

Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempfi*) as a function of ambient CO₂: pH-dependent limitations of cellular protein biosynthesis?

M. Langenbuch and H. O. Pörtner

Alfred-Wegener-Institut für Polar- und Meeresforschung, Ökophysiologie und Ökotoxikologie, Postfach 120161,
D-27515 Bremerhaven, Germany

*Author for correspondence (e-mail: hpoertner@awi-bremerhaven.de)

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Summary

Scenarios of rising CO₂ concentration in surface waters due to atmospheric accumulation of anthropogenic CO₂, or in the deep sea due to anticipated industrial dumping of CO₂, suggest that hypercapnia (elevated partial pressure of CO₂) will become a general stress factor in aquatic environments, with largely unknown effects on species survival and well being, especially in cold and deep waters. For an analysis of CO₂ effects at the cellular level, isolated hepatocytes were prepared from two representatives of the Antarctic fish fauna, *Pachycara brachycephalum* and *Lepidonotothen kempfi*. Correlated changes in energy and protein metabolism were investigated by determining the rates of oxygen consumption at various levels of P_{CO₂}, of intra- and extracellular pH, and after inhibition of protein synthesis by cycloheximide. A decrease in extracellular pH

(pHe) from control levels (pHe 7.90) to pHe 6.50 caused a reduction in aerobic metabolic rate of 34–37% under both normocapnic and hypercapnic conditions. Concomitantly, protein biosynthesis was inhibited by about 80% under conditions of severe acidosis in hepatocytes from both species. A parallel drop in intracellular pH probably mediates this effect. In conclusion, the present data indicate that elevated P_{CO₂} may limit the functional integrity of the liver due to a pronounced depression in protein anabolism. This process may contribute to the limits of whole-animal tolerance to raised CO₂ levels.

Key words: hypercapnia, CO₂, Antarctic fish, *Pachycara brachycephalum*, *Lepidonotothen kempfi*, oxygen consumption, metabolic rate, respiratory acidosis, protein synthesis, hepatocyte.

Introduction

It has long been known that periodically or permanently elevated CO₂ partial pressure (hypercapnia) is a natural phenomenon in some marine biotopes such as sediments of the intertidal zone, estuaries (Diaz and Rosenberg, 1995) or hypoxic bottom waters (Knoll et al., 1996). In most of the pelagic zones of the sea, however, CO₂ levels have remained more or less constant. In those habitats subjected to CO₂ oscillations, animals rely on adaptational mechanisms that enable them to compensate for such oscillations. Some of these mechanisms may be phylogenetically old and plesiomorphic, since high CO₂ partial pressures typically go hand in hand with low oxygen levels and are thus characteristic for the earth's early atmosphere (Bernier and Kothalova, 2001) and, accordingly, early aquatic environments.

The currently rising concentrations of anthropogenic CO₂ in atmosphere and surface waters (Haugan and Drange, 1996), and anticipated scenarios of industrial CO₂ disposal in the deep sea, suggest that CO₂ will again become a more general stress factor in aquatic environments, with as yet unknown effects in species that were not previously affected. Plans to dispose of anthropogenic CO₂ in the deep sea (an idea originally

forwarded by Marchetti, 1977, 1979) will expose animals living in the aphotic zone to elevated CO₂ levels, with the highest concentrations at the site of disposal (Auerbach et al., 1996).

The most notable animal groups that will be threatened by increasing ocean P_{CO₂} levels include cold-water deep-sea fishes, which at present live in an environment that is characterized by great temporal and horizontal spatial stability of physical and chemical conditions (Childress, 1995). Therefore, it is strongly suggested that deep sea fauna will be sensitive to any change that occurs suddenly and is well beyond the range of conditions under which this fauna has evolved (Hädrich, 1996). General questions arise as to which physiological mechanisms define the limits of tolerance to elevated CO₂ levels, and whether the physiologically 'old' mechanisms of adaptation displayed by organisms from unstable, hypoxic environments are still in use and effective in extant fauna from stable deep sea and pelagic waters.

Apart from investigations of the mechanisms of acid–base regulation, little is known about the influence of hypercapnia on key physiological processes that define the long-term

survival and productivity of a species. Relevant data have mostly been collected for invertebrates that dwell in environments that are regularly prone to combined hypercapnia and hypoxia exposures. These animals can decrease their energy demand far below the standard metabolic rate. The phenomenon of hypercapnia-induced metabolic depression has been extensively investigated in the sipunculid *Sipunculus nudus*, a marine worm from intertidal and subtidal sandy sediments. Under conditions of severe respiratory acidosis, the oxygen consumption of whole animals is reduced (Pörtner et al., 1998) and linked to a decrease in the metabolic rate of isolated muscle tissue of up to 45% (Reipschläger and Pörtner, 1996; Langenbuch and Pörtner, 2002). These reductions in cellular energy turnover are partly realized by diminished costs of net H⁺ excretion and slower regulation of intracellular pH (pHi; Pörtner et al., 2000). Concomitant shifts in cellular N metabolism indicate that a change in the use of amino acid substrates as well as a decrease of protein biosynthesis rates occurs under acidosis (Langenbuch and Pörtner, 2002). Furthermore, a drop in neuronal and motor activity, caused by the accumulation of the neurotransmitter adenosine, supports metabolic depression during hypercapnia and particularly during exposure to combined anoxia and hypercapnia (Reipschläger et al., 1997). The latter data clearly show that the problem of CO₂ tolerance is not only defined at the cellular level. Limiting factors or processes can become effective at all levels of organisation, from specific cellular metabolic pathways to the functional integration of different tissue types into the whole organism under the control of the central nervous system. Comparable data do not exist at this level of detail for any other animal phyla, including the vertebrates.

In order to identify and characterise the key processes determining the CO₂ tolerance of vertebrates, and deep sea fishes, especially, it is crucial to choose an appropriate model organism. Due to extreme difficulties in the collection and handling of deep sea animals, we decided instead to study a representative of the Antarctic benthic fauna that displays physiological characteristics very similar to those in the deep sea, namely hypometabolism and life in the permanent cold. Analysis of the influence of elevated P_{CO₂} on the cellular physiology of the Antarctic eelpout *Pachycara brachycephalum* was the main focus of the present study. This member of the cosmopolitan family Zoarcidae is endemic to the Southern Ocean, lives at aphotic depths as far down as 1800 m and displays a sluggish benthic lifestyle, which contributes to an exceptionally low metabolic rate that is typical of zoarcids (Wells, 1987; Anderson, 1994; van Dijk et al., 1999). To investigate whether effects seen in this species are representative of cold-water fish, the analyses were repeated with specimens of *Lepidonotothen kempfi*. This benthopelagic species probably has a circum-Antarctic distribution and lives at depths between 100 and 900 m (Gon and Heemstra, 1990).

As a first step, we examined whether cellular effects similar to those seen in isolated tissue of the marine invertebrate *S.*

nudus are elicited by experimental hypercapnia in cold-water fish. To this end, correlated changes in pH, P_{CO₂} and aerobic metabolic rate were investigated in isolated hepatocytes from *P. brachycephalum* and *L. kempfi*. We also determined whether and to what extent a shift in the partitioning of cellular energy occurs under conditions of raised CO₂ concentration, including effects on the essential and energetically costly process of protein biosynthesis. Cycloheximide, a specific inhibitor of eucaryotic protein synthesis by inactivating peptidyl transferase activity of the ribosomal 60S subunit, was used to determine what proportion of changes in overall oxygen consumption of the cell were due to protein synthesis.

Materials and methods

Animals

Specimens of *Lepidonotothen kempfi* Norman (125–525 g; 27–32 cm) and *Pachycara brachycephalum* Pappenheim (50–70 g; 22–30 cm) were caught in April 2002 during an Antarctic expedition with RV 'Polarstern' (ANT XIX/5). Releaser traps were used to catch Antarctic eelpout near King George Island (Admiralty Bay and Maxwell Bay) at depths of 300 and 400 m. The Notothenioid *L. kempfi* was collected from bottom trawls at depths of 200–680 m in the northern part of the Scotia Arc near South Georgia and the South Sandwich Islands. All animals were maintained for 2–3 weeks on board in an air-conditioned container equipped with aquaria and aerated recirculated natural seawater at 1±1°C before the start of the experiments to ensure they were in good, healthy physiological condition. *P. brachycephalum* has been maintained under these conditions for up to 2 years, and significant growth was observed during this time, indicating that their exposure to atmospheric pressure does not lead to any significant physiological disturbances. Fish were not fed prior to experimentation.

Preparation and incubation of isolated hepatocytes

Experiments were carried out onboard RV 'Polarstern'. Fish were anaesthetized with a lethal dose of MS222 (0.5 g l⁻¹) in seawater. To date no effect of the substance on standard metabolic rate or protein synthesis in fish has been reported (Hove and Moss, 1997). However, rainbow trout immersed for periods longer than 15 min had significantly decreased hepatic glycogen concentrations, probably mediated by catecholamine release (Palace et al., 1990; Perrier and Bernier, 1998). To avoid this effect we minimized exposure times to MS222 to around 5 min.

The liver was excised and placed on ice in medium 1 (Hepes 10 mmol l⁻¹, NaCl 240 mmol l⁻¹, KCl 5 mmol l⁻¹, KH₂PO₄ 0.4 mmol l⁻¹, Na₂HPO₄ 0.3 mmol l⁻¹, NaHCO₃ 4 mmol l⁻¹, glucose 5.6 mmol l⁻¹, pH 7.40). Using a small disposable syringe, medium 1 was injected into the hepatic vascular system and the liver blanched immediately due to the clearance of blood from the organ. The perfusate was discarded. Subsequently, the liver was perfused for 20–30 min by repeated injection of 3 ml of ice-cold medium 2 (medium 1

containing 750 U ml⁻¹ Sigma collagenase type IV and 1% bovine serum albumin). Afterwards the liver tissue was finely minced with scissors and incubated for another 45 min on ice in medium 2. Once every 10 min, the tissue was gently torn apart with a plastic Pasteur pipette. After digestion, the resultant cell slurry was poured through a 150 µm nylon mesh to remove large fragments. Remaining blood cells were removed by centrifugation (2–4 min, 80 g, 2°C) and washing of the cellular pellet with medium 3 (medium 1 with 1% BSA). The supernatant and the layer of red cells on top of the hepatocytes were removed using a Pasteur pipette. Isolated hepatocytes were resuspended in 2–3 ml of medium 4 (medium 1 containing 1% BSA, 2 ml 100 ml⁻¹ Life Technologies amino acid mix and 2 mmol l⁻¹ MgSO₄). Cell density was determined in a Fuchs–Rosenthal counting chamber and adjusted to 3.5×10⁷ cells ml⁻¹ (*L. kempfi*) or 1.8×10⁷ cells ml⁻¹ (*P. brachycephalum*), respectively. Cell viability was assessed by examining for the exclusion of Trypan Blue. On average, more than 96% of the *L. kempfi* cells and 93% of the *P. brachycephalum* cells excluded the dye. In all analyses cells were kept in medium 4 on ice for 1 h before further experimentation.

To measure the respiration of hepatocytes at different extracellular pH values (pHe) and P_{CO₂}, cells were collected from 130–150 µl samples of the cell suspension and centrifuged for 4 min (80 g, 2°C). Pellets were resuspended in medium 1 adjusted to the respective pH (pHe=7.90, 7.20 or 6.50) and P_{CO₂} (normocapnia: 100% air, P_{CO₂}=0.03 kPa; hypercapnia: 99% air/1% CO₂, P_{CO₂}=1.01 kPa) and incubated for 50 min on ice in Eppendorf tubes filled to the top. Oxygen consumption measurements were conducted in low amino acid medium 1 (in contrast to the storage medium 4 of the cellular stock solution) to achieve conditions similar to those presumed for the whole animal during standard metabolism. Conditions of low pH or hypercapnic stress have been shown to suppress feeding activity in a variety of organisms (e.g. Bamber, 1987, 1990) and hepatocytes were prepared from non-fed fish.

Media used for incubation were bubbled continuously using a 2M303/a-F Wösthoff (Germany) gas mixing pump. The pH of the medium was titrated to the specific value required using HCl and NaOH, after equilibrating the solutions with the respective gas mixture. Values were maintained constant during further bubbling. A pHe of 7.90 was chosen as a control value, as found in blood plasma from different Antarctic fishes (Kunzmann, 1991). Acidotic pH values as low as 6.50 mimic elevated environmental P_{CO₂} values and were intended to fall slightly below the range of values reached naturally in fish plasma, e.g. under conditions of severe metabolic acidosis (Heisler, 1986a). To determine the extent of inhibition of oxygen consumption by cycloheximide, cells were treated as described above, but the incubation medium contained 35.5 µmol l⁻¹ cycloheximide (from a stock solution in dimethylsulphoxide) and cells were incubated for 60 min on ice before oxygen consumption was determined.

Measurements of cellular oxygen consumption

Oxygen consumption of isolated hepatocytes was measured using a micro-optode system (PreSens, Neuburg, Germany). The sensor consists of a fiber optic cable supplied with a standard glass fiber plug to connect to the optode array. The tip of the sensor is coated with a ruthenium-II-luminophor complex immobilized in a polymer matrix. Light is emitted from a blue-light diode and the resulting fluorescence signal is detected and enhanced by a photomultiplier. Oxygen acts as a dynamic fluorescence quencher of the luminophor. The intensity, lifetime and modulation of the phase angle of the fluorescence signal are influenced by the number of oxygen molecules present and can be measured to calculate oxygen saturation in the medium (Klimant et al., 1995).

All experiments were performed at 2°C. The cell suspension was constantly stirred in glass microvials (0.25 ml, Supelco, Bellefonte, USA) in a cooling waterbath to ensure a uniform sample temperature. Micro-optodes were implanted in small plastic syringes that could be attached to the top of the glass vials using specially designed adapter lids, thereby providing a gas-tight measuring chamber. A two-point calibration of the system was performed using saturated ascorbic acid solution for 0% and air-bubbled medium for 100% air saturation.

After incubation, cells were recollected by centrifugation and pellets were resuspended in 150 µl of fresh medium of the desired pH and P_{CO₂} (with or without cycloheximide). Using a small Hamilton syringe, the cell suspension into the glass vial and the exact volume and cell number of the solution was noted. After closing the system and stabilising the phase angle signal, measurements were continued for at least 20 min.

Cellular oxygen consumption rates were determined under control conditions (pH 7.90, normocapnia) and compared to the rates under increasing acidotic stress (pH 7.20 and pH 6.50). All experiments were also performed at normocapnic and hypercapnic P_{CO₂} and at the respective pH. The inhibiting effect of cycloheximide was quantified at pH 7.90 and pH 6.50 under both normocapnic and hypercapnic conditions.

Intracellular acid–base variables

The intracellular pH of *P. brachycephalum* isolated hepatocytes was measured after incubation using the homogenate technique (Pörtner et al., 1990). Unfortunately, sample numbers of *L. kempfi* and thus the amount of hepatic tissue obtained was far too low to permit parallel analyses of pHi in this species. Pellets of 45–55 mg cellular fresh mass were resuspended in a 300 µl Eppendorf tube in a solution containing KF (160 mmol l⁻¹) and nitrilotriacetic acid (1 mmol l⁻¹). The tubes were filled to the top with approximately 250 µl of the solution to avoid air bubbles, closed and mixed on a vortex mixer. Intact cells were disrupted by freeze thawing and sonification (2 min, 2°C). After brief centrifugation (30 s, 20 000 g, 2°C), the supernatant was used for pHi measurements. Apparent intracellular HCO₃⁻ concentrations were calculated using values of pK^{'''} and the solubility coefficient αCO₂ determined according to Heisler (1986b). Well-equilibrated cellular suspensions have markedly

reduced diffusion limitations compared to tissues, so intracellular P_{CO_2} levels were assumed to equal extracellular P_{CO_2} levels for the calculation of bicarbonate concentrations.

Statistics

For each treatment (normocapnia and hypercapnia), oxygen consumption rates under control and experimental conditions were compared using two-factorial analyses of variance (ANOVA) or analyses of covariance (ANCOVA). When a significant influence of a single variable was indicated by ANOVA/ANCOVA, the different experimental treatments were compared using the Tukey Test. For the effects of cycloheximide on oxygen consumption rates, values are expressed as a percentage of the respective control value, to facilitate visual comparisons between different experimental conditions. Original data were analysed by Student's t -test for paired samples. Significant changes of normalized data were determined using the Mann-Whitney rank sum test. In all cases, $P < 0.05$ was accepted as the level of significant difference. All values are presented as means \pm standard deviation (S.D.) for preparations of hepatocytes from 4–6 individuals. Two replicate samples were measured per preparation for each treatment (combination of pHe and P_{CO_2}).

Results

Steady-state intracellular acid–base variables

By controlling pHe and P_{CO_2} of the medium it was possible to clamp steady-state intracellular variables such as pHi, P_{CO_2} and $[\text{HCO}_3^-]_i$. Changes in *P. brachycephalum* cellular acid–base variables are summarized in Table 1. As shown in Fig. 1, pHi decreased with medium pH in a significant and linear way (ANOVA; $F=339.535$, $P=0.003$). In most instances, pHi values were slightly, but not significantly (ANOVA; $F=3.986$, $P=0.184$), higher in hypercapnic than in normocapnic samples, with smaller differences at lower pHe values. This observation is in line with previous data obtained

Table 1. Intra- and extracellular acid–base variables of isolated hepatocytes prepared from *P. brachycephalum*

pHe	pHi	P_{CO_2} (kPa)	$[\text{HCO}_3^-]_i$ (mmol l ⁻¹)	$[\text{HCO}_3^-]_e$ (mmol l ⁻¹)
7.90	7.65 \pm 0.08	1.02	10.45	48.95
7.90	7.58 \pm 0.05	0.03	0.39	1.54
7.20	7.23 \pm 0.05	1.02	4.12	8.68
7.20	7.17 \pm 0.07	0.03	0.15	0.27
6.50	6.98 \pm 0.05	1.02	2.61	1.68
6.50	6.98 \pm 0.07	0.03	0.08	0.05

pHi values are means \pm S.D., $N=5-6$.

Intracellular CO_2 partial pressure was assumed to equal medium P_{CO_2} .

Apparent mean intracellular bicarbonate concentrations were calculated from mean pHi values by use of the Henderson–Hasselbach equation (see Materials and methods).

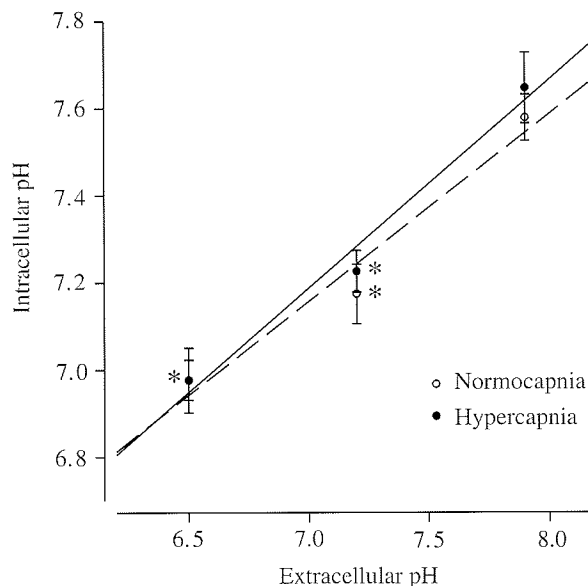


Fig. 1. Steady state intracellular pH values (pHi) of isolated hepatocytes from *P. brachycephalum* under conditions of normo- and hypercapnia, measured after 50 min of exposure to media of different pH. *pHi significantly different from the control value at pHe 7.90. Values are means \pm S.D., $N=5-6$.

using *S. nudus* isolated muscle tissue (Langenbuch and Pörtner, 2002). Intracellular accumulation of bicarbonate, especially under hypercapnic conditions (2.6 mmol l⁻¹ at pHe 6.50, 10.5 mmol l⁻¹ at pHe 7.90; see Fig. 4), was not as marked as in the isolated invertebrate muscle tissue, where intracellular bicarbonate values increased from 2.3 mmol l⁻¹ (pHe 6.70) to 15.3 mmol l⁻¹ (pHe 7.90), due to higher regulated values of intracellular pH.

Dependence of oxygen consumption rates on experimental pH values

Rates of oxygen consumption by isolated hepatocytes from both *L. kempfi* and *P. brachycephalum* (Fig. 2A,B) were significantly influenced by decreasing medium pH (ANOVA; $F=23.390$, $P < 0.001$ for *P. brachycephalum*; $F=63.119$, $P < 0.001$ for *L. kempfi*). The parallel lowering in pHi, seen in *P. brachycephalum* hepatocytes, correlated in a linear and significant way ($F=37.038$, $P < 0.001$) with decreasing rates of oxygen consumption (see Fig. 3). Under normocapnia and at a low pHe of 7.20 (pHi=7.17 \pm 0.07), the rate of oxygen consumption by hepatocytes from Antarctic eelpout decreased to approximately 85% of the rate measured under control conditions (pHe 7.90, $P_{\text{CO}_2}=0.03$ kPa) and fell even further to 66% at pHe 6.50 (pHi=6.98 \pm 0.07). The results obtained for hepatocytes from *L. kempfi* were similar. Oxygen consumption rates decreased by approx. 17% at pHe 7.20 and 33% at pHe 6.50. The decline in aerobic metabolic rates was similar during hypercapnic incubation of hepatocytes from both species. On average, the rates were approx. 23% below control

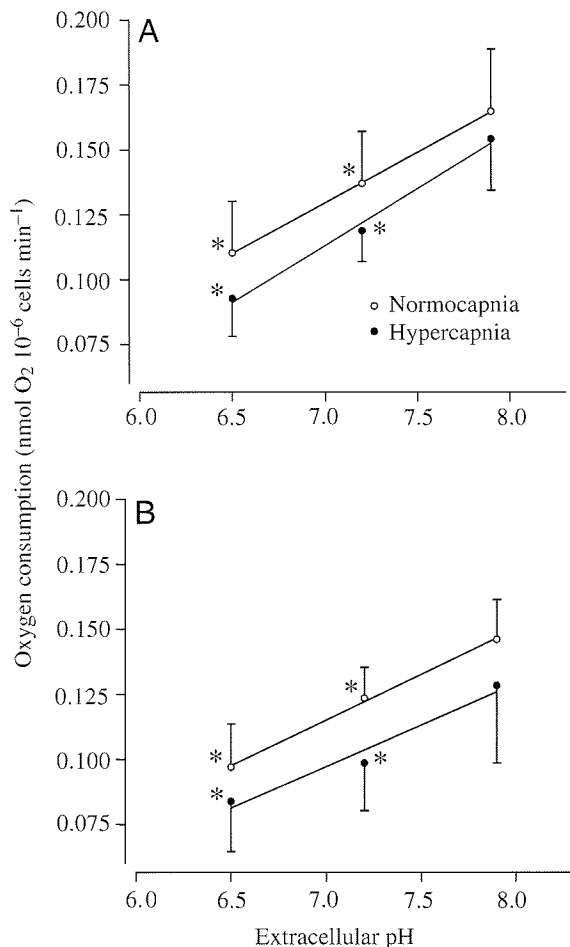


Fig. 2. Rates of oxygen consumption by isolated hepatocytes from *L. kempfi* (A) and *P. brachycephalum* (B) at different extracellular pH values during normocapnic or hypercapnic incubation. *Oxygen consumption rate significantly different from the respective control value at pHe 7.90. Values are means \pm S.D., $N=6-7$ for *L. kempfi* and $N=4$ for *P. brachycephalum*.

levels at pHe 7.20 and 37% below at pHe 6.50. Comparison of normocapnic and hypercapnic data clearly demonstrated that for identical pHe values there was a significant difference in oxygen consumption under the two P_{CO_2} treatments in both fishes (ANOVA; $F=11.025$, $P=0.002$ for *P. brachycephalum*; $F=12.220$, $P<0.001$ for *L. kempfi*). In contrast, the influence of pHi on oxygen consumption of hepatocytes from *P. brachycephalum* displayed no significant dependence on P_{CO_2} treatment, despite a similar mean difference between normocapnic and hypercapnic oxygen consumption rates at a specific pHi value (Fig. 3, ANCOVA; $F=0.002$, $P=0.964$). However, the pHi determinations showed relatively high standard deviations, possibly due to methodological reasons, so differences between normocapnic and hypercapnic data might have been seen with larger (but unavailable) sample numbers.

Changes in other acid-base variables, P_{CO_2} or extra-/intracellular $[HCO_3^-]$, were not consistently related to oxygen

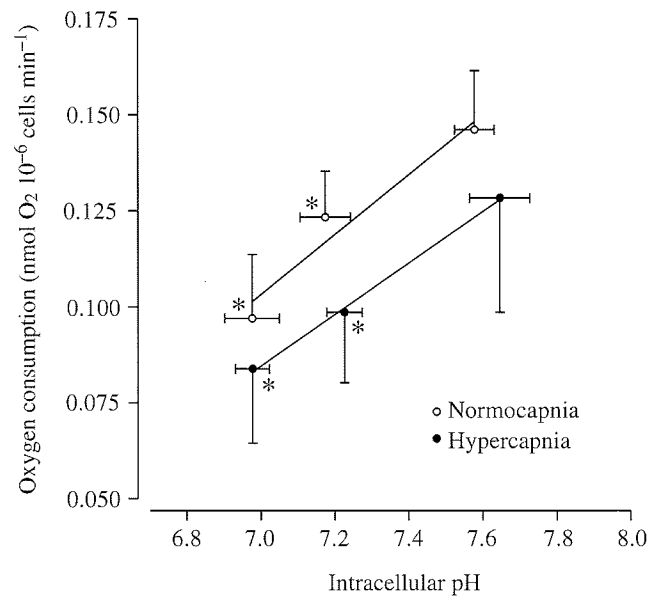


Fig. 3. Relationship between rates of oxygen consumption by isolated hepatocytes from *P. brachycephalum* and intracellular pH, measured at different values of extracellular pH during normocapnic or hypercapnic incubation. *Oxygen consumption rate significantly different from the respective control value at pHe 7.90. Values are means \pm S.D., $N=4$.

consumption under any of the experimental conditions analysed. As seen in Fig. 4, intracellular and especially extracellular bicarbonate concentrations differed considerably between both P_{CO_2} treatments at the same pHe, but nevertheless the changes in oxygen consumption rates were similar.

Inhibition of protein biosynthesis by cycloheximide

Depression of cellular oxygen consumption after specific inhibition of protein biosynthesis by cycloheximide is an indication of the contribution of protein anabolism to overall metabolic rate. In preliminary titration experiments the effect of the cycloheximide concentration on the rate of oxygen consumption was established (data not shown). We incubated cells with $35.5 \mu\text{mol l}^{-1}$ (Lefebvre et al., 1993), 0.7 mmol l^{-1} and 1 mmol l^{-1} (Wieser and Krumschnabel, 2001) cycloheximide. The measured reduction of oxygen consumption rate did not increase with increased inhibitor concentrations above the value obtained at $35.5 \mu\text{mol l}^{-1}$ cycloheximide. Therefore, we decided to work with the lowest tested inhibitor concentration to minimize side effects due to unspecific inhibition of cellular processes.

In normocapnic compared to hypercapnic hepatocytes, there were no detectable significant differences in the response of cellular oxygen consumption rates to cycloheximide under control (pHe 7.90) or acidotic conditions (pHe 6.50) ($P=0.862$ and $P=0.664$, respectively, for *P. brachycephalum*; $P=0.716$ and $P=0.879$, respectively, for *L. kempfi*). Our experiments clearly showed that under

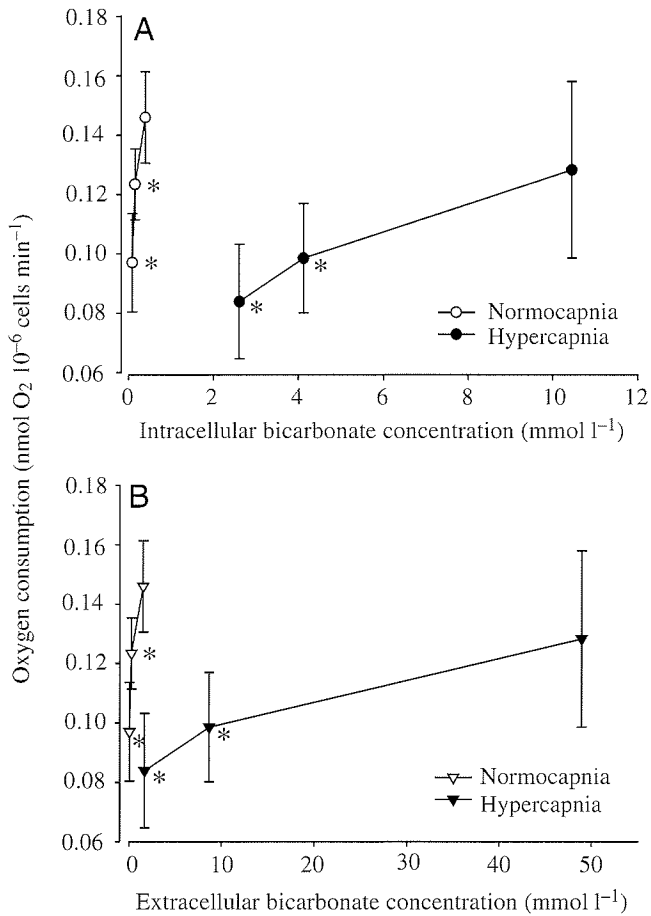


Fig. 4. Correlated changes in rates of oxygen consumption and intracellular (A) and extracellular (B) bicarbonate concentrations determined in hepatocytes isolated from *P. brachycephalum*. Intracellular values were measured after 50 min of exposure to normocapnic and hypercapnic media of different pH. *Oxygen consumption rate significantly different from the respective control value at pHe 7.90. Values are means \pm S.D., $N=4$.

control (pHe 7.90, P_{CO_2} 0.03 kPa) and hypercapnic conditions (pHe 7.90, P_{CO_2} 1.01 kPa) in *L. kempfi* and *P. brachycephalum* liver cells, approx. 20% of the aerobic energy metabolism is due to cellular protein synthesis (Fig. 5). The decline in oxygen consumption to 83.1% (*L. kempfi*) and 77.2% (*P. brachycephalum*) of the respective control values, measured after incubation of hepatocytes with $35.5 \mu\text{mol l}^{-1}$ cycloheximide, was significant in both cases ($P < 0.001$ for *L. kempfi* and *P. brachycephalum*). Under conditions of severe acidosis (pHe 6.50, pHi 6.98 ± 0.07 in *P. brachycephalum*) cycloheximide-induced inhibition of cellular oxygen consumption was only 3–5%, i.e. no significant change from the respective control value in *L. kempfi* ($P=0.139$). The decrease was also small, but was significant, in *P. brachycephalum* ($P=0.016$). In both species and under normocapnic and hypercapnic conditions the effect of cycloheximide on oxygen consumption at pHe 7.90 and 6.50 (*L. kempfi*: $P=0.008$ for

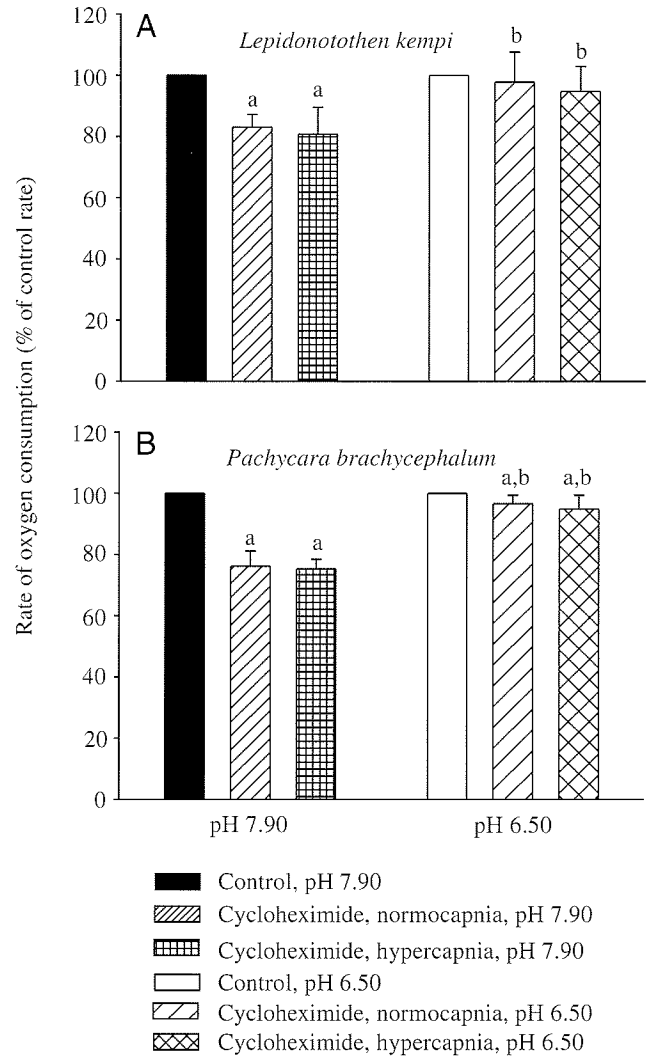


Fig. 5. Rates of oxygen consumption by isolated hepatocytes from *L. kempfi* (A) and *P. brachycephalum* (B), measured after exposure to media of different pH and P_{CO_2} . Control values at the respective pHe were determined after incubation of cells without cycloheximide. To measure the decrease of oxygen consumption rate induced by inhibition of protein biosynthesis, cells were incubated for 60 min with $35.5 \mu\text{mol l}^{-1}$ cycloheximide prior to analysis of metabolic rate. ^aOxygen consumption rates significantly different from the respective control value at pHe 7.90 or pHe 6.50; ^boxygen consumption rates significantly different from the respective cycloheximide-inhibited value at pHe 7.90 (significant differences within normalized data were identified using the Mann–Whitney rank sum test; all results were confirmed by an additional analysis of original data with the Student's *t*-test for paired samples). Values are means \pm S.D., $N=6$ for both species.

normocapnia, $P=0.032$ for hypercapnia; *P. brachycephalum*: $P < 0.001$ for normocapnia, $P < 0.001$ for hypercapnia). Provided that there is no pH-dependent shift in the costs of peptide synthesis, these data suggest that protein biosynthesis was downregulated by approx. 80% at pHe 6.50 in both species, regardless of the level of P_{CO_2} .

Discussion

Antarctic fish thrive at constant low temperatures around 0°C and, accordingly, display low standard metabolic rates compared to fish from warmer waters. With values of 0.50 mmol O₂ kg⁻¹ h⁻¹ (*Pachycara brachycephalum*; van Dijk et al., 1999) and 0.54 mmol O₂ kg⁻¹ h⁻¹ (*Rhizophila dearborni*; Wells, 1987), Antarctic Zoarcidae show exceptionally low whole animal metabolic rates even among fish species endemic to the southern ocean. Wells (1987) measured rates of 1.09–2.03 mmol O₂ kg⁻¹ h⁻¹ in nototheniid fish from McMurdo Sound. In contrast, resting metabolic rates of deep sea fish are as low as 0.11 mmol O₂ kg⁻¹ h⁻¹ (*Coryphaenoides acrolepis*; Smith and Hessler, 1974) or 0.13 mmol O₂ kg⁻¹ h⁻¹ (*Sebastolobus altivelis*; Smith and Brown, 1983). These low metabolic rates are closest to those found in *P. brachycephalum*, which is therefore a deep water Antarctic fish model for deep sea fish. Similarly at the cellular level, hepatocytes from the Antarctic eelpout display somewhat lower oxygen consumption rates under control conditions (0.15±0.02 nmol O₂ 10⁻⁶ cells min⁻¹) than cells from the Nototheniid *L. kempfi* (0.17±0.02 nmol O₂ 10⁻⁶ cells min⁻¹). Unfortunately, data for deep sea fish are not available, but Antarctic fish hepatocytes only consume a small fraction of the amount of oxygen required by hepatocytes from temperate fish at their respective habitat temperature (goldfish: 0.74±0.06 nmol O₂ 10⁻⁶ cells min⁻¹ at 20°C, trout: 5.27±0.60 nmol O₂ 10⁻⁶ cells min⁻¹ at 15°C; Wieser and Krumschnabel, 2001). Values obtained in polar zoarcids may therefore also be representative for cold and deep water fish, with respect to the effects of elevated CO₂ levels.

Similar to previous findings in muscle tissue of a CO₂ tolerant marine invertebrate, *S. nudus*, rate of oxygen consumption by hepatocytes from both Antarctic fish species mirror a drastic reduction in energy demand, depending on pH, but without any influence by intra- or extracellular levels of P_{CO₂} and [HCO₃⁻] (see Fig. 4). In *P. brachycephalum* hepatocytes, intracellular pH changed in parallel with pHe. As shown in Fig. 3, both CO₂ treatments yielded some, but not significant, difference in energy turnover at identical pH_i values, whereas for the same pHe values, the differences in oxygen consumption rates were significant (Fig. 2). It cannot currently be excluded that both intra- and extracellular pH have a specific influence on cellular oxygen demand. At pHe 6.50 oxygen consumption fell by up to 34% in hepatocytes from both Antarctic fish species, signalling a severe reduction in hepatic cellular energy turnover during acidosis. This clearly agrees with previous findings of metabolic depression by respiratory acidosis in other animal models, including the marine invertebrate *S. nudus* (Langenbuch and Pörtner, 2002) or brine shrimp embryos (*Artemia franciscana*; Hand and Gnaiger, 1988). Similar to *Artemia* embryos, where hypercapnia and the subsequent decrease in pH_i serve as a signal for the massive downregulation in metabolic rate, the decrease in hepatic pH_i observed in *P. brachycephalum* elicited metabolic depression, which may temporarily contribute to protect the functional integrity of hepatocytes. In

support of this conclusion, Sakaida and Coworkers (1992) found reduced mortality of rat hepatocytes after treatment with cyanide (chemical anoxia) when cells were cultured at pH 6.00 rather than at control pH. Evidently, similar cellular effects are still present in species that live permanently in highly oxygenated environments like the Antarctic oceans and display a sluggish mode of life and low levels of energy turnover compared to other, warmer water fish. Nevertheless, the question arises as to whether the observed decrease in cellular oxygen consumption is an integral part of a regulated whole-body metabolic depression that will extend the time period that an animal can survive on stored fuel supplies during periods of environmental stress (Guppy et al., 1994), or whether it should be seen as a single rudimentary mechanism which is 'old' on an evolutionary time scale.

Previous analyses of the influence of variable acid–base parameters on trout hepatocyte metabolism revealed a similar effect of reduced pHe and pH_i on the depression of cellular metabolism (production of CO₂ and glucose from lactate; Walsh et al., 1988). Moreover, specific effects of CO₂ on the pathways of lactate metabolism have been postulated (Walsh et al., 1988), which indicate that the shifting acid–base equilibria may be associated with specific changes in metabolic equilibria. When interpreting such data it needs to be borne in mind that lactate may change cellular energy turnover (Pinz and Pörtner, 2003). Specific CO₂ effects are not evident in total energy turnover analysed by oxygen consumption (this study). Overall, a reduction of aerobic metabolism in fish hepatocytes is brought about by the synergistic inhibition by acid–base parameters of both energy-producing (see Walsh et al., 1988) and energy-consuming processes (see below). For comparison, and in contrast to our study, short-term exposure of goldfish or trout hepatocytes to the low pHe of 6.60 did not cause significant metabolic depression (Krumschnabel et al., 2001).

Inhibition of protein synthesis

Under conditions of environmental stress and thus limited energy supply, survival is supported by a parallel and coordinated reduction in ATP-producing and -consuming processes, which includes protein biosynthesis as a major contributor to cellular ATP turnover. In fish, the central site of secondary metabolism is liver, which is responsible for the synthesis and secretion of a variety of essential proteins (e.g. lipoproteins or fibrinogen), and therefore relies upon a highly active protein synthesis machinery (Houlihan, 1991) and may be most severely affected by a non-transient reduction in protein biosynthesis rates due to permanent disturbances of cellular energy metabolism.

Growing evidence indicates that the costs of protein synthesis may have been overestimated in many previous studies, due to secondary effects on cellular energy metabolism by use of overly high cycloheximide concentrations. In isolated goldfish and trout hepatocytes, oxygen consumption rates followed an inverse hyperbolic function with rising cycloheximide concentrations, reaching a maximum effect at

not less than 15 mmol l⁻¹. However, incorporation of labelled amino acids into cellular protein was already fully blocked by 25 µmol l⁻¹ cycloheximide (Wieser and Krumschnabel, 2001). To ensure accurate measurements of the percentage contribution of protein synthesis to metabolic rate we established that the inhibiting effect was saturated at 35.5 µmol l⁻¹ without further reduction in the rate of oxygen consumption at higher levels. These findings show that at this concentration of inhibitor, unspecific inhibition of other cellular processes could very well have been excluded.

A significant decrease of cellular oxygen consumption rates to approx. 80% of the control rates was observed upon inhibition by 35.5 µmol l⁻¹ cycloheximide under control conditions (normocapnia, pHe 7.90) in hepatocytes from both *L. kempfi* and *P. brachycephalum*. These values mirror protein synthesis costs that account for 20% of the hepatic basal energy metabolism and are well within the range of values given for other ectotherm hepatocytes in the literature. A study by Land et al. (1993) on turtle hepatocytes reported a 36% contribution of protein synthesis to metabolic rate while Fuery et al. (1998) determined a value of 12% in liver slices of *Bufo marinus*. These differences are not surprising as both oxygen consumption and rate of protein synthesis in liver, can vary enormously, depending on nutritional and hormonal status (Reeds et al., 1985).

Under conditions of severe acidosis (pHe 6.50, pHi=6.98±0.07) the fraction of cycloheximide-sensitive oxygen consumption decreased significantly from 20% (pHe 7.90) to 3–5% in hepatocytes from both species, reflecting, on average, an 80% reduction in protein synthesis of both *L. kempfi* and *P. brachycephalum* liver cells. An even more pronounced reduction was observed in *Artemia franciscana* embryos, where protein synthesis was suppressed to 3% of control values after 4 h of anoxia or aerobic acidosis due to a global arrest of transcription and translation (Hofmann and Hand, 1994; van Breukelen et al., 2000). A less extreme response to anoxia was seen in hepatocytes from an anoxia-tolerant turtle. The downregulated process of protein synthesis contributed 33% to the global 88% metabolic depression measured under conditions of anoxia (Land et al., 1993; Land and Hochachka, 1994). Compared to the turtle data, the contribution of protein synthesis inhibition to the reduction in metabolic rate in hepatocytes of Antarctic fish was much higher. Almost 60% of the depression in oxygen consumption under acidosis can be attributed to the decline in protein synthesis in both species.

Interestingly, the capacity for protein synthesis was maintained in anoxia-tolerant goldfish hepatocytes even in the metabolically depressed state. In contrast, the more anoxia-sensitive trout hepatocytes experienced a massive downregulation of protein synthesis during anoxia (by 50%, Wieser and Krumschnabel, 2001). Unfortunately, pH effects were not investigated in these studies, so cause and effect relationships remain elusive. The percentage changes in protein synthesis of trout hepatocytes and the even larger changes in liver cells from Antarctic fish may suggest a high

sensitivity to hypercapnia-induced acidosis in the polar fish. Here pH-dependent inhibition of protein biosynthesis is responsible for the largest fraction of the decline in cellular energy turnover at low pH. Perturbations in the activity of Na⁺/K⁺-ATPase or mitochondrial proton leak, both other major contributors to aerobic energy demand of a single cell, were likely to have been very small. In a short-term scenario, the maintenance of transmembrane ion gradients may be more important than the synthesis of new proteins for maintaining the integrity of the hepatocytes. However, it appears likely that a pronounced limitation of protein synthesis will compromise the functional integrity of the organ on an extended time scale.

Summary and conclusions

The present study examined the correlated changes in acid–base variables and in oxygen consumption of hepatocytes from Antarctic fish during normocapnia and hypercapnia. The aerobic energy metabolism of fish hepatocytes was pH-dependent, with a significant and large reduction in the fraction of cellular energy turnover allocated to protein synthesis during extra- and intracellular acidosis. One has to keep in mind that liver accounts for only 2–3% of total body mass in *L. kempfi* and *P. brachycephalum*. Thus, it is difficult to extrapolate the present findings to the whole animal level. Effects seen in isolated hepatocytes may contribute to whole organism metabolic depression under conditions of uncompensated extra- and intracellular acidosis, provided that other tissues, especially the much larger fraction of muscle tissue, respond in similar ways to the liver. Accordingly, it remains unclear from the present data if the 33–34% decrease in hepatic aerobic metabolic rate contributes to whole organism energy savings. At the cellular level, the mechanisms leading to a marked reduction in cellular protein synthesis and, thereby, energy turnover may be similar in marine invertebrates like the CO₂-tolerant intertidal worm *S. nudus* (Langenbuch and Pörtner, 2002) and in fishes. The relevance of the present cellular findings for whole organism responses to CO₂ in Antarctic fish is currently under investigation in this laboratory. An estimation of the adaptational flexibility of Antarctic (and deep sea) fish under conditions of CO₂ stress requires the analysis of whole body metabolic rates as well as the identification of key physiological processes and mechanisms at all organizational levels (e.g. energy and protein metabolism in muscle tissue, cardiorespiratory system, neurotransmitter patterns in the brain). Additional long-term dose–response studies are required to establish critical *in vivo* thresholds for the growth and reproductive success of various groups of animals. The main objective in all of these studies should be the development of a comprehensive picture of short-, medium- and, especially, long-term effects of hypercapnia on animal physiology that will enable us to predict possible long-term consequences for the complex network of marine ecosystems due to future passive or active (dumping) accumulation of anthropogenic CO₂ in the oceans.

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