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Distribution of sea urchins living near shallow water CO_2 vents is dependent upon species acid–base and ion-regulatory abilities

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ABSTRACT

To reduce the negative effect of climate change on Biodiversity, the use of geological CO_2 sequestration has been proposed; however leakage from underwater storages may represent a risk to marine life. As extracellular homeostasis is important in determining species' ability to cope with elevated CO_2, we investigated the acid–base and ion regulatory responses, as well as the density, of sea urchins living around CO_2 vents at Vulcano, Italy. We conducted *in situ* transplantation and field-based laboratory exposures to different pCO_2/pH regimes. Our results confirm that sea urchins have some ability to regulate their extracellular fluid under elevated pCO_2. Furthermore, we show that even in closely-related taxa divergent physiological capabilities underlie differences in taxa distribution around the CO_2 vent. It is concluded that species distribution under the sort of elevated CO_2 conditions occurring with leakages from geological storages and future ocean acidification scenarios, may partly be determined by quite subtle physiological differentiation.

1. Introduction

Increased anthropogenic CO_2 emissions accompanied the advent of industrialisation and have resulted over the past two centuries in a net increase in atmospheric CO_2 (Solomon et al., 2007). This in turn increased oceanic CO_2 levels, resulting in a reduction in both pH and carbonate ion (CO_3^{2-}) concentration (Zeebe and Wolf-Gladrow, 2001). This phenomenon is termed ocean acidification (OA) (Caldeira and Wickett, 2003; Orr et al., 2005; Raven et al., 2005) and is considered a threat to marine life. The use of geological CO_2 sequestration (so called Carbon Capture and Storage – CCS) has been proposed to reduce (or slow down) the impact of global climate change on global biodiversity (Gibbins et al., 2006; Blackford et al., 2009). However, leakages from CCS represent a potential risk to marine life, as they may lead to localized acute and extreme CO_2 release events, with potentially negative biological consequences (Seibel and Walsh, 2003; Barry et al., 2004; Blackford et al., 2009; Small et al., 2010; Christen et al., 2012; Donohue et al., 2012). There is evidence that increased CO_2 levels in sea water and the resultant reduction in pH may impair physiological, ecological and behavioural functions of marine animals (Widdicombe and Spicer, 2008; Melzner et al., 2009; Munday et al., 2009). The capacity for extracellular acid–base regulation is thought to be important in determining a species' ability to cope with elevated CO_2 (Pörtner et al., 1998; Widdicombe and Spicer, 2008; Melzner et al., 2009; Whiteley, 2011), with echinoderms and molluscs being amongst the phyla exhibiting the poorest regulatory abilities, and thus being amongst the most vulnerable. The vulnerability of echinoderms and molluscs to OA is highlighted by the results of a series of multispecies mesocosm laboratory experiments (Widdicombe et al., 2009; Hale et al., 2011; Christen et al., 2012) and observations made on assemblages associated with natural CO_2 vents (Hall-Spencer et al., 2008; Cigliano et al., 2010; Kroeker et al., 2011; Johnson et al., 2012). The abundance of echinoids and bivalves, in particular, is negatively related to increased seawater pCO_2 (or reduced pH), indicating that in a future high-CO_2 world taxa distribution may in part be determined by their homeostatic abilities and associated energy costs.

Most studies investigating how elevated CO_2 conditions will impact the function of marine organisms are laboratory-based (although cf. Thomsen et al., 2010; Lombardi et al., 2011; Rodolfo-Metalpa et al., 2011), making it difficult to directly relate laboratory results to the effect that elevated CO_2 (i.e. OA and CCS leakages) will have on marine biota *in situ*. Field experiments, at sites with naturally-elevated CO_2 conditions, such as shallow-water

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CO_2 vents, are potentially useful analogues for investigating the effect of future dissolved CO_2 levels on marine organisms and ecosystems (Hall-Spencer et al., 2008; Cigliano et al., 2010; Thomsen et al., 2010; Lombardi et al., 2011; Rodolfo-Metalpa et al., 2011; Kroeker et al., 2011, 2012).

As mentioned previously, echinoderms, and echinoids in particular, are considered particularly vulnerable to low pH and carbonate saturation status (e.g. Kurihara and Shirayama, 2004a, 2004b; Miles et al., 2007; Widdicombe and Spicer, 2008; Melzner et al., 2009; Todgham and Hofmann, 2009; Dupont et al., 2010; Spicer et al., 2011; Stumpp et al., 2011; Catarino et al., 2012). However, contrary to the prevailing view that echinoids possess limited (or no) extracellular regulatory ability, recent studies seem to indicate the presence of a diverse suite of acid–base and osmo–iono responses to elevated CO_2 (low pH) in some sea urchin species (Miles et al., 2007; Vidolin et al., 2007; Spicer et al., 2011; Stumpp et al., 2011; see also Spicer et al., 1988), For example, values of the non-bicarbonate buffer line slope (β_{NB}, expressed here as mmol l^{-1} pH^{-1}, Stumpp et al., 2012) for the species of sea urchins investigated to date appear to vary between 0.4 and 5.6 (see Spicer et al., 1988, 2011; Miles et al., 2007; Stumpp et al., 2011), suggesting considerable variation in aspects of the acid–base regulatory ability within this group, perhaps as extreme as the comparison between gastropod and bivalves with cephalopods (i.e. both ends of the range of homeostatic properties of the acid–base status for invertebrates, see Melzner et al., 2009 for review). Clearly we do not yet have a good working knowledge of acid–base regulation in echinoids, let alone understand the physiological responses closely-related species will show to elevated CO_2, and the importance of such responses to species' ecology.

This aim of this study is to better understand the physiological responses of vulnerable shallow-water marine organisms *in situ* to the exposure to elevated CO_2 (low pH) predicted to occur under future OA, and potential CCS leakage scenarios. Consequently, we investigated the extracellular acid–base balance and ionic regulation of two sea urchin species both *in situ*, and after transplantation around shallow water CO_2 vents at Vulcano Island, Italy. First, we determined the distribution and density of both species at key points along the CO_2/pH gradient at the vent, selecting comparable habitats suitable for sea urchins settling. We then characterised baseline extracellular acid–base and ionic values in untreated, field-collected individuals as a back-drop to investigating the acid–base and ionic regulatory responses of sea urchins exposed in *in situ* transplantation (2–4 d) and in a short-term field-laboratory experiments (0–24 h) to different pCO_2/pH conditions.

2. Materials and methods

2.1. Species studied

Only two echinoid species are common around the shallow water CO_2 vents of Vulcano Island; the black sea urchin, *Arbacia lixula* (Linnaeus 1758) and the purple sea urchin, *Paracentrotus lividus* (Lamarck 1816). Both species regularly co-occur in the infralittoral zone along the Mediterranean and north-east Atlantic coasts (Privitera et al., 2008). Although both species are found in coralline algal barrens, macroalgae and seagrass habitats (Privitera et al., 2008; Bonaviri et al., 2011; Pinna et al., 2012), *A. lixula* preferentially feeds on encrusting coralline algae (Privitera et al., 2008), and at least partially on sessile animals (Wangensteen et al., 2011), whilst *P. lividus* favours erect fleshy algae (Privitera et al., 2008; Bonaviri et al., 2011). Sparid fishes and starfish are natural predators of both sea urchins, as are some labrid fishes which prey on their juvenile forms (Hereu et al., 2005; Bonaviri et al., 2009). Of the two species only *P. lividus* is harvested, although no harvesting was recorded in the study area, which is private property inaccessible to bathers.

2.2. Study site and sea urchin survey

The study area is Levante Bay (38°25'N, 14°57'E) located on the north-east side of the volcanic island, Vulcano (Italy), where an active shallow-water CO_2 vent creates a natural pH and pCO_2 gradient along the north-westerly side of the bay (Johnson et al., 2011; Arnold et al., 2012; Lidbury et al., 2012; Boatta et al., in press, see Fig. 1). The stations identified for the experiment were characterised by different concentrations of CO_2 and thus different levels of pH (A: non-acidified water, B and C: acidified water; see Table 1, Fig. 1), but were similar in depth (approx. 2–3 m). The densities of sea urchins (*A. lixula* and *P. lividus*) were estimated at stations A and B, whilst at station C there was no suitable habitat for sea urchins to settle on and so, unsurprisingly, no sea urchins were found. Counts of both sea urchin species were obtained from a visual census and density expressed as numbers of individuals encountered while snorkelling a 2×5 m transect over comparable rocky substrata with macroalgal coverage (Kingsford and Battershill, 1998; Edgar and Barret, 1997). Three transects (in total 12 replicates at 1–3 m water depth) were surveyed in two areas chosen haphazardly within stations A and B.

2.3. Environmental monitoring

Seawater pH, temperature and salinity were measured at different stations and on numerous occasions throughout the duration of the observations and experiments described below (approx. three weeks). These values are compared with a monitoring programme carried out at this same site by Boatta et al. (in press), which took place from September 2009 to July 2011. This was a good cross-check on whether the environmental conditions to which we exposed the sea urchins during the experiments, described below, were representative of the mean and variation characterising the pH gradient. For the *in situ* transplantation and field-laboratory experiments the environmental monitoring was carried out as follows. pH_{NSB} was measured using a pH electrode, always maintained at ambient seawater temperature (Seven Easy pH InLab micro-electrode, Mettler-Toledo Ltd., Beaumont Leys, UK), coupled to a pH meter (Sevengo, Mettler-Toledo Ltd., Beaumont Leys, UK) and calibrated using Mettler-Toledo pH standards (pH 4.01, 7.00, 9.21 at 25 °C) also maintained at seawater temperature. Temperature was measured using a digital thermometer (HH806AU, OMEGA Eng. Ltd., Manchester, UK). Salinity was measured using a hand-held conductivity meter (TA 197 LFMulti350, WTW, Weilheim, Germany). Mean total alkalinity values given here were taken from Johnson et al. (2011). Additional carbonate system parameters (dissolved inorganic carbon (DIC), pCO_2, calcite and aragonite saturation, $[HCO_3^-]$ and $[CO_3^{2-}]$) were calculated from pH and TA measurements using the software program CO2SYS (Pierrot et al., 2006) with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and $[KSO_4]$ using Dickson (1990).

Our results for the carbonate system along the pCO_2/pH gradient of the vent (Table 1) indicate that the mean values for water chemistry parameters recorded during the *in situ* transplantations and the field-laboratory experiments were broadly comparable (see Table 1) to those of Boatta et al. (in press) with one important exception, as a shift in pCO_2/pH during our study resulted in the seawater chemistry of station C resembling more closely the profile of station B. So sea urchins were transplanted to station C to carry out the 2–4 d *in situ* exposure. Also seawater was pumped from station C to the field-laboratory for use in the 0–24 h experiment, field-laboratory experiments.

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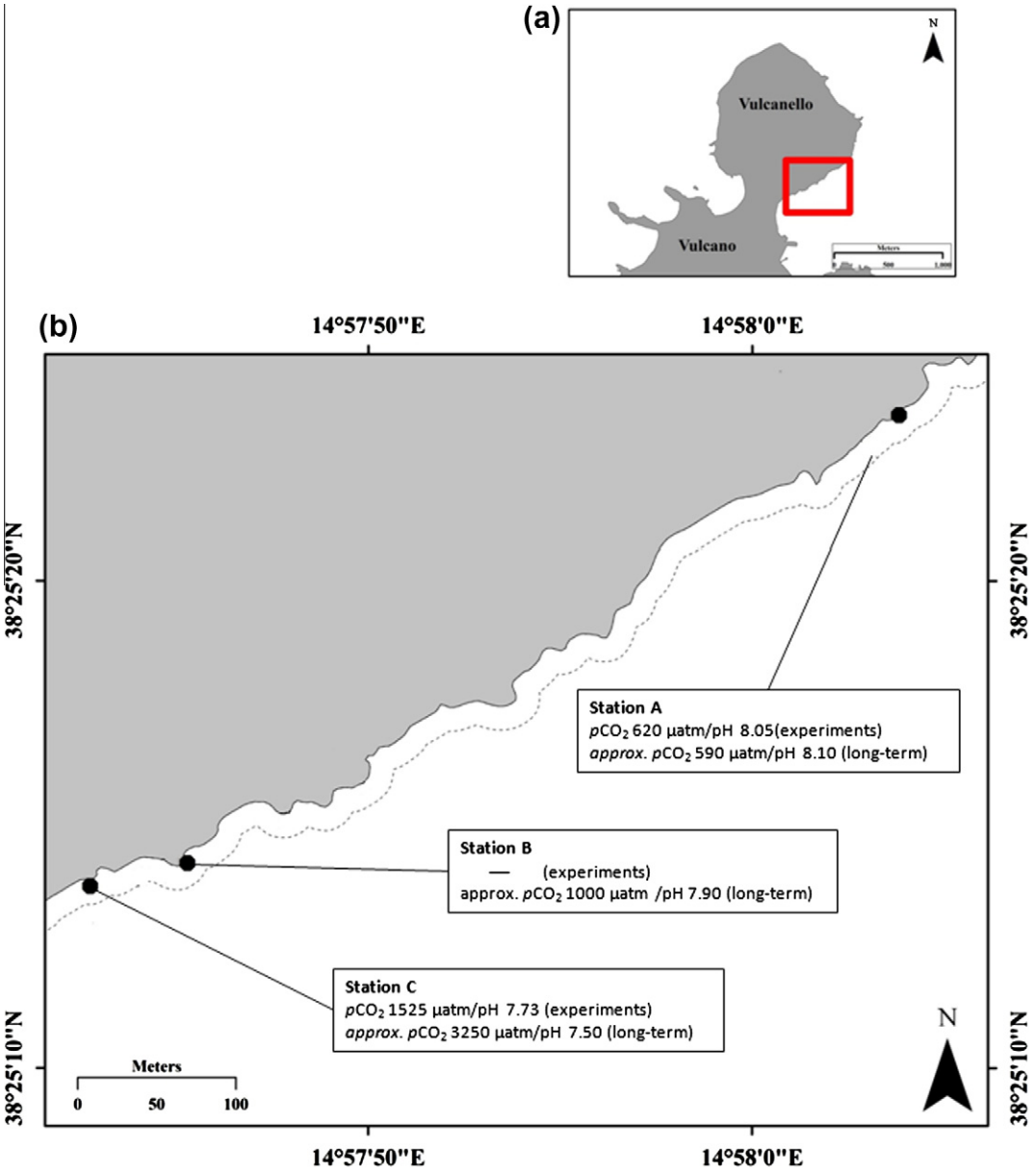

Fig. 1. Maps of the study area showing the (a) northern region of the island of Vulcano (Sicily, Italy), the red square indicates the study area in Cala di Levante; and (b) pCO_2/pH gradient around the CO_2 vent; the grey shaded area represents land, the continuous line represents the coastline, and the dashed line defines the 5 m seawater belt parallel to the coastline were the pCO_2/pH gradient is present. Station A: Caging experiment and sea-urchin density visual census site with normal pH; station B: Sea-urchin density visual census site in acidified waters; station C: Caging experiment site in acidified waters. In the boxes for each station, mean values for pCO_2 and pH are reported for measurements obtained *in situ* throughout the duration of the 'experiments' (approx. three weeks), and from a 'long-term' monitoring programme carried out at this same site (see Boatta et al. (in press) in this special issue).

2.4. Sea urchins collection and experimental set-up

Sea urchins were collected by hand from station A by snorkelers, and immediately transferred to a bucket (vol. = 12 l) whilst still underwater to avoid any negative effects of the exposure to air on sea urchin physiology (see Spicer et al., 1988; Burnett et al., 2002). Buckets containing sea urchins were kept immersed for <5 min before removal to the experimental field laboratory (near station C) which minimised potential thermal fluctuations. Upon arrival sea urchins were used in one of three different ways:

(i) To characterise the physiological parameters to establish baseline levels for untreated, field-collected individuals as described below, *A. lixula* ($N = 23$) and *P. lividus* ($N = 34$). Immediately upon arrival at the field laboratory coelomic fluid was collected, physiological and morphometric measurements made, and sampling of calcified structures was carried out (all as described in detail below).

(ii) For a transplantation caging experiment to investigate sea urchins *in situ* physiological responses over 2 and 4 d exposure to either control or elevated pCO_2. After collection from station A two groups of approx. 10 individuals *per* species, *per* treatment were haphazardly selected and placed individually in cages ($20 \times 20 \times 20$ cm, wooden framed with plastic garden mesh $\varnothing = 0.5$ cm). Five or six stones of comparable size were placed in each cage; stones were collected from the same area were sea urchins were collected and were covered by a natural film of bacteria/microalgae. This

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Table 1
Mean ± SD of seawater physico-chemical parameters measured in, or calculated for, the field and field-laboratory control and acidified areas. Seawater chemistry parameters, dissolve inorganic carbon (DIC), CO_2 partial pressure (pCO_2), calcite and aragonite saturation (Ω_{calc} and Ω_{ara}), bicarbonate and carbonate ions concentration ($[HCO_3^-]$ and $[CO_3^{2-}]$), were calculated from pH and total alkalinity (TA) using CO2SYS (Pierrot et al., 2006) with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and $[KSO_4]$ using Dickson (1990) are indicated by an asterisk (*). TA values are from Johnson et al., 2011 (†).

Parameter	Field		Field-laboratory	
	Station A – control (*in situ* transplantation experiment)	Station C – acidified (*in situ* transplantation experiment)	Control (0–24 h experiment)	Acidified (0–24 h experiment)
Salinity	38	38	38	38
Temperature (°C)	19.51 ± 0.24	19.85 ± 0.91	22.12 ± 0.20	23.01 ± 1.11
TA (μEq kg^{-1})†	2625	2736	2625	2736
pH	8.05 ± 0.04	7.73 ± 0.09	8.06 ± 0.05	7.69 ± 0.12
DIC (μmol kg^{-1})*	2391.66 ± 22.87	2646.40 ± 36.09	2369.41 ± 28.95	2646.95 ± 45.15
pCO_2 (μatm)*	620.09 ± 68.83	1525.76 ± 397.40	619.26 ± 8212	1754.60 ± 583.18
Ω_{calc}*	4.14 ± 0.34	2.29 ± 0.40	4.50 ± 0.43	2.34 ± 0.49
Ω_{ara}*	2.70 ± 0.21	1.50 ± 0.26	2.96 ± 0.28	1.54 ± 0.32
HCO_3^- (μmol kg^{-1})*	2194.44 ± 35.13	2499.65 ± 41.43	2158.26 ± 44.93	2495.88 ± 50.53
CO_3^{2-} (μmol kg^{-1})*	177.25 ± 14.40	96.13 ± 17.18	192.56 ± 16.48	99.99 ± 20.94

provided both a suitable substrata for the sea urchins to attach onto and a source of food to graze on. After 2 or 4 d of exposure to either control or acidified conditions (station A and C respectively), eight individuals *per* species, *per* treatment were haphazardly recovered by snorkelling, immediately transported to the field laboratory in a bucket of sea water. Upon arrival, extracellular (coelomic) fluid was collected and physiological, morphometric and body mass measurements were obtained (as described below).

(iii) To investigate more acute physiological responses in the sea urchins to aid interpretation of the trajectory observed at 2 and 4 d, a field-laboratory was constructed to conduct repeated measures at different times over a 24 h period of sea urchins exposed to either control or elevated pCO_2. On arrival at the field laboratory eight specimens *per* species, *per* treatment were immediately transferred to individually-identifiable open containers (vol. = 1 l) nested within an opaque experimental aquaria (vol. = 80 l), fitted with a lid. This small set-up was coupled to an electrical water pump (2CPM80E HP 0.5, Pedrollo, Verona, Italy) for the continuous circulation of sea water within the aquaria (flow rate: max 130 ± 5 l min^{-1}). Sea water was supplied from either the control or acidified area, *via* an adjustable-length hose, for the entire duration of the trials, thus maintaining pH, temperature and salinity conditions near-identical to that of the relative field stations. Coelomic fluid from individual sea urchins exposed to low or elevated pCO_2 conditions was sampled at 0, 1.5, 3, 6, 12 and 24 h for the determination of acid–base balance parameters (see Section 2.5), and at 0, 3, 6, 12 and 24 h for the determination of ions concentrations (see Section 2.5). All sampling took place on partly immersed individuals. Morphometric measurements, detailed below, were undertaken at the end of the experiment. All experiments were run in parallel, but separately, for the two species investigated.

2.5. Sampling and analysis of coelomic fluid

Upon arrival at the field laboratory, sea urchins were either sampled immediately or after exposure, always within 3 s of being handled under water. A clean, clear and anaerobically-obtained, coelomic fluid sample (vol. = 100 μl) was obtained from each individual by inserting the needle of a gas-tight syringe (Hamilton, gas-tight 1710RN, Bonaduz, Switzerland, vol. = 100 μl), to a depth of about 1 cm through the peristomial membrane and the pharyngeal peritoneum, into the perivisceral coelom. The residence time of samples within the syringe was <10 s, before use in the determination of acid–base parameters or storage for subsequent analysis (see below).

To measure coelomic fluid pH (pH_{cf}), total CO_2 [TCO_2] and major cations the following procedure was carried out. To determine TCO_2, a 50 μl subsample was immediately introduced anaerobically into a previously calibrated carbon dioxide analyser (965D, Ciba Corning Diagnostics Cor., Cambridge, USA). The remaining fluid (vol. = 50 μl) was transferred immediately to a 0.5 ml microcentrifuge tube (polyethylene Beckman type, Fisher Scientific, Loughborough, UK) and pH_{cf} measured (< 10 s after extraction) by immersing a micro-pH probe (Micro-InLab pH combination electrode, Mettler Toledo) in the fluid creating an anaerobic sealed area between the bottom of the tube and the tip of the pH probe (see also Miles et al., 2007; Spicer et al., 2007; Marchant et al., 2010; Small et al., 2010; Donohue et al., 2012). The micro-pH probe was coupled to a pH meter (Seven Easy pH Meter, Mettler Toledo), calibrated as described above. The remaining coelomic fluid (vol. = 50 μl, min 10 μl) was sealed in the microcentrifuge tube, stored for 2 weeks at ambient temperature and subsequently used for cation analysis upon return to Plymouth, UK. These samples of coelomic fluid (10 μl) were carefully diluted to a final volume of 2 ml using ultra-pure water. The resultant dilutions were then analysed for $[Ca^{2+}]$, $[Mg^{2+}]$, $[Sr^{2+}]$, $[Na^+]$ and $[K^+]$, using an ICP optical emission spectrometer (725-ES, Varian Medical Systems Inc., Palo Alto, USA). Values were expressed as mmol l^{-1}. To execute these measurements as rapidly as possible two operators always worked together.

2.6. Calculation of coelomic fluid pCO_2 and $[HCO_3^-]$

Coelomic fluid pCO_2 and $[HCO_3^-]$ were calculated using the Henderson–Hasselbach equation in the following forms (see Spicer et al., 2007):

$$pCO_2 = TCO_2/\alpha(10^{(pHcf-pK'1)} + 1) \quad (1)$$

$$[HCO_3^-] = TCO_2 - \alpha pCO_2 \quad (2)$$

where α is the solubility coefficient of CO_2 of sea water taken as 0.337 mmol l^{-1} kPa^{-1} at 15 °C approx. 35 salinity, and pK'_1 is the negative log of the first apparent dissociation constant of carbonic acid taken as 6.04 at 15 °C (Truchot, 1976). Truchot's pK_1 for haemolymph for the crab, *Carcinus maenas* was chosen because: (i) the pK_1 for *A. lixula* could not be determined at the time of this experiment due to logistic difficulties and it is not available in the literature, (ii) pK_1 for *P. lividus* whilst available in the literature was determined in an Atlantic population and under a different salinity-temperature regime than used here, (iii) Truchot

determined values over a salinity-temperature range similar to that of this present study.

2.7. Measurement of echinoid mass, morphometrics and mineralisation of calcified structures

Immediately post-sampling, individual sea urchins were weighed with a digital high-precision scale (BA 210-S, Sartorius Mechatronics, Göttingen, Germany, to 0.1 mg accuracy), taking great care to avoid loss of coelomic fluid from the wound created by the sampling described above.

The height and diameter of the test were then measured using precision callipers. Finally, individuals were sacrificed and the entire test, all undamaged primary spines (not from the ambulacral region) and entire Aristotle's lanterns samples were collected for cations analyses upon return to Plymouth, UK. Upon arrival, individual tissue samples were scrubbed clean of all organic material using a plastic soft brush and plastic dissection tools before being freeze-dried at −50 °C for 48 h with a freeze-drier apparatus (Edwards Super Modulyo, Edwards Vacuum, Crawley, UK). Samples were weighed with a high precision digital scale (PS-200, Fisher Scientific Ltd., Corby, UK, to 0.1 mg accuracy) before being digested individually in a glass beaker (vol. = 50 ml) containing 3 ml of nitric acid (70% concentration, trace analysis grade, Fisher Scientific UK Ltd., Loughborough, UK). The beaker was covered with a watch glass and left at room temperature for 60 min to allow readily oxidised material to be digested. The beaker containing the digestant was then placed in a high-Throughput Microwave Reaction System Run (MARSXpress, CEM Corporation, Matthews, USA) and gently heated to boiling for at least 1 h to ensure full digestion. The sample was then transferred to an acid-washed 25 ml volumetric flask and diluted to 25 ml with ultra-pure water to obtain solutions with concentration of the cations of interests within the range detectable by an atomic absorption spectrometer. The sample was then analysed for $[Ca^{2+}]$, $[Mg^{2+}]$ and $[Sr^{2+}]$ using an ICP optical emission spectrometer (725-ES, Varian Medical Systems Inc., Palo Alto, USA). Data were expressed as mmol of ion kg^{-1}.

2.8. Statistical analysis

Differences in the density and distribution of *A. lixula* and *P. lividus*, separately were tested using two univariate PERMANOVA applying the following two factorial experimental design with 'pH station' (two levels, fixed) and 'Area' (two levels, random, nested in pH) (Anderson, 2001).

Two different statistical approaches were employed to analyse the short-term laboratory (0–24 h) and longer-term *in situ* (2–4 d) exposure experiments. For the short-term exposure experiment, as measurements were repeated on the same individuals at each time interval, a two-way nested orthogonal experimental design was employed to investigate the relationship between pCO_2/pH exposure and duration of exposure and the physiological parameters measured, with individuals set as random factors nested in the pCO_2/pH treatment. For the long-term exposure experiment a two-way orthogonal experimental design was employed to investigate the relationship between pCO_2/pH exposure and duration of exposure and the physiological parameters measured. For both experimental designs, relationships were explored using GLM with the spheroid volume of the main body of the sea urchin or mass as a covariate. We included spheroid volume, as a proxy for coelomic fluid volume, as potentially more relevant to the investigation of acid–base and ionic regulation. When mass and volume as the covariate were found not to have a significant effect on acid–base and ionic regulation traits, they were removed from analysis. In addition, where a significant relationship between pCO_2/pH and duration of exposure was detected comparisons amongst treatments for any given life-history and physiological parameter were conducted using Estimate Marginal Mean tests with Bonferroni adjustment for multiple comparisons.

Data met assumption for normality of distribution for most parameters (minimum $Z_{32} = 1.309$, $P = 0.065$, Kolmogorov–Smirnov test), with the following exceptions: in *P. lividus* TCO_2/pCO_2/$[HCO_3^-]$ for the 0–24 h exp., in *A. lixula* TCO_2 and pCO_2 for the 2–4 d exp., and finally in untreated field-collected individuals pCO_2 (maximum $Z_{96} = 1.813$, $P < 0.0001$, Kolmogorov–Smirnov test). Assumption of homogeneity of variance was met for all traits investigated (minimum $F_{3,28} = 2.657$, $P = 0.068$, Levene's test), with the following exceptions: in *P. lividus* pH for the 2–4 d exp., TCO_2/pCO_2/$[HCO_3^-]$ for the 0–24 h exp., in *A. lixula* pCO_2 for the 2–4 d exp., pH/TCO_2/pCO_2/$[HCO_3^-]$ for the 24 h exp., and finally for untreated field-collected individuals TCO_2 and HCO_3^- (maximum $F_{1,56} = 6.218$, $P = 0.016$, Levene's test). However, our experimental designs included between 4 and 12 treatment combinations for the short-term field laboratory and long-term *in situ* experiments with a minimum of seven replicates *per* pCO_2/pH ∗ duration combination. Thus we assume that our test should be tolerant to deviation from the assumption of normality and homogeneity (Sokal and Rohlf, 1995; Underwood, 1997). For the testing of physiological parameters in untreated field-collected individuals, where assumptions of the test used were not met, a Kruskal–Wallis test or a Welch ANOVA test as appropriate were run to validate the results of the *t*-test. All statistical analyses were conducted using SPSS v.19.

3. Results

No mortality was recorded for any of the collections and experiments undertaken, with all sea urchins appearing to be in a good health (actively moving spines and tubular feet, and showing no spine loss) throughout the duration of the experiments.

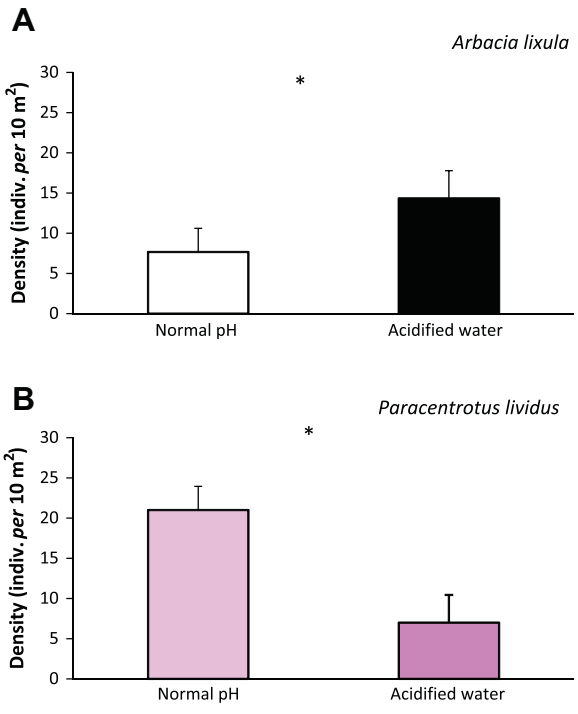

Fig. 2. The densities of (A) the black sea urchin, *Arbacia lixula* and, (B) the purple sea urchin, *Paracentrous lividus* separately for each species under control (white and pink respectively) and acidified conditions (black and purple respectively). ∗ indicate significant differences ($P < 0.05$) in the densities recorded at each pCO_2/pH area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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3.1. Sea urchins' density along the pCO_2/pH gradient

Mean density of *Arbacia lixula* was approx. eight indiv. *per* 10 m^2 in the control area (station A) and 14 indiv. *per* 10 m^2 inside the acidified area (station B) (Fig. 2A). This difference in density between the two sites characterised by different pH levels, was significant (Pseudo-$F_{1,11}$ = 200.00, P = 0.005). In *Paracentrotus lividus*, however, mean density was approx. 21 indiv. *per* 10 m^2 in the control area (station A) and seven indiv. *per* 10 m^2 inside the acidified area (station B) (Fig. 2B). Again, the difference in density between the sites was significant (Pseudo-$F_{1,11}$ = 21.51, P = 0.046) but where *A. lixula* was most abundant in the acidified area, *P. lividus* was more abundant in the control area. For both species, the term 'Area (pH)' had no significant effect (maximum Pseudo-$F_{2,11}$ = 2.12, P = 0.174).

3.2. Acid–base balance and cation status in field-collected, untreated individuals

Baseline parameters for extracellular fluid acid–base status in field-collected untreated individuals of the sea urchin *A. lixula* and *P. lividus* are presented in Fig. 3, and those for extracellular fluid ionic status are presented in Table 2. In summary, *A. lixula* showed lower mean coelomic fluid pH, TCO_2 and bicarbonate

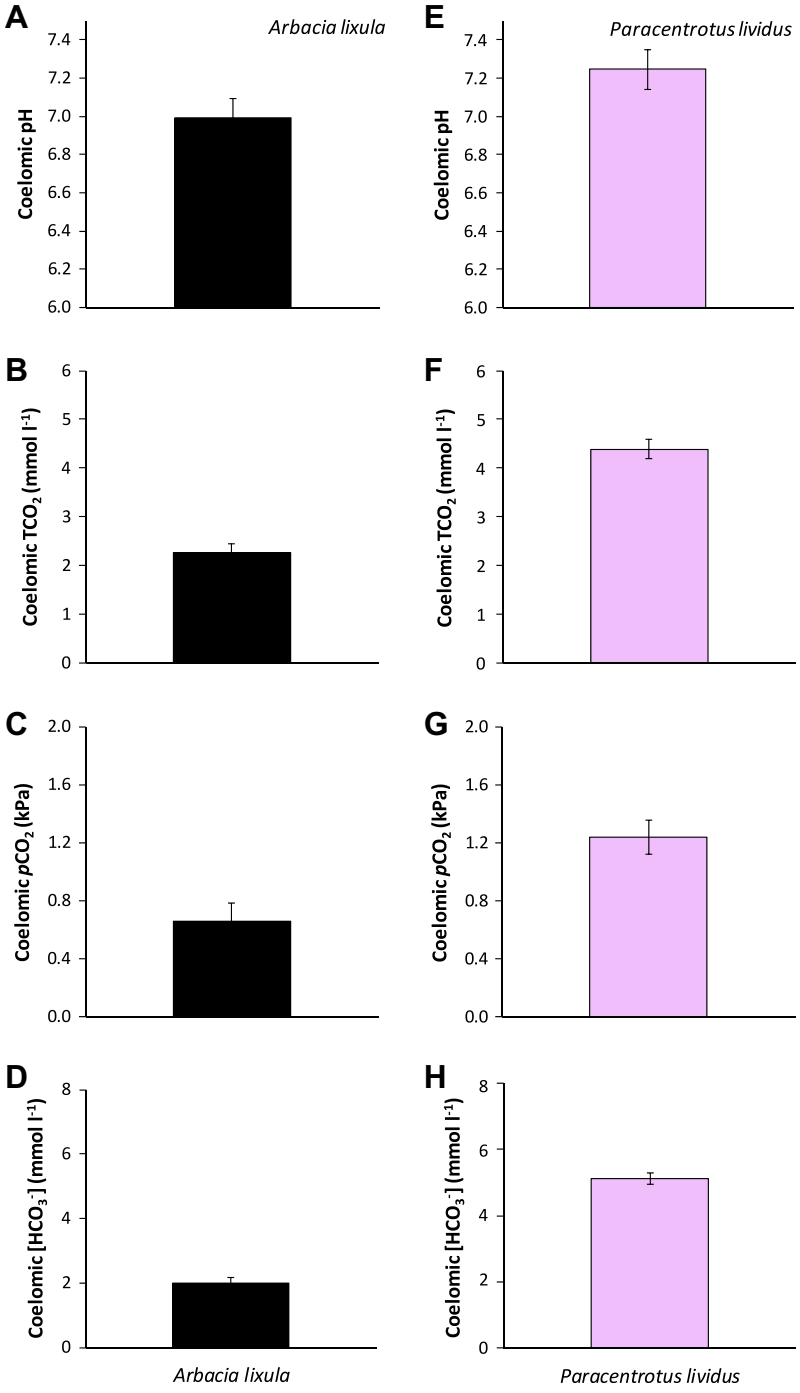

Fig. 3. Baseline parameters for extracellular fluid acid–base status (pH, TCO_2, pCO_2, [HCO_3^-]) in untreated field-collected sea urchins of *A. lixula* (A–D in black) and *P. lividus* (E–H in pink). Histograms are means ± SE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Ionic content of the coelomic fluid and calcified tissues of field-collected untreated individuals of the black sea urchin, *Arbacia lixula* (Linnaeus 1758) and the purple sea urchin, *Paracentrotus lividus* (Lamarck 1816). Values are mean ± SE. Data are expressed as mmol l^{-1} for coelomic fluid and mmol kg^{-1} for the other parameters.

Structure	Inn	*Arbacia lixula*	*Paracentrotus lividus*
Coelomic			
	Ca^{2+}	14.52 ± 0.49	8.74 ± 0.24
	Mg^{2+}	65.22 ± 4.3	53.94 ± 1.30
	Sr^{2+}	0.19 ± 0.01	0.09 ± 0.002
	Na^{+}	558 ± 20	462 ± 11
	K^{+}	14.29 ± 0.69	1319 ± 0.34
Test			
	Ca^{2+}	6435 ± 205	6345 ± 115
	Mg^{2+}	636 ± 18	678 ± 16
	Sr^{2+}	16.55 ± 0.53	17.95 ± 0.38
Lantern			
	Ca^{2+}	6326 ± 118	6475 ± 83
	Mg^{2+}	641 ± 15	725 ± 19
	Sr^{2+}	16.37 ± 0.32	18.14 ± 0.35
Spines			
	Ca^{2+}	6644 ± 608	7550 ± 51
	Mg^{2+}	400 ± 46	279 ± 6.88
	Sr^{2+}	15.17 ± 1.54	16.77 ± 0.25

levels, as well as higher ionic concentrations in the coelomic fluid and largely lower concentrations of ions in the exoskeleton compared to *P. lividus*.

3.3. Coelomic fluid acid–base balance and cation status of individuals exposed in situ to elevated pCO_2 conditions

After 2 and 4 d of exposure to elevated pCO_2 and low pH conditions *in situ* the mean coelomic fluid pH (pH_{cf}) of *A. lixula* ranged between 6.80 and 6.87, and between 7.18 and 7.34 for *P. lividus*. In both species, pH_{cf} measured across all treatments were comparable (see Fig. 4A and E). In fact there was no significant effect of exposure to different pCO_2, exposure duration or their interaction for pH_{cf} of either species (max $F_{1,32} = 1.252$, $P = 0.272$). In addition, mean pH_{cf} values from the *in situ* exposure were broadly comparable to those for field-collected untreated individuals (see Fig. 3). Furthermore, in *A. lixula* after 4 d exposure to elevated pCO_2/low pH, mean coelomic TCO_2 and [HCO_3^-] was 4.11 mmol l^{-1} and 3.42 mmol l^{-1} respectively (Fig. 4B and C), which was significantly greater than values measured at all other treatments (approx. 1.93 mmol $l^{-1}$and 1.61 mmol l^{-1} respectively) (min $F_{1,32} = 17.986$, $P < 0.0001$). Mean coelomic pCO_2 (0.61–1.84 kPa) was significantly greater in individuals kept in elevated pCO_2/low pH conditions after 4 d exposure when compared to individuals kept for 2 d to elevated pCO_2/low pH and 4 d under control pCO_2/pH conditions (Fig. 4C), as indicated by significant interactions between exposure to pCO_2 and exposure duration (min $F_{1,32} = 9.514$, $P = 0.005$). No effect of spheroid volume on any parameters investigated was detected ($P > 0.05$). In *P. lividus*, mean coelomic TCO_2 and [HCO_3^-] ranged between 2.51 and 6.91 mmol l^{-1} and 2.29 and 4.99 mmol l^{-1} respectively (Fig. 4F and H), and increased with exposure duration only in individuals in elevated pCO_2/low pH conditions (Fig. 4F and H), as indicated by the presence of significant interactions between exposure to elevated pCO_2 and exposure duration (minimum $F_{1,32} = 23.152$, $P < 0.0001$). No significant effect of exposure to elevated pCO_2 and exposure duration on coelomic pCO_2 and spheroid volume on any parameter investigated was detected (Fig. 4G, $P > 0.05$).

Ion concentrations for coelomic fluid and calcified tissues are summarised in Table 3. In *A. lixula*, no significant differences in coelomic fluid, test, Aristotle's lantern or spines ion content were detected as a result of exposure to different pCO_2/pH levels, exposure duration or their interaction. In fact there was no significant effect at all (maximum $F_{1,29} = 4.008$, $P = 0.055$). In *P. lividus*, after 2 and 4 d of exposure to elevated pCO_2/low pH conditions, mean [Na^+] in the coelomic fluid increased from approx. 472 to approx. 535 mmol l^{-1}, with exposure having a significant effect on this parameter ($F_{1,29} = 4.458$, $P = 0.043$, see Table 3). In addition, exposure to elevated pCO_2/low pH conditions was accompanied by a significant increase in mean [Ca^{2+}] of the test (from approx. 6094 mmol kg^{-1} to approx. 6545 mmol kg^{-1}) and mean [Mg^{2+}] (from approx. 664 mmol $kg^{-1}$to approx. 695 mmol kg^{-1}) (min $F_{1,29} = 7.888$, $P = 0.009$, see Table 3). Finally, mean [Sr^{2+}] in the spines of *P. lividus* ranged between 16.88 and 20.81 mmol kg^{-1}, with the mean value at 2 d of exposure under control pCO_2/pH conditions being significantly greater than those measured at the other treatments ($F_{2,39} = 34.066$, $P < 0.001$, see Table 3). There was no significant difference in any of the other comparisons for *P. lividus* ($P > 0.05$).

3.4. Coelomic fluid acid–base balance and cation status of individuals exposed in the field-laboratory set-up to elevated pCO_2 conditions

The effects of exposure to elevated pCO_2 and low pH at 0, 1.5, 3, 6, 12, and 24 h are presented in Fig. 5. In summary, *A. lixula* coelomic acid–base parameters largely did not vary between pCO_2 treatments throughout the entire duration of the exposure, with the exception of coelomic TCO_2 and [HCO_3^-] at 3 and 12 h, which increased from an average of 2.04 to 3.36 mmol l^{-1} and from an average of 1.83 to 3.03 mmol l^{-1} respectively (Fig. 5B and C). The differences among these values were significant, as indicated by the presence of interactions between exposure to elevated pCO_2/low pH and exposure duration (min $F_{5,84} = 3.937$, $P = 0.003$). Individuals of *P. lividus* kept under elevated pCO_2/low pH conditions, however, exhibited a significant decrease in pH at 1.5, 12 and 24 h, from a mean of pH 7.23 to 6.94 (Fig. 5E). There was also a significant increase in coelomic TCO_2 and [HCO_3^-] at 3, 6, 12 and 24 h of exposure (Fig. 5F and H), as well as pCO_2 at 1.5, 12 and 24 h (Fig. 5G), as indicated by the presence of significant interactions between exposure to elevated pCO_2/low pH and exposure duration for all these parameters (min $F_{1,84} = 3.665$, $P = 0.005$). Finally spheroid volume had a significant positive effect on *A. lixula* pH_{cf}, ($F_{1,84} = 6.224$, $P = 0.015$).

Values for the coelomic fluid ion concentration are presented in Table 4. Mean [Mg^{2+}] of coelomic fluid of *A. lixula* decreased from 72 to 62 mmol l^{-1} and [Na^+] decreased from 567 to 537 mmol l^{-1} upon exposure to elevated pCO_2/low pH conditions. These differences were significant (Table 4, min $F_{1,64} = 4.17$, $P = 0.045$). Mean [Ca^{2+}], [Sr^{2+}] and [K^+] were not affected by elevated pCO_2/low pH, duration of exposure or their interaction. In *P. lividus*, mean concentrations for all ions measured were significantly greater under elevated pCO_2/low pH conditions (Table 4, minimum $F_{1,65} = 9.266$, $P < 0.0001$). Finally, duration of exposure had a negative effect on coelomic [Mg^{2+}] decreasing from 73 to 60 mmol l^{-1} between 3 and 24 h under elevated pCO_2/low pH (Table 4, minimum $F_{4,65} = 4.638$, $P = 0.004$). No other comparisons were significantly different ($P > 0.05$).

4. Discussion

4.1. Sea urchins density under different pCO_2/pH regimes

Around the area of the shallow-water CO_2 vent of Vulcano Island, the black sea urchin, *A. lixula* and the purple sea urchin, *P. lividus* both show significant differences in their distributional patterns in relation to the spatial difference in seawater pCO_2. Whilst there is a reduction in density of *P. lividus* as one gets closer

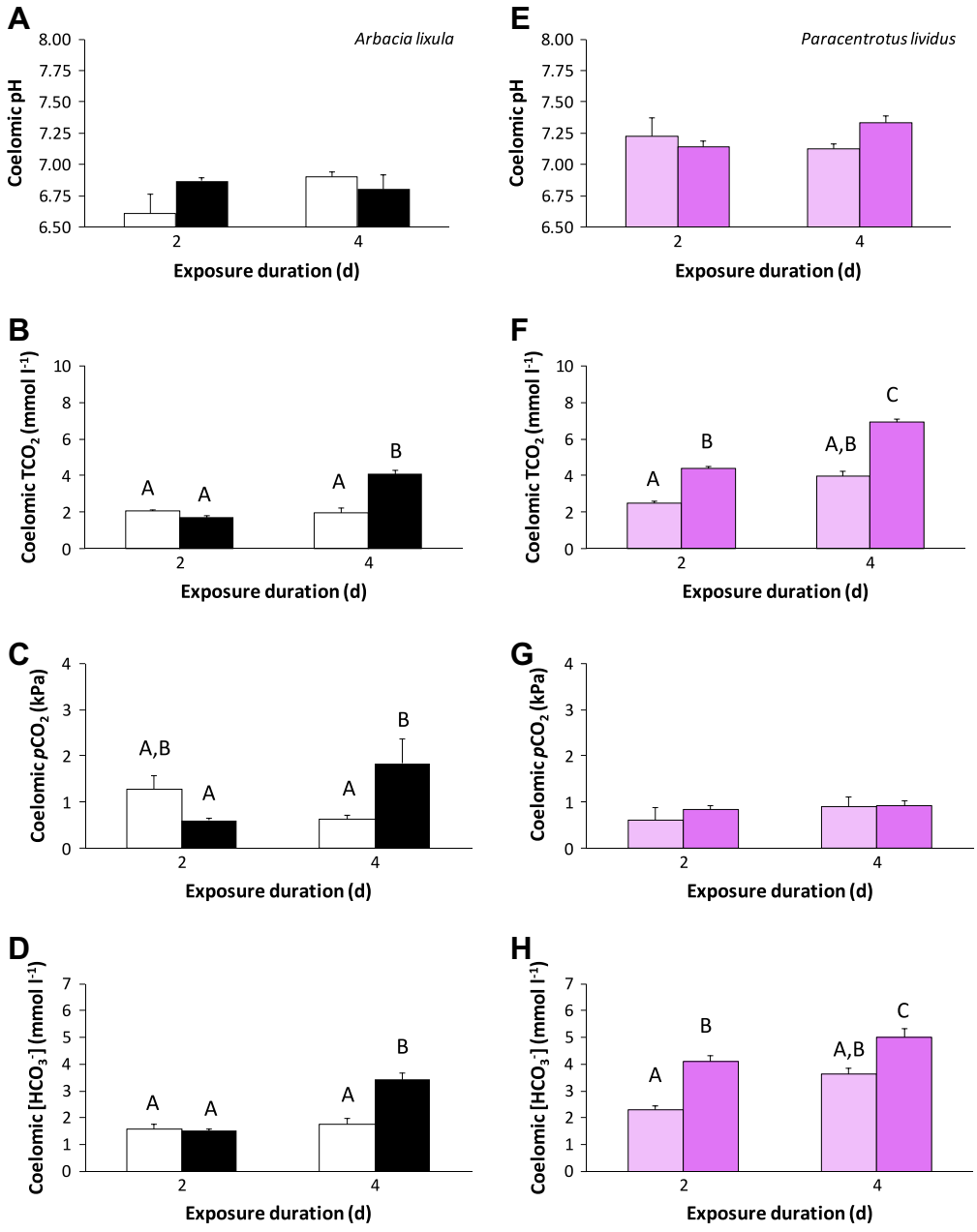

Fig. 4. Extracellular fluid acid–base status (pH, TCO_2, pCO_2, $[HCO_3^-]$) in sea urchins of *A. lixula* (A–D) and *P. lividus* (E–H) sampled at 2 d or 4 d of exposure to *in situ* control (white and pink respectively) and acidified conditions (black and purple respectively). Values are mean ± SE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to the CO_2 vents (−67%), a pattern already documented for other vents (Hall-Spencer et al., 2008; see also Johnson et al., 2012; Suggett et al., 2012 for the Vulcano CO_2 vents although it is difficult to directly compare our results to these studies as they included unsuitable habitats for sea urchins in their investigation, see Johnson, 2012), *A. lixula* occurs at greatest densities in areas characterised by higher pCO_2/lower pH (+87%). Increasing abundance with increasing pCO_2/decreasing pH has been documented previously for species of 'tolerant' phyla, such as crustaceans, polychaetes, and nematodes (e.g. Cigliano et al., 2010 for field observations, e.g. Hale et al., 2011; Christen et al., 2012 for laboratory mesocosm experiments). To our knowledge, however, this is the first time it has been recorded for echinoids, which contains species that were previously reported to be more sensitive. Increases in density of a given taxa/group across a pH gradient have been thought to be generated directly by their ability to respond physiologically to elevated CO_2 conditions, and/or indirectly colonisation of new 'ecological space' due to the loss of less tolerant taxa or changes to species interactions (Hale et al., 2011; Christen et al., 2012; Johnson et al., 2012; Kroeker et al., 2012), indirect effects likely caused by species different level of physiological vulnerability to elevated CO_2 and low pH. Physiological impairment due to elevated pCO_2/low pH is attributed to alteration of cellular homeostasis (e.g. Reipschläger et al., 1997; Pörtner et al., 1998) and energy metabolism (Pörtner et al., 1998; Beniash et al., 2010; Lannig et al., 2010; Melatunan et al., 2011; Dickinson et al., 2012) leading to altered energy budgets (see Wood et al., 2008; Findlay et al., 2010; Stumpp et al., 2011; Melatunan et al., 2012), ultimately determining taxon distribution in response to elevated pCO_2 conditions (see Bozinovic et al., 2011). We now explore the relationship between changes in sea urchin distribution along the pCO_2/pH gradient and the different physiological responses of these two species. However,

Table 3
Ionic content of the coelomic fluid and calcified tissues of the sea urchins, (A) *A. lixula* and, (B) *P. lividus* exposed *insitu* to different pCO_2/pH conditions. Values are mean ± SE. Data are expressed as mmol l^{-1} for coelomic fluid and mmol kg^{-1} for the other parameters. Different letters show significant differences ($P < 0.05$) among means of a same trait measured under different treatments.

Duration of exposure (d)	Tissue							
	Coelomic fluid		Test		Lantern		Spine	
	2	4	2	4	2	4	2	4
A								
Control								
Ca^{2+}	15.46 ± 0.69	14.92 ± 0.28	5757 ± 508	3701 ± 242	6861 ± 140	7750 ± 1134	7278 ± 167	7069 ± 93
Mg^{2+}	63.88 ± 4.94	68.45 ± 4.26	573.60 ± 51.90	549.00 ± 19.20	705.80 ± 21.2	774.00 ± 113.00	453.7 ± 11.10	452.96 ± 9.16
Sr^{3+}	0.14 ± 0.009	0.12 ± 0.009	14,95 ± 1.33	14.52 ± 0.69	16.52 ± 1.42	20.13 ± 2.91	16.83 ± 0.37	16.56 ± 0.23
Na^{+}	549.62 ± 8.25	565.7 ± 11.9	–	–	–	–	–	–
K^{+}	14.51 ± 0.55	13.81 ± 0.64	–	–	–	–	–	–
Acidified								
Ca^{2+}	11.98 ± 2.69	13.97 ± 2.70	6221 ± 220	6361 ± 107	6904 ± 102	6908 ± 223	7247 ± 98	7326 ± 67
Mg^{2+}	54.12 ± 5.53	61.16 ± 8.42	619.80 ± 24.90	643.80 ± 17.00	686.1 ± 10.3	699.20 ± 24,20	470.75 ± 7.26	470.96 ± 9.35
Sr^{2+}	0.10 ± 0.016	0.11 ± 0.02	15.80 ± 0.54	16.00 ± 0.65	17.93 ± 0.28	27.76 ± 0.59	26.97 ± 0.23	17.15 ± 0.20
Na^{+}	467.70 ± 47.30	502.10 ± 56.80	–	–	–	–	–	–
K^{+}	12.02 ± 1.25	16.63 ± 2.16						
B								
Control								
	9.48 ± 0.22	9.33 ± 0.20	6148 ± 131^{A}	6039 ± 172^{A}	6477 ± 190	6364 ± 196	9090 ± 1398	7404 ± 371
Mg^{2+}	55.72 ± 1.34	54.46 ± 2.42	677.70 ± 15.60^{A}	652.228.5^{A}	709.50 ± 24.80	727.30 ± 34.60	336.80 ± 57.80	272.75 ± 7.03
Sr^{2+}	0.08 ± 0.013	0.09 ± 0.001	17.12 ± 0.57	16.98 ± 0.46	17.13 ± 0.36	18.22 ± 0.79	20.81 ± 3.29^{A}	16.88 ± 0.20^{B}
Na^{+}	471.33 ± 6.72^{A}	472.67 ± 9.16^{A}	–	–	–	–	–	–
K^{+}	11.63 ± 0.33	12.55 ± 0.15	–	–	–	–	–	–
Acidified								
Ca^{2+}	15.09 ± 3.10	10.23 ± 0.56	6571 ± 103^{B}	6518 ± 234^{B}	6425 ± 263	6514 ± 268	74570 ± 93	7462 ± 107
Mg^{2+}	61.78 ± 6.04	59.94 ± 3.04	707.70 ± 10.80^{B}	682.30 ± 23.70^{B}	713.00 ± 25.90	747.90 ± 10.38	289.01 ± 8.59	287.60 ± 8.78
Sr^{2+}	0.11 ± 0.019	0.10 ± 0.006	18.23 ± 0.34	18.03 ± 0.68	17.73 ± 0.68	18.55 ± 0.814	17.28 ± 0.27^{B}	17.20 ± 0.42^{B}
Na^{+}	541.90 ± 52.40^{B}	527.90 ± 28.20^{B}	–	–	–	–	–	–
K^{+}	13.65 ± 1.49	13.76 ± 0.83						

first we must consider some ecological factors that could determine sea urchins' distribution.

4.2. Rejection of feeding biology and predators as determinants of sea urchin distribution around CO_2 vents

Differences in the distribution of the two sea urchins investigated here may be related to different feeding preferences. Gut contents analyses suggest that *A. lixula* preferentially feeds on calcitic algae (Privitera et al., 2008), although a recent study using stable isotopes has shown that its feeding niche may be broader including sessile animals (Wangensteen et al., 2011). On the contrary, *P. lividus* more strictly favours erect fleshy algae (Privitera et al., 2008; Bonaviri et al., 2011; Wangensteen et al., 2011). Along the pCO_2/pH gradient created by the CO_2 vent of Vulcano, Johnson et al. (2012) reported that calcified algae are most abundant in the control pCO_2/pH areas, whilst fleshy macroalgae (in particular brown macroalgae) become progressively more abundant in the acidified areas. This pattern is similar to that reported from other CO_2 vents (e.g. Porzio et al., 2011; Fabricius et al., 2011). Based on their different feeding ecology, we might have predicted *A. lixula* to be most abundant in the control pCO_2 areas where calcified algae are most abundant, and *P. lividus* to be most abundant in the more acidified areas where macroalgae are dominant and productivity enhanced (see Johnson et al., 2012; Russell et al., in press). However, the opposite is true. Although we cannot completely exclude that *A. lixula* could also be able to exploit non-calcifying invertebrates in acidified waters, as could do in control areas, current evidence to date support the idea that feeding biology can be rejected as a major determinant of sea urchins' distribution in the area around the CO_2 vent of Vulcano.

We may also reject the idea that differences in the activity levels of natural predators shaped sea urchins' local distribution. There are no changes in distribution or behaviour of natural predators in the areas around the vent compared with the vent area (Milazzo and Azzurro, *pers. obs.*), and human harvesting of *P. lividus* does not occur here. Furthermore, it is important to consider that the Vulcano CO_2 vent system does not allow for replication (being one pCO_2/pH gradient only) and that despite in this work we measured sea urchins density in comparable habitats, suitable for sea urchins settling across the CO_2 gradient, the habitat structure in Vulcano is relatively patchy. Despite these limitations, and although we advise this type of investigations are conducted in multiple sites to verify their consistency, the distribution of these sea urchin species around this natural CO_2 vent appear to be influenced (and possibly determined), as with other species, by their respective physiologies (e.g. Stillman, 2002; Calosi et al., 2007, 2008, 2010; Bozinovic et al., 2011; Lai et al., 2011; Rastrick and Whiteley, 2011; Whiteley et al., 2011).

4.3. Acid–base and ionic status of field-collected untreated sea urchins

Field-collected individuals of *A. lixula* and *P. lividus* have different physiologies. The coelomic fluid of field-collected *A. lixula* is more acidic (mean = 6.99) than *P. lividus*, being approx. 0.3 units lower. Indeed it is the lowest echinoid extracellular pH recorded under control seawater pCO_2/pH conditions (cf. Spicer et al., 1988, 2011; Spicer, 1995; Burnett et al., 2002; Catarino et al., 2012; Stumpp et al., 2012), with the exception of the sand dollar, *Echinarachnius parma* (Cole, 1940). The pH of sea urchin coelomic fluid is usually well below that of sea water, due to the accumulation of CO_2 and organic acid metabolites, as aerobic and anaerobic pathways operate even in normoxic echinoids (Farmanfarmaian, 1966; Ellington, 1982; Shick, 1983; Bookbinder and Shick, 1986). In addition, the CO_2 capacity of the coelomic fluid of *P. lividus* is amongst the highest ever recorded in a sea urchin (see Spicer et al. 1988, 1995, 2011; Miles et al., 2007; Stumpp et al., 2011), and comparable to that of a population of the green sea urchin,

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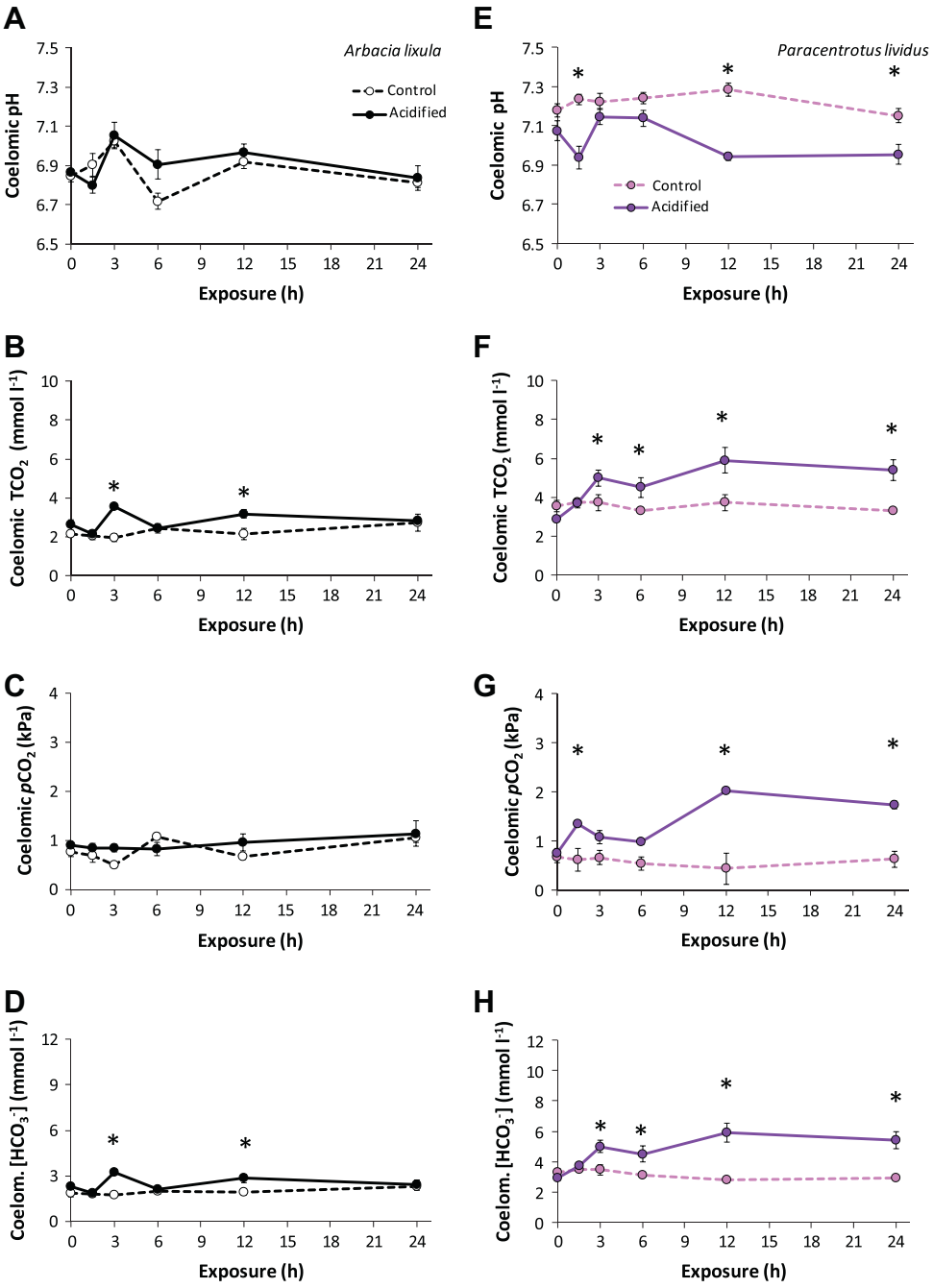

Fig. 5. Coelomic fluid acid–base status (pH, TCO_2, pCO_2, $[HCO_3^-]$) in sea urchins of *A. lixula* (A–D) and *P. lividus* (E–H) exposed for 24 h to control (white and pink respectively, dotted line) and acidified conditions (black and purple respectively, full line) under field-laboratory conditions. Repeated coelomic fluid sampling of the same individuals was undertaken at 0, 3, 6, 12, 24 h from the starting of the exposure. Values are mean ± SE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Strongylocentrotus droebachiensis from Maine (USA) (6.00 mmol l^{-1} – Cole, 1940). Also the CO_2 capacity of the coelomic fluid of *P. lividus* is about double that of *A. lixula*, the latter being more comparable to that recorded in the purple-tipped sea urchin, *Psammechinus miliaris* by Spicer et al. (1988). Mean coelomic $[HCO_3^-]$ and pCO_2 of *P. lividus* are, to date, amongst the highest measured in a sea urchin under control seawater pCO_2/pH conditions (Spicer, 1995, 2011; Miles et al., 2007; Stumpp et al., 2011), whilst mean coelomic $[HCO_3^-]$ and pCO_2 in *A. lixula* were less than half of those measured in *P. lividus*. Nonetheless mean pCO_2 values in *P. lividus* were at the higher end of those reported to date for other echinoids. Our data being the first on the acid-base regulation of *P. lividus* and *A. lixula,* differences in coelomic pCO_2 may be explained by differences in the experimental regime we used when compared to those of other studies (i.e. semi-natural conditions *vs.* laboratory maintenance and experiments), as well as by the natural variability among different species of echinoids. This may mean that *P. lividus* may incur greater regulatory costs. *Arbacia lixula* may instead have evolved low coelomic fluid pH, thus possibly keeping regulatory cost lower in area with fluctuating pH and CO_2.

A. lixula displays a greater capacity to control tissue water than other sea urchins for which we have data, namely the rock boring sea urchin, *Echinometra lucunter* and the variegated sea urchin,

Table 4
Ionic content of the coelomic fluid of sea urchins, (A) *A. lixula* and, (B) *P. lividus* exposed to different pCO_2/pH conditions in the laboratory. Values are mean ± SE. Data are expressed as mmol l^{-1} for coelomic fluid and mmol kg^{-1} for the other parameters. (†) Mean values for *P. lividus* of the group of individuals used for the acidified treatment could not be taken at time 0 under, therefore here we give mean values for those individuals of *P. lividus* subsequently kept at control conditions.

Duration *of* exposure (d)	0	3	6	12	24
A					
Control					
Ca^{2+}	14.52 ± 0.49	13.84 ± 1.32	15.09 ± 0.68	14.58 ± 1.28	15.86 ± 0.63
Mg^{2+}	65.22 ± 4.3	69.36 ± 7.01	82.19 ± 4.75	76.42 ± 6.58	70.33 ± 4.67
Sr^{2+}	0.19 ± 0.01	0.11 ± 0.01	0.13 ± 0.006	0.12 ± 0.01	0.12 ± 0.007
Na^{+}	558.3 ± 19.7	525.1 ± 49.1	598 ± 19	567.3 ± 45.3	586.83 ± 9.45
K^{+}	14.29 ± 0.69	12.89 ± 1.03	14.35 ± 0.41	14.2 ± 1.13	14.29 ± 1.01
Acidified					
Ca^{2+}	16.83 ± 2.44	11.21 ± 0.73	15.3 ± 2.22	10.47 ± 1.39	15.98 ± 2.57
Mg^{2+}	61.15 ± 0.76	61.91 ± 2.92	61.12 ± 0.72	61.08 ± 1.91	63.09 ± 2.2
Sr^{3+}	0.13 ± 0.01	0.1 ± 0.006	0.12 ± 0.01	0.09 ± 0.006	0.13 ± 0.012
Na^{+}	533.12 ± 6.01	537 ± 26.7	534.7 ± 11.6	533.7 ± 1.69	547.1 ± 16.5
K^{+}	13.69 ± 0.22	13.57 ± 0.86	12.84 ± 0.21	12.91 ± 0.50	14.31 ± 0.44
B					
Control					
Ca^{2+}	8.74 ± 0.24	9.51 ± 0.25	9.47 ± 0.135	10.14 ± 0.77	8.75 ± 0.24
Mg^{2+}	53.94 ± 1.30	57.01 ± 0.88	57.88 ± 0.231	59.12 ± 3.78	53.26 ± 1.06
Sr^{2+}	0.09 ± 0.002	0.10 ± 0.01	0.09 ± 0.001	0.10 ± 0.008	0.09 ± 0.002
Na^{+}	461.7 ± 11	485.08 ± 7.56	503.84 ± 2.97	511.2 ± 32.8	463.08 ± 9.48
K^{+}	13.19 ± 0.34	12.08 ± 0.27	13.06 ± 0.22	12.83 ± 0.83	11.97 ± 0.3
Acidified					
Ca^{2+}	8.74 ± 0.24†	22.25 ± 2.53	30.98 ± 8.62	24.35 ± 8.35	14.43 ± 0.35
Mg^{2+}	53.94 ± 1.30†	72.88 ± 0.95	70.53 ± 1.63	65.89 ± 0.76	65.17 ± 0.47
Sr^{2+}	0.09 ± 0.002†	016 ± 0.01	0.21 ± 0.04	0.16 ± 0.04	0.11 ± 0.002
Na^{+}	461.7 ± 11†	573.53 ± 9.48	575.3 ± 13.3	541.15 ± 4.51	546.48 ± 3.84
K^{+}	13.19 ± 0.34†	15.73 ± 0.47	15.50 ± 0.55	14.45 ± 0.15	15.33 ± 0.53

Lytechinus variegatus (Freire pers. obs.) and displayed a greater coelomic fluid Ca^{2+}, Mg^{2+}, Sr^{2+} and Na^{+} content when compared *to P. lividus*, however, details as to why this occurs remains unclear. Strong iono-regulatory capacity is generally coupled with capacity for acid–base regulation (Seibel and Walsh, 2003; Pörtner et al., 2004; Widdicombe and Spicer, 2008; Melzner et al., 2009; Whiteley, 2011). Thus *A. lixula*'s ability to maintain ionic gradients for major cations may indicate better developed acid–base regulatory ability compared to *P. lividus*. These differences in baseline acid–base and ionic status support the idea that the sea urchins investigated here may be differently equipped to respond to elevated environmental pCO_2 that occur with CO_2 leakages. However, the capacity for regulation of acid–base and ionic status, rather than the status *per se*, is more important when understanding differences taxa homeostatic ability.

4.4. In-situ mid-term acid–base and ionic regulation following acclimatisation to elevated pCO_2/low pH

After 2 and 4 d exposure *in situ* to elevated pCO_2/low pH, both sea urchin species fully compensated their coelomic fluid pH. However, in *P. lividus* buffering is achieved, *via* an increase in extracellular $[HCO_3^-]$ (see Fig. 6B), with no significant changes observed in coelomic pCO_2. This suggests a metabolic component to the compensation. In *A. lixula*, complete compensation at day 2 cannot be attributed to changes in $[HCO_3^-]$ or metabolic alkalosis. Full non-bicarbonate compensation of extracellular-fluid pH was recently described in the velvet fiddler crab, *Necora puber* (Small et al., 2010) and the burrowing shrimp, *Upogebia deltaura* (Donohue et al., 2012) under comparable pCO_2/pH conditions after 30 d of exposure. In these crustaceans, non-bicarbonate full compensation of haemolymph pH was suggested to be linked to an increase in protein content and represent a longer-term compensatory mechanism, as crustaceans are known at least in the short-term to rely on the HCO_3^- buffering (Truchot et al., 1976; Cameron and Iwama, 1987; Whiteley, 1999, 2011; Spicer et al., 2007). In *A. lixula*, the situation is reversed as non-bicarbonate buffering is observed at day 4 when the sea urchin switches to HCO_3^- compensation, despite $[HCO_3^-]$ in this species being lower than that of *P. lividus*. Considering the low protein and lipid content of sea urchin coelomic fluid (see Booloottian, 1966; Binyon, 1972), it is unlikely that increases in these parameters explain the initial buffering capacity of *A. lixula*, although at this stage there is no plausible alternative. Furthermore, at day 4 *A. lixula* also experienced an increase in coelomic fluid pCO_2 whilst maintaining its coelomic fluid pH constant, which is caused by a significant increase in $[HCO_3^-]$ but also partially by a possible metabolic compensation (Fig. 6A). In addition, in *A. lixula* there were no significant changes in ions in coelomic fluid or carbonated tissues. Since coelomic $[Ca^{2+}]$, $[Mg^{2+}]$ and $[Sr^{2+}]$ does not increase, and test concentrations for these ions did not change significantly, it is likely that sea urchins do not incur dissolution of their carbonate structures (test and lantern in particular). Thus the HCO_3^- increase observed (approx. 1.7 mmol l^{-1}) at day 4 may be due to the uptake of this ion from sea water as proposed for other species (e.g. Cameron, 1985; Small et al., 2010; Donohue et al., 2012). An alternative explanation could be that $[HCO_3^-]$ increases as the consequence of an increase in activity of the enzyme carbonic anhydrase (CA), but no direct measure of CA activity is available for this study. In *P. lividus* the increase in HCO_3^- is approx. 1.8 and 1.4 mmol l^{-1} at 2 and 4 d respectively. The significant increase in test's $[Ca^{2+}]$ and $[Mg^{2+}]$ suggests that not only does this species not experience test dissolution (confirming Catarino et al., 2012 results) but possibly net calcification may increase. This is the first time that both the carbonated compartments of a sea urchin that can contribute to internal HCO_3^- buffering *via* dissolution (test and Aristotle's lantern) and its coelomic fluids are sampled in order to establish the origin of the HCO_3^- used for buffering. Furthermore, increased calcification in organisms exposed to elevated pCO_2/low pH has already been documented in various taxa of marine organisms (see Ries et al., 2009; Findlay et al., 2011), including the sea urchin, *Arbacia punctulata* (Ries et al., 2009) which was exposed at pCO_2/pH/Ω_{ara} values comparable to that

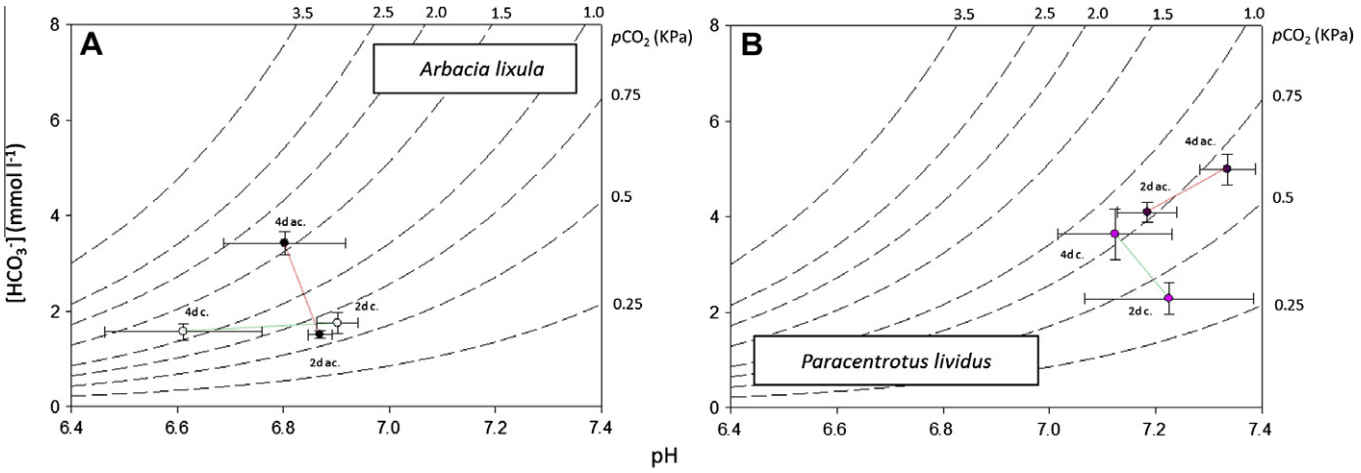

Fig. 6. Davenport diagram showing the effects of elevated pCO_2/pH on the acid base status of coelomic fluid from the sea urchins *A. lixula* and *P. lividus* measured *in situ* at 2 d and 4 d from the beginning of exposure to control (white and pink respectively) and acidified conditions (black and purple respectively). Points are means ± SE for each time point. Full isopleths show calculated pCO_2 values for each [HCO_3^-] and pH combination. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

measured in the acidified site during our experiments and those from the long-term monitoring by Boatta et al. (in press). If chronic hyper-calcification does occur in *P. lividus*, the high energetic cost of this process may not be a sustainable strategy, altering trade-offs between maintenance and growth/reproduction (see Wood et al., 2008; Stumpp et al., 2012; but cf. Findlay et al., 2010). The small changes in HCO_3^- buffering observed here indicate that other compensatory mechanisms may be operating, e.g. the electroneutral Na^+/H^+ exchangers. The observed increase in coelomic [Na^+] is normally established by the Na^+/K^+ ATPase pumps, but here can be considered as another evidence of increased sea water uptake in sea urchins kept under elevated pCO_2/low pH conditions, this indicating that also *P. lividus* can concentrate its coelomic fluid. Our results corroborate Freire et al. (2011)'s conclusion that echinoderms may be able to up-regulate Na^+ uptake.

4.5. Acid–base and ionic-regulatory acclimation in sea urchins exposed to elevated pCO_2/low pH conditions

The difference in the acid–base balance abilities in *A. lixula* and *P. lividus* after 2 and 4 d *in situ* were more pronounced in the laboratory experiments. These experiment are particularly useful (due to the fine time resolution) for helping us understand the different physiological pathways species follow to acclimatise to elevated pCO_2 before reaching at 2 and 4 d full pH_{cf} compensation.

A. lixula again displays the capacity to compensate extracellular pH at each time point of observation during the 24 h of exposure to elevated pCO_2/low pH conditions. Respiratory acidosis appears to occur in this species at 1.5 and 6 h (see Fig. 7A) of exposure to elevated pCO_2 but is immediately compensated by a metabolic alkalosis at 3 and 12 h, as indicated in the Davenport diagram (at these time points there is an approx. 1.7 mmol l^{-1} increase in coelomic [HCO_3^-] with no significant increase in coelomic pCO_2 (see Fig. 7A). Then there is no change between 24 h and 2–4 d of exposure to elevated pCO_2 in this species, apart from a small increase in [HCO_3^-] between 2 and 4 d. However, caution is necessary when comparing data from the *in situ* and field laboratory experiment together due to the different experimental approaches employed. In addition, coelomic fluid [Mg^{2+}] and [Na^+] were significantly lower under elevated pCO_2/low pH conditions, which may indicate either a partial loss of the ionic regulatory ability or an attempt to lower the concentration of this cation in the coelomic fluid in hypercap-

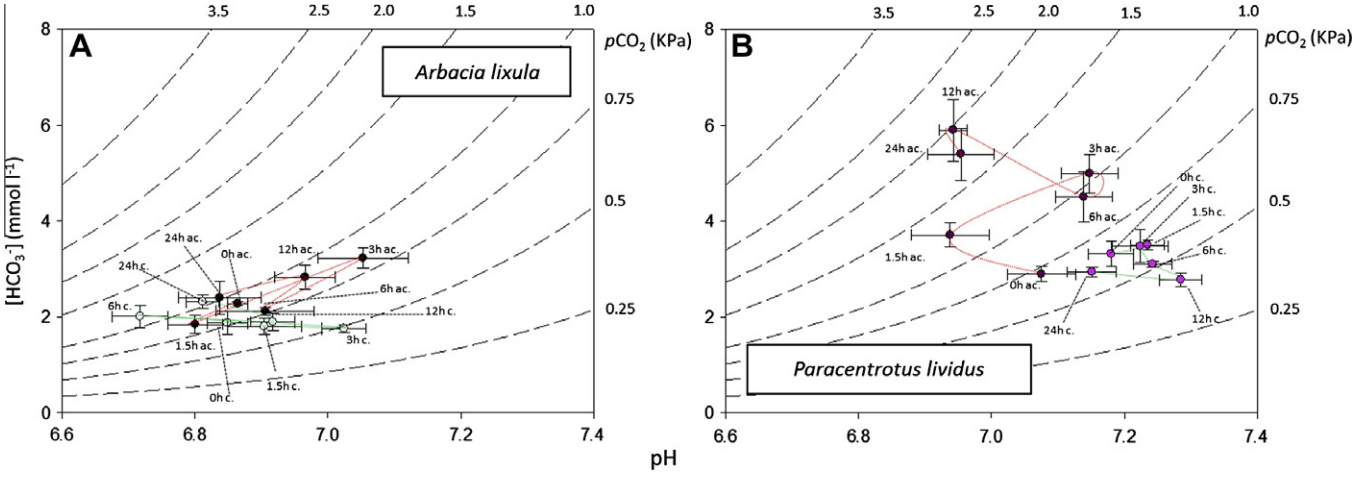

Fig. 7. Davenport diagrams showing the effects of elevated pCO_2/pH on the acid base status of the coelomic fluid from sea urchins *A. lixula* (A) and *P. lividus* (B) measured at the field laboratory at 0, 1.5, 3, 6, 12, 24 h from the beginning of exposure control (white and pink respectively) and acidified conditions (black and purple respectively). Points are means ± SE for each time point. Full isopleths show calculated pCO_2 values for each [HCO_3^-] and pH combination. Note the difference in scale of the *Y*-axis for *A. lixula* and *P. lividus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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nic sea urchins. In any case, *A. lixula* does not appear to undergo any dissolution of the carbonated tissues during the first 24 h of exposure to elevated pCO_2.

On the other hand *P. lividus* displays a severe respiratory acidosis at 1.5 h which is compensated, *via* a possible respiratory alkalosis at 3–6 h (Fig. 7B). This is followed by a second respiratory acidosis at 12–24 h which is further compensated by another respiratory alkalosis occurring between 24 h and 2 d (Figs. 7B and 6B). The levels of bicarbonate observed in the coelomic fluid of *P. lividus* under these conditions are among the highest so far recorded for any species of echinoid, although we should remember that they are transient. In fact, at 2 and 4 d of exposure *in situ*, extracellular [HCO_3^-] returns to values comparable to those recorded in field-collected individuals. We suggest that between 24 h and 2–4 d, *P. lividus* potentially undergoes a switch in its buffering mode, as it stops relying on bicarbonate buffering with [HCO_3^-] (probably too energetically expensive to sustain long-term), reducing from approx. 5.99 mmol l^{-1} (at 12 h, see Fig. 7B) to approx. 4.54 mmol l^{-1} (at 2 and 4 d, see Fig. 6B). This further corroborates the idea that also sea urchins may switch between buffering modes, as seen in crustaceans (Spicer et al., 2007; Small et al., 2010; Donohue et al., 2012).

5. Conclusions

The decrease in density in the high CO_2 areas in *P. lividus* could be interpreted as the long-term consequence of its relatively poorer ability to regulate extracellular acid–base balance. Despite a greater capacity for bicarbonate buffering, *P. lividus* is more at risk from the negative effects of elevated pCO_2 due to the possible long-term costs of maintaining high [HCO_3^-]. This may have repercussions for the functioning of the marine ecosystems to which this species belongs, as well as for its fisheries and aquaculture industries. On the other hand, *A. lixula* possesses low pH_{cf} and (in the short term) the ability for full pH compensation, *via* an unidentified non-bicarbonate mechanism: possibly linked to lower metabolic rate function. Its ability to regulate extracellular ions may also bestow on *A. lixula* a greater resilience to high CO_2. We should highlight that in our experiments Ω_{calc} and Ω_{ara} were not undersaturated, and so our results can be considered to be solely related to the level of environmental pCO_2 and pH reported. Furthermore, our findings lend further support to the idea that echinoids do possess a certain degree for extracellular acid–base and ionic regulatory ability, but also that a considerable degree of variation exists in these traits (Spicer et al., 1988; Spicer, 1995; Miles et al., 2007; Vidolin et al., 2007; Freire et al., 2011; Stumpp et al., 2012). Thus differences in the ecophysiology of individual species (as with the sea urchins in this study) will likely play an important role in defining the ability of assemblages to cope with elevated pCO_2/low pH. It is likely that such differences will be due to changes in energy budgets, with follow-on constrains on species future abundance and distribution.

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