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Cold induced changes of adenosine levels in common eelpout (*Zoarces viviparus*): a role in modulating cytochrome *c* oxidase expression

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SUMMARY

Exposure of ectothermic organisms to variations in temperatures causes a transient mismatch between energy supply and demand, which needs to be compensated for during acclimation. Adenosine accumulation from ATP breakdown indicates such an imbalance and its reversal reflects a restoration of energy status. We monitored adenosine levels in blood serum and liver of common eelpout (*Zoarces viviparus*) during cold exposure *in vivo*. Furthermore, we tested its effect on the pattern of thermal acclimation in hepatocytes isolated from cold- (4°C) *versus* warm- (11°C) exposed fish. Adenosine levels increased during cold exposure *in vivo* and reached a transient maximum after 24 h in serum, but remained permanently elevated in liver. Whole animal cold acclimation induced a rise of liver citrate synthase activity by 44±15%, but left cytochrome *c* oxidase activity (COX) and RNA expression of the respective genes unchanged. Cold incubation of hepatocytes from warm-acclimated fish failed to cause an increase of mitochondrial enzyme activities despite increased COX4 mRNA levels. Conversely, warm acclimation of hepatocytes from cold-acclimated fish reduced both enzyme activities and COX2 and COX4 mRNA levels by 26–37%. Adenosine treatment of both warm- and cold-acclimated hepatocytes suppressed COX activities but activated COX mRNA expression. These effects were not receptor mediated. The present findings indicate that adenosine has the potential to regulate mitochondrial functioning *in vivo*, albeit the pathways resulting in the contrasting effects on expression and activity need to be identified.

Key words: hepatocytes, primary culture, adenosine, temperature acclimation, cytochrome *c* oxidase, citrate synthase, RNase protection assay.

INTRODUCTION

Temperature has a large impact on many biological processes, for example on the velocity of enzymatic reactions and diffusion rates, and hence is one of the most important abiotic factors for ectothermic animals. Because of thermal specialization, marine water breathers live within limited thermal windows. It has recently been demonstrated that the limits of thermal sensitivity are set at the highest level of biological organisation, the functioning of the whole organism, with a key role of oxygen supply through ventilation and circulation (Pörtner, 2001; Pörtner, 2002). These limits exert their effects on the growth rate of individual specimens and the abundance of a population, thereby shaping the biogeography of a species (Pörtner and Knust, 2007).

Many temperate zone poikilotherms possess the ability to acclimate to various temperatures and thereby shift their thermal tolerance windows. The limits are indicated by an onset of functional hypoxia, which has to be overcome during acclimation. The basis of thermal windows and acclimation is determined at the molecular level, including membrane composition, protein structure and enzyme functioning, with mitochondria having a key role (Pörtner et al., 2005). For example, during cold exposure of temperate zone fish, muscle mitochondrial density and/or oxidative capacity increase to compensate for the decelerating effects of low temperatures on metabolic rate and capacity (Egginton and Sidell, 1989; Guderley, 2004). This effect is associated with increments in the activities of mitochondrial enzymes such as citrate synthase (CS) and cytochrome *c* oxidase (COX), two commonly used indicators for mitochondrial capacities (Battersby and Moyes, 1998; St-Pierre et al., 1998). However, liver was found to display different patterns of thermal acclimation. In temperate zone eelpout and cod, liver CS activities were elevated in the cold, whereas the activities of COX remained largely unaltered (Lannig et al., 2003; Lucassen et al., 2003; Lucassen et al., 2006). This may indicate a functional adaptation of mitochondria, to adjust to tissue-specific demands in the cold, such as enhanced lipid synthesis (Pörtner et al., 2005).

Adjustments of mitochondrial content and function are not only induced by cold or warm acclimation, but also by various other physiological challenges. For example, an increase of capacity accompanies endurance exercise training or shivering thermogenesis, and a decrease occurs during hypoxia. The primary effector has not been established for any of these examples, but it is an attractive hypothesis that bioenergetic disturbances themselves contribute to these adjustments. Several bioenergetic factors have been discussed to elicit proliferation (Hood, 2001; Leary and Moyes, 2000) and only recently, nitric oxide emerged as a good candidate in mammals (Nisoli et al., 2004). It still needs to be established, whether the same factor(s) and pathway(s) are involved in ectotherms and in response to all physiological challenges.

One potential bioenergetic signal to modulate thermal adjustments is adenosine. Adenosine is predominantly produced following a breakdown of cytosolic ATP levels and thus indicates an acute insufficiency of energy metabolism. It can be released from the cells by specialized nucleoside transporters and thus affect the whole organism (Buck, 2004). Adenosine induces a number of physiological effects that commonly balance energy demand and supply at low energy availability, and is therefore often described

as a 'retaliatory metabolite' (Newby et al., 1990). These effects include a reduction of protein synthesis rate (Tinton et al., 1995), a decrease of oxygen consumption (Krumschnabel et al., 2000) or a stimulation of anaerobic glycolysis (Lutz and Nilson, 1997). Adenosine is usually examined in the context of hypoxic or anoxic exposure (Lutz and Kabler, 1997; Reipschläger et al., 1997; Renshaw et al., 2002). However, since thermal acclimation in fact involves compensation for temperature-induced hypoxia (Pörtner, 2002) adenosine may also play a role in temperature adaptation.

The purpose of this study was to examine (1) if acute temperature changes affect adenosine concentrations in ectothermic marine fish, (2) if adjustments in mitochondrial function observed *in vivo* can be induced in isolated fish hepatocytes with temperature as a single factor, (3) if adenosine participates in the regulation of mitochondrial adjustments to temperature, (4) how adenosine action on mitochondrial functions might be mediated. This study was performed in the common eelpout, *Zoarces viviparus*, which has become a model organism to study thermal acclimation and limitation (Pörtner and Knust, 2007). Here we describe, for the first time, that adenosine is a suitable effector to modulate the thermally induced cellular acclimation response.

MATERIALS AND METHODS

Animals

Adenosine concentrations were determined in tissues of laboratory-born offspring (first generation) of common North Sea eelpout *Zoarces viviparus* L., caught in the German Bight near Helgoland. Fish were raised in the aquarium of the Alfred Wegener Institute, Bremerhaven at 11.0±0.5°C and 30‰ salinity and fed twice per week with small shrimps (*Neomysis integer*, *Crangon crangon*). Animals (mass: 13.5±1.2 g, mean ± s.e.m.) were acutely transferred to 4.0±0.5°C and sampled before (t=0) and after 1 and 3 days of cold incubation. For the preparation of hepatocytes North Sea eelpout, caught in the German Bight in spring 2004 (mass: 67.3±25.9 g) were acclimated to either 4.0±0.5°C (representing mean winter habitat temperature) or 11.0±0.5°C (close to optimum temperature) and a salinity of 30‰ for at least 2 months. During the acclimation period specimens were fed once per week with *Crangon crangon*.

Determination of adenosine concentrations

Blood was collected from fish anaesthetised with 0.5 g l^{-1} MS-222 (3-amino-benzoic-methanosulfonate) by opening the caudal vein. Livers were excised, immediately frozen in liquid nitrogen and animals were killed by a cut through the spinal cord. Blood was stored on ice for 4 h to allow coagulation, centrifuged for 10 min at 5000 ***g*** and the serum was transferred into fresh reaction tubes. Preliminary tests confirmed the adequacy of this sampling procedure, which kept adenosine levels close to or below detection limits in unstressed animals.

Serum samples were mixed with 0.2 volumes ice-cold TCA (15% trichloracetic acid), sonicated for 1 min at 0°C in a bath sonicator (Branson, Danbury, CT, USA) and centrifuged (4 min at 16 000 ***g***, 0°C) to precipitate proteins. The supernatants were neutralized with 4 volumes of tri-*n*-octylamine/1,1,2-trichlortrifluorethane mix (1:4), centrifuged for 3 min at 16 000 ***g***, 0°C, and the upper phase was collected. Frozen liver samples were pulverized under liquid nitrogen, suspended in 3.5 volumes ice-cold TCA and processed as described for serum samples, but the pH of the extracts was adjusted to 9.0–9.4 with 2 mol l^{-1} NaOH.

Adenosine was determined by capillary electrophoresis (Beckman, Fullerton, CA, USA) using a method modified after Casey et al. (Casey et al., 1999). Extracts were supplied with 0.4 mmol l^{-1} uric acid as an internal standard and filtered through a 0.2 μm syringe filter. Samples were separated on a 50 μm diameter uncoated fused silica capillary with a current of 30 kV at 40°C. Adenosine peaks were identified by migration time and sample spiking. Adenosine concentrations were calculated from the area ratio of adenosine:uric acid using a calibration curve created with concentrations between 0.5–50 μmol l^{-1} adenosine.

Isolation of hepatocytes

Hepatocytes were isolated following a procedure modified after Mommsen et al. (Mommsen et al., 1994). For each cell culture two fish from the same acclimation temperature were prepared simultaneously to obtain a sufficient number of cells. Animals were anaesthetised with 0.5 g l^{-1} MS-222 and killed by a cut through the spinal cord. The liver of the first fish was carefully excised and weighted, transferred immediately to ice-cold solution 1 (magnesium-free Hank's medium, containing 240 mmol l^{-1} NaCl, 10 mmol l^{-1} Hepes, 5.5 mmol l^{-1} glucose, 5.4 mmol l^{-1} KCl, 4.2 mmol l^{-1} $NaHCO_3$, 0.4 mmol l^{-1} KH_2PO_4, 0.3 mmol l^{-1} Na_2HPO_4, pH 7.4) and perfused through the vena hepatica with solution 1 to remove blood cells. The second liver was prepared accordingly. Subsequently both livers were transferred to one vial and each organ was perfused two times with 2 ml g^{-1} fresh mass ice-cold collagenase solution [solution 1 + 1% bovine serum albumin (BSA) + 750 i.u. ml^{-1} collagenase type IV]. Between perfusions livers were gently massaged for about 10 min. After finely chopping the tissue the homogenate was gently shaken on ice for 60 min. Finally, the suspension was filtered through 250 μm mesh-size gauze, and hepatocytes were collected by centrifugation (4 min at 70 ***g***, 0°C) and washed by repeated centrifugation (2 min at 70 ***g***, 0°C) in solution 1 containing 1% BSA to remove collagenase, lipids and erythrocytes. Cells were resuspended in culture medium (Leibovitz L-15 medium + 103 mmol l^{-1} NaCl + 10 mmol l^{-1} Hepes + 1% BSA + 5 mmol l^{-1} glucose + 1% penicillin–streptomycin; pH 7.8 at 4°C) and shaken on ice until being dispersed for primary cell culture. Cell density and viability was determined in a Fuchs-Rosenthal haemocytometer dish by Trypan Blue exclusion.

Cell culture and incubation conditions

Cells were incubated in polystyrene 6-well plates. To each well 2 ml of culture medium was added and plates were pre-cooled to 4.0°C. The hepatocyte suspension was equally portioned between wells to a concentration of 2×10^6 viable cells per well. Culture dishes were briefly shaken by hand to spread cells on the bottom. Cells were incubated under air atmosphere at 4.0°C or 11.0°C (±0.1°C), and 5 μmol glucose were added per 10^6 cells per day. In parallel to the control group, cells were exposed to adenosine at both acclimation temperatures using the same basic culture conditions. Cells were supplied with 100 nmol ml^{-1} adenosine directly after dispersion and once every 24 h thereafter. To investigate the potential role of adenosine receptors, one group of cells was incubated for 30 min with 100 nmol ml^{-1} 8-phenyltheophylline (8-PT), a selective adenosine A_1-receptor antagonist, always prior to addition of adenosine, and in another group adenosine was replaced with 100 nmol ml^{-1} 5′-(*N*-ethylcarboxamido)adenosine (NECA), a non-selective adenosine receptor agonist. Owing to limited cell numbers, incubations with 8-PT or NECA were only performed with cells from warm-acclimated fish at 11°C.

For sampling of hepatocytes, culture dishes were transferred onto ice without shaking and 1 ml of culture medium was removed. Cells were resuspended in the remaining culture medium and precipitated

by centrifugation (2 min at 1000 ***g***, 0°C). After residual medium was carefully removed, cells were immediately frozen in liquid nitrogen. Samples were collected after 48 and 72 h of incubation. Since no differences were seen between the two time points (multi-factorial ANOVA) values were pooled and treated as replicate samples.

In an additional incubation series the viability of hepatocytes during cell culture was determined by Trypan Blue exclusion. Therefore, cells incubated at 4 or 11°C were harvested after 48 and 72 h by resuspension in their culture medium. Three sub-samples were taken for each well and mixed with 1:6 volumes of 0.4% Trypan Blue dye (Sigma, Steinheim, Germany). Numbers of cells that excluded or took up the dye were counted in a Fuchs-Rosenthal haemocytometer dish.

Enzyme activity

Cells were homogenized in 150 μl ice-cold buffer (20 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, 0.1% Tween 20, pH 7.4) by shaking on a Vortex Genie2 (Scientific Industries, New York, NY, USA) for 2 min at the highest level. COX and CS activities were determined at 20°C in a thermostatted spectrophotometer (Beckman, Fullerton, CA, USA) according to Lucassen et al. (Lucassen et al., 2003). Prior to measurement of CS activity, homogenates were sonicated in a bath sonicator (Branson, Danbury, CT, USA) for 5 min at 0°C.

RNA isolation and construction of probes

Total RNA from hepatocytes was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol for animal cells. RNA was quantified in a spectrophotometer (Eppendorf, Hamburg, Germany) with A_{260}:A_{280} ratios always >1.8. Additionally, the integrity of RNA was verified by formaldehyde agarose gel electrophoresis (Sambrook et al., 1989).

For sequencing of the *Z. viviparus* COX4 gene, mRNA was isolated from total RNA with the Oligotex kit (Qiagen, Hilden, Germany). RT–PCR was performed according to the method of Lucassen et al. (Lucassen et al., 2003) with gene-specific primers (Table 1) designed homologous to published sequences, using the MacVector 7.0 program package (Accelrys, Oxford, UK). The PCR reaction containing 1.5 mmol l^{-1} $MgCl_2$ was performed with 32 cycles of 45 s denaturation at 94°C, 2 min annealing at 57±6°C and 1 min elongation at 72°C. Separation, cloning and analyses of PCR fragments was performed as described by Mark et al. (Mark et al., 2006).

The 5′ and 3′ termini of COX4 mRNA were identified with the RLM–RACE kit (Ambion, Austin, TX, USA) following the manufacturer's manual. Gene-specific backward primers for 5′-RACE and forward primers for 3′-RACE were designed according to the partial sequence determined for COX4 of *Z. viviparus*. Forward primers for amplification of the 5′ terminus and backward primers for the 3′ terminus, corresponding to the adapter sequence, were provided with the kit. Cloning and analysis of positive clones was performed as for RT–PCR fragments (Mark et al., 2006).

For construction of the COX4-specific probe, primers for a 183 bp fragment were designed appropriate to the coding sequence and used for RT–PCR as described above. A 255 bp COX2 fragment was isolated from an existing 507 bp fragment [(Lucassen et al., 2003); accession no.: AY227660] by PCR with the primers given in Table 1. Existing fragments were applied for the construction of CS (369 bp; accession no.: AY382597) and β-actin (215 bp; accession no.: AY227657) probes (Lucassen et al., 2003; Mark et al., 2006).

Quantification of RNA

Gene-specific RNA transcripts of CS and the two COX subunits were quantified with a ribonuclease protection assay (RPA) using the RPA III kit (Ambion, Austin, TX, USA), with β-actin as an internal standard to correct for loading differences. The construction of templates from the PCR clones described above and the *in vitro* synthesis of [α-^{32}P]UTP-labelled antisense probes was performed according to the method of Lucassen et al. (Lucassen et al., 2003). To obtain equal intensities for protected fragments, specific radioactivity was applied as follows: 1000 Ci mmol l^{-1} for CS and COX4 probes, and 45 Ci mmol l^{-1} for COX2 and β-actin probes.

The RPA was adjusted to low RNA amounts (2 μg) following the manufacturer's instructions. Accordingly, the amount of each radiolabelled probe was lowered to 20 000 cts min^{-1} per sample, and hepatocyte RNA was supplied with an equal quantity of yeast RNA, to improve the formation of pellets. Sample RNA was simultaneously hybridized to all antisense probes at 42°C. RNAse treatment was performed with an RNaseA/T1 dilution of 1:100. RNA:RNA hybrids were precipitated and separated by denaturing PAGE (Lucassen et al., 2003). Radioactivity was detected and quantified in a phosphor storage imaging system (Fuji, Tokyo, Japan) using the AIDA software package (raytest, Straubenhardt, Germany).

Table 1. List of primers for RT-PCR and RACE

Fragment	Term	Sequence	Position	Length
COX4	Cox4-F5-M	CTGAAGGAGAAGGAGAAGG	221–240	157
	Cox4-B4	CRGTGAARCCGATRAAGAAC	377–358	
COX4-3′	Cox4-RACE-F1	TGAAGGAGAAGGAGAAGGGC	233–252	517
	Cox4-RACE-F2	CTGTGGTTGGAGGGATGTTC	353–372	
COX4-5′	Cox4-RACE-B1	GCGGTACAATCCAATCTTCTCCTC	297–274	297
	Cox4-RACE-B2	CAACCACAGATTTCCACTCTTGC	361–339	
	Cox4-RACE-B3	CGATAAAGAACATCCCTCCAACCAC	379–355	
COX4-c	Cox4-F8	CCTTCGCCTTGTTGGAAAACG	66–86	183
	Cox4-B6	TTCTCCTTCTCCTTCAGGGACAC	248–226	
COX2-a	Cox2-F3	TTATCCTTATCGCCCTGCCC	145–164	255
	Cox2-B2	CATCAGCGGAGACTAAAACG	399–380	

All sequences are written from 5′ to 3′. The positions correspond to the position of the following genes: *Tunnus obesus* cytochrome *c* oxidase subunit IV (COX4) [(Hüttemann, 2000), GenBank accession no. AF204870]; *Z. viviparus* cytochrome *c* oxidase subunit II (COX2-a) [(Lucassen et al., 2003), GenBank accession no. AY227660] and subunit IV (COX4-3′; COX4-5′; COX4-a) (GenBank accession no. EF175142).

Statistical analyses

Statistical significance was tested at the $P \leq 0.05$ level. Outliers were identified on the 95% significance level with Nalimov's test (Noack, 1980). Adenosine concentrations were analysed using one-factor ANOVA and the *post-hoc* Student–Newman–Keuls test. Differences in freshly isolated hepatocytes were analysed using a *t*-test. The impact of acclimation temperature in primary cell culture was observed with multi-factorial ANOVA. The effects of different treatments during cell culture in each acclimation group were analysed in a pairwise manner using repeated measures ANOVA and the *post-hoc* Student–Newman–Keuls test. Linear

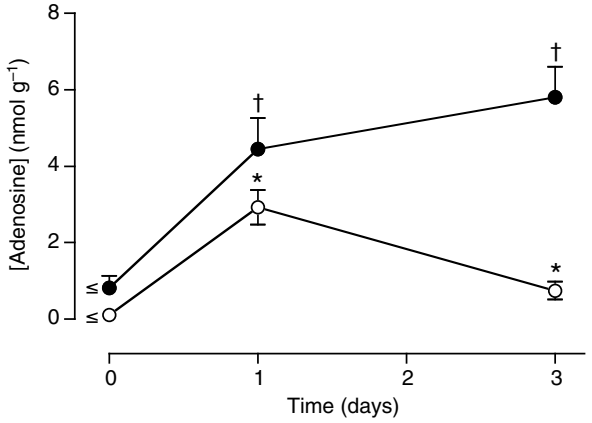

Fig. 1. Adenosine levels in blood serum (open circles) and liver (filled circles) of eelpout (*Z. viviparus*) during 1 or 3 days of cold exposure. The fish were acclimated to 11°C (*t*=0) and transferred to 4°C. Data points marked by ≤ include samples with adenosine concentration below detection limit. For these samples the detection limit of the method was used for calculations. *Significant difference from other time points; †significant difference from control (*t*=0). Values are means ± s.e.m. (*N*=9–10).

regressions and squared correlation coefficients were calculated using SigmaStat 3.0. Data are given as means ± s.e.m. (*N*=4–10).

RESULTS

Adenosine levels during cold acclimation

Adenosine concentrations in blood serum and liver of unstressed, long-term warm-acclimated *Zoarces viviparus* were found to be below or close to the detection limit (Fig. 1; 0 days). Adenosine levels in serum were significantly increased after 24 h of acute cold exposure. After 3 days, serum adenosine concentrations were found to be significantly lower than on the first day, but they were still elevated compared to the warm-acclimated group. Adenosine levels in the liver of *Z. viviparus* were also significantly increased after 24 h of cold exposure. Concentrations remained high during 3 days of cold exposure (Fig. 1).

Primary culture of hepatocytes

Cellular viability directly after isolation was always >90%. Hepatocyte appearance under the microscope remained stable under culture conditions and cells showed very little uptake of Trypan Blue dye. The fraction of cells that took up the dye was similar at both incubation temperatures and never exceeded 16.8%. The total number of viable cells was maintained at initial levels at both temperatures during the whole incubation period (Fig. 2). The activities and mRNA levels of both mitochondrial enzymes, as well as total RNA contents, were widely maintained at a high level in cells incubated at their origin temperature under control conditions, indicating a good conservation of cellular functions.

Effects of temperature and adenosine on enzyme activities

The effect of temperature acclimation *in vivo* on mitochondrial enzyme activities was determined in freshly isolated hepatocytes of *Z. viviparus* acclimated at 4 and 11°C. Citrate synthase (CS) activity per 10^6 cells was significantly higher, by 44±10%, in cells from cold-acclimated compared with those from warm-acclimated animals (Fig. 3A), whereas activities of cytochrome *c* oxidase (COX) remained unaffected by whole animal acclimation (Fig. 3D). During cell culture, the original acclimation temperature *in vivo* influenced

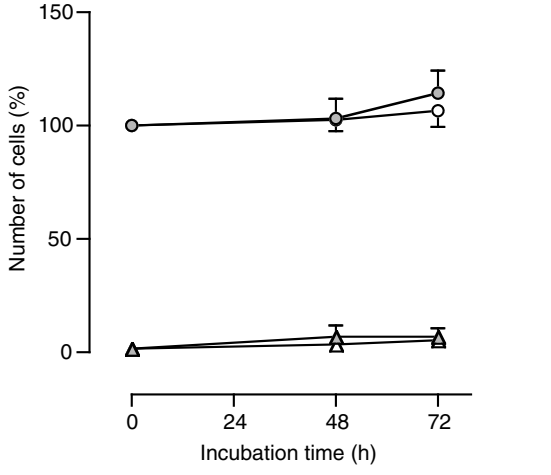

Fig. 2. Cellular viability during primary culture of hepatocytes from common eelpout (*Z. viviparus*). The number of viable cells (circles), expressed relative to the initial cell number, and the fraction of dead cells (triangles) in total cell counts were determined for cells from warm-acclimated animals during primary culture at either 11°C (shaded symbols) or 4°C (open symbols). Values are means ± s.e.m. (*N*=3).

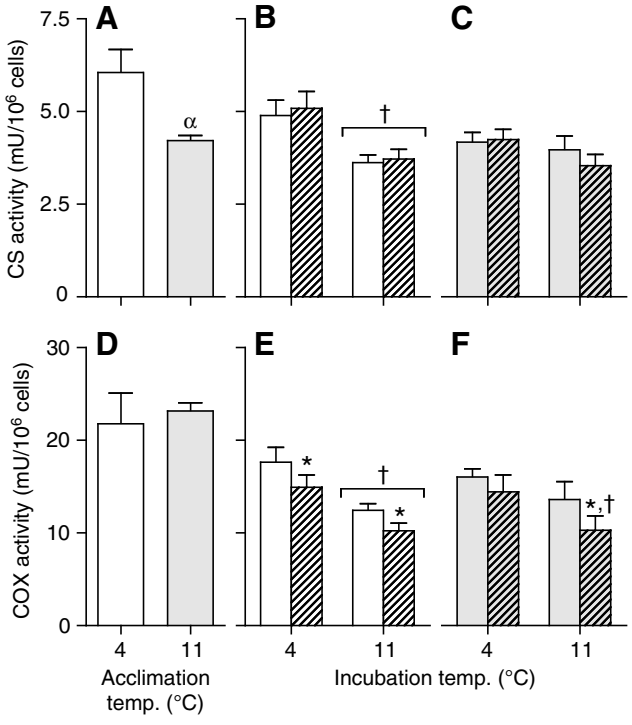

Fig. 3. Activities of CS (A–C) and COX (D–F) in hepatocytes prepared from cold- (white bars) and warm- (grey bars) acclimated eelpout (*Z. viviparus*). Activities were determined in freshly isolated hepatocytes (A,D; *N*=4) and in cells after incubation at either 4 or 11°C (B,C,E,F; *N*=5–8) under control conditions (white bars) or with the addition of adenosine (hatched bars). αSignificant difference between acclimations; *significant difference from the control group incubated at the same temperature; †significant difference from the corresponding group incubated at 4°C. Values are means ± s.e.m.

the response of both enzymes to incubation temperature. Warm incubation of hepatocytes from 4°C-acclimated eelpout caused a significant decrease of CS and COX activities, by 26±4% and 30±4%, respectively, compared with their cold-incubated counterparts (Fig. 3B,E). By contrast, enzyme activities in cells isolated from warm-acclimated fish were not altered by cold or warm incubation (Fig. 3C,F). Adenosine treatment of isolated hepatocytes had no impact on CS, but significantly affected COX activities. In cells from cold-acclimated eelpout, adenosine treatment resulted in a reduction of COX activities by 16±8% at 4°C and by 18±8% at 11°C compared with their respective control cells (Fig. 3E). In cells from warm-acclimated *Z. viviparus* COX activities remained more or less unaffected by the 4°C incubation temperature, whereas adenosine treatment during warm incubation significantly reduced COX activities, by 25±12% compared to the untreated control at 11°C (Fig. 3F).

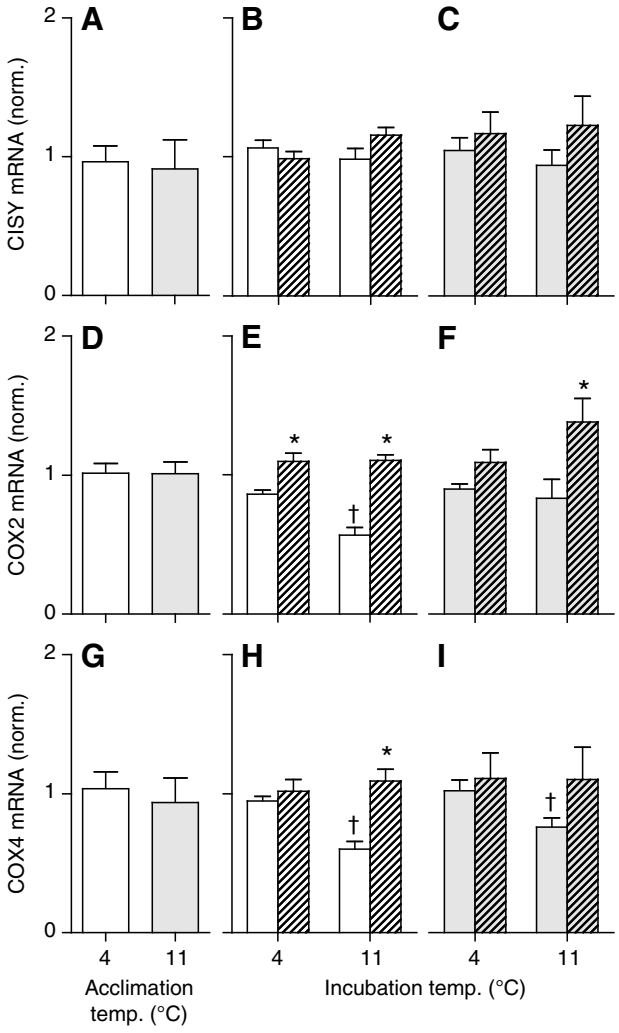

Fig. 4. Relative expression of CS mRNA and of the different COX subunit mRNAs in hepatocytes of cold- (white bars) and warm- (grey bars) acclimated eelpout (*Z. viviparus*). Relative amounts of CS (A–C), COX2 (D–F) and COX4 (G–I) mRNA were determined in freshly isolated hepatocytes (A,D,G; *N*=4) and in cells after incubation at either 4 or 11°C (B,C,E,F,H,I; *N*=5–8) under control conditions (white bars) or with the addition of adenosine (hatched bars). mRNA levels in equal amounts of total RNA were determined by RPA and normalized to the mean of freshly isolated hepatocytes from warm-acclimated fish. *Significant difference from control group incubated at the same temperature; †significant difference from corresponding group incubated at 4°C. Values are means ± s.e.m.

Specific mRNA expression

Expression of CS mRNA was neither affected by whole animal acclimation or incubation temperature, nor by adenosine. The mRNA levels for CS were very similar in all treatments (Fig. 4A–C).

The expression of COX mRNA was measured by use of the mitochondrial encoded COX2 and the nuclear encoded COX4 subunits, both displaying a similar pattern. In accordance with unchanged enzyme activities, long-term temperature acclimation of *Z. viviparus* (*in vivo*) resulted in equal mRNA levels for both subunits in freshly isolated hepatocytes (Fig. 4D,G). However, temperature significantly affected the response of hepatocytes to different incubation conditions. In cells from cold-acclimated *Z. viviparus*, warm incubation without adenosine induced a significant reduction in COX2 and COX4 mRNA levels, by 34±6% and 37±6%, respectively (Fig. 4E,H). By contrast, adenosine treatment, which had caused a drop in COX activities, significantly increased the expression of COX2, by 27±7% at 4°C and 95±7% at 11°C, and of COX4 by 81±14% at 11°C. The increase in COX2 and COX4 expression was less pronounced in hepatocytes from warm-acclimated eelpout, but followed the same pattern. COX4 mRNA levels were maintained at significantly, by 34±10%, higher levels in cold- than in warm-incubated cells under control conditions, and were increased to levels found in 4°C controls, when treated with adenosine at 11°C (Fig. 4I). For COX2 expression, no effect occurred as a result of incubation temperature, but adenosine treatment at 11°C resulted in mRNA levels 66±20% higher than in control cells (Fig. 4F).

Effects of adenosine receptor antagonists and agonists

A potential role for adenosine–receptor interactions in eliciting adenosine effects was tested by the addition of 8-PT, an antagonist for adenosine A_1 receptors, and 5′-(*N*-ethylcarboxamido)adenosine

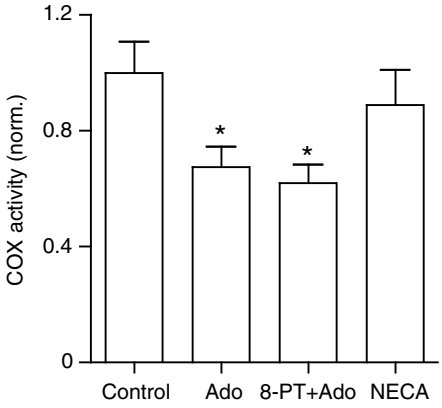

Fig. 5. Influence of an adenosine receptor antagonist and agonist on adenosine action. COX activities were determined in isolated hepatocytes after 48 or 72 h of incubation and normalized to the mean of the control group. Cells were treated with the adenosine receptor antagonist 8-PT combined with adenosine (ado) or with the adenosine agonist NECA instead of adenosine and are compared to their analogous control and adenosine-treated groups. All reagents were supplied at a concentration of 100 nmol ml^{-1}. Cells were prepared from warm-acclimated *Z. viviparus* and incubated at 11°C. *Significant difference from control. Values are means ± s.e.m. (*N*=4–6).

(NECA), a potent non-selective agonist for adenosine receptors (Ralevic and Burnstock, 1998). 8-PT- and NECA-treated samples were compared to the analogous control and adenosine-treated groups of the respective preparations.

Hepatocytes treated with the receptor blocker 8-PT in addition to adenosine (Fig. 5) exhibited COX activities identical to the ones measured in cells treated with adenosine alone, both significantly lower than the activities determined for the control group. This suggests that adenosine A_1 receptors are not involved in the adenosine effects described above. This assumption is supported by the observation that the adenosine agonist had no effect either (Fig. 4). COX activities in cells treated with NECA instead of adenosine were almost identical to those of the control group (P=0.984). Thus, a receptor-mediated hormone-like action of adenosine on COX activity can be excluded as unlikely.

DISCUSSION

Temperature effects on adenosine concentration

Adenosine accumulation often occurs from the degradation of intra- and extracellular ATP, when energy demand exceeds energy supply. The metabolite can be released from the cell by specialized nucleoside transporters and can be distributed to the whole organism (Buck, 2004). Therefore, adenosine might act as a hormone-like effector in response to bioenergetic challenges such as temperature changes. However, although there is no literature available concerning the effect of temperature, elevated adenosine levels have frequently been reported following anoxic or hypoxic conditions. In the brain of epaulette shark, adenosine levels increased during acute anoxia, whereas the levels of phosphorylated adenylates (ATP+ADP+AMP) remained virtually unchanged (Renshaw et al., 2002). Since acute temperature changes result in functional hypoxia (Pörtner, 2001; Pörtner, 2002), similar effects may occur during thermal response. Sartoris et al. (Sartoris et al., 2003) found unchanged ratios of phosphorylated adenylates during thermal acclimation of common eelpout. In the present study, adenosine concentrations were found to increase significantly in blood serum and liver during 24 h after a temperature shift from optimum (11°C) to a typically experienced low temperature (4°C). Increased levels were still visible after 3 days of incubation (Fig. 1). Changes in adenosine concentrations are typically small but significant and therefore elicited by only minor reductions in ATP levels. These observations suggest that adenosine might be a suitable signal to support metabolic adaptation in response to such bioenergetic disturbances.

Adenosine concentrations observed in eelpout after cold exposure were similar to those previously reported for the heart of short-horned sculpin during acute anoxia (MacCormack and Driedzic, 2004). Hypoxic conditions usually cause an increase in adenosine levels for minutes or hours (Renshaw et al., 2002; Lutz and Kabler, 1997). By contrast, cold-induced adenosine accumulation in *Z. viviparus* persisted for at least 3 days (Fig. 1), possibly as long as the hypoxic challenge remained uncompensated for. After 3 days of cold exposure liver adenosine concentrations remained high, whereas serum adenosine had already significantly decreased (Fig. 1). In our previous study on eelpout, cold compensation of energy metabolism in liver, monitored by mitochondrial enzymes, was shown to become visible after 4 days (Lucassen et al., 2003). In line with thermally induced hypoxia and functional insufficiency an extended exposure to temperature change may result in a prolonged elevation of adenosine levels until the seasonal acclimation process is well underway. This would allow adenosine to contribute to the cold acclimation process.

Impact of temperature and adenosine on mitochondrial enzymes

To test for a potential role of adenosine as a systemic effector in thermal adaptation, we investigated its influence on the acclimation response of isolated hepatocytes from cold- *versus* warm-acclimated fish. Therefore, we monitored the activity and expression of the mitochondrial key enzymes citrate synthase (CS) and cytochrome c oxidase (COX) in isolated hepatocytes. Elevated activities of the mitochondrial matrix enzyme CS in conjunction with conserved capacities of membrane bound COX typify the cold acclimation response of eelpout liver (Lucassen et al., 2003). By contrast, both enzymes typically increase in parallel in red and white muscle (Lannig et al., 2003; Lucassen et al., 2006). In line with earlier observations, cold acclimation of *Z. viviparus in vivo* had no impact on COX activities (Fig. 3D), but resulted in an increase of CS activities (Fig. 3A). The mRNA levels of CS (Fig. 4A) and of two COX subunits, the mitochondrial-encoded subunit COX2 (Fig. 4D) and the nuclear-encoded subunit COX4 (Fig. 4G), were not affected by *in vivo* temperature acclimation. The acclimation profile of freshly isolated hepatocytes is thus in line with the situation in liver of thermally acclimated specimens (Lucassen et al., 2003).

The consecutive response of isolated hepatocytes to temperature clearly depended on the previous acclimation temperature of the cells *in vivo*. Warm incubation of cells from cold-acclimated fish induced a muscle type reduction of the activities of both mitochondrial enzymes (Fig. 3B,E) and a decrease of mRNA expression of both COX subunits (Fig. 4E,H). In line with an earlier study on catfish hepatocytes (Koban, 1986), this suggests that fish liver cells without any systemic input may display basic adjustment to warming.

By contrast, cold incubation of hepatocytes from warm-acclimated eelpout left enzyme activities unchanged (Fig. 3C,F) and solely increased the mRNA levels of COX4 (Fig. 4I). These findings are in line with the concept that a cold-induced mismatch of energy demand and supply becomes effective at a high organisational level, the intact animal, as a consequence of limitations in oxygen supply (Pörtner, 2001; Pörtner, 2002). The decrease in the metabolic rate of isolated cells in the cold parallels the reduction of energy demand and occurs at ample oxygen supply. This experimental situation alleviates the pressure to acclimate, an observation, which indicates that cold acclimation *in vivo* occurs in response to systemic signal(s).

Adenosine probably participates as a modulator of thermal acclimation. When applied to isolated hepatocytes, the metabolite had no effect on CS, but distinctly affected COX activities. Although *in vivo* acclimation of *Z. viviparus* changed neither the initial activities nor the mRNA expression of COX, the cellular response to adenosine was found to depend on the thermal origin of the cells. Responsiveness to adenosine was enhanced in hepatocytes from cold-acclimated fish, however, the effects were more pronounced at higher incubation temperatures.

The adenosine effect comprised two major components, a decrease of COX activity (Fig. 3E,F) and a concomitant increase of COX mRNA expression (Fig. 4E,F,H,I). The activity of COX, the terminal oxygen-consuming step of the respiratory chain, is often used as an approximation for the aerobic capacity of the cells (Kadenbach et al., 2000). Thus, it can be assumed that adenosine reduces the capacity of aerobic energy production. This is in line with observations by Krumschnabel et al. (Krumschnabel et al., 2000), who found reduced oxygen consumption rates under the acute effect (10–30 min) of adenosine in trout hepatocytes in parallel to a decrease of protein synthesis rate. They assumed that the deceleration of oxygen uptake was due to diminished cellular ATP

demand caused by adenosine. Such an effect may be paralleled by the reduction of COX capacities as observed in the present study.

The suppressing effect on aerobic capacities is contrasted by the stimulating effect of adenosine on the expression of COX. Since this effect was more pronounced in cells incubated at 11°C, adenosine treatment of isolated hepatocytes abolished the warming induced reduction of COX expression. As a result, COX mRNA remained at similar levels at both incubation temperatures (Fig. 4E,H,I), resembling the expression pattern obtained during *in vivo* temperature acclimation (Fig. 4D,G). Adenosine treatment thus results in a discrepancy between the levels of transcription (increased) and the capacity of the enzyme (decreased). Similarly, loose coordination between message levels and enzyme functional capacity was found in the time-course of whole animal acclimation (Lucassen et al., 2003). Adenosine may thus influence the coordination of transcriptional and translational activities or cause posttranslational modification of the enzyme proteins. Besides, the discrepancy might result from incomplete response of the cell culture within the experimental time. However, while establishing the cell culture system we found steady state levels for COX activities established within the first 48 h, which were conserved for the following 3 days (data not shown). Because of restricted survival time of eelpout hepatocytes in primary culture, a further extension of the incubation period was not feasible. A more delayed response of the functional levels compared to the mRNA response seems to be rather unlikely.

The question arises of how adenosine exerts these effects. A receptor-mediated action was investigated by application of the adenosine A_1 receptor antagonist 8-PT and the non-selective receptor agonist NECA. Both ligands have been used to block and stimulate adenosine receptors in other fish species, respectively (Krumschnabel et al., 2000; Rosati et al., 1995). However, 8-PT could not prevent the effects of adenosine, and NECA failed to reduce COX activities in eelpout liver cells (Fig. 4). Furthermore, hepatocytes continuously consumed adenosine, a process stimulated at elevated temperature (data not shown). These observations suggest diffusive entry, and intracellular action of adenosine. Assuming a receptor-mediated action in accordance with earlier studies on fish hepatocytes, adenosine concentrations about one order of magnitude higher than the observed concentrations have been used in our cell culture system. Higher adenosine levels might be necessary to compensate possible insensitivity of the isolated cells, but also facilitate the diffusive entry of the metabolite. Since the actual concentration in incubated cells was not measurable because of the small sample size, we cannot exclude higher intracellular adenosine levels *in vitro* compared to *in vivo*, which might have influenced the response of the cells *in vitro*.

Although no data exist for an intracellular action in fish, adenosine may act through different mechanisms in hepatocytes. First, adenosine can be reconverted to AMP by adenosine kinase and give rise to subsequent ATP synthesis (Bontemps et al., 1983). ATP is known as an allosteric inhibitor of COX but also acts as a noncompetitive inhibitor of CS in fish (Hochachka and Lewis, 1970), thus adenosine treatment might result in reduced activities of both enzymes which has, however, not been observed here. Accumulation of ATP upon thermal acclimation of the cells appears unlikely. Second, high intracellular adenosine concentrations prevent the hydrolysis of *S*-adenosylhomocystein (SAH), a competitive inhibitor of most *S*-adenosylmethionine (SAM)-dependent methyltransferases (Kloor and Oswald, 2004), which are involved in the methylation of many molecules, e.g. proteins, DNA and RNA (Chiang et al., 1996). In knockout mice deficient for the synthesis of hepatic SAM, the levels of COX1 and COX2 proteins were found to be only half of those in wild-type mice, whereas the levels of COX2 mRNA remained unaltered, indicating a translational downregulation of COX (Santamaria et al., 2003). Although the underlying mechanisms still need to be investigated, the inhibition of SAM-dependent methyltransferases by adenosine may account for the mismatch between the expression and activity levels of COX, observed in adenosine-treated eelpout hepatocytes. The adenosine-related increase in the expression of both the nuclear and the mitochondrial encoded COX subunits is most remarkable and suggests coordinated regulation of nuclear and mitochondrial genes, thereby substantiating the observed effects. However, with the data at hand and the sparse literature available, the mechanism of how adenosine affects COX transcription remains to be elaborated.

Conclusions and perspectives

In summary, the lack of cold acclimation in isolated fish hepatocytes *in vitro* and the differences between warm acclimation patterns in whole animals *versus* cells isolated from cold-acclimated specimens indicate the involvement of systemic control in thermal acclimation. The accumulation of adenosine observed during cold exposure *in vivo* would allow for a role for adenosine in thermal acclimation. Our findings suggest that adenosine specifically modulates mitochondrial functioning. mRNA from nuclear and mitochondrial encoded COX subunits were found to be increased under adenosine treatment, resulting in an expression pattern in isolated hepatocytes similar to the one found during whole animal acclimation. By contrast, functional levels of COX were decreased in the presence of adenosine, possibly mediated by the inhibition of methyltransferases. The detailed mechanisms of action including the stimulating effect of adenosine on COX transcription await further investigation. Since *in vivo* thermal acclimation of liver mitochondria involves the modulation of CS, the lack of an effect of adenosine on CS activity and expression levels indicate that further signals remain to be identified.

LIST OF ABBREVIATIONS

8-PT	8-phenyltheophylline
COX	cytochrome *c* oxidase
COX2/4	cytochrome *c* oxidase subunit 2/4
CS	citrate synthase
MS-222	3-amino-benzoic-methanosulfonate
NECA	5′-(*N*-ethylcarboxamido)adenosine
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RPA	ribonuclease protection assay

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REFERENCES

Battersby, B. J. and Moyes, C. D. (1998). Influence of acclimation temperature on mitochondrial DNA, RNA, and enzymes in skeletal muscle. *Am. J. Physiol.* **275**, R905-R912.

Bontemps, F., Van den Berghe, G. and Hers, H. G. (1983). Evidence for a substrate cycle between AMP and adenosine in isolated hepatocytes. *Proc. Natl. Acad. Sci. USA* **80**, 2829-2833.

Buck, L. T. (2004). Adenosine as a signal for ion channel arrest in anoxia-tolerant organisms. *Comp. Biochem. Physiol.* **139B**, 401-414.

Casey, T. M., Dufall, K. G. and Arthur, P. G. (1999). An improved capillary electrophoresis method for measuring tissue metabolites associated with cellular energy state. *Eur. J. Biochem.* **261**, 740-745.

Chiang, P. K., Gordon, R. K., Tal, J., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K. and McCann, P. P. (1996). S-Adenosylmethionine and methylation. *FASEB J.* **10**, 471-480.

Egginton, S. and Sidell, B. D. (1989). Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am. J. Physiol.* **256**, R1-R9.

Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biol. Rev. Camb. Philos. Soc.* **79**, 409-427.

Hochachka, P. W. and Lewis, J. K. (1970). Enzyme variants in thermal acclimation. Trout liver citrate synthases. *J. Biol. Chem.* **245**, 6567-6573.

Hood, D. A. (2001). Invited review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J. Appl. Physiol.* **90**, 1137-1157.
Hüttemann, M. (2000). New isoforms of cytochrome c oxidase subunit IV in tuna fish. *Biochim. Biophys. Acta* **1492**, 242-246.
Kadenbach, B., Hüttemann, M., Arnold, S., Lee, I. and Bender, E. (2000). Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome c oxidase. *Free Radic. Biol. Med.* **29**, 211-221.
Kloor, D. and Osswald, H. (2004). S-Adenosylhomocysteine hydrolase as a target for intracellular adenosine action. *Trends Pharmacol. Sci.* **25**, 294-297.
Koban, M. (1986). Can cultured teleost hepatocytes show temperature acclimation? *Am. J. Physiol.* **250**, R211-R220.
Krumschnabel, G., Biasi, C. and Wieser, W. (2000). Action of adenosine on energetics, protein synthesis and K(+) homeostasis in teleost hepatocytes. *J. Exp. Biol.* **203**, 2657-2665.
Lannig, G., Eckerle, L. G., Serendero, I., Sartoris, F.-J., Fischer, T., Knust, R., Johansen, T. and Pörtner, H.-O. (2003). Temperature adaptation in eurythermal cod (*Gadus morhua*): a comparison of mitochondrial enzyme capacities in boreal and Arctic populations. *Mar. Biol.* **142**, 589-599.
Leary, S. C. and Moyes, C. D. (2000). The effects of bioenergetic stress and redox balance on the expression of genes critical to mitochondrial function. In *Enviromental Stressors and Gene Responses* (ed. K. B. Storey and J. Storey), pp. 209-229. Amsterdam: Elsevier Science.
Lucassen, M., Schmidt, A., Eckerle, L. G. and Pörtner, H. O. (2003). Mitochondrial proliferation in the permanent versus temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. *Am. J. Physiol.* **285**, 1410-1420.
Lucassen, M., Koschnick, N., Eckerle, L. G. and Pörtner, H. O. (2006). Mitochondrial mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones. *J. Exp. Biol.* **209**, 2462-2471.
Lutz, P. L. and Kabler, S. (1997). Release of adenosine and ATP in the brain of the freshwater turtle (*Trachemys scripta*) during long-term anoxia. *Brain Res.* **769**, 281-286.
Lutz, P. L. and Nilsson, G. E. (1997). Contrasting strategies for anoxic brain survival – glycolysis up or down. *J. Exp. Biol.* **200**, 411-419.
MacCormack, T. J. and Driedzic, W. R. (2004). Cardiorespiratory and tissue adenosine responses to hypoxia and reoxygenation in the short-horned sculpin *Myoxocephalus scorpius*. *J. Exp. Biol.* **207**, 4157-4164.
Mark, F. C., Lucassen, M. and Pörtner, H. O. (2006). Thermal sensitivity of uncoupling protein expression in polar and temperate fish. *Comp. Biochem. Physiol.* **1D**, 365-374.
Mommsen, T. P., Moon, T. W. and Walsh, P. J. (1994). Hepatocytes: isolation, maintenance and utilization. In *Biochemistry and Molecular Biology of Fishes, Analytical Techniques*. Vol. 3 (ed. P. W. Hochachka and T. P. Mommsen), pp. 355-372. Amsterdam: Elsevier Science.
Newby, A. C., Worku, Y., Meghji, P., Nakazawa, M. and Skladanowski, A. C. (1990). Adenosine: a retaliatory metabolite or not? *News Physiol. Sci.* **5**, 67-70.
Nisoli, E., Clementi, E., Moncada, S. and Carruba, M. O. (2004). Mitochondrial biogenesis as a cellular signaling framework. *Biochem. Pharmacol.* **67**, 1-15.
Noack, S. (1980). *Statistische Auswertung von Meß- und Versuchsdaten mit Taschenrechner und Tischcomputer*. Berlin, New York: Walter de Gruyter Verlag.
Pörtner, H. O. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88**, 137-146.
Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol.* **132A**, 739-761.
Pörtner, H. O. and Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science* **315**, 95.
Pörtner, H. O., Lucassen, M. and Storch, D. (2005). Metabolic biochemistry: its role in thermal tolerance and in the capacities of physiological and ecological function. In *The Physiology of Polar Fishes* (*Fish Physiology 21*) (ed. J. F. Steffensen, A. P. Farrell, W. S. Hoar and D. R. Randall), pp. 79-154. San Diego: Elsevier Academic Press.
Ralevic, V. and Burnstock, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50**, 413-492.
Reipschlager, A., Nilsson, G. E. and Portner, H. O. (1997). A role for adenosine in metabolic depression in the marine invertebrate *Sipunculus nudus*. *Am. J. Physiol.* **272**, R350-R356.
Renshaw, G. M., Kerrisk, C. B. and Nilsson, G. E. (2002). The role of adenosine in the anoxic survival of the epaulette shark, *Hemiscyllium ocellatum*. *Comp. Biochem. Physiol.* **131B**, 133-141.
Rosati, A. M., Traversa, U., Lucchi, R. and Poli, A. (1995). Biochemical and pharmacological evidence for the presence of A1 but not A2a adenosine receptors in the brain of the low vertebrate teleost *Carassius auratus* (goldfish). *Neurochem. Int.* **26**, 411-423.
Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*. New York: Cold Spring Habor Laboratory Press.
Santamaria, E., Avila, M. A., Latasa, M. U., Rubio, A., Martin-Duce, A., Lu, S. C., Mato, J. M. and Corrales, F. J. (2003). Functional proteomics of nonalcoholic steatohepatitis: mitochondrial proteins as targets of S-adenosylmethionine. *Proc. Natl. Acad. Sci. USA* **100**, 3065-3070.
Sartoris, F. J., Bock, C. and Pörtner, H. O. (2003). Temperature-dependent pH regulation in eurythermal and stenothermal marine fish: an interspecies comparison using ^{31}P-NMR. *J. Therm. Biol.* **28**, 363-371.
St-Pierre, J., Charest, P.-M. and Guderley, H. (1998). Relative contribution of quantitative and qualitatve changes in mitochondria to metabolic compensation during seasonal acclimatisation of rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **201**, 2961-2970.
Tinton, S. A., Chow, S. C., Buc-Calderon, P. M., Kass, G. E. and Orrenius, S. (1995). Adenosine inhibits protein synthesis in isolated rat hepatocytes. Evidence for a lack of involvement of intracellular calcium in the mechanism of inhibition. *Eur. J. Biochem.* **229**, 419-425.