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Effects of environmental hypercapnia on animal physiology: A ^{13}C NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*

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Abstract

Global climate change is associated with a progressive rise in ocean CO_2 concentrations (hypercapnia) and, consequently, a drop in seawater pH. However, a comprehensive picture of the physiological mechanisms affected by chronic CO_2 stress in marine biota is still lacking. Here we present an analysis of protein biosynthesis rates in isolated muscle of the marine invertebrate *Sipunculus nudus*, a sediment dwelling worm living at various water depths. We followed the incorporation of ^{13}C-labelled phenylalanine into muscular protein via high-resolution NMR spectroscopy. Protein synthesis decreased by about 60% at a medium pH of 6.70 and a consequently lowered intracellular pH (pHi). The decrease in protein synthesis rates is much stronger than the concomitant suppression of protein degradation (60% versus 10–15%) possibly posing a threat to the cellular homeostasis of structural as well as functional proteins. Considering the progressive rise in ocean CO_2 concentrations, permanent disturbances of cellular protein turnover might seriously affect growth and reproductive performance in many marine organisms with as yet unexplored impacts on species density and composition in marine ecosystems.

Keywords: Hypercapnia; Protein synthesis; ^{13}C NMR spectroscopy; Marine invertebrate; Respiratory acidosis; Metabolic depression

1. Introduction

Growth of living organisms is usually defined as an increase in cell number and/or cell size in parallel with a positive change in caloric content (Mommsen and Moon, 2001). This long-term process largely depends on cellular protein synthesis, builds a key prerequisite for successful reproduction and thus supports the maintenance of populations. During individual growth, protein synthesis will exceed protein breakdown. Once net growth has ceased, protein turnover is in a steady state where most functional proteins are continually replaced. The energetic expenditure that is required to fuel this process is enormously high: calculations by Hawkins (1991) demonstrated a minimal fraction of about 20% for the contribution of protein synthesis to whole body maintenance metabolism in a wide range of species, comprising mammals, fish and mussels. Numerous biotic and abiotic factors control the partitioning of resources into growth ranging from the individuals' genetic background through animal density and food quality to temperature and environmental pollutants (Mommsen and Moon, 2001).

Under conditions of environmental stress, many organisms display specific strategies of metabolic energy conservation and extended passive survival that involve the depression of both energy producing and energy consuming cellular processes (for a review see Hand and Hardewig, 1996). In the light of its large contribution to maintenance costs protein synthesis is one target for inhibition during metabolic depression. Oxygen limitation, for instance, has been demonstrated to depress protein synthesis in rat liver (Surks and Berkowitz, 1971). Protozoan cells like *Tetrahymena* decrease protein synthesis by up to 70% during starvation (Cuny et al., 1985).

Hypercapnia (elevated CO_2 partial pressure) correlates with adverse factors like hypoxia and elevated temperature and, until now, represents a transient stress factor in some marine environments like the sediments of the intertidal zone (Diaz and Rosenburg, 1995), hypoxic bottom waters (Knoll et al., 1996) and

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intertidal pools (Truchot and Duhamel-Jouve, 1980). Currently rising concentrations of CO_2 in atmosphere and surface waters (Haugan and Drange, 1996; Brewer, 1997; Caldeira and Wickett, 2003) as well as anticipated scenarios of anthropogenic CO_2 disposal in the deep sea (Marchetti, 1979; Auerbach et al., 1996) indicate that hypercapnia will play a more important and permanent role in shaping the structure and functioning of marine ecosystems. Understanding the effects of CO_2 requires a thorough understanding of the physiological mechanisms through which CO_2 exerts its effects on organismic, population and thus, ecosystem functioning (Pörtner et al., 2004, 2005).

In marine invertebrates, hypercapnia has been found to elicit metabolic depression (through an acidification of body fluids; Reipschläger et al., 1997; Pörtner et al., 1998, 2000) as well as growth reductions, observed by Shirayama (2002), Michaelidis et al. (2005). An extreme case of hypercapnia and/or anoxia induced hypometabolism can be found in brine shrimp (*Artemia franciscana*) embryos associated with an almost complete shutdown of protein synthesis due to global arrest of translation and a drastic reduction of transcription (Hofmann and Hand, 1994; Van Breukelen et al., 2000). However, the effects of hypercapnia alone on protein synthesis have neither been studied in adult brine shrimp nor in marine ectotherms in general and the effective physiological parameters have not been identified. In this context, the question needs to be addressed, whether the effects of CO_2 on growth occur through an imbalance between whole body protein synthesis and degradation.

The present paper addresses the effects of elevated CO_2 levels on protein synthesis in the marine invertebrate, *Sipunuclus nudus*, a model species in previous efforts to investigate CO_2 effects in a non-calcifying organism. *S. nudus* is adapted to regular CO_2 oscillations in some of its natural habitats. Previous studies have focused on short to medium-term effects such as acid–base regulation and metabolic rate (see above) or changes in aerobic energy metabolism of specific tissues (Langenbuch and Pörtner, 2002, 2003). Long-term consequences include enhanced mortality (Langenbuch and Pörtner, 2004), possibly due to disturbances of protein metabolism. A decrease in N-excretion and in O/N ratios of isolated muscle tissue paralleled metabolic depression at reduced extracellular pH (pHe) and suggested associated changes in amino acid catabolism (Langenbuch and Pörtner, 2002).

In the present study we analysed the cellular background of metabolic depression at high water PCO_2 with a focus on the possible down-regulation of protein biosynthesis. To this end we measured the incorporation of ^{13}C-labelled phenylalanine (Phe) into the cellular protein pool. We report a pH-dependent decline in protein biosynthesis rates at high CO_2 levels. Results are

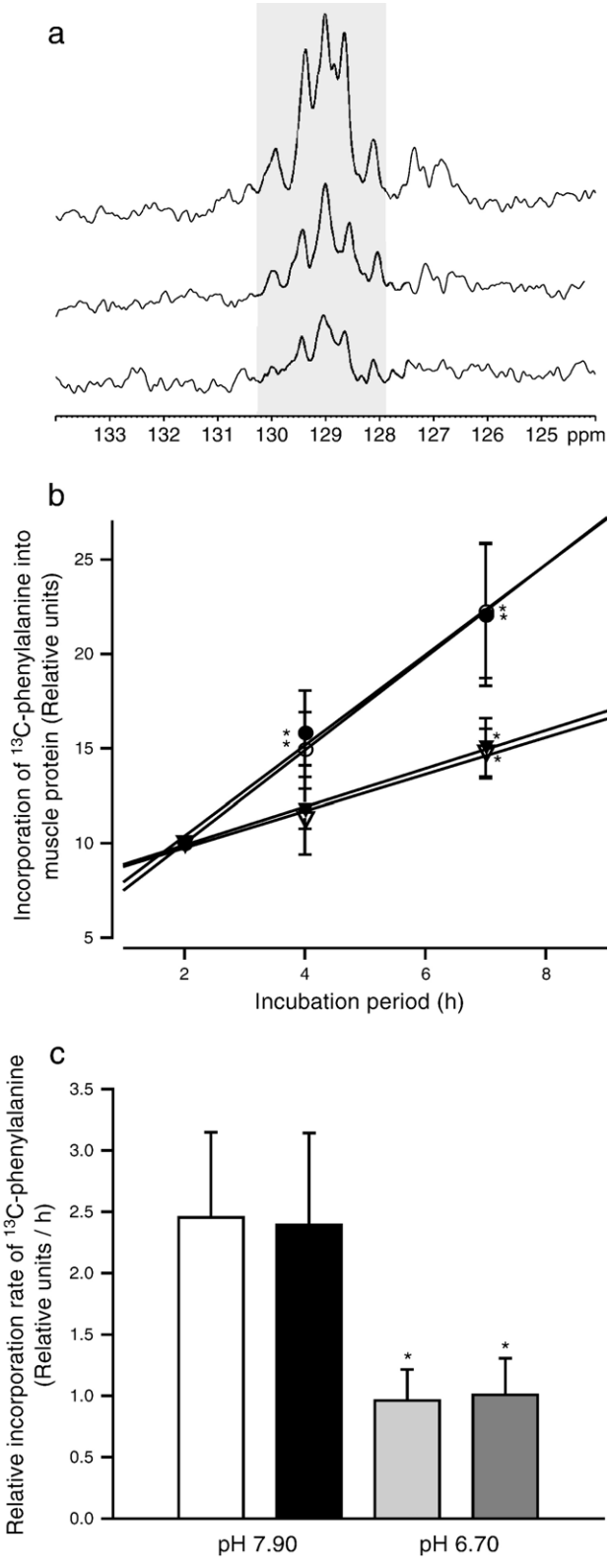

Fig. 1. Impact of PCO_2 and pHe on incorporation of ^{13}C labelled phenylalanine into muscle protein. a) Intercepts of ^{13}C NMR spectra of protein extracts from tissue samples of one animal depicting increasing signals of aromatic carbon nuclei from incorporated ^{13}C-Phe after labelling periods of 2 h, 4 h and 7 h, respectively. Peak area has been integrated over the range of the shaded area. b) Incorporation of labelled Phe into muscle protein over time. The relative amount of ^{13}C-Phe as computed from peak area integration of protein ^{13}C NMR spectra is plotted over time. Different symbols represent results for various experimental pH/PCO_2 conditions (i.e. filled circle, pH 7.90, PCO_2 0.01 kPa; hollow circle, pH 7.90, PCO_2 1.01 kPa; filled triangle, pH 6.70, PCO_2 0.03 kPa, hollow triangle, pH 6.70, PCO_2 1.01 kPa). * Indicates values of incorporated ^{13}C-Phe significantly different from the respective control value after 2 h of labelled substrate incubation. c) Relative incorporation rates of ^{13}C-Phe into muscle protein as calculated from regression lines in Fig. 1b. White and black bars represent values at control pH 7.90 and low (0.03 kPa) or high (1.01 kPa) PCO_2, respectively; light and dark grey bars depict the respective data for pH 6.70. Note that the significant 60% decrease (indicated by *) in incorporation rates is (as in b) directly attributable to the pH treatment without any influence of the respective PCO_2 level.

discussed in the context of data from our previous work to shed light on the effects of hypercapnia on overall protein metabolism as well as animal survival in their natural environment.

2. Materials and methods

2.1. Animals

Large specimens (20–60 g) of *S. nudus* L. were dug up from sandy sediments of the intertidal zone in Locquémeau, Brittany (France) in November 2000. The animals were kept in aquaria with natural seawater and a bottom layer of sand (10–15 cm) from the original habitat for several weeks before the beginning of the experiments. Aquaria were supplied with aerated and recirculated seawater at 10–15 °C.

2.2. Preparation and incubation of isolated muscle tissue

For the preparation of isolated body wall musculature individuals were killed by "decapitation" behind the base of the introvert retractor muscles. The animals were opened dorsally and all inner organs including the ventral nerve cord were removed. The body wall musculature was cut transversally to obtain three more or less equally sized pieces. The tissue then was fixed with a fine needle and thread onto a plastic frame to ensure full equilibration with the ambient medium. Previous analyses had shown that the energy status of the isolated tissue remains undisturbed during 48 h at least (Pörtner et al., 2000).

Each tissue preparation was first subjected to an incubation period of 45 h at the respective pH and PCO_2 to enable new steady state intracellular acid–base parameters. For each incubation, frames with fixed tissue of four animals were divided between three racks (constituting three time points in the subsequent phenylalanine incubation). The racks were placed together in a closed recirculation system containing a volume of 8 L of 34‰ artificial seawater (455 mM NaCl, 10 mM KCl, 24 mM $MgCl_2$, 10 mM $CaCl_2$, 28 mM $MgSO_4$) with 0.1 g L^{-1} streptomycin, 10^5 I.U. L^{-1} penicillin and 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonicacid] at a temperature of 15±0.5 °C. The medium was equilibrated and bubbled continuously with either normocapnic (40% air, 60% nitrogen; PCO_2 0.03 kPa) or hypercapnic (40% air, 59% nitrogen, 1% CO_2; PCO_2 1.01 kPa) gas mixtures supplied by a gas mixing pump (2M303/a-F, Wösthoff, Germany). Moderate hypoxia (847 kPa PO_2) was chosen since normoxic PO_2 levels can be damaging to this sediment dwelling animal. Both CO_2 treatments were carried out at medium pH (pHe) 7.90 (control conditions) and 6.70 (severe acidosis). Normocapnic as well as hypercapnic solutions were equilibrated with the corresponding gas mixture for several hours and pH was adjusted by the addition of solid $NaHCO_3$. The appropriate amount was calculated from the Henderson–Hasselbalch equation using a value of pK′ determined according to Heisler (1986). Medium pH was checked at the beginning and at the end of the experiment to make sure that variations remained within ±0.03 pH units of the initial value.

Subsequently, tissues were subjected to a second incubation in artificial seawater at the respective pH and PCO_2 values, which contained 3 mM U-$^{13}C_9$ L-phenylalanine (97–98% uniformly labelling of all carbon isotopes; Cambridge Isotope Laboratories). Each rack carrying four samples was incubated in a closed and continuously bubbled container of 150 mL volume for 2 h, 4 h or 7 h, respectively. Finally, tissue samples were quickly washed with artificial seawater devoid of phenylalanine, freeze-clamped and stored under liquid nitrogen for further analyses.

Table 1
Intra- and extracellular acid–base variables of *S. nudus* isolated muscle tissue under norm and hypercapnia, measured after long-term exposure to media of different pH and PCO_2 (data adopted from Langenbuch and Pörtner, 2002)

pHe	*P*CO2e (kPa)	pHi	*P*CO2i (kPa)	[HCO3 i] (mM)
7.9	0.03	7.29±0.02	0.31±0.05	1.35±0.18
7.9	1.01	7.42±0.03	2.58±0.27	15.31±1.27
7.2	0.03	7.06±0.04	0.49±0.05	1.25±0.21
7.2	1.01	7.18±0.01	1.38±0.13	4.56±0.52
6.7	0.03	6.91±0.03	0.46±0.16	0.81±0.24
6.7	1.01	6.99±0.03	0.30±0.03	2.25±0.24

Values are means±S.D., N=5–6, except for extracellular pH and PCO_2, which characterize the respective media. Apparent mean intracellular bicarbonate concentrations were calculated from measured pHi using values of pK′′′ and the solubility coefficient α_{CO2} determined according to Heisler (1986).

2.3. Tissue extracts

To obtain perchloric acid (PCA) extracts, tissue samples were ground under liquid nitrogen and the frozen powder (0.8–1.2 g) was added to 2.5 mL of precooled 12% PCA in 15 mL test tubes. After adding 3.5 mL ice-cold PCA, the suspension was homogenized for 3–10 s on ice using an Ultra-Turrax T8 (IKA, Germany). After centrifugation (15 min, 1500 *g*, 0 °C), the aqueous layer was removed, neutralized to pH 7.5 by the addition of 5 M KOH and lyophilized. The residual precipitated protein as well as cell remnants were resuspended in 8 mL H_2O, neutralized again and the supernatant also lyophilized for NMR spectroscopic studies. In so doing, every tissue sample yields a protein fraction as well as cytosolic fraction containing water-soluble metabolites, but no protein.

2.4. NMR spectroscopy

Lyophilized protein and cytosolic PCA extracts were redissolved in 1 mL D_2O per 1.38 g tissue wet weight. Spectra were recorded on a Bruker 9.4 T Avance NMR spectrometer, operating at frequencies of 400 MHz for ^1H and 100.6 MHz for ^{13}C measureents. All experiments were performed using a 5 mm ^1H/BBI probe. ^1H: 400 scans; flip angle 90°; relaxation delay 11.0 s; spectral width 8250 Hz; data size 64 K. ^{13}C: 6100 scans for cytosolic extracts, 16400 scans for protein fractions; flip angle 90°; relaxation delay 2.0 s; spectral width 24000 Hz; data size 64 K; composite pulse decoupling.

The assignment of Phe signals in one-dimensional ^{13}C NMR spectra was confirmed by additional two-dimensional chemical shift correlation $^1H/^{13}C$ NMR experiments. ^{13}C enrichment in cellular pools of free and incorporated Phe was determined from peak area integration of aromatic carbon nuclei signals in ^{13}C NMR spectra (see Fig. 1a). For the comparison of treatments

data were calibrated to an arbitrary value of 10 relative units per gram wet weight used for extract preparation in all 2 h samples.

2.5. Statistics

For each treatment (hypercapnia, normocapnia), relative Phe incorporation as well as protein synthesis rates under control and experimental conditions were compared using two-factorial analysis of variance (ANOVA). When a significant influence of a single variable was indicated by ANOVA, the different treatments were compared using the Tukeys test. In all cases, $P<0.05$ was accepted to indicate a significant difference. All values are calculated as means±SD, $N=4–5$.

3. Results

In accordance with our previous work (Langenbuch and Pörtner, 2002) identical tissue incubation conditions were chosen to clamp steady-state intracellular acid–base variables by controlling pHe and PCO_2 of the medium. In *S. nudus*, falling medium pH causes a linear decrease in pHi with hypercapnic values always significantly higher than normocapnic values due to the larger accumulation of intracellular HCO_3^- under hypercapnia (see Langenbuch and Pörtner, 2002; cf. Table 1).

Using this procedure, we tracked the incorporation of uniformly labelled [U-^{13}C]-L-phenylalanine into muscle protein by use of NMR spectroscopy under various clearly defined acid–base conditions. PCA extraction of frozen tissue yielded fractionated samples of low molecular weight cytosolic compounds as well as of tissue protein (mostly myofibrillar) that were used to record ^{1}H and ^{13}C NMR spectra.

For an analysis of the pH dependence of tissue protein synthesis rates muscle samples were exposed to an excess of labelled Phe throughout the incorporation period to equilibrate all intra- and extracellular pools of the amino acid (according to the "flooding dose" approach; Davis et al., 1999). A linear increase of the ^{13}C-Phe signal in muscle protein fractions over time (cf. Fig. 1b) as well as identical cytosolic labelling conditions (data not shown) in all experimental treatments indicate that protein synthesis was not at any time limited by substrate availability and that recordings occurred under conditions where the applied concentration was well above saturation levels for protein synthesis. Intracellular levels of marked phenylalanine result several-fold higher than control levels (virtually zero) within 120 min. An excessive steady state value of ^{13}C-Phe could be observed inside the cytosol after about 4 h under all conditions. The pattern of accumulation of labelled Phe into the cytosolic compartment of muscle tissue was essentially the same in control as well as in all three experimental approaches (ANOVA; $P=0.625$) indicating a significant increase in cytosolic Phe under all conditions (ANOVA; $P<0.001$). In addition, we were not able to detect a diversion of labelled amino acid into catabolic processes of energy metabolism. The cytosolic pattern of ^{13}C-signals did not change even in spectra of samples exposed to labelled substrate for 7 h.

Relative protein biosynthesis rates were quantified following the incorporation of labelled Phe into the tissue protein pool (Fig. 1a,b). ^{13}C-Phe accumulated linearly in cellular protein of control (pHe 7.90) as well as acidotic (pHe 6.70) muscle samples under normo- (0.03 kPa PCO_2) and hypercapnia (1.01 kPa PCO_2). Nevertheless, the increase of incorporated ^{13}C-Phe under control conditions was almost three times faster than under acidosis. Starting from ten relative units levels reached about 15 U after 4 h at pHe 7.90 while at low pHe only approximately 11.5 U were reached. The same picture emerges from a direct comparison of the slopes of regression curves that mirror the relative rates of incorporation of labelled Phe into cellular protein (see Fig. 1c). At pHe 7.90 an incorporation rate of 2.45±0.69 relative units/h was found in normocapnic tissue samples while this rate was significantly depressed by approximately 61% to a level of 0.96±0.25 relative units/h at pHe 6.70 (ANOVA; $P=0.011$). In contrast to the effects of experimental pH (control as well as acidotic) the two PCO_2 treatments at constant pH did not result in different incorporation rates of labelled Phe, evident from a rate of 2.39±0.75 relative units/h under high pHe and PCO_2 conditions. Under hypercapnia, pH dependent values also decreased by about 56% at low pHe to 1.01±0.30 relative units/h (ANOVA; $P=0.004$). Statistical analyses revealed no significant differences between normo- and hypercapnic samples of muscle tissue with respect to the effects of pHe (ANOVA; $P=0.877$ for pHe 7.90; $P=0.906$ for pHe 6.70). Considering the present results, this analysis clearly identifies pH rather than PCO_2 to be effective at the level of cellular protein synthesis.

4. Discussion

The present study aims to delineate the effects of respiratory versus non-respiratory acid–base disturbances on cellular protein synthesis of isolated muscle tissue of the marine invertebrate *S. nudus*. Data collected in isolated muscle tissue can be regarded representative for the whole organism as body wall musculature comprises two thirds of total body tissue in *S. nudus* (Pörtner, 1987).

S. nudus displays various degrees of metabolic depression depending on the degree of CO_2 elevation. At moderate levels of 1.01 kPa PCO_2 animals were still able to completely compensate for the initial disturbance of intracellular pH (pHi) within 48 h, despite a constantly reduced value of extracellular pH (pHe). The 20% reduction of aerobic energy turnover observed under these conditions in whole animals as well as in isolated muscle preparations (Reipschläger et al., 1997; Pörtner et al., 1998) can be largely attributed to the pHe dependent inhibition of Na^+/H^+exchangers (Grinstein and Rothstein, 1986) and other membrane proteins involved in pHi regulation (Pörtner et al., 2000). However, a further depression of aerobic metabolic rates to about 55% of normocapnic control values occurred under severe acidosis (at pHe 6.70 which is associated with a net drop in pHi by about 0.4 U in muscle tissue; cf. Table 1; Langenbuch and Pörtner, 2002). This depression can only be explained by the down regulation of additional ATP-consuming cellular processes. A depression of both anabolic (this paper) as well as catabolic (Langenbuch and Pörtner, 2002) processes of protein metabolism occurs in isolated muscle tissue of *S. nudus*. The present analysis clearly revealed a significant 60% reduction in tissue protein biosynthesis rates due to low pH

and independent of the respective CO_2 treatment. The effective decline in overall protein turnover thus contributes to energy conservation during metabolic depression.

Up to now, a direct influence of pH on global rates of cellular protein synthesis has, for instance, been found in fish tissues (Reid et al., 1997), rat cardiomyocytes (Schlüter et al., 1998) and in *Artemia franciscana* dormant embryos (Kwast and Hand, 1996; Hand, 1997). The mechanisms of down-regulation have only been examined in the case of the global arrest of protein synthesis during diapause of embryonic brine shrimps, but not in other metazoans and not in adult specimens altogether. Hofmann and Hand (1992, 1994) suggested that a pHi dependent covalent modification of components of the translational machinery is responsible for shut-down during entry into the quiescent state. Vayda et al. (1995) studied a plant model system (potato tubers) and showed that the pH dependent formation of a stable association of the translational elongation factor eEF1a with polysomes in the cytoplasm gave rise to an inhibition of protein biosynthesis. Furthermore, an acidosis induced phosphorylation of the translational elongation factor eEF2 was demonstrated in a mouse embryonic fibroblast cell line, blocking the function of intact ribosomes by preventing the translocation of peptidyl tRNAs from the A to the P-site of the ribosomal complex (Dorovkov et al., 2002). A second target for translational control of protein synthesis is the modification of initiation factors as has been shown for the inhibition of protein biosynthesis by phosphorylation of eIF2a in hibernating mammals (Frerich et al., 1998; Van Breukelen and Martin, 2001). Until now, however, none of these studies showed a significant influence of intra- or extracellular pH on the modification process. It needs to be investigated whether similar mechanisms might be responsible for the global decrease in protein synthesis rates of *S. nudus* muscle tissue under hypercapnia induced acidosis.

During periods of moderate CO_2 stress as in their natural habitat (e.g. during low tide), *S. nudus* is probably still able to fully restore pHi. Thus, the observed decrease in overall oxygen consumption is then mainly mediated by reduced energetic costs of proton homeostasis (Pörtner et al., 2000) as well as by reduced nervous and muscular activity (Reipschläger et al., 1997). The latter will, however, limit ventilatory movements, and thereby cause respiratory depression and further CO_2 accumulation. From present data we cannot yet estimate the CO_2 levels beyond which protein synthesis rates begin to decrease. Further study will have to show whether this effect sets in gradually or whether pH-disturbances only become effective beyond certain threshold levels.

For a complete picture of protein metabolism catabolic processes also have to be included. In *S. nudus*, in contrast to other invertebrate phyla, ammonia is the only end product of N-metabolism (Urich, 1990). Recent study revealed a significant 10–15% decrease in ammonia excretion at the lowest pHe of 6.70 tested under both hypercapnic as well as normocapnic conditions (Langenbuch and Pörtner, 2002). This slight depression of protein degradation reflects metabolic depression and, at the same time, reduces the requirement for high rates of protein synthesis under conditions of reduced energy turnover, comparable to the extension of protein half-life observed under hypoxia (Anchordoguy et al., 1993).

The low O/N ratios of 4.0–4.5 determined in normoxic, normocapnic control samples (Langenbuch and Pörtner, 2002) clearly show that energy requirements of muscle tissue are exclusively covered by protein catabolism (Cowey and Corner, 1963; Snow and Williams, 1971; Mayzaud and Conover, 1988). A further drop of O/N ratios to values around 3.0 indicated a change in the mixture of metabolized amino acids to preferred oxidative decarboxylation and catabolism of dicarboxylic, low O/N amino acids like asparagine, glutamic acid or glutamine. The benefit of this shift is an enhanced net-production of bicarbonate (Pörtner, 1995) supporting the compensation of pHi under acidic conditions. Elevated intracellular bicarbonate concentrations might also activate soluble adenylyl cyclases (sAC) as in different mammalian tissues (Chen et al., 2000). The subsequent increase in intracellular cAMP levels might result in a suppression of protein translation via PHAS proteins as described by Lawrence et al. (1997). However, genetic evidence for an existence of sACs in invertebrates has not yet been reported.

In summary, we conclude that at decreased values of pHe as well as pHi (cf. Table 1) an overall reduction of protein turnover occurs which contributes to the conservation of body stores during metabolic depression under hypercapnia (Langenbuch and Pörtner, 2004). Here we observed a strong and over-proportional reduction of protein synthesis (by 60%; cf. Fig. 1c) compared to the reduction of protein/amino acid degradation (by 10–15%; Langenbuch and Pörtner, 2002). Chronic hypercapnia might thus not only lead to a rapid constraint on protein synthesis and associated processes like growth and reproduction but, on top of that, might also cause a lack of key metabolic enzymes. These changes require investigation as they may contribute to elevated mortality of *S. nudus* specimens kept long term under hypercapnia (Langenbuch and Pörtner, 2004). However, changes observed here are mediated by a drop in extracellular pH larger than the 0.8 U fall in water pH expected by the year 2300 (Caldeira and Wickett, 2003). A much smaller increase in CO_2 levels may still be harmful as indicated by recent evidence for enhanced mortality in calcifying molluscs and echinoderms at low but permanently elevated CO_2 levels of 560 ppm, just 200 ppm beyond present concentrations (Shirayama, 2002). Although sensitivities likely differ between organisms depending on their levels of organisation and energy turnover further mechanisms beyond a pH dependent disturbance of protein metabolism may contribute and require further investigation (Pörtner et al., 2004, 2005).

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