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ACID–BASE REGULATION, METABOLISM AND ENERGETICS IN *SIPUNCULUS NUDUS* AS A FUNCTION OF AMBIENT CARBON DIOXIDE LEVEL

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Summary

Changes in the rates of oxygen consumption and ammonium excretion, in intra- and extracellular acid–base status and in the rate of H^+-equivalent ion transfer between animals and ambient water were measured during environmental hypercapnia in the peanut worm *Sipunculus nudus*. During exposure to 1 % CO_2 in air, intracellular and coelomic plasma P_{CO_2} values rose to levels above those expected from the increase in ambient CO_2 tension. Simultaneously, coelomic plasma P_{O_2} was reduced below control values. The rise in P_{CO_2} also induced a fall in intra- and extracellular pH, but intracellular pH was rapidly and completely restored. This was achieved during the early period of hypercapnia at the expense of a non-respiratory increase in the extracellular acidosis. The pH of the extracellular space was only partially compensated (by 37 %) during long-term hypercapnia. The net release of basic equivalents under control conditions turned to a net release of protons to the ambient water before a net, albeit reduced, rate of base release was re-established after a new steady state had been achieved with respect to acid–base parameters.

Hypercapnia also affected the mode and rate of metabolism. It caused the rate of oxygen consumption to fall, whereas the rate of ammonium excretion remained constant or even increased, reflecting a reduction of the O/N ratio in both cases. The transient intracellular acidosis preceded a depletion of the phosphagen phospho-L-arginine, an accumulation of free ADP and a decrease in the level of Gibbs free energy change of ATP hydrolysis, before replenishment of phosphagen and restoration of pHi and energy status occurred in parallel.

In conclusion, long-term hypercapnia *in vivo* causes metabolic depression, a parallel shift in acid–base status and increased gas partial pressure gradients, which are related to a reduction in ventilatory activity. The steady-state rise in H^+-equivalent ion transfer to the environment reflects an increased rate of production of protons by metabolism. This observation and the reduction of the O/N ratio suggest that a shift to protein/amino acid catabolism has taken place. Metabolic depression prevails, with completely compensated intracellular acidosis during long-term hypercapnia eliminating intracellular pH as a significant factor in the regulation of metabolic rate *in vivo*. Fluctuating levels of the phosphagen, of free ADP and in the ATP free energy change values independent of pH are interpreted as being correlated with oscillating ATP turnover rates during early hypercapnia and as reflecting a tight coupling of ATP turnover and energy status *via* the level of free ADP.

Key words: acid–base regulation, ammonia excretion, buffer value, CO_2 partial pressure, environmental hypercapnia, extracellular pH, free ADP, Gibb's free energy change of ATP hydrolysis, H^+ transfer, intracellular pH, metabolic depression, nitrogen metabolism, phospho-L-arginine, ventilation, peanut worm, *Sipunculus nudus*.

Introduction

The sipunculid worm *Sipunculus nudus* is exposed to low tide for periods of different duration in its natural environment, the intertidal zone. During low tides, the infauna and epifauna experience hypoxia as well as an increase in CO_2 partial pressures (hypercapnia) as a result of reduced gas exchange caused by the restricted access to surface water. It is well established that intertidal animals are able to tolerate extended periods of hypoxia by decreasing their metabolic rate. Extreme hypoxia in *Sipunculus nudus* causes energy expenditure to fall by 50–70 % (Hardewig *et al.* 1991). Metabolic depression also affects the acid–base status and leads to a reduced rate of H^+-equivalent ion exchange (Pörtner *et al.* 1991) associated with shifted set points of acid–base regulation. During anoxic exposure, intracellular pH is regulated at a value approximately 0.3 units below that maintained under normoxia (Pörtner, 1993).

The potential contribution of CO_2 to metabolic depression in intertidal animals has not been investigated, and the effects of CO_2 on the mechanisms of acid–base regulation in these animals are insufficiently understood. Lindinger *et al.* (1984) reported that hypercapnia causes a respiratory acidosis in the extracellular fluid (haemolymph) and a mixed respiratory and non-respiratory acidosis in the intracellular space of the bivalve *Mytilus edulis*. Both intra- and extracellular acidification were only partially compensated in this species. A number of studies conducted on brine shrimp *Artemia* embryos and land snails have shown that, during hypercapnia in whole animals, CO_2 elicits a depression of metabolic rate (Busa and Crowe, 1983; Barnhart and McMahon, 1988; Hand and Gnaiger, 1988; Barnhart, 1989; Rees and Hand, 1990). In contrast, Silver and Jackson (1985) and Jackson *et al.* (1988) reported that experimentally elevated CO_2 levels had no influence on the metabolic rate of freshwater turtles *Chrysemys picta bellii*. For brine shrimp embryos, the depressant effect of CO_2 on metabolism is interpreted to be largely *via* acidotic changes in intracellular pH (Busa and Crowe, 1983; Hand and Gnaiger, 1988). Such a role of pHi in the downregulation of metabolic rate, however, has been questioned for other organisms (Brooks and Storey, 1989; Rees and Hand, 1991; Scholnick *et al.* 1994), among them *Sipunculus nudus* (Reipschläger and Pörtner, 1996).

Parallel work on *S. nudus* has been carried out in isolated muscle preparations (Reipschläger and Pörtner, 1996). The aim of the present study was to delineate the time course of the effects of hypercapnia on acid–base status, to quantify the responses of the acid–base regulatory system and to analyse changes in oxygen consumption, ammonia excretion and energy status associated with elevated ambient CO_2 tensions in *Sipunculus nudus in vivo*. Focusing on the specific effects of gaseous CO_2, analyses were always performed under conditions of sufficient access to oxygen for the animals. Intra- and extracellular pH, P_{CO_2} and bicarbonate levels were determined and utilized, together with tissue buffer values estimated under well-defined metabolic control conditions, in a quantitative treatment of the overall acid–base balance. To evaluate the specific effects of CO_2, the importance of changing bicarbonate levels in the water at constant P_{CO_2} was also investigated during the analyses of the whole-animal acid–base status.

Materials and methods

Experimental animals

Specimens of *Sipunculus nudus* L. (26–74 g) were collected by digging them up from intertidal flats at Locquémeau, Brittany, France, in September and April. The animals were kept in tanks with a 10–20 cm layer of sand from the original habitat for at least 1 week prior to experimentation. The holding tanks were supplied with filtered and recirculated artificial sea water at 10–15 °C. Water bicarbonate level was adjusted to a constant level of approximately 2.3 mmol l^{-1} by titration with HCl.

Experimental procedure

Prior to experimentation, the animals were catheterized by introducing a 2–3 cm length of PE 60 or PE 90 tubing (total length 75 cm) into the coelomic cavity after puncturing the posterior end of the body. The tubing was secured with cyanoacrylate glue. The long-term viability of the cannulated specimens and the stability of steady-state control acid–base parameters had been established previously (Pörtner *et al.*, 1991). After 24 h of acclimation to the experimental conditions under normoxic normocapnia (P_{O_2}≈100 mmHg or 13.3 kPa, P_{CO_2}≈0.6 mmHg or 0.08 kPa), the animals were exposed to environmental hypercapnia (1 % CO_2) by equilibration of the water with gas mixtures prepared from pure N_2, O_2 and CO_2 using precision gas-mixing pumps (Wösthoff, Bochum, Germany, type M 303/a-F). 1 % CO_2 was chosen as a high CO_2 level experienced by the animals in their natural environments. During experimentation in normocapnic sea water, animals were exposed to different water levels of water bicarbonate, originating from titration with hydrochloric acid or solid sodium bicarbonate (see below). The incubation water was exchanged with fresh sea water at intervals of 24 h.

H^+-equivalent ion transfer processes, contributing to acid–base regulation of the whole animal, were quantified in the closed, darkened seawater recirculation system described by Pörtner *et al.* (1991). In brief, the system contained between 300 and 470 ml of artificial sea water thermostatted to 15±0.05 °C. Animal chambers (selected to match the size of the animals) were connected to a gas-exchange column. Gases fed into the column were thermostatted to 15 °C and humidified to minimize evaporative water loss. The water was recirculated at a rate of 100 ml min^{-1} using a roller pump (type MP-GE, Ismatec, Zürich, Switzerland). In accordance with the direction of water flow in the natural burrow, water passed along the body surface from the anterior to the posterior end of the animal. After placing the animal in the chamber, the cannula was fed through the aeration column. During the acclimation period, water P_{O_2} values ranged between 100 and 120 mmHg (13.3–16 kPa) and P_{CO_2} between 0.4 and 0.7 mmHg (0.05–0.09 kPa). The initial seawater bicarbonate level was adjusted to 2.3 mmol l^{-1}, but was allowed to rise to 3 mmol l^{-1} during the experiment as a result of the release of bicarbonate by the animal. After 24–48 h of control incubation, the gas supply was switched to 1 % CO_2 in air and the incubation was continued for 6–7 days. After this period, the experiment was terminated or continued with an additional normocapnic control period of up to 4 days (see Fig. 5).

Water from the aeration column was recirculated continuously at a rate of 6.6 ml min^{-1} by means of a second roller pump (Ismatec IP 4, Zürich), feeding a ‘Δ-bicarbonate system’ (Heisler, 1989) to monitor changes in water pH and, thus, bicarbonate levels. Bicarbonate level was calculated from P_{CO_2} and pH according to Heisler (1984, 1989). During passage through the system, the water was thermostatted to 30±0.05 °C and equilibrated with humidified gas at constant 1 % CO_2. pH was measured by means of a glass electrode and a double-electrolyte bridge Ag/AgCl reference with a sleeve

diaphragm connected to a high-impedance isolation amplifier (model 87, Knick, Berlin, Germany). The signal was recorded on a chart recorder and fed, after A/D conversion, into a microcomputer system for on-line analysis. The accuracy of the calculation was checked by the addition of known amounts of HCl and $NaHCO_3$ to the system and by analysis of the total CO_2 in the water sampled from the Δ-bicarbonate system.

Intracellular acid–base parameters were determined in animals incubated in darkened aquaria containing 5 l of artificial sea water. After exposure to 1 % CO_2 under normoxic conditions for various times, coelomic fluid samples were withdrawn and analysed as described below. Animals were dissected as quickly as possible, and the body wall musculature was freeze-clamped by means of a Wollenberger clamp precooled in liquid nitrogen. Muscle samples were wrapped in aluminium foil and stored under liquid nitrogen until analysed.

Rates of oxygen consumption and ammonia excretion were studied by closed-system respirometry at 15 °C. Changes in water oxygen partial pressure were monitored for 2–3 h using a Clarke-type electrode (Eschweiler, Kiel, Germany). Water samples were taken from the respiration chamber at the beginning and at the end of this period and were analysed for ammonia as described below.

To obtain recordings with an acceptable signal-to-noise ratio, ventilatory frequency was monitored in cannulated animals dwelling in a 10 cm bottom layer of sand in darkened aquaria containing 15 l of aerated natural sea water at 15 °C. Each animal was connected to a pressure transducer (UFI, type 1050, Morro Bay, CA, USA) *via* the indwelling catheter. The ventilatory activity was monitored as the frequency of coelomic fluid pressure fluctuations (see Zielinski and Pörtner, 1996) by using a MacLab system (ADI Instruments, Hastings, UK) under control conditions, during long-term hypercapnia and during subsequent recovery and taking into account the protocol of oxygen consumption measurements. Moreover, some animals were maintained under normocapnia and infused with 10 μl g^{-1} body mass of saline containing 1.5 mmol l^{-1} adenosine following the rationale of Reipschläger *et al.* (1997).

Analyses

Coelomic fluid samples were collected repeatedly from the animals (approximately 0.7 ml from a total volume of 13.5–18 ml) in the Δ-bicarbonate system. Samples were analyzed for extracellular pH, P_{CO_2} and P_{O_2} using a thermostatted microelectrode assembly (15±0.1 °C, BMS 3, Radiometer, Copenhagen, Denmark). To avoid depleting the animals of coelomic fluid, samples used for blood gas analyses (0.08–0.25 ml) were re-infused into the animals directly after measurements had been completed. The electrodes were calibrated using precision phosphate buffers (Radiometer, Copenhagen, Denmark) or humidified mixtures of O_2, CO_2 and N_2 provided by gas-mixing pumps. The CO_2 content (C_{CO_2}) of plasma and water samples was analysed by means of a Capnicon III apparatus (Cameron Instruments, Port Aransas, TX, USA) or a Hach-Carle series 100 gas chromatograph, both calibrated with $NaHCO_3$ standard solutions. Apparent bicarbonate levels were determined as $C_{CO_2}-\alpha P_{CO_2}$, where α is the solubility of CO_2 in the respective compartment (calculated according to Heisler, 1986). Water bicarbonate levels were obtained from pH recordings, as described above.

Intracellular acid–base variables were analysed using the homogenate technique (Pörtner *et al.* 1990). In brief, tissue samples were ground under liquid nitrogen taking care that no condensation of CO_2 or water occurred. Frozen tissue powder (100–200 mg) was added to 0.2 ml of a solution containing KF (160 mmol l^{-1}) and nitrilotriacetic acid (1 mmol l^{-1}) in a 0.6 ml Eppendorf tube (total volume of medium determined by mass). The Eppendorf tube was filled to the top with KF/nitrilotriacetic acid solution, closed and its contents mixed on a vortex mixer. After brief centrifugation, the supernatant was analysed for total CO_2 content and pH. Intracellular C_{CO_2} and P_{CO_2} values were calculated from the total tissue CO_2 content determinations taking into account the fractional values of water content and extracellular water in the tissue of this species determined at the same temperature (Pörtner, 1987) (see Pörtner *et al.* 1990 for the calculations involved). The remaining tissue powder was extracted in perchloric acid (Beis and Newsholme, 1975, modified according to Pette and Reichmann, 1982). Buffer value determinations in tissue homogenates followed the technique of Heisler and Piiper (1971) as modified by Pörtner (1990). Ammonium concentrations in water and plasma samples and the levels of phospho-L-arginine, L-arginine, inorganic phosphate and ATP in the muscle samples were analysed using established enzymatic procedures (see Bergmeyer *et al.* 1986; for the procedure used to determine inorganic phosphate concentration, see Pörtner, 1990). The octopine dehydrogenase used to assay octopine, phospho-L-arginine and arginine was prepared and purified for these determinations from the adductor muscles of *Pecten maximus* following the procedure described by Gäde and Carlsson (1984).

The levels of free ADP and AMP were calculated on the basis of the equilibrium of arginine kinase and myokinase. Equilibrium constants for both enzymes were corrected for experimental temperature and pH-dependence with reference to the changing proton and magnesium binding of the adenylates and the proton turnover of the arginine kinase reaction (Pörtner, 1993; Pörtner *et al.* 1996).

Changes over time were tested for significance at the 5 % level by using one-way or repeated-measures analysis of variance (ANOVA) and by performing the Bonferroni/Dunn *post-hoc* test or contrasts for group comparisons, the latter in repeated-measures experiments using SuperAnova, Abacus Concepts. Values are presented as means ± S.E.M. throughout. Comparisons of data for normocapnic and hypercapnic animals under steady-state conditions and of energetic parameters in isolated tissue and whole animals were performed using Student's paired and unpaired *t*-tests.

Results

Sipunculus nudus has been shown previously to maintain

more or less constant blood gas and acid–base variables, with constant rates of H^+-equivalent ion exchange between the animals and the ambient water during extended control periods of up to 7 days (Pörtner *et al.* 1991). Therefore, the observed changes elicited by the experimental variations in water parameters can be expected to represent true experimental changes not caused by the length of the incubation period.

The time course of changes in the extracellular acid–base status was investigated in animals dwelling in a water recirculation system combined with the Δ-bicarbonate analysis system (Fig. 1). Control extracellular acid–base parameters at time 0 before the onset of hypercapnia were pH=7.87±0.04, P_{CO_2}=1.95±0.11 mmHg (0.26±0.01 kPa) and $[HCO_3^-]$=7.24±1.02 mmol l^{-1}; the rate of H^+-equivalent ion exchange was –0.06 mmol h^{-1} kg^{-1}. Transmission of the change in ambient P_{CO_2} to the extracellular space was complete after 6–12 h (Fig. 1; see also Fig. 5). Seawater pH fell from 8.08 to 7.04 during saturation of the water with 1 % CO_2. Extracellular pH decreased to a minimum of 7.25±0.04 after 6 h, but later stabilized at approximately pH 7.45 after 72 h. Obviously, only partial extracellular compensation of the respiratory acidosis (by 37 %) had been achieved. During this time, coelomic plasma bicarbonate concentration rose to a new steady-state level of 12 mmol l^{-1} when coelomic fluid P_{CO_2} stabilized at approximately 10 mmHg.

The time course of changes in intracellular acid–base status was investigated in animals dwelling in darkened aquaria containing a water volume of 5 l (Fig. 2). With the onset of hypercapnia, intracellular pH fell to 7.24 over 3–6 h and remained constant for up to 12 h. The intracellular acidosis was completely compensated within 48 h of hypercapnia. Intracellular P_{CO_2} had reached steady-state values of 10.5–11 mmHg after some initial oscillations between 3 and 12 h. Intracellular bicarbonate concentration levelled off after

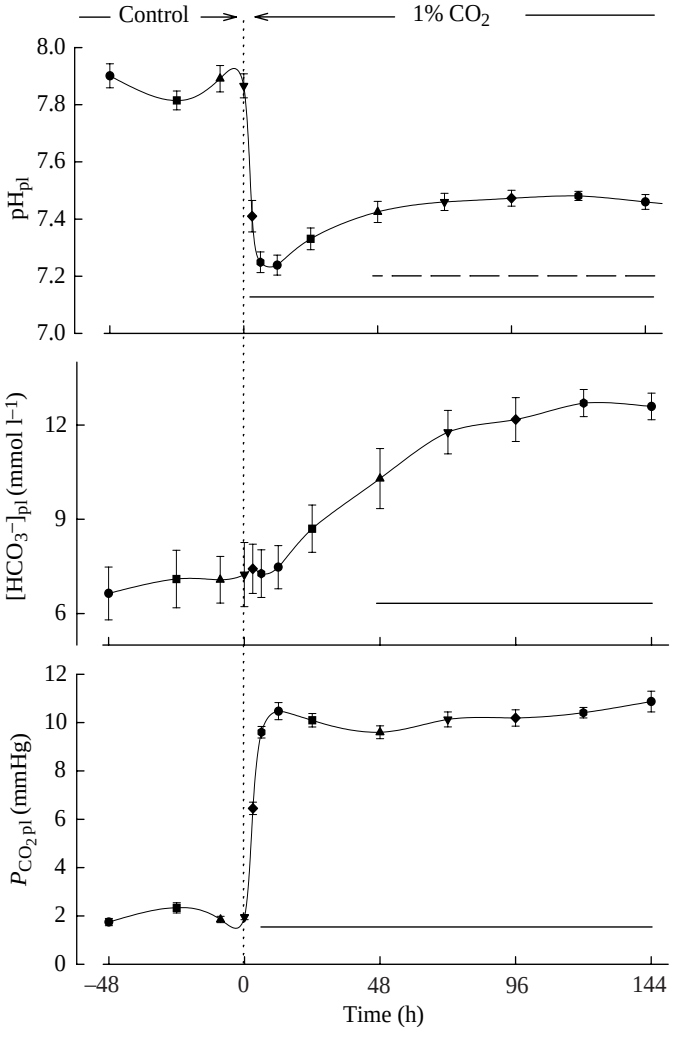

Fig. 1. Acid–base variables (pH, $[HCO_3^-]$, P_{CO_2}) in the coelomic plasma of *Sipunculus nudus* under control conditions and during the subsequent 144 h of hypercapnia (1 % CO_2). Values are means ± S.E.M., *N*=4. A horizontal line indicates the range of values significantly different from the control values; a broken line indicates the maintenance of a significant difference from pH values during short-term hypercapnia, i.e. at 6 and 12 h of hypercapnia).

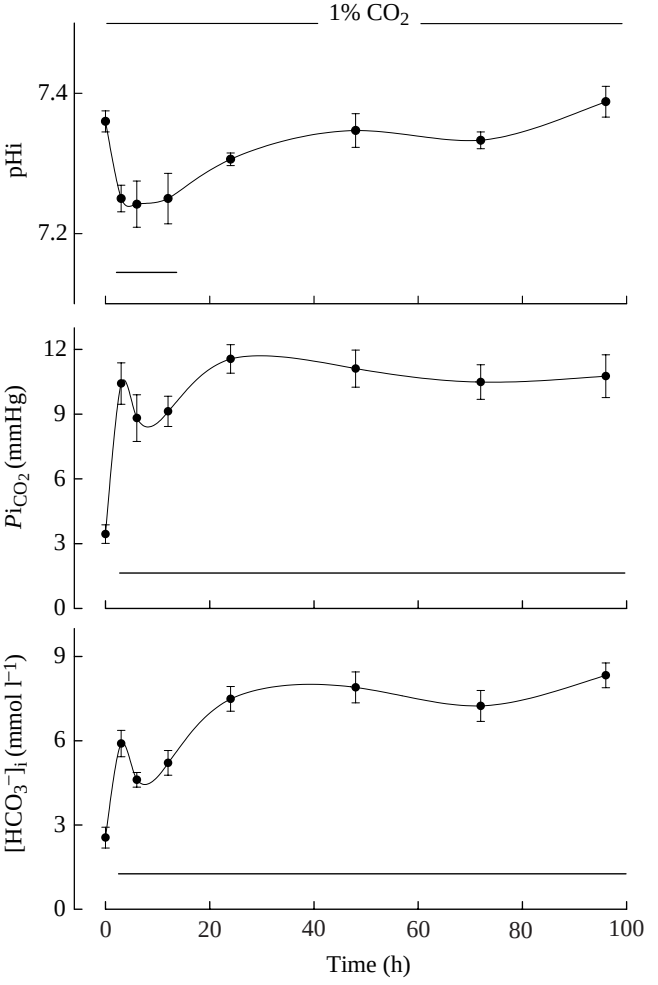

Fig. 2. Intracellular acid–base variables (pH, P_{CO_2}, $[HCO_3^-]$) in the body wall musculature of *Sipunculus nudus* under control conditions (0 h) and during the subsequent 96 h of hypercapnia (1 % CO_2). Values are means ± S.E.M., *N*=5 or 6. A horizontal line indicates the range of values significantly different from control values.

Table 1. *Comparison of steady-state values of intra- and extracellular acid–base and gas variables in* Sipunculus nudus *before and during long-term hypercapnia*

	Normocapnia	Hypercapnia
Aquarium		
pHe	8.02±0.03 (5)	7.44±0.02* (5)
pHi	7.36±0.01 (5)	7.33±0.01 (5)
ΔpH	0.65±0.03 (5)	0.12±0.01* (5)
Pe_{CO_2} (mmHg)	1.6±0.2 (5)	8.0±0.4* (5)
Pi_{CO_2} (mmHg)	3.4±0.4 (5)	10.5±0.8* (5)
ΔP_{CO_2} (mmHg)	1.8±0.4 (5)	2.5±0.7 (5)
$[HCO_3^-]_e$ (mmol l^{-1})	7.2±0.2 (5)	10.6±0.3* (5)
$[HCO_3^-]_i$ (mmol l^{-1})	2.6±0.4 (5)	7.2±0.5* (5)
$\Delta[HCO_3^-]$ (mmol l^{-1})	4.6±0.3 (5)	3.7±0.7 (5)
Water recirculation system		
Pe_{CO_2}	1.94±0.08 (7)	10.6±0.4* (6)
Pw_{CO_2}	0.57±0.03 (6)	7.8±0.1* (4)
ΔP_{CO_2}	1.37	2.81
Pe_{O_2}	25.8±3.8 (7)	14.2±2.1* (6)
Pw_{O_2}	110.0±3.6 (4)	111.0±2.7 (4)
ΔP_{O_2}	84.2	96.8

Values are means ± S.E.M. (*N*).

*Significantly different from the normocapnic value, $P<0.05$.

Experimental hypercapnia was induced by treatment with 1 % CO_2 for 120 h.

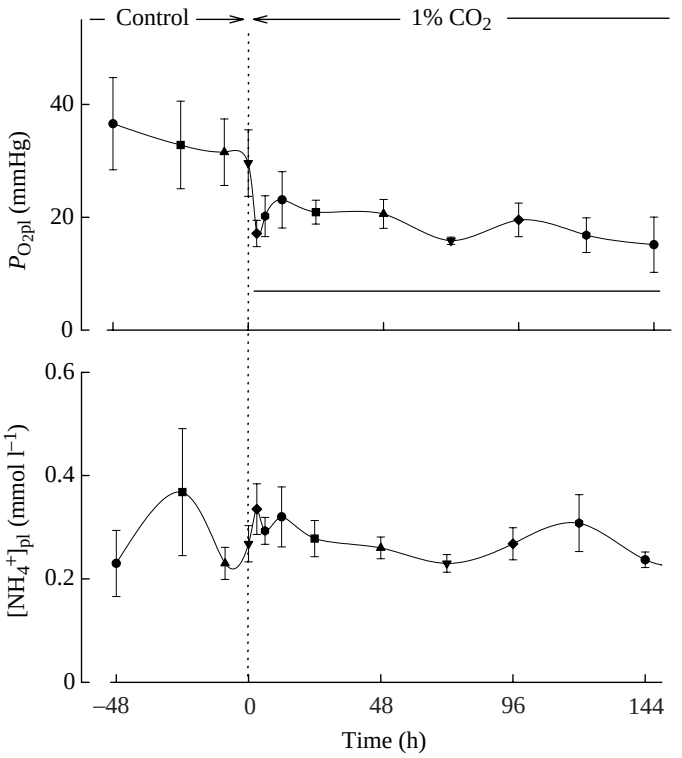

Fig. 3. P_{O_2} and ammonium levels in the coelomic plasma of *Sipunculus nudus* under control conditions and during the subsequent 144 h of hypercapnia (1 % CO_2). Values are means ± S.E.M., $N=4$. A horizontal line indicates the range of values significantly different from control values.

24 h, and values remained more or less contant between 7.2 and 8.3 mmol l^{-1} thereafter. In order to compare the data collected in low-volume recirculation systems and with that from relatively large aquaria, extracellular data were recorded for steady-state normocapnia and hypercapnia in some animals killed for pHi analysis (Table 1).

Fig. 3 shows the effect of hypercapnia on coelomic P_{O_2} (data collected as for Fig. 1). An initial sharp drop from control levels of 29.6±5.9 mmHg (3.95±0.79 kPa) at time 0 h to 17.1±2.3 mmHg (2.28±0.31 kPa) was followed by a transient rise and slow fluctuations around a mean value of approximately 17 mmHg. Plasma ammonium levels also showed oscillations but remained more or less constant under control and hypercapnic conditions. Table 1 compares steady-state water and intra- and extracellular acid–base parameters and gas partial pressures under control conditions and during long-term hypercapnia. Only those values collected in the same experimental arrangement are compared. The differences between intra- and extracellular P_{CO_2} as well as between extracellular and water P_{CO_2} increased during hypercapnia, as did the P_{O_2} difference between the extracellular fluid and the water.

The observed net release of base equivalents under control conditions was similar to the pattern described previously (Pörtner *et al.* 1991; Fig. 4). Hypercapnia reversed the flux of acid–base equivalents, resulting in a net uptake of bicarbonate-equivalent ions. This transient net bicarbonate uptake resulted in a transient reduction in the rate of water alkalization. When intra- and extracellular acid–base parameters had reached new steady-state values, the direction of the net proton flux returned to the original direction of base release, although at a reduced rate reflected by the increase in the rate of ammonium excretion.

This finding was confirmed during analysis of the return to normocapnia in a separate experiment (Fig. 5A–D). Changes seen during long-term hypercapnia were very similar to those depicted in Fig. 4. The return to normocapnia led to a transient accumulation of bicarbonate in the coelomic plasma linked to an overshoot in extracellular pH. P_{CO_2} fell to control normocapnic values within 12 h. Plasma pH returned to control values within 72–96 h. Coelomic bicarbonate concentration approached, but did not reach, normocapnic levels during this period (Fig. 5A–C). Finally, the return to normocapnia caused an increase in the net release of base equivalents (Fig. 5D). Taking into account the reduced rate of net base release during hypercapnia, the animals lost exactly the amount of base that they had accumulated during hypercapnia.

The transient intracellular acidosis observed during the first day of hypercapnia was followed by a delayed decrease in the level of phospho-L-arginine, indicated by a reduced

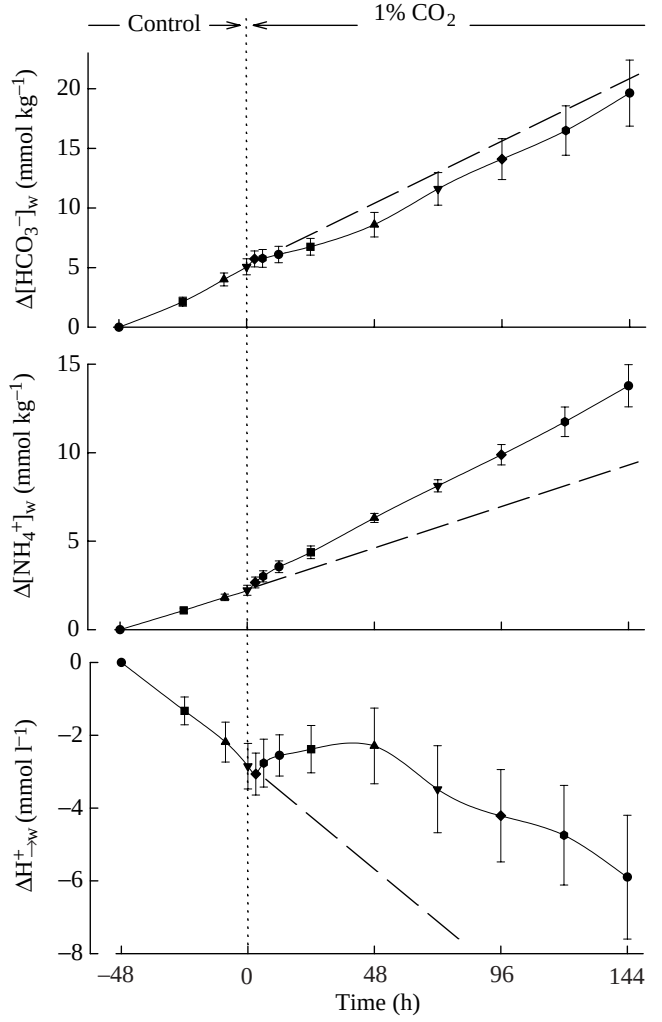

Fig. 4. H^+-equivalent ion transfer ($\Delta H^+_{\rightarrow w}$) between the animals (extracellular data in Fig. 1) and the ambient water, calculated from changes in ammonium and bicarbonate levels in the water. Control rates are extrapolated (dashed line) for the experimental period. Values are means ± S.E.M, *N*=4. Note the increase in the rate of ammonium excretion during hypercapnia (cf. Fig. 8).

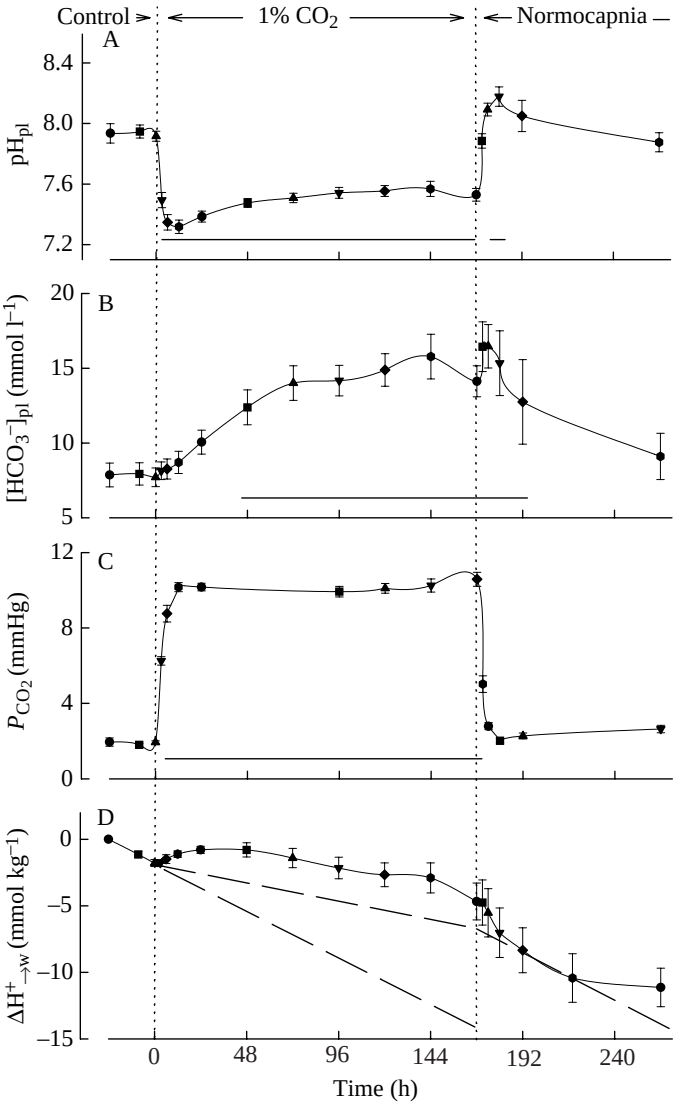

Fig. 5. (A–C) Acid–base variables (pH, $[HCO_3^-]$, P_{CO_2}) in the coelomic plasma of *Sipunculus nudus* under control conditions, during 168 h of hypercapnia (1 % CO_2) and during the subsequent return to control conditions. Note the overshoot in extracellular pH and bicarbonate levels during the return to normocapnia. A horizontal line indicates the range of values significantly different from control values. (D) H^+-equivalent ion transfer ($\Delta H^+_{\rightarrow w}$) between the animals and the ambient water calculated from changes in water ammonium and bicarbonate levels. The control rate and the hypercapnic rate are extrapolated (dashed lines) for the subsequent experimental period. Values are mean ± S.E.M., *N*=4.

ratio [phospho-L-arginine]/[phospho-L-arginine + L-arginine] (Fig. 6). The return to control pH during long-term exposure to hypercapnia resulted in a return of the phospho-L-arginine level to the control level. Fluctuations in phosphagen levels were reflected by a significant transient rise in inorganic phosphate concentrations. Fig. 7 shows that ATP levels remained unaffected and that a slight accumulation of free ADP and AMP occurred during the period when phospho-L-arginine levels fell. Levels of free ADP and AMP were insignificantly different from control levels but were significantly different from the values reached during long-term hypercapnia. The value of the Gibb's free energy change of ATP hydrolysis initially fell significantly during hypercapnic acidosis. The decrease was mostly dependent upon the rise in inorganic phosphate level and was reversed when the phosphate level returned to the control value during long-term hypercapnia. Minimum levels of free ADP, free AMP and inorganic phosphate, and maximum values of the ratio [phospho-L-arginine]/[phospho-L-arginine + L-arginine] and the ATP free energy change were seen after 96 h of hypercapnia.

The time courses of intra- and extracellular acid–base changes can only be compared with caution owing to different experimental conditions. Quantitative analysis of extracellular acid–base changes demonstrates that the extracellular acidosis exceeded the extent of acidification expected from CO_2

accumulation. For this analysis, tissue non-bicarbonate buffer values were determined as 16.0±1.3 mmol kg^{-1} pH $unit^{-1}$ (mean ± S.E.M., *N*=18), and a coelomic fluid buffer value of 4.6 mmol kg^{-1} pH^{-1} was adopted from Pörtner *et al.* (1984). After 3 h, a non-respiratory acidification of 1.9 mmol H^+ l^{-1} in excess of respiratory acidification was observed in the coelomic plasma. At this time, the intracellular acidosis was almost maximal, but with a base excess of 0.7 mmol l^{-1} instead of a deficit. The maximum extent of extracellular non-respiratory acidification (resulting in a base deficit of 2.9 mmol l^{-1} after 6 h of hypercapnia) coincided with the maximum drop in extracellular pH. At the same time, intracellular pH had reached its minimum value, representing a slight base excess of 0.2 mmol l^{-1} cell water, which increased to 0.9 mmol l^{-1} after 12 h. Under steady-state conditions (after approximately 48 h of hypercapnia), non-respiratory base equivalents accumulated in the intra- and extracellular body fluids amounted to 1.77 mmol kg^{-1} ($\Delta HCO_3^-{}_{non\text{-}resp.}$=[0.58×(0.73 mmol l^{-1} coelomic plasma) + 0.19×(4.93 mmol l^{-1} cell water of the musculature)]/ 0.77=1.77 mmol kg^{-1}) (for details of the equation and body fluid fractions, see Pörtner, 1987). Changes in 85 % of the animal's body mass were considered in this analysis, which compares well with the net uptake of base equivalents of

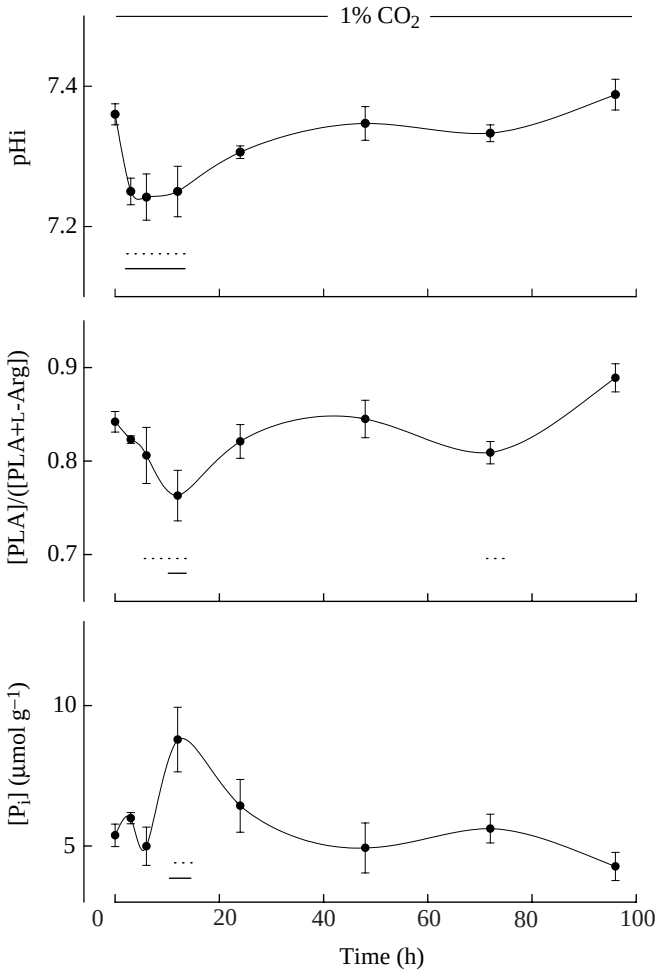

Fig. 6. Changes in pHi, in the ratio [phospho-L-arginine]/[phospho-L-arginine + L-arginine] and in the inorganic phosphate (P_i) concentration in the body wall musculature of *Sipunculus nudus* under control conditions (pHi=7.36±0.02, [phospho-L-arginine]/[phospho-L-arginine + L-arginine]=0.84±0.01; $[P_i]$=5.4±0.4 μmol g^{-1} wet mass) and during the subsequent 96 h of hypercapnia (1 % CO_2). Values are means ± S.E.M., *N*=5 or 6. A horizontal line indicates the range of values significantly different from control values; a broken line indicates that values are significantly different from that measured after long-term hypercapnia, i.e. at 96 h of hypercapnia. PLA, phospho-L-arginine; L-Arg; L-arginine.

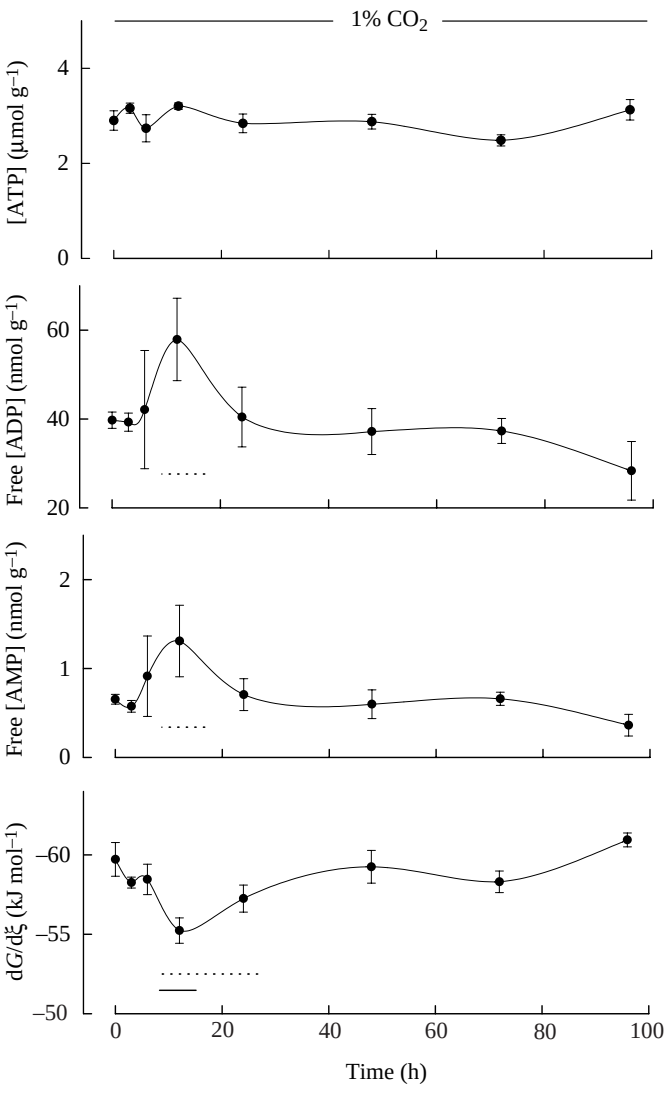

Fig. 7. ATP levels, the concentrations of unbound ADP and AMP and the levels of ATP free energy, in the body wall musculature of *Sipunculus nudus* under control conditions ([ATP]=2.90±0.21 μmol g^{-1} wet mass; $[ADP]_{free}$=40±2 nmol g^{-1} wet mass; $[AMP]_{free}$= 0.65±0.06 nmol g^{-1} wet mass; ATP free energy, $dG/d\xi$= −59.7±1.1 kJ mol^{-1} and during the subsequent 96 h of hypercapnia (1 % CO_2). Values are means ± S.E.M., *N*=5 or 6. A horizontal line indicates the range of values significantly different from control values; a broken line indicates that values are significantly different from that measured after long-term hypercapnia, i.e. at 96 h of hypercapnia.

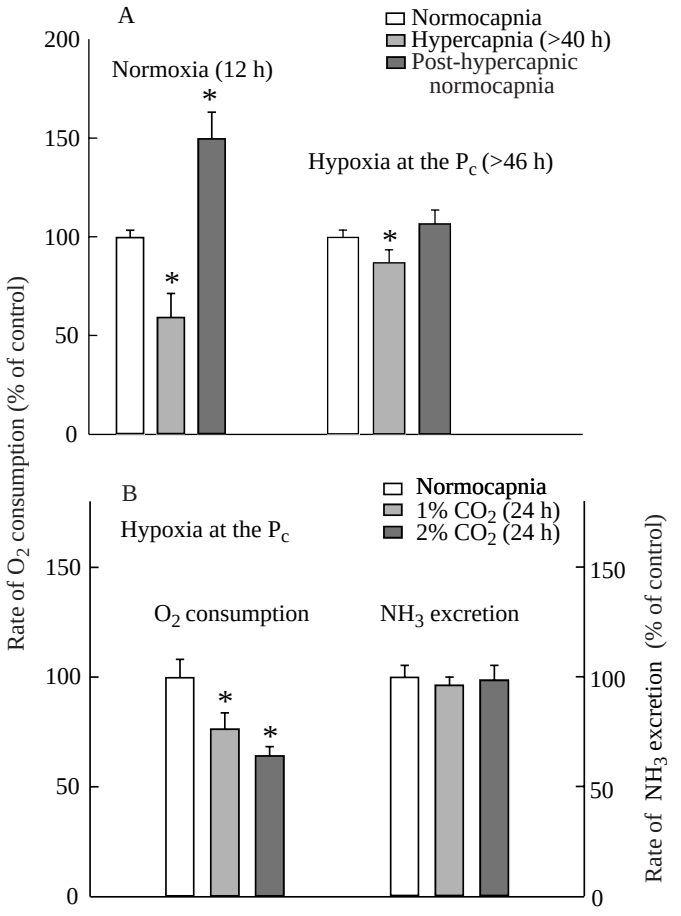

Fig. 8. (A) Rates of oxygen consumption during normocapnia, after subsequent exposure to hypercapnia (1 % CO_2 for >40 h) and after the return to normocapnia. This protocol ensured that acid–base parameters had reached new steady-state values under hypercapnia. Note the increase in the rate of oxygen consumption during the first hours of post-hypercapnic normocapnia. (B) Rates of oxygen consumption and ammonia excretion under 1 and 2 % CO_2 in air. The data measured in a closed system confirm the reduction in the O/N ratio derived from the drop in metabolic rate and from the increase in the rate of ammonium excretion shown in Fig. 4 (open system). An asterisk indicates a significant difference from the control values. Values are means + S.E.M., N=5–6. P_c, critical P_{O_2}.

1.8 mmol kg^{-1} body mass derived from the changes in water parameters of the Δ-bicarbonate system.

Closed-system respirometry revealed that hypercapnic exposure caused metabolic depression in *Sipunculus nudus* (Fig. 8). The decrease in the rate of oxygen consumption was maintained after steady-state conditions had been attained with respect to the levels of intra- and extracellular acid–base parameters. A decrease in the rate of oxygen consumption was observed under both normoxia and hypoxia (the level of P_{O_2} chosen was slightly above the critical P_{O_2}, defined as the ambient oxygen level below which anaerobiosis sets in; Pörtner and Grieshaber, 1993; Fig. 8A). The return to normocapnia was correlated with a significant rise in the rate of oxygen consumption above control values after 12 h. This overshoot was reversed after normocapnic recovery for at least 46 h. The reduction in the rate of oxygen consumption was related to the level of ambient hypercapnia. Hypercapnia of 2 % was almost twice as effective as exposure to 1 % CO_2 (Fig. 8B). The increase in the rate of ammonia/ammonium excretion in the (open) water recirculation system could not be confirmed during closed-system respirometry. However, the rate of ammonia excretion remained constant and independent of the level of ambient CO_2, thereby confirming that the O/N ratio (8.4±0.6 under control conditions), reflecting the contribution of protein/amino acid metabolism to the overall metabolic rate, fell in animals exposed to hypercapnia by 19±9 % (1 % CO_2) or by 36±2 % (2 % CO_2) (mean ± S.E.M., N=5).

The ventilatory frequency of animals dwelling in sand reflected the changes in metabolic rate and fell significantly during hypercapnia (1 % CO_2) from a control value of 2.27±0.16 to 1.78±0.24 min^{-1} (mean ± S.E.M., N=4); control values were exceeded between 9 and 12 h of recovery at a level of 2.83±0.53 min^{-1} (N=3). Adenosine infusions caused a small and insignificant drop in ventilatory frequency from 2.12±0.24 to 1.97±0.08 min^{-1} during the period 1.5–3 h after infusion, when a maximum drop in the rate of oxygen consumption had been observed in previous experiments (Reipschläger *et al.*, 1997).

Discussion

Acid–base regulation

Like the findings of Lindinger *et al.* (1984) in *Mytilus edulis*, acid–base regulation during hypercapnia in *Sipunculus nudus* occurs as an accumulation of bicarbonate in body fluid compartments to compensate for the respiratory acidosis. In *S. nudus*, all of the bicarbonate originates from ion exchange with the ambient water, whereas in *M. edulis* the dissolution of the shell is largely responsible for this process. Starting at lower bicarbonate levels in the intra- than in the extracellular space, total base accumulation occurred to a similar extent in both compartments in *S. nudus*. The relative capacity of pH regulatory mechanisms appears to be larger in the tissues, since compensation of the intracellular acidosis was faster and occurred initially at the expense of an increase in the extracellular acidosis. A base deficit transiently developing in the coelomic plasma indicates that transfer mechanisms between the coelomic fluid and water are slower than the net base exchange rate between the body wall musculature and coelomic fluid. Finally, the intracellular acidosis was more or less completely compensated during hypercapnia, while accumulation of bicarbonate in the extracellular space was not sufficient for full recovery of pHe.

The final steady-state value of extracellular pH is a direct function of the acid–base characteristics of the medium. In support of this conclusion, changes in water bicarbonate levels under normocapnia were seen to modulate extracellular bicarbonate levels (Table 2). Accordingly, an increasing degree of extracellular compensation is expected in hypercapnic sea water at higher bicarbonate levels. Heisler (1984, 1986) suggested that a maximum extracellular

Table 2. *Coelomic plasma and water bicarbonate levels in* Sipunculus nudus *before and during experimental hypercapnia or after titration of water bicarbonate levels under normocapnia in the water recirculation system*

	$[HCO_3^-]$ (mmol l^{-1})			
Experiment	Water	Plasma normocapnia	Plasma hypercapnia	$\Delta[HCO_3^-]_{e-w}$
I	3.4	6.8±0.3	–	3.4
	2.9	5.7±0.3	–	2.8
	1.0	5.2±0.3	–	4.2
	0.6	3.9±0.3*	–	3.3
II	2.8	7.1±0.7	–	4.3
	3.3	–	12.7±0.4*	9.4

Data are from two experiments, experiment I and II.

*Significant difference from control values (normocapnia at 2.8 or 2.9 mmol l^{-1} HCO_3^- in experiments I and II, respectively).

Note the maintenance of the bicarbonate gradient ($\Delta[HCO_3^-]_{e-w}$) during changes in $[HCO_3^-]_w$ and normocapnia and the increase in the bicarbonate concentration difference during hypercapnia.

Experimental hypercapnia was induced by treatment with 1 % CO_2 for 120 h.

Values are means ± S.E.M., N=4.

bicarbonate concentration may determine the extent to which extracellular respiratory acidification can be compensated in fish. This maximum concentration may be related to a maximum concentration difference or ratio between the extracellular space and the ambient water beyond which passive loss is equivalent to the rate of uptake. Water-breathers in general maintain lower extracellular bicarbonate levels than air-breathers and, for this reason, concentration gradients are smaller and extracellular compensation is more complete. Under 1 % CO_2, extracellular compensation is usually almost complete in marine fish (e.g. Toews *et al.* 1983; Claiborne and Evans, 1992), less complete in crabs (e.g. Cameron and Iwama, 1987) and even less complete in the sipunculid worm, although partial compensation of the extracellular acidosis required the concentration difference between plasma and water bicarbonate to rise from 4.3 to approximately 10 mmol l^{-1} (Table 2). Compensation was also incomplete in *M. edulis*, despite the use of shell carbonate for buffering (Lindinger *et al.* 1984). Some animals (e.g. channel catfish, blue crab and possibly *M. edulis*) are able to shift the bicarbonate threshold to higher levels according to the degree of hypercapnia (Lindinger *et al.* 1984; Cameron and Iwama; 1989, Heisler, 1993). The range of concentrations used in the present experiments does not allow us to identify a maximum extracellular level in *S. nudus.* Further research is required to compare vertebrate and invertebrate capacities of acid–base regulation.

If an elastic bicarbonate threshold exists for the extracellular compartment, it may also exist intracellularly. Intracellular pH depends upon extracellular pH in the isolated byssus retractor muscle of *Mytilus edulis* (Zange *et al.* 1990), in isolated rat heart (Sugden and Fuller, 1991) and also in the isolated body wall musculature of *Sipunculus nudus* (Reipschläger and Pörtner, 1996). The maintenance of intracellular pH under normocapnic control conditions was associated with a HCO_3^- concentration difference of approximately 4 mmol l^{-1} between the intra- and extracellular spaces. Although, at present, we cannot evaluate why and how such a gradient is maintained, it is intriguing that similar quantities of bicarbonate were accumulated intra- and extracellularly and, in consequence, the same concentration difference was seen during long-term hypercapnia (Table 1). Adopting the principles elaborated above for the extracellular compartment, intracellular compensation appears to be easier since baseline pH and, thus, bicarbonate levels are low, leaving more scope for an increase in bicarbonate concentration. The Henderson–Hasselbalch equation predicts that less bicarbonate is required to be accumulated intracellularly to re-establish a low control pHi, whereas the higher set point of extracellular pH requires more bicarbonate to be accumulated per pH unit of compensation than in the intracellular space.

The correlated time courses of intra- and extracellular compensation strongly suggest that intracellular compensation was supported by the increase in extracellular pH and bicarbonate levels, even though extracellular compensation remained incomplete. Intracellular pH remained at its minimum value and was more or less constant as long as extracellular pH and bicarbonate levels were minimal. pHi started to rise only when extracellular parameters also increased. In isolated body wall musculature, steady-state pHi values were reduced when hypercapnic tissues were exposed to an extracellular pH close to the lowest values observed *in vivo* (see Fig. 1). Control values of intracellular pH in hypercapnic muscle preparations were re-established only at extracellular pH values comparable to that attained after compensation *in vivo* (Fig. 1; Reipschläger and Pörtner, 1996). As a corollary, only with partial compensation of the extracellular acidosis during hypercapnia and the associated accumulation of bicarbonate are intact animals able to adjust pHi to pre-hypercapnic values. Accordingly, intracellular compensation is limited by the maximum extracellular bicarbonate level, which is a function of the level of hypercapnia. These interrelationships emphasize the importance of an integrated control of extracellular and intracellular acid–base status under control conditions and during environmental stress.

The present data support the more general hypothesis that, rather than maintaining absolute levels of pH, actual values depend upon steady-state ionic equilibria between the intra- and extracellular compartments and the ambient media. Only in media reflecting a ‘normal’ environment in terms of ion composition and pH are set points of acid–base parameters typical of control conditions observed.

Compensation of the intracellular acidosis was not observed in *M. edulis* during hypercapnia, possibly owing to the limited duration of the hypercapnic exposure (24 h). However, a

metabolic acidosis developed in *M. edulis* during hypercapnia, suggesting metabolic depression so severe that ventilation may have become insufficient and that anaerobic metabolism may have been utilized for energy production (Lindinger *et al.* 1984). In contrast, ambient P_{O_2} was above the critical P_{O_2} in the present study (see Pörtner *et al.* 1985), and coelomic fluid P_{O_2} was maintained at a high level during hypercapnia in *S. nudus*, suggesting that the animals were completely aerobic.

Metabolic depression and the mode of metabolism

Although control values of pHi regulation were successfully re-established during long-term hypercapnia, elevated CO_2 levels caused a decrease in the rate of oxygen consumption under both normoxia and at levels of hypoxia slightly above the critical P_{O_2} (Fig. 8A), indicating that the depressant effect of CO_2 is independent of ambient oxygen level. *S. nudus* is an oxyconformer, probably with a variable contribution to oxygen consumption from an alternative oxidase (Pörtner and Grieshaber, 1993). Nonetheless, metabolic depression was independent of the actual rate of oxygen consumption, which varied with ambient P_{O_2}. The hypercapnia-induced fall in metabolic rate in *Sipunculus nudus* occurred even at normal pHi. Therefore, as verified in a companion study (Reipschläger and Pörtner, 1996), a contribution of intracellular pH to metabolic depression is most unlikely in this species. However, extracellular pH *in vivo* remained reduced under these conditions, and a separate study conducted using isolated body wall musculature has demonstrated that a decrease in extracellular pH rather than intracellular pH caused metabolic depression in *Sipunculus nudus* (Reipschläger and Pörtner, 1996).

The data obtained in the water recirculation system show an increase in the rate of ammonium accumulation in the water during hypercapnia, with constant ammonium levels in the coelomic plasma (Figs 3, 4). Under the conditions of an open system, this could be caused either by an increased trapping of ammonia in the ambient water at low pH or by an increase in the rate of ammonia production and excretion, reflecting a drop in the O/N ratio and, thus, a shift to the catabolism of protein or amino acids. Reinvestigation of ammonia release in a closed system could not confirm the rise in ammonium production during hypercapnia (Fig. 8B). This may be due to ammonium accumulation in the plasma, owing to the development of a non-equilibrium condition in the closed chamber, or to seasonal differences (collection of animals at different times of the year, see Materials and methods). However, the O/N ratio drops during hypercapnia. A value of 8.4 during normocapnia indicates that metabolism in *Sipunculus* relies largely on amino acids as fuel, and this reliance is even greater during hypercapnia with even lower O/N ratios. The mechanism causing the increase in amino acid catabolism is unclear; however, if metabolic depression does include a reduction in the rate of protein synthesis, as described for hypoxic animals (Hofmann and Hand, 1990; Land *et al.* 1993), a diminished recruitment of amino acids for protein synthesis could be associated with their increased use as metabolic substrates under conditions when the rate of amino acid release from protein degradation is more or less unchanged.

Amino acid degradation leads to the production of metabolic bicarbonate and ammonium (Atkinson and Camien, 1982). When water-breathers release ammonia, the remaining H^+ neutralizes the accumulating bicarbonate (see Pörtner, 1989). If ammonium is released by ion exchange instead, an increase in metabolic bicarbonate production associated with increased amino acid degradation might support the rise in intra- and extracellular bicarbonate levels and not only reduce the total workload for ion-exchange mechanisms but also accelerate the establishment of new acid–base equilibria. The detailed mechanisms regulating the metabolic contribution to acid–base regulation require further investigation. Nonetheless, net H^+-equivalent ion exchange, which is equivalent to a net release of base under normocapnic control conditions, shifts to a reduced rate of base release during hypercapnia. Thereby, it reflects an increased net production of protons in metabolism, as expected from the proton balance of overall amino acid catabolism, which leads to ammonium formation in excess of bicarbonate formation and, consequently, net acid excretion (see Pörtner, 1989, 1995).

Metabolic changes and tissue energetics

The decrease in overall metabolic rate was related to a fall in plasma P_{O_2} (Fig. 3) and a rise in P_{CO_2}, resulting in an increased difference between the animals and their environment and, at least for CO_2, also between the intra- and extracellular spaces (Table 1). These changes suggest that a depression of gas exchange has occurred, a conclusion also supported by the finding of a decrease in ventilatory frequency during hypercapnia. A reduction in ventilatory activity would suggest that a central nervous mechanism contributes to metabolic depression. In a companion study (Reipschläger *et al.* 1997), we have demonstrated that adenosine accumulates in the nervous tissue of animals during hypercapnia and contributes to metabolic depression. Adenosine infusion in animals dwelling in sand caused a slight but insignificant drop in ventilatory frequency.

During the transition to long-term hypercapnia, oscillations in plasma O_2 and tissue CO_2 levels were similar, with an initial sharp drop in plasma P_{O_2} and an associated rise in tissue P_{CO_2} (Figs 2, 3). A subsequent rise in plasma P_{O_2} and drop in tissue P_{CO_2} compensated for these initial changes. Although oxygen consumption data are not available for this period, the rationale outlined above suggests that ventilation and metabolic rate rose again after an initial drop; however, this compensatory effect was only transient and was followed by the documented long-term metabolic depression. Recordings of ventilation frequency reflected the transient rise in metabolic rate after 12 h of hypercapnia, but did not reach significance levels (results not shown).

The fluctuations in the levels of high-energy phosphates support these conclusions and also suggest that an immediate drop in metabolic rate occurred with the onset of hypercapnia, followed by a compensatory rise before energy turnover fell

again during the transition to steady-state hypercapnic depression. This hypothesis is supported by the time courses of changes in pHi and phospho-L-arginine levels and by the discrepancy observed between them. Phosphagens such as phospho-L-arginine (PLA) are rapidly available energy reserves which will buffer any drastic change in energy demand even when catabolism remains fully aerobic. Protons are involved in the transphosphorylation of the phosphagen and, therefore, influence the equilibrium of arginine kinase (simplified):

$$PLA^- + MgADP^- + H^+ \leftrightharpoons MgATP^{2-} + \text{L-arginine}^+$$

$$MgATP^{2-} \leftrightharpoons MgADP^- + xP_i^{2-} + (1-x)P_i^- + xH^+ .$$

An acidosis, therefore, could be expected to cause a drop in phospho-L-arginine levels associated with a decrease in the ratio [phospho-L-arginine]/[phospho-L-arginine + L-arginine]. Finally, transphosphorylation and subsequent utilization of MgATP would lead to a net release of inorganic phosphate.

However, phosphagen levels did not follow the sharp drop in pHi and decreased significantly only after 12 h, although intracellular pH had already reached close to minimum values after 3–6 h. Moreover, the delayed decrease in phosphagen levels was associated with a delayed rise in free ADP and AMP levels which, in combination with the rise in phosphate levels, led to a significant drop in the Gibb's free energy of ATP hydrolysis (Figs 6, 7). The effect of pH itself on the latter parameter remained small and almost negligible under these circumstances (see Pörtner, 1993). In accordance with the picture arising from the changes in gas partial pressure gradients, we suggest that the delayed drop in phospho-L-arginine level and the rise in free ADP and AMP levels are caused by a transient increase in metabolic rate after an initial drop during early hypercapnia. These initial oscillations in high-energy phosphate levels were followed by long-term metabolic depression, which allowed phospho-L-arginine levels to rise again. Similarly, a transient drop in phospho-L-arginine levels followed by a compensatory rise was also seen during the transient overshoot in oxygen consumption caused by a temperature rise in the Antarctic limpet *Nacella concinna* (S. Zielinski, L. Peck and H. O. Pörtner, unpublished results).

In conclusion, the immediate fall in energy turnover during early hypercapnia may have prevented the acidosis from causing a parallel drop in phospho-L-arginine levels. In support of this hypothesis, phosphagen levels also remained constant in isolated hypercapnic body wall musculature despite the development of an intracellular acidosis (Reipschläger and Pörtner, 1996). These observations are in accordance with the conclusions by Meyer *et al.* (1991), who also suggested that the development of an acidosis need not necessarily cause phosphagen depletion.

The observation that the decrease in intracellular pH during hypercapnia did not cause a depletion of phospho-L-arginine indicates that intracellular pH and phosphagen metabolism remain uncoupled when metabolic rate is low. The levels of the phosphagen and the Gibb's free energy change of ATP hydrolysis both fell only after an initial delay, probably at higher metabolic rates, associated with the respective accumulation of free ADP and inorganic phosphate. In support of this conclusion, the Gibb's free energy change of ATP hydrolysis was significantly higher in isolated body wall musculature (-66 ± 2 kJ mol^{-1}, [phospho-L-arginine]/[phospho-L-arginine + L-arginine]$=0.96\pm0.01$; [P_i]$=0.8\pm0.5$ μmol g^{-1} wet mass, [free ADP]$=6\pm2$ nmol g^{-1} wet mass at minimal metabolic rate; data from Reipschläger and Pörtner, 1996) than in the body wall of intact animals (-60 ± 1 kJ mol^{-1}, [phospho-L-arginine]/[phospho-L-arginine + L-arginine]$=0.84\pm0.01$; [P_i]$=5.4\pm0.4$ μmol g^{-1} wet mass, [free ADP]$=40\pm1$ nmol g^{-1} wet mass; this study), very probably because of a higher metabolic rate *in vivo*. All of these values were obtained at similar intracellular pH values under conditions which can be termed aerobic control conditions. The comparison emphasizes that aerobic metabolic rate and the energy status of a tissue are inversely coupled with large factorial rises in the level of free ADP at higher metabolic rates. This may hold true even for interspecies comparisons considering, for example, the high values of 100 nmol free ADP g^{-1} wet mass in resting squid mantle musculature (Finke *et al.* 1996), which exhibits high rates of oxygen consumption under control conditions.

Conclusions

Acid–base regulation in *S. nudus* during hypercapnia occurred as an accumulation of bicarbonate to a similar extent in the extra- and intracellular spaces. The amount of bicarbonate accumulated was sufficient to compensate for hypercapnia-induced intracellular acidification, but left the extracellular space partially uncompensated. Although extracellular compensation was incomplete, it provided a preconditioning for the re-establishment of control intracellular pH values. Accordingly, steady-state extracellular pH and intra- and extracellular P_{CO_2} and bicarbonate levels shifted in association with a decreased steady-state rate of metabolism in *S. nudus in vivo*. A reduction in ventilation frequency caused a rise in gas partial pressure gradients between the intra- and extracellular body compartments and between the extracellular space and the ambient water.

The compensation of the intracellular acidosis during long-term hypercapnia eliminates intracellular pH as an important factor in long-term metabolic down-regulation. Companion studies have demonstrated that metabolic depression during steady-state hypercapnia is mediated by a decrease in extracellular pH effective at the cellular level (Reipschläger and Pörtner, 1996) and by a depression of central nervous activity mediated by adenosine accumulation (Reipschläger *et al.* 1997). The overshoot in metabolic rate seen during recovery from hypercapnic exposure resembles the payment of an oxygen debt after anoxic exposure; however, the mechanism remains unexplained. Hypercapnic depression caused metabolism to shift to an increased proportion of protein and amino acid catabolism, as indicated by the drop in O/N ratios and the increased release of proton equivalents seen as a net change in the steady-state transepithelial transfer of acid–base

equivalent ions. The mechanisms eliciting this shift also require further investigation.

Fluctuations in gas partial pressures, levels of high-energy phosphates and ventilatory frequency suggest that the period of transition to hypercapnia was characterized by an initial drop in metabolic rate and ventilation frequency, followed by a transient compensatory rise before the initiation of long-term metabolic depression. The mechanism behind the oscillatory transition to long-term hypercapnia remains obscure. Different time courses of changes in pHi and phospho-L-arginine levels indicate that intracellular pH and phosphagen metabolism remain uncoupled when metabolic rate is low. A small rise in metabolic rate is accompanied by a large factorial rise in free ADP levels, which appears to be the key factor modulating phosphagen levels even under fully aerobic conditions.

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