Polar Biol (2001) 24: 869–874
DOI 10.1007/s003000100298

ORIGINAL PAPER

Christian Bock · Franz J. Sartoris · Rolf-M. Wittig
Hans-O. Pörtner

Temperature-dependent pH regulation in stenothermal Antarctic and eurythermal temperate eelpout (Zoarcidae): an in-vivo NMR study

Accepted: 12 May 2001 / Published online: 6 July 2001

Abstract Temperature-dependent adjustments of intracellular pH are thought to play a major role in the maintenance of protein function. Comparative studies were carried out in two species from the same fish family (Zoarcidae), the stenothermal Antarctic eelpout (*Pachycara brachycephalum*) and the eurythermal eelpout (*Zoarces viviparus*), to find out whether pH regulation is modified by temperature in the closely related species and to what extent the respective pattern differs between eurytherms and stenotherms. Previous invasive studies had compared individual animals sampled at various temperatures and suggested that a decrease in intracellular pH (pHi) values occurs at rising temperatures, as predicted by the alpha-stat hypothesis of acid-base regulation. The present study used non-invasive in vivo ^{31}P-NMR spectroscopy in non-anaesthetized, unrestrained fish for long-term online recordings in individual specimens. Control spectra obtained at $T = 0$°C for *P. brachycephalum* and at 12°C for *Z. viviparus* indicated low stress conditions, as well as a high stability of energy and acid-base status over time periods longer than 1 week. Temperature changes had no influence on the concentration of high-energy phosphates like phosphocreatine or ATP. Temperature-induced pH changes were monitored continuously in a range between 0 and 6°C for polar, and 12 and 18°C for temperate eelpout. A pHi change of around –0.015 pH units/°C was observed within both species, in accordance with the alpha-stat hypothesis; however, extrapolation to the same temperature revealed different set points of pH regulation in the two species. These findings confirm that an alpha-stat pattern of pH regulation can be found in stenothermal Antarctic animals, at set points deviating from an alpha-stat pattern, however, in a between-species comparison.

This paper presents results of the Midterm Symposium of the SCAR programme "Ecology of the Antarctic Sea Ice Zone" (EASIZ). The manuscript was edited by W. Arntz and A. Clarke.

C. Bock (✉) · F.J. Sartoris · Rolf-M. Wittig · Hans-O. Pörtner
Alfred Wegener Institute for Polar and Marine Research,
Columbusstrasse, 27568 Bremerhaven, Germany
E-mail: cbock@awi-bremerhaven.de
Tel.: +49-471-48311380
Fax: +49-471-48311149

Introduction

The alpha-stat hypothesis of acid-base regulation implies that pH regulation in poikilotherms should maintain a constant degree of protonation (α) of imidazole groups in proteins despite changes in body temperature (Reeves 1985). A pH_i change of around –0.018 pH units/°C is expected to support the alpha-stat pattern and ensure protein function at fluctuating temperatures. With some exceptions, an alpha-stat pattern of intracellular pH regulation has been found in several marine ectotherms (invertebrates and fish) from temperate latitudes (for a review see Pörtner et al. 1998) where animals are exposed to wide diurnal and seasonal temperature fluctuations. The question arises as to whether the same holds true for Antarctic species which live within a very narrow temperature window throughout the year. Answering this question will lead to a more profound insight into the overall homeostatic importance of temperature-dependent pH regulation and the relevance of this process in polar species.

In vivo ^{31}P-NMR (nuclear magnetic resonance) spectroscopy has become a helpful tool for physiological studies on fish, and has been applied especially to the determination of changes in energy metabolism and intracellular pH during hypoxia in freshwater species (e.g. Van den Thillart et al. 1989a, b; Van Ginneken et al. 1995; Borger et al. 1998). A few in-vivo NMR studies have been carried out on marine fish – the goby *Pomatoschistus* sp. (Grøttum et al. 1998) and *Harpagifer antarcticus* (Moerland and Eggington 1998). The latter study focused on intracellular pH and temperature, although online pH measurements at different temperatures were not performed. These investigations used

small animals (≤ 5 cm body length) that were anaesthetized and oriented in a vertical position in narrow-bore standard high-resolution NMR spectrometers. These measurements may not reflect the normal physiological status of the animals. Therefore, we developed an experimental set-up for NMR studies of marine fish using a horizontal magnet which allows for controlled and well-defined physiological conditions and continuous measurements during long-term experiments.

Invasive studies compared pH values measured in different individuals of the eurythermal North Sea eelpout (*Zoarces viviparus*) sampled at various temperatures, and supported the conclusion that a decrease in intracellular pH values occurs, as predicted by the alpha-stat hypothesis (Van Dijk et al. 1997). The aim of the present study was to carry out long-term online recordings during changing temperatures in the same individuals and to investigate the α-stat pattern of pH regulation, not only in eurythermal eelpout (*Z. viviparus*) but also in the stenothermal Antarctic eelpout (*Pachycara brachycephalum*). Species of the fish family Zoarcidae can be found around the world in all latitudes, including the deep sea and polar regions. This family is therefore most suitable for the comparison of closely related species from different latitudes. The investigation of pH_i regulation is suitable for answering the question as to whether and to what extent adaptive flexibility with respect to temperature change found in eurythermal temperate zone fish is still present in stenothermal Antarctic fish.

Materials and methods

Antarctic eelpout (*P. brachycephalum*) were caught in Admiralty Bay, Antarctica during the cruise ANT XV/III (1998) and kept in seawater aquaria at 0°C. Three to six animals (average length 30 cm, average weight 60 g) were used for the in vivo studies. Temperate eelpout were collected in the Baltic Sea by commercial fishermen in 1998 and kept in aquaria at 12°C. Animals were fed once a week and starved for 1 week before experiments. The animals were placed in a Plexiglas flow-through chamber with variable slide barriers on each side to position the animals in the centre of the chamber (Fig. 1a). Animals were conscious (not anaesthetized), unrestrained and free to move inside the chamber during the whole experimental time. The chamber was inserted into the spectrometer and a constant flow of seawater (1.5 l/min) was supplied by hydrostatic pressure from a seawater reservoir (ca. 50 l). After passing the chamber, the water was caught in a second tank and pumped back to the reservoir (Fig. 1b). Temperature was measured directly inside the chamber and in the water tanks using a fluoroptic thermometer (Luxtron 504, Polytec, Waldheim, Germany). Temperature data were sampled continuously (sampling rate 10/min, MacLab system, AD Instruments, Australia) and stored on a computer. Temperature control (stability ± 0.3°C in the range between 0° and 30°C) was achieved using a cryostat connected to the upper water reservoir and by feeding the tubings through jackets perfused with coolant.

All experiments were carried out using a 4.7 T magnet with actively shielded gradient coils (Bruker Biospec 47/40 DBX System), equipped with a 20-cm actively decoupled ^{1}H-resonator for signal excitation during imaging and a 5-cm triple-tuned (^{1}H-^{31}P-^{13}C) surface coil (passively decoupled) for signal reception. The coils were oriented orthogonal to each other to prevent cross talking. The 5-cm surface coil was placed directly onto the chamber wall and positioned close to the tail of the animal for ^{31}P-NMR spectroscopy (Fig. 1a).

Pilot scans were collected just before temperature variation and directly after reaching the desired temperature using a multi-slice gradient echo technique in all three directions to control the position of the animal (matrix 256×192, FOV = 12 cm, slice thickness 2 mm, TR = 100 ms, TE = 10 ms, scan time 25 s). For anatomical studies, multi-slice RARE images were performed in coronal and transversal directions (matrix 256×192, FOV = 12 cm, 4 slices with a slice thickness of 1 mm, TR = 2000 ms, TE = 10 ms, rare factor of 8, scan time 1.5 min).

In vivo ^{31}P-NMR spectra were acquired continuously over 1,200 scans, consisting of a 100 μs bp pulse, a repetition delay of 0.52 s resulting in a measurement time of 10 min. All spectra were processed automatically applying a user program with size = 16 K, line broadening = 5 Hz and an automatic baseline correction. The spectra were calibrated to phosphocreatine (PCr) used as an internal standard. Typical line width of PCr was 15 Hz. Signal integration and chemical shifts were calculated using an automatic fit routine (mdcon, Bruker Analytical, Rheinstetten) and compared with results determined by automatic peak picking. A calibration curve for the calculation of pH values from the chemical shift of the inorganic phosphate (Pi) signal was obtained from standard solutions at different pH, and temperatures at 0 and 20°C, with a simulated intracellular ion content. The temperature-dependent pKs for inorganic phosphate and temperature correction factors were obtained from Kost (1990).

The animals were allowed to recover for at least 24 h under control conditions inside the magnet before starting temperature incubations. Steady-state conditions were confirmed by constant in vivo ^{31}P-NMR spectra. Temperature was increased in two steps, from 0 to 3°C and from 3 to 6°C in steps of 1°C over 3 h (12 to 18°C for *Z. viviparus*). When a new steady state was reached (12°C) the temperature was kept constant for at least 12 h. Temperature was decreased to 0°C again at the end of the experiment and the animal was brought back into the aquarium.

Results

The experimental set-up allowed the investigation of Antarctic eelpout under control conditions at 0°C for more than 1 week (data not shown). MR images were obtained from unrestricted and unanaesthetized fish without any movement artefacts, and they revealed that the fish were usually resting on the ground oriented towards the incoming water (Fig. 2). Almost no surrounding water was detected due to the high flow rate in comparison with the detection time of the surface coil. Using a cross coil set-up reduced the surrounding water signal and therefore increased the signal to noise ratio compared with measurements with a single resonator. Different organs are visible inside the fish (assigned as liver, gills, stomach and gall bladder), indicating the good anatomical resolution of the MR images.

Recovery from handling stress occurred within 2 h after setting up the experiment, indicated by constant in vivo ^{31}P-NMR spectra (data not shown). Figure 3 shows a typical in vivo ^{31}P-NMR spectrum of Antarctic eelpout (*P. brachycephalum*) at 0°C compared to temperate eelpout (*Z. viviparus*) at 12°C obtained in the same set-up. Low stress is indicated by the high phosphocreatine over inorganic phosphate ratio (mean value around 11) and the high β-ATP signal compared to the α- and

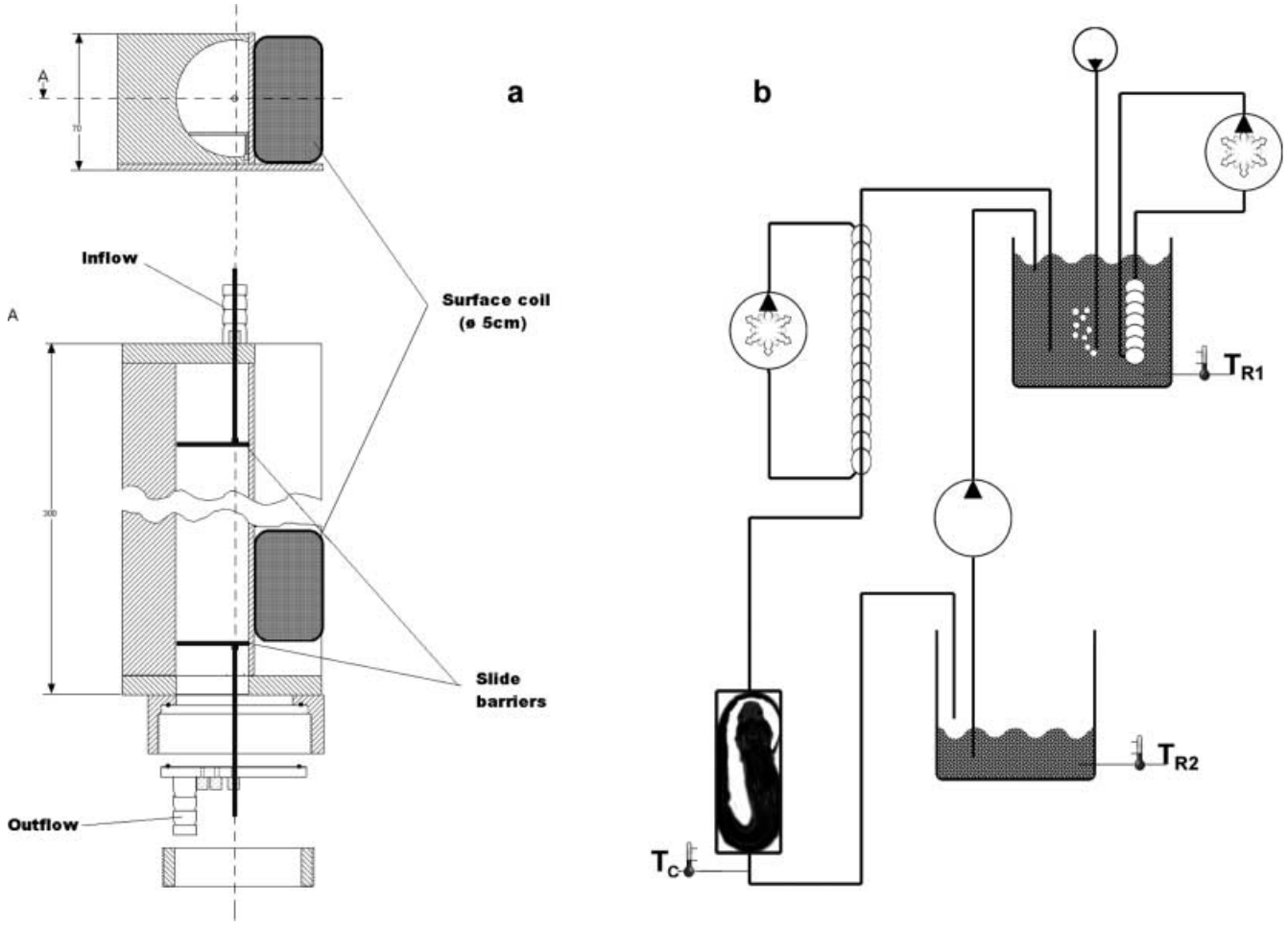

Fig. 1 Flow-through chamber (**a**) for in-vivo NMR measurements of unanaesthetized and unimmobilized fish (max. length 40 cm). The *graph* shows the direction of flow, the position of the surface coil for NMR spectroscopy and the slide barriers to optimize the position of the animals in the sensitive volume of the surface coil. **b** Experimental set-up. The chamber was placed into the spectrometer and a constant flow of seawater (up to 1.5 l/min) was supplied by hydrostatic pressure. Seawater (approx. 50 l) was bubbled with air in the header tank; temperature was measured in the chamber (T_C) and in the water reservoirs (T_{R1} and T_{R2}) using a fluoroptic thermometer connected to a MacLab system. Temperature control was achieved by a cryostat connected to the upper water reservoir and the cooling jackets of the tubings

γ-ATP signals, which also include ADP. In addition to PCr, inorganic phosphate and the three ATP resonances, glycerophosphoethanolamine (GPE), glycerophosphocholine (GPC), phosphoethanolamine (PE) and phosphocholine (PC) signals could be identified in eelpout spectra. Furthermore, a high level of an unknown phosphate compound (X) could be detected in the phosphodiester region (PD). In contrast, only a small amount of GPC and GPE, as well as PC and PE, were visible in *Z. viviparus*, and signal X was not found at all in the in vivo ^{31}P-NMR spectrum of the eurythermal eelpout.

A typical time course of intracellular pH variations at rising temperatures is presented in Fig. 4. An intracellular pH of about 7.4 was measured at 0°C. The scatter of individual values resulted from the small inorganic phosphate signal under control conditions and the insensitivity of the automatic fit routine for small signals in combination with small changes in chemical shifts. An acidification could be observed immediately after changing the temperature from 0°C to 3°C, as well as from 3°C to 6°C. At both temperatures a new steady state value was reached within 3 h. Figure 5 summarizes the mean values of intracellular pH of *P. brachycephalum* at 0, 3 and 6°C compared to pH changes observed in *Z. viviparus* at 12, 15 and 18°C. The decrease in pH with temperature calculated from these data was about ΔpH/°C = –0.015/°C in *P. brachycephalum* and ΔpH/°C = –0.014/°C in *Z. viviparus*.

Discussion

The aim of this study was the online monitoring and comparison of changes in intracellular pH and energy status with temperature in the stenothermal Antarctic eelpout *P. brachycephalum* and the eurythermal temperate eelpout *Z. viviparus*. All animals survived heat exposure and no mortalities occurred during the subsequent recovery period. 6°C is well below the critical

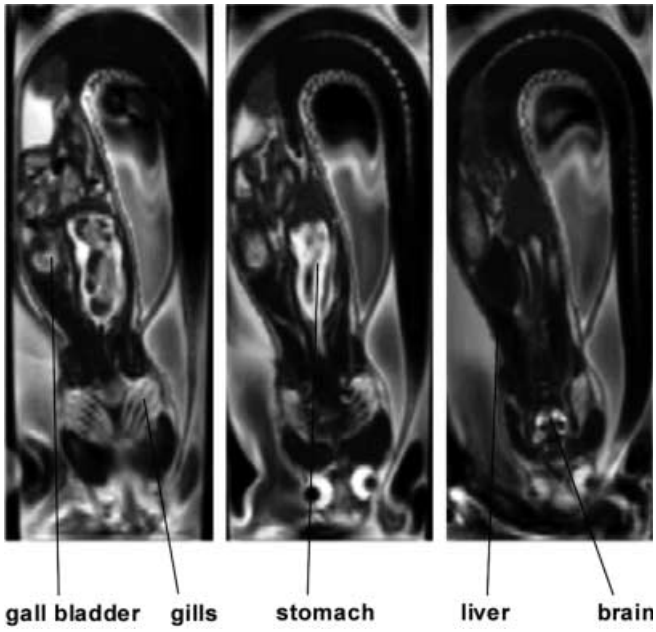

Fig. 2 In-vivo MR images from the Antarctic eelpout *Pachycara brachycephalum* under control conditions (obtained with RARE; parameters: matrix 256×192, FOV = 12 cm, 4 slices with a slice thickness of 1 mm, TR = 2,000 ms, TE = 10 ms, rare factor of 8, scan time 1.5 min). Different organs are visible inside the fish (assigned as gall bladder, gills, stomach, liver and brain), indicating the good quality of the MR images, obtained in seawater as a highly conductive medium

temperature of *P. brachycephalum* found at about 9°C, according to changes in oxygen consumption and anaerobic metabolism (Van Dijk et al. 1999). Since no anaesthetics were used, their potential influence on blood and tissue acid-base parameters as reported for rainbow trout (Iwama et al. 1989) and in the Antarctic fish *Pagothenia borchgrevinki* (Ryan 1992) could be excluded.

Spectra obtained from *Pachycara brachycephalum*, as well as from *Z. viviparus*, indicated low stress conditions, reflected by low Pi signals and stable resting pH_i. In contrast to *Z. viviparus*, high amounts of phosphomono- and diesters could be detected in *Pachycara brachycephalum*. Van den Thillart et al. (1989a) found phosphodiester signals in in vivo ^{31}P-NMR spectra of carp and goldfish. They also recorded ^{31}P-NMR spectra from different tissue extracts and concluded that these signals mainly accrued from the lateral red muscle. The lateral red muscle of fish displays a higher free fatty acid content, a much greater lipase activity and a faster lipid turnover than the white muscle (George 1962); the authors therefore interpreted these phosphodiesters to be intermediate products of phospholipid formation (Van den Thillart et al. 1989a). This may suggest that Antarctic eelpout have a higher free fatty acid content, reflecting a faster lipid turnover or greater lipase activity than in temperate species. A higher free fatty acid content, as well as a faster lipid turnover, might be associated with metabolism used to preserve membrane fluidity (Storelli et al. 1998). Alternatively, the high phosphodiester content may reflect the higher mitochondrial density in Antarctic eelpout (Johnston et al. 1988).

pH plays an important role in the maintenance of protein function during temperature change. The constant dissociation state of histidine residues within proteins, especially in active sites of enzymes, is seen as a key factor in this process (α-stat hypothesis, Reeves 1985). A shift in intracellular pH with changing body temperature may compensate for the temperature-dependent change of pK values of histidine imidazole groups ($\Delta pK/\Delta T \sim -0.018$/°C). The temperature-dependent flexibility of acid-base regulation is interpreted as reflecting metabolic adjustments and thereby adaptive flexibility with respect to temperature; however, this pattern is only found within the range of thermal tolerance (e.g. Sommer et al. 1997). In our study, we did not observe any significant changes in the levels of high-energy phosphates like PCr and ATP during temperature incubations between 0 and 6°C (12 and 18°C in temperate eelpout), thus confirming the good physiological conditions inside the magnet and also reflecting the insensitivity of white muscle energy status to thermal stress, possibly supported by an α-stat pattern of pH_i regulation.

A mean pH_i change of –0.015 pH/°C, as observed below the critical temperature in *Pachycara brachycephalum*, is close to the value expected according to an α-stat pattern of pH regulation. Furthermore, our data agree with those pH values and temperature-induced pH changes found by use of invasive methods (at –0.015 pH/°C in *Pachycara brachycephalum* white muscle; Van Dijk et al. 1999). In contrast to *Z. viviparus* where the contribution of passive mechanisms to the pH change is small (Van Dijk et al. 1997), a large fraction of the temperature-induced pH change in *Pachycara brachycephalum* is caused by passive physico-chemical buffering (–0.011 pH/°C; Pörtner and Sartoris 1999). Such a large contribution of passive buffering is typically found in polar stenotherms (Pörtner and Sartoris 1999).

Moerland and Eggington measured the pH_i of the stenothermal Antarctic teleost *H. antarcticus* using ^{31}P-NMR, and concluded that their data were not consistent with the α-stat hypothesis (Moerland and Eggington 1998). This conclusion arose from a comparison of pH_i values between unrelated species, since in their own study pH_i was only determined at one temperature (1°C) and compared with published data from different organisms obtained at different temperatures. pH_i values compared between species were relatively independent of temperature below 17°C. In fact, the comparison of pH_i values from Antarctic eelpout (0–6°C) with those found in the closely related Baltic eelpout (12–18°C) also reveals a lower slope (dotted line, –0.009 pH/°C, Fig. 5). At first sight, this is in line with the conclusion of Moerland and Eggington. Nevertheless, both eelpout species showed a pH_i change in accordance with α-stat pH regulation (see Fig. 5, –0.015 and –0.014 pH/°C, respectively), disclosing a misleading generalisation

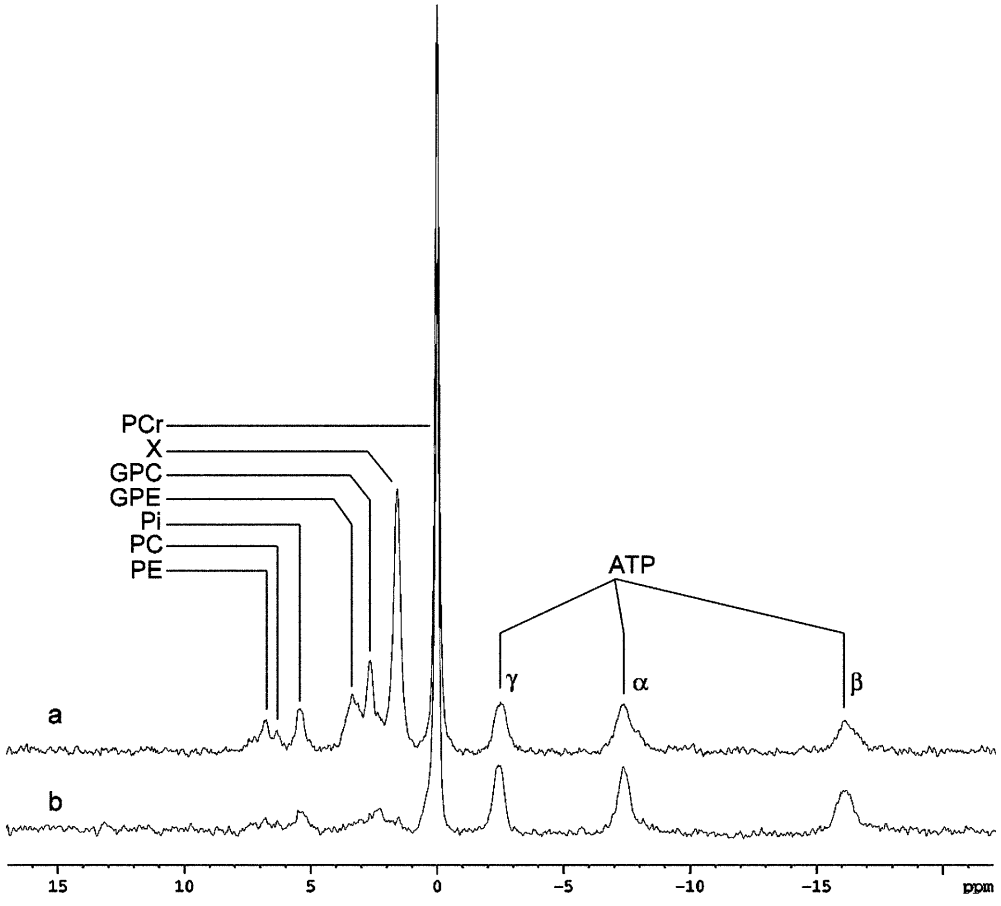

Fig. 3 In vivo ^{31}P-NMR spectra of *Pachycara brachycephalum* (*a*) and *Zoarces viviparus* (*b*) muscle tissue under control conditions. The spectrum of Antarctic eelpout showed additional signals in the phosphodiester region, assigned as *GPE*, *GPC*, and their monomers *PE* and *PC*. An unknown signal *X* was also observed

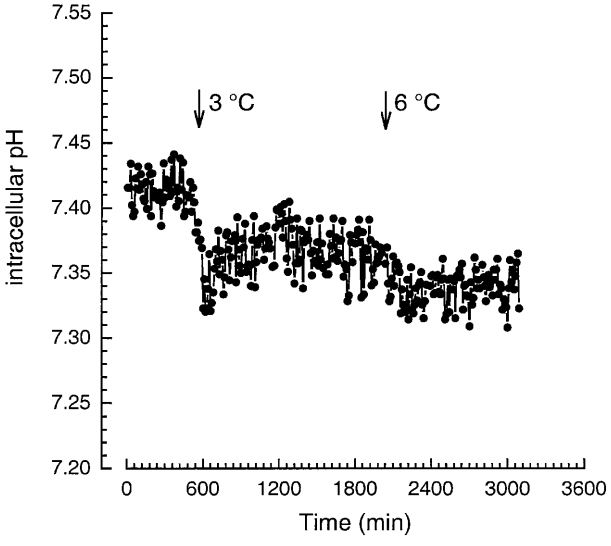

Fig. 4 Typical time course of pH changes at rising temperatures obtained from in vivo ^{31}P-NMR spectra of *Pachycara brachycephalum*. A slight acidification could be observed developing with increasing temperatures (0 to 3°C and 3 to 6°C)

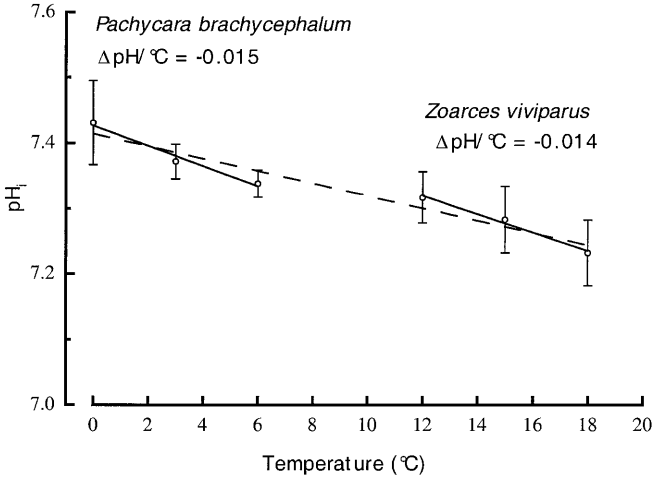

Fig. 5 Intracellular pH values derived from in-vivo ^{31}P-NMR spectra of Antarctic eelpout at 0°C ($n=6$), 3°C ($n=5$) and 6°C ($n=3$) in comparison to pH values from Baltic Sea eelpout at 12°C ($n=3$), 15°C ($n=3$) and 18°C ($n=2$, mean ± range). Spectra obtained 3 h after the change of temperature were used for the calculation of mean values. The decrease in pH with increasing temperature occurred by $\Delta pH/°C = -0.015$ for *Pachycara brachycephalum* and $\Delta pH/°C = -0.014$ for *Zoarces viviparus* and reflects α-stat pH regulation, whereas the comparison between species shows a slope (*broken line*, $\Delta pH/°C = -0.009$) lower than expected from the α-stat pattern (see text)

based on comparisons between species. It appears that cold-adapted species operate at a lower set point of intracellular pH than expected from an extrapolation of pH values from temperate or warm-adapted species. Alternatively, Pörtner and Sartoris (1999) suggested that a higher mitochondrial density causes lower mean values of intracellular pH. Further study must elaborate on whether this may explain the apparent non α-stat pattern seen in the between-species comparisons.

A recent in vivo ^{31}P-NMR study on common carp at different temperatures also found an α-stat pattern of pH regulation (Borger et al. 1998). These authors reported an immediate change in pH upon exposure to the new temperature and a pH significantly different from control conditions after a long-term temperature change by 8°C (25°C to 33°C) over 4 weeks (Borger et al. 1998). This confirms and reflects exactly our finding of a rapid temperature-dependent pH_i change in Antarctic eelpout. The immediate pH_i change with temperature in *Pachycara brachycephalum* is in line with a large contribution of passive mechanisms to temperature-induced pH_i changes.

In conclusion, and in contrast to previous reports in the literature, our study demonstrates that pH_i regulation in at least some Antarctic fish occurs as predicted by the α-stat hypothesis. This observation emphasizes the importance of a finely tuned adjustment of intracellular pH, even in polar animals, which experience minor temperatures changes compared to species living in temperate zones. Future research should compare the role of intracellular pH in the regulation of metabolic pathways and energy turnover in closely related species from various, including polar, latitudes.

Acknowledgements Support by a BMBF grant (project 03PL02A, NMR laboratory) is gratefully acknowledged. A contribution to the ELOISE project, Effects of climate-induced change on marine coastal fishes (CLICOFI, ENV-CT97-0596), funded by the European Union. ELOISE publication no. 141.

References

Borger R, Boeck G de, Van Audekerke J, Dommisse R, Blust R, Van der Linden A (1998) Recovery of the energy metabolism after a hypoxic challenge at different temperature conditions: a ^{31}P-nuclear magnetic resonance spectroscopy study with common carp. Comp Biochem Physiol A 120:143–150

George JC (1962) A histochemical study of red and white muscle of mackerel. Am Midl Nat 68:487–494

Grøttum JA, Erikson U, Grasdalen H, Staurnes M (1998) In vivo ^{31}P-NMR spectroscopy and respiration measurements of anaesthetized goby (*Pomatoschistus* sp.) pre-exposed to ammonia. Comp Biochem Physiol A 120:469–475

Iwama GK, McGeer JC, Pawluk MP (1989) The effects of five fish anaesthetics on acid-base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. Can J Zool 67:2065–2073

Johnston IA, Camm JP, White MG (1988) Specialisation of swimming muscles in the pelagic Antarctic fish, *Pleurogramma antarcticum*. Mar Biol 100:3–12

Kost GJ (1990) pH standardization for phosphorus-31 magnetic resonance heart spectroscopy at different temperatures. Magn Res Med 14:496–506

Moerland T, Eggington S (1998) Intracellular pH of muscle and temperature: insight from in vivo ^{31}P NMR measurements in a stenothermal Antarctic teleost (*Harpagifer antarcticus*). J Therm Biol 23:275–282

Pörtner HO, Sartoris FJ (1999) Invasive studies of intracellular acid-base parameters: quantitative analyses during environmental and functional stress. In: Eggington S, Taylor EW, Raven JA (eds) Regulation of acid-base status in animals and plants. Society for Experimental Biology Seminar Series. Cambridge University Press, Cambridge, pp 69–98

Pörtner HO, Hardewig I, Sartoris FJ, Van Dijk PLM (1998) Energetic aspects of cold adaptation; critical temperatures in metabolic, ionic and acid base regulation? In: Pörtner HO, Playle R (eds) Cold ocean physiology. Society for Experimental Biology Seminar Series. Cambridge University Press, Cambridge, pp 88–120

Reeves RB (1985) Alphastat regulation of intracellular acid-base state? In: Gilles R (ed) Circulation, respiration, and metabolism. Springer, Berlin Heidelberg New York, pp 414–423

Ryan SN (1992) Susceptibility of the Antarctic fish *Pagothenia borchgrevinki* to MS-222 anaesthesia. Polar Biol 11:583–589

Sommer A, Klein B, Pörtner HO (1997) Temperature induced anaerobiosis in two populations of the polychaete worm *Arenicola marina*. J Comp Physiol 167B:25–35

Storelli C, Acierno R, Maffia M (1998) Membrane lipid and protein adaptations in Antarctic fish. In: Pörtner HO, Playle R (eds) Cold ocean physiology. Society for Experimental Biology Seminar Series. Cambridge University Press, Cambridge, pp 166–189

Van Dijk PLM, Hardewig I, Pörtner HO (1997) Temperature dependent shift of pH_i in fish white muscle: contribution of passive and active processes. Am J Physiol 272:R84–R89

Van Dijk PLM, Tesch C, Hardewig I, Pörtner HO (1999) Physiological disturbances at high temperatures: a comparison between stenothermal Antarctic and eurythermal temperate eelpouts (Zoarcidae). J Exp Biol 202:3611–3621

Van Ginneken V, Van den Thillart G, Addink A, Erkelens C (1995) Fish muscle energy metabolism measured during hypoxia and recovery: an in vivo ^{31}P-NMR study. Am J Physiol 268:R1178–1187

Van den Thillart G, Van Waarde A, Muller HJ, Erkelens C, Addink A, Lugtenburg J (1989a) Fish muscle energy metabolism measured by in vivo ^{31}P-NMR during anoxia and recovery. Am J Physiol 256:R922–R929

Van den Thillart G, Körner F, Van Waarde A, Erkelens C, Lutgenburg J (1989b) A flow through probe for in vivo ^{31}P NMR spectroscopy of unanaesthetized aquatic vertebrates at 9.4 Tesla. J Magn Res 84:573–579