Temperature-dependent changes in energy metabolism, intracellular pH and blood oxygen tension in the Atlantic cod

F. J. Sartoris*, C. Bock, I. Serendero, G. Lannig
and H. O. Pörtner

Alfred-Wegener-Institut für Polar- und Meeresforschung, Postfach 120161, Columbusstraße, 27568 Bremerhaven, Germany

The effect of acute increase in temperature on oxygen partial pressure (Po_2) was measured in the gill arches of Atlantic cod *Gadus morhua* between 10 and 19° C by use of oxygen micro-optodes. Oxygen saturation of the gill blood under control conditions varied between 90 and 15% reflecting a variable percentage of arterial or venous blood in accordance with the position of each optode in the gill arch. The data obtained suggested that arterial Po_2 remained more or less constant and arterial oxygen uptake did not become limiting during warming. A progressive drop in venous Po_2, however, was observed at >10° C indicating that excessive oxygen uptake from the blood is not fully compensated for by circulatory performance, until finally, Po_2 levels fully collapse. In a second set of experiments energy and acid–base status of white muscle of Atlantic cod *in vivo* was measured by magnetic resonance (^{31}P-NMR) spectroscopy in unanaesthetized and unimmobilized fish in the temperature range between 13 and 21° C. A decrease in white muscle intracellular pH (pH_i) with temperature occurred between 10 and 16° C (ΔpH per ° C $= -0.025$ per ° C). In white muscle temperature changes had no influence on high-energy phosphates such as phosphocreatine (PCr) or ATP except during exposure to high critical temperatures (>16° C), indicating that white muscle energy status appears to be relatively insensitive to thermal stress if compared to the thermal sensitivity of the whole animal. The data were consistent with the hypothesis of an oxygen limitation of thermal tolerance in animals, which is set by limited capacity of oxygen supply mechanisms. In the case of Atlantic cod circulatory rather than ventilatory performance may be the first process to cause oxygen deficiency during heat stress.   © 2003 The Fisheries Society of the British Isles

Key words: blood oxygen level; energy metabolism; *Gadus morhua*; microoptodes; NMR; temperature.

INTRODUCTION

A marine species, which will probably be affected by climate change is the Atlantic cod *Gadus morhua* L., an inhabitant of the continental shelf from the shoreline to 600 m depth or more. Its distribution covers both sides of the North Atlantic and adjacent seas, from the Bay of Biscay to Novaya Zemlya,

*Author to whom correspondence should be addressed. Tel.: +49 471 4831 1312; fax: +49 471 4831 1149; email: fsartoris@awi-bremerhaven.de

as well as the Baltic and White Seas. Although most North Atlantic cod stocks are currently considered to be overfished, temperature change may have contributed to the decrease in abundance (Brander, 1996). A decrease in Atlantic cod recruitment in the North Sea has been associated with higher-than-average temperatures during the last decade (O'Brian *et al.*, 2000). The effect of temperature change, however, is not necessarily the same in all areas of distribution (Daan, 1974; Boutilier, 1998). Since ambient temperature is the most important factor determining Atlantic cod migration and distribution in the Atlantic, global warming might have opposite effects on Atlantic cod populations on the northern and southern edges of their distribution (Pörtner *et al.*, 2001; Sirabella *et al.*, 2001).

As water temperature rises towards the upper tolerance limits, the animals are faced with a decrease in dissolved oxygen and, at the same time, with an increased oxygen demand due to elevated maintenance costs. Therefore, the balance between energy consumption and oxygen-dependent energy production may become disturbed at extreme temperatures. Nonetheless, regulated physiological parameters such as metabolic rate, blood and tissue oxygenation, acid–base status and cellular energy levels may display substantial changes before harmful effects occur (Pörtner, 1993; Pörtner & Grieshaber, 1993).

Recently, whole animal aerobic scope has been addressed as the first process to become limiting as temperature reaches low or high extremes, reflecting limited capacity of respiratory systems to ensure sufficient oxygen supply to tissues at 'pejus' temperatures (T_p, 'getting worse') (Frederich & Pörtner, 2000; Pörtner, 2001). Loss in aerobic scope at T_p is followed by transition to anaerobic metabolism at critical temperature limits (T_c, onset of anaerobic metabolism) (Zielinski & Pörtner, 1996; Sommer *et al.*, 1997; Frederich & Pörtner, 2000; Pörtner, 2001). In the case of the crustacean *Maja squinado* the envelope characterized by T_p agreed well with the ambient temperature range of this species and therefore, is interpreted to indicate the limits of long-term survival in the natural environment and to be ecologically relevant. Oxygen limitation at extreme temperatures appears as the unifying principle determining temperature-dependent limits of geographical distribution of marine ectotherms (Pörtner *et al.*, 1998, 2000, 2001). Moreover, the physiological and biochemical processes of temperature adaptation would explain trade-offs within energy budgets with the respective consequences for temperature-dependent changes in growth and reproduction (Pörtner *et al.*, 2001).

In the present study 'online' analyses of blood oxygen tensions in gill vessels of Atlantic cod were carried out using implanted optical oxygen sensors. The aim was to determine the temperature dependence of blood oxygen transport. These measurements occurred without fixation or anaesthetization of the fish and handling stress could be reduced to a minimum. In parallel experiments, the technique of *in vivo* magnetic resonance (^{31}P-NMR) spectroscopy was used to investigate the energetic consequences of thermal stress by measuring changes in the levels of intracellular pH and high-energy phosphates in white muscle of Atlantic cod. Previously, *in vivo* ^{31}P-NMR in fishes, particularly freshwater species, has been applied to determine changes in energy metabolism and intracellular pH (pH$_i$, van den Thillart *et al.*, 1989*a*, *b*; van Ginneken *et al.*, 1995, 1996; Borger *et al.*, 1998). Marine fishes such as *Harpagifer antarcticus*

Nybelin have also been investigated (Moerland & Egginton, 1998). By use of a horizontal magnet such measurements became possible in unrestrained and non-anaesthetized marine fishes during long-term experiments (>1 week) under controlled and well-defined physiological conditions (Bock *et al.*, 2001, 2002).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Atlantic cod were caught by bottom trawling at 20–40 m depth in the German Bight near Helgoland (54°09′ N; 7°53′ E) during the summer of 1998 (fish used in the NMR studies) and in February and May of 1999 (fish used for the oxygen tension measurements). In the laboratory the fish were kept for several weeks in large aquaria (1 m^3) in natural sea water (salinity = 32) at a temperature of 10–12° C and a 12L : 12D photoperiod. The animals used in the *in vivo* NMR studies had a mean ± S.D. total length (L_T) of 40 ± 5 cm while the L_T lengths of the fish used for the oxygen tension measurements was 37 ± 7 cm. The fish were fed twice a week with frozen cockles *Cerastoderma edule*. Feeding was terminated 3 days prior to the start of experimentation.

GILL BLOOD OXYGEN PARTIAL PRESSURE (P_{O_2})

Analyses were carried out following the principles of cannulation outlined by Larsen *et al.* (1997). Initially, the fish were anaesthetized with MS222 (0·08 g l^{-1}) and during preparation, the gills were perfused with aerated sea water containing 0·05 g l^{-1} MS222. Subsequently, the fish were placed in the experimental chamber and exposed to a constant flow of aerated sea water at 5 l min^{-1}. Water temperature and salinity were controlled and recorded online throughout the whole experimental period. The oxygen tension in the gill arches was measured using oxygen microoptodes (Pre Sens, Neuburg a. d. Donau, Germany). The fibre tip of the optode was coated with an oxygen-sensitive layer containing a luminescent oxygen indicator. In addition, the tip was coated with Teflon to avoid clotting and oxygen-independent fluorescent signals caused by the accumulated material. Prior to insertion the optode was calibrated and drift and temperature dependence were recorded. The optode was inserted *c.* 0·5 cm into the efferent branchial blood vessel and fixed with a drop of cyanoacrylate glue. Due to the small diameter of the optode tip (<100 μm) it was safe to assume that blood flow was not hampered by the implanted sensor. Oxygen levels in the blood under control conditions varied between 90 and 15% air saturation indicating variable contributions of venous blood to P_{O_2} readings depending on the position of the optode in the respective gill arch. The good long-term stability (linear drift <0·44% h^{-1}) of the optodes enabled online monitoring of P_{O_2} for >1 week with a measurement frequency of once per minute.

After a recovery period of 24 h water temperature was changed at 1° C h^{-1}. In preliminary experiments temperature was increased until a 'critical temperature' was reached, defined as the temperature when a drastic decrease in blood oxygen tension could be observed. At this temperature limit (19–22° C) recovery of the fish was often not possible even if the fish was immediately transferred to the control temperature. Since one of the intentions of this study was to define a physiological threshold temperature with ecological relevance such as the T_p (Frederich & Pörtner, 2000) incubation was always terminated at 19° C. At the end of the experiments optodes were removed and recalibrated. The signal was corrected for the temperature-dependent drift of the optode redetermined in aerated sea water.

IN VIVO ^{31}P-NMR STUDIES

All experiments were carried out using a 4·7 T magnet with a horizontal, 40 cm diameter bore with actively shielded gradient coils (Bruker Biospec 47/40 DBX System).

A 5 cm surface coil was placed on top of the chamber and positioned directly above the tail of the animal for ^{31}P-NMR spectroscopy of muscle tissue. The position of the phosphocreatine signal was calibrated to 0 ppm relative to an external methylenediphosphate (MDP) standard. *In vivo* ^{31}P-NMR spectra were acquired continuously over 1200 scans using 100 s bp pulse (60°), repetition time of 0·6 s, size = 8K, sweep width 4096 Hz resulting in a measurement time of 10 min. Spectra were processed using an automatic fit routine as described in Bock *et al.* (2001).

The fish were anaesthetized in aerated sea water containing MS222 (0·08 g l^{-1}) and placed in a Perspex flow-through chamber (Bock *et al.*, 2002). The fish were orientated towards the incoming water. Although the space available inside the chamber was minimized the fish could swim slowly when facing the incoming water. After full recovery from anaesthesia the chamber was closed. A constant flow of sea water of 2·5 l min^{-1} was maintained, which was high enough to ensure sufficient oxygen supply at each temperature but low enough to limit swimming speed to a minimum level. The animals remained unrestrained and were free to swim slowly inside the chamber. The fish were allowed to recover for at least 24 h under control conditions before the start of the experimental protocol. Steady state conditions, as defined by permanently low inorganic phosphate (Pi) signals and constant phosphocreatine : inorganic phosphate (PCr : Pi) ratios, were confirmed by repeated recordings of *in vivo* ^{31}P-NMR spectra. Temperature control (stability ± 0·5° C in the range between 10 and 19° C) was achieved as described by Bock *et al.* (2002). In accordance with preliminary trials and depending on the response of the animals, temperature was increased in two or three steps of 1° C over 3 h, from 10 to 13° C, then from 13 to 16° C and then from 16 to 19° C. When a new steady state was reached (13, 16 and 19° C) the temperature was kept constant for at least 12 h. Some (three out of nine animals) did not survive temperatures >16° C. For the remainder, the temperature was brought back to 10° C at the end of the experiment and the animals were returned to the aquarium.

For pH$_i$ analyses, subsequent spectra were added until the Pi signal could be clearly distinguished from noise and the position of the signal was determined relative to PCr. pH values were calculated and corrected for temperature from chemical shift data as described by Bock *et al.* (2001). The Gibbs' free energy change of ATP hydrolysis (dG/dζ) was calculated according to the methodology outlined by Pörtner *et al.* (1996). Free inorganic phosphate was assumed to be 1 mmol l^{-1} in resting animals (van Dijk *et al.*, 1999). The quantification of the concentrations of total Pi, creatine (Cr) and ATP was complicated by movement artefacts, since the sensitive volume perceived by the surface coil depends on the spatial orientation of the fish. To avoid problems arising from fish movements the following methodology was developed. In an initial experiment the movement of an Atlantic cod in the experimental chamber under control conditions had been restrained so that the fish stopped swimming, which allowed NMR spectra to be obtained without changes in spatial orientation. From this spectra the α-, β- and γ-ATP signals were quantified using the MDP concentration. The MDP standard was calibrated against the signal obtained from a 15 mmol l^{-1} PCr standard solution. The α- and γ-ATP signals include α- and β-ADP signals; therefore changes in these integrals will be solely dependent on the movement of the fish under the experimental conditions applied. Changes in the β-ATP signal (or PCr signal) without concomitant changes in the α- and γ-ATP signals, therefore excludes movement artefacts and allows for the quantification of ATP degradation due to metabolic processes. In turn, changes in α- and γ-ATP signals relative to MDP can be used to correct for the influence of movements by the fish.

STATISTICAL ANALYSIS

Data were checked for outliers beyond the 95% CL using Nalimov's test (Noack, 1980). Statistical significance was tested at the $P \leq 0·05$ level using ANOVA and the *post-hoc* Student–Newman–Keuls test for independent samples. Regression coefficients were calculated using Sigma Plot 2000 (SPSS). All means are given ±S.D.

RESULTS

GILL OXYGEN TENSION

All fish survived the experiments when temperatures remained below thermal tolerance limits. In a preliminary experiment the stability of the microoptodes was tested for >1 week demonstrating the suitability of this technique for the online measurement of Po_2 in blood vessels of fishes. After removing the optode from the gill vessel it was possible to recalibrate and use it again. As the optode preparation reads mixed arterial and venous blood, true venous Po_2 readings are at the low end of the range. Based on this qualitative assumption and with regard to the different temperature influences on blood with high or low oxygen contents, the different saturation levels were interpreted as variable contributions of venous blood. The temperature dependence of high, *i.e.* arterial, Po_2 in gill blood of Atlantic cod under control conditions at 10° C is shown in Fig. 1(b). Periods with relatively constant Po_2 values were interspersed with periods characterized by large fluctuations. With rising temperature only a slight decrease occurred until Po_2 fell abruptly at $\geq 23°$ C [Fig. 1(a)]. Along with the sudden
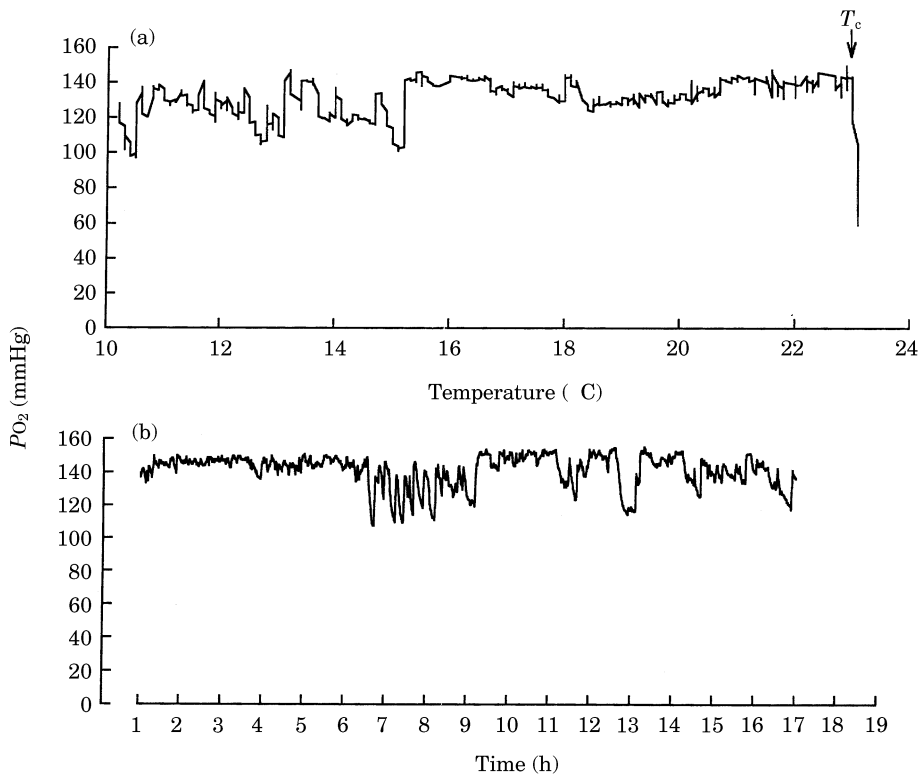

FIG. 1. (a) Changes in oxygen partial pressure (Po_2) over time in Atlantic cod gill blood during warming by $1°\,C\,h^{-1}$. The decrease in Po_2 at 23° C was as abrupt as was the final decrease in intracellular pH (pH_i) observed in the nuclear magnetic resonance (NMR) studies. The decrease was not predictable from the moderate change in blood Po_2 observed during progressive warming. (b) Typical time course of changes in Po_2 over time in gill blood of an individual Atlantic cod under control conditions at 10° C.
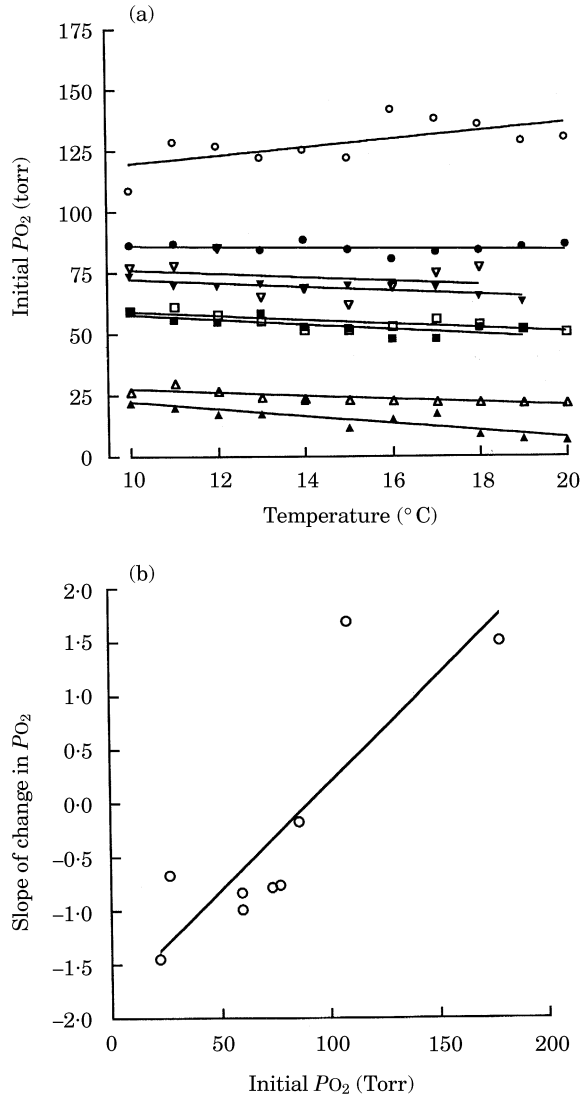
FIG. 2. Oxygen partial pressure decrease in Atlantic cod gill blood during warming by $1°C\,h^{-1}$. (a) The lower the initial P_{O_2}, the larger the temperature-dependent decrease during warming for each individual fish. Each of the lines refer to an individual Atlantic cod with a different initial P_{O_2}. (b) The relationship between the initial P_{O_2} value in the gill and the slope of temperature-dependent decrease in blood P_{O_2} ($y = 0.02x - 1.81$, $r^2 = 0.85$, $P < 0.01$).

drops in PCr, ATP and pH_i levels at high thermal limits, as observed in the ^{31}P-NMR studies, this incident was sudden and not predictable from any progressive changes in oxygen tension below the heat tolerance limit. This was not only the case when arterial blood was recorded but was also seen in venous blood (unpubl. data). The data reveal that the temperature influence on arterial P_{O_2} is much less than on venous P_{O_2} which displayed a significant linear decrease during progressive warming (Figs 2 and 3). For each individual fish it appears that the
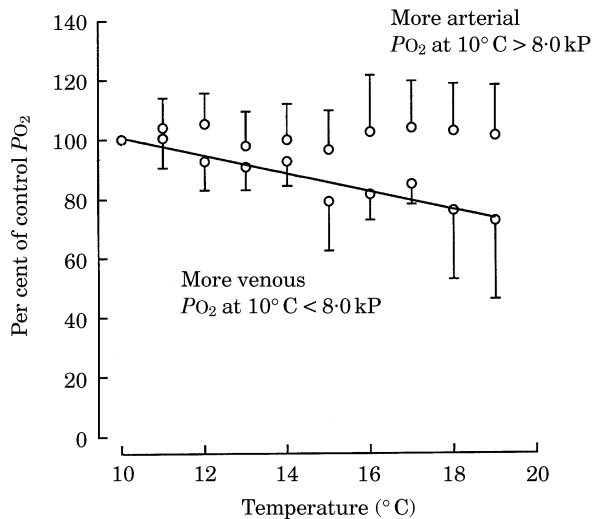
FIG. 3. Mean ± S.D. oxygen partial pressure decrease in Atlantic cod gill blood during warming by $1° C h^{-1}$. With a large initial fraction of venous blood contributing to PO_2 readings, stronger temperature dependence became visible ($y = -3.02x + 130.78$, $r^2 = 0.94$, $P < 0.001$).

lower the initial PO_2, the larger is the temperature-dependent decrease during warming. Overall, a larger contribution of venous blood [at PO_2 values < 8.0 kPa (60 torr)] showed an increasing PO_2 decrement during warming, while with a larger influence of arterial blood [PO_2 values > 8.0 kPa (60 torr)] temperature and blood oxygen tension largely remained unrelated (Figs 2 and 3).

TISSUE ENERGETICS AND ACID–BASE STATUS

The experimental set up used in ^{31}P-NMR experiments allowed for online recording of pH_i and levels of ATP and PCr in white muscle of unrestricted and non-anaesthetized Atlantic cod for >1 week. Recovery from slight anaesthetization and handling stress occurred within 2 h. *In vivo* ^{31}P-NMR spectra of white muscle of Atlantic cod immediately after placing the fish in the flow-through chamber are shown in Fig. 4(a) and after resting conditions were reached at 10° C in Fig. 4(b). Stress-free conditions are indicated by a high PCr : Pi ratio. The PCr : Pi ratio of free-swimming Atlantic cod spectra was extremely high (40–50) and, in consequence, determination of control pH_i was hampered by the low Pi signal. Integration of the three ATP signals yielded similar values. Since the α- and γ-ATP signals comprise α- and β-ADP, a high β-ATP signal indicates high ATP and low free ADP values. Both factors attest to excellent and stable physiological status under control conditions.

Reliable determinations of white muscle pH_i from chemical shifts values were only possible after averaging numerous spectra. A typical time course of the change in intracellular pH with increasing temperature is presented in Fig. 5. A mean pH_i of *c.* 7.45 was measured at 10° C. The mean values of intracellular pH of Atlantic cod at 10, 13, 16 and 19° C are shown in Fig. 6(a). The decrease in
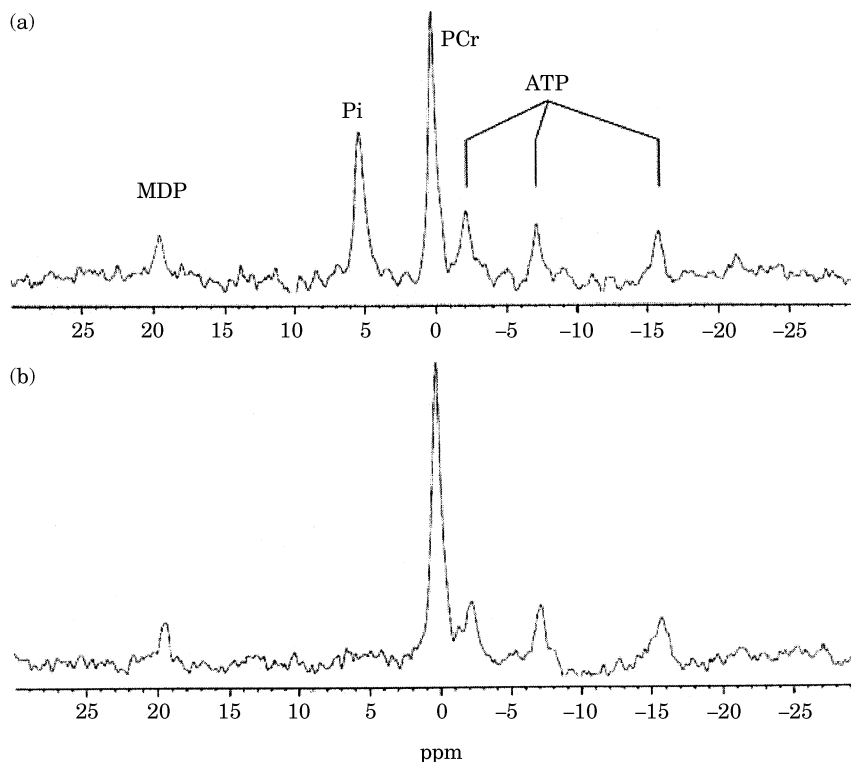
FIG. 4. *In vivo* ^{31}P-NMR spectra of white muscle of Atlantic cod: (a) obtained immediately after placing the fish in the flow-through chamber and (b) resting condition of the unrestrained Atlantic cod. Note the high phosphocreatine (PCr) level compared to the inorganic (Pi) level, which indicate low muscle tonus and low stress conditions.

pH with temperature calculated from these data was *c.* -0.025 ΔpH per $^\circ$C between 10 and 16°C. The mean change in intracellular pH for each temperature step decreased from -0.029 per $^\circ$C (10–13°C) to -0.022 per $^\circ$C (13 and 16°C) and tended to level off between 16 and 19°C. Temperature changes had no influence on PCr or ATP levels (mean $\pm$ s.d. control values were $27.3 \pm 4.2 \, \mu\text{mol g}^{-1}$ for PCr and $4.8 \pm 0.8 \, \mu\text{mol g}^{-1}$ for ATP) except beyond a high tolerance threshold reached at $\geq 16^\circ$C which was variable between individual fish. The PCr and ATP levels dropped drastically, in similar ways to those observed for Po$_2$ levels. There was a significant trend for the levels of free ADP to increase with rising temperature. The dynamics of the Gibbs' free energy change of ATP hydrolysis followed the same pattern as PCr and ATP levels [Fig. 6(b)], with a severe drop (from 62 to 56 kJ mol^{-1}) that became visible once the upper tolerance limit was surpassed. Within 1 h the fish lost balance and died. Concomitant with this disturbance of energy balance a decrease in muscle pH$_i$ could be observed as shown in Fig. 5 for an individual Atlantic cod reaching a tolerance limit at 16°C. Direct cooling (within 15 min) could not reverse this process, indicating an irreversible heat damage.
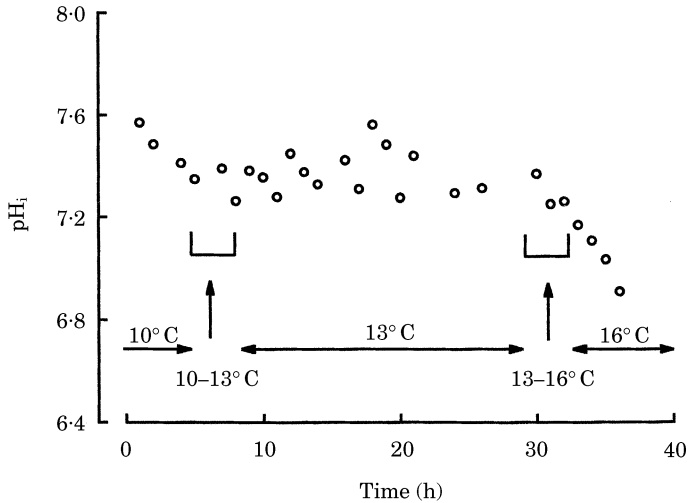
FIG. 5. Typical time course of changes in intracellular pH (pH_i) in the white muscle of an individual Atlantic cod (each symbol is the mean value of five spectra). The abrupt decrease in pH_i seen after a few hours of incubation at 16° C was not preceded by changes in the energy status of the muscle. At variable temperatures this pattern was typical for all individuals but indicated large differences between critical thermal limits of individual fish (see Fig. 2).

DISCUSSION

METHODOLOGY

For a comparison of NMR data and the level of blood oxygenation, the oxygen tensions in gill blood of Atlantic cod were measured with microoptodes. In preliminary experiments it was found that the method was suitable for prolonged measurements. The patterns observed close to lethal limits matched those observed in the NMR at similar temperatures. This indicated that the range of tolerance is not largely influenced by the implantation of the optode. The optode preparation probably reads mixed arterial and venous blood with true venous Po_2 readings at the low end of the depicted range (Fig. 3).

The use of a horizontal magnet made it possible to measure intracellular pH and energy metabolites in non-anaesthetized and unrestrained slowly swimming Atlantic cod with a mean L_T of $c.$ 40 cm, at the expense of some decrease in signal to noise ratio over time owing to spontaneous activity. Thereby, any potential influence of long-term anaesthesia on blood, tissue and acid–base parameters as reported for rainbow trout *Oncorhynchus mykiss* (Walbaum) (Iwama *et al.*, 1989) and the Antarctic fish *Pagothenia borchgrevinki* (Boulenger) (Ryan, 1992) could be avoided. The spectra obtained after 10 min showed a satisfactory signal to noise ratio. The levels of PCr ($27 \cdot 3 \pm 4 \cdot 2 \, \mu mol \, g^{-1}$), ATP ($4 \cdot 8 \pm 0 \cdot 8 \, \mu mol \, g^{-1}$) and the Gibbs' free energy of ATP hydrolysis ($61 \cdot 7 \pm 0 \cdot 44 \, kJ \, mol^{-1}$) matched the values found by van Dijk *et al.* (1999) in eelpouts (Zoarcidae) (PCr $\cong 25 \, \mu mol \, g^{-1}$; ATP $\cong 3 \cdot 7 \, \mu mol \, g^{-1}$; $\Delta G/\Delta \xi \cong 61 \cdot 0 \, kJ \, mol^{-1}$). In Atlantic cod, however, the PCr : Pi ratio was higher (40–50) (Fig. 4) than reported by Borger *et al.* (1998) for common carp *Cyprinus*
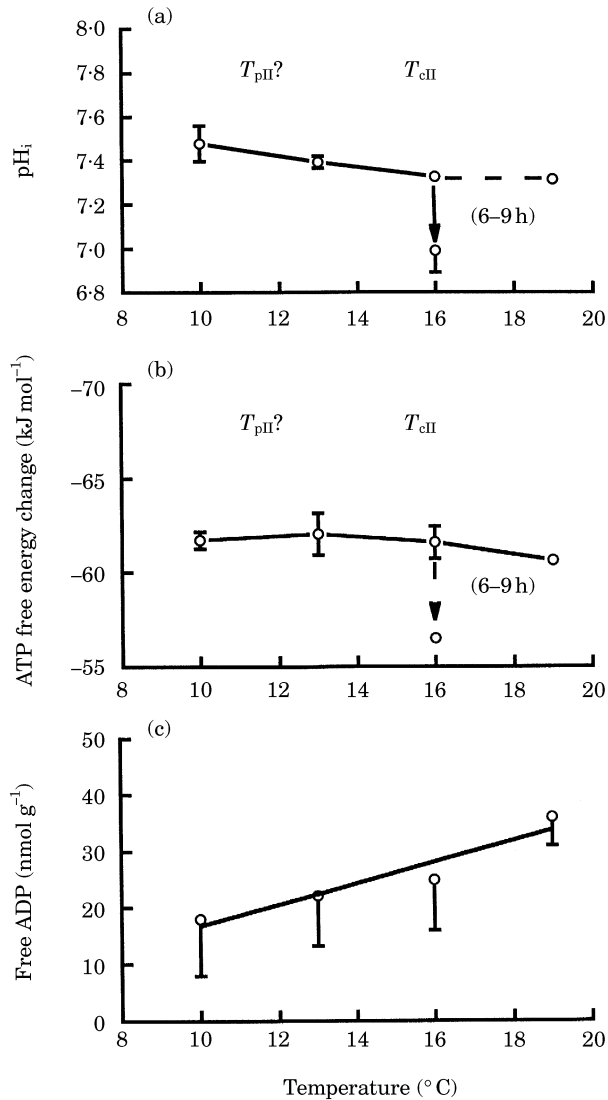
FIG. 6. Relationship between intracellular pH (pH_i) (a), Gibbs' free energy change (b) and the levels of free ADP ($y = -2 \cdot 15 + 1 \cdot 89x$, $r^2 = 0 \cdot 95$, $P \leq 0 \cdot 05$) (c) during warming in white muscle of Atlantic cod ($n = 4$ at 10, 13 and 19° C and $n = 9$ at 16° C). Temperature was increased in two or three steps of 1° C over 3 h, from 10 to 13° C, then from 13 to 16° C and then from 16 to 19° C. When a new steady state was reached (13, 16 and 19° C) the temperature was kept constant for at least 12 h. pH regulation followed the alphastat pattern consistently between 10 and 16° C in all investigated fish but not beyond 16° C. Some (three out of nine fish) did not survive temperatures >16° C which is reflected by a sudden drop in pH_i and Gibbs' free energy change (after 6–9 h at 16° C) indicating the upper critical temperature (T_{cII}) to be reached at 16° C (see Fig. 5).

carpio L. (PCr:Pi $\cong$ 30) and by Bock *et al.* (2001) for eelpouts (PCr:Pi $\cong$ 15). The high PCr:Pi ratio in Atlantic cod results as a consequence of extremely low Pi values. In consequence, pH_i determination was hampered by the low Pi signal and required extensive averaging of numerous spectra. This reflects

a thoroughly resting musculature, but was somewhat unexpected since very high ratios of PCr:Pi (Chiba *et al.*, 1988, 1990*a*, *b*) are usually found under anaesthesia due to the relaxation effect on muscle tonus. In Atlantic cod high PCr:Pi values and physiological pH_i values were maintained for >1 week. These emphasizes the good physiological condition of the animals during long-term experimentation.

BLOOD OXYGEN STATUS

During warming arterial P_{O_2} remained largely constant and thus arterial oxygen uptake (*i.e.* ventilation) did not become limiting even during severe heat stress [Fig. 1(a)]. This contrasts with the situation in crustaceans where a breakpoint in ventilation rate and corresponding changes in arterial haemolymph P_{O_2} and heart rate could be observed at T_p (Frederich & Pörtner, 2000). In Atlantic cod, the increased oxygen demand accompanying a temperature increment results in a depletion of the venous oxygen reserve as evidenced by a progressive drop in assumed venous P_{O_2}. This finding strongly indicates that excessive oxygen uptake from the blood is not fully compensated for by circulatory performance (Figs 2 and 3). With a progressive decrease in venous oxygen reserves with increasing temperature, a clear T_p cannot be identified. Similar to the observation of a drastic change in pH and energy status in the NMR spectra, final thermal limitation is indicated by a sudden drop in oxygen tension. This is probably a consequence of circulatory collapse at the T_c resulting from a progressive insufficient oxygen supply not being able to meet the rising oxygen demand anymore. The P_{O_2} pressure head driving diffusion into the cell should rise to cover this demand; however, the progressive fall in venous P_{O_2} indicates an increasingly inadequate pressure head for maintenance of full mitochondrial aerobic scope.

It can be concluded that circulatory rather than ventilatory performance sets the limit of thermal tolerance in Atlantic cod. The heart of Atlantic cod relies on oxygen delivered by the venous blood. Since the oxygen tension in the venous blood decreases with increasing temperature this might have influenced the cardiac performance of the heart. In the rainbow trout the venous P_{O_2} threshold to support cardiac performance was between 7·8 and 9·9 torr (1·0 and 1·32 kPa) (Steffensen & Farrell, 1998). As the optode preparation reads mixed arterial and venous blood true venous P_{O_2} readings are most likely at the low end of the range shown in Fig. 3, close to a low initial P_{O_2} value of 25 torr (3·3 kPa). Considering the sudden fall in blood P_{O_2} at the T_c, a venous P_{O_2} of *c.* 8 torr (1·1 kPa) should be close to the limiting value just prior to the T_c in Atlantic cod, a similar estimate to that reported by Steffensen & Farrell (1998). The development of low venous P_{O_2} values probably relates to the observation that heart rate in fishes increases with temperature (Farrell & Jones, 1992) but does not exceed a maximum value (120 bpm in salmonids) even at high temperatures (Farrell, 1991). A limiting role of the circulatory system is also suggested by a capacity-limited increase in blood flow during warming in the Antarctic eelpout *Pachycara brachycephalum* (Pappenheim) (Mark *et al.*, 2002). The temperature-dependent capacity limit of blood circulation in Atlantic cod remains to be investigated and compared with the patterns of venous P_{O_2} for

a quantification of the T_p. The limiting role of circulatory performance with a non-limiting capacity for gill oxygen uptake in Atlantic cod also emphasizes the temperature-dependent use of different haemoglobin isoforms as a means to optimize oxygen transport in Atlantic cod (O. Brix, pers. comm.).

ENERGY AND ACID–BASE STATUS

Not only the limited capacity of the circulatory system but also temperature-dependent changes in intracellular pH may be an indicator of T_p (as indicated by deviation from alphastat pH regulation, Mark *et al.*, 2002) as well as T_c (indicated by a sudden drop in pH_i, PCr and ATP and in Gibbs' free energy of ATP hydrolysis) thresholds in Atlantic cod. Even if the literature is not uniform concerning the validity of the alphastat hypothesis, the work recently of Ultsch & Jackson (1996) and Pörtner *et al.* (1998) demonstrated that at least intracellular pH is generally regulated according to alphastat especially in the normal temperature range of the species and in between the T_c range (Sommer *et al.*, 1997; Bock *et al.*, 2001; Mark *et al.*, 2002). The magnitude of temperature-induced pH changes indicates an alphastat pattern of pH_i regulation in Atlantic cod, which is visible up to $16°$ C with a trend towards lower slopes at higher temperatures (Fig. 6). A slight deviation from alphastat pH regulation may already set in, but not very clearly at $>13°$ C. Adopting the principles elaborated by Mark *et al.* (2002) in a parallel study on *P. brachycephalum*, the T_p in Atlantic cod would be found close to $16°$ C, just below the T_c. These relationships remain to be investigated. In general, changes in temperature-dependent pH regulation might be a suitable early physiological indicator of thermal limitation as also shown in lugworms *Arenicola marina* (Sommer *et al.*, 1997). Mark *et al.* (2002) showed that the deviation from alphastat pH regulation occurred at the upper T_p in *P. brachycephalum*. The width of the window between T_p (deviation from alphastat pH regulation) and T_c (drastic decrease of pH owing to anaerobic metabolism) might thus be reflected in patterns of changes in intracellular pH. The mechanism behind the shift in temperature-dependent pH regulation at T_p but below the T_c is not yet understood, but probably involves a change in membrane properties.

The importance of alphastat pH regulation for the maintenance of energy homeostasis has been outlined by Zielinski & Pörtner (1996) and by Pörtner *et al.* (1998). With alphastat control of intracellular pH, the levels of ATP free energy should be maintained during cooling. The temperature-dependent changes in intracellular pH and the maintenance of Gibbs' free energy change in the white muscle of Atlantic cod during warming within the thermal tolerance window supports this conclusion.

At T_c, which was found at a temperature variable between individuals but $>16°$ C, the onset of anaerobic metabolism and a sudden drop of pH_i and Gibbs' free energy change suggest complete loss of aerobic scope and insufficient oxygen supply to even cover standard metabolic rate. In accordance with earlier studies (van Dijk *et al.*, 1999) no progressive changes in the levels of white muscle high-energy phosphates such as PCr and ATP during temperature perturbations were observed except during exposure to temperature extremes beyond the T_c when after variable periods of incubation the fish lost balance

and PCr and ATP started to decrease. At this 'point of no return' even immediate cooling could not reverse this process and the fish died within 1 h. Recordings of Po_2 in the blood indicated complete failure of the circulatory system at this point. The acidosis and energetic collapse observed, match the definition of the T_c, which were defined based on work on invertebrates and fishes (Pörtner *et al.*, 1998; Pörtner, 2001). As found earlier in marine invertebrates, the T_c indicates transition to anaerobic energy production and thus to a time-limited situation. It is equivalent to the long-term lethal temperature. Since the period of hypoxia tolerance is limited, and will be even more so at high temperatures, the period of survival beyond the T_c appears very short in Atlantic cod once venous oxygen stores are depleted.

In an earlier study of eelpouts (van Dijk *et al.*, 1999), oxygen limitation became visible first in aerobic organs such as the liver before effects could be seen in white muscle. Only when circulation and ventilation collapse and the animal is close to death, do drastic changes occur in the white muscle. This is probably due to the lower metabolic rate and thus O_2 demand in white muscle. In conclusion, white muscle in resting fishes appears to be less sensitive to thermal stress than more aerobic organs such as the liver or brain. Future studies of thermal tolerance in fishes should therefore focus on the role of aerobic organs in setting the thermal limits of ectotherms. As a precondition, methodological problems associated with non-invasive NMR studies in smaller organs *in vivo* need to be solved.

By applying new methods for the long-term study of physiological parameters in non-anaesthetized Atlantic cod from the North Sea some evidence for an oxygen limitation of thermal tolerance in this species could be provided. In general, the temperature limits of physiological performance should correspond to the geographical distribution limits according to environmental temperature. The correlation between temperature and the hierarchy and time limits of thermal limitation (T_p and T_c) as outlined by Pörtner (2001) indicates that from an ecological point of view the T_p probably sets the distribution limits of a species. Although, not as clearly defined as in a crustacean (Frederich & Pörtner, 2000) or in Antarctic eelpout (Mark *et al.*, 2002), the present data indicate that T_p in Atlantic cod may be reached between 13 and 16° C, before a capacity limitation of circulation causes a fatal drop in venous oxygen levels and, finally, collapse and death at the T_c. This finding is in line with the conclusion that animals refused food above 16° C, possibly as a consequence of lost aerobic scope.

References

Bock, C., Sartoris, F. J., Wittig, R. M. & Pörtner, H. O. (2001). Temperatures dependent pH regulation in stenothermal Antarctic and eurythermal temperate eelpout (Zoarcidae): an *in vivo* NMR study. *Polar Biology* **24,** 869–874.

Bock, C., Sartoris, F. J. & Pörtner, H. O. (2002). In vivo MR spectroscopy and MR imaging on non-anaesthetized marine fish: techniques and first results. *Magnetic Resonance Imaging* **20,** 165–172.

Borger, R., de Boeck, G., van Auderke, J., Dommisse, R., Blust, R. & van den 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. *Comparative Biochemistry and Physiology A* **120,** 143–150.

Boutilier, R. G. (1998). Physiological ecology in cold ocean fisheries: a case study in Atlantic cod. In *Cold Ocean Physiology* (Pörtner, H. O. & Playle, R., eds), pp. 463–489. Cambridge: Cambridge University Press.

Brander, K. (1996). Effects of climate change on cod (*Gadus morhua*) stocks. In *Global Warming: Implications for Freshwater and Marine Fish* (Wood, C. M. & McDonald, D. G., eds), pp. 255–278. Cambridge: Cambridge University Press.

Chiba, A., Hamaguchi, M., Kosaka, M., Asai, T., Tokuno, T. & Chichibu, S. (1988). Anesthetic effects of MS 222 on the loach measured with ^{31}P-NMR. *Medical Biology* **117,** 445–448.

Chiba, A., Hamaguchi, M., Kosaka, M., Asai, T., Tokuno, T. & Chichibu, S. (1990a). In vivo ^{31}P-NMR analysis of the electric anesthetized loach, *Cobitis biwae*. *Comparative Biochemistry and Physiology A* **97,** 385–389.

Chiba, A., Hamaguchi, M., Kosaka, M., Tokuno, T., Asai, T. & Chichibu, S. (1990b). Energy metabolism in unrestrained fish with in vivo ^{31}P-NMR. *Comparative Biochemistry and Physiology A* **96,** 253–255.

Daan, N. (1974). Growth of North Sea Cod, *Gadus morhua*. *Netherlands Journal of Sea Research* **8,** 27–48.

van Dijk, P. L. M., Hardewig, I., Tesch, C. & Pörtner, H. O. (1999). Physiological disturbances at critically high temperatures: a comparison between stenothermal Antarctic, and eurythermal temperate eelpouts (Zoarcidae). *Journal of Experimental Biology* **202,** 3611–3362.

Farrell, A. P. (1991). From hagfish to tuna – a perspective on cardiac function. *Physiological Zoology* **64,** 1137–1164.

Farrell, A. P. & Jones, D. R. (1992). The heart. In *Fish Physiology*, Vol. XII (Hoar, W. S., Randall, D. J. & Farrell, A. P., eds), pp. 1–88. New York: Academic Press, Inc.

Frederich, M. & Pörtner, H. O. (2000). Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in the spider crab, *Maja squinado* (Decapoda). *American Journal of Physiology* **279,** R1531–R1538.

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. *American Journal of Physiology* **268,** R1178–R1187.

van Ginneken, V., van den Thillart, G., Addink, A. & Erkelens, C. (1996). Synergistic effect of acidification and hypoxia: in vivo ^{31}P-NMR and respirometric study in fishes. *American Journal of Physiology* **271,** R1746–R1752.

Iwama, G. K., McGeer, J. C. & Pawluk, M. P. (1989). The effects of five fish anaesthetics on acid-base balance, hematocrit, blood gases, cortisol, and adrenalin in rainbow trout. *Canadian Journal of Zoology* **67,** 2065–2073.

Larsen, B. K., Pörtner, H. O. & Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Marine Biology* **128,** 337–346.

Mark, F., Bock, C. & Pörtner, H. O. (2002). Oxygen limited thermal tolerance in Antarctic fish investigated by magnetic resonance imaging (MRI) and spectroscopy ^{31}P-MRS. *American Journal of Physiology* **283,** R1254–R1262.

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

Noack, S. (1980). *Statistische Auswertung Von Meß- und Versuchsdaten mit Taschenrechner und Tischcomputer*. Berlin: Walter de Gruyter.

O'Brian, C. M., Fox, C. J., Planque, B. & Casey, J. (2000). Climate variability and North Sea cod. *Nature* **404,** 142.

Pörtner, H. O. (1993). Multicompartmental analyses of acid-base and metabolic homeostasis during anaerobiosis: invertebrate and lower vertebrate examples. In *Surviving Hypoxia: Mechanisms of Control and Adaptation* (Hochachka, P. W., Lutz, P. L., Sick, T., Rosenthal, M. & van den Thillart, G., eds), pp. 139–156. Boca Raton, FL: CRC Press Inc.

Pörtner, H. O. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88,** 137–146.

Pörtner, H. O. & Grieshaber (1993). Characteristics of the critical Po_2 (s): gas exchange, metabolic rate and the mode of energy production. In *The Vertebrate Gas Transport Cascade: Adaptations to Environment and Mode of Life* (Bicudo, J. E. P. W., ed.), pp. 330–357. Boca Raton, FL: CRC Press Inc.

Pörtner, H. O., Finke, E. & Lee, P. G. (1996). Metabolic and energy correlates of intra-cellular pH in progressive fatigue of squid (*L. brevis*) mantle muscle. *American Journal of Physiology* **271,** R1404–R1414.

Pörtner, H. O., Hardewig, I., Sartoris, F. J. & van Dijk, P. (1998). Energetic aspects of cold adaptation: critical temperatures in metabolic, ionic and acid-base regulation? *Cold Ocean Physiology* (Pörtner, H. O. & Playle, R., eds), pp. 88–120. Cambridge: Cambridge University Press.

Pörtner, H. O., van Dijk, P. L. M., Hardewig, I. & Sommer, A. (2000). Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. In *Antarctic Ecosystems: Models for Wider Ecological Understanding* (Davison, W. & Williams, C. H., eds), pp. 109–122. Christchurch: Caxton Press.

Pörtner, H. O., Berdal, B., Blust, R., Brix, O., Colosimo, A., De Wachter, B., Giuliani, A., Johansen, T., Fischer, T., Knust, R., Lannig, G., Naevdal, G., Nedenes, A., Nyhammer, G., Sartoris, F. J., Serendero, I., Sirabella, P., Thorkildsen, S. & Zakhartsev, M. (2001). Climate induced effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). *Continental Shelf Research* **21,** 1975–1997.

Ryan, S. N. (1992). Susceptibility of the Antarctic fish *Pagothenia borchgrevinki* to MS-222 anaesthesia. *Polar Biology* **11,** 583–589.

Sirabella, P., Giuliani, A., Colosimo, A. & Dippner, J. (2001). Breaking down the climate effects on cod recruitment. *Marine Ecology Progress Series* **216,** 213–222.

Sommer, A., Klein, B. & Pörtner, H. O. (1997). Temperature induced anaerobiosis in two populations of the polychaete worm *Arenicola marina*. *Journal of Comparative Physiology B* **167,** 25–35.

Steffensen, J. F. & Farrell, A. P. (1998). Swimming performance, venous oxygen tension and cardiac performance of coronary-ligated rainbow trout, *Oncorhynchus mykiss*, exposed to hypoxia. *Comparative Biochemistry and Physiology A* **119,** 585–592.

van den Thillart, G., van Waarde, A., Muller, H. J., Erkelens, C., Addink, A. & Lugtenburg, J. (1989*a*). Fish muscle energy metabolism measured by in vivo ^{31}P-NMR during anoxia and recovery. *American Journal of Physiology* **256,** R922–R929.

van den Thillart, G., Körner, F., van Waarde, A., Erkelens, C. & Lutgenburg, J. (1989*b*). A flow through probe for *in vivo* ^{31}P NMR spectroscopy of unanesthetized aquatic vertebrates at 9.4 Tesla. *Journal of Magnetic Resonance* **84,** 573–579.

Ultsch, G. R. & Jackson, D. C. (1996). pH and temperature in ectothermic vertebrates. *Bulletin of the Alabama Museum of Natural History* **18,** 1–41.

Zielinski, S. & Pörtner, H. O. (1996). Energy metabolism of the intertidal worm *Sipunculus nudus* below a critical temperature. *Journal of Comparative Physiology B* **166,** 492–500.