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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 $pCO_2$ ($pCO_2_{sw}$) 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 $pCO_2_{sw}$ 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 $[HCO_3^-]$ ($pH_e$, $pCO_2_e$, and $[HCO_3^-]_e$, respectively) in line with fluctuations in $pCO_2_{sw}$. The less tolerant species, *P. lividus*, had the greatest capacity for $[HCO_3^-]$ buffering in response to acute $pCO_2_{sw}$ 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 $pCO_2_{sw}$ may be as important as chronic responses to mean changes in $pCO_2_{sw}$ 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.
Natural variability in pCO$_2$ and acid–base balance

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 (Manzano et al., 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 pCO$_2$, pCO$_2$ pH, and total alkalinity (Ap) (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$_2$ can provide valuable insights into how species will respond to future OA. Understanding how organisms tolerate natural variations in carbonate 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$_{2sw}$, and [HCO$_3^{-}$]) 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$_3^{-}$], 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.).

<table>
<thead>
<tr>
<th>Seawater parameter</th>
<th>Control treatment</th>
<th>Fluctuating treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (NBS)</td>
<td>8.04 ± 0.01*</td>
<td>7.72 ± 0.07*</td>
</tr>
<tr>
<td>pH max</td>
<td>8.12</td>
<td>8.04</td>
</tr>
<tr>
<td>pH min</td>
<td>7.95</td>
<td>7.14</td>
</tr>
<tr>
<td>$A_t$ (mEq kg$^{-1}$)</td>
<td>2.53 ± 0.01</td>
<td>2.54 ± 0.01</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>19.6 ± 0.2</td>
<td>19.5 ± 0.2</td>
</tr>
<tr>
<td>Salinity</td>
<td>382 ± 0.1</td>
<td>382.2 ± 0.1</td>
</tr>
<tr>
<td>$pCO_2$ (μatm)</td>
<td>608.8 ± 19.1*</td>
<td>1742.3 ± 352.7*</td>
</tr>
<tr>
<td>$pCO_2$ max</td>
<td>7793</td>
<td>5497.4</td>
</tr>
<tr>
<td>$pCO_2$ min</td>
<td>492.5</td>
<td>599</td>
</tr>
<tr>
<td>$\Omega_{CO_2}$</td>
<td>3.97 ± 0.09*</td>
<td>2.34 ± 0.28*</td>
</tr>
<tr>
<td>$\Omega_{HCO_3}$</td>
<td>1.52 ± 0.18*</td>
<td></td>
</tr>
<tr>
<td>$HCO_3^-$ (μmol kg$^{-1}$)</td>
<td>2102 ± 9*</td>
<td>2295 ± 28*</td>
</tr>
<tr>
<td>$CO_2^-$ (μmol kg$^{-1}$)</td>
<td>170.1 ± 4.1*</td>
<td>100.2 ± 12.0*</td>
</tr>
</tbody>
</table>

*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 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 $pCO_2$ on sea urchin coelomic fluid acid–base balance, individuals were randomly assigned to one of two treatments: a stable control $pCO_2$ and naturally fluctuating $pCO_2$ ($n = 9$ individuals per species per treatment). Exposure to naturally fluctuating $pCO_2$ was achieved by pumping seawater into a holding tank from Levante Bay (coordinates = 38°25.176′N, 14°57.695′E) using a subsurface 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 CO$_2$ gradient (Hall-Spencer et al., 2008; Johnson et al., 2012; Boatta et al., 2013; Milazzo et al., 2014). The site from which fluctuating $pCO_2$ was obtained was ca. 300 m from the main gas vents away from the influence of H$_2$S and heavy metals (Boatta et al., 2013; Vizzini et al., 2013). Exposure to stable $pCO_2$ 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 $pCO_2$ or stable $pCO_2$ 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$_2$, 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.

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

**Determinant 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 fluid 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, 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]$\_L$), NBL, and the first apparent dissociation constants of carbonic acid ($P_k^1$) in urchin coelomic fluid. NBLs 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–Hassebalch equation in the following forms:

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

$$[HCO_3^-] = 10^{pH-pK^1} \cdot apCO_2,$$

where $\alpha$ 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):

$$pK^1 = pH \left( \frac{Log_{10}(TCO_2 - apCO_2)}{apCO_2} \right).$$
\[ \text{HCO}_3^- = \text{TCO}_2 - \alpha \text{pCO}_2. \] (5)

**Determination of coelomic fluid protein concentration**

[Protein] concentration was determined by the Bradford coomassie blue assay; 10 µl of each coelomic fluid sample was diluted in to 90 µl of H,O, 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, \( \text{pCO}_2 \), \([\text{HCO}_3^-]\), and [protein], of field collected control *A. lixula* and *P. lividus* were compared using a one-way ANOVA. To test if the possible relationship between \( \text{pCO}_2 \), pH, or \([\text{HCO}_3^-]\), (dependant factors) and \( \text{pCO}_2 \) (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 \( \text{pCO}_2 \), linear regressions were also performed for each acid–base parameter vs. \( \text{pCO}_2 \) 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 *Lividus* pH, \( \text{pCO}_2 \), and \([\text{HCO}_3^-]\), with \( \text{pCO}_2 \) 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 \( \text{pCO}_2 \). The standard deviation of pH, \( \text{pCO}_2 \), and \([\text{HCO}_3^-]\), 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, and \([\text{HCO}_3^-]\), 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 \( \text{pCO}_2 \) (\( F_{1,17} = 0.628, p > 0.05 \)). The [protein] 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 \( \text{pCO}_2 \) on urchin coelomic fluid acid–base balance**

There was a significant three-way interaction between site, species, and \( \text{pCO}_2 \). The relationship between \( \text{pCO}_2 \) and \( \text{pCO}_2 \) in *P. lividus* was significantly different from 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 \( \text{pCO}_2 \) on pH, \( (F_{6a.1} = 10.633, p < 0.01 \), Figure 1). The relationship between \( \text{pCO}_2 \) and pH, in *P. lividus* was significantly different between the fluctuating and the control treatments (\( F_{118,10.1} = 10.581, p < 0.01 \), Figure 1), while *A. lixula* again showed no significant difference between the two treatments (\( F_{118,10} = 0.106, p = 0.745 \), Figure 1).

The relationship between \( \text{pCO}_2 \) and \([\text{HCO}_3^-]\) also demonstrated a significant three-way interaction between site, species, and \( \text{pCO}_2 \). However, the relationship between \( \text{pCO}_2 \) and \([\text{HCO}_3^-]\) is different between the fluctuating and the control treatments in both *Lividus* (\( F_{115,7.1} = 8.905, p < 0.01 \), Figure 1) and *A. lixula* (\( F_{137,9.1} = 7.719, p < 0.01 \), Figure 1). This is because both species show some significant increase in \([\text{HCO}_3^-]\), as seawater \( \text{pCO}_2 \) 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 \( \text{pCO}_2 \) (\( F_{44,8.1} = 475.914, p < 0.001 \), Figure 1).

While fluctuating \( \text{pCO}_2 \) 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, \( \text{pCO}_2 \), and \([\text{HCO}_3^-]\) of all individuals against \( \text{pCO}_2 \) (Table 3). The pH, of both *A. lixula* and *P. lividus* showed a negative relationship with \( \text{pCO}_2 \); however, the average B for the regression of pH, against \( \text{pCO}_2 \) of *Lividus* (\( -0.091 \pm 0.006 \text{unit–pH} \), unit–p–CO\(_2\)\(^{-1}\), was significantly greater than that of *A. lixula*, \( -0.055 \pm 0.005 \text{unit–pH} \), unit–p–CO\(_2\)\(^{-1}\), \( t_{16} = 4.645, p < 0.001 \), Figure 2). This indicates that individual decreases in pH, in response to fluctuations in \( \text{pCO}_2 \) were greater (i.e. steeper response gradient) in *P. lividus* than in *A. lixula*. The \( \text{pCO}_2 \), and \([\text{HCO}_3^-]\), of both *P. lividus* and *A. lixula* had a positive relationship against \( \text{pCO}_2 \). The average B for \( \text{pCO}_2 \) against \( \text{pCO}_2 \) of *P. lividus*, \( 0.174 \pm 0.017 \text{unit–CO}_2\)\(^{-1}\) was significantly higher than that of *A. lixula*, \( 0.102 \pm 0.014 \text{unit–CO}_2\)\(^{-1}\), \( t_{16} = -3.193, p = 0.006 \), as was the average B for \([\text{HCO}_3^-]\) against \( \text{pCO}_2 \) of *P. lividus* (\( 0.368 \pm 0.042 \text{mmol l}^{-1}\) \( \text{pCO}_2\)\(^{-1}\), *A. lixula* was \( -0.065 \pm 0.025 \text{mmol l}^{-1}\) \( \text{pCO}_2\)\(^{-1}\), \( t_{16} = -6.203, p < 0.001 \)). This indicates that individual increases in both \( \text{pCO}_2 \) and \([\text{HCO}_3^-]\) in response to increases in \( \text{pCO}_2 \) were

<table>
<thead>
<tr>
<th>Table 2. Acid–base balance of control field collected <em>A. lixula</em> and <em>P. lividus</em>.</th>
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</thead>
<tbody>
<tr>
<td><strong>A. lixula</strong></td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>( \text{pCO}_2 ) (kPa)</td>
</tr>
<tr>
<td>([\text{HCO}_3^-]) (mmol l(^{-1}))</td>
</tr>
<tr>
<td>Protein (µg ml(^{-1}))</td>
</tr>
<tr>
<td>( \text{pK}_a )</td>
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</table>

Data are means ± s.e.

*Significant differences between species (p < 0.05).
greater in *P. lividus* than *A. lixula* (Figure 2). Finally, when exposed to fluctuating $pCO_{2\text{sw}}$, the standard deviation of $[HCO_3^-]$ 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 $pCO_{2\text{sw}}$ 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 $pCO_{2\text{sw}}$ was greater than that of *A. livida* because *P. lividus* relies on limited bicarbonate buffering capabilities for acid–base regulation, whereas *A. livida* 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...
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 pCO₂ and in naturally acidified environments have focused on stable mean differences in pCO₂sw conditions (e.g. Suggett et al., 2012; Calosi et al., 2013a, b). Understanding how species respond to natural pCO₂sw 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 pCO₂ (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 pCO₂, some species of sea urchins have been shown to exhibit bicarbonate buffering to maintain acid–base homeostasis in response to stable elevated pCO₂ in laboratory (Miles et al., 2007) and field conditions (Calosi et al., 2013a). In the present study, there was no significant effect of stable pCO₂sw 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 pCO₂sw 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 [HCO₃⁻] and pCO₂sw, there were also significant fluctuations in pH and pCO₂sw. In contrast to P. lividus, A. lixula exhibited comparatively lower fluctuations in [HCO₃⁻] and experienced no significant fluctuations in pCO₂ or pH relative to fluctuating pCO₂sw. 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 pCO₂sw followed the non-bicarbonate buffering line (Figure 3) and therefore is due to non-bicarbonate buffering rather than HCO₃⁻ regulation. This mechanism in A. lixula therefore appears to result in greater pH stability under fluctuating pCO₂sw conditions. The acid–base responses observed in the present study make an interesting comparison with those of stable mean elevations in pCO₂sw in the same species. When exposed to chronic exposure to high pCO₂sw 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 compensation by P. lividus in the present study indicates that this species is unable to respond efficiently to short-term fluctuations in pCO₂sw. It is therefore apparent that under fluctuating pCO₂sw conditions, the ability of P. lividus to compensate pH breaks down, while the non-bicarbonate buffering capacity of A. lixula allows the maintenance of pH homeostasis. The maintenance of pH 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 pCO2sw conditions.

...conditions; triangles indicate fluctuating non-bicarbonate buffering line. Squares indicate stable control pH s.e. of each species at each time point. Solid line indicates...

...pH response show by *P. lividus* (Figure 3). *Paracentrotus lividus* has a significantly lower [protein], 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], between sea urchin species may be the main reason for differences in acid–base regulatory capacity, with species exhibiting higher [protein], having greater capacity for pH regulation (Spicer et al., 1988), as demonstrated in the present study.

Differences in [HCO3−], 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 pCO2 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 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 pCO2 may also be an important aspect of determining species distributions (Binyon, 1972; Stickle and Diehl, 1987) including across natural CO2 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 HCO3− regulation. Therefore, species such as *A. lixula*, which demonstrate a higher capacity of non-bicarbonate buffering and therefore a reduced reliance on HCO3− regulation, may be more tolerant to not only chronic stable high pCO2 conditions but also acute fluctuations in pCO2sw associated with natural coastal systems. This may be important in determining the resilience of a species as the ability to mobilize HCO3− in an attempt to compensate pH, 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 pCO2sw (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 pCO2sw that ultimately controls its distribution at the vents. Importantly, the present study shows that natural acute fluctuations in pCO2sw 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 pCO2sw 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 pCO2sw as with chronic changes in mean pCO2sw. 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$_2$ 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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**References**


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