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Deep-water prawn *Pandalus borealis* displays a relatively high pH regulatory capacity in response to CO_2-induced acidosis

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ABSTRACT: Ocean acidification and possible leakage from subsea CO_2 storage may create unfavourable conditions for marine organisms. Deep-living animals are generally believed to be more vulnerable to elevated partial pressure of CO_2 (pCO_2, environmental hypercapnia) than shallow-living species, but so far only a limited number of studies have investigated the effects of environmental hypercapnia on deep-living animals. In the present study, the deep-water prawn *Pandalus borealis* (Krøyer, 1838) was subjected to time-dependent exposure to hypercapnic seawater (pCO_2 ~ 9100 µatm; pH_{NBS} 6.86) for up to 16 d. Surprisingly, animals were able to partially compensate extracellular acidosis under these severe conditions by accumulating buffering bicarbonate ions at levels comparable to those reported for shallow-living decapod crustaceans. pH regulation was achieved without significantly increasing the ion-regulating activity of total ATPase and Na^+/K^+-ATPase. Oxygen consumption rate was not substantially affected by exposure. A significant increase in ammonia excretion rate was found within the initial phase of exposure, possibly reflecting H^+ buffering by ammonia as a result of increased protein metabolism. The results from this study suggest that the deep-water prawn *P. borealis* has relatively well-developed mechanisms to counteract CO_2-induced acidosis. This finding further indicates greater variation in CO_2 tolerance of deep-water organisms than previously assumed and greater tolerance in species with relatively high activity levels compared to less active species. More studies on species from different taxonomic groups and habitat depths are therefore needed to improve the knowledge on how deep-living organisms will respond to the challenges of a high CO_2 world.

KEY WORDS: CO_2 · Deep sea · *Pandalus borealis* · Carbon capture and storage · CCS · Acid–base regulation · Decapod crustaceans · Ocean acidification

INTRODUCTION

In addition to the threat of global warming, the marine environment is currently facing 2 potential challenges related to the increasing emissions of anthropogenic CO_2: ocean acidification and possible leakage from sub-seabed storage of anthropogenic CO_2. The former describes the increased absorption of CO_2 by the oceans, which results in an increased H^+ concentration and a reduced ocean pH (Haugan & Drange 1996, Caldeira & Wickett 2003). In addition, the introduction of carbon capture and storage (CCS) of anthropogenic CO_2 in geological formations under the seabed may constitute a future threat to the ecosystems above the storage sites. Although this is believed to offer safe containment of the captured CO_2, leakage may potentially occur through cracks and faulty seals, creating detrimental conditions for organisms living close to the leakage site (Turley et al. 2004).

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Recent studies have revealed that elevated seawater CO_2 may alter both community structures and biodiversity in benthic communities (Cigliano et al. 2010, Hale et al. 2011). To better understand the ecological consequences of predicted ocean acidification scenarios and possible seepage from anthropogenic CO_2 storage sites, it is important to perform physiological studies to determine how the negative effects of CO_2 affect the physiological status of different species.

Since CO_2 readily diffuses over biological membranes, an increase in the partial pressure of CO_2 (pCO_2) in seawater results in a corresponding increase in the extracellular and intracellular fluids of the organisms living in the water (Gutknecht et al. 1977). The CO_2 molecules react with water in the body fluids to form carbonic acid (H_2CO_3), which further dissociates and results in an increased concentration of hydrogen ions. The primary physiological effect of environmental hypercapnia is therefore acidosis of the body fluids. While lower invertebrates often do not substantially regulate the pH of their blood during hypercapnic exposure (Pörtner et al. 1998, Miles et al. 2007, Thomsen & Melzner 2010), teleost fish and higher invertebrates such as decapod crustaceans and cephalopods often obtain partial or full compensation of extracellular acidosis by excreting protons and/or accumulating buffers (Heisler 1984, Pane & Barry 2007, Spicer et al. 2007, Gutowska et al. 2010a). Melzner et al. (2009) suggested that this may be an adaptation related to their high metabolic rates, as a well-developed acid–base regulatory machinery is necessary to counteract body fluid acidosis resulting from strenuous activity. This may also suggest that more active species are better equipped to handle the acidic effects of CO_2 than more sluggish species. Compensation of CO_2-induced acidosis is normally achieved by accumulating bicarbonate from the surrounding water to neutralize the excess H^+ (Claiborne et al. 2002, Pane & Barry 2007, Spicer et al. 2007, Gutowska et al. 2010a, Small et al. 2010). Acid–base regulation is tightly connected to osmoregulation, and both processes are believed to take place through the same, or similar, ion-regulating proteins in the gills (Wheatly & Henry 1992, Whiteley 2011). Hydrogen ions seem to be excreted through electroneutral Na^+/H^+ exchangers, while the accumulation of bicarbonate occurs in exchange for chloride (Cameron 1978, Wheatly & Henry 1992). The driving force for ion exchange, for both osmoregulatory and acid–base regulatory purposes, is basolateral Na^+/K^+-ATPase, which extrudes 3 Na^+ in exchange for 2 K^+ for every molecule of ATP (Skou & Esmann 1992, Lucu & Towle 2003). Vesicular H^+-ATPase plays an important role in ion regulation, as it is thought to be connected to chloride/bicarbonate exchange. Both ATPases have also been found to be highly involved in ammonia excretion in both fish and crustaceans (Onken & Putzenlechner 1995, Weihrauch et al. 2004, 2009). Ammonia (NH_3), formed from the catabolism of proteins and amino acids, may act as a buffer of H^+ by forming ammonium ions (NH_4^+), which are excreted into the surrounding medium (Lindinger et al. 1984). The enzyme glutamate dehydrogenase (GDH, EC.1.4.1.3) is a deaminating enzyme which removes the amino group of glutamate, resulting in the formation of α-ketoglutarate and ammonia, and its activity has been found to be related to ammonia excretion in marine invertebrates (Bidigare & King 1981). It is therefore possible that this enzyme plays a role in acid–base regulation in marine organisms during CO_2-induced acidosis. In decapods, the activity of this enzyme is highest in muscle tissue (King et al. 1985).

The deep sea is defined to begin at the shelf break (~200 m depth) as it coincides with the transition from shallow- to deep-living fauna (Thistle 2003). Although many marine species are known to be sensitive to elevated CO_2 levels (Michaelidis et al. 2005, Miles et al. 2007), deep-living animals have been considered particularly vulnerable (Seibel & Walsh 2001). Contrary to shallow-living species, deep-living animals do not often experience large fluctuations in environmental conditions and are believed to have low tolerance for environmental disturbances because of their low metabolic rates and intracellular buffering capacities (Castellini & Somero 1981, Seibel & Walsh 2001). The few laboratory experiments performed until now have involved deep-living species with low activity levels. The results have shown that they are not able to compensate hypercapnic acidosis, most likely because of a reduced capacity to accumulate bicarbonate ions (Pane & Barry 2007, Hammer et al. 2011, Taylor et al. 2013). The presence of thriving deep-sea communities in areas with naturally high seawater CO_2, such as volcanic vents and acidic upwelling areas, however, also suggests that certain deep-sea species may evolve to tolerate such conditions (Tunnicliffe et al. 2009, Jantzen et al. 2013).

The deep-water shrimp *Pandalus borealis* spends its early stages in relatively shallow water (5 to 20 m) and is often found associated with estuaries. Juveniles then move into deeper water and become stenohaline and stenothermal as adults. Adult *P. borealis* have a circumboreal distribution and are

most commonly found at depths between 50 and 500 m. *P. borealis* is predominantly a benthic, opportunistic omnivore, but like many deep-living species, it feeds in the water column during diel migrations (Shumway et al. 1985). This may suggest that it is a relatively active deep-water species and may therefore have a higher tolerance to the physiological effects of CO_2 compared to more sluggish species. A previous study on juvenile *P. borealis* showed that while survival was not significantly affected, this species did display a delayed zoeal progression (development time) at CO_2 levels relevant for predicted ocean acidification scenarios (pH 7.6) (Bechmann et al. 2011).

The objective of the present study was to investigate the effects of environmental hypercapnia in an active, deep-living benthic species and to find out whether responses to elevated pCO_2 in these animals are similar to responses in shallow-living animals.

MATERIALS AND METHODS

Collection and treatment of animals

Adult *Pandalus borealis* were collected using a shrimp trawl in the Åsenfjord, Norway (150 to 200 m depth), in December 2010. We conducted short trawls (15 min) to reduce the number of prawns caught and added a 60 l barrel at the bottom end of the trawl to minimize damage to the animals. Prior to the experiments, prawns were held in 200 l basins supplied with fresh seawater (Trondheimsfjord, 70 m depth) maintained at 7°C in a climate-controlled room for 1 mo. Animals were fed commercial trout feed twice a week prior to the experiments and once a week during the experiments. Only males, with an average wet weight of 4.33 g (2.65 to 6.82 g), were used for the experiment. During the experiment, animals were kept in constant darkness.

The experiments were conducted according to the Animal Welfare Act and approved by the Norwegian National Animal Research Authority (FOTS reference no. 1911).

Experimental set-up

The experimental set-up was similar to the semi-flow-through system described by Hammer et al. (2012). In short, fresh seawater saturated with 100% CO_2 gas (Mapcon, Yara Praxair) was introduced in a set ratio to normocapnic (normal CO_2) seawater (50 and 500 ml min^{-1}) to yield the target pH and pCO_2, respectively, in a 100 l header tank. The mixture was further pumped (7.5 l min^{-1}) to a chamber from which the exposure water was distributed to 5 exposure chambers. The exposure chambers were 30 l aquaria containing a maximum of 5 animals per aquarium at a time. Excess water in the exposure chambers flowed back into the header tank. A water volume corresponding to the volume of the entire exposure system was exchanged 3 times a day.

Control animals were held in an identical set-up as described above but with the introduction of fresh, normocapnic seawater rather than hypercapnic seawater for 8 d.

Oxygen consumption and ammonia excretion

Oxygen consumption was determined by closed respirometry. The respiration chambers consisted of 1.5 l glass containers containing 1 animal at a time. The animals were acclimated overnight to minimize the effect of handling on their metabolic rates. Between measurements, the chambers were connected to the exposure system described above. Triplicates of water samples were carefully collected in 100 ml ground-glass Erlenmeyer flasks filled to the brim and stoppered shortly before (time 0) and 1 h after the chambers were disconnected from the system. Dissolved oxygen in the samples was measured using a YSI self-stirring biological oxygen demand probe (Model 5905) with a YSI dissolved oxygen meter (Model 58), correcting for salinity, temperature and barometric pressure. Water samples collected from the respiration chambers were filtered (Whatman GF/F) and stored in 50 ml polypropylene centrifuge tubes at −20°C prior to analysis. The concentration of ammonia-nitrogen was determined according to the indophenol blue method (Norwegian Standard 4746, 1975). Both oxygen consumption and ammonia excretion were calculated as the difference in concentrations between time 0 and time 1 h, and both were related to the wet mass (M_{wet}) of the animals.

Seawater pH, total alkalinity and pCO_2

Seawater pH was determined in control and CO_2-enriched water using a combined pH microelectrode (MeterLab pHC3359-8, Radiometer Analytical) with a standard pH meter (MeterLab PHM210, Radiometer Analytical), calibrated with National Bureau of Standards (NBS) buffers (pH 4 and 7). The apparent

activity coefficient of H^+ was determined by 4-point titration of 20 ml of seawater by 5 ml of standard acid with normalities in the range of 0.01 to 0.016 N HCl and was used to calculate total alkalinity of seawater (Anderson et al. 1999). Concentrations of seawater carbonate species were calculated using CO2SYS software (Pierrot et al. 2006) with dissociation constants for the NBS scale from Mehrbach et al. (1973) as refit by Dickson & Millero (1987).

Extra- and intracellular acid–base parameters

Hemolymph was collected from the abdominal sinus after the animal had been removed from the exposure chamber and weighed. Blood sampling was completed within 1 min after the animals were removed from the water. Extracellular ph (pH_e) was measured immediately after sampling at 7°C, as described for seawater. Although pH measurements were performed in an open system, the pH remained stable within the time of the reading. Total CO_2 (TCO_2) was measured using a TCO_2 analyzer (Model 965, Ciba-Corning) on 50 µl hemolymph samples which had previously been centrifuged for 2 min at 12000 rpm (12000 × *g*). Centrifugation had a negligible effect on the TCO_2 of the sample. The instrument was calibrated with dilutions of a commercial CO_2 standard (Reagecon, 2 g l^{-1}). Extracellular pCO_2 and bicarbonate concentration were calculated by rearranging the Henderson-Hasselbalch equation and using the CO_2 solubility and apparent pK value reported by Truchot (1976).

Immediately after the animal was euthanized, intracellular pH (pH_i) of the abdominal muscle was determined according to the homogenate method developed by Pörtner et al. (1990). A thin (~2 mm) sample of the posterior adductor muscle was freeze-clamped and flash-frozen in liquid N_2 and subsequently ground to a powder in liquid N_2. The powder was then dissolved in 1 ml of ice-cold media containing reagents which inhibit homogenate metabolism (130 mmol l^{-1} potassium fluoride, 6 mmol l^{-1} nitrilotriacetic acid, pH 7.2). Following brief mixing and centrifugation (5000 × *g* for 1 min), the resultant pH of the supernatant was measured as described for pH_e and used as a proxy for pH_i of the tissue. We did not assess the contribution of extracellular fluid because Pörtner et al. (1990) showed a negligible effect. Subsequent studies using this method on marine invertebrates have also assumed a negligible effect of pH_e on pH_i (Sartoris & Pörtner 1997, Michaelidis et al. 2005).

Extracellular osmolality

The osmolality of the hemolymph was measured using a vapour pressure osmometer (Model 5500, Wescor) on duplicates of samples that had been stored in liquid paraffin at −20°C.

ATPase activity

Gill tissue was homogenized in 10× w/v homogenizing buffer (50 mM imidazole, 250 mM sucrose, 1 mM EDTA, 5 mM mercaptoethanol, 0.1% sodium deoxycholate, protease inhibitor cocktail [Catalog No. P8340, Sigma], pH 7.4) using an Ultraturrax homogenizer (15 strokes), centrifuged at 1500 × *g* for 10 min at 0°C and then stored at −80°C prior to analyses. The homogenate was further diluted 1:2 in 100 mM Tris-acetate buffer (pH 8.6 with 0.1% Triton X-100), and Na^+/K^+-ATPase activity was determined according to the method of Holliday (1985) adapted for 96-well plates as the difference between inorganic phosphate (P_i) liberated in assay buffer A (167 mM NaCl, 50 mM KCl, 33.3 mM imidazole, pH 7.20) and assay buffer B (217 mM NaCl, 1.67 mM ouabain, 33.3 mM imidazole, pH 7.2). A third assay, where the homogenate was added immediately after the addition of stop solution, was applied to determine the phosphate present in the homogenate and/or released from non-enzymatic hydrolysis of ATP. This strategy also enabled us to determine the total ATPase activity as the concentration of P_i produced in assay buffer B minus the P_i concentration from the third assay. The optical density (OD) was read at 700 nm using a Cary 50 microplate reader (Varian). The concentration of P_i liberated was calculated from a standard curve of potassium dibasic phosphate. Samples were run in triplicate, and concentration of liberated P_i was normalized to concentration of protein measured by using Bradford reagent (Sigma) with BSA (Bio-Rad) as the standard.

GDH activity

Immediately after an animal was killed, the intestine was removed, and the tissue from the abdominal muscle was flash-frozen in liquid nitrogen and stored at −80°C until analysed. The tissue was then homogenized 1:4 v/w in 100 mM Tris-acetate buffer, pH 8.4, with 0.1% Triton X-100 using a Precellys® 24 homogenizer (2 × 15 s) and centrifuged at 10000 × *g* at 0°C for 10 min. GDH (EC.1.4.1.3) activity was assayed using a modified version of the GDH step in the glut-

Table 1. Physico-chemical parameters (mean ± SEM) of normo- and hypercapnic seawater. Water temperature: 7.11 ± 0.03°C; salinity: 35. pH_{NBS}: pH on the National Bureau of Standards scale; pCO_2: partial pressure of CO_2; HCO_3^-: bicarbonate; TCO_2: total CO_2; TA: total alkalinity; ΩCa: calcite saturation state; ΩAr: aragonite saturation state; SW: seawater. *Values significantly different from controls

	pH_{NBS}	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1} SW)	TCO_2 (µmol kg^{-1} SW)	TA (µmol kg^{-1} SW)	ΩCa	ΩAr
Normocapnia	8.06 ± 0.06	511 ± 5.9	2186 ± 13.9	2317 ± 15.4	2450 ± 17.5	2.32 ± 0.04	1.60 ± 0.03
Hypercapnia	6.86 ± 0.01*	9085 ± 147*	2410 ± 13.8*	2853 ± 21.5*	2428 ± 13.7	0.16 ± 0.01*	0.11 ± 0.01*

aminase activity assay described by Gella & Pascual (1982). By removing GDH from the assay and adding glutamate, results were comparable to those of a commercial GDH assay kit (Catalog No. K729-100, BioVision). A 5 µl aliquot of homogenate was added to the wells of a 96-well plate, and the volume was adjusted to 50 µl with the assay buffer. One well was assigned as a positive control, to which 2 µl of GDH-positive control (Part No. K729-100-4, BioVision) was added, and the volume was adjusted to 50 µl using the assay buffer. Then, 100 µl of reaction buffer (2.2 mM NAD, 0.28 ADP, 0.555 g l^{-1} BSA, 0.555 mM iodonitrotetrazolium chloride, 3 g l^{-1} Cremophor EL, 55 mM Tris-acetate, pH 8.6, 133.5 mM glutamate, 55.5 µM phenazine methosulfate) was added to the wells containing the test samples and positive control. The samples were mixed and then incubated for 3 min at 37°C before following the production of NADH at 37°C for 15 min at 450 nm using a Cary 50 microplate reader (Varian). The linear range of NADH produced from 0 to 5 min was used to calculate NADH production, and a standard curve was used to calculate the NADH produced in the assay.

Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test. Data with non-normal distributions were log-transformed. We then used ANOVA on normally distributed and log-transformed data with Dunnett's post hoc test, setting a significance level of $p \leq 0.05$. All values are presented as means with standard errors of the mean (SEM). Differences in seawater carbonate chemistry parameters were tested using unpaired *t*-tests.

RESULTS

Table 1 depicts seawater pH and carbonate chemistry parameters under normal and high CO_2 conditions in the present study.

We observed clear time-dependent changes in extra- and intracellular acid–base parameters in *Pandalus borealis* subjected to environmental hypercapnia (Fig. 1). After 12 h of exposure, mean extracellular pCO_2 had increased significantly to 1.06 ± 0.09 kPa, compared to 0.25 ± 0.02 in control animals (Fig. 1a). Values remained significantly higher compared to controls for all groups. Although time-dependent exposure to environmental hypercapnia induced a drop in pH_e, the new steady state value, approximately 0.15 unit below control values, was not significantly different compared to the pH_e of 7.76 ± 0.04 in control animals (Fig. 1b). The extracellular bicarbonate concentration of control animals was 5.07 ± 0.94 mmol l^{-1}. Bicarbonate concentrations were significantly higher in all time groups exposed to elevated seawater CO_2, and the highest concentrations, at 15.10 ± 2.93 mmol l^{-1}, were found in animals exposed for 1 d (Fig. 1c). Bicarbonate concentrations were significantly elevated in animals exposed for 1 and 16 d. Unexpectedly, the pH_i generally increased with time of exposure, from 7.05 ± 0.02 in control animals, and was significantly higher at pH 7.20 ± 0.02 after 8 d (Fig. 1d). pH_i returned to control values at the end of exposure (16 d).

Extracellular osmolality decreased slightly compared to the control value of 1014 ± 7 mOsm kg^{-1} throughout the exposure period, and after 16 d of exposure, extracellular osmolality was 982 ± 5 mOsm kg^{-1} (Fig. 2). However, extracellular osmolality never differed significantly from control values.

Mass-specific oxygen consumption was generally lower in prawns exposed to elevated CO_2, but only those exposed for 4 d displayed significantly lower oxygen consumption compared to controls (Fig. 3a). Ammonia excretion was significantly higher compared to controls (0.54 ± 0.10 µg M_{wet}^{-1} h^{-1}) in animals exposed for 1 and 2 d when mean ammonia excretion was 1.87 ± 0.63 and 1.64 ± 0.48 µg M_{wet}^{-1} h^{-1}, respectively (Fig. 3b). Except for a decrease after 4 d, ammonia excretion was generally higher in exposed animals but returned to control values in animals exposed for 16 d.

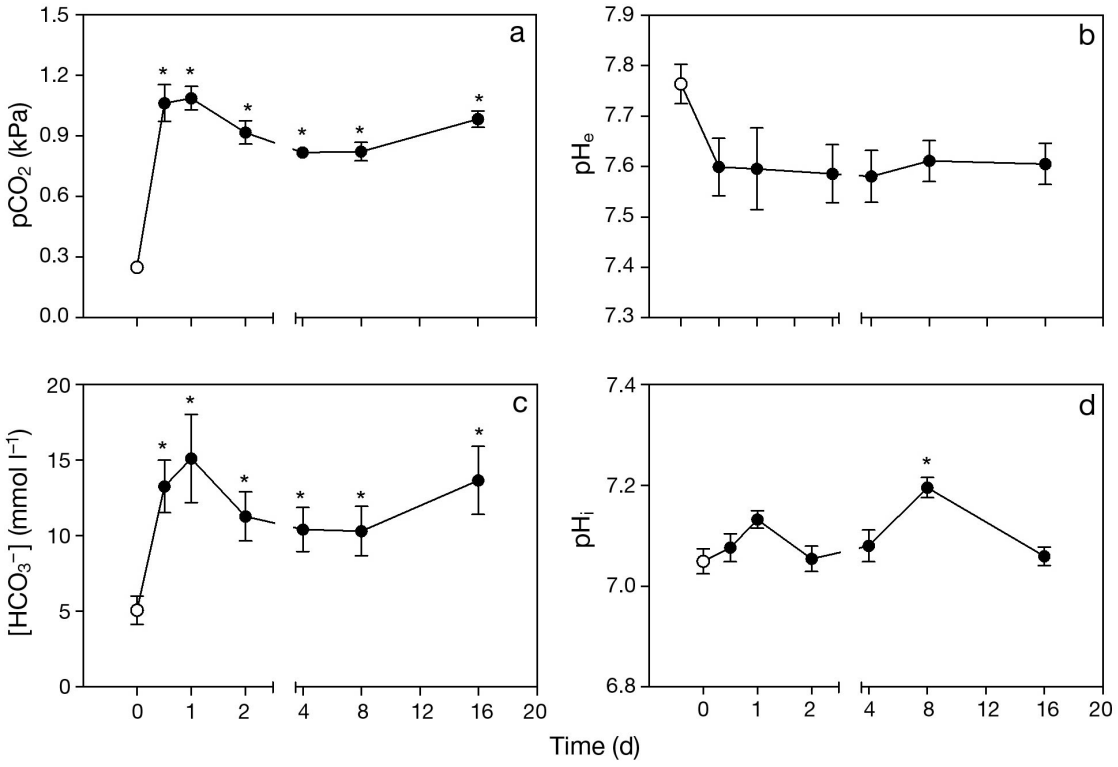

Fig. 1. *Pandalus borealis*. Acid–base parameters of hemolymph: (a) partial pressure of CO_2 (pCO_2), (b) extracellular pH (pH_e), (c) bicarbonate (HCO_3^-) concentration and (d) intracellular pH (pH_i) of prawn subjected to control conditions (○) and time-dependent exposure to environmental hypercapnia (●, pCO_2 = 9085 µatm and pH_{NBS} = 6.86). Values are means ± SEM, n = 5–11. *Values significantly different from control values ($p \leq 0.05$)

The activity of total ATPase and Na^+/K^+-ATPase generally increased with time of exposure but never differed significantly from controls (Fig. 4). With the exception of animals exposed for 1 and 2 d, the activity of total ATPase increased with time of exposure from 39.3 ± 3.49 µmol P_i mg^{-1} h^{-1} in control animals to 76.8 ± 17.0 µmol P_i mg^{-1} h^{-1} after 8 d, when the highest activity was found. A similar pattern of change was found for Na^+/K^+-ATPase, where animals exposed to hypercapnic exposure for 4 d displayed the highest activity (7.93 ± 1.5 µmol P_i mg^{-1} h^{-1}) compared to 4.33 ± 0.76 µmol P_i mg^{-1} h^{-1} in control animals.

The GDH activity was also only slightly affected, with generally higher activities in exposed animals compared to controls (Fig. 5). The highest activity occurred after 8 d, when GDH activity was 90 ± 18 mU ml^{-1} mg^{-1} compared to 67 ± 17 mU mg^{-1} in control animals.

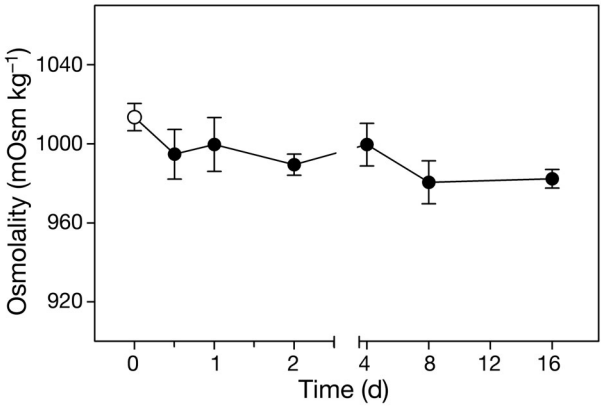

Fig. 2. *Pandalus borealis*. Extracellular osmolality of prawn subjected to time-dependent exposure to environmental hypercapnia (●, partial pressure of CO_2 = 9085 µatm and pH_{NBS} = 6.86). ○: controls. Values are means ± SEM, n = 4–7

DISCUSSION

Deep-living animals are generally considered more sensitive to elevated levels of CO_2 compared to shallow-living species (Seibel & Walsh 2001, 2003). However, the results of our study challenge this view, as the exposure of *Pandalus borealis* to environmental hypercapnia (pCO_2 = 9100 µatm, 90 Pa) did not significantly reduce pH_e, which is often seen in marine invertebrates (Pörtner et al. 1998, Michaelidis 2002, Miles et al. 2007). One possible

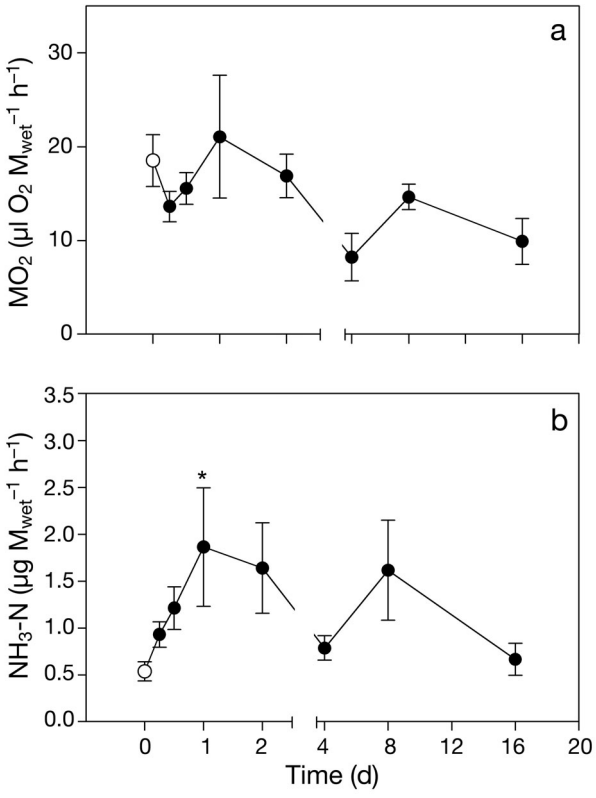

Fig. 3. *Pandalus borealis*. (a) Oxygen consumption (MO_2) and (b) ammonia-nitrogen (NH_3-N) excretion subjected to control conditions (○) and time-dependent exposure to environmental hypercapnia (●, partial pressure of CO_2 = 9085 µatm and pH_{NBS} = 6.86). M_{wet}: wet mass. Values are means ± SEM, n = 4–11. *Values significantly different from control values ($p \leq 0.05$)

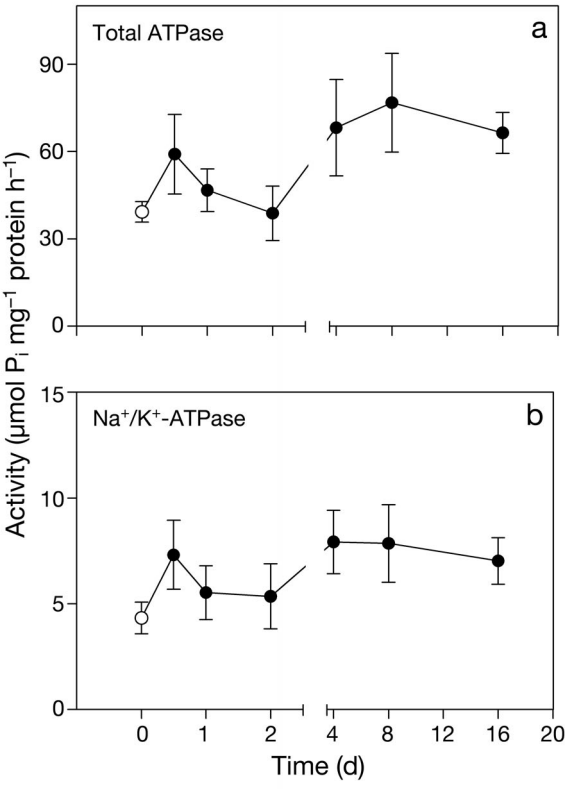

Fig. 4. *Pandalus borealis*. Activity of (a) total ATPase and (b) Na^+/K^+-ATPase in gills of prawn subjected to control conditions (○) and time-dependent exposure to environmental hypercapnia (●, partial pressure of CO_2 = 9085 µatm and pH_{NBS} = 6.86). P_i: inorganic phosphate. Values are means ± SEM, n = 4–11

explanation for the lack of pH drop may be that the first samples were collected after 12 h, which has been reported as sufficient time for other decapod species to achieve partial or full compensation during similar exposure conditions (Pane & Barry 2007, Spicer et al. 2007, Small et al. 2010, Appelhans et al. 2012). The significant increase in extracellular bicarbonate concentration observed in hypercapnic *P. borealis* after only 12 h supports this explanation. Within 1 d of exposure, mean bicarbonate concentration was 15.1 mmol l^{-1}, 3 times higher than in control animals. Bicarbonate ions are important extra- and intracellular buffers, and accumulation of bicarbonate from the ambient water is the most common strategy used by marine ectotherms to counteract extracellular acidosis (Cameron 1985, Wheatly & Henry 1992, Whiteley 2011).

The increase in bicarbonate concentration by *Pandalus borealis* is comparable to responses in shallow-living decapods exposed to similar conditions (Tru-

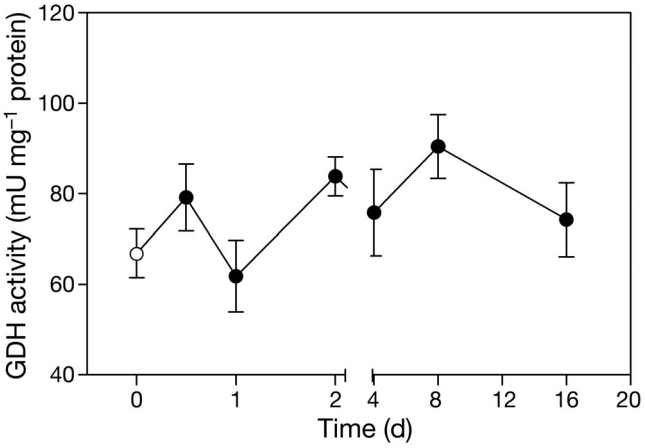

Fig. 5. *Pandalus borealis*. Activity of glutamate dehydrogenase (GDH) in abdominal muscle of prawn subjected to control conditions (○) and time-dependent exposure to environmental hypercapnia (●, partial pressure of CO_2 = 9085 µatm and pH_{NBS} = 6.86). Values are means ± SEM, n = 6–11

chot & Fontaine 1975, Pane & Barry 2007, Spicer et al. 2007) and indicates a relatively high compensatory capacity in *P. borealis* in response to CO_2-induced acidosis. In a recent study, the subtidal burrowing shrimp *Upogebia deltaura* was able to compensate hypercapnic acidosis at pH 7.64 but not at pH 7.35, and no animals survived pH 6.71 for more than 13 d (Donohue et al. 2012). This comparison suggests that *P. borealis* may display even higher tolerances to elevated seawater CO_2 than certain subtidal shrimps.

Failure to accumulate sufficient levels of bicarbonate ions resulted in an uncompensated extracellular acidosis in the deep-sea crab *Chionoecetes tanneri* in response to seawater CO_2 levels similar to those used in the present study (Pane & Barry 2007). The authors did, however, point out that the low bicarbonate acquisition of *C. tanneri* could possibly be explained by the low temperature in the experiment (3°C). The activity of an enzyme is usually affected by temperature, and a low temperature could therefore mean a reduced ion pumping activity in the gills. This could further suggest that pH regulation can be achieved in *C. tanneri* but over a longer time period than 24 h.

The different response to CO_2 exposure observed between the 2 deep-living species could also be explained by the fact that *Chionoecetes tanneri* is found at much greater depths than *Pandalus borealis* (~1000 m vs. 50 to 500 m, respectively) and is therefore adapted to an even more stable environment. The results most likely reflect the different activity levels of the 2 species. Diel migrations to shallow water may result in acidic metabolite build-up because of increased swimming activity. Thus, *P. borealis* may be pre-adapted to counteract the acidifying effect of CO_2 during hypercapnic exposure. Although pH_e was not completely restored to control values in *P. borealis*, this modest elevation may be because of the level of exposure, and a full compensation of acidosis could be expected at a more moderate CO_2 level.

Unexpectedly, pH_i increased with time of hypercapnic exposure to values significantly higher than those of controls after 8 d. This increase contrasts most findings for marine invertebrates, where a transient or prolonged acidosis is observed (Pörtner et al. 1998, Michaelidis et al. 2005, Hammer et al. 2011) and may suggest that compensatory mechanisms lead to an alkalosis of the tissue during exposure to environmental hypercapnia. One explanation may come from the contribution of the extracellular fluid to the tissue homogenate. In animals with low buffering capacities in the extracellular fluid, this effect is expected to be negligible on pH_i. However, high bicarbonate concentration found in the extracellular fluid of *Pandalus borealis* following CO_2 exposure may affect the pH in the muscle tissue sample, manifesting itself as an increase in pH_i. The results also suggest that in this species, the pH of the intracellular compartments may not be as tightly controlled as would be expected from the high extracellular acid–base regulatory capacity. Maintenance of pH_i is of crucial importance due to the pH sensitivity of many enzymes, and acidosis can thus lead to reduced rates of biochemical processes and the induction of metabolic depression (Guppy & Withers 1999). The pH_i of control *P. borealis* (pH 7.05) in the present study was lower than that found by Sartoris & Pörtner (1997) and that reported by Wheatly & Henry (1992) for other decapods (~pH 7.20 to 7.30). The reason for this difference is unclear, but to confirm the results, pH_i was determined on samples from a large number of animals, resulting in a relatively uniform pH_i value of all control animals.

Osmoregulation and acid-base regulation of extracellular fluids are closely connected in decapod crustaceans, as both processes are thought to occur through similar ion-transporting proteins located in the gills. For instance, when transferred to dilute seawater, the acid–base status of osmoregulating species is affected; during re-establishment of a new steady state osmolality, an initial drop in pH is followed by a small alkalosis in subtidal decapods (Whiteley et al. 2001). The velvet swimming crab *Necora puber* was able to fully compensate extracellular acidosis during exposure to hypercapnic seawater of pH 6.74, but hypercapnic exposure induced large, transient drops in extracellular osmolality at moderate CO_2 levels (pH 7.3) (Spicer et al. 2007). Dissanayake et al. (2010) observed a complete compensation of pH_e achieved without any associated change in extracellular osmolality in 2 species of palaemonid prawns exposed to a more moderate level of CO_2 (~3000 µatm). However, hypercapnic *Palaemon elegans* displayed lower hemolymph ion concentrations, while *P. serratus* displayed elevated concentrations, compared to their respective controls. This may reflect increased ion exchange to regulate pH_e and suggests that the mechanisms used may be slightly different in the 2 species. The extracellular osmolality of *Pandalus borealis* decreased slightly with time of exposure, but no significant changes were found. The results of the present study show that *P. borealis* is able to partially compensate pH_e without affecting osmolality substantially in response to CO_2-induced acidosis. It is possible, however, that the extracellular ion distribution is also altered in *P. borealis.*

The activity of the enzyme Na^+/K^+-ATPase is the driving force for ion regulation, and the activity of branchial Na^+/K^+-ATPase often increases in crustaceans subjected to changes in ambient salinity, particularly reduced salinity (Holliday 1985, Lucu & Towle 2003). It was therefore expected that the Na^+/K^+-ATPase activity would also increase in response to environmental hypercapnia to increase the acid–base regulatory capacity. Increased Na^+/K^+-ATPase activity in response to elevated seawater CO_2 levels has been observed in teleost fish (Deigweiher et al. 2008). However, the effect of hypercapnic exposure on gill Na^+/K^+-ATPase activity in decapods has been poorly investigated. Pane et al. (2008) found significantly decreased Na^+/K^+-ATPase activity in Dungeness crabs *Cancer magister* exposed to pCO_2 of ~10 000 µatm for 24 h, and no change was found in the deep-sea crab *Chionoecetes tanneri* subjected to the same conditions. Using both microarray and quantitative PCR, Fehsenfeld et al. (2011) found only small changes in gene expression of proteins known to be important in osmoregulation and acid–base regulation, while other membrane proteins were differentially regulated in response to elevated seawater pCO_2 (3900 µatm) under low salinity conditions. However, prolonged exposure of *Carcinus maenas* to higher CO_2 levels (7500 µatm) at normal seawater conditions (35‰) resulted in significant upregulation of several branchial ion-regulating proteins but not Na^+/K^+-ATPase (K. M. Hammer et al. unpubl.). While ion regulation, and thus acid–base regulation, is thought to occur mainly in the posterior gills of decapod crabs, anterior gills were recently also shown to be important for acid–base regulation, and there is a differential regulatory response in the gills under both normal and elevated CO_2 levels (Fehsenfeld & Weihrauch 2013).

Although an increase in both total ATPase and Na^+/K^+-ATPase activity was observed in hypercapnic *Pandalus borealis* in the present study, neither parameter changed significantly in activity. However, gene expression and protein synthesis of other important acid–base regulating proteins may be altered in response to CO_2-induced acidosis to increase the regulatory capacity of *P. borealis*. Further studies on the mechanistic responses of this species are therefore needed to better understand the relatively strong ability for this deep-water species to regulate pH_e.

An unexpected finding for *Pandalus borealis*, not related to the CO_2 exposure, was that Na^+/K^+-ATPase amounted to only approximately 10% of the total ATPase activity, as this enzyme has been reported to constitute about 80% of total ATPase activity in euryhaline crabs (Castilho et al. 2001). Increasing the concentration of the Na^+/K^+-ATPase-specific inhibitor ouabain did not alter the results, suggesting that other ATPases may be highly active in the gills of *P. borealis*. The results of the present study and the findings of Pane et al. (2008) may indicate that when exposed to environmental hypercapnia, decapod crustaceans can maintain pH_e without increasing the activity of ion-transporting enzymes above the basal metabolic level. However, the activity of other enzymes involved in acid–base regulation, such as carbonic anhydrase, could be altered to increase acid–base regulation during hypercapnic exposure (Burnett et al. 1981, Henry & Cameron 1983). Mechanisms other than branchial ion regulation are also likely important in decapod response to CO_2-induced acidosis.

The acidic effect of elevated levels of CO_2 is often associated with metabolic depression. However, previous studies show variable effects of elevated pCO_2 on metabolic rate in different species (Reipschläger & Pörtner 1996, Michaelidis et al. 2005, Wood et al. 2008, Thomsen & Melzner 2010). The metabolic rate of teleosts and higher invertebrates, such as cephalopods, remains close to normal during hypercapnic exposure, possibly because of their ability to regulate body fluid pH (Deigweiher et al. 2008, Gutowska et al. 2008). In the present study, hypercapnic *Pandalus borealis* displayed a general decrease in oxygen consumption, but only animals subjected to 4 d of exposure had significantly reduced rates compared to controls. The decrease could possibly be explained by the severity of exposure, resulting in a reduced pH_e. In this study, all controls were held for 8 d in the experimental set-up. Therefore, we may have missed changes in metabolic rates not related to seawater CO_2 concentrations. This issue could have been resolved by measuring the metabolic rates of control animals throughout the experiment.

Ammonia excretion in *Pandalus borealis* was significantly higher in animals exposed for up to 2 d, while prawns exposed for longer periods displayed excretion rates similar to those in controls. Increased ammonia excretion rate indicates an increased rate of protein, and/or amino acid, catabolism and has been observed in marine ectotherms during hypercapnic exposure (Lindinger et al. 1984, Pörtner et al. 1998, Thomsen & Melzner 2010). This increase may be explained by elevated energy demand during these conditions, similar to that experienced by crabs exposed to low salinity (Weihrauch et al. 2009). Ammonia is a weak base and can further neutralize excess protons by accepting a proton and forming

NH_4^+ at low pH (Roos & Boron 1981). Lindinger et al. (1984) suggested that ammonia excreted as NH_4^+ could serve as an important mechanism of acid excretion in hypercapnic *Mytilus edulis*. The mechanisms involved in ammonia excretion in both decapod crustaceans and teleost fish have been debated. While passive diffusion of ammonia may take place, strong evidence exists for the involvement of both Na^+/K^+ (NH_4^+)-ATPase and vesicular H^+-ATPase, as well as secondary sodium transporters in ammonia excretion. In addition, studies point towards an important role of Rhesus-like proteins (Rh proteins) as ammonia transporters in gills of decapods (Weihrauch et al. 2004, 2009). Fehsenfeld & Weihrauch (2013) recently found that the various gills of shore crabs *Carcinus maenas* acclimated to diluted seawater displayed differential ammonia excretion rates and, further, that these rates were differentially affected by elevated levels of CO_2.

Ammonia excretion may also contribute to acid–base regulation in *Pandalus borealis*, but it does not appear as quantitatively important as other processes such as bicarbonate accumulation.

The enzyme GDH is involved in the deamination of glutamate, and its activity often reflects ammonia excretion in marine crustaceans (Bidigare & King 1981). However, the non-significant increase in GDH activity in *Pandalus borealis* suggests that this enzyme does not play an important role in acid–base regulation. It is possible, however, that other deaminating enzymes, such as phosphate-dependent glutaminase, are more important for acid–base regulation in crustaceans, as seen in the mammalian kidney (Atkinson & Bourke 1984).

Although *Pandalus borealis* displayed a relatively high capacity to counteract CO_2-induced acidosis in the present experiment, the relatively short duration of the exposure (16 d) limits any conclusions on long-term exposure response to elevated CO_2 levels. Several recent studies reveal possible detrimental side effects from the compensatory increase in bicarbonate at CO_2 levels predicted within the next century, such as hypercalcification (Checkley et al. 2009, Gutowska et al. 2010b) and disturbance of neurological functions. In several species of tropical coral reef fish, high levels of bicarbonate may cause neurological disorders, such as disrupted chemosensory functions and anti-predator behaviours (Munday et al. 2009). One study links this response to a disruptive effect of elevated bicarbonate concentrations on the GABA receptor (Nilsson et al. 2012).

Deep-water animals generally have lower metabolic rates than shallow-living species. Researchers debate the reason for this difference, suggesting temperature, scarcity of food and light conditions as important parameters. Although all 3 factors may influence metabolic rates of deep-living animals, light limitation offers the most promising explanation, as only species using vision to find food show the depth-related decline in metabolic rates, and this decline mostly applies only to fish (Drazen & Seibel 2007). In fact, several studies show that deep-living invertebrates, which rely on smell rather than vision to locate prey, generally do not display depth-related declines in metabolic rates when standardised for temperature and body size (Childress et al. 1990). They may thus have the same enzyme activities as shallow-living counterparts and may therefore also display high compensatory abilities towards environmental stressors. Although the metabolic rates of *Pandalus borealis* were quite low, these rates may be explained by feeding rates, as animals were only fed once a week. One may argue whether *P. borealis* can be considered a typical deep-water species, as it can occur as shallow as 50 m at sufficiently low seawater temperature (<8°C) (Shumway et al. 1985). This exception may apply to several deep-sea species which occur in shallower waters if conditions are favourable. Given that *P. borealis* is also considered a stenohaline and stenothermal species, one would expect this species to be a poor ionoregulator and thus to be particularly vulnerable to conditions of elevated seawater CO_2, as reviewed by Whiteley (2011). The results of our study may nonetheless suggest that deep-water species with relatively high activity levels often encounter metabolic acidosis and therefore have regulatory mechanisms to handle the acidic effect of CO_2.

Although adult *Pandalus borealis* appear to tolerate high levels of CO_2, earlier developmental stages may be more vulnerable to similar conditions. Previous studies on larvae and juveniles show that although predicted ocean acidification scenarios (pH 7.6) do not significantly affect survival in larval *P. borealis*, they do delay development (Bechmann et al. 2011, Arnberg et al. 2012). The additive effect of global warming adds further concern, as increased temperature elicits greater effects than reduced pH (Arnberg et al. 2012).

CONCLUSION

In conclusion, our study indicates that the deep-water prawn *Pandalus borealis* has a capacity to counteract extracellular acidosis similar to that of

intertidal species. This capacity was unexpected and suggests that this deep-living species has relatively highly evolved acid–base regulatory mechanisms that can handle CO_2-induced acidosis, in contrast to common assumptions for deep-living species. If broadly applicable, this finding may indicate that elevated pCO_2 has less of an effect on active deep-living species than previously assumed. Further studies are thus needed to improve the understanding of how deep-living animals will handle hypercapnic conditions associated with ocean acidification and potential leaks from sub-seabed storage of anthropogenic CO_2.

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