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RESEARCH ARTICLE

Energy metabolism and regeneration are impaired by seawater acidification in the infaunal brittlestar *Amphiura filiformis*

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ABSTRACT

Seawater acidification due to anthropogenic release of CO_2 as well as the potential leakage of pure CO_2 from sub-seabed carbon capture storage (CCS) sites may impose a serious threat to marine organisms. Although infaunal organisms can be expected to be particularly impacted by decreases in seawater pH, as a result of naturally acidified conditions in benthic habitats, information regarding physiological and behavioral responses is still scarce. Determination of P_{O_2} and P_{CO_2} gradients within burrows of the brittlestar *Amphiura filiformis* during environmental hypercapnia demonstrated that besides hypoxic conditions, increases of environmental P_{CO_2} are additive to the already high P_{CO_2} (up to 0.08 kPa) within the burrows. In response to up to 4 weeks exposure to pH 7.3 (0.3 kPa P_{CO_2}) and pH 7.0 (0.6 kPa P_{CO_2}), metabolic rates of *A. filiformis* were significantly reduced in pH 7.0 treatments, accompanied by increased ammonium excretion rates. Gene expression analyses demonstrated significant reductions of acid–base (*NBCe* and *AQP9*) and metabolic (*G6PDH, LDH*) genes. Determination of extracellular acid–base status indicated an uncompensated acidosis in CO_2-treated animals, which could explain the depressed metabolic rates. Metabolic depression is associated with a retraction of filter feeding arms into sediment burrows. Regeneration of lost arm tissues following traumatic amputation is associated with significant increases in metabolic rate, and hypercapnic conditions (pH 7.0, 0.6 kPa) dramatically reduce the metabolic scope for regeneration, reflected in an 80% reduction in regeneration rate. Thus, the present work demonstrates that elevated seawater P_{CO_2} significantly affects the environment and the physiology of infaunal organisms like *A. filiformis*.

KEY WORDS: Acid–base regulation, Carbon capture storage, Behavior, Hypercapnia, Ocean acidification, Invertebrates

INTRODUCTION

The effects of elevated seawater P_{CO_2} (hypercapnia) on marine organisms have moved into the research focus because of rising atmospheric CO_2 concentrations that have led to a drop in ocean average surface pH by 0.1 units since industrialization and are expected to cause a further decline by 0.3 to 0.5 pH units till the end of the century, a phenomenon known as ocean acidification (Caldeira and Wickett, 2003; Dupont and Pörtner, 2013). In this context, carbon capture storage (CCS) has been discussed as a potent technique to remove CO_2 from the atmosphere to be stored in sub-seabed sediments (Haugen and Eide, 1996). For example, the Skagerrak and Kattegat region is debated as a suitable area for CCS (Haugen et al., 2011). However, the potential risks of seepage of pure CO_2 may represent an enormous local challenge to benthic and infaunal organisms as a result of strong local pH fluctuations (IPCC, 2005).

Water-breathing animals exchange CO_2 across epithelia by maintaining a diffusion gradient with ~0.2–0.4 kPa higher P_{CO_2} values in tissues compared with the surrounding water (Evans et al., 2005; Melzner et al., 2009). In order to maintain this diffusion gradient, the increase of seawater P_{CO_2} will result in an increase of P_{CO_2} in body tissues and fluids. Such hypercapnic conditions can cause an extracellular acidosis if not actively compensated by H^+ secretion or/and HCO_3^- accumulation in body fluids (Heisler, 1989). Earlier studies using *Sipunculus nudus* as a marine model organism demonstrated that an uncompensated extracellular acidosis can trigger metabolic depression (Reipschläger and Pörtner, 1996; Reipschläger et al., 1997; Pörtner et al., 1998). CO_2-induced acid–base disturbances have been demonstrated to alter the physiology and developmental features of marine invertebrates (Thomsen and Melzner, 2010; Hu et al., 2011; Stumpp et al., 2011b; Stumpp et al., 2012). For example, echinoderms, crustaceans and mollusks have been shown to alter growth/developmental rates, oxygen consumption and gene expression in response to hypercapnia (Kurihara et al., 2007; Dupont et al., 2010; Lannig et al., 2010; Walther et al., 2010; Hu et al., 2011; Stumpp et al., 2011a; Stumpp et al., 2011b; Stumpp et al., 2012; Dupont and Thorndyke, 2014). Because of the very low P_{O_2} partial pressures (Vopel et al., 2003) in burrows that are very likely accompanied by high P_{CO_2} and low pH, burrowing species are already experiencing higher acidification compared with other benthic species. Additionally, benthic habitats are often confronted with strong fluctuations in P_{O_2} and P_{CO_2}, leading to naturally acidified conditions, which will be amplified by ocean acidification (Melzner et al., 2012). It can be expected that increases in seawater P_{CO_2} will strongly affect CO_2 and pH gradients within sediment burrows, leading to strong acid–base challenges to infaunal organisms.

The infaunal brittlestar *Amphiura filiformis* (O. F. Müller 1776) is an important species in many polar and temperate marine benthic habitats, with densities of up to 3500 individuals per square meter (Rosenberg et al., 1997). *Amphiura filiformis* lives in semi-permanent sediment burrows and feeds on particulate organic matter (POM) by extending two to three arms into the water column (Loo et al., 1996). This species is an important prey for many predators like crustaceans and fish, leading to sublethal injury (e.g. loss of exposed arms) (Duineveld and Van Noort, 1986). As arms are essential organs for suspension feeding (Woodley, 1975), respiration (Ockelmann, 1978) and ventilation of the burrow (Nilsson, 1999), long-term selection pressure on *A. filiformis* has led to the ability to autotomize their arms in case of an attack by a predator, and to a high potential for regeneration of these lost tissues (Dupont and Thorndyke, 2006). The process itself and the physiological

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List of abbreviations

ADM	ash dry mass
AFDM	ash-free dry mass
CCS	carbon capture storage
DM	dry mass
FM	fresh mass
pH_e	external pH
POM	particulate organic matter
RMR	routine metabolic rate

properties of regeneration were investigated in earlier studies, suggesting that energetic costs for the regeneration of arms are significant (Fielman et al., 1991; Pomory and Lawrence, 1999). Moreover, depending on the position of autotomy, the available energy can be used for either growth or differentiation of the regenerating arm piece (Dupont and Thorndyke, 2006). Previous studies demonstrated differential responses of regeneration rates in brittlestars exposed to seawater acidification (Wood et al., 2008; Wood et al., 2011). The arctic brittlestar *Ophiocten sericeum* decreased regeneration rates under acidified conditions whereas *A. filiformis* increased regeneration rates under acidified conditions of pH 7.3. However, in both species, reduced seawater pH led to an increase in metabolic rates, which has been hypothesized to support increased energetic demands to maintain calcification.

The present work aimed at investigating whether elevated seawater P_{CO_2} levels, relevant for ocean acidification and potential CO_2 seepage from CCS sites, may impact energy metabolism and regeneration capacities of the infaunal brittlestar *A. filiformis*. To test how changes in seawater P_{CO_2} affect the micro-environment surrounding *A. filiformis*, we determined abiotic factors (e.g. P_{O_2}, pH and P_{CO_2}) within their burrows. This information is crucial in order to estimate the actual P_{CO_2} levels experienced by the animal, and helps to explain how elevated seawater P_{CO_2} could affect the physiology of infaunal organisms. Furthermore, it can be assumed that under control conditions *A. filiformis* already experience increased hypercapnic and hypoxic conditions within their burrows due to respiration and metabolic release of CO_2. This would probably lead to an additive effect of increased seawater P_{CO_2} to the naturally increased P_{CO_2} levels within burrows. We hypothesized that decreased seawater pH imposes a significant challenge to the energy metabolism of these animals as a result of low acid–base regulatory abilities. According to earlier studies conducted on other invertebrate species (Reipschläger and Pörtner, 1996; Michaelidis et al., 2007; Thomsen and Melzner, 2010; Stumpp et al., 2012), we expected that *A. filiformis* may tolerate moderate acidification but aerobic metabolism cannot support energetic demands during severe acidification over longer periods, leading to the onset of metabolic depression. This may particularly affect the regeneration process as it is believed to be associated with high energetic costs.

RESULTS

CO_2 perturbation experiments

In order to investigate the effects of seawater acidification on physiology, behavior and abiotic parameters inside sediment burrows, we performed four pH perturbation experiments (Table 1). The first experiment (experiment 1), addressing the effects of acidification on metabolic rates, NH_4^+ excretion, gene expression and composition of body parts, used seawater pH values of 8.0, 7.3 and 7.0 corresponding to P_{CO_2} levels of 526, 3396 and 6644 µatm in the seawater above the sediment. pH in the benthic layer of the Gullmarsfjord was estimated using data from SMHI Database Svenskt Havrarkiv (from March to September 1959–1987) and varies between 7.8 in autumn and winter and 8.1 in spring. To address the extracellular acid–base status of *A. filiformis* exposed to different pH conditions (experiment 2), we used pH values of 8.0, 7.6 and 7.3 corresponding to P_{CO_2} levels of 492, 1473 and 3213 µatm. The pH levels used in these two experiments simulate potential scenarios in the context of ocean acidification in benthic habitats (e.g. pH 8.0, 7.6 and 7.3) as well as a more extreme pH level of 7.0, which simulates acidification by leakage of pure CO_2 from sub-seabed CCS sites. To investigate the effects of acidification on abiotic conditions inside the sediment burrow micro-habitat (experiment 3) and regeneration capacity (experiment 4), we performed two additional experiments using the lower pH level of 7.0, which corresponded to a P_{CO_2} of 6400 µatm.

Abiotic parameters within burrows (experiment 1)

O_2 and CO_2 profiles determined for *A. filiformis* burrows demonstrate a progressive decrease of P_{O_2} and an increase of P_{CO_2} with depth (Fig. 1A,B). O_2 levels decreased down to 50.11±7.3 mmol l^{-1} (20% air saturation) and CO_2 levels increased to 0.13±0.009 kPa (pH 7.64±0.03) at a depth of 3 cm (Fig. 1A,B). No pH-induced differences in O_2 profiles were recorded in the sediment (Fig. 1C). During environmental acidification (pH 7), burrow water pH decreased to 6.98±0.02 (P_{CO_2} 0.65±0.05 kPa) (Fig. 1A). Total alkalinity measured from burrow water (3 cm depth) was 2.17±0.26 under control and 2.17±0.48 under low pH conditions. Decreased seawater pH induced by hypercapnic conditions led to increases in burrow water P_{CO_2} in an additive fashion. However, independent of the degree of seawater acidification (hypercapnic conditions), we observed a constant P_{CO_2} gradient of ~0.05 kPa between burrow water at 3 cm depth and the surrounding seawater.

Table 1. Seawater physiochemical conditions of the four different hypercapnia experiments

	Measured				Calculated	
	pH	Temperature (°C)	Salinity	Alkalinity (mmol)	P_{CO_2} (µatm)	Total CO_2 (mmol)
Experiment 1	7.93±0.02	8.70±0.27	31.20±0.10	2.18±0.03	695.78±39.81	2.09±0.03
	7.03±0.03	8.60±0.22	31.16±0.09	2.18±0.03	6347.76±556.30	2.44±0.04
Experiment 2	8.06±0.05	10.23±0.90	31.19±0.07	2.22±0.02	526.34±19.01	2.10±0.02
	7.29±0.04	10.17±0.95	31.22±0.06	2.23±0.04	3395.83±236.59	2.36±0.05
	7.30±0.04	10.25±0.86	31.17±0.06	2.20±0.03	6643.84±185.20	2.49±0.02
Experiment 3	8.04±0.08	8.47±0.14	31.57±0.17	2.25±0.04	492.46±78.79	2.13±0.05
	7.63±0.07	8.42±0.16	31.46±0.15	2.27±0.02	1473.40±241.60	2.28±0.02
	7.30±0.03	8.42±0.18	31.64±0.08	2.29±0.02	3213.10±218.46	2.40±0.02
Experiment 4	7.99±0.08	11.10±0.14	31.55±0.35	2.20±0.04	614.60±126.43	2.09±0.01
	7.01±0.01	11.05±0.07	31.65±0.49	2.19±0.01	6399.70±252.10	2.46±0.03

pH is measured on the NBS scale.

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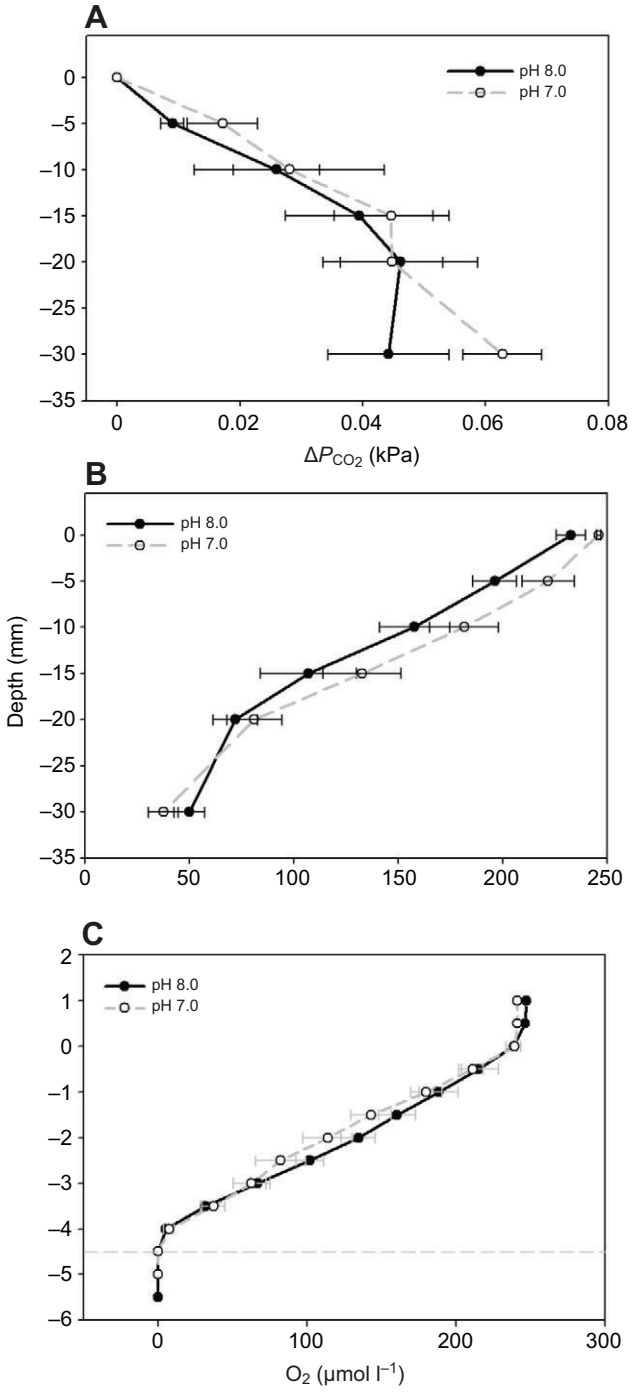

Fig. 1. Abiotic parameters in *Amphiura filiformis* burrows. (A) P_{CO_2} profiles (ΔP_{CO_2} relative to P_{CO_2} at 0 mm depth) in burrows of *A. filiformis*. CO_2 concentrations were calculated from total alkalinity and pH measured in different depth and pH treatments. Values are given as means ± s.e.m. (*N*=4). (B,C) Oxygen profiles in burrows of *A. filiformis* (B) and in the sediment surface (C). Oxygen concentrations were measured in different depth and CO_2 treatments. Values are given as means ± s.e.m. (*N*=4).

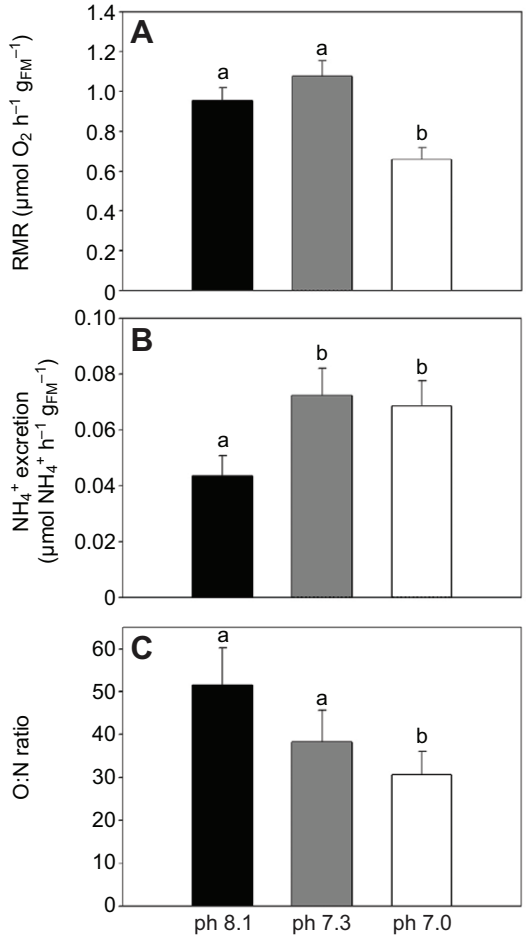

Fig. 2. Effects of seawater acidification on metabolism and NH_4^+ excretion. (A) Routine metabolic rate (RMR) and (B) NH_4^+ excretion rate in *A. filiformis* exposed to three pH levels over a period of 4 weeks. g_{FM}, g fresh mass. (C) O:N ratio. Values are given as means ± s.e.m. Significant differences between treatments are indicated by different letters (one-way ANOVA, $P<0.05$, $N=4$).

Routine metabolic rate, ammonium excretion and O:N ratio (experiment 2)

Routine metabolic rate (RMR) was significantly influenced by decreased pH over the time course of 4 weeks (Fig. 2A), with a significant decrease at pH 7.0 down to 0.66±0.06 μmol O_2 g_{FM}^{-1} h^{-1} (where g_{FM} is grams of fresh mass) compared with 0.95±0.06 to 1.07±0.07 μmol O_2 g_{FM}^{-1} h^{-1} at pH 8.1 and pH 7.3, respectively. Ammonium excretion rates significantly increased with increasing P_{CO_2} from 0.044±0.007 μmol NH_4^+ g_{FM}^{-1} h^{-1} under pH 8.1 conditions to 0.0723±0.01 NH_4^+ g_{FM}^{-1} h^{-1} and 0.069±0.009 NH_4^+ g_{FM}^{-1} h^{-1} at pH 7.3 and 7.0, respectively (Fig. 2B). Accordingly, the O:N ratio decreased significantly with decreasing pH from 51.57±8.59 at pH 8.1 down to 30.56±5.46 at pH 7.0 (Fig. 2C). We did not observe any mortality during the entire experimental period.

Gene expression (experiment 2)

In disc tissues of animals maintained for 4 weeks at pH 7.3 or pH 7.0 (Fig. 3A) the only significant change in gene expression was observed for the NHE3 regulator *NHE3reg*, which was 0.36±0.09 log_2-fold (22%) upregulated in response to pH 7.3. No significant differences were observed for any other genes.

In arm tissues (Fig. 3B) several significant changes were observed: among the ion-regulatory genes, *NBCe* and *AQP9* were 1.0±0.46 and 1.72±0.95 log_2-fold downregulated in pH 7.0 treatment. Among the metabolic genes, *G6PDH* transcript abundance was significantly affected in both treatments, by 0.96±0.37and 1.61±0.55log_2-fold at pH 7.3 and 7.0, respectively. *LDH* expression was significantly reduced by 0.52±0.24 log_2-fold

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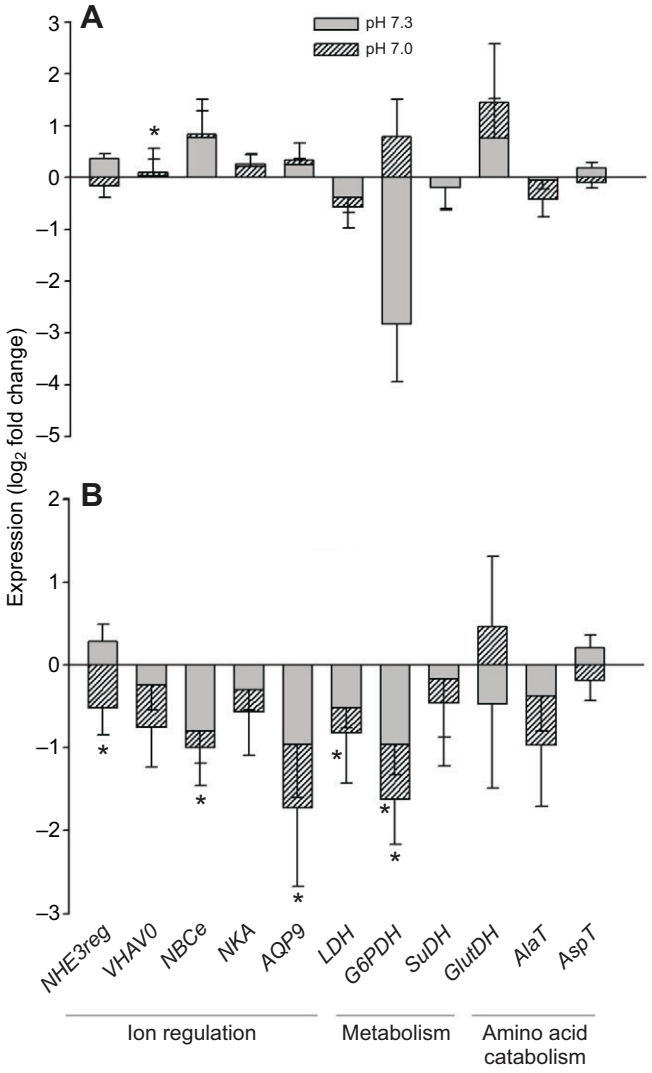

Fig. 3. Gene expression profiles in disc and arm tissues. Expression profiles for selected genes determined for disc (A) and arm (B) tissues of *A. filiformis* after exposure to three different pH levels (pH 8.1, 7.3 and 7.0) for 4 weeks. Expression of the candidate genes was normalized to ubiquitin-conjugated enzyme (*UCE*) gene expression. Asterisks denote a significant change (one-way ANOVA, $P<0.05$) in gene expression ($N=4$).

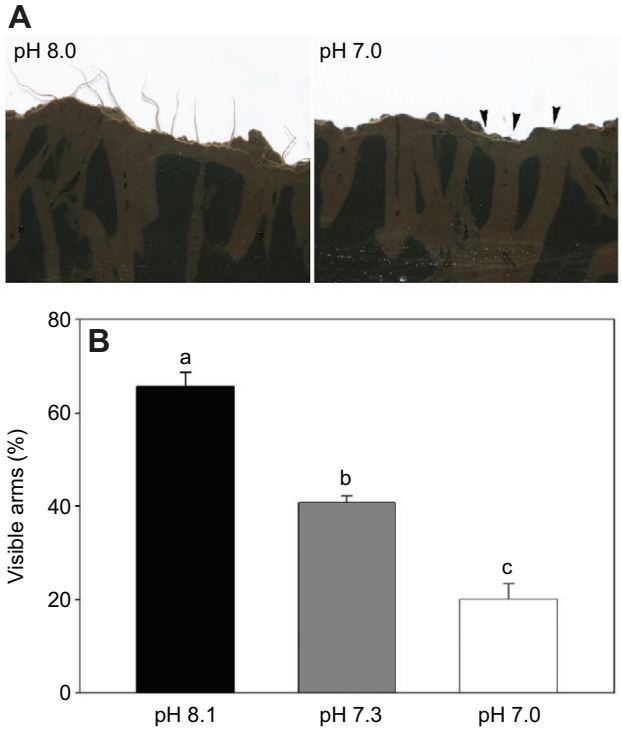

Fig. 4. Amount of arms visible during the 4 week incubation in the different pH treatments. (A) Photographs taken in 'thin aquaria' of pH 8.1- and pH 7.0-treated animals after 2 weeks incubation time. Arrows indicate the position of visible arms lying on the sediment surface in the low pH treatment. (B) Percentage visible arms in the different pH treatments. Values are given as means ± s.e.m. Significant differences between treatments are indicated by different letters (one-way ANOVA, $F=65.202$, $P<0.001$, followed by *post hoc* Holm–Sidak test, $N=4$).

at pH 7.3. No significant changes were detected between the different pH treatments for genes involved in amino acid catabolism including amino acid-specific trans-aminases.

Biometrics and behavior (experiment 2)

Along the experimental period no significant changes were detected in fresh mass (FM), dry mass (DM), ash-free dry mass (AFDM) and the ratio between ash dry mass (ADM) and DM for arms and bodies, respectively (supplementary material Tables S1 and S2). However, a significant decrease of visible actively filter-feeding arms was observed in decreased pH, with only 43% of visible arms in pH 7.3 and 27% in pH 7.0 seawater (Fig. 4), whereas animals in pH 8.1 exposed up to 73% of their arms into the water column.

Extracellular acid–base status (experiment 3)

In order to test how far *A. filiformis* are able to control their extracellular pH homeostasis, we used pH-sensitive optodes to measure extracellular pH (pH_e) in the coelomic cavity of control and CO_2-treated animals over a time course of 15 days (Fig. 5). Under pH 8.1 (0.05 kPa P_{CO_2}) conditions, pH_e was ~0.2 to 0.3 units below the environmental pH. When exposed to low pH conditions, the pH_e dropped within 48 h to 7.64±0.06 and 7.52±0.05 at an ambient pH of 7.63 (0.15 kPa P_{CO_2}) and 7.3 (0.32 kPa P_{CO_2}), respectively (Fig. 5A). Over the course of 10 days the pH_e remained relatively stable at the respective pH levels. The calculation of HCO_3^- levels in the coelomic fluid indicates that under pH 8.1 conditions *A. filiformis* has high extracellular HCO_3^- levels (6–7 mmol l^{-1}) compared with the surrounding seawater (2–2.5 mmol l^{-1}). When exposed to lowered sea water pH, animals significantly increase their extracellular fluid [HCO_3^-] to 8–9 mmol l^{-1} within 48 h (Fig. 5B). In the following days, extracellular fluid [HCO_3^-] was maintained at elevated levels in animals treated with decreased pH, compared with the control group.

Regeneration and RMR (experiment 4)

Regeneration rate (mm day^{-1}) was calculated as the coefficient of the significant linear regression between regenerate length (mm) and time (days). Regeneration rate was significantly (3.5 times) faster (ANCOVA; $F=73.03$, $P<0.0001$) in pH 8.1 (0.083±0.004 mm day^{-1}; $F=369.36$, $P<0.0001$) compared with pH 7.0 (0.024±0.002 mm day^{-1}; $F=216.58$, $P<0.0001$) (Fig. 6A,B). The differentiation index (DI as a percentage) was calculated as the proportion in length of the regenerate that is completely differentiated [DI=(DL/RL)×100, where DL is differentiated length and RL is total regenerate length, see Materials and methods], which serves as an indicator of functional recovery of the regenerate (see Dupont and Thorndyke, 2006). This calculated DI was 59±12 under control conditions after the

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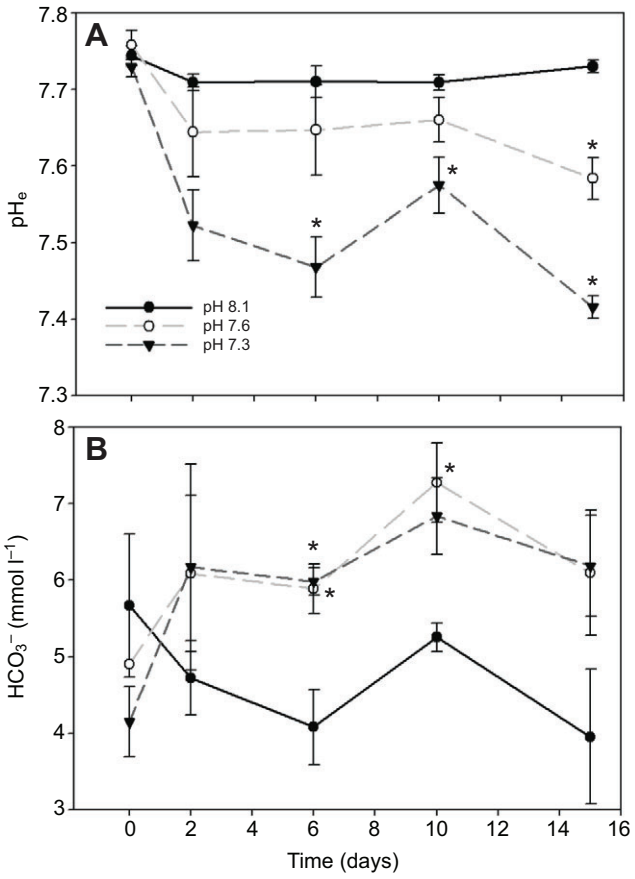

Fig. 5. Extracellular acid–base status during hypercapnia treatment in the coelomic cavity of *A. filiformis*. Animals were exposed to three different pH levels (pH 8.1, 7.6, 7.3) for 15 days. Extracellular pH (pH_e, A) and body fluid HCO_3^- levels (B) were determined before the start of the experiment and at four time points during the incubation period. Asterisks denote significant differences between treatment groups (one-way ANOVA, P<0.05). Values are given as means ± s.e.m. (N=6).

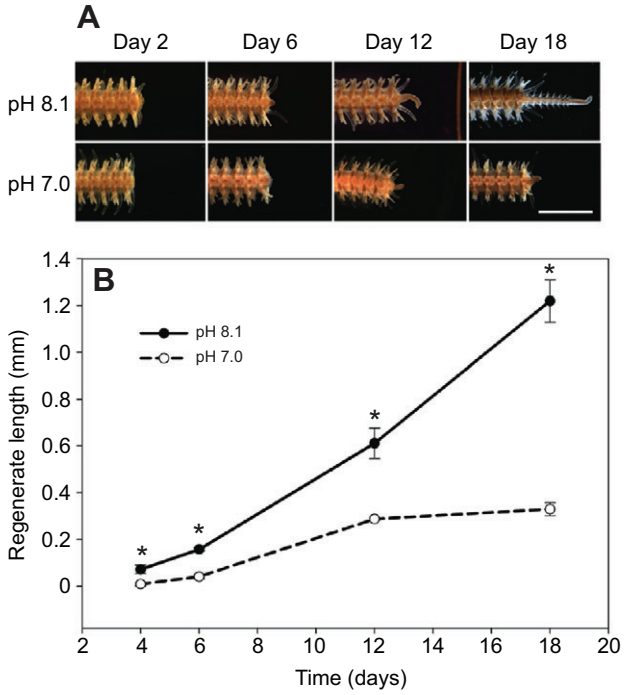

Fig. 6. Effects of seawater acidification on regeneration. (A) Images showing sequential regeneration and regenerate length for animals exposed to pH 8.1 and pH 7.0 conditions. (B) Regeneration during pH 8.1 and pH 7.0 treatments along the experimental period of 18 days. Values are given as means ± s.e.m. (N=4–6). Asterisks denote a significant difference between treatments (P<0.05).

regeneration period of 18 days whereas no differentiation was observed in low pH-treated animals. In both treatments the formation of a blastema had been observed; however, regeneration seemed to be delayed or depressed in animals treated with decreased pH (Fig. 6).

The wound-healing and regeneration process is accompanied by significant changes in RMR (Fig. 7). Under control pH conditions, animals increased RMR immediately after amputation from 0.93±0.06 to 1.04±0.05 µmol O_2 g_{FM}^{-1} h^{-1}. These animals further increased their RMR up to 6 days post-amputation, reaching up to 1.27±0.07 µmol O_2 g_{FM}^{-1} h^{-1}. In the following days, RMR progressively declined, returning to starting levels at 18 days post-amputation (Fig. 7A). In contrast, RMR of animals regenerating in pH 7.0 seem to initially increase as observed for pH 8.0 animals. However, instead of continuing to increase until 6 days post-amputation, RMR declined after 2 days post-amputation and stayed at background levels of ~0.9–1.0 µmol O_2 g_{FM}^{-1} h^{-1} (Fig. 7B).

DISCUSSION

Abiotic conditions within burrows

While surrounding seawater has a pH of 8.1, *A. filiformis* experience a pH lower than 7.7 and strong hypoxic conditions within their sediment burrows. Oxygenation of *A. filiformis* burrows and the oxygenation of the surrounding sediment reported in the literature corroborate our findings, demonstrating that oxygen concentrations decrease with depth in burrows (Vopel et al., 2003). This is a consequence of anoxic conditions within the sediment itself and the metabolism of the animal that demands continuous uptake of O_2. Two-dimensional pH distributions in marine sediments demonstrated rapid changes in pH by ±2 units within millimeters ranging from pH 6 to pH 8 (Zhu et al., 2006). Such acidified conditions in marine sediments can be explained by the aerobic decomposition of organic matter producing CO_2 and the reoxidation of anaerobic metabolites such as NH_4^+, HS^- and Fe_2^+ (Zhu et al., 2006).

Our study also demonstrates that acidification in the overlaying seawater led to an additive acidification in the burrow water to maintain a CO_2 gradient of ~0.05 kPa between burrow water (3 cm depth) and the overlaying water column. The finding that burrows contained live animals suggests that despite the potential ability of *A. filiformis* to ventilate their burrows with their arms (see Vopel et al., 2003), P_{CO_2} levels within burrows were significantly affected by CO_2-induced seawater acidification. Ventilation to control abiotic conditions inside sediment burrows has been reported for other marine taxa including polychaetes (Marinelli, 1994), crustaceans (Stamhuis and Videler, 1998) and some burrowing fish species (Atkinson et al., 1987). The fact that acidified conditions negatively affected the sweeping activity of arms in the water column could suggest that active ventilation of the burrows by the animal may be affected as well. This indicates the importance of considering not only the environmental and local variations in abiotic parameters (McElhany and Busch, 2013) but also variations seen by many organisms in their micro-habitats. In this context the present work demonstrates that *A. filiformis* is already being confronted with pH levels predicted for the coming 100 years in open ocean surface waters. pH 7.7 is the extreme of pH natural variability experienced today and pH levels down to 7.3 may be the extreme of near-future natural variability. Moreover,

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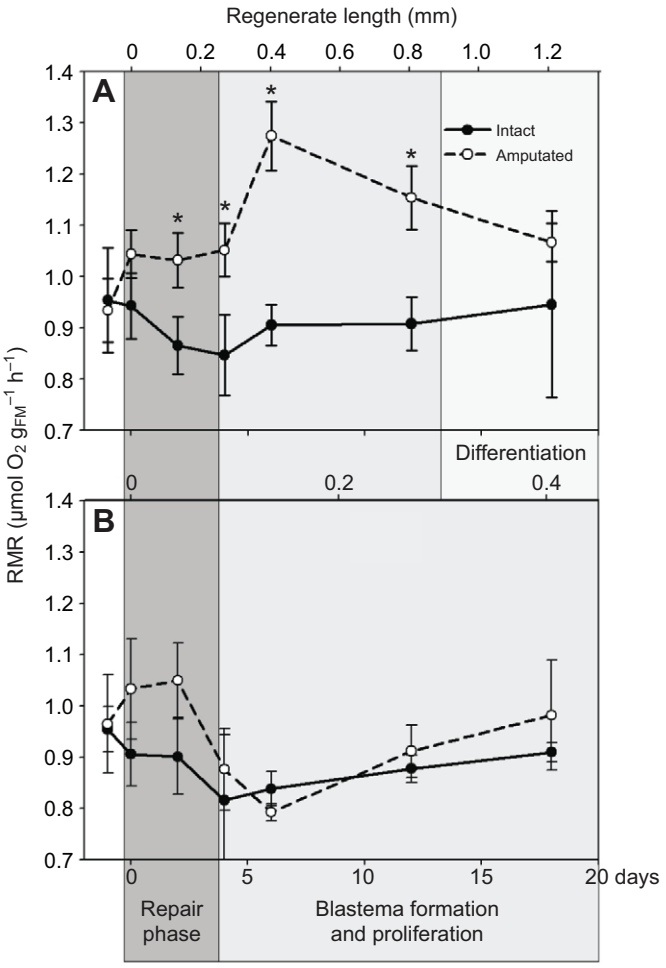

Fig. 7. RMR during regeneration under acidified conditions. Comparison of RMR along a regeneration period of 18 days for *A. filiformis* exposed to pH 8.1 (A) and pH 7.0 (B) conditions. Intact animals were used as the control group. RMR as a function of regenerate length is given by the *x*-axis at the top. Different gray shades indicate phases of the healing and regeneration process. Asterisks denote significant differences (Student's *t*-test, $P<0.05$) between treatments. Values are given as means ± s.e.m. ($N=6$).

potential leakage of pure CO_2 from CCS sites can easily lead to a local acidification of burrow water to <pH 7.3 and can be expected to significantly affect physiological processes of burrowing organisms like *A. filiformis*, which are discussed below.

Physiology and behavior

Our pH_e measurements suggest that *A. filiformis* is a weak acid–base regulator, similar to other echinoderms, which cannot fully compensate for an extracellular acidosis during environmental hypercapnia. However, in accordance to other echinoderms (see Stumpp et al., 2012), *A. filiformis* was able to accumulate up to 1.5–2.5 mmol l^{-1} HCO_3^- in body fluids in response to an extracellular acidosis. In contrast, organisms that can fully restore pH_e during acidosis (e.g. fish and crustaceans) can actively accumulate HCO_3^- in the 10–20 mmol l^{-1} range at hypercapnia levels of 0.5−1 kPa P_{CO_2} (Larsen et al., 1997; Pane and Barry, 2007). An uncompensated acidosis has been demonstrated to induce metabolic depression (Pörtner et al., 2000; Pörtner et al., 2004). Metabolic depression is a major strategy in most invertebrate phyla and all vertebrate classes to survive environmental stress (Dezwaan and Wijsman, 1976; Guppy, 2004; Ramnanan and Storey, 2006). A number of recent studies indicated that mild hypercapnia does not affect or even stimulates metabolism whereas more severe hypercapnic conditions can lead to depressed metabolic rates (Thomsen and Melzner, 2010; Dorey et al., 2013). In accordance with these observations, our work demonstrated that moderate seawater acidification (pH 7.3) does not elicit metabolic depression but has the tendency to increase metabolic rates during a 4 week acclimation period. A similar moderate acidification-induced stimulation of metabolic rates has been previously observed in several marine invertebrates, including brittlestars *A. filiformis* and *Ophiocten sericeum* (Wood et al., 2008; Wood et al., 2011), the bivalve *Mytilus edulis* (Thomsen and Melzner, 2010), pluteus larvae of sea urchins (Stumpp et al., 2011b) and the Arctic pteropod *Limacina helicina* (Comeau et al., 2010). These studies suggested increased energetic demands to fuel compensatory mechanisms to maintain calcification rates and acid–base homeostasis. However, our study also demonstrated that prolonged exposure to stronger acidification (pH 7.0) resulted in a 31% reduction of RMR in *A. filiformis*. Similar observations were made in other marine invertebrates like the worm *Sipunculus nudus*, where hypercapnia-induced reductions in pH_e (and not in internal pH) were demonstrated to elicit metabolic depression as indicated by reduced oxygen consumption rates in isolated muscle tissues (Reipschläger and Pörtner, 1996). Furthermore, recent work conducted on sea urchin larvae demonstrated that moderate acidification increase metabolic rates whereas pH levels <6.5 significantly reduced respiration rates (Dorey et al., 2013). In the present work the depression of metabolic rates in the low pH treatment is further underlined by a general downregulation pattern in arm tissues of genes involved in energy-consuming (e.g. acid–base genes) as well as energy-providing (metabolic genes) processes. Although not tested at the protein level, these results suggest that genes coding for metabolic and acid–base regulatory processes are expressed at lower rates under acidified conditions. Such a modulation of physiological processes (e.g. reduction of protein synthesis and downregulation of energy-providing processes) has been described to be a general feature of organisms undergoing metabolic depression (Guppy and Withers, 1999). Interestingly, expression profiles of body tissues did not demonstrate such a clear downregulation pattern as observed for arm tissues. This differential, tissue-specific response could have two possible explanations: (i) body tissues maintain routine functionality despite metabolic depression in arm tissues or (ii) arm tissues are the major site of energy metabolism, respiration and ion regulation whereas body tissues mainly serve reproduction and nutrient absorption. The latter is supported by behavioral observations demonstrating that during exposure to acidified conditions, *A. filiformis* retract their arms into burrows. This behavioral response could be interpreted as an energy-saving mechanism to reduce additional energetic costs for suspension feeding by sweeping their arms in the water column. Reduced activity may also translate into decreased bioturbation of sediments, which has been hypothesized to affect nutrient flux between water and sediment (Wood et al., 2009). Moreover, retraction of arms during hypercapnic exposure may lead to reduced feeding rates as well as reduced ventilation of burrows, which could have a negative feedback on the animal's energy budget and hypercapnic conditions inside the sediment burrows. Thus, determinations of feeding rates in combination with energetic expenses during environmental hypercapnia represent an important task for future research.

Besides increased metabolic rates at moderate pH and decreased metabolic rates at low pH levels, the present work demonstrates that *A. filiformis* increases NH_4^+ excretion rate at both moderate and high

hypercapnia levels. Higher NH_4^+ excretion rates in response to decreased seawater pH significantly decreased the O:N ratio from control to low pH treatments. Measured metabolic and NH_4^+ excretion rates as well as O:N ratios are comparable to published values for *A. filiformis* and other brittlestars from temperate latitudes (Davoult et al., 1991; Christensen and Colacino, 2000; Talbot and Lawrence, 2002; Vopel et al., 2003) as well as other echinoderms such as the sea urchins *Psammechinus miliaris* (Otero-Villanueva et al., 2004) and *Sterechinus neumayeri* (Hill and Lawrence, 2006). Moreover, the O:N ratio in *P. miliaris* maintained on an algal diet was 37 while urchins fed with mussel or salmon tissue decreased the ratio to 29 and 12, respectively (Otero-Villanueva et al., 2004). This indicates that diet composition directly translates into altered O:N ratios. Based on histological studies, Wood et al. (Wood et al., 2008) suggested that unfed *A. filiformis* increases protein metabolism by the breakdown of muscle tissues to fuel increased energetic demands in response to seawater acidification. It is likely that enhanced catabolism and cell turnover/dedifferentiation of muscle tissues following traumatic amputation in brittlestars and crinoids is related to cell differentiation to produce stem cells to regenerate lost tissues rather that fueling calcification processes (Candia Carnevali and Bonasoro, 2001; Biressi et al., 2010). However, despite increased NH_4^+ excretion rates, the present study could not demonstrate any changes in ratios between ADM and DM between pH treatments, which would have indicated an increased utilization of muscle tissue or other amino acid compounds as an energy source. However, higher NH_4^+ excretion rates can be directly linked to acid–base compensatory processes (Shih et al., 2008; Wu et al., 2010). It has been hypothesized that secretion of NH_4^+ derived from protein catabolism serves as an additional acid extrusion mechanism in the mussel *M. edulis* (Thomsen and Melzner, 2010), the sea urchin *Strongylocentrotus droebachiensis* (Stumpp et al., 2012) and the worm *S. nudus* (Langenbuch and Pörtner, 2002). In this context the importance of Rhesus proteins needs to be mentioned as these were recently discovered to be associated with acid–base regulatory abilities in various marine taxa (Weihrauch et al., 2009; Nawata et al., 2010; Hu et al., 2013). Future studies are needed to broaden our understanding regarding the pathways of NH_4^+-based acid–base regulatory mechanisms in marine invertebrates.

Regeneration

The present work demonstrates increased demands of aerobic metabolism during the course of regeneration of autotomized arm tissues. This supports hypotheses formulated in earlier studies which suggested that regeneration in brittlestars is linked to significant energetic costs (Fielman et al., 1991; Pomory and Lawrence, 1999). In particular, the initial phase of regeneration, which is characterized by cell proliferation (Thorndyke et al., 2001; Dupont and Thorndyke, 2006), seems to require significant energetic costs that derive from aerobic metabolism. Environmental acidification leads to a reduction of the metabolic scope of regeneration.

This reduction in metabolic scope for regeneration processes is associated with a significant decrease in regeneration rate in *A. filiformis* exposed to decreased pH. This contrasts with the finding of Wood et al. (Wood et al., 2008) of increased regeneration rates in the same species exposed to decreased seawater pH. These different results are probably due to methodological differences: in the study by Wood et al. (Wood et al., 2008), amputations were not standardized whereas in the present study arms were cut at 1 cm from the disc in all animals, which minimized differences in regeneration rate (Dupont and Thorndyke, 2006). However, the results of the present work are in general accordance with observations made in brittlestars exposed to osmotic challenges. For example, the brittlestar *Ophiophragmus filograneus* has been demonstrated to reduce regeneration rates down to 25% compared with control animals when exposed to hyposaline conditions (Talbot and Lawrence, 2002). Interestingly, under this hypoosmotic stress, reduced regeneration rates of *O. filograneus* were accompanied by reduced metabolic rates and increased NH_4^+ excretion, in a similar manner to that observed in the present study. It can be hypothesized that ionic disturbances in general, including acid–base and osmotic challenges, may trigger very similar physiological responses at certain threshold levels.

Conclusions

Our work highlights the importance of considering micro-habitats when designing experiments on ocean acidification. The infaunal brittlestar *A. filiformis* experiences naturally low oxygen and pH conditions within sediment burrows. A decrease in seawater pH is additive to the naturally low pH within the burrows. More studies on abiotic parameters in sediment burrows are needed in order to fully understand the effects of acidification on these microhabitats, and how far organisms are able to control and cope with this microenvironment.

Our results also indicate that *A. filiformis* cannot tolerate prolonged exposure to pH 7.0 as indicated by the onset of metabolic depression. This effect on aerobic metabolism reduces the metabolic scope for regeneration, which, in turn, has severe repercussions in terms of the regenerative capacity of autotomized arm tissues. Our results suggest that adult *A. filiformis* are relatively robust to CO_2-induced seawater acidification (down to pH 7.3, predicted extreme of natural variability by 2100) as predicted for the coming century as a consequence of anthropogenic CO_2 emissions. However, stronger acidification down to a pH of 7.0, as may be locally expected for potential leakage from CCS sites, can significantly affect the fitness of this ecologically important species.

MATERIALS AND METHODS

Animals and sampling site

Sediment containing *Amphiura filiformis* was collected at 30–35 m depth, using a box corer, in the vicinity of The Sven Lovén Centre for Marine Sciences (SLC), Kristineberg, Sweden, in September 2011. Individuals were immediately collected from sediment cores by gentle rinsing to avoid breaking of arms, and maintained in natural flowing deep seawater at 12°C, pH 8.0 and a salinity of 31. Animals were acclimated to lab facilities 3 weeks prior to the start of experiments.

In total we conducted four separate experiments to determine behavior, respiration and ammonia excretion rates, growth, extracellular acid–base status and regeneration capacity in *A. filiformis*, and abiotic conditions within the sediment burrows. Depending on the experiment, we let the animals bury in their natural sediment (experiment 1 and 3) or not (experiment 2 and 4). We applied between two (experiment 3 and 4) and three (experiment 1 and 2) pH treatments based on the following assumptions. Natural seawater pH of 8.1 was used as the control condition. Medium pH drops down to pH 7.6 and 7.3 can be expected to occur within the next few centuries as a result of rising atmospheric P_{CO_2} conditions and were used as simulated ocean acidification scenarios. Low pH treatments (pH 7.0) were applied as a potential carbon capture storage scenario and can also be expected to occur in sediment burrows in predicted ocean acidification scenarios (in experiments without sediment).

All experiments were performed in accordance with the Swedish law for animal welfare and were approved by the animal welfare officers of the University of Gothenburg.

Experiment 1: determination of abiotic parameters within burrows

Intact specimens with a disc diameter of 5–6 mm and fresh mass ranging from 216 to 275 mg that showed no evidence of recent regeneration events

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and no apparent gonads were used in all experiments. O_2 and pH in *A. filiformis* burrows were measured using fiber optic oxygen and pH sensors (PreSens oxygen micro optode, type PSt1; PreSens pH microsensors NTH-HP5-L5) mounted on a micromanipulator and connected to a fiber optic oxygen and pH transmitter, respectively (Oxy4 Micro and pH Micro, PreSens, Regensburg, Germany). The sensors were calibrated according to the manufacturer's instructions.

'Thin aquaria' filled with sieved mud (dimensions 8×18×1 cm) as described previously (Rosenberg et al., 1991) were equipped with 10–12 animals each, allowing us to observe the vertical position and burrows within the sediment. Experimental thin aquaria were supplied with seawater from independent header tanks at a flow rate of 40–45 ml min^{-1}, and were adjusted to the respective pH using a computerized feedback system as described above (pH 8.1 and 7.0). All experiments were set up as two controls (pH 8.1; >90% O_2 saturation) and two parallels where pH was lowered to pH 7 (0.63 kPa CO_2). Experiments were repeated two to three times, resulting in a total independent replicate number of N=4–6. O_2 and pH in different depth levels (0, 5, 10, 15, 20 and 30 mm) of burrows were measured visually (accuracy ±100 µm). In total, we measured at least 10 different burrows, containing live animals for each depth level. Seawater was sampled from the burrows using a gastight syringe (Hamilton) mounted on a micromanipulator. Alkalinity from seawater samples was essentially determined spectrophotometrically (ND-2000, NanoDrop Technology, Wilmington, DE, USA) according to Sarazin et al. (Sarazin et al., 1999) in a volume of 1.5 µl. The carbonate system speciation was calculated as described above.

Experiment 2: 4 week low pH exposure within natural sediments

Animals were maintained in a flow-through seawater system consisting of 12 PVC aquaria (5 l volume) in a 10°C climate chamber at the SLC. A light regime with a 12 h:12 h light:dark cycle was chosen. Natural flowing deep seawater with a salinity of 31±0.07 was distributed to the experimental aquaria at a rate of 50 ml min^{-1}. Before the start of the experiments the animals were maintained in these tanks under control conditions for 10 days. Each of the experimental tanks contained 15 individuals that were allowed to bury in 4 cm of sieved (0.7 mm) sediment from the collection site. Three pH levels (control pH 8.1, medium pH 7.3 and low pH 7.0) with four replicates each were continuously maintained in the respective aquaria using a computerized feedback system (AquaMedic) that regulates pH (NBS scale) by addition of pure gaseous CO_2 directly into the seawater (±0.05 pH units). pH was controlled independently in each aquarium and aquaria were randomly arranged with respect to pH level. pH, temperature and salinity were monitored daily and total alkalinity was determined once a week during the incubation time and was essentially measured following Sarazin et al. (Sarazin et al., 1999). pH was monitored using a Metrohm (827 pH lab) pH_{NBS} meter. The carbonate system speciation was calculated from pH_{NBS} and alkalinity using CO2SYS (Lewis and Wallace, 1998) (Table 1) with dissociation constants from Mehrbach et al. (Mehrbach et al., 1973) refitted by Dickson and Millero (Dickson and Millero, 1987). The measured carbonate chemistry is in accordance with field conditions with pH_{NBS} levels of 8.0–8.1 at 10°C and an alkalinity ranging from 2.15 to 2.3 mmol.

The total duration of the experiment was 4 weeks and behavioral observations, oxygen consumption, NH_4^+ excretion measurements (see below for methods) and tissue sampling were performed once every 7 days after the start of the experiment (four times in total). Before each sampling time point, the total number of visible arms that were extended into the water column or laying on the sediment was determined for each tank. Three specimens from each aquarium (three pH levels, four replicates, four time points, three animals per time point and aquarium) were gently taken from the sediment and rinsed with seawater from the respective P_{CO_2} treatment to remove particles from the animals. Two animals were used for respiration and NH_4^+ excretion measurements and the remaining animal was used for gene expression analysis. Metabolic and NH_4^+ excretion rates from each replicate aquarium and sampling time point were averaged over the time course of 4 weeks (N=4). For gene expression analyses, arms were removed from the body using forceps and both body parts (arms and body) were shock-frozen in liquid nitrogen and stored at −80°C.

Experiment 3: determination of extracellular (perivisceral coelomic fluid) acid–base status

To investigate the effects of CO_2-induced seawater acidification on pH_e homeostasis, specimens were maintained in a flow-through seawater system consisting of nine PVC aquaria (5 l volume; for more details see experiment 1). Before the start of the experiments the animals were acclimated in these tanks under control conditions for 5 days. Each of the experimental tanks contained 48 individuals. For practical reasons and to avoid sediment effects on pH exposure, animals were kept without sediment. Three pH levels (control pH 8.1, medium pH 7.6 and low pH 7.3) with three replicates each were continuously maintained in the respective aquaria as described above. Seawater physiochemical parameters were measured and calculated as in experiment 1 and are given in Table 1. The total duration of the experiment was 15 days and extracellular pH measurements were performed at five time points: 0, 2, 6, 10 and 15 days. In total, two specimens (one for pH and one for alkalinity measurements) from each aquarium (three pH levels, three replicates, five time points, three animals per time point and aquarium) were gently taken from the aquaria and excess water was removed on a paper tissue. pH_e was determined within 30 s at 10°C by insertion of fiber optic pH sensors (PreSens pH microsensors NTH-HP5-L5; tip diameter 150 µm) into the coelomic cavity. The sensors were calibrated according to the manufacturer's instructions at the same ambient temperature of 10°C. For total alkalinity measurements, 5 µl of coelomic fluid were quickly sampled from the coelomic cavity using a gastight syringe with a 21 gauge needle. The entire sampling of coelomic fluid was completed in less than 1 min. The coelomic fluid was transferred to an Eppendorf tube and centrifuged for 60 s (6000 rpm; 4300 ***g***) using a minifuge (Capsulefuge PMC-880 Gilson); 3 µl of the supernatant was used for further analyses. Alkalinity from coelomic fluid samples was determined spectrophotometrically (ND-2000, NanoDrop Technology) according to Sarazin et al. (Sarazin et al., 1999) in a volume of 1.5 µl. The carbonate system was calculated as described in the previous section.

Experiment 4: effects of seawater hypercapnia on arm regeneration

To determine the impact of low pH and regeneration on metabolic rate, 48 brittlestars were divided into two groups for the hypercapnia treatment (pH 8.1 and pH 7.0) consisting of 12 control (intact) and 12 animals with amputations. Regeneration rates are dependent on the position of amputation (arm loss) (Dupont and Thorndyke, 2006) and three arms were amputated at 1 cm from the disc to favor fast regeneration. Intact animals and those with amputations were placed in the same aquaria of the respective P_{CO_2} treatment and continuously supplied with fresh seawater (40–45 ml min^{-1}). For practical reasons and to avoid sediment effects on pH exposure, animals were kept without sediment and were separated individually in 6-well plates (each well had a volume of 15 ml) covered with a net (mesh size 1.5 mm) to avoid interaction between individuals and to be able to track individuals along the experimental period. Respiration rates were determined (see below) as described before at 0, 1, 2, 4, 6, 12 and 18 days post-amputation in both regenerating and intact animals. The fresh mass of each individual was determined before the start of the experiment. In three experiments conducted at different times but with the same experimental design, amputated brittlestars maintained under both pH (8.1 and 7.0) levels were removed from the aquaria every 4, 6, 12 and 18 days, anesthetized with iso-osmotic 4% $MgCl_2$, and the total length of the regenerate and the length of the differentiated part of the regenerate (fully formed segments with clearly developed ossicles, podia and spines) were measured using a dissecting microscope (Leica MZ 16 A) (see Dupont and Thorndyke, 2006).

Determination of oxygen consumption and NH_4^+ excretion rates

Animals were placed in glass respiration chambers with a volume of 26 ml containing 0.45 µm filtered seawater equilibrated with the appropriate P_{CO_2} level. Respiration chambers were closed, submerged in a water bath at 10°C and oxygen saturation was measured continuously (once every 30 s) for 2–3 h using fiber optic oxygen sensors (PreSens oxygen micro optode, type PSt1) placed in the lid of respiration chambers that were connected to a OXY-4 mini multichannel fiber optic oxygen transmitter (PreSens). The

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sensors were calibrated according to the manufacturer's instructions. Preliminary experiments demonstrated that the brittlestars could not sufficiently mix the water volume. Therefore, two syringes were connected via gas-tight connectors to the respiration chambers to gently mix the water every 20 min. This resulted in a linear decrease of oxygen concentration during the incubation period. We avoided decreases in oxygen concentration below the 70% air saturation level. After the respiration measurement, animals were removed from the chambers and the missing water volume was refilled with 0.45 µm filtered seawater; respiration chambers were then closed and monitored for 6–12 h for detection of background respiration. Bacterial respiration never exceeded 10% of animal respiration. Prior to the start of the incubation, animals were acclimated for 30 min in the respiration chambers. For calculation of oxygen consumption rates, the linear decrease in oxygen concentration between the start and end of the measurement period was considered. Subsequently, body mass of individuals was measured. Oxygen consumption rates ($\dot{M}_{O_2}$) are expressed as µmol O_2 g_{FM}^{-1} h^{-1}.

Ammonium excretion rates were determined from NH_4^+ concentration measurements prior to and following incubation of brittlestars for respiration measurements. Before closing and after opening respiration chambers, a 1 ml seawater sample was removed for NH_4^+ measurements. NH_4^+ concentration in seawater samples was determined using the indophenol method and measurements were conducted using an automated spectrophotometer (TRAACS 2000, Bran&Luebbe, Norderstedt, Germany) calibrated with a standard series of known NH_4^+ concentrations.

Additionally, a separate glass chamber without a brittlestar was also incubated to determine background readings of filtered seawater for ammonium excretion and respiration rates. Ammonia (NH_3) was not measured as NH_3 concentrations at pH values of 8–7.1 are negligible (0.2–2% of total ammonium/ammonia) (Körner et al., 2001). Ammonium excretion rates are expressed as µmol NH_4^+ g_{FM}^{-1} h^{-1}. The oxygen to nitrogen ratio, which serves as an indicator of protein metabolism, was calculated according to the following equation:

$$O : N = 2\dot{M}_{O_2} (NH_4^+ \text{ excretion})^{-1} .$$

Biometric analyses

Biometric parameters of animals from the CO_2 pertubation experiment (experiment 2) were analyzed using whole-animal FM, DM, AFDM and ash content (ADM) of several body parts (body and arms).

Mass was determined using a precision scale (AT261 Deltarange, Mettler, Giessen, Germany). After determination of wet mass following gentle drying of animals on a paper towel, body parts were dried at 60°C for 20 h (Modell 400, Memmert, Schwabach, Germany) and mass was determined again. ADM was determined by placing arms and body parts in a drying oven at 550°C for 20 h (B170, Nabertherm, Bremen, Germany) prior to mass determination. AFDM was then calculated by subtracting ADM from DM of the arms and bodies, respectively. Biometric measurements including determination of disc diameter, WM, DM and ADM were conducted with brittlestars that were used for respiration and ammonium excretion trials after the physiological measurements were completed. For statistical analyses, values of single individuals (two per replicate) were averaged and the N=4 experimental aquaria per pH level were considered the unit of replication.

Preparation of mRNA

Frozen arm and disc tissues from *A. filiformis* were homogenized in Tri reagent (Sigma-Aldrich, T-9424). Samples were centrifuged for 10 min at 12,000 ***g*** and the pellet was discarded to remove all calcium carbonate from tissues. Total RNA was purified using the Ribopure column system (Ambion, Austin, TX, USA) following the manufacturer's protocol. DNA contamination was removed with DNase I. The amount of mRNA was determined by spectrophotometry (ND-2000, NanoDrop Technology). All mRNA pellets were stored at −20°C.

Real-time quantitative PCR (qPCR)

The mRNA expressions of target genes were measured by qPCR using the Applied Biosystems 7300 Real Time PCR System. *Amphiura filiformis* sequences were obtained from a 454 sequencing library of regenerating arm tissues (O.O.-M., unpublished data). Sequences used for gene expression studies were translated into amino acid sequences, entered in a BLAST

Table 2. Primer sequences used for qRT-PCR

Gene	Abbreviation	Function	Primer sequence 5′–3′	Amplicon length
Ion-regulation				
Na^+/H^+-exchanger 3 regulator	*NHE3reg*	Regulation of NHE3	F: GAAAAAGGAAAACACGGACAATTTATAC	
			R: CCTGGTTTCACTCCCGCTAA	77
V-type H^+-ATPase subunit V0	*VHAV0*	Active proton secretion	F: AACAATTCCACTATGGCGTCTTCT	
			R: GCTACGCATTCTGCTATCCATACTAT	88
Na^+/HCO_3^- cotransporter	*NBCe*	Secondary active ion transport	F: CCCAAGTTGTCGGCTGTAGAG	
			R: GCATTGGAATAACCAGTAATAATTTGTC	104
Na^+/K^+-ATPase	*NKA*	Electrochemical gradient	F: CATGCCACACTGCTTTCTTTG	
			R: GTGAGTTCTTCCTGGTCTTGCA	81
Aquaporin 9	*AQP9*	Channel protein	F: GATGAACGGCTCCATACCATCT	
			R: TTGGCACACTTCTACTGATGCTTT	86
Metabolism				
Lactate dehydrogenase	*LDH*	Anaerobic metabolism	F: ATGTGTTCAGCCAGGAGTAACAAG	
			R: GCACCAGGATCTATAGGAAAGCAT	84
Glucose 6-phosphate dehydrogenase	*G6PDH*	Glycolysis	F: GGTTCCACCGCCAAAAATATC	
			R: TAAAGCCTTAAACGAACGTAAAGCA	79
Succinate dehydrogenase	*SuDH*	Respiratory chain/Krebs cycle	F: GAACAGTTTGCTACGTGCGAAT	
			R: CAACATGGCAGGCATGTTTT	75
Amino acid catabolism				
Glutamate dehydrogenase	*GlutDH*	Protein metabolism	F: GGTGCTACGGAGAAGGATATAGTGA	
			R: TGCTGTTCTCATAATTTGCCTAGCT	81
Alanine transaminase	*AlaT*	Protein metabolism	F: TGAAGAGAGTGGTTGGAGTCTACAGA	
			R: CTACCATAGCAACTGGTTTACAGTGTTT	89
Aspartate transaminase	*AspT*	Protein metabolism	F: CATGGGTCTCTATGGTGAACGA	
			R: GTGACTCACATCTCTTGGCTTCAT	80
Reference gene				
Ubiquitin-conjugated enzyme	*UCE*	Protein degradation	F: TTTCACAACTAAGATCTATCATCCAAACA	
			R: TGGTGACCACTGTGACCTCAAG	82

F, forward; R, reverse.

search and aligned with sequences from other echinoderms (supplemental material Fig. S1). Primers were designed (Table 2) using primer analysis software (Primer Express version 2.0, Applied Biosystems) with the default parameters of the TaqMan MGB Probe and Primer design procedure. PCRs contained 2 ng of cDNA, 50 nmol l^{-1} of each primer and the SYBR Green MasterMix (Applied Biosystems) in a final volume of 10 µl. All qPCRs were performed as follows: 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (the standard annealing temperature of all primers). PCR products were subjected to a melting-curve analysis to verify that only a single product was present. Control reactions were conducted with DNase-treated RNA samples to determine levels of background and genomic DNA contamination. The standard curve of each gene was confirmed to be in a linear range with ubiquitin conjugated enzyme (*UCE*) as the reference gene. The expression of this reference gene was checked for stability and has been demonstrated to be stable in other marine species across ontogenetic stages and during CO_2 treatments (Hu et al., 2011; Hu et al., 2013; Tseng et al., 2013).

Statistical analyses

For metabolic and NH_4^+ excretion rates, each of the four experimental replicates per treatment was used and averaged over the four time points. Normality of distributions was assessed via the Kolmogorov–Smirnov test. Differences between hypercapnia treatment groups were analyzed using one-factorial ANOVA followed by *post hoc* Tukey tests using Sigma Stat (Systat Software). A two-way ANOVA was performed for biometric and extracellular pH HCO_3^- data with time and pH as variables followed by *post hoc* Holm–Sidak test. Percentage values of visible arms in relation to all arms were arcsine transformed prior to statistical analysis (one-way ANOVA). The threshold for significance was $P<0.05$. Student's *t*-test was used to compare metabolic rates of animals with amputated arms and intact animals. The data in the text and figures are presented as the mean ± s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.S., M.Y.H. and S.D. designed the study, conducted experiments, analyzed the data and compiled the manuscript with the help of all other co-authors. I.C. conducted CO_2 perturbation experiments including seawater carbonate chemistry analyses and analyzed the data. O.O.-M. provided sequence information and contributed to molecular analyses.

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Supplementary material

Supplementary material available online at
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