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Comparison of enzyme activities linked to acid–base regulation in a deep-sea and a sublittoral decapod crab species

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ABSTRACT: When compared to the sublittoral Dungeness crab *Cancer magister*, the deep-sea Tanner crab *Chionoecetes tanneri* exhibited lower activities of enzymes involved in some of the processes essential for efficient acid–base regulation. Tissue enzymatic activities were compared between Dungeness crabs held in normoxia and Tanner crabs held in hypoxia—both treatments mimicking typical habitat oxygen levels. In the posterior gill, activities of all forms of ATPase and carbonic anhydrase (CA) were approximately 2- to 13.2-fold lower in Tanner crabs than in Dungeness crabs. CA activity in the heart and white muscle was also significantly lower in hypoxic deep-sea Tanner crabs, while ATPase activity in these 2 tissues was similar between the 2 treatments. Diagnostically, enzymatic activities were compared when both species were held in normoxic seawater, with additional significant differences found in specific white muscle ATPase fractions (amiloride- and N-ethylemaleimide [NEM]-sensitive ATPases) and tissue buffering (β) capacity. When both species were acclimated to normoxia, *C. tanneri* exhibited mass specific rates of oxygen consumption significantly lower (4.5-fold) than *C. magister*. Under short-term, strongly hypercapnic conditions (1% CO_2), the Dungeness crab displayed reduced (30 to 40%) branchial ATPase activities, while enzymatic activities in the Tanner crab gill, muscle and heart were refractive to short-term (24 h) hypercapnia, suggesting a minimal ability to tune branchial function to changing environmental conditions. These results support our hypothesis that the deep-sea Tanner crab has a reduced capacity for active transport of acid–base relevant ions, particularly at the gill, and is therefore at a marked disadvantage with respect to iono- and acid–base regulatory capacity. These results add to a growing database documenting the limited ability of deep-sea megafauna to compensate for internal acid–base disruptions associated with introduction of anthropogenic CO_2 into the deep sea.

KEY WORDS: CO_2 · Deep sea · Physiology · Decapod crustacean · Acid–base regulation

INTRODUCTION

Direct, large-scale sequestration of carbon dioxide (CO_2) into the deep sea has been advanced as a possible solution to rising atmospheric CO_2 levels (Marchetti 1977, 1979). Additionally, the chronic absorption of atmospheric CO_2 by surface ocean waters coupled with ocean mixing is causing a significant increase in anthropogenic CO_2 in the deep-sea (Feely et al. 2004), with projected large increases in the future.

Caution has been advised regarding the possible detrimental effects of anthropogenically induced deep-sea hypercapnia on characteristically hypometabolic deep-sea fauna (Seibel & Walsh 2001, Pörtner et al. 2004). Deep-sea fauna are also predicted to be highly sensitive to hypercapnia due to long-term adaptation to a naturally invariant physio-chemical environment (Gage & Tyler 1991, Kennett & Ingram 1995, Pörtner et al. 2004).

In a companion paper (Pane & Barry 2007), we showed that a deep-sea decapod crustacean, the grooved Tanner crab *Chionoecetes tanneri*, was more sensitive to short-term, severe hypercapnia than the sublittoral Pacific Dungeness crab *Cancer magister*.

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During 24 h of high-level hypercapnia, *C. tanneri* made no compensation for a 0.4 unit reduction in extracellular (hemolymph) pH. In contrast, *C. magister* completely restored extracellular pH to normocapnic levels during the same hypercapnic challenge (Pane & Barry 2007).

Since the metabolically costly process of acid–base regulation (particularly during hypercapnic challenge) requires extensive ion transport capacity (Ahearn et al. 1999), we have hypothesized that the limited acid–base regulatory function exhibited by *Chionoecetes tanneri* is a function of reduced capacity to actively transport acid–base relevant ions across membranes, particularly the branchial epithelium. A previous report comparing 2 species of deep-sea crabs of the genus *Chaceon* with the sublittoral *Callinectes sapidus* concluded that activities of specific metabolic enzymes were generally much lower in the deep-sea decapod species (Walsh & Henry 1990).

The current report continues the comparison between *Chionoecetes tanneri* and *Cancer magister* (see Pane & Barry 2007) to test the hypothesis that activities of acid–base relevant enzymes are lower in deep-sea decapod species. We have compared Dungeness crabs held in normoxic seawater to Tanner crabs held in hypoxic seawater. These conditions best mimic typical *in situ* oxygen levels, giving the more physiologically relevant comparison. We have also eliminated oxygen as a variable and compared enzymatic activities from individuals of both species held in normoxic seawater. Enzymatic rates presented include total ATPase, Na^+-K^+-ATPase, P-type (membrane-bound and N-ethylemaleimide [NEM]) ATPases, amiloride-sensitive ATPases, and carbonic anhydrase. The relevant acid–base impact of the ATPases we selected involves general creation and maintenance of ion (particularly Na^+) gradients necessary for efficient acid–base regulation (Henry & Wheatly 1992, Wheatly & Henry 1992, Pequeux 1995). Carbonic anhydrase participates directly in acid–base regulation by catalyzing the reversible hydration of CO_2 to form carbonic acid and subsequently bicarbonate ions and protons (Henry & Cameron 1983, Henry & Wheatly 1992).

Additionally, whole animal oxygen uptake rates were used to assess the extent of hypometabolism in the deep-sea *Chionoecetes tanneri*, and *in vitro* tissue buffering capacity was measured to further compare acid–base regulatory capacities between the 2 crab species. To evaluate any direct effect of elevated CO_2 on acid–base regulatory capacity, crabs of both species were exposed to high levels of CO_2 in short-term (24 h) experiments. The merits of using the 2 species, *C. tanneri* and *Cancer magister*, in such a comparative approach are outlined in Pane & Barry (2007).

MATERIALS AND METHODS

Animal collection and holding. A detailed account of collection and laboratory holding of the deep-sea decapod crab *Chionoecetes tanneri* can be found in Pane & Barry (2007). Briefly, *C. tanneri* of both sexes (200 to 800 g) were collected by remote operated vehicle from the seafloor at depths of 950 to 1050 m within Monterey Canyon (see Fig. 1 in Pane & Barry 2007). Crabs were transferred to the laboratory and held in a recirculated seawater system in darkened chambers at 3°C, a pH of 7.85 ± 0.10, and a salinity of 35 ± 1. Laboratory holding and experimentation were conducted under normobaric conditions. Oxygen levels were either 90 ± 5% saturation (normoxia ≈ 350 µM), or 10 ± 5% saturation (hypoxia ≈ 40 µM). The hypoxic treatment mimicked *in situ* oxygen levels typical of the oxygen minimum zone at depths between roughly 400 and 1200 m off the California coast (Childress 1995).

Pacific Dungeness crabs *Cancer magister* of both sexes (500 to 900 g) were captured from 30 to 40 m depth by local fishermen using baited traps. Crabs were transferred to the laboratory and held in flowing seawater at 10 ± 1°C, with a pH of 7.90 ± 0.10, salinity of 35 ± 1, and an oxygen saturation of 90 ± 5%, under a 12 h light:12 h dark photoperiod. During the holding period, all crabs were fed to satiation with chopped squid. Food was withheld 48 h prior to, and throughout, all 24 h hypercapnic exposures. Experiments starving 2 other species of deep-sea crab, *Geryon longipes* and *Bythograea thermydron*, for 34 d have shown no effect of starvation on the metabolic function of muscle tissue (Company et al. 2008).

Hypercapnic exposure. Short-term hypercapnia was accomplished via 24 h exposures to flowing seawater equilibrated with 1% CO_2 delivered from calibrated gas cylinders (Airgas) containing either 1% CO_2, 3% O_2, balance N_2 (hypoxia); or 1% CO_2, 20% O_2, balance N_2 (normoxia); as described in detail in Pane & Barry (2007). *Cancer magister* (at 10°C) and *Chionoecetes tanneri* (at 3.5°C) held in the laboratory under normoxia were exposed to seawater equilibrated with the cylinder containing 20% O_2, while *C. tanneri* (again at 3.5°C) held in the laboratory under hypoxia was exposed to seawater equilibrated with the cylinder containing 3% O_2. During hypercapnic exposure, individuals of each species were held in individual (12 or 20 l) darkened and sealed chambers with no head space and served by a water flow of 50 ml min^{-1}. Oxygen concentrations in these 2 treatments were 95 and 14%, respectively. It is following these 24 h exposures that tissues were sampled for measurements of ATPase activity and buffering capacity.

All prehypercapnic data were collected by sampling a subset of individuals prior to the appropriate CO_2 exposure.

For all sampling, crabs were anaesthetized for 3 min in a slurry of ice and seawater, then sacrificed by immediate removal of the dorsal carapace. The heart and the eighth (posterior) gill on the right side were excised immediately, snap-frozen in liquid nitrogen, and stored at –80°C for future analyses. Simultaneously, the most anterior walking leg on the left side was removed and snap-frozen in liquid nitrogen. The leg was then removed from the liquid nitrogen, and, while frozen, the exoskeleton was split and the white muscle was removed, snap-frozen, and stored at –80°C.

Table 1. Inhibitors used to measure specific fractions of ATPase activity in heart, white muscle, and gill tissue from *Chionoecetes tanneri* and *Cancer magister*. See 'Discussion' for a biochemical/physiological description of each inhibitor of ATPase activity. NEM: N-ethylemaleimide; imidazole buffer: 50 mM imidazole, pH 7.4; DI: deionized; DMSO: dimethyl sulfoxide

ATPase fraction	Inhibitor	Concentration of inhibitor (nM)	Incubation time (min)	Solvent
Na^+-K^+-ATPase	Ouabain	2.6	30	Imidazole buffer
	Ouabagenin	2.5	30	DI water
P-type ATPase	NEM	2.5	20	DI water
Amiloride-sensitive ATPase	Amiloride	0.5	30	10% DMSO in imidazole buffer

Tissue buffering capacity. *In vitro* buffering capacities (β) of crab heart and muscle were determined by basic titration according to the method of Castellini & Somero (1981). Heart (~50 to 150 mg) and white muscle (~200 to 500 mg) tissues were weighed to the nearest milligram, thawed in 10 ml of 0.9% NaCl, and homogenized with a Brinkman PT 10/35 tissue homogenizer. Homogenates were equilibrated to room temperature (19°C), and the pH of the homogenate was measured (IQ 240; Scientific Instruments). As necessary, 0.02 N HCl was added to bring the starting pH to 6. Homogenates were then titrated, with constant stirring, to a pH of 7 with 0.2 N (muscle) or 0.05 N (heart) NaOH solutions calibrated to a temperature-appropriate pH of normality (pN) against a standardized solution of HCl (Fisher Scientific). Buffering capacities were then expressed as Slykes, or µmoles of base needed to effect a 1 unit of pH change (6 to 7) in 1 g of wet tissue.

ATPase activities. ATPase activities of white muscle, heart and gill tissues were measured using a coupled pyruvate kinase/lactate dehydrogenase assay following changes in NADH absorption at 340 nm, as described in McCormick (1993), with modifications according to Goffredi & Childress (2001). Tissue aliquots (50 to 150 mg) were thawed in ~5 vol of ice-cold buffer composed of sucrose (250 mM), Na_2EDTA (10 mM), imidazole (50 mM), and sodium deoxycholate (1 mg ml^{-1}), pH = 7.40, then gently homogenized on ice in a glass douncer. Homogenates were centrifuged at 5000 × *g* for 30 s at 4°C, and supernatants were incubated at room temperature with various inhibitors (Table 1). The assay was then started by the addition of supernatant/inhibitor solutions to the ATPase activity solution containing imidazole (50 mM), phosphoenolpyruvate (PEP; 2.1 mM), reduced nicotinamide adenine dinucleotide (NADH; 0.17 mM), adenosine triphosphate (ATP; 0.53 mM), lactate dehydrogenase (LDH; EC 1.1.1.27; 3.5 U ml^{-1}), pyruvate kinase (PK; EC 2.7.1.40; 3.8 U ml^{-1}), NaCl (47.25 mM), $MgCl_2$ (2.6 mM), and KCl (21 mM), pH 7.4. Oxidation of NADH was followed spectrophotometrically (Spectramax 340 PC; Molecular Devices) for 10 min at 25°C, against standardized solutions of adenosine diphosphate (ADP). ATPase activity was normalized to protein concentration, as measured using Coomassie Blue and albumin protein standards (Bradford 1976).

Total ATPase activity was measured in each supernatant after incubation with double-distilled water. The ATPase activities of specific fractions were then derived by the subtraction of inhibited ATPase rates from total ATPase activity. Inhibition of activity was calculated as a decrease in activity beyond that caused by the solvent.

Inhibitor concentrations and incubation times were minimized (Table 1), while still achieving maximal inhibition using dose- and time-dependent curves (data not shown).

Carbonic anhydrase activity. Tissue carbonic anhydrase (CA) activity was measured as described by Henry et al. (1990), with the exceptions that homogenization was by hand in a glass douncer and homogenates (rather than supernatants) were assayed. Data acquisition (ΔpH) followed the methods of Blanchard & Grosell (2006).

Whole animal oxygen uptake. Temperature-dependent rates of oxygen consumption (MO_2) were determined via closed-cell respirometry on both crab species acclimated to normoxia. Crabs were placed individually in darkened 20 l chambers and allowed to settle for 4 to 8 h under flowing seawater of the appropriate temperature (3.8°C for *Chionoecetes tanneri* and 9.5°C for *Cancer magister*). Gentle recirculation within the chamber was driven by a submersible pump. Flow was then stopped, the chambers sealed, and oxygen drawdown was measured over a 40 to 120 min period using an Aanderaa immersion oxygen optrode (Aanderaa

Data Instruments) coupled to a PC running LabVIEW software (National Instruments). Appropriate oxygen solubility constants from Boutilier et al. (1984) and wet weights were used to calibrate mass-specific MO_2. Rates of oxygen consumption were corrected for 'blank' oxygen consumption by the experimental apparatus (~0.1% of whole animal rates) in the absence of crabs. MO_2 was measured in both species in normocapnic seawater with ~50 to 80% oxygen saturation.

Statistical analyses. All measured values are presented as means ± 1 standard error of the mean (SEM; n = number of crabs). The Shapiro-Wilks test for normality and the Levene test for homogeneity of variance were conducted on raw data prior to statistical treatment. Data meeting these assumptions were analyzed for significant differences using a 1-way ANOVA followed by a Bonferroni's *post hoc* multiple comparison test. Data not meeting parametric assumptions were compared with a Kruskal-Wallis test followed by multiple comparison testing according to the method of Dunn (1964), as described by Zar (1984). The $p < 0.05$ level was used for statistical significance in all cases. Oxygen consumption rates in the 2 species and CA activities in various tissues were compared by an unpaired 2-tailed Student's *t*-test.

RESULTS

ATPase activities

Prior to hypercapnic exposure, all forms of ATPase activity in the posterior gills of the crab species studied (including total ATPase, Na^+-K^+-ATPase, and amiloride-sensitive ATPase) were significantly higher in *Cancer magister* than in *Chionoecetes tanneri* acclimated to either oxygen regime (Fig. 1). ATPase activities in *C. tanneri* posterior gills were 2- to 5-fold lower than in *C. magister* gills.

In the posterior gill of *Cancer magister*, 24 h of high-level hypercapnia had an inhibitory effect on all forms of ATPase activity (Fig. 1). Conversely, ATPase activities in *Chionoecetes tanneri* gills were unaffected by hypercapnia (Fig. 1).

Muscle ATPase activity followed a different pattern, with similar pre- and post-hypercapnic levels of total ATPase activity across the 3 treatments (Fig. 2). Pre-hypercapnia, however, both amiloride- and NEM-sensitive ATPase activities were significantly lower in white muscle from *Chionoecetes tanneri* acclimated to normoxia (Fig. 2).

Measurements of total and amiloride-sensitive ATPase activity for crab heart tissues showed no difference across the 3 treatments either prior to or following short-term hypercapnic exposure (Fig. 3).

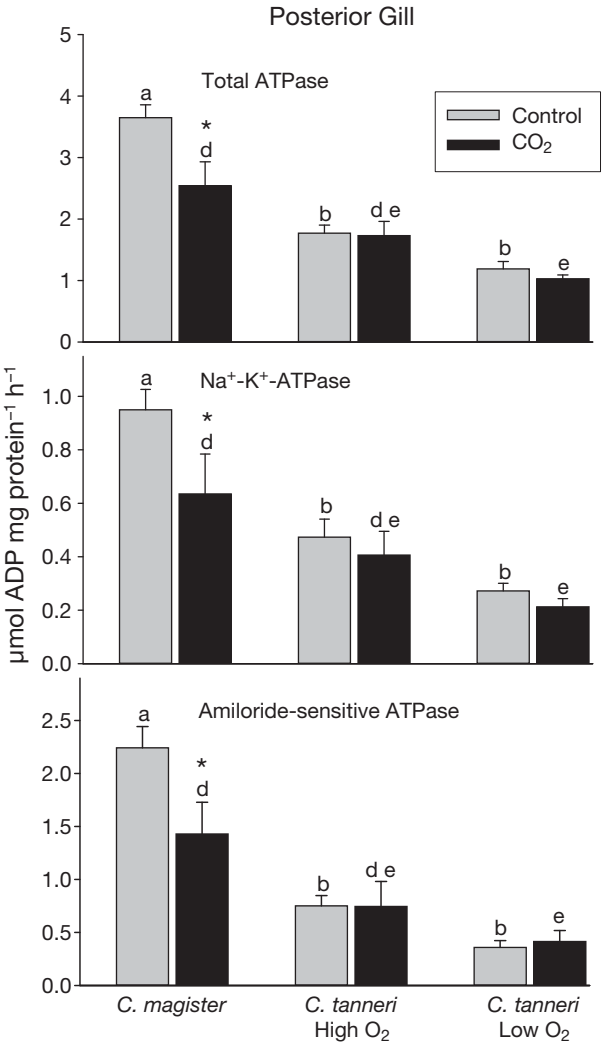

Fig. 1. *Cancer magister* and *Chionoecetes tanneri*. ATPase activities in the posterior gills of *C. magister* and of *C. tanneri* held under 2 different oxygen regimes, both before and after short-term (24 h), high-level (1%) hypercapnic exposure. Bars not sharing the same letter within the same treatment (pre- or post-hypercapnia) are significantly different. Asterisk denotes a significant difference between pre- and post-hypercapnic responses. Data are means ± 1 SEM (n = 4 to 6): total ATPase, Na^+-K^+-ATPase and amiloride-sensitive ATPase

Tissue buffering capacities

Prior to hypercapnic exposure, *in vitro* non-bicarbonate buffering capacity of crab muscle tissue (β) mimicked the pattern for NEM- and amiloride-sensitive muscle ATPase activity (Fig. 4; cf. Fig. 2). The buffering capacity of pre-hypercapnic white muscle was significantly lower (~30% reduction) in normoxic *Chionoecetes tanneri* than of muscle tissue from *Cancer magister*. However, 24 h of short-term hypercapnia negated this difference, resulting in similar post-hypercapnic β-levels among the 3 treatments.

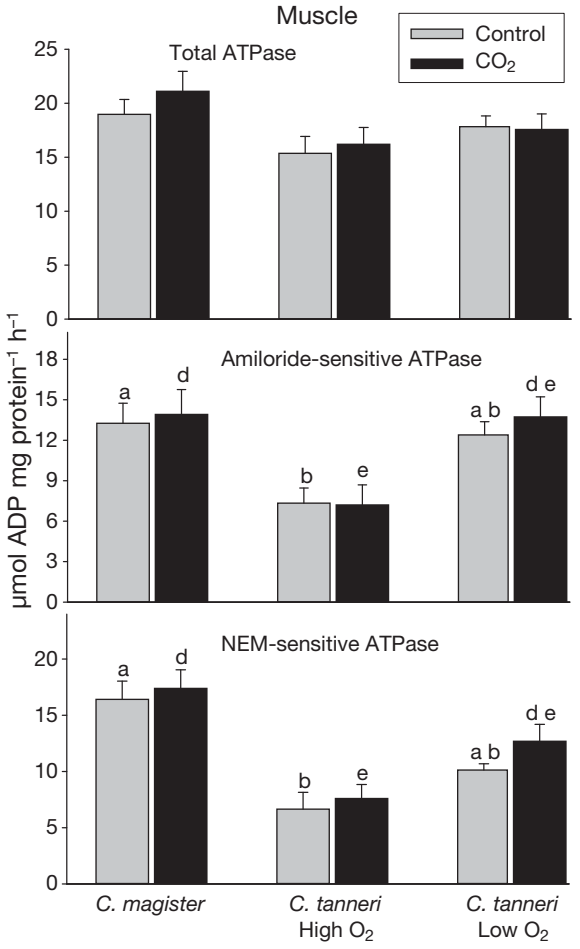

Fig. 2. *Cancer magister* and *Chionoecetes tanneri*. ATPase activities in white muscle of *C. magister*, and of *C. tanneri* held under 2 different oxygen regimes, both before and after short-term (24 h), high-level (1%) hypercapnic exposure. Bars not sharing the same letter within the same treatment (pre- or post-hypercapnia) are significantly different. Data are means ± 1 SEM (n = 4 to 6): total ATPase, amiloride-sensitive ATPase and N-ethylemaleimide (NEM)-sensitive ATPase

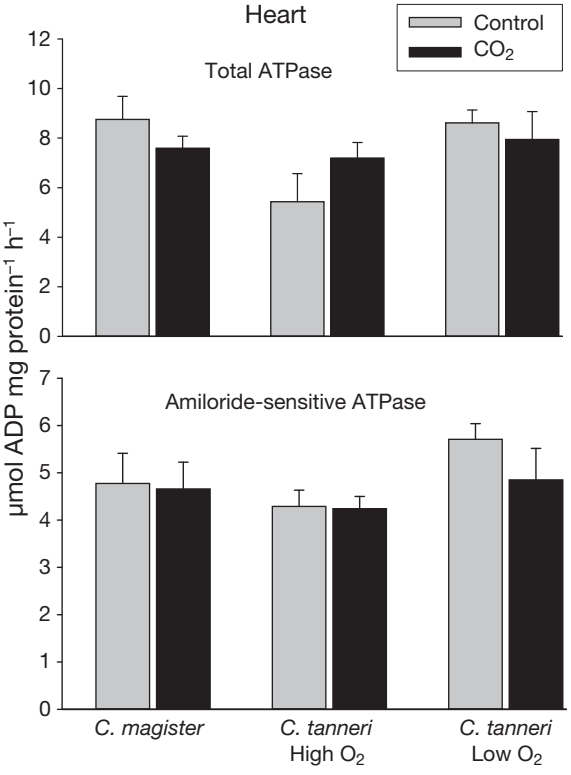

Fig. 3. *Cancer magister* and *Chionoecetes tanneri*. ATPase activities in hearts of *C. magister* and of *C. tanneri* held under 2 different oxygen regimes, both before and after short-term (24 h), high-level (1%) hypercapnic exposure. Data are means ± 1 SEM (n = 4 to 6): total ATPase and amiloride-sensitive ATPase

The buffering capacity of crab heart tissue did not vary significantly among the 3 treatments prior to or following acute exposure to elevated levels of CO_2 (Fig. 4).

Carbonic anhydrase activities

Normocapnic levels of tissue CA activity were compared between *Cancer magister* held in normoxic seawater and *Chionoecetes tanneri* kept in low oxygen (10% saturation), mimicking typical *in situ* conditions. In white muscle and heart tissues, *C. magister* CA activity was approximately 3 times higher than that measured for *C. tanneri*, with the difference approaching a 14-fold increase in posterior gills (Fig. 5).

Whole animal oxygen uptake

Chionoecetes tanneri acclimated to normoxia exhibited mass-specific rates of oxygen consumption (MO_2) significantly lower (4.5-fold) than did *Cancer magister* similarly acclimated. MO_2 in *C. tanneri* was 0.51 ± 0.09 μmol O_2 g^{-1} h^{-1} (n = 8) versus 2.31 ± 0.51 μmol O_2 g^{-1} h^{-1} (n = 6) in *C. magister*.

DISCUSSION

Oxygen uptake experiments using whole animals confirmed the hypometabolism of the deep-sea Tanner crab *Chionoecetes tanneri* when compared to the nearshore Dungeness crab *Cancer magister*. Though a temperature difference of about 5.5 to 6.0°C was a necessary respirometric variable due to thermal tolerances, particularly in Tanner crabs, direct temperature corrections of the respirometric data to a median temperature of 6.7°C, using either a Q_{10} of 2.0 or the method of Gillooly et al. (2001), yield either a 3.0- or 2.8-fold difference, respectively, in MO_2 between the 2 species.

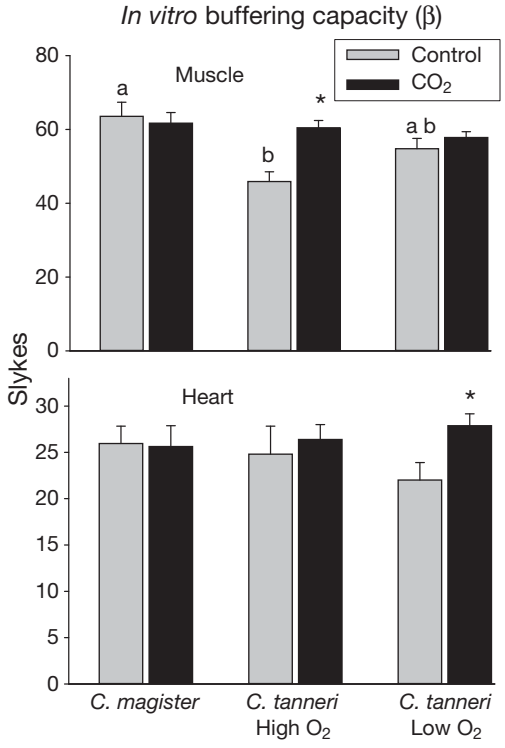

Fig. 4. *Cancer magister* and *Chionoecetes tanneri*. *In vitro* non-bicarbonate buffering capacity (β) of tissues from *C. magister*, and from *C. tanneri* held under 2 different oxygen regimes, both before and after short-term (24 h), high-level (1%) hypercapnic exposure. Bars not sharing the same letter within the same treatment (pre- or post-hypercapnia) are significantly different. Asterisk denotes a significant difference between pre- and post-hypercapnic responses. Data are means ± 1 SEM (n = 5 to 6): white muscle and heart

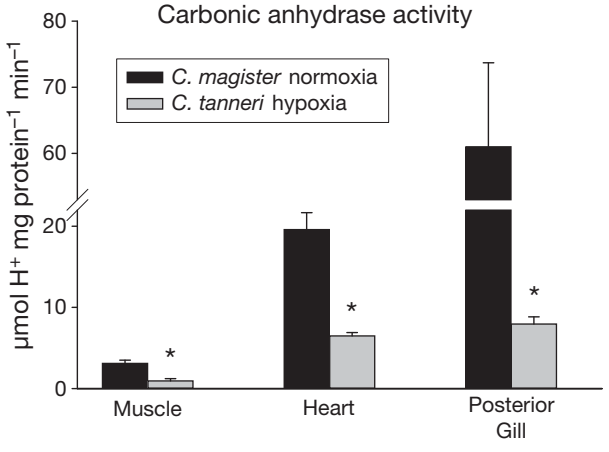

Fig. 5. *Cancer magister* and *Chionoecetes tanneri*. Carbonic anhydrase (CA) activity in tissues from *C. magister*, and from *C. tanneri* held under 2 different oxygen regimes. Asterisk denotes a tissue-specific significant difference in CA rates between species. Data are means ± 1 SEM (n = 6 to 7). Note the break in the *y*-axis

Our respirometric data coincide closely with previously published values. In the genus *Chionoecetes*, Paul & Fuji (1989) measured an oxygen consumption rate of ~0.6 µmol O_2 g^{-1} h^{-1} in *C. bairdi* at a similar temperature (4 to 5°C). For the Dungeness crab, our experimental rate of 2.3 µmol O_2 g^{-1} h^{-1} falls between 2 other Dungeness respiration rates of 1.4 (Johansen et al. 1970) and 3.0 µmol O_2 g^{-1} h^{-1} (McDonald et al. 1980), with all 3 values recorded at 10°C.

In sighted pelagic animals, the visual/predator hypothesis states that limited light with depth reduces visual predation pressure and selects for reduced locomotory abilities and metabolic capacities at depth (Seibel et al. 1997, Childress & Seibel 1998). Predominantly applicable to pelagic animals, the suitability of this theory to sluggish and hypoactive deep-sea benthic fauna such as *Chionoecetes tanneri* is less clear. While Tanner crabs have a much lower respiration rate than Dungeness crabs, even after temperature correction—data that coincide with other observations of deep-sea benthic animal metabolic rates up to an order of magnitude less than those of more near-shore counterparts (Henry et al. 1990)—the comparison in the present study is somewhat limited by phylogenetic constraints.

Rather than speculating on the putative evolutionary origins of the different metabolic rates that we observed in the 2 species, we focus here on the ecological practicality of the differences observed in MO_2. Hypometabolism in *Chionoecetes tanneri* will greatly constrain its ability to actively regulate internal acid–base status during hypercapnic exposure, a process requiring substantial metabolic energy. Many of the pre-hypercapnic differences described below, wherein Tanner crabs consistently displayed reduced enzymatic activities, may be directly attributable to hypometabolism.

When we compared the 2 most physiologically relevant treatments, normoxic Dungeness crabs versus hypoxic Tanner crabs, the deep-sea Tanner crab exhibited lower activities of branchial enzymes involved in some of the processes essential for efficient acid–base regulation. Prior to hypercapnic exposure (i.e. under normocapnic conditions), activities of all forms of ATPase and CA in the posterior gill were approximately 2- to 13.2-fold lower in Tanner crabs than in Dungeness crabs (Figs. 1 & 5). A similar scope of 1.5- to 20-fold lower activity was found in an assortment of metabolic enzymes from the deep-sea crabs *Chaceon fenneri* and *Chaceon quinquedens*, when compared to the sublittoral *Callinectes sapidus* (Walsh & Henry 1990).

In decapod crabs, the gill is the primary site for key acid–base and ionoregulatory ion transport processes (Cameron 1986, Henry & Wheatly 1992, Pequeux 1995).

In particular, the posterior gills (Gills 5 to 8) bear the brunt of this work. Unlike marine fishes (Marshall & Grosell 2005), crabs do not supplement acid–base relevant branchial ion transport with gastrointestinal tract or renal ion transport to any substantial degree (Pequeux 1995). Reductions in ATP-dependent and acid–base relevant enzymatic activity in the Tanner crab gill, then, are likely to cause substantial limitations in acid–base regulatory capacity.

The mechanisms of branchial transmembrane and transepithelial transport of acid–base relevant ions are highly conserved across animal phyla and represent one of the most important, but metabolically costly, means of defending intracellular pH (Seibel & Walsh 2001, 2003). One example of transepithelial branchial ion transport is proton exchange between extracellular fluid and seawater, whereby protons are excreted from the gill to combat internal acidosis. Protons are supplied by CA, which hydrates CO_2 to form carbonic acid, which then dissociates to H^+ and HCO_3^- ions available for transport. Very low CA activity in the posterior gills (and other tissues) of the deep-sea Tanner crab (Fig. 5) coincides with physiological data demonstrating uncompensated extracellular acidosis in this species exposed to short-term, high-level hypercapnia (Pane & Barry 2007).

Another important component to branchial proton excretion is sodium-proton antiport, accomplished by the sodium proton exchanger NHE. Like many transport processes, sodium-proton exchange relies on Na^+ gradients established in large by Na^+-K^+-ATPase. Both the more traditional cardiac glycoside-sensitive fraction of Na^+-K^+-ATPase activity assayed using the inhibitors ouabain and ouabagenin (Goffredi & Childress 2001; Fig. 1) and an amiloride-sensitive fraction (Soltoff & Mandel 1983, Ellis-Davies et al. 1996; Fig. 1) were markedly lower in hypoxic Tanner crabs than in normoxic Dungeness crabs, further suggesting inefficient acid–base (and ionoregulatory) transport processes.

In the low micromolar range, amiloride is a potent inhibitor of sodium-proton exchange. In the low millimolar range, however, amiloride targets Na^+-K^+-ATPase, directly inhibiting its activity (Soltoff & Mandel 1983). At an amiloride concentration of 2.5 mM, we observed a 20 to 60% inhibition of Na^+-K^+-ATPase activity across species, tissues, and treatments. Using 2 mM amiloride, Soltoff & Mandel (1983) observed a 30 to 45% inhibition of Na^+-K^+-ATPase activity in isolated rabbit kidney proximal tubules. We have therefore used amiloride as an additional pharmacological tool to demonstrate reduced ATP-dependent enzymatic activity in gill and muscle tissue of deep-sea Tanner crabs.

It is important to note, however, that amiloride is a highly non-specific inhibitor of Na^+-K^+-ATPase. There is some evidence that an amiloride-sensitive fraction of Na^+-K^+-ATPase activity may overlap with the more traditional cardiac glycoside (ouabain or ouabagenin)-sensitive ATPase activity (Epstein & Lechene 1988), but the mechanisms of amiloride inhibition are, to date, poorly characterized. This leaves the possibility that amiloride, applied to locomotory or contractile tissue such as white muscle and heart, respectively, may inhibit a substantial portion of ATPase activity related to these muscular activities, but may not be particularly associated with transmembrane transport of acid–base relevant ions. However, when applied to white muscle, amiloride and the highly specific P-type inhibitor NEM show very similar patterns of inhibition (Fig. 2), suggesting that these 2 inhibitors may target similar (P-type) fractions of ATPase activity.

A second diagnostic comparison was made between the 2 species of crab, both held in normoxia. In this comparison, significant pre-hypercapnic differences appeared between the species in white muscle enzymatic activities (Fig. 2) and non-bicarbonate tissue buffering (β) capacity (Fig. 4). In addition to the white muscle amiloride-sensitive ATPase fraction described above, the more broad P-type ATPase activity, strongly sensitive to NEM inhibition (Lin & Randall 1993, Gerencser & Zhang 2001, Goffredi & Childress 2001), was significantly lower in normoxic Tanner crab white muscle (Fig. 2). Na^+-K^+-ATPase activity, being an abundant P-type (membrane bound) ATPase, likely comprises a large fraction of the NEM-sensitive activity observed, though there are several other P-type ATPases with certain acid–base and iono-regulatory importance. These include H^+-ATPase, shown to be NEM sensitive (Lin & Randall 1993) and critical to proton export for intracellular pH regulation, as well as P-type Cl^--ATPase (Gerencser & Zhang 2001), which is also NEM sensitive and critical to anion (and indirectly HCO_3^-) balance. P-type metal ATPases such as Zn-, Cu-, or Co-ATPase also have key ionoregulatory functions, and may be secondarily important in acid–base balance.

It is of interest that significantly reduced amiloride- and NEM-sensitive ATPase activity in Tanner crab muscle occurred only in the normoxic comparison. Similar results were found for Tanner crab muscle buffering capacity, suggesting that exposure to relatively high levels of oxygen effected these changes. We speculate that the reduced β levels in Tanner crab muscle are a direct function of normoxia. Tanner crabs acclimated to typical hypoxic *in situ* conditions of the oxygen minimum zone would be exposed to anomalously high oxygen levels when exposed to normoxic seawater, resulting in a highly aerobic physiological state. Passive buffering is one of the more straightforward means of defending intracellular pH (Seibel

& Walsh 2001), and resting β levels in muscle are inversely correlated to the extent to which a tissue experiences anaerobisis (Castellini & Somero 1981, Henry & Wheatly 1992, Wheatly & Henry 1992). Therefore, our Tanner crabs held at relatively high oxygen values (normoxia) and presumably not under the threat of anaerobisis, exhibited a lower resting muscle β than the more metabolically active Dungeness crabs held under normoxia, or Tanner crabs held in hypoxic seawater.

The observed reductions in amiloride- and NEM-specific muscle ATPase under prehypercapnic conditions (Fig. 2) may also be a function of normoxic conditions in the laboratory. However, the connection between oxygen levels and depressed rates of muscle ATPase activity is less clear and a subject for further study, including a detailed analysis of ion gradients in the muscle of Tanner crabs held under different oxygen regimes.

A notable physiological effect of short-term hypercapnia was a 30 to 40% reduction in all forms of posterior gill ATPase activity in Dungeness crabs (Fig. 1). A number of studies have shown that CO_2 can cause metabolic suppression in invertebrates (Barnhart & McMahon 1988, Barnhart 1989, Rees & Hand 1990, Michaelidis et al. 2005), though substantial reductions in extracellular (blood or hemolymph) pH appear to drive this reduction (Michaelidis et al. 2005). Within 24 h, Dungeness crabs exposed to high-level CO_2 (1%) completely restored extracellular pH to normocapnic values (Pane & Barry 2007), indicating that CO_2 may have a more direct inhibitory effect on branchial ATPase activity in this species. Alternatively, branchial ATPase activity in the sublittoral Dungeness crab may be indirectly inhibited by the marked shift in proton (and other ion) gradients across the gill caused by hypercapnic exposure.

No reduction in ATPase activity following short-term (24 h) hypercapnia was observed in any Tanner crab tissue. This may be a direct function of hypometabolism in this species, where such a minimal aerobic scope for activity limits the ability to tune metabolic rate to changing environmental conditions.

In a previous study (Pane & Barry 2007), both hypoxic- and normoxic-adapted Tanner crabs experienced an uncompensated 0.4 unit drop in extracellular pH during the same CO_2 exposure regime. In light of these previous findings, the increase in passive buffering capacity (β) of muscle from Tanner crabs acclimated only to normoxia (Fig. 4) may indicate an effort in this treatment to defend intracellular pH after 24 h of hypercapnia. In Tanner crabs acclimated to hypoxia, pre-hypercapnic β-levels were higher and subsequently unchanged by hypercapnia (Fig. 4), suggesting that hypoxia pre-conditioned the buffering capacity of Tanner crab muscle. Such an effect is consistent with the relationship described above, wherein the hypoxic Tanner crab, presumably better conditioned to anaerobic events, maintains a higher muscle buffering capacity and is therefore better suited to defend intracellular pH by passive means during extracellular acidosis (Seibel & Walsh 2001, 2003).

CONCLUSIONS

The specific effects of hypercapnia on crab tissue were less revealing than the marked pre-hypercapnic, interspecific differences between the deep-sea Tanner crab and the Dungeness crab. We have previously reported (Pane & Barry 2007) that extracellular acid–base regulatory strategies between the 2 species during short-term hypercapnia are a study in contrast. The Dungeness crab perfectly compensated extracellular pH during hypercapnia, while the Tanner crab left extracellular pH undefended. Presumably, Tanner crabs use their limited metabolic energy to defend the more critical intracellular pH, as is the case in other sluggish aquatic animals (Brauner et al. 2004); this is an area we are currently investigating.

Even with adequate intracellular pH regulation, the current data provide a very strong explanation for inefficient regulation of hemolymph pH in Tanner crabs. The reduced scope of enzymatic (particularly branchial) activities in Tanner crabs, consistent with hypometabolism, render these animals incapable of efficient transport of ionic and acid–base equivalents to combat short-term hypercapnia. Under a carbon sequestration scenario to offset rising atmospheric CO_2 levels, this is exactly the situation that deep-sea benthic fauna may face. This scenario calls for the injection of large amounts of liquid CO_2 into the deep sea, either on the bottom or as a CO_2-rich mid-depth plume (Adams et al. 1998). Either scenario or the mixing of anthropogenic carbon from surface waters to the deep sea will result in significant reductions in ocean pH, particularly near CO_2 injection sites.

While our results indicate that Tanner crabs will be unable to defend extracellular pH during acute hypercapnia, the story is less clear for putative chronic (permanent) hypercapnia. If chronic hypercapnia proves to be equally disturbing to the extracellular acid–base status of this deep-water species, the pursuant costs of such a compromise are likely to include impaired oxygen transport, tissue oxygenation and overall metabolic capacity, reduced growth and reproductive capacity, and possible implications for prey capture and predator avoidance.

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