$, $p = 0.005$); change in body height was correlated (negatively) with pH 6.6 in both fed (Fig. 5b, Mann-Whitney U test, $U = 49$, $p = 0.015$) and fasted (Fig. 5b, Mann-Whitney U test, $U = 3$, $p = 0.006$) individuals during the 30-day treatment period. After 30 days, no significant effects of feeding regime alone on width or height were observed (i.e., within each treatment, no difference among fed and fasted individuals).

Significantly greater width was seen after 140 days treatment exposure in fed urchins as compared with fasted urchins (Fig. 5c) at pH 7.2 (Mann-Whitney U test, $U = 7$, $p = 0.046$), 7.6 (Mann-Whitney U test, $U = 54$, $p = 0.003$), pH 7.9 (Mann-Whitney U test, $U = 62$, $p = 0.002$) and

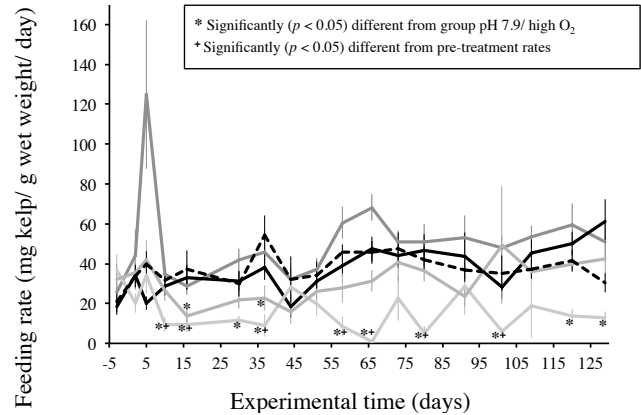


Fig. 4. *S. fragilis* feeding rates before and during exposure to extreme (pH 6.6), high (pH 7.2), moderate (pH 7.6) and control (pH 7.9) $p\text{CO}_2$ at in situ O_2 levels, and to control $p\text{CO}_2$ (pH 7.9) at 100 % air saturation. Feeding rates are shown as mean \pm SEM.

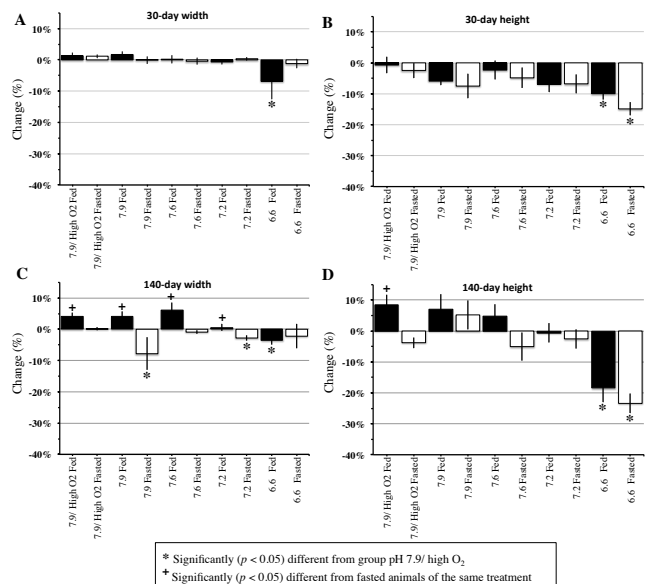


Fig. 5. *S. fragilis* changes in width (A and C) and height (B and D) are shown following 30 (A and B) and 140 (C and D) days of exposure to extreme (pH 6.6), high (pH 7.2), moderate (pH 7.6) and control (pH 7.9) $p\text{CO}_2$ at in situ O_2 levels, and to control $p\text{CO}_2$ (pH 7.9) at 100 % air saturation, following a feeding (filled bars) or fasting (unfilled bars) regime. Change in size is shown as mean \pm SEM.

pH 7.9/high O_2 (Mann-Whitney U test, $U = 2$, $p = 0.007$). Conversely, urchins at pH 6.6 (Fig. 5c, Mann-Whitney U test, $U = 21$, $p = 0.570$) did not show correlation of width and feeding after 140 days of treatment exposure. Changes in height (Fig. 5d) were significantly correlated (positively) with feeding only in urchins exposed to pH 7.9 at high O_2 levels (Fig. 5d, Mann-Whitney U test, $U = 4$, $p = 0.015$). After 140 days, fed (Fig. 5d, Mann-Whitney U test, $U = 35$, $p = 0.005$) and fasted (Fig. 5d, Mann-Whitney U test,

$U = 42$, $p = 0.003$) urchins at pH 6.6 showed correlation (negative) of pH and change in height.

3.2.5 Reproduction

Fasted individuals from all treatments showed GSI values statistically equivalent to zero (Fig. 6, two-tailed, one-sample t-tests, $p > 0.05$). *S. fragilis* exposed to pH 6.6 and fed for 140 days showed significantly lower GSI (Mann-Whitney U test, $U = 29$, $p < 0.05$) when compared with individuals from all other treatments (Fig. 6).

4 Discussion

On exposure to elevated $p\text{CO}_2$, *S. fragilis* shows PCF chemistry consistent with CO_2 -derived respiratory acidosis. Metabolic compensation of respiratory acidosis is typical of aquatic regulators and is characterized by large gains in bicarbonate concentration and movement in an alkalotic direction; there was evidence of this type of compensation only in individuals from the pH 7.5 (moderate $p\text{CO}_2$) treatment; in this case compensation was statistically complete only after 31 days of exposure; but in the absence of significant gains in HCO_3^- . At high and extreme $p\text{CO}_2$, however, significant bicarbonate gains were observed with no significant change in pH. A drop in the ratio of PCF $p\text{CO}_{2(\text{in})} : p\text{CO}_{2(\text{out})}$ created an even more challenging situation for individuals to eliminate CO_2 along its concentration gradient- the usual strategy for marine animals.

The low PCF non-bicarbonate buffer capacity of *S. fragilis* likely underlies its limited capacity for compensating a respiratory acidosis. As compared with findings for shallower living *Strongylocentrotidae* (Holtmann et al., 2013; Spicer et al., 2007, 2011; Stumpp et al., 2012), deep-sea dwelling *S. fragilis* appears less capable of acid-base balance regulation. This result is not surprising, considering *S. fragilis*' marked lack of available internal body compartments for ion exchange and thereby movement of H^+ for acid-base balance regulation (Pörtner, 1993). *S. fragilis* is largely devoid of internal structures, visibly possessing only a thin, relatively straight-thru gastrointestinal tract and variable gonadal mass (typically with GSI of approx. 5%) within otherwise non-compartmentalized extracellular fluids.

The pH along the bathymetric range of *S. fragilis* drops from 7.8 to near 7.6 in the OMZ. On exposure to environmental acidification of only 0.1 pH units below this in situ value, *S. fragilis* was unable to fully compensate a respiratory acidosis after 30 days. The enquiry of the consequences of prolonged systemic acidosis is addressed by experiment two, in which results show that among the parameters measured the effects of a presumably slight extracellular acidosis are negligible at pH 7.6 and are also very minimal at pH 7.2.

The results of experiment two suggest *S. fragilis* is not vulnerable to adverse physiological effects of OA at realis-

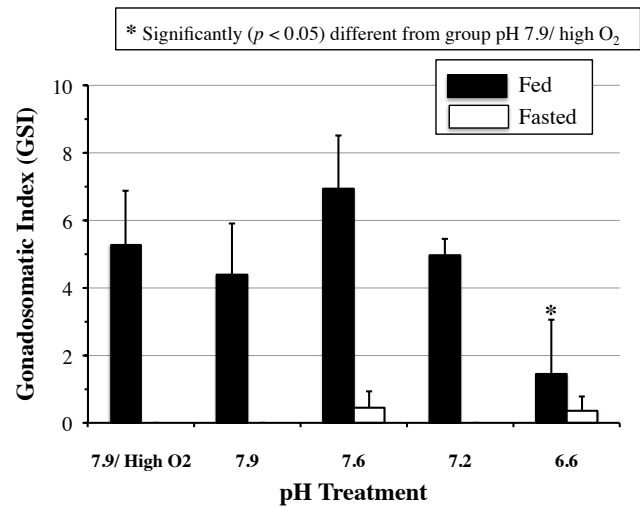


Fig. 6. *S. fragilis* Gonadosomatic Index (GSI) after 140 days of exposure to extreme (pH 6.6), high (pH 7.2), moderate (pH 7.6) and control (pH 7.9) $p\text{CO}_2$ at in situ O_2 levels, and to control $p\text{CO}_2$ (pH 7.9) at 100% air saturation, following feeding and fasting regimes. GSI is shown as mean \pm SEM.

tic near-future $p\text{CO}_2$ levels. Unlikely instances of extreme $p\text{CO}_2$ exposure – for example, deep-sea carbon sequestration – would leave *S. fragilis* vulnerable to impaired righting ability, reduced feeding, decreased somatic growth and compromise of reproductive capacity. Effects on righting time and feeding are minimal and transient at pH 7.2, a level of OA feasible in the OMZ by the end of the century (Caldeira and Wickett, 2003; Doney et al., 2009a, b). At pH 6.6 the effects on righting time and feeding are more catastrophic and persistent, and are accompanied by decreased somatic growth and a significant decline in reproductive capacity.

Not surprising but perhaps worrisome for the persistence of *S. fragilis* at its current bathymetric range is the adverse impact of fasting on GSI. The ability to search for, consume, and assimilate prey items is of utmost concern for reproduction, nutrition, and ultimately the persistence of a detritivore such as *S. fragilis*. Presumably, reproduction is prioritized over other processes in the energy budget, such that the population persists. However, some studies show that reproductive growth in fact tends *not* to be prioritized (Stumpp et al., 2012) unless food quality (i.e., protein content) is high (Poorbagher et al., 2010). As species from the genus *Strongylocentrotidae* often reach an age of over 10 years, with occasional reports of over 100 years (Bodnar, 2013; Ebert, 2008; Ebert and Southon, 2003; Russell et al., 1998), perhaps the maintenance of adult health is more important for lifetime reproductive success than loss of a single spawning season. Gonads are also suggested to be used as energy reserves in urchins during acclimation (Dupont et al., 2012), in which case our relatively short exposure periods may have led to

over-estimation of the long-term effects of persistent elevated $p\text{CO}_2$ on fitness.

Our results suggest *S. fragilis* is unlikely to experience adverse effects during this century; however, we have not investigated multi-generation adaptation in *S. fragilis*. The capacity of *S. fragilis* to adapt to increasing $p\text{CO}_2$ and low O_2 is paramount in determining future distribution patterns. Our study is limited by a relatively short (from a generational perspective) exposure period. Another study has shown adult *S. droebachiensis* acclimate to moderately elevated $p\text{CO}_2$ in a matter of a few months, but with carry-over effects that make larvae and juveniles more susceptible to negative impacts of ocean acidification (Dupont et al., 2012). Furthermore, we have only examined one taxon of a complex ecosystem (Barry et al., 2011; Loreau et al., 2001; Russell et al., 2012). It is possible that while *S. fragilis* may perform better in the seawater chemistry of its shallower range, competition in fact could be a more considerable limitation at that depth range. The OMZ, while generally considered an energetically limited, arduous area of the ocean to inhabit, has seemingly few competing taxa.

Our results offer an alternative to the hypothesis that deep-sea taxa are more sensitive to environmental change than related shallow-living taxa, due to a comparative constancy of conditions. The large bathymetric range of *S. fragilis* has certainly afforded this species an advantage in terms of acclimation to a range of $p\text{CO}_2$ levels. Furthermore, the seasonal upwelling seen in *S. fragilis*' geographic range can introduce additional variability that may offer further benefits to its capacity for acclimation to changing environmental conditions. The effects of global climate change on deep-sea ecosystems and the services they provide to society is undoubtedly a complex issue in need of further investigation. While the impacts of OA on individual animal physiology and population dynamics are becoming better understood, consequent changes in taxa interactions and resultant community structure are important next considerations.

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