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## Contribution to Special Issue: 'Towards a Broader Perspective on Ocean Acidification Research' Original Article

# Temporal fluctuations in seawater $p\text{CO}_2$ may be as important as mean differences when determining physiological sensitivity in natural systems

Daniel P. Small<sup>1\*</sup>, Marco Milazzo<sup>2</sup>, Camilla Bertolini<sup>3</sup>, Helen Graham<sup>4,5</sup>, Chris Hauton<sup>6</sup>, Jason M. Hall-Spencer<sup>7</sup>, and Samuel P. S. Rastrick<sup>6,8</sup>

<sup>1</sup>Biology Department, St Francis Xavier University, 2320 Notre Dame Avenue, Antigonish, NS, Canada B2G 2W5

<sup>2</sup>Department of Earth and Marine Science, Università degli studi di Palermo, CoNISMa, Via Archirafi 20, I-90123 Palermo, Italy

<sup>3</sup>School of Biological Sciences, Medical Biology Centre, Queen's University Belfast, 97 Lisburn Road, Belfast, Northern Ireland BT9 7BL, UK

<sup>4</sup>School of Marine Science and Technology, Ridley Building, Newcastle University, Newcastle upon Tyne, Tyne and Wear NE1 7RU, UK

<sup>5</sup>Uni Research Environment, Postboks 7810, 5020 Bergen, Norway

<sup>6</sup>Ocean and Earth Science, National Oceanography Centre Southampton, University of Southampton Waterfront Campus, European Way, Southampton SO14 3ZE, UK

<sup>7</sup>Marine Biology and Ecology Research Centre, School of Marine Science and Engineering, Plymouth University, Drake Circus, Plymouth, Devon PL4 8AA, UK

<sup>8</sup>Institute of Marine Research, PO Box 1870 Nordness, 5870 Bergen, Norway

\*Corresponding author: e-mail: [dsmall@stfx.ca](mailto:dsmall@stfx.ca)

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Most studies assessing the impacts of ocean acidification (OA) on benthic marine invertebrates have used stable mean pH/ $p\text{CO}_2$  levels to highlight variation in the physiological sensitivities in a range of taxa. However, many marine environments experience natural fluctuations in carbonate chemistry, and to date little attempt has been made to understand the effect of naturally fluctuating seawater  $p\text{CO}_2$  ( $p\text{CO}_{2\text{sw}}$ ) on the physiological capacity of organisms to maintain acid–base homeostasis. Here, for the first time, we exposed two species of sea urchin with different acid–base tolerances, *Paracentrotus lividus* and *Arbacia lixula*, to naturally fluctuating  $p\text{CO}_{2\text{sw}}$  conditions at shallow water  $\text{CO}_2$  seep systems (Vulcano, Italy) and assessed their acid–base responses. Both sea urchin species experienced fluctuations in extracellular coelomic fluid pH,  $p\text{CO}_2$ , and  $[\text{HCO}_3^-]_e$  ( $\text{pH}_e$ ,  $p\text{CO}_{2e}$ , and  $[\text{HCO}_3^-]_e$ , respectively) in line with fluctuations in  $p\text{CO}_{2\text{sw}}$ . The less tolerant species, *P. lividus*, had the greatest capacity for  $[\text{HCO}_3^-]_e$  buffering in response to acute  $p\text{CO}_{2\text{sw}}$  fluctuations, but it also experienced greater extracellular hypercapnia and acidification and was thus unable to fully compensate for acid–base disturbances. Conversely, the more tolerant *A. lixula* relied on non-bicarbonate protein buffering and greater respiratory control. In the light of these findings, we discuss the possible energetic consequences of increased reliance on bicarbonate buffering activity in *P. lividus* compared with *A. lixula* and how these differing physiological responses to acute fluctuations in  $p\text{CO}_{2\text{sw}}$  may be as important as chronic responses to mean changes in  $p\text{CO}_{2\text{sw}}$  when considering how  $\text{CO}_2$  emissions will affect survival and success of marine organisms within naturally assembled systems.

**Keywords:** acid–base balance, natural variability, ocean acidification, sea urchin, volcanic vents.

## Introduction

Most studies investigating the effects of elevated  $p\text{CO}_2$  associated with ocean acidification (OA) on marine invertebrates have focused on stable mean differences in seawater  $p\text{CO}_2$  ( $p\text{CO}_{2\text{sw}}$ ). Such studies have indicated a range of variability in tolerances to elevated  $p\text{CO}_2$  between species and phyla (e.g. Harvey *et al.*, 2013; Kroeker *et al.*, 2013), which drive shifts in species distributions along natural  $p\text{CO}_{2\text{sw}}$  gradients (e.g. Calosi *et al.*, 2013a, b). To understand species responses to elevated  $p\text{CO}_2$ , we need to know how organisms tolerate natural variations in carbonate conditions (Hofmann *et al.*, 2011). While large areas of Open Ocean vary little in seawater  $p\text{CO}_2$  and pH, wide fluctuations occur in coastal marine habitats (e.g. Price *et al.*, 2012; Reum *et al.*, 2014). Upwelling systems, such as the eastern Pacific, have relatively high  $p\text{CO}_2$  levels and aragonite under-saturation (Manzello, 2010; Harris *et al.*, 2013). Although nearshore carbonate chemistry has wide seasonal (Hauri *et al.*, 2012; Harris *et al.*, 2013) and daily fluctuations (Hofmann *et al.*, 2011), the underlying trend is for increasing  $\text{CO}_2$  enrichment and aragonite under-saturation due mainly to burning fossil fuels (IPCC, 2013). In some instances, coastal systems, such as those in fjords and estuaries, can exceed mean atmospheric  $\text{CO}_2$  due to high respiration rates in organic-rich conditions (Borges and Abril, 2011; Reum *et al.*, 2014). In addition to carbon flux from coastal respiration, photosynthesis and calcification can also drive short-term fluctuations in  $p\text{O}_2$ ,  $p\text{CO}_2$ , pH, and total alkalinity ( $A_T$ ) (e.g. Truchot and Duhamel-Jouve, 1980; Mucci *et al.*, 2011). As our understanding of nearshore carbonate chemistry increases, we must take this into account when selecting the appropriate  $p\text{CO}_2$  levels in OA experiments (Andersson and Mackenzie, 2012; McElhany and Busch, 2013; Reum *et al.*, 2014). Understanding how organisms cope with their current environment can provide valuable insights into how species will respond to future OA. Specifically, the fluctuations in  $p\text{CO}_2$  experienced by coastal species in many habitats are far greater in magnitude than the predicted long-term increase in mean  $\text{CO}_2$  due to OA (Hofmann *et al.*, 2010; Joint *et al.*, 2011). Gaining a better understanding of the effect of fluctuating carbonate chemistry on marine organisms may provide basic insights into variations in OA tolerance found in physiological observations (Dupont and Pörtner, 2013).

The ability to maintain extracellular homeostasis is important in determining tolerance to elevated  $p\text{CO}_{2\text{sw}}$  (Whiteley, 2011). Most organisms are able to regulate their extracellular pH through acid–base buffering mechanisms, but this can be energetically costly (Pörtner *et al.*, 2004). Differences in extracellular pH regulatory capabilities and the ability to regulate metabolic rates between species indicate that certain taxa, e.g. teleost fish, brachyuran crustaceans, and cephalopod molluscs, will be more tolerant of elevated  $p\text{CO}_2$  than others, including bivalve molluscs and echinoderms (Melzner *et al.*, 2009). Interspecies variability of acid–base regulation capabilities in closely related and/or co-habiting species may underpin comparative sensitivities to OA (e.g. Pane and Barry, 2007; Bressan *et al.*, 2014), which are important if we are to predict community and ecosystem responses to OA. It has been recognized that species living in distinctly different  $p\text{CO}_2$  environments have differences in acid–base regulatory capabilities, for example, shallow water species are more able to compensate for acid–base disturbances than deep-water species (Pane and Barry, 2007). While such studies demonstrate variations in acid–base regulatory capabilities within phyla, they do not reveal the community or ecosystem effects of differing species sensitivities. It is these

differences in sensitivities to elevated  $p\text{CO}_2$  which drive decreases in species richness and increased dominance of tolerant species at naturally acidified vent sites (Kroeker *et al.*, 2011).

Understanding how closely related species living in the same habitat deal with natural  $p\text{CO}_2$  conditions would form a basis to our understanding of how communities will be affected by future climate change. Differences in physiological regulatory capabilities have been suggested to drive differences in distribution patterns between closely related species across natural gradients in  $p\text{CO}_{2\text{sw}}$ , such as those found at volcanic  $\text{CO}_2$  vent sites (e.g. Suggett *et al.*, 2012; Calosi *et al.*, 2013a, b). However, to date, all such studies on comparative growth and physiology have focused on chronic mean differences in  $p\text{CO}_{2\text{sw}}$  (e.g. Suggett *et al.*, 2012; Calosi *et al.*, 2013a, b), despite  $p\text{CO}_{2\text{sw}}$  at vent sites exhibiting considerable variation within the space of hours to days (Boatta *et al.*, 2013). To date, we have little understanding of how short-term natural fluctuations in  $p\text{CO}_{2\text{sw}}$  affect animal acid–base regulation and so distribution at vent sites. An example of this can be seen in the distributions of the sea urchin species used in the present study, *Arbacia lixula* and *Paracentrotus lividus*, at the  $\text{CO}_2$  vent sites off Isola Vulcano (Sicily, Italy). Despite a greater capacity for bicarbonate buffering in response to stable elevated  $p\text{CO}_{2\text{sw}}$ , *P. lividus* decreases in population density at higher  $p\text{CO}_{2\text{sw}}$  closer to the vent sites compared with *A. lixula* (Calosi *et al.*, 2013a). This indicates that the ability to buffer stable increases in mean  $p\text{CO}_{2\text{sw}}$  via increased  $[\text{HCO}_3^-]$  is not the primary physiological response driving distribution at the vent sites. The present study investigates the hypothesis that for species such as *P. lividus*, an increase in acute natural fluctuations in  $p\text{CO}_{2\text{sw}}$  may pose as great or a greater challenge to maintaining acid–base regulation as chronic mean elevations in  $p\text{CO}_{2\text{sw}}$  due to the constant need to up- or down-regulate possibly costly bicarbonate buffering responses. Changes in the distribution of related species across present natural  $p\text{CO}_{2\text{sw}}$  gradients at volcanic seep sites, or, in response to future climate change may have as much to do with physiological responses to increased acute fluctuations in  $p\text{CO}_{2\text{sw}}$  as with chronic mean elevations in  $p\text{CO}_{2\text{sw}}$ .

To test this hypothesis, *P. lividus* and *A. lixula* collected from control areas were exposed to acute natural fluctuations in  $p\text{CO}_{2\text{sw}}$  generated by the volcanic  $\text{CO}_2$  vent. The coelomic fluid acid–base balance ( $\text{pH}_e$ ,  $p\text{CO}_{2e}$ , and  $[\text{HCO}_3^-]_e$ ) of *P. lividus* and *A. lixula* was measured every 6 h over the course of 4 d (90 h, total of 16 time points) in an attempt to understand the effect of fluctuating  $p\text{CO}_{2\text{sw}}$  conditions on the distribution of *A. lixula* compared with *P. lividus*. Individual responses in acid–base parameters to changes in  $p\text{CO}_{2\text{sw}}$  were determined along with differences in bicarbonate and non-bicarbonate buffering capacity between the two species. Bicarbonate and non-bicarbonate buffering capacity were determined by davenport models where bicarbonate buffering is indicated by increases in  $[\text{HCO}_3^-]_e$  above the non-bicarbonate buffer line (NBL) and changes in non-bicarbonate buffering are indicated by changes in the slope of the NBL. Changes in non-bicarbonate buffering were also linked to changes coelomic fluid protein concentration.

## Material and methods

### Animal collection

On 18 May 2013, *A. lixula* and *P. lividus* ( $n = 18$  per species) were collected from a 2–3 m depth in Ponente Bay, Vulcano well away from the effects of seabed  $\text{CO}_2$  vents (coordinates =  $38^\circ 25.185' \text{N}$ ,  $14^\circ 57.074' \text{E}$ ). Sea urchins were immediately transported in fully

**Table 1.** Seawater chemistry throughout the exposure period, measured in the experimental tanks at each time point (means  $\pm$  s.e.).

Seawater parameter	Control treatment	Fluctuating treatment
pH (NBS)	8.04 $\pm$ 0.01*	7.72 $\pm$ 0.07*
pH max	8.12	8.04
pH min	7.95	7.14
A <sub>T</sub> (mEq kg <sup>-1</sup> )	2.53 $\pm$ 0.01	2.54 $\pm$ 0.01
Temperature (°C)	19.6 $\pm$ 0.2	19.5 $\pm$ 0.2
Salinity	38.2 $\pm$ 0.1	38.2 $\pm$ 0.1
pCO <sub>2</sub> (μatm)	608.8 $\pm$ 19.1*	1742.3 $\pm$ 352.7*
pCO <sub>2</sub> max	779.3	5497.4
pCO <sub>2</sub> min	492.5	599
Ω <sub>Cal</sub>	3.97 $\pm$ 0.09*	2.34 $\pm$ 0.28*
Ω <sub>Ara</sub>	2.59 $\pm$ 0.06*	1.52 $\pm$ 0.18*
HCO <sub>3</sub> <sup>-</sup> (μmol kg <sup>-1</sup> )	2102 $\pm$ 9*	2295 $\pm$ 28*
CO <sub>3</sub> <sup>2-</sup> (μmol kg <sup>-1</sup> )	170.1 $\pm$ 4.1*	100.2 $\pm$ 12.0*

\*Significant differences between treatments ( $p < 0.05$ ).

aerated aquaria (vol. = 15 l) to a volcanic CO<sub>2</sub> vent gradient in Levante Bay, Vulcano (coordinates = 38°25.189' N, 14°57.711' E). The volume of each sea urchin was calculated by water displacement. Individuals were then blotted dry with a paper towel and wet body mass determined using a high-precision balance (SI-603, Denver Instrument, Bohemia, NY, USA). Individuals were then sealed in mesh containers (vol. = 1 l, mesh diameter = 1 cm, see Calosi et al., 2013a) and placed into large aerated holding tanks (vol. = 60 l) containing control seawater collected from a control site in Levante Bay (salinity: 38, temperature: 19°C, coordinates = 38°25.215' N, 14°57.797' E, Table 1) for a recovery period of 6 h.

### Experimental exposure

To investigate the effect of natural variations in pCO<sub>2sw</sub> on sea urchin coelomic fluid acid–base balance, individuals were randomly assigned to one of two treatments; a stable control pCO<sub>2sw</sub> and naturally fluctuating pCO<sub>2sw</sub> ( $n = 9$  individuals per species per treatment). Exposure to naturally fluctuating pCO<sub>2sw</sub> was achieved by pumping seawater into a holding tank from Levante Bay (coordinates = 38°25.176' N, 14°57.695' E) using a submersible water pump (FSP400DW, Adeo Services, Lille, France, max flow = 7500 l h<sup>-1</sup>). The release of CO<sub>2</sub> from the vent site generated a natural pH and pCO<sub>2</sub> gradient (Hall-Spencer et al., 2008; Johnson et al., 2012; Boatta et al., 2013; Milazzo et al., 2014). The site from which fluctuating pCO<sub>2sw</sub> was obtained was ca. 300 m from the main gas vents away from the influence of H<sub>2</sub>S and heavy metals (Boatta et al., 2013; Vizzini et al., 2013). Exposure to stable pCO<sub>2sw</sub> was achieved by pumping water directly from a site 390 m from the main CO<sub>2</sub> vent (Boatta et al., 2013). Sea urchins were exposed to either fluctuating pCO<sub>2sw</sub> or stable pCO<sub>2sw</sub> for 90 h. Measurements of pH, temperature, and salinity in the tanks were taken at each experimental time point with a hand-held multiparameter instrument (Professional Plus YSI, YSI Inc., Yellow Springs, OH, USA). Water samples for total alkalinity (A<sub>T</sub>) were collected daily from inflowing water from each site during the experiment. One-hundred-millilitre water sample was passed through 0.2 μm pore size filters, poisoned with 0.05 ml of 50% HgCl<sub>2</sub> to avoid biological alteration, and then stored in the dark at 4°C. Three replicate subsamples were analysed at 25°C using a titration system. The pH was measured at 0.02 ml increments of 0.1 N HCl. Total alkalinity was calculated from the Gran function applied to pH variations

from 4.2 to 3.0, as mEq kg<sup>-1</sup> from the slope of the curve HCl volume vs. pH. Total alkalinity measurements were corrected using standards provided by A.G. Dickson (batch 99 and 102). Parameters of the carbonate system (pCO<sub>2</sub>, CO<sub>3</sub><sup>2-</sup>, HCO<sub>3</sub><sup>-</sup>) and saturation state of calcite and aragonite were calculated from pH, A<sub>T</sub>, temperature and salinity using the free-access CO<sub>2</sub>SYS (Lewis and Wallace, 1998) with constants provided by Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and KSO<sub>4</sub> constants from Dickson (1990). Water chemistry throughout the exposure period is shown in Table 1.

### Determination of coelomic fluid acid–base balance

The acid–base balance of sea urchin coelomic fluid was determined following the methods in Calosi et al. (2013a). Briefly, coelomic fluid (vol. = 100 μl) was extracted anaerobically from each individual using a gas-tight syringe (Gas-tight 1710 100 μl syringe with an RN 22S gauge needle, Hamilton Co., Bonaduz, Switzerland) inserted 1 cm into the coelomic cavity via the peristomial membrane at 6 h intervals for 90 h (4 d, 16 time points). Total bound and dissolved CO<sub>2</sub> (TCO<sub>2</sub>) was analysed instantly in a subsample of coelomic fluid (vol. = 30 μl) using a TCO<sub>2</sub> analyser (956D TCO<sub>2</sub> Analyser, Corning Diagnostics, Cambridge, MA, USA). For determination of coelomic pH, the remaining coelomic fluid (vol. = 70 μl) was rapidly (within 5 s) injected into a 0.5 ml micro-centrifuge tube (TUL-649-010L, Fisher Scientific, Loughborough, UK) and placed on a micro pH probe (Micro-Inlab pH combination electrode, Mettler Toledo, Leicester, UK) connected to a pH meter (Seven Easy pH meter, Mettler Toledo) forming a gas-tight seal between the air and sample (after, Rastrick et al., 2014).

An additional 150 μl of coelomic fluid was extracted from nine freshly collected reference individuals from the collection site of *A. lixula* and *P. lividus*, and immediately frozen for later determination of coelomic fluid protein concentration ([protein]<sub>e</sub>), NBL, and the first apparent dissociation constants of carbonic acid (pK<sub>1</sub>) in urchin coelomic fluid. NBL were determined after Rastrick et al. (2014), modified from Spicer et al. (1988). Briefly, the 100 μl coelomic fluid sample from each reference individual was injected into a glass diffusion chamber and equilibrated to 0.1, 1.0, and 2.5% CO<sub>2</sub> (mixed with O<sub>2</sub>-balanced N<sub>2</sub>) in turn, using gas supplied by a gas mixing pump (Wösthoff pump, Wösthoff GmbH, Bochum, Germany). The sample was constantly measured for pH using a micro pH electrode connected to a pH meter. Samples were agitated with a magnetic stirrer and considered equilibrated when the pH value stopped decreasing (circa 15 min). At equilibration pH, the pH value was recorded and TCO<sub>2</sub> measured in a subsample (vol. = 30 μl) using a TCO<sub>2</sub> analyser. This was repeated for each pCO<sub>2</sub> level. NBLs were constructed using the Henderson–Hasselbalch equation in the following forms:

$$p\text{CO}_2 = \frac{\text{TCO}_2}{\alpha(10^{\text{pH}-\text{p}K'_1} + 1)}, \quad (1)$$

$$[\text{HCO}_3^-] = 10^{\text{pH}-\text{p}K'_1} \alpha p\text{CO}_2, \quad (2)$$

where  $\alpha$  is the CO<sub>2</sub> solubility coefficient in seawater at 20°C (0.3293 mmol l<sup>-1</sup> kPa<sup>-1</sup>; Spicer et al., 1988; after Harvey, 1955) and pK<sub>1</sub>' calculated as 6.03 for *P. lividus* and 5.91 for *A. lixula* at 20°C using Equation (3):

$$pK'_1 = \text{pH} \left( \frac{\text{Log}_{10}(\text{TCO}_2 - \alpha p\text{CO}_2)}{\alpha p\text{CO}_2} \right), \quad (3)$$

$[\text{HCO}_3^-]_e$  and  $p\text{CO}_{2e}$  in experimental individuals were calculated from  $\text{pH}_e$  and  $\text{TCO}_{2e}$  determined above using Equations (4) and (5), respectively (Truchot, 1976);

$$p\text{CO}_2 = \frac{\text{TCO}_2}{\alpha(10^{\text{pH}-\text{p}K'_1} + 1)}, \quad (4)$$

$$\text{HCO}_3^- = \text{TCO}_2 - \alpha p\text{CO}_2. \quad (5)$$

### Determination of coelomic fluid protein concentration

$[\text{Protein}]_e$  concentration was determined by the Bradford coomassie blue assay; 10  $\mu\text{l}$  of each coelomic fluid sample was diluted in to 90  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , 5  $\mu\text{l}$  of each diluted sample was plated in to a 96 micro well plate with 250  $\mu\text{l}$  of Coomassie reagent (23200, Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). After 10 min, the absorbance was read at 595 nm (iMark Microplate Absorbance Reader, Bio-Rad Laboratories, Hercules, CA, USA) and compared with albumin protein standards at nine concentrations from 0 to 2000  $\mu\text{g ml}^{-1}$  (23209, Thermo Scientific) treated in the same way.

### Statistical analysis

The  $\text{pH}_e$ ,  $p\text{CO}_{2e}$ ,  $[\text{HCO}_3^-]_e$ , and  $[\text{protein}]_e$  of field collected control *A. lixula* and *P. lividus* were compared using a one-way ANOVA. To test if the possible relationship between  $p\text{CO}_{2e}$ ,  $\text{pH}_e$ , or  $[\text{HCO}_3^-]_e$  (dependant factors) and  $p\text{CO}_{2\text{sw}}$  (covariate) varied between site (control and fluctuating) and species (fixed factors), a repeated-measures, nested GLMM (with individual urchin as a random factor nested within site as a fixed factor) was performed, which considers the fact that the individuals sampled at the two sites are not true replicates (e.g. Collard *et al.*, 2016). To understand the species-specific differences in response to fluctuating  $p\text{CO}_{2\text{sw}}$ , linear regressions were also performed for each acid–base parameter vs.  $p\text{CO}_{2\text{sw}}$  on each individual. The slope of the individual regressions ( $B$ ) was obtained, and the average for each species calculated. The average  $B$  for changes in *P. lividus*  $\text{pH}_e$ ,  $p\text{CO}_{2e}$ , and  $[\text{HCO}_3^-]_e$  with  $p\text{CO}_{2\text{sw}}$  was compared against that of *A. lixula* using a Student's  $t$ -test to determine any significant differences in coelomic fluid acid–base response with variations in  $p\text{CO}_{2\text{sw}}$ . The standard deviation of  $\text{pH}_e$ ,  $p\text{CO}_{2e}$ , and  $[\text{HCO}_3^-]_e$  for each individual were also compared using a Student's  $t$ -test to determine differences in acid–base variation between *A. lixula* and *P. lividus*. All statistical procedures were performed using SPSS software (V18, SPSS, Chicago, IL, USA); all values are presented as means  $\pm$  standard error.

## Results

### Urchin coelomic fluid acid – base parameters before exposure

The acid–base balance of coelomic fluid from field collected ( $\text{pH} = 8.05$ ) *A. lixula* was significantly different from that of *P. lividus* (Table 2). In summary,  $\text{pH}_e$  and  $[\text{HCO}_3^-]_e$  were significantly lower in *A. lixula* than *P. lividus* ( $F_{\text{min}1,17} = 101.692$ ,  $p < 0.001$ ), while there was no significant difference in  $p\text{CO}_{2e}$  ( $F_{1,17} = 0.628$ ,  $p > 0.05$ ). The  $[\text{protein}]_e$  of *A. lixula* was almost threefold higher than in *P. lividus* ( $F_{1,17} = 10.272$ ,  $p < 0.01$ ), and the calculated NBL for *A. lixula* ( $y = -1.504 \times +12.606$ ,  $R^2 = 0.8254$ ) was significantly lower than that of *P. lividus* ( $y = -0.493 \times +7.6358$ ,  $R^2 = 0.7299$ ,  $t_{10} = 8.925$ ,  $p < 0.001$ ).

### The effect of fluctuating $p\text{CO}_{2\text{sw}}$ on urchin coelomic fluid acid – base balance

There was a significant three-way interaction between site, species, and  $p\text{CO}_{2\text{sw}}$  on  $p\text{CO}_{2e}$  ( $F_{39,4,1} = 5.587$ ,  $p < 0.05$ , Figure 1). The relationship between  $p\text{CO}_{2\text{sw}}$  and  $p\text{CO}_{2e}$  in *P. lividus* was significantly different between the fluctuating and the control treatments ( $F_{137,9,1} = 7.650$ ,  $p < 0.01$ , Figure 1). In contrast, *A. lixula* showed no significant difference between the two treatments ( $F_{137,9,1} = 0.333$ ,  $p = 0.565$ , Figure 1).

There was also a significant three-way interaction between site, species, and  $p\text{CO}_{2\text{sw}}$  on  $\text{pH}_e$  ( $F_{44,6,1} = 10.633$ ,  $p < 0.01$ , Figure 1). The relationship between  $p\text{CO}_{2\text{sw}}$  and  $\text{pH}_e$  in *P. lividus* was significantly different between the fluctuating and the control treatments ( $F_{118,0,1} = 10.581$ ,  $p < 0.01$ , Figure 1), while *A. lixula* again showed no significant difference between the two treatments ( $F_{118,0,1} = 0.106$ ,  $p = 0.745$ , Figure 1).

The relationship between  $p\text{CO}_{2\text{sw}}$  and  $[\text{HCO}_3^-]_e$  also demonstrated a significant three-way interaction between site, species, and  $p\text{CO}_{2\text{sw}}$  ( $F_{56,8,1} = 61.378$ ,  $p < 0.001$ , Figure 1). However, the relationship between  $p\text{CO}_{2\text{sw}}$  and  $[\text{HCO}_3^-]_e$  is different between the fluctuating and the control treatments in both *P. lividus* ( $F_{157,7,1} = 8.905$ ,  $p < 0.01$ , Figure 1) and *A. lixula* ( $F_{157,9,1} = 7.719$ ,  $p < 0.01$ , Figure 1). This is because both species show some significant increase in  $[\text{HCO}_3^-]_e$  as seawater  $p\text{CO}_2$  increases; however, this response was significantly greater in *P. lividus* compared with *A. lixula* (where a bicarbonate response was extremely limited) when exposed to natural fluctuations in  $p\text{CO}_{2\text{sw}}$  ( $F_{44,8,1} = 475.914$ ,  $p < 0.001$ , Figure 1).

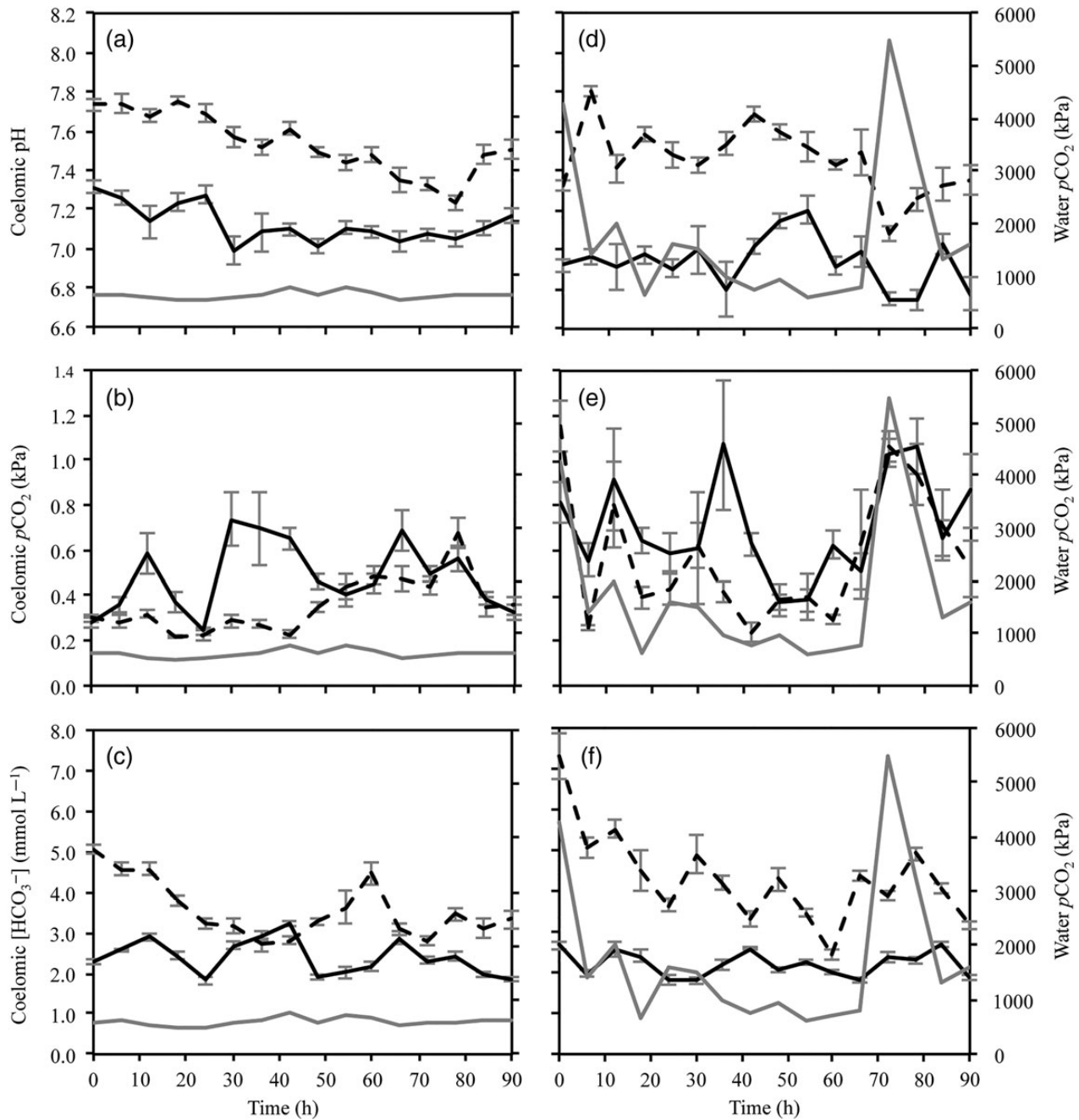
While fluctuating  $p\text{CO}_{2\text{sw}}$  had a significant effect on the coelomic acid–base status of both species, the effect was greater on *P. lividus* than *A. lixula*, as demonstrated by comparing the average slopes of the regressions ( $B$  value) performed on the  $\text{pH}_e$ ,  $p\text{CO}_{2e}$ , and  $[\text{HCO}_3^-]_e$  of all individuals against  $p\text{CO}_{2\text{sw}}$  (Table 3). The  $\text{pH}_e$  of both *A. lixula* and *P. lividus* showed a negative relationship with  $p\text{CO}_{2\text{sw}}$ ; however, the average  $B$  for the regression of  $\text{pH}_e$  against  $p\text{CO}_{2\text{sw}}$  of *P. lividus*,  $-0.091 \pm 0.006$  unit- $\text{pH}_e$  unit- $p\text{CO}_{2\text{sw}}^{-1}$ , was significantly greater than that of *A. lixula*,  $-0.055 \pm 0.005$  unit- $\text{pH}_e$  unit- $p\text{CO}_{2\text{sw}}^{-1}$  ( $t_{16} = 4.645$ ,  $p < 0.001$ , Figure 2). This indicates that individual decreases in  $\text{pH}_e$  in response to fluctuations in  $p\text{CO}_{2\text{sw}}$  were greater (i.e. steeper response gradient) in *P. lividus* than in *A. lixula*. The  $p\text{CO}_{2e}$  and  $[\text{HCO}_3^-]_e$  of both *P. lividus* and *A. lixula* had a positive relationship against  $p\text{CO}_{2\text{sw}}$ . The average  $B$  for  $p\text{CO}_{2e}$  against  $p\text{CO}_{2\text{sw}}$  of *P. lividus*,  $0.174 \pm 0.017$  kPa  $p\text{CO}_{2\text{sw}}^{-1}$ , was significantly higher than that of *A. lixula*,  $0.102 \pm 0.014$  kPa  $p\text{CO}_{2\text{sw}}^{-1}$  ( $t_{16} = -3.193$ ,  $p = 0.006$ ), as was the average  $B$  for  $[\text{HCO}_3^-]_e$  against  $p\text{CO}_{2\text{sw}}$  (*P. lividus* =  $0.368 \pm 0.042$  mmol  $\text{l}^{-1}$   $p\text{CO}_{2\text{sw}}^{-1}$ , *A. lixula* =  $-0.065 \pm 0.025$  mmol  $\text{l}^{-1}$   $p\text{CO}_{2\text{sw}}^{-1}$ ,  $t_{16} = -6.203$ ,  $p < 0.001$ ). This indicates that individual increases in both  $p\text{CO}_{2e}$  and  $[\text{HCO}_3^-]_e$  in response to increases in  $p\text{CO}_{2\text{sw}}$  were

**Table 2.** Acid–base balance of control field collected *A. lixula* and *P. lividus*.

	<i>A. lixula</i>	<i>P. lividus</i>
$\text{pH}_e$	7.31 $\pm$ 0.03*	7.73 $\pm$ 0.03*
$p\text{CO}_{2e}$ (kPa)	0.28 $\pm$ 0.02	0.30 $\pm$ 0.02
$[\text{HCO}_3^-]_e$ (mmol $\text{l}^{-1}$ )	2.29 $\pm$ 0.09*	5.07 $\pm$ 0.11*
Protein $_e$ ( $\mu\text{g ml}^{-1}$ )	334.8 $\pm$ 66.2*	122.4 $\pm$ 19.8*
$\text{p}K'_1$	5.91	6.03

Data are means  $\pm$  s.e.

\*Significant differences between species ( $p < 0.05$ ).



**Figure 1.** The coelomic fluid pH (a and d),  $p\text{CO}_2$  (kPa, b and e), and  $[\text{HCO}_3^-]$  ( $\text{mmol L}^{-1}$ , c and f) of *P. lividus* and *A. lixula* during 4 d exposure to stable control  $p\text{CO}_{2\text{sw}}$  conditions (a, b, and c) and fluctuating  $p\text{CO}_{2\text{sw}}$  conditions (d, e, and f). Each time point represents means  $\pm$  s.e. Grey lines indicate seawater  $p\text{CO}_{2\text{sw}}$ . Black lines indicate *A. lixula*. Dashed lines indicate *P. lividus*.

greater in *P. lividus* than *A. lixula* (Figure 2). Finally, when exposed to fluctuating  $p\text{CO}_{2\text{sw}}$ , the standard deviation of  $[\text{HCO}_3^-]_e$  in individual *P. lividus* was significantly higher than that in *A. lixula* ( $t = 7.377$ ,  $p < 0.001$ ).

No mortality was observed throughout the experimental period nor were there issues with urchin viability or infection encountered due to the repeated sampling of coelomic fluid (see Calosi et al., 2013a); there was also no significant progressive acidosis throughout the experimental period showing that repeated sampling had no effect on acid–base balance.

## Discussion

The present study demonstrates that naturally fluctuating  $p\text{CO}_{2\text{sw}}$  produced different magnitudes of bicarbonate acid–base responses in two species of sea urchin. The acid–base response of *P. lividus* in relation to fluctuating  $p\text{CO}_{2\text{sw}}$  was greater than that of *A. lixula* because *P. lividus* relies on limited bicarbonate buffering capabilities for acid–base regulation, whereas *A. lixula* relied on non-bicarbonate buffering capacity. The distributions of closely related species at  $\text{CO}_2$  vents are driven by differences in acid–base regulatory capabilities (Calosi et al., 2013a). However, despite variations

**Table 3.** Acid–base balance of *A. lixula* and *P. lividus* throughout the experimental period.

Treatment	Species			
	<i>A. lixula</i>		<i>P. lividus</i>	
	Control	Fluctuating	Control	Fluctuating
$\text{pH}_e$	$7.12 \pm 0.05$	$6.94 \pm 0.05^*$	$7.53 \pm 0.04$	$7.45 \pm 0.05^*$
Max	7.31	7.19	7.75	7.80
Min	6.99	6.74	7.23	7.08
$p\text{CO}_{2e}$ (kPa)	$0.48 \pm 0.06$	$0.71 \pm 0.11^*$	$0.36 \pm 0.03$	$0.58 \pm 0.10^*$
Max	0.74	1.07	0.68	1.15
Min	0.24	0.37	0.22	0.24
$[\text{HCO}_3^-]_e$ ( $\text{mmol l}^{-1}$ )	$2.40 \pm 0.10$	$2.21 \pm 0.09^*$	$3.57 \pm 0.17$	$4.37 \pm 0.23^*$
Max	3.23	2.67	5.07	7.31
Min	1.83	1.81	2.70	2.44

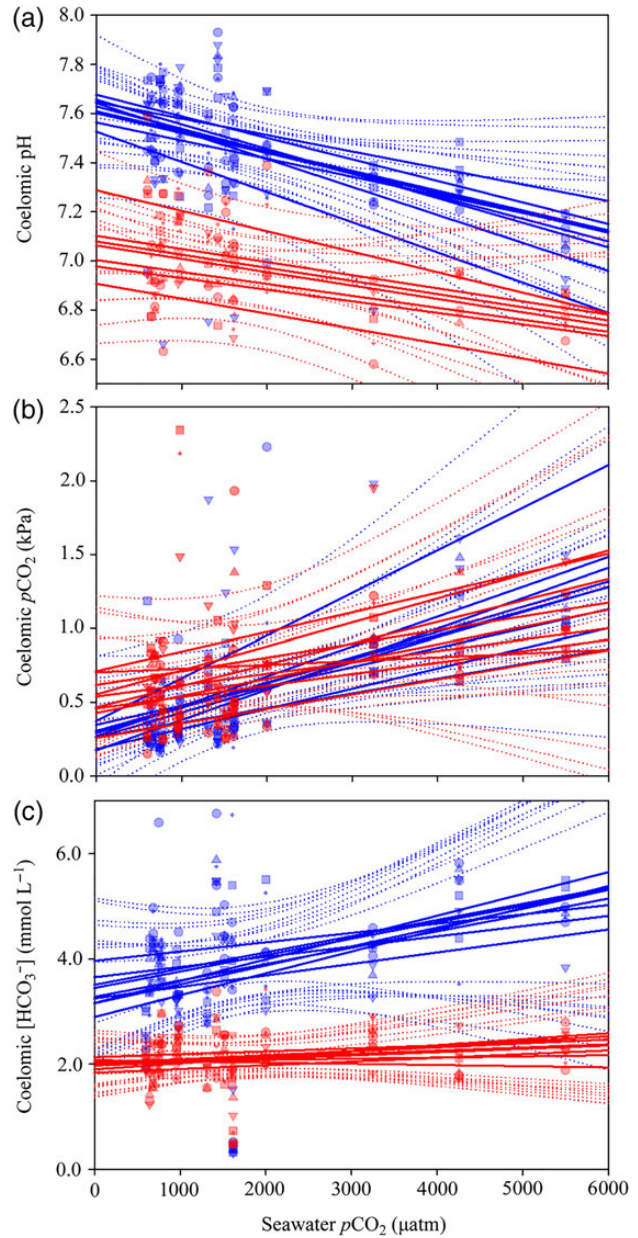
Data are presented as means  $\pm$  s.e., along with the minimum and maximum values observed.

\*Significant effect of seawater  $p\text{CO}_2$  on acid–base parameters ( $p < 0.05$ ).

in carbonate chemistry being demonstrated in various ecosystems (Hofmann *et al.*, 2011) including volcanic vent sites (Boatta *et al.*, 2013), most studies pertaining to the comparative physiological responses between species to elevated  $p\text{CO}_2$  and in naturally acidified environments have focused on stable mean differences in  $p\text{CO}_{2\text{sw}}$  conditions (e.g. Suggett *et al.*, 2012; Calosi *et al.*, 2013a, b). Understanding how species respond to natural  $p\text{CO}_{2\text{sw}}$  conditions will help explain some of the variation in physiological responses and may provide better predictions on how species will respond to future change (e.g. Kroeker *et al.*, 2011).

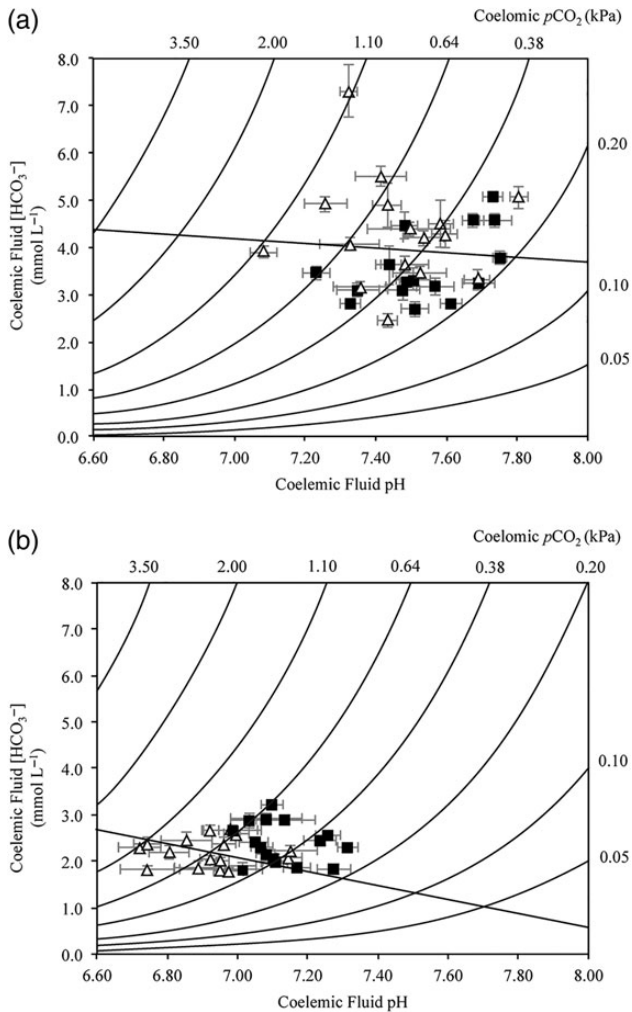
Echinoderm coelomic fluid acid–base parameters often conform according to the surrounding seawater (Farmanfarmaian, 1966; Miles *et al.*, 2007) and they have minimal acid–base regulatory capacity when exposed to elevated  $p\text{CO}_2$  (Spicer *et al.*, 1988; Miles *et al.*, 2007; Appelhans *et al.*, 2012; Calosi *et al.*, 2013a). Despite the apparent poor acid–base and ion-regulatory mechanisms, when exposed to elevated  $p\text{CO}_2$ , some species of sea urchins have been shown to exhibit bicarbonate buffering to maintain acid–base homeostasis in response to stable elevated  $p\text{CO}_2$  in laboratory (Miles *et al.*, 2007) and field conditions (Calosi *et al.*, 2013a). In the present study, there was no significant effect of stable  $p\text{CO}_{2\text{sw}}$  on the coelomic fluid acid–base balance of either *P. lividus* or *A. lixula* during control incubations.

The differences in acid–base responses to fluctuating  $p\text{CO}_{2\text{sw}}$  between the two species in the present study can be related to the differing mechanisms of acid–base regulation employed. *Paracentrotus lividus* relied on uncompensated bicarbonate acid–base buffering as while they exhibited a significant relationship between  $[\text{HCO}_3^-]_e$  with  $p\text{CO}_{2\text{sw}}$ , there were also significant fluctuations in  $\text{pH}_e$  and  $p\text{CO}_{2e}$ . In contrast to *P. lividus*, *A. lixula* exhibited comparatively lower fluctuations in  $[\text{HCO}_3^-]_e$  and experienced no significant fluctuations in  $p\text{CO}_{2e}$  or  $\text{pH}_e$  in relation to fluctuating  $p\text{CO}_{2\text{sw}}$ . The bicarbonate buffering capacity of *A. lixula* was to the extent of being almost non-existent. The acid–base response of *A. lixula* to decreasing  $p\text{CO}_{2\text{sw}}$  closely follows the non-bicarbonate buffering line (Figure 3) and therefore is due to non-bicarbonate buffering rather than  $\text{HCO}_3^-$  regulation. This mechanism in *A. lixula* therefore appears to result in greater  $\text{pH}_e$  stability under fluctuating  $p\text{CO}_{2\text{sw}}$  conditions. The acid–base responses observed in the present study make an interesting comparison with those of stable mean elevations in  $p\text{CO}_{2\text{sw}}$  in the same species. When exposed to



**Figure 2.** Individual regressions of acid–base parameters coelomic pH (a), coelomic  $p\text{CO}_2$  (kPa, b), coelomic  $[\text{HCO}_3^-]$  ( $\text{mmol l}^{-1}$ , c) vs. fluctuating  $p\text{CO}_{2\text{sw}}$  of *A. lixula* and *P. lividus*. Red indicates *A. lixula* and blue indicates *P. lividus*. Solid lines indicate individual regressions; dotted lines indicate 95% confidence limits of the regressions. Points indicate raw data from each individual at each  $p\text{CO}_{2\text{sw}}$ , represented by different symbols.

chronic exposure to high  $p\text{CO}_{2\text{sw}}$ , *A. lixula* also demonstrated a lower bicarbonate response than *P. lividus*; however, in both species, acid–base disturbances are fully compensated (Calosi *et al.*, 2013a). Conversely, the lack of full  $\text{pH}_e$  compensation by *P. lividus* in the present study indicates that this species is unable to respond efficiently to short-term fluctuations in  $p\text{CO}_{2\text{sw}}$ . It is therefore apparent that under fluctuating  $p\text{CO}_{2\text{sw}}$  conditions, the ability of *P. lividus* to compensate  $\text{pH}_e$  breaks down, while the non-bicarbonate buffering capacity of *A. lixula* allows the maintenance of  $\text{pH}_e$  homeostasis. The maintenance of  $\text{pH}_e$  homeostasis in *A. lixula*



**Figure 3.** Davenport diagrams representing coelomic fluid acid–base balance of (a) *P. lividus* and (b) *A. lixula*. Each point represents mean  $\pm$  s.e. of each species at each time point. Solid line indicates non-bicarbonate buffering line. Squares indicate stable control pH conditions; triangles indicate fluctuating  $p\text{CO}_{2\text{sw}}$  conditions.

may also, in part, be alleviated by comparatively low routine  $\text{pH}_e$  levels in this species. Throughout control incubations and in field collected individuals from the control site, *A. lixula* consistently had a lower  $\text{pH}_e$  ( $7.31 \pm 0.03$ ) than *P. lividus* ( $7.73 \pm 0.03$ ) with no difference in  $p\text{CO}_{2e}$ . Comparatively low  $\text{pH}_e$  under natural and control conditions have been reported previously for *A. lixula* (Calosi et al., 2013a) and have been reported in other marine organisms (e.g. Gutowska et al., 2010; Donohue et al., 2012). Although the reasons for this, and possible physiological implications, are outside the scope of the present study, what is important is the ability of *A. lixula* to maintain  $\text{pH}_e$  homeostasis and avoid acidosis of the coelomic compartment (i.e. a decrease in  $\text{pH}_e$  away from its routine value) in response to fluctuations in than  $p\text{CO}_{2\text{sw}}$  with almost no change in  $[\text{HCO}_3^-]$ .

This greater capacity for non-bicarbonate buffering in *A. lixula* is reflected by the steeper NBL of *A. lixula* compared with *P. lividus*, and the acid–base responses in *A. lixula* being closely clustered around the NBL compared with the high variation in bicarbonate response show by *P. lividus* (Figure 3). *Paracentrotus lividus* has a

significantly lower  $[\text{protein}]_e$  than that of *A. lixula*, which while low compared with other species of marine invertebrates, is representative of the low protein concentrations seen in sea urchins (e.g. Spicer et al., 1988; Miles et al., 2007). Comparative differences in  $[\text{protein}]_e$  between sea urchin species may be the main reason for differences in acid–base regulatory capacity, with species exhibiting higher  $[\text{protein}]_e$  having greater capacity for  $\text{pH}_e$  regulation (Spicer et al., 1988), as demonstrated in the present study.

Differences in  $[\text{HCO}_3^-]_e$  response to fluctuating conditions may be related to routine rates of activity and metabolism, as seen in crustaceans where species with routinely higher rates of activity and metabolism have higher non-bicarbonate buffering capacities due to higher levels of extracellular protein (haemocyanin; Watt et al., 1999; Whiteley, 2011). Whether the same conditions apply to urchins is currently unknown, but if true, may explain differences in  $p\text{CO}_2$  sensitivity between the two species. The energetic consequences of relying on bicarbonate buffering in *P. lividus* may be greater than that of non-bicarbonate buffering in *A. lixula*. For example,  $\text{Na}^+/\text{K}^+$ -ATPase is one of a number of transport mechanisms utilized in invertebrate acid–base regulation (Reipschläger and Pörtner, 1996), the activity of which is energetically expensive accounting for up to 40% of the total energy demand in the sea urchin *Strongylocentrotus purpuratus* (Leong and Manahan, 1997). While there are differences between acid–base regulatory mechanisms in the two species of urchins in the present study, it must also be noted that differences in energy acquisition and utilization between the two species under elevated  $p\text{CO}_2$  may also be an important aspect of determining species distributions (Binyon, 1972; Stickle and Diehl, 1987) including across natural  $\text{CO}_2$  gradients. *Arbacia lixula* is regarded as omnivorous, while *P. lividus* is an herbivorous grazer (Agnetta et al., 2013) indicating higher protein acquisition in *A. lixula* compared with *P. lividus*. Non-bicarbonate buffering capacity, while perhaps initially expensive, may in the long term be less energetically expensive than constant  $\text{HCO}_3^-$  regulation. Therefore, species such as *A. lixula*, which demonstrate a higher capacity of non-bicarbonate buffering and therefore a reduced reliance on  $\text{HCO}_3^-$  regulation, may be more tolerant to not only chronic stable high  $p\text{CO}_{2\text{sw}}$  conditions but also acute fluctuations in  $p\text{CO}_{2\text{sw}}$  associated with natural coastal systems. This may be important in determining the resilience of a species as the ability to mobilize  $\text{HCO}_3^-$  in an attempt to compensate  $\text{pH}_e$  in response to OA may have energetic consequences that in the long term give an advantage to species that favour non-bicarbonate protein buffering and so exhibited smaller and presumably less costly fluctuations in acid–base statues. Given the ability of *P. lividus* to compensate stable increases in  $p\text{CO}_{2\text{sw}}$  (Calosi et al., 2013a), it would be expected to maintain population density closer to the vent sites. However, this present study shows that it may be the inability of *P. lividus* to compensate for short-term fluctuations in  $p\text{CO}_{2\text{sw}}$  that ultimately controls its distribution at the vents. Importantly, the present study shows that natural acute fluctuations in  $p\text{CO}_{2\text{sw}}$  associated with natural coastal systems and predicted to increases in the future are just as important in modulating this response as a chronic overall increase in  $p\text{CO}_{2\text{sw}}$  associated with OA.

In conclusion, this study shows that the differential sensitivity of closely related species to future climate change (i.e. the “winners” and “losers”) may have as much to do with physiological compensatory responses to natural acute fluctuations in  $p\text{CO}_{2\text{sw}}$  as with chronic changes in mean  $p\text{CO}_{2\text{sw}}$ . Furthermore, many coastal marine habitats experience periodic and acute fluctuations in seawater carbonate chemistry, the level of variation within which is



predicted to increase. Understanding species physiological responses to variations in  $p\text{CO}_{2\text{sw}}$  in these habitats is essential in understanding the species and community responses to OA as closely related species, such as *P. lividus* and *A. lixula*, have trophic and ecological interactions which are important in characterizing the ecology of the systems in which they reside (Bulleri *et al.*, 1999; Privitera *et al.*, 2008).

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