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Original Article

Temporal fluctuations in seawater pCO_2 may be as important as mean differences when determining physiological sensitivity in natural systems

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Most studies assessing the impacts of ocean acidification (OA) on benthic marine invertebrates have used stable mean pH/pCO_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 pCO_2 (pCO_{2sw}) 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 pCO_{2sw} conditions at shallow water CO_2 seep systems (Vulcano, Italy) and assessed their acid–base responses. Both sea urchin species experienced fluctuations in extracellular coelomic fluid pH, pCO_2, and $[HCO_3^-]$ (pH_e, pCO_{2e}, and $[HCO_3^-]_e$, respectively) in line with fluctuations in pCO_{2sw}. The less tolerant species, *P. lividus*, had the greatest capacity for $[HCO_3^-]_e$ buffering in response to acute pCO_{2sw} 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 pCO_{2sw} may be as important as chronic responses to mean changes in pCO_{2sw} when considering how 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 pCO_2 associated with ocean acidification (OA) on marine invertebrates have focused on stable mean differences in seawater pCO_2 (pCO_{2sw}). Such studies have indicated a range of variability in tolerances to elevated pCO_2 between species and phyla (e.g. Harvey *et al.*, 2013; Kroeker *et al.*, 2013), which drive shifts in species distributions along natural pCO_{2sw} gradients (e.g. Calosi *et al.*, 2013a, b). To understand species responses to elevated pCO_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 pCO_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 pCO_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 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 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 pO_2, pCO_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 pCO_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 pCO_2 experienced by coastal species in many habitats are far greater in magnitude than the predicted long-term increase in mean 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 pCO_{2sw} (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 pCO_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 pCO_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 pCO_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 pCO_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 pCO_{2sw}, such as those found at volcanic 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 pCO_{2sw} (e.g. Suggett *et al.*, 2012; Calosi *et al.*, 2013a, b), despite pCO_{2sw} 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 pCO_{2sw} 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 CO_2 vent sites off Isola Vulcano (Sicily, Italy). Despite a greater capacity for bicarbonate buffering in response to stable elevated pCO_{2sw}, *P. lividus* decreases in population density at higher pCO_{2sw} closer to the vent sites compared with *A. lixula* (Calosi *et al.*, 2013a). This indicates that the ability to buffer stable increases in mean pCO_{2sw} via increased $[HCO_3^-]$ 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 pCO_{2sw} may pose as great or a greater challenge to maintaining acid–base regulation as chronic mean elevations in pCO_{2sw} 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 pCO_{2sw} 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 pCO_{2sw} as with chronic mean elevations in pCO_{2sw}.

To test this hypothesis, *P. lividus* and *A. lixula* collected from control areas were exposed to acute natural fluctuations in pCO_{2sw} generated by the volcanic CO_2 vent. The coelomic fluid acid–base balance (pH_e, pCO_{2e}, and $[HCO_3^-]_e$) 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 pCO_{2sw} conditions on the distribution of *A. lixula* compared with *P. lividus*. Individual responses in acid–base parameters to changes in pCO_{2sw} 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 $[HCO_3^-]_e$ 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 CO_2 vents (coordinates = 38°25.185′N, 14°57.074′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 ± s.e.).

Seawater parameter	Control treatment	Fluctuating treatment
pH (NBS)	8.04 ± 0.01*	7.72 ± 0.07*
pH max	8.12	8.04
pH min	7.95	7.14
A_T (mEq kg^{-1})	2.53 ± 0.01	2.54 ± 0.01
Temperature (°C)	19.6 ± 0.2	19.5 ± 0.2
Salinity	38.2 ± 0.1	38.2 ± 0.1
pCO_2 (μatm)	608.8 ± 19.1*	1742.3 ± 352.7*
pCO_2 max	779.3	5497.4
pCO_2 min	492.5	599
Ω_{Cal}	3.97 ± 0.09*	2.34 ± 0.28*
Ω_{Ara}	2.59 ± 0.06*	1.52 ± 0.18*
HCO_3^- (μmol kg^{-1})	2102 ± 9*	2295 ± 28*
CO_3^{2-} (μmol kg^{-1})	170.1 ± 4.1*	100.2 ± 12.0*

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

aerated aquaria (vol. = 15 l) to a volcanic CO_2 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_{2sw} on sea urchin coelomic fluid acid–base balance, individuals were randomly assigned to one of two treatments; a stable control pCO_{2sw}, and naturally fluctuating pCO_{2sw} ($n = 9$ individuals *per* species *per* treatment). Exposure to naturally fluctuating pCO_{2sw} 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^{-1}). The release of CO_2 from the vent site generated a natural pH and pCO_2 gradient (Hall-Spencer *et al.*, 2008; Johnson *et al.*, 2012; Boatta *et al.*, 2013; Milazzo *et al.*, 2014). The site from which fluctuating pCO_{2sw} was obtained was ca. 300 m from the main gas vents away from the influence of H_2S and heavy metals (Boatta *et al.*, 2013; Vizzini *et al.*, 2013). Exposure to stable pCO_{2sw} was achieved by pumping water directly from a site 390 m from the main CO_2 vent (Boatta *et al.*, 2013). Sea urchins were exposed to either fluctuating pCO_{2sw} or stable pCO_{2sw} 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_T) 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% $HgCl_2$ 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^{-1} 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_2, CO_3^{2-}, HCO_3^-) and saturation state of calcite and aragonite were calculated from pH, A_T, temperature and salinity using the free-access CO_2SYS (Lewis and Wallace, 1998) with constants provided by Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987) and KSO_4 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_2 (TCO_2) was analysed instantly in a subsample of coelomic fluid (vol. = 30 μl) using a TCO_2 analyser (956D TCO_2 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 microcentrifuge tube (TUL-649-010L, Fisher Scientific, Loughborough, UK) and placed on a micro pH probe (Micro-Inlab pH combination electrode, Metter Toledo, Leicester, UK) connected to a pH meter (Seven Easy pH meter, Metter 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 ($[protein]_e$), NBL, and the first apparent dissociation constants of carbonic acid (pK_1) 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_2 (mixed with O_2-balanced N_2) 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_2 measured in a subsample (vol. = 30 μl) using a TCO_2 analyser. This was repeated for each pCO_2 level. NBLs were constructed using the Henderson–Hasselbalch equation in the following forms:

$$pCO_2 = \frac{TCO_2}{\alpha(10^{pH-pK'_1} + 1)}, \quad (1)$$

$$[HCO_3^-] = 10^{pH-pK'_1}\alpha pCO_2, \quad (2)$$

where α is the CO_2 solubility coefficient in seawater at 20°C (0.3293 mmol l^{-1} kPa^{-1}; Spicer *et al.*, 1988; after Harvey, 1955) and pK'_1 calculated as 6.03 for *P. lividus* and 5.91 for *A. lixula* at 20°C using Equation (3):

$$pK'_1 = pH\left(\frac{\mathrm{Log}_{10}(TCO_2 - \alpha pCO_2)}{\alpha pCO_2}\right), \quad (3)$$

$[HCO_3^-]_e$ and pCO_{2e} in experimental individuals were calculated from pH_e and TCO_{2e} determined above using Equations (4) and (5), respectively (Truchot, 1976);

$$pCO_2 = \frac{TCO_2}{\alpha(10^{pH-pK'_1}+1)}, \quad (4)$$

$$HCO_3^- = TCO_2 - \alpha pCO_2. \quad (5)$$

Determination of coelomic fluid protein concentration

$[Protein]_e$ concentration was determined by the Bradford coomassie blue assay; 10 μl of each coelomic fluid sample was diluted in to 90 μl of H_2O, 5 μl of each diluted sample was plated in to a 96 micro well plate with 250 μl 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 μg ml^{-1} (23209, Thermo Scientific) treated in the same way.

Statistical analysis

The pH_e, pCO_{2e}, $[HCO_3^-]_e$, and $[protein]_e$ of field collected control *A. lixula* and *P. lividus* were compared using a one-way ANOVA. To test if the possible relationship between pCO_{2e}, pH_e, or $[HCO_3^-]_e$ (dependant factors) and pCO_{2sw} (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 pCO_{2sw}, linear regressions were also performed for each acid–base parameter vs. pCO_{2sw} 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* pH_e, pCO_{2e}, and $[HCO_3^-]_e$ with pCO_{2sw} 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 pCO_{2sw}. The standard deviation of pH_e, pCO_{2e}, and $[HCO_3^-]_e$ 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 ± standard error.

Results

Urchin coelomic fluid acid–base parameters before exposure

The acid–base balance of coelomic fluid from field collected (pH = 8.05) *A. lixula* was significantly different from that of *P. lividus* (Table 2). In summary, pH_e and $[HCO_3^-]_e$ were significantly lower in *A. lixula* than *P. lividus* ($F_{min1,17} = 101.692$, $p < 0.001$), while there was no significant difference in pCO_{2e} ($F_{1,17} = 0.628$, $p > 0.05$). The $[protein]_e$ 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 pCO_{2sw} on urchin coelomic fluid acid–base balance

There was a significant three-way interaction between site, species, and pCO_{2sw} on pCO_{2e} ($F_{39.4,1} = 5.587$, $p < 0.05$, Figure 1). The relationship between pCO_{2sw} and pCO_{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 pCO_{2sw} on pH_e ($F_{44.6,1} = 10.633$, $p < 0.01$, Figure 1). The relationship between pCO_{2sw} and 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 pCO_{2sw} and $[HCO_3^-]_e$ also demonstrated a significant three-way interaction between site, species, and pCO_{2sw} ($F_{56.8,1} = 61.378$, $p < 0.001$, Figure 1). However, the relationship between pCO_{2sw} and $[HCO_3^-]_e$ 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 $[HCO_3^-]_e$ as seawater pCO_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 pCO_{2sw} ($F_{44,8,1} = 475.914$, $p < 0.001$, Figure 1).

While fluctuating pCO_{2sw} 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 pH_e, pCO_{2e}, and $[HCO_3^-]_e$ of all individuals against pCO_{2sw} (Table 3). The pH_e of both *A. lixula* and *P. lividus* showed a negative relationship with pCO_{2sw}; however, the average B for the regression of pH_e against pCO_{2sw} of *P. lividus*, -0.091 ± 0.006 unit-pH_e unit-$pCO_{2sw}{}^{-1}$, was significantly greater than that of *A. lixula*, -0.055 ± 0.005 unit-pH_e unit-$pCO_{2sw}{}^{-1}$ ($t_{16} = 4.645$, $p < 0.001$, Figure 2). This indicates that individual decreases in pH_e in response to fluctuations in pCO_{2sw} were greater (i.e. steeper response gradient) in *P. lividus* than in *A. lixula*. The pCO_{2e} and $[HCO_3^-]_e$ of both *P. lividus* and *A. lixula* had a positive relationship against pCO_{2sw}. The average B for pCO_{2e} against pCO_{2sw} of *P. lividus*, 0.174 ± 0.017 kPa $pCO_{2sw}{}^{-1}$, was significantly higher than that of *A. lixula*, 0.102 ± 0.014 kPa pCO_{2sw}^{-1} ($t_{16} = -3.193$, $p = 0.006$), as was the average B for $[HCO_3^-]_e$ against pCO_{2sw} (*P. lividus* $= 0.368 \pm 0.042$ mmol l^{-1} pCO_{2sw}^{-1}, *A. lixula* $= -0.065 \pm 0.025$ mmol l^{-1} pCO_{2sw}^{-1}, $t_{16} = -6.203$, $p < 0.001$). This indicates that individual increases in both pCO_{2e} and $[HCO_3^-]_e$ in response to increases in pCO_{2sw} were

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

	A. lixula	*P. lividus*
pH_e	7.31 ± 0.03*	7.73 ± 0.03*
pCO_{2e} (kPa)	0.28 ± 0.02	0.30 ± 0.02
$[HCO_3^-]_e$(mmol l^{-1})	2.29 ± 0.09*	5.07 ± 0.11*
$Protein_e$ (μg ml^{-1})	334.8 ± 66.2*	122.4 ± 19.8*
pK'_1	5.91	6.03

Data are means ± s.e.
*Significant differences between species ($p < 0.05$).

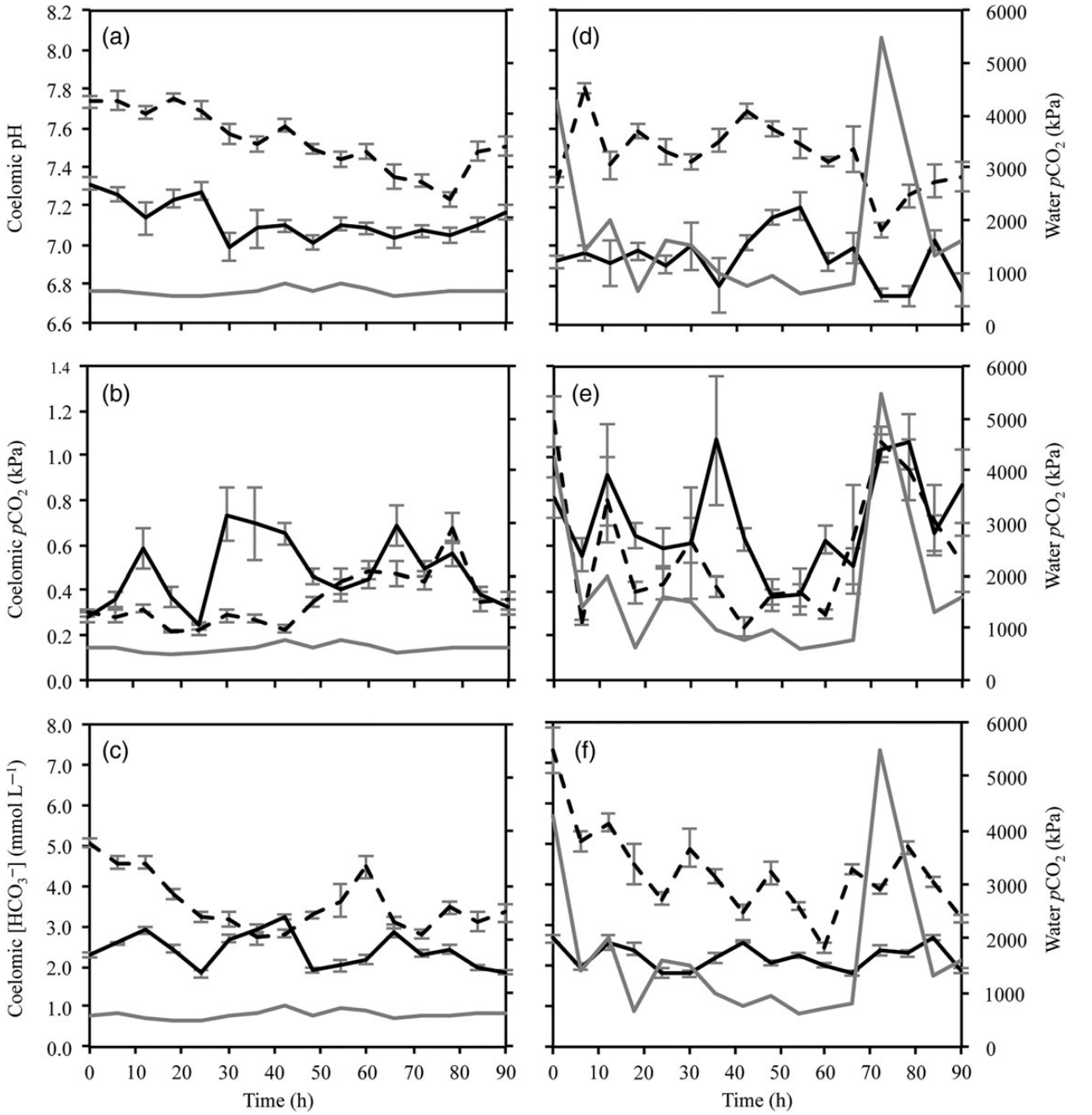

Figure 1. The coelomic fluid pH (a and d), pCO_2 (kPa, b and e), and $[HCO_3^-]$ (mmol l^{-1}, c and f) of *P. lividus* and *A. lixula* during 4 d exposure to stable control pCO_{2sw} conditions (a, b, and c) and fluctuating pCO_{2sw} conditions (d, e, and f). Each time point represents means ± s.e. Grey lines indicate seawater pCO_{2sw}. Black lines indicate *A. lixula*. Dashed lines indicate *P. lividus*.

greater in *P. lividus* than *A. lixula* (Figure 2). Finally, when exposed to fluctuating pCO_{2sw}, the standard deviation of $[HCO_3^-]_e$ in individual *P. lividus* was significantly higher than that in *A. lividus* ($t = 7.377$, $p < 0.001$).

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 pCO_{2sw} 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 pCO_{2sw} 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 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.

	Species			
	A. lixula		*P. lividus*	
Treatment	**Control**	**Fluctuating**	**Control**	**Fluctuating**
pH_e	7.12 ± 0.05	6.94 ± 0.05*	7.53 ± 0.04	7.45 ± 0.05*
Max	7.31	7.19	7.75	7.80
Min	6.99	6.74	7.23	7.08
pCO_{2e} (kPa)	0.48 ± 0.06	0.71 ± 0.11*	0.36 ± 0.03	0.58 ± 0.10*
Max	0.74	1.07	0.68	1.15
Min	0.24	0.37	0.22	0.24
$[HCO_3^-]_e$(mmol l^{-1})	2.40 ± 0.10	2.21 ± 0.09*	3.57 ± 0.17	4.37 ± 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 ± s.e., along with the minimum and maximum values observed.
*Significant effect of seawater pCO_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 pCO_2 and in naturally acidified environments have focused on stable mean differences in pCO_{2sw} conditions (e.g. Suggett *et al.*, 2012; Calosi *et al.*, 2013a, b). Understanding how species respond to natural pCO_{2sw} 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 pCO_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 pCO_2, some species of sea urchins have been shown to exhibit bicarbonate buffering to maintain acid–base homeostasis in response to stable elevated pCO_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 pCO_{2sw} 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 pCO_{2sw} 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 $[HCO_3^-]_e$ with pCO_{2sw}, there were also significant fluctuations in pH_e and pCO_{2e}. In contrast to *P. Lividus*, *A. lixula* exhibited comparatively lower fluctuations in $[HCO_3^-]_e$ and experienced no significant fluctuations in pCO_{2e} or pH_e in relation to fluctuating pCO_{2sw}. 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 pCO_{2sw} closely follows the non-bicarbonate buffering line (Figure 3) and therefore is *due* to non-bicarbonate buffering rather that HCO_3^- regulation. This mechanism in *A. lixula* therefore appears to result in greater pH_e stability under fluctuating pCO_{2sw} conditions. The acid–base responses observed in the present study make an interesting comparison with those of stable mean elevations in pCO_{2sw} in the same species. When exposed to

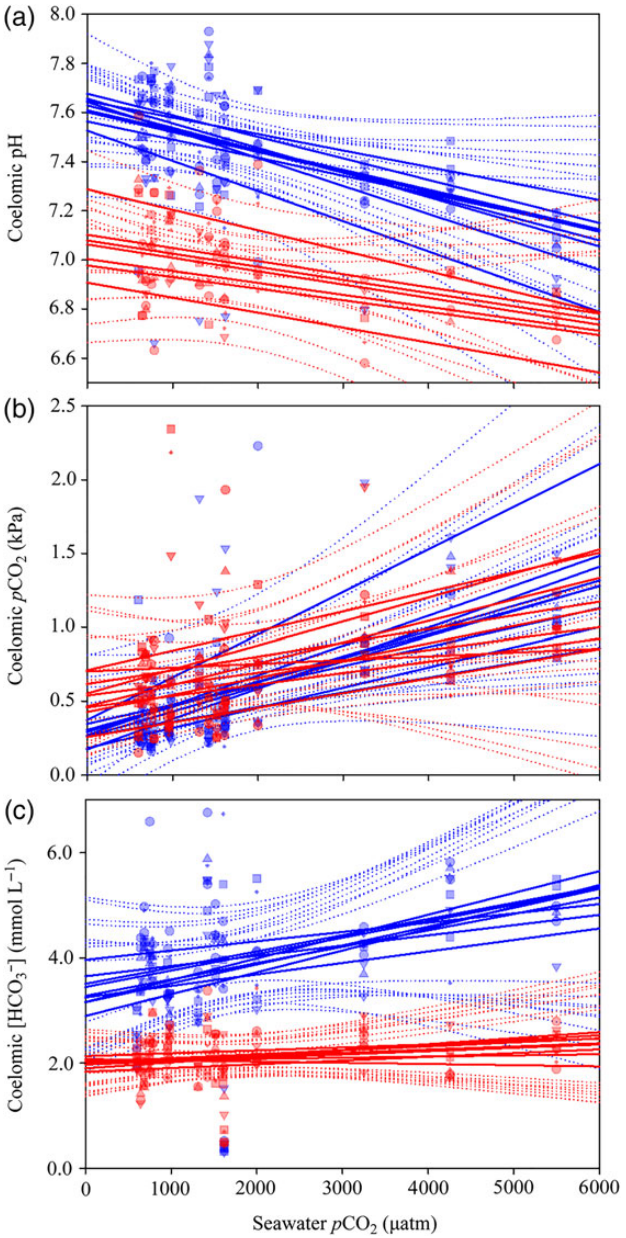

Figure 2. Individual regressions of acid–base parameters coelomic pH (a), coelomic pCO_2 (kPa, b), coelomic $[HCO_3^-]$ (mmol l^{-1}, c) vs. fluctuating pCO_{2sw} of *A. lixula* and *P. lividus*. Red indicates *A. lixula* and blue indicates *P. lividus*. Solid lines indicate individual regressions; dotted lines indicate 95% confidence limits of the regressions. Points indicate raw data from each individual at each pCO_{2sw}, represented by different symbols.

chronic exposure to high pCO_{2sw}, *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 pH_e compensation by *P. lividus* in the present study indicates that this species is unable to respond efficiently to short-term fluctuations in pCO_{2sw}. It is therefore apparent that under fluctuating pCO_{2sw} conditions, the ability of *P. lividus* to compensate pH_e breaks down, while the non-bicarbonate buffering capacity of *A. lixula* allows the maintenance of pH_e homeostasis. The maintenance of pH_e homeostasis in *A. lixula*

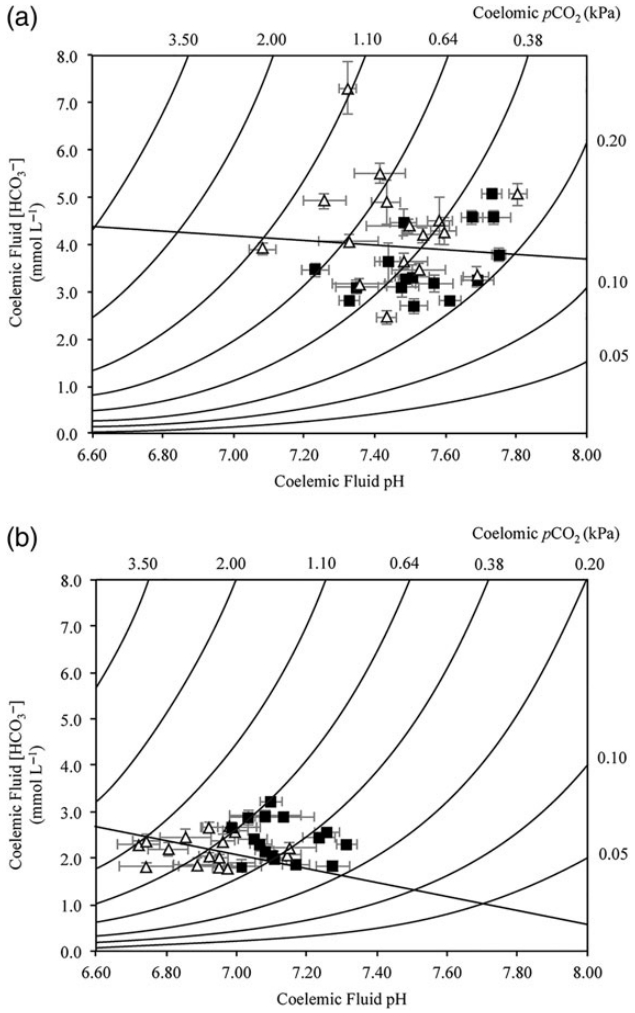

Figure 3. Davenport diagrams representing coelomic fluid acid–base balance of (a) *P. lividus* and (b) *A. lixula*. Each point represents mean ± s.e. of each species at each time point. Solid line indicates non-bicarbonate buffering line. Squares indicate stable control pH conditions; triangles indicate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

may also, in part, be alleviated by comparatively low routine pH_e levels in this species. Throughout control incubations and in field collected individuals from the control site, *A. lixula* consistently had a lower pH_e (7.31 ± 0.03) than *P. lividus* (7.73 ± 0.03) with no difference in pCO_{2e}. Comparatively low 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 pH_e homeostasis and avoid acidosis of the coelomic compartment (i.e. a decrease in pH_e away from its routine value) in response to fluctuations in than pCO_{2sw} with almost no change in $[HCO_3^-]$.

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 $[protein]_e$ 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 $[protein]_e$ between sea urchin species may be the main reason for differences in acid–base regulatory capacity, with species exhibiting higher $[protein]_e$ having greater capacity for pH_e regulation (Spicer *et al.*, 1988), as demonstrated in the present study.

Differences in $[HCO_3^-]_e$ 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 pCO_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, Na^+/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 purporatus* (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 pCO_2 may also be an important aspect of determining species distributions (Binyon, 1972; Stickle and Diehl, 1987) including across natural 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 HCO_3^- regulation. Therefore, species such as *A. lixula*, which demonstrate a higher capacity of non-bicarbonate buffering and therefore a reduced reliance on HCO_3^- regulation, may be more tolerant to not only chronic stable high pCO_{2sw} conditions but also acute fluctuations in pCO_{2sw} associated with natural coastal systems. This may be important in determining the resilience of a species as the ability to mobilize HCO_3^- in an attempt to compensate 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 presumable less costly fluctuations in acid–base statues. Given the ability of *P. lividus* to compensate stable increases in pCO_{2sw} (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 pCO_{2sw} that ultimately controls its distribution at the vents. Importantly, the present study shows that natural acute fluctuations in pCO_{2sw} associated with natural costal systems and predicted to increases in the future are just as important in modulating this response as a chronic overall increase in pCO_{2sw} 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 pCO_{2sw} as with chronic changes in mean pCO_{2sw}. 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 pCO_{2sw} 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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