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Mitochondrial mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones

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Summary

Adjustments in mitochondrial properties and capacities are crucial in acclimatization to seasonal cold as well as in evolutionary cold adaptation of marine ectotherms. To examine whether gene expression mechanisms contribute to different settings of aerobic capacities in populations of cod (*Gadus morhua*) along a latitudinal cline, maximum activities of key enzymes of mitochondrial metabolism and their respective mRNA levels were compared in white muscle and liver of cold (4°C) and warm (10°C) acclimated individuals from cod populations of the North Sea and the Barents Sea, respectively. In white muscle, cold acclimation caused a parallel increase in citrate synthase (CS) and in cytochrome *c* oxidase (COX) activities, but with a much larger effect in the cold eurythermal Arctic population. In liver, cold acclimation was accompanied by increments in CS activities, but differences between populations were minor. Overall COX activities in liver were not affected by cold acclimation, but were higher in the cold adapted population. In both populations increments in muscle CS capacities were tightly correlated with elevated mRNA levels, suggesting transcriptional control of citrate synthase levels in muscle. In liver, CS mRNA levels differed between populations but were not affected by cold acclimation, so that post-transcriptional control may contribute to elevated functional levels in this tissue. Mitochondrial-encoded COX2 mRNA levels were not limiting for functional activities in both tissues, in favour of post-transcriptional control or limitations by other transcripts of the COX complex. Altogether, the differentiation in gene expression between both populations was more strongly expressed at 4°C. The comparison of functional levels and transcript levels may reflect genetic differentiation at functional sites, in line with genetic differences between the two populations previously established by non-coding genetic markers.

Key words: cold acclimation, cytochrome *c* oxidase, citrate synthase, RNase protection assay, cod, *Gadus morhua*.

Introduction

Temperature has a large impact on all biological processes and is, therefore, a key factor shaping marine ecosystems. Animal organisms, due to their inherently high levels of organisational complexity, specialize to environmental temperature more than unicellular bacteria and algae (Pörtner, 2002a). Accordingly, thermal tolerance windows differ between ectothermal animal species depending on latitude or on seasonal temperature acclimatisation, and are therefore related to geographical distribution.

Ectothermic animals experience a decelerating effect of cold temperature on cellular processes and compensate in order to maintain functional balance between energy allocation and demand, e.g. by adjusting the velocity of chemical and enzymatic reactions, the rates of diffusion, membrane fluidity and the flexibility of protein structure. They are thereby able to shift the window of thermal tolerance according to the ambient temperature regime. Mitochondria are known to play a key role in this process since they are the primary site of ATP production in animals, their density and capacity thus reflecting aerobic energy turnover. Adaptation to cold in a (southern hemisphere) latitudinal cline is associated with rising mitochondrial densities in fish muscle; however, capacities of mitochondrial respiration were found to fall in parallel with decreasing habitat temperature (Johnston et al., 1998). In contrast, acclimation to seasonal cold (studied mostly in northern hemisphere fish) is well known to cause a rise in aerobic capacity, reflected in rising mitochondrial densities and/or increasing capacities of mitochondrial enzymes (cf. Guderley, 1998; Guderley, 2004; Pörtner, 2002b). As a trade-off, the high energy demand for the synthesis of mitochondria and the generation and maintenance of transmembrane proton gradients despite proton leakage causes mitochondrial maintenance costs to rise during acclimatisation to cold (cf. Pörtner et al., 1998; Pörtner et al., 2000; Pörtner, 2002b).

Adjustments in aerobic capacity through mitochondrial proliferation or degradation are thus crucial events in thermal

adaptation and the shift of thermal limits. Measurements of key enzymes of the citric acid cycle, mostly citrate synthase (CS), on the one hand, and oxidative electron transport components, mostly cytochrome *c* oxidase (COX), on the other, are widely used to monitor the long-term acclimation of fish mitochondria and tissue aerobic capacity. Increments in these parameters likely reflect modifications in both the properties and the numbers of the mitochondria present (Guderley, 1998).

Regulatory patterns during cold acclimation have been addressed in studies comparing enzyme capacities with transcript levels of the respective genes. For example, significant increments in red and white muscle COX activities were observed upon cold acclimation in trout; however, levels of COX1 mRNA or other messengers of mitochondrial-encoded genes under study remained unaffected by cold (Battersby and Moyes, 1998). In contrast, both mitochondrial-encoded COX1 and COX2 and nuclear-encoded COX4 mRNA were overcompensated relative to enzyme capacities in white muscle and liver of the eurythermal common eelpout during cold acclimation (Hardewig et al., 1999). A loose relationship between mRNA levels and maximum activities was also found for citrate synthase in eelpout liver upon cold acclimation (Lucassen et al., 2003). In this case, an intermediate rise in transcript levels occurred during acclimation and might have supported rapid establishment of the new steady state. Therefore, interspecific as well as tissue-specific variability in acclimation to thermal challenges exist, and all levels of enzyme expression (transcription, translation, protein modification) seem to be involved in the adaptation process. The contribution of all of these processes has to be elucidated in each individual case, before any statements on unifying principles can be made.

Elevated standard metabolic rates in cold adapted populations of eurythermal animals indicate that cold compensated metabolic rates are typical for this group. A recent analysis suggests that the patterns of cold stenothermy in Antarctic ectotherms *versus* cold eurythermy in northern hemisphere ectotherms extend to the respective differences in metabolic capacities as well as in metabolic scopes and activity levels (Pörtner, 2002b). Cold eurythermy patterns were also shown to develop in northern populations of the same species in a latitudinal cline of the northern hemisphere, possibly as a consequence of evolutionary temperature adaptation, linked to genetic differentiation between populations. In line with a rise in metabolic costs, increments in mitochondrial aerobic capacities have been observed in marine invertebrates and in cod during adaptation to eurythermal cold in a (northern hemisphere) latitudinal cline (Tschischka et al., 2000; Sommer and Pörtner, 2002; Fischer, 2002).

In a recent study, the effects of cold acclimation *versus* those of cold adaptation were evaluated by studying the capacities of aerobic enzymes in populations of eastern Atlantic cod along a latitudinal cline (Lannig et al., 2003). These comparisons showed significantly higher capacities of aerobic enzymes in the northernmost population from the Barents Sea, associated with higher rates of oxygen consumption after cold acclimation (Fischer, 2002). According to microsatellite analyses the cod populations from the southern North Sea (NSC) and from the Barents Sea (north-eastern Arctic cod, NEAC) are genetically distinct (Nielsen et al., 2001; Hutchinson et al., 2001). The cod population from the North Sea experiences large seasonal temperature fluctuations between 4°C to 18°C, whereas cold adapted cod from the Barents Sea live in more stable colder habitats at temperatures between 2°C and 4°C. Nonetheless, NEAC migrate to somewhat warmer waters for spawning and display growth optima close to 10°C (Pörtner et al., 2001). These patterns indicate that the species is typically eurythermal in many areas of its distribution range.

Therefore, the present study was designed to investigate at which levels the differences in aerobic metabolism as observed in the two populations are regulated and whether the genetic differentiation as found for non-coding regions extends to differences in the gene expression of central metabolic enzymes. We studied two populations of cod, one from the North Sea and the other one from the Barents Sea, after acclimation to the same low (4°C) and high (10°C) temperatures. By comparing the patterns of gene expression in the genetically distinct populations the present data set extends our previous study on differences in enzyme capacities. We analysed maximum activities as well as transcript levels of CS and COX in liver and white muscle. Since no RNA probes were available for citrate synthase from cod we isolated and characterised a fragment of the citrate synthase gene from *G. morhua.*

Materials and methods

Animals

Gadus morhua L. from the southern North Sea population (NSC: final body mass prior to tissue sampling: 464.5±50.5 g) were caught during spring 2003 in the German Bight near Helgoland. North eastern Arctic cod (NEAC: final body mass: 309.8±24.3 g), hatched from brood stocks by the University of Bergen, were provided in 2001 and raised at the Alfred Wegener Institute until beginning of the experiment. All fishes were kept at 10.0±0.5°C and 30‰ salinity under a 12:12 h light-dark cycle and were fed with shrimps and mussels twice a week. Fish were acclimated to 4.0±0.5°C (cold acclimated) and 10.0±0.5°C (warm acclimated) for at least 6 weeks. Starting from 10°C, the temperature was changed at a rate of 1°C day^{-1} until the final temperature was reached. Feeding was terminated 5 days prior to sample preparation.

RNA-isolation

Animals were slightly anaesthetized with MS-222 (0.3 g l^{-1}) before being killed. Samples from liver and white muscle (lateral, taken from close behind the anus) were quickly removed and frozen instantaneously in liquid nitrogen. For the quantitative isolation of total RNA from frozen liver tissue the RNeasy kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. Total RNA from muscle was isolated using the peqGold Trifast kit (Peqlab, Erlangen,

Germany) based on the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987), since this kit could be easily adapted to larger tissue sizes, as required for tissues with low RNA contents. mRNA was purified from total RNA using the Oligotex kit (Qiagen) according to the manufacturer's instructions. RNA quality was checked as described earlier (Lucassen et al., 2003).

Construction of probes and sequence determination

Species-specific probes for the quantification of RNA transcripts of citrate synthase (CISY) and cytochrome *c* oxidase (COX) genes were constructed by use of reverse transcription followed by polymerase chain reaction (RT-PCR) as described earlier (Lucassen et al., 2003). For CISY the primer pair CISY-F9/CISY-B16 (Lucassen et al., 2003) was used. For the mitochondrial-encoded COX subunit 2 (COX2) GmCOX2-F5 (5′-CCGCATCTTACTATTAAAGCAATGG-3′) was designed as forward and GmCOX2-B8 (5′-GGATTCAACTGGGACAACCATACG-3′) as backward primers according to the published mitochondrial sequence of *G. morhua* (Johansen and Bakke, 1996) (GenBank X99772). Briefly, reverse transcription and PCR were performed as described earlier (Lucassen et al., 2003). The citrate synthase fragment was amplified in 32 cycles with an annealing temperature of 58°C (± 6°C) for 80 s. For the COX2 fragment a touchdown program was used with an annealing temperature from 57 to 49°C (–1°C per cycle) and a 12°C gradient, followed by 25 cycles at 52±6°C annealing temperature. After amplification a final prolonged elongation step of 8 min at 72°C was introduced to prepare the PCR fragments for the cloning. The gel-purified fragments were cloned with the TOPO TA Cloning kit (Invitrogen, Karlsruhe, Germany) according to the manual. After separation of some clones plasmids were isolated from overnight cultures using the Qiaprep Spin Miniprep kit (Qiagen). To verify the presence and size of inserts, the isolated plasmids were analysed by restriction digestion with *Eco*RI. For each fragment the DNA sequence of positive clones were determined for both strands by MWG-Biotech (Ebersberg, Germany). Primer construction, assembling, analyses and alignments of the sequences were performed using the MacVector 7.2 program package (Oxford Molecular Ltd, Oxford, UK). The DNA sequence for citrate synthase has been submitted to GenBank and can be obtained under the following accession number: GmCISY: DQ059757.

Quantification of RNA

Gene-specific mRNA was quantified in ribonuclease protection assays (RPA) performed with the RPA-III kit from Ambion (Austin, TX, USA), according to the manufacturer's instructions. 10 μg total RNA was simultaneously hybridized at 42°C to antisense probes for CS, COX2 and 18S-rRNA. For 18S-rRNA, a commercial plasmid containing a highly conserved 80-bp fragment (pTRI RNA 18S, Ambion) was used as reference. Probes (antisense) were synthesized by *in vitro* transcription with T7 or T3 RNA Polymerase (Invitrogen) (Lucassen et al., 2003) and labelled with [α-^{32}P]uridine 5′-triphosphate (Amersham Bioscience, Freiburg, Germany). To equalize protected fragment intensities, a specific radioactivity of 1000 Ci mmol^{-1} (1 Ci=3.7×10^{10} Bq) was used for CISY, 45 Ci mmol^{-1} for COX2 and about 0.1 Ci mmol^{-1} for 18S-rRNA, respectively. After RNase treatment the RNA:RNA hybrids were co-precipitated with yeast RNA (5 μg). The RNA was separated by denaturing PAGE as described earlier (Lucassen et al., 2003), and radioactivity was detected and quantified using a phosphor storage image system (FLA-5000; Fuji, Tokyo, Japan) and the AIDA software package (raytest, Straubenhardt, Germany).

Protein isolation and enzyme assays

Frozen tissues were powdered under liquid nitrogen and homogenized briefly in 9 vol. ice-cold buffer (20 mmol l^{-1} Tris/HCl, pH 7.4, 1 mmol l^{-1} EDTA, 0.1% Triton X-100) with an Ultra Turrax. Cellular debris was removed by 10 min centrifugation at 1000 ***g*** and 4°C. The supernatant was carefully transferred into a new tube, avoiding co-transferring the upper lipid layer present in the liver preparations. COX activity was determined according to published methods (Moyes et al., 1997) in 20 mmol l^{-1} Tris/HCl, pH 8.0 containing 0.5% Tween 20 and 0.05 mmol l^{-1} reduced cytochrome *c*. Cytochrome *c* was reduced by the addition of excess sodium dithionite, which was removed by G-25 gel filtration. The decrease in extinction at λ=550 nm was monitored in a thermostatted spectrophotometer (Beckman, Fullerton, CA, USA) at 10.0°C. Enzyme activity in units per gram tissue was calculated using an extinction coefficient (ϵ_{550}) for cytochrome *c* of 19.1 mol^{-1} cm^2. The extraction of CS followed the protocol for COX with 75 mmol l^{-1} Tris/HCl, pH 7.6, 1 mmol l^{-1} EDTA as extraction buffer. The powdered tissue was mixed briefly with the ice-cold buffer using an Ultra Turrax. Homogenization was completed by ultrasonic treatment for 5 min at 0°C (Branson Sonifier 450: output control 8, duty cycle 50%). After centrifugation (10 min, 1000 ***g***, 4°C) the extract was withdrawn from below the lipid layer. CS activity was determined according to published methods (Sidell et al., 1987) in 75 mmol l^{-1} Tris/HCl, pH 8.0, 0.25 mmol l^{-1} 5,5′-dithio-bis(2 nitrobenzoic acid) (DTNB), 0.4 mmol l^{-1} acetyl CoA, 0.4 mmol l^{-1} oxalacetate. The increase in extinction at λ=412 nm was measured in a thermostatted spectrophotometer at 10°C. Enzyme activity in units per gram tissue was calculated using an extinction coefficient (ϵ_{412}) of 13.6 mol^{-1} cm^2 for the dye complex. Protein content was measured in the extracts using the Bradford method (Bradford, 1976) and a BSA standard.

Lipid extraction

Since tissue size, RNA content and protein concentration varied considerably between individual livers, the lipid content was determined according to a modified protocol (Folch et al., 1957). About 500 mg liver were extracted with a total of 20 vol. chloroform:methanol (2:1) using a glass homogenizer and a Teflon pestle (1 min, 1200 r.p.m.). The extraction was completed by ultrasonic treatment for 5 min at 0°C (Branson

Sonifier 450: output control 8, duty cycle 50%). After centrifugation for 10 min at 3000 ***g*** the supernatant was transferred into a weighted glass centrifugation tube and treated with 0.2 vol. 0.88% KCl. The phases were separated through centrifugation (10 min, 3000 ***g***), and the upper water phase together with any traces from the interphase was carefully removed from the glass tube. The solvent of the lower phase was evaporated under N_2 stream until no further reduction in mass could be determined.

Statistics

Statistical significance was tested at the $P \leqslant 0.05$ level using analysis of variance (one-factor ANOVA) and the *post hoc* Student–Newman–Keuls test for independent samples. Linear regressions and squared correlation coefficients were calculated using SigmaStat 3.0. Data were tested for outliers at the 95% significance level using Nalimov's test (Noack, 1980). Data are given as means ± s.e.m. (N=5–6).

Results

Temperature dependent expression of citrate synthase and cytochrome c oxidase

For reliable mRNA quantification species specific RNA probes are a prerequisite. Therefore, a 473-bp cDNA fragment of the citrate synthase gene was isolated by RT-PCR from *G. morhua* using primers originally designed for zoarcid genes (Lucassen et al., 2003) and characterised after cloning and sequencing. The CS-sequence of *G. morhua* (GenBank accession number: DQ059757) is similar to the sequences of other vertebrate citric synthases and shares about 95% identity to other fish (90% identity to mammals) at the protein and 90% (80%) at the DNA level (data not shown). These observations are in line with the central role of citrate synthase in metabolism and anabolism and the nuclear localization of its genes. These observations also substantiate that the isolated cDNA fragment, subsequently used for the preparation of RNA probes, represents the citrate synthase gene. In a similar way, a 168-bp probe has been isolated from the mitochondrially encoded COX2 using published information about the mitochondrial genome of *G. morhua*. The consensus DNA sequences from isolated clones were identical to the published sequence (data not shown) and could therefore be used for mRNA detection and quantification.

mRNA expression of the nuclear-encoded CS gene and the mitochondrial-encoded COX2 gene of *G. morhua* from North Sea (NSC) and Barents Sea (NEAC) were analysed after acclimation to 4°C and 10°C, respectively. In muscle (Fig. 1A), CS transcript levels increased significantly with acclimation to low temperature, both in the NSC (about 1.8-fold) and the NEAC population (about 2.5-fold). Moreover, cold acclimated NEAC even showed significantly higher levels of the transcript than cold acclimated NSC, whereas the warm acclimated groups from both populations were indistinguishable. For the COX2 gene no such clear relationship between transcript levels and temperature became evident (Fig. 1B). Whereas both groups of NEAC expressed COX2 at the same level as the cold acclimated group from the North Sea, COX2 mRNA levels from warm acclimated NSC tended to be higher than in all other groups. The significance of this trend was revealed when comparing the warm acclimated groups of the different populations by use of Student's *t*-test.

In liver, temperature acclimation had no effect on the transcript levels of the citrate synthase gene in either the North Sea or the Arctic populations (Fig. 2). However, transcript levels were lower by a factor of two in the Arctic cod than in

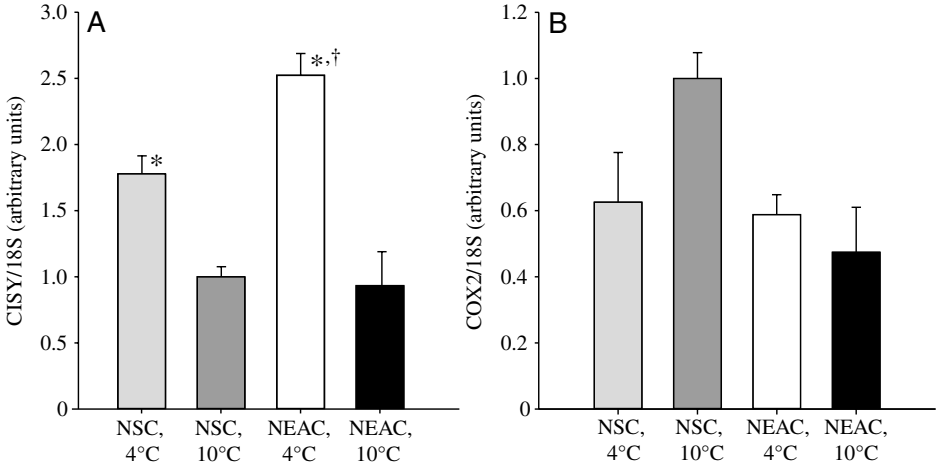

Fig. 1. Effects of temperature acclimation on mRNA levels of (A) citrate synthase and (B) cytochrome *c* oxidase subunit 2 in white muscle of *G. morhua* from North Sea (NSC) and Barents Sea (NEAC). The relative quantities of CISY and COX2 were determined from ribonuclease protection assays. Data were corrected for loading differences using the 18S rRNA signal. Values are means ± s.e.m. (N=5–6) and expressed relative to the warm-acclimated control group (NSC, 10°C), which was set to 1. The data were tested for outliers at the 95% significance level using Nalimov's test (Noack, 1980). *Significant difference from the warm-acclimated control group within a population; †significant difference between populations at the same acclimation temperature.

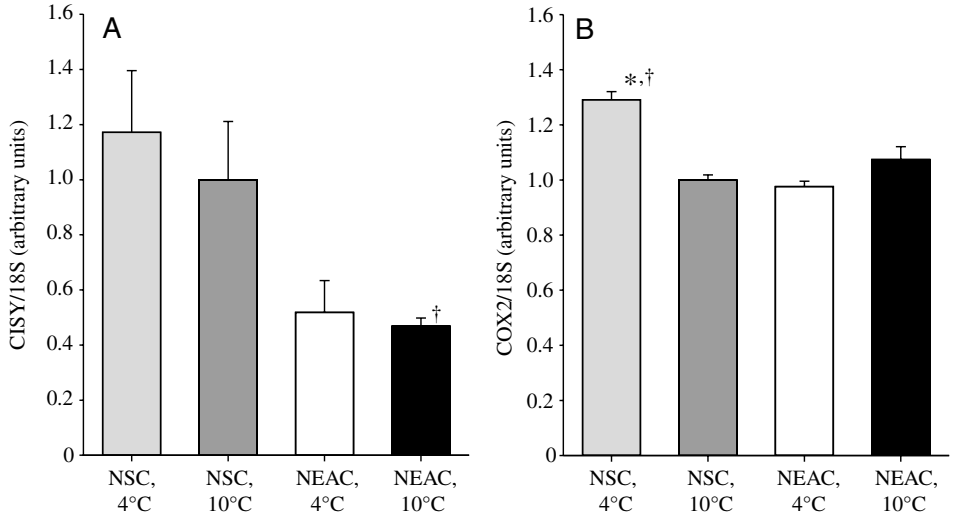

Fig. 2. mRNA levels of (A) citrate synthase and (B) cytochrome *c* oxidase subunit 2 in the liver of *G. morhua* from North Sea (NSC) and Barents Sea (NEAC), respectively, acclimated to 4°C and 10°C. The relative quantities of CISY and COX2 were determined from ribonuclease protection assays. Data were corrected for loading differences using the 18S rRNA signal. Values are means ± s.e.m. (*N*=5–6) and expressed relative to the warm-acclimated control group (NSC, 10°C), which was set to 1. The data were tested for outliers at the 95% significance level using Nalimov's test (Noack, 1980). *Significant difference from the warm-acclimated control group within a population; †significant difference between populations at the same acclimation temperature.

the North Sea cod to begin with. Transcript levels of COX2 remained unchanged in the Arctic population upon thermal acclimation and increased only slightly (by a factor of 1.3) but significantly in the cold acclimated North Sea cod compared to any other group.

Analysis of enzyme activities

Maximum activities of citrate synthase and cytochrome c oxidase were determined at 10°C in all experimental groups. According to Fig. 3, both enzyme activities rose significantly with acclimation to cold in white muscle of both populations (Fig. 3). CS and COX activities were highest in cold acclimated Arctic cod, so that significant differences between the populations became apparent upon cold acclimation. At (warm) control temperatures no such difference was detectable.

In liver, a contrasting picture became visible when CS and COX activities were expressed relative to tissue fresh mass (Fig. 4A,C): maximum activities decreased in both populations with acclimation to cold, and this decrement was again more pronounced in the Arctic population. Since this decline of mitochondrial capacity in the cold was quite unexpected (see Introduction) and since the liver is known as a huge lipid store in *G. morhua*, we analysed lipid contents and hepatosomatic indices (Table 1). Although variable between specimens, liver size was significantly increased in cold acclimated Arctic cod. A similar trend was visible in the North Sea population. In parallel, lipid content seemed to increase with cold acclimation, although this trend was not significant. Therefore, we calculated total liver enzyme activities for each specimen based on 100 g fish (Fig. 4B,D): in this case CS activities rose significantly upon cold acclimation within both populations. Acclimation to cold had no effect on total COX activities within both populations, whereas activities in the Arctic population tended to be higher regardless of acclimation temperature.

Discussion

According to previous evidence cod populations collected from different areas within a latitudinal cline of the Eastern Atlantic are functionally different (cf. Pörtner et al., 2001; Pörtner, 2002b). When analysed at the same temperature, specific growth rates in cod were found lower in populations from higher latitudes. This was hypothesized to be due to the cost of eurythermal cold adaptation, reflected in higher metabolic rates in NEAC than in NSC at low acclimation temperatures (cf. Pörtner et al., 2005). Use of non-coding markers revealed that the north-eastern Arctic population is genetically distinct from the more southern populations (Hutchinson et al., 2001; Nielsen et al., 2001). These differences may extend to the functional level. Therefore, the study of cod populations from the opposite margins of the thermal window of this species (Pörtner et al., 2001) may be promising for an identification of evolutionary aspects of temperature-related differentiation between populations of the same species at functional and molecular levels.

Liver, which is a metabolically active tissue, and white muscle, which represents the largest fraction of body mass, both have a large impact on whole animal energy budget. Liver size and composition vary with growth rate, temperature and season in cod (Holdway and Beamish, 1984; Pelletier et al., 1994) and liver is used for lipid storage, especially in gadoids. In the present study, hepatosomatic indices increased with cold acclimation in

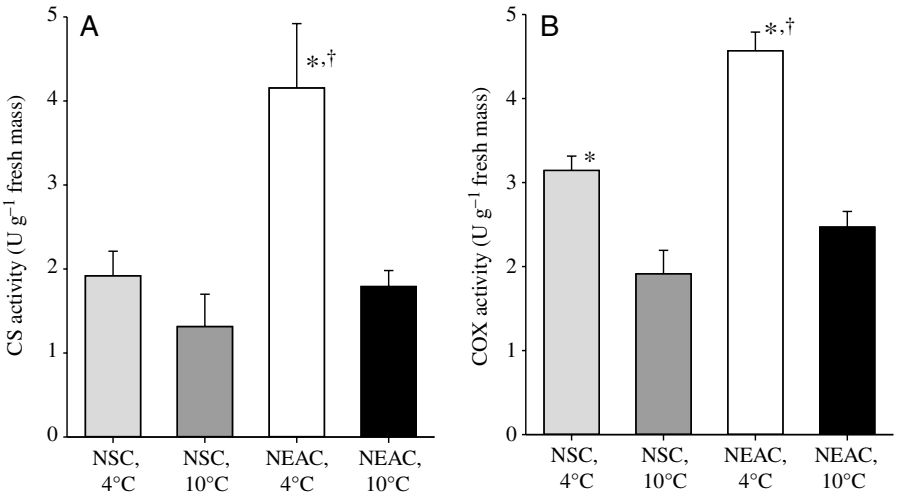

Fig. 3. Effects of temperature acclimation on maximum activities of (A) citrate synthase and (B) cytochrome *c* oxidase in white muscle of *G. morhua.* Activities were measured at 10°C. Values are means ± s.e.m. (*N*=5–6). The data were tested for outliers at the 95% significance level using Nalimov'stest (Noack, 1980). *Significant difference from the warm-acclimated control group within a population; †significant difference between populations at the same acclimation temperature.

both populations and were highest in NEAC at 4°C (Table 1). The increase in liver size was accompanied by increments in total lipid content. In consequence, other tissue components were effectively diluted when evaluated on a per g fresh mass basis. Lower levels of mass-specific total RNA (Table 1) and protein (data not shown) were found in cold acclimated fish, similar to findings by Pelletier et al. (Pelletier et al., 1994). These results imply that higher lipid contents in the cold have reduced the available space for the metabolically active fraction (total protein, enzymes, RNA, etc.) of the hepatocytes. Comparison of the liver data at the whole animal level is therefore essential for an evaluation of metabolic capacities. This comparison revealed enhanced liver sizes, resulting in enhanced liver metabolic capacities in the cold (see below). Since all fish were of comparable body size and mass, no further consideration of allometric scaling was needed (Pelletier et al., 1993; Lannig et al., 2003). HSI and condition factors (Table 1) were lower in the present study than found in the literature for growing fish but showed the same trends with respect to temperature effects (Holdway and Beamish, 1984; Lannig et al., 2003). Potential effects of high growth rates on mitochondrial activities, which have been described for white muscle in some fish species (cf. Guderley, 1998), should not have influenced our data. Indeed, no correlation between condition factors and muscle enzyme activities could be detected.

Effects of cold on mitochondrial enzymes

In ectothermic animals, long-term temperature shifts lead to compensatory changes in the levels of glycolytic and mitochondrial enzymes (Pörtner, 2002b; Guderley, 2004). An extensive literature links cold temperatures with enhanced aerobic capacities in tissues of fish including cod (Lannig et al., 2003), particularly in skeletal muscle. At the whole animal level these adjustments enhance functional capacity and alleviate the

Table 1. *Hepatosomatic indices, condition factors, lipid contents in liver and RNA content of G. morhua acclimated to 4°C and 10°C*

Species	Acclimation temperature (°C)	HSI (%)	Condition factor (g cm^{-3}×1000)	Total lipids (%)	Liver RNA (Q) (μg mg^{-1} fresh mass)	Muscle RNA (T) (μg mg^{-1} fresh mass)
G. morhua (NSC)	4.0	2.19±0.41	9.20±0.29	25.4±7.1	1.27±0.22*	0.44±0.06
	10.0	1.04±0.05	8.46±0.60	14.9±2.0	2.97±0.33	0.41±0.09
G. morhua (NEAC)	4.0	3.26±0.61*	8.32±0.28	35.0±7.0	1.65±0.22	0.58±0.09
	10.0	1.27±0.10	8.17±0.74	24.3±9.1	2.32±0.29	0.40±0.03

NSC, North Sea cod; NEAC, north-eastern Arctic cod.
Values are means ± s.e.m. (*N*=5–6).
Hepatosomatic indices (HSI)=(liver:body mass)×100.
Condition factors were calculated as previously described (Lucassen et al., 2003). Condition factor=[mass (g)×total length (cm)$^{-3}$]×1000.
Liver lipid content is relative to fresh mass.
RNA content in liver was determined using the RNeasy kit from Qiagen (Q) and in muscle, the Trifast kit (T).
*Significant difference from the warm-acclimated control group within a population.

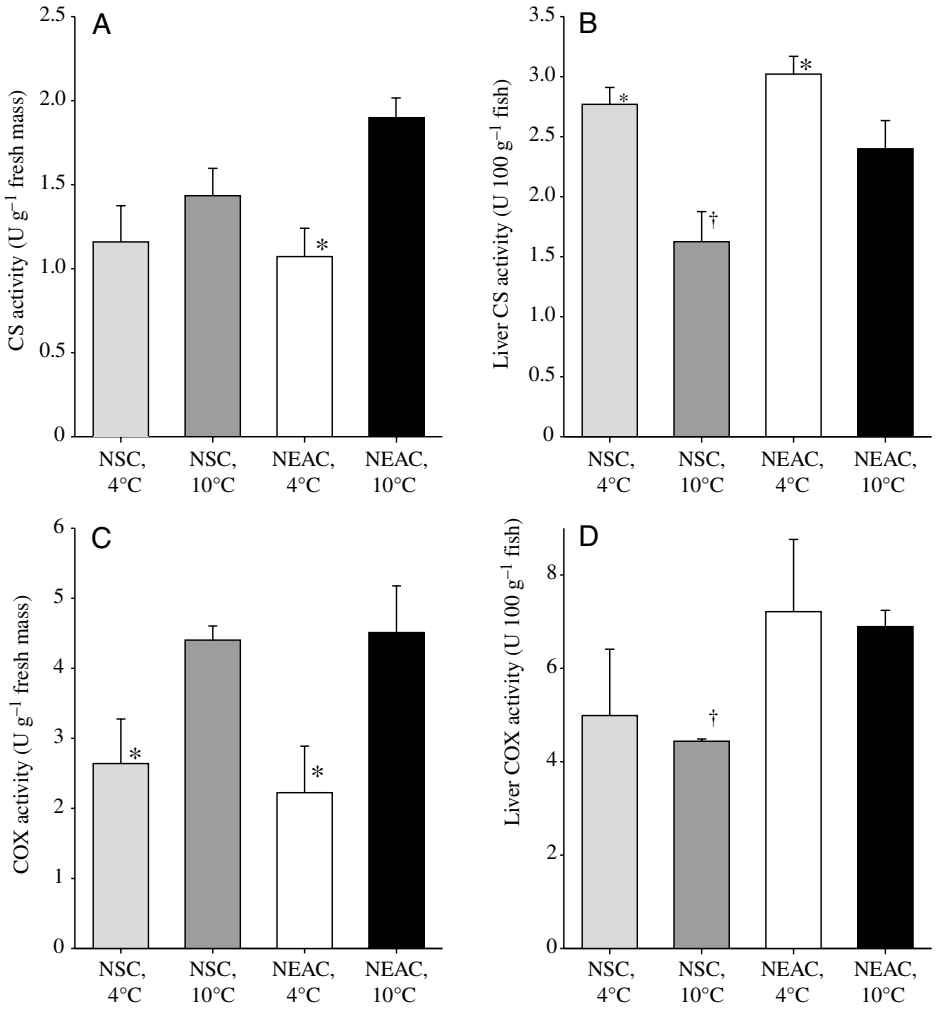

Fig. 4. Maximum activities of (A,B) citrate synthase and (C,D) cytochrome *c* oxidase in liver of *G. morhua* after acclimation to 4°C and 10°C. Activities were measured at 10°C. The values in A and C are expressed relative to wet mass. For the calculation of activities in total liver (B,D) individual enzyme activities determined per g wet mass were multiplied by the hepatosomatic index thus yielding an activity value for all liver in 100 g fish. Values are means ± s.e.m. (*N*=5–6). The data were tested for outliers at the 95% significance level using Nalimov'stest (Noack, 1980). *Significant difference from the warm-acclimated control group within a population; †significant difference between populations at the same acclimation temperature.

threat of functional hypoxia in the cold (Pörtner, 2002b). The present paper compares acclimation phenomena in cod at the temperature of optimal growth for both populations (10°C) (Pörtner et al., 2001; Fischer, 2002), which is close to mean habitat temperature for NSC, and at 4°C, which is the normal habitat temperature for NEAC but close to the lower temperature boundary for NSC. Increased capacities for CS and COX were found upon cold acclimation in white muscle of both populations, indicating higher aerobic capacities in the cold. However, whereas both enzymes displayed similar activities in both populations when acclimated to 10°C, the cold adapted population displayed increased capacities relative to the southern population when acclimated to cold. These results are in line with the view that metabolic cold compensation is present, especially in cold eurythermal animals (cf. Pörtner et al., 1998; Pörtner et al., 2000).

For total liver, the capacities for CS increased upon cold acclimation, whereas COX activities remained constant (Fig. 4). Similar to the present data, previous findings in cod and eelpout showed that liver citrate synthase activity rather than cytochrome *c* oxidase activity more clearly reflected mitochondrial responses to cold exposure (Lannig et al., 2003; Lucassen et al., 2003). Increased liver citrate synthase activities at constant levels of cytochrome *c* oxidase activity, as found in both populations, would indicate a relative increase in matrix over membrane functions in liver mitochondria. Similar to these results the ratio of CS over COX activities in liver rose by a factor of 2 in different populations of temperate eelpout (Lucassen et al., 2003).

In contrast, a fixed ratio of both enzymes was found in white muscle of both cod populations studied here and might be mainly due to changes in mitochondrial volume density

without concomitant changes in mitochondrial size and cristae surface density. These findings indicate a fixed ratio of matrix to cristae enzymes in muscle. Similarly, a parallel rise in the activities of both enzymes was seen in white and red muscle of trout during cold acclimation (Battersby and Moyes, 1998) and in red and white muscle of striped bass by stereological analyses (Egginton and Sidell, 1989). This pattern may thus be unifying and may mirror the key function of muscle mitochondria in energy supply.

Accordingly, cold acclimation in eurythermal species may cause similar relative changes in the tissue-specific capacities of metabolic functions. Liver mitochondria especially might be involved in anabolic processes with citrate synthase, providing excess citrate for example for lipid synthesis (cf. Pörtner, 2002a). In line with this view, lipid content was found to be elevated in cod liver during cold acclimation (see above). These considerations might imply that energy requirements are only one factor shaping acclimation of liver to seasonal cold. Shifting functional requirements for mitochondria in cold *versus* warm acclimated tissues, especially liver, warrants further investigation.

Despite the lack of phenotypic responses to cold with respect to COX capacity, higher maximum activities were found in liver of the cold adapted population regardless of acclimation temperature (Fig. 4D). These results indicate higher aerobic capacities in liver of the cold adapted population which, however, is not reflected in largely elevated whole-animal oxygen consumption rates in the warm (10°C) (Fischer, 2002). In the cold, however, largely enhanced aerobic capacities in muscle might reflect higher overall costs of mitochondrial maintenance in the cold adapted population and a shift in whole animal energy budget, in line with elevated rates of oxygen consumption in NEAC in the cold (see above).

Regulation of mitochondrial adjustments to cold

Investigation of RNA expression in relation to enzyme activities should provide some insight into the sites and levels of the regulation of mitochondrial proliferation and their involvement in the differentiation of populations. mRNA levels of CS rose significantly with cold acclimation in white muscle of both populations (Fig. 1), again displaying significant differences at the lower acclimation temperature only. Therefore, increments of the functional protein are closely related to increased transcript levels. This correlation becomes evident when plotting normalized citrate synthase activities against normalized mRNA levels (Fig. 5A; with fresh mass as the common denominator). For CS a significant correlation between maximum activities and mRNA could be detected, the slope of the regression line following the line of identity. Considering the similarity of total RNA contents within and between the two populations a fixed ratio between enzyme activity and specific mRNA levels reflects mostly transcriptional control of CS levels in white muscle under steady state conditions. Higher transcription rates or higher mRNA stability in NEAC than in NSC would explain the observed patterns and may also reflect the presence of different CS alleles in the two populations. A good example of such a phenomenon is available for lactate dehydrogenase-B (LDH-B) in *Fundulus heteroclitus* liver (Schulte et al., 1997; Schulte et al., 2000). Here the twofold difference in enzyme concentrations between northern and southern populations was associated with a twofold difference in both the abundance of the transcripts and the transcription rate of the gene. These findings suggested that the concentration differences in LDH-B enzyme were the result of changes at the level of transcription (Crawford and Powers, 1992). Investigation of the 5′ flanking region revealed remarkable differences between fish populations and a role for stress-responsive elements in mediating the modified transcription pattern (Schulte et al., 1997; Schulte et al., 2000). Further studies of the flanking regions (both 5′ and 3′) of the CS gene in cod are required to specify the reasons for the observed differences in transcript levels between cod populations.

In cod liver such a strong relationship between message levels and functional activities did not exist. Although significant differences were found in the relative expression of CS in the two populations (Fig. 2), whole animal transcript levels were not directly related to maximum enzyme activities. Considering other vertebrate genome projects and our own attempts to isolate cDNA probes, there is no evidence for further CS isoforms in vertebrate organisms. Therefore, while mere transcriptional control dominates the regulation of CS in muscle, further post-transcriptional processes might be involved in liver. Similar to our findings, variable ratios between CS activities and mRNA were found in a comparison of muscle fibres in different tuna and billfish species, indicating an important role for post-transcriptional processes in the fine tuning of enzyme functioning to tissue requirements (Dalziel et al., 2005). A fluctuating ratio of mRNA levels and citrate synthase activities under steady state conditions was also found in liver of boreal eelpout (Lucassen et al., 2003). These patterns may therefore be common and reflect dual function of this enzyme in liver (see above), which may require more complex, but unknown ways of regulation.

The expression of COX was investigated using the mitochondrial-encoded subunit COX2. Considerable variability existed in the transcript levels for muscle, and no clear temperature induced alterations were found. In liver the transcript levels were remarkably stable, again showing only minor temperature effects. No direct correlation could be detected when COX activities were plotted against COX2 mRNA levels (Fig. 5B). Thus the increments in muscle COX activities were not associated with elevated transcript levels, indicating that mitochondrial-encoded COX2 mRNA was not limiting for setting activity levels. Similarly, the differences between populations observed at the functional level in liver were not paralleled by different transcript levels. Therefore, transcriptional control of COX gene expression does not occur through mitochondrial-encoded COX2. Instead, transcriptional control through other probably nuclear-encoded subunits or post-translational control may dominate the functional expression of COX. In support of this assumption, Battersby

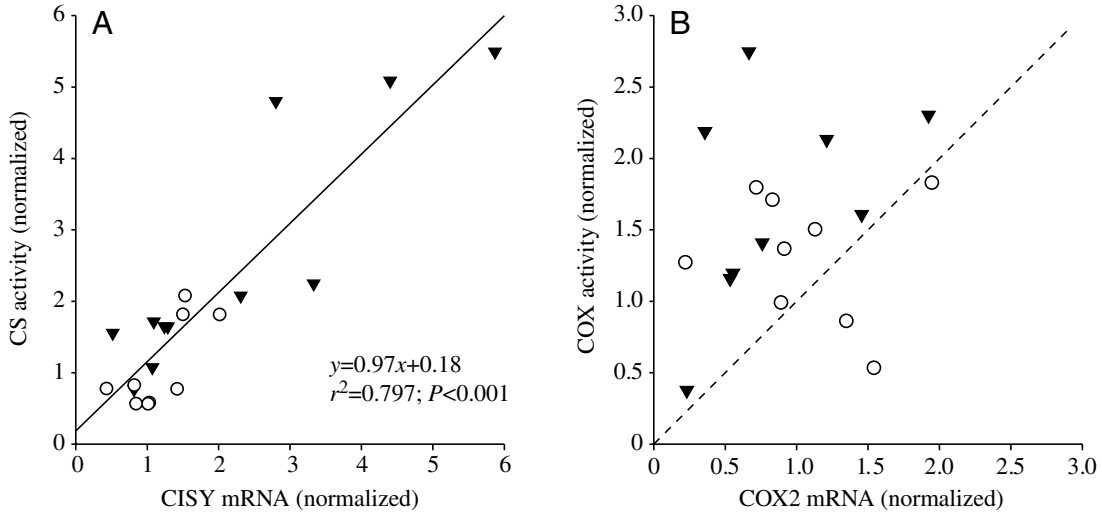

Fig. 5. Enzymatic activities *versus* mRNA levels in liver of cold acclimated *G. morhua*. (A) Citrate synthase (CS); (B) Cytochrome *c* oxidase (COX). Enzyme activities (per g fresh mass; 10°C assay temperature) and mRNA levels (per g fresh mass) were normalized by setting the mean activity of warm-acclimated *G. morhua* from the North Sea population to 1. Cold acclimation led to increments in maximum activities of both enzymes, reflecting a higher aerobic capacity in the Arctic population. Citrate synthase activities were significantly correlated with the increase in mRNA levels in both populations, in line with transcriptional control of this enzyme. No such correlation was found for cytochrome *c* oxidase activity and COX2 mRNA levels despite a significant increase in mRNA levels for the Arctic population. Open circles, *G. morhua* (North Sea, NSC); filled triangles, *G. morhua* (north eastern Arctic Sea NEAC). Broken line, line of identity for COX (y=1.0x).

and Moyes found evidence for regulation by nuclear-encoded genes (Battersby and Moyes, 1998) and, similar to our findings in cod, no limitation of functional activities by different mitochondrial-encoded transcripts. In line with these findings, the expression of the nuclear-encoded COX4 subunit in boreal eelpout was found more cold compensated than the mitochondrial-encoded subunits COX1 and COX2 (Hardewig et al., 1999). Further study is required to specify the role of the nucleus in setting COX activity.

Furthermore, the modulation of membrane composition during thermal adaptation has to be considered for membrane proteins like COX (cf. Hazel, 1995). Wodtke demonstrated for carp red muscle that the specific activity of COX was increased in cold acclimated membranes, merely through changes in lipid and fatty acid composition (Wodtke, 1981a; Wodtke, 1981b). However, no evidence was found for homeoviscous adaptation of mitochondrial membrane lipids in liver and heart of cold acclimated sea bass *Dicentrarchus labrax* (Trigari et al., 1992). As a consequence, cytochrome *c* oxidase activity per mg mitochondrial protein was decreased at lower acclimation temperatures. Accordingly, adaptation in membrane properties in cod muscle mitochondrial membranes may suffice to explain the observed increase in COX activities and the maintained functional activities in liver.

Conclusions and perspectives

Exclusively transcriptional control of CS levels was found in cod white muscle and may typify tissues where energy requirements only define mitochondrial functions. In liver a role of CS in anabolism may cause a more complex regulatory pattern. Post-transcriptional control is likely involved in phenotypic acclimation to moderate cooling, as seen for COX in this and in previous studies in fish.

The observed differences in the expression of CS are indicative of differences between populations at the genome level, which relate to the differentiation at the functional level. These differences are operative at the cold end of the thermal tolerance window and may reflect the stable separation of both populations. Selection of regulatory loci may be involved in establishing higher aerobic capacities seen in the cold adapted population. Clearly, chromosomal analyses of the entire CS gene are required for an in-depth characterization of the genetic differences between both populations.

Enhanced expression capacity of aerobic genes in the cold may support increased mitochondrial capacities and typify cold adapted eurytherms in general, at least those which display significant metabolic cold compensation, as seen in north eastern Arctic cod. Enhanced gene expression capacity may provide the functional basis for the cold compensated capacity for aerobic exercise suggested to exist in NEAC compared to North Sea cod (Pörtner, 2002b). This occurs at the expense of reduced growth performance. Very likely, the genetic differences reflected in our present data are only representative of a larger array of genetic differences that underlie the observed functional patterns. Therefore, future comparative studies in these populations using large-scale genomic studies will be promising for elucidating the impact of abiotic factors like temperature on functional and genetic differentiation of populations. The hypothesis that eastern Atlantic cod extends its geographical distribution over a larger latitudinal range than would be possible for one genetically uniform population by differentiation into

genetically and functionally distinct populations, needs to be tested.

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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.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Crawford, D. L. and Powers, D. A. (1992). Evolutionary adaptation to different thermal environments via transcriptional regulation. *Mol. Biol. Evol.* **9**, 806-813.

Dalziel, A. C., Moore, S. E. and Moyes, C. D. (2005). Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types. *Am. J. Physiol.* **288**, R163-R172.

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.

Fischer, T. (2002). The effects of climate induced temperature changes on cod (G*adus morhua* L.): linking ecological and physiological investigations. PhD thesis, Universität Bremen, Germany.

Folch, J., Lees, M. and Sloane, S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Guderley, H. (1998). Temperature and growth rates as modulators of the metabolic capacities of fish muscle. In *Cold Ocean Physiology* (ed. H.-O. Pörtner and R. Playle), pp. 58-87. Cambridge: Cambridge University Press.

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

Hardewig, I., Van Dijk, P., Moyes, C. and Pörtner, H.-O. (1999). Temperature-dependent expression of cytochrome-c oxidase in Antarctic and temperate fish. *Am. J. Physiol.* **277**, R508-R516.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.

Holdway, D. A. and Beamish, F. W. H. (1984). Specific growth rate and proximate body composition of atlantic cod (*Gadus morhua* L.). *J. Exp. Mar. Biol. Ecol.* **81**, 147-170.

Hutchinson, W., Carvalho, G. and Rogers, S. (2001). Marked genetic structuring in localised spawning populations of cod *Gadus morhua* in the North Sea and adjoining waters, as revealed by microsatellites. *Mar. Ecol. Prog. Ser.* **223**, 251-260.

Johansen, S. and Bakke, I. (1996). The complete mitochondrial DNA sequence of Atlantic cod (*Gadus morhua*): relevance to taxonomic studies among codfishes. *Mol. Mar. Biol. Biotechnol.* **5**, 203-214.

Johnston, I. A., Calvo, J., Guderley, H., Fernandez, D. and Palmer, L. (1998). Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J. Exp. Biol.* **201**, 1-12.

Lannig, G., Eckerle, L., 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.

Lucassen, M., Schmidt, A., Eckerle, L. G. and Pörtner, H.-O. (2003). Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. *Am. J. Physiol.* **285**, R1410-R1420.

Moyes, C. D., Mathieu-Costello, O. A., Tsuchiya, N., Filburn, C. and Hansford, R. G. (1997). Mitochondrial biogenesis during cellular differentiation. *Am. J. Physiol.* **272**, C1345-C1351.

Nielsen, E. E., Hansen, M. M., Schmidt, C., Meldrup, D. and Gronkjaer, P. (2001). Fisheries. Population of origin of Atlantic cod. *Nature* **413**, 272.

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

Pelletier, D., Guderley, H. and Dutil, J. (1993). Does aerobic capacity of fish muscle change with growth rates? *Fish Physiol. Biochem.* **12**, 83-93.

Pelletier, D., Dutil, J. D., Blier, P. and Guderley, H. (1994). Relation between growth rate and metabolic organization of white muscle, liver and digestive tract in cod, *Gadus morhua*. *J. Comp. Physiol. B* **164**, 173-190.

Pörtner, H.-O. (2002a). 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. (2002b). Physiological basis of temperature-dependent biogeography: trade-offs in muscle design and performance in polar ectotherms. *J. Exp. Biol.* **205**, 2217-2230.

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

Pörtner, H.-O., Van Dijk, P., Hardewig, I. and Sommer, A. (2000). Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. In *Antarctic Ecosystems: Models for Wider Ecological Understanding* (ed. W. Davison and C. Williams), 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. et al. (2001). Climate induced temperature 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*). *Cont. Shelf Res.* **21**, 1975-1997.

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.

Schulte, P., Gomez-Chiarri, M. and Powers, D. (1997). Structural and functional differences in the promoter and 5′ flanking region of Ldh-B within and between populations of the teleost *Fundulus heteroclitus*. *Genetics* **145**, 759-769.

Schulte, P. M., Glemet, H. C., Fiebig, A. A. and Powers, D. A. (2000). Adaptive variation in lactate dehydrogenase-B gene expression: role of a stress-responsive regulatory element. *Proc. Natl. Acad. Sci. USA* **97**, 6597-6602.

Sidell, B. D., Driedzic, W. R., Stowe, D. B. and Johnston, I. A. (1987). Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. *Physiol. Zool.* **60**, 221-232.

Sommer, A. and Pörtner, H.-O. (2002). Metabolic cold adaptation in the lugworm *Arenicola marina*: comparison of a north sea and a white sea population. *Mar. Ecol. Prog. Ser.* **240**, 171-182.

Trigari, G., Pirini, M., Ventrella, V., Pagliarani, A., Trombetti, F. and Borgatti, A. R. (1992). Lipid composition and mitochondrial respiration in warm- and cold adapted sea bass. *Lipids* **27**, 371-377.

Tschischka, K., Abele, D. and Pörtner, H.-O. (2000). Mitochondrial oxyconformity and cold adaptation in the polychaete *Nereis pelagica* and the bivalve *Arctica islandica* from the Baltic and White Seas. *J. Exp. Biol.* **203**, 3355-3368.

Wodtke, E. (1981a). Temperature adaptation of biological membranes. Compensation of the molar activity of cytochrome c oxidase in the mitochondrial energy-transducing membrane during thermal acclimation of the carp (*Cyprinus carpio* L.). *Biochim. Biophys. Acta* **640**, 710-720.

Wodtke, E. (1981b). Temperature adaptation of biological membranes. The effects of acclimation temperature on the unsaturation of the main neutral and charged phospholipids in mitochondrial membranes of the carp (*Cyprinus carpio* L.). *Biochim. Biophys. Acta* **640**, 698-709.