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Effects of temperature acclimation on lactate dehydrogenase of cod (*Gadus morhua*): genetic, kinetic and thermodynamic aspects

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Summary

The aim of this study was to determine the effects of seasonal temperature variation on the functional properties of lactate dehydrogenase (LDH) from white muscle and liver of Norwegian coastal cod (*Gadus morhua*) and the possible relevance of LDH allelic variability for thermal acclimation. Two groups of fishes were acclimated to 4°C or 12°C for one year. Polymorphism was observed in only one (*Ldh-B*) of the three *Ldh* loci expressed in cod liver and/or muscle. Isozyme expression remained unchanged regardless of acclimation temperature (T_A). The products of locus *Ldh-B* comprise only 14–19% (depending on the tissue) of total LDH activities and, consequently, differences between phenotypes are negligible in terms of their effect on LDH total performance. No kinetic (K_m^{PYR}, K_{si}^{PYR}, V_{max}) or thermodynamic (E_a, ΔG) differences were found among *Ldh-B* phenotypes. Clear kinetic differences were observed between LDH isoforms in the two tissues. However, the Arrhenius activation energy (E_a) for pyruvate reduction was the same for both tissues (E_a=47 kJ mol^{-1}) at T_A=12°C. Factors T_A, tissue and phenotype did not reveal a significant effect on the Gibbs free energy change (ΔG) of the reaction (55.5 kJ mol^{-1}). However, at T_A=4°C, the E_a was increased (E_a=53–56 kJ mol^{-1}) and the temperature dependence of the constant of substrate inhibition for pyruvate (K_{si}^{PYR}) decreased in both muscle and liver.

In conclusion, the strategies of LDH adjustment to seasonal temperature variations in cod involve changes in LDH concentration (quantitative), adjustment of thermodynamic (E_a) and kinetic (K_{si}^{PYR}) properties of the LDH (modulative) but not the expression of alternative isoforms (qualitative). We assume that the observed increase in E_a and the decrease of temperature dependence of K_{si}^{PYR} at low T_A is the result of structural changes of the LDH molecule (temperature-driven protein folding). We propose a new mechanism of metabolic compensation of seasonal temperature variations – cold acclimation results in changes in the kinetic and thermodynamic properties of LDH in a way that favours aerobic metabolism through reduction of the competition of LDH for pyruvate in normoxic conditions.

Key words: allozyme, Arrhenius activation energy, fish, *Gadus morhua*, glycolysis, isozyme, kinetics, lactate dehydrogenase, LDH, liver, metabolic compensation, muscle, temperature acclimation.

Introduction

Lactate dehydrogenase (LDH; l-lactate NAD$^+$ oxidoreductase; E.C. 1.1.1.27) catalyses the terminal step in anaerobic glycolysis and, together with the other glycolytic enzymes, is located in the cytosol. On the one hand, the level of LDH activity and the functional properties of this enzyme reflect the capacity for anaerobic energy production and, thereby, the level of resistance to oxygen deficiency during hypoxia, vigorous exercise or thermal stress (Pörtner, 2002b; Somero, 1998). On the other hand, LDH also serves to remove lactate during aerobic recovery, especially in tissues such as liver or heart.

During adaptation or acclimation to low temperatures, the capacity of anaerobic metabolism may be subject to adaptive modification. While compensation of aerobic enzymes and mitochondrial densities is a well-established feature of cold adaptation (cf. Pörtner, 2002b for a review) the picture is less clear for anaerobic capacity. In polar fish such as Antarctic notothenioids, excess of aerobic design at low activity lifestyles would explain why anaerobic capacity is generally reduced. Oxidative enzymes show relatively high degrees of cold compensation in notothenioids, while glycolytic enzymes do not (Crockett and Sidell, 1990; Dunn and Johnston, 1986; Johnston, 1987). In contrast to the low lactate levels in fatigued notothenioids, a significant anaerobic capacity was recently

found in Antarctic eelpout (*Pachycara brachycephalum*; Hardewig et al., 1998), a benthic and rather immobile species. Cold-compensated anaerobic capacity was also found in some, but not all, temperate freshwater fish (Van Dijk et al., 1998). As a corollary, glycolytic capacity may still be cold compensated in strictly benthic zoarcids, but no longer in more active polar fishes, which, therefore, tend to express a more aerobic mode of metabolism. The functional modification of LDH depending on the ambient temperature regime has been a focal issue in the study of thermal adaptation, including analyses of substrate affinities, thermodynamic properties and changes in enzyme structure that may cause the functional modifications observed (Danilenko et al., 1998; Fields et al., 2001, 2002; Fields and Somero, 1998; Hochachka and Somero, 1984; Klyachko et al., 1995b; Ozernyuk et al., 1994; Persikov et al., 1999; Sharpe et al., 2001; Zasedateleva et al., 1999).

The question arises as to whether temperature-dependent changes in anaerobic capacity or functional properties of LDH can also be seen in temperate or sub-Arctic to Arctic cold water fish like the cod *Gadus morhua*. This demersal species displays moderate levels of aerobic capacity and uses significant anaerobic metabolism at high, beyond critical, swimming speeds (Reidy et al., 1995). Therefore, the present study was designed to investigate the changes in the capacity and functional properties of LDH in cold- *vs* warm-acclimated cod from a temperate population.

In many fishes, LDH is coded by three independent loci (De Panepucci et al., 1984; Ferreria et al., 1991; Zietara et al., 1996). Simultaneous expression of LDH isozymes (A, B and C) and allozymes of LDH-B yields a complicated electrophoretic pattern (overall LDH suite), which is sometimes difficult to interpret. The protein (LDH) is a tetramer, and the two alleles result in a five-band electrophoretic pattern of allozymes (in the case of a heterozygote; Hillis et al., 1996; Utter et al., 1987). LDH in cod (*Gadus morhua*) is represented by three loci; *Ldh-A* and *Ldh-B* have been identified in liver, muscle, heart and eye, while *Ldh-C* is only observed in liver (Mork et al., 1985; Zietara and Skorkowski, 1993). Although an alternative allele is found in *Ldh-A* in cod from Iceland (Mork et al., 1985), *Ldh-B* is the most variable locus for this species. Polymorphism in locus *Ldh-B* can be described by two alleles (*a* and *b,* with a relative migration distance of 70 and 100 during gel electrophoresis with histidine buffer, pH 7.0) in cod and is mainly represented by three common phenotypes; *Ldh-B*(*a/a*), *Ldh-B*(*a/b*) and *Ldh-B*(*b/b*). In Norwegian waters, however, two rare alleles *Ldh-B*(*c*) (with a relative migration distance of 85) and *Ldh-B*(*d*) (with a relative migration distance of 140) have been observed as rare heterozygotes, combined with the allele *Ldh-B*(*b*) (Mork et al., 1985).

Some polymorphic loci display a latitudinal cline in allele frequency, which correlates with a change in mean water temperature (Hummel et al., 1997; Kitto et al., 1983; Powers and Place, 1978; Powers and Schulte, 1998). This observation indicates the adaptive significance of changes in allozyme composition for ectothermic animals under particular environmental conditions. Highly significant heterogeneity between areas was found at the *Ldh-B* locus, which distinguished North-Eastern Arctic cod (NEAC) from other cod stocks (Mork et al., 1985). *Ldh-B* may also differ between Norwegian coastal cod and NEAC (Jorstad, 1984; Mork and Giaever, 1999) together with differences in other genetic markers like haemoglobin, phosphoglucose isomerase (Dahle and Jorstad, 1993; Jorstad, 1984; Mork et al., 1985) and synapophysin (Fevolden and Pogson, 1997).

Although it is more common for isozymes to show distinct differences in functional characteristics than for enzymes coded by alternative alleles (allozymes), the latter may also differ in their kinetic properties (Henry and Ferguson, 1987; Jollivet et al., 1995; Place and Powers, 1984). In some circumstances, these kinetic differences could lead to frequency-dependent selection, which is potentially capable of maintaining balanced polymorphism (Clarke and Allendorf, 1979). In the present paper, we looked at LDH acclimation capacity in cod, including aspects of acclimation from the population level down to the thermodynamic level of the enzyme. We have chosen to work on crude homogenates (e.g. with the overall LDH suite), because it is well known that purified LDH-B allozymes have significant kinetic differences, as shown in the mummichog *Fundulus heteroclitus* (Place and Powers, 1984). Accordingly, the following questions were addressed: what are the implications of changes in LDH-B allozyme content for the final functional properties of the overall LDH suite in cod, and which mechanisms are involved in modifying LDH activity during seasonal temperature variation?

Materials and methods

Animals

Norwegian coastal cod (*Gadus morhua* Linnaeus 1758; from Bergen, Norway) in their first year were raised at ambient temperatures in a semi-natural pond northwest of Bergen (Institute of Marine Research, Norway). The one-year-old cod were brought to the Department of Fisheries and Marine Biology, University of Bergen (Norway), where the experiments were conducted. The fish were pre-acclimated by keeping them at approximately 10°C from December 1998 until the start of experimentation (January 1999).

Acclimation

The cod were tagged individually with pit-tags and then randomly separated into three groups and acclimated to 4°C (two tanks) and 12°C (one tank) in a flow-through system with natural seawater. The water for the experiment was pumped from ~100 m depth of the fjord outside Bergen, where salinity and temperature remain constant throughout the year. The 4°C acclimation groups consisted of 39 and 41 fishes in each tank (80 in total), and the 12°C acclimation group comprised 42 animals. 4°C is a common temperature for cod in boreal regions, and 12°C is close to the optimal temperature for somatic growth of cod (Jobling, 1988; Pörtner et al., 2001).

Temperature was maintained at ±1°C during the experimental period from 19 January 1999 until 7 March 2000 (408 days). The fishes were fed in excess with commercial dry pellets for marine fishes (NorAqua, Trondheim, Norway) by an automatic feeder. A simulated natural light regime was used corresponding to ambient light conditions in Bergen (60°N). The oxygen concentration in the outlet water was recorded daily and remained above 86% saturation. Water salinity was 33‰ throughout the experiment.

Physiological conditions

The growth rate was measured individually once every six weeks, and condition factor (*k*-factor), hepatosomatic index (HI), and gonadosomatic index (GI) were measured at the end of the experiment. The rate of oxygen consumption ($\dot{M}_{O_2}$; μmol min^{-1} g^{-1}) was measured in a flow-through respirometer using the microoptode technique (Van Dijk et al., 1999). $\dot{M}_{O_2}$ data that are presented in the article have been collected in a separate acclimation experiment on Southern North Sea cod in the Alfred Wegener Institute Foundation for Polar and Marine Research (AWI; Bremerhaven, Germany) by T. Fischer (unpublished data; N=8).

Sampling

At the end of the experiment (7 March 2000) the fishes were killed by the addition of MS222 (tricaine methane sulphonate) to the water (1 g l^{-1}). Total fresh mass, length, liver mass and gonad mass were measured, and tissues (blood, white muscle and liver) were sampled. The sampling time was minimised and it generally took less than 3 min from the killing of the fish until sample fixation. The samples were freeze clamped in liquid nitrogen (–196°C), transported on dry ice (–78.5°C) and stored in a deep freeze at –80°C.

Genetic variation

According to previous studies on genetic variation in Atlantic cod, the allozyme of hemoglobin (Hb-I) from blood and five allozyme markers (LDH-B, GPI, GPD, IDH and PGM) from white muscle were chosen as markers for population genetic analysis (Gjosaeter et al., 1992; Jorstad, 1984; Mork and Giaever, 1999; Renaud et al., 1986). Genetic variation in Hb-I was freshly analysed on agar gel in Smithies buffer pH 8.6, as described by Fyhn et al. (1994). The muscle allozyme markers were analysed by starch gel electrophoresis, performed in histidine buffer pH 7.0 and stained as described by Jorstad (1984). The allozyme frequencies were analysed by computer program BIOSYS-1 (University of Illinois).

LDH isoelectric focusing

Both starch and agar gel electrophoresis were used to detect allele frequencies in six selected loci (including *Ldh-B*). However, later the samples were screened again by isoelectric focusing (IEF) but only for LDH. The results of allozyme identification were completely identical between methods.

20% tissue homogenates (w/v) were prepared (Ultra-Turrax T25) in ice-cold 20% sucrose water solution, and then the samples were centrifuged at 15×10^3 ***g*** for 20 min. Supernatant was collected and used for IEF. IEF was carried out in PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden) at 6°C. The samples (1 μl) were applied in the middle of PhastGel IEF 3.5–9.5 (Amersham Pharmacia Biotech). The focusing was carried out using the standard pH 3–9 protocol provided by Amersham Pharmacia Biotech: pre-focusing (75 AVh at 780 V, 1.5 W, 2.2 mA, 6°C); focus with applicator (100 AVh at 200 V, 0.1 W, 0.4 mA, 6°C); focus after removal of the applicator (480 AVh at 2000 V, 3.5 W, 2.5 mA, 6°C). LDH staining solution for one PhastGel contained 10 ml of 131 mmol l^{-1} D/L-lactic acid (racemic liquid mixture) in 0.2 mol l^{-1} Tris pH 8.0; 250 μl NAD^+ (10 mg ml^{-1}); 250 μl MTT (methyl-thiazolyl blue; 5 mg ml^{-1}); 125 μl PMS (phenazine methosulphate; 5 mg ml^{-1}) (Hillis et al., 1996). Staining solution was freshly prepared and all staining procedures were performed in the dark at room temperature. Staining time was between 10 min and 15 min depending on the appearance of the bands but before the background got too dark.

We used an IEF calibration pI kit (pH 3.5–9.3; Amersham Pharmacia Biotech). The calibration markers were always applied to the first and the last lanes of each gel. After focusing they were cut off and stained separately according to the 'fast Coomassie staining protocol for PhastGel IEF' provided by Amersham Pharmacia Biotech (development technique file No 200; PhastSystem). After the gel was dried it was scanned (with a regular scanner), and the isoelectric points (pIs) of every single band on the gel were calculated using computer program ImageMaster TotalLab V1.11 (Amersham Pharmacia Biotech).

Ldh locus classification (*Ldh-A*, *-B* and *-C*) was adopted from Grant and Stahl (1988) and Zietara and Skorkowski (1993).

LDH kinetic studies with saturating cofactor levels

LDH is an equilibrium enzyme, which catalyses the reaction:

$$\text{Lactate} + \text{NAD}^+ \underset{}{\overset{\text{LDH}}{\rightleftharpoons}} \text{Pyruvate} + \text{NADH} + \text{H}^+ \,. \qquad (1)$$

The equilibrium of this reaction is shifted to the formation of lactate and NAD^+, e.g. $K_{eq}=4\times10^{-5}$ mol l^{-1} for the marine fish *Fundulus heteroclitus* (Place and Powers, 1984). Therefore, we expressed LDH activity in terms of the initial rate of pyruvate and NADH consumption.

1% tissue homogenates (w/v) were prepared (Ultra-Turrax T25) in ice-cold buffer (Hepes, 100 mmol l^{-1} KCl, pH 7.00 at 20°C) and then the homogenates were centrifuged at 15×10^3 ***g*** for 20 min at 4°C (Yang and Somero, 1993). The supernatant was used for kinetic analyses. The concentration of the enzyme was adjusted to be sufficiently low that the reaction proceeded at a constant rate over kinetic time and caused a decrease in optical density by less than 10% of initial values. The kinetics were read (every 15 s for 3 min) using Bio-Rad (Hercules, CA,

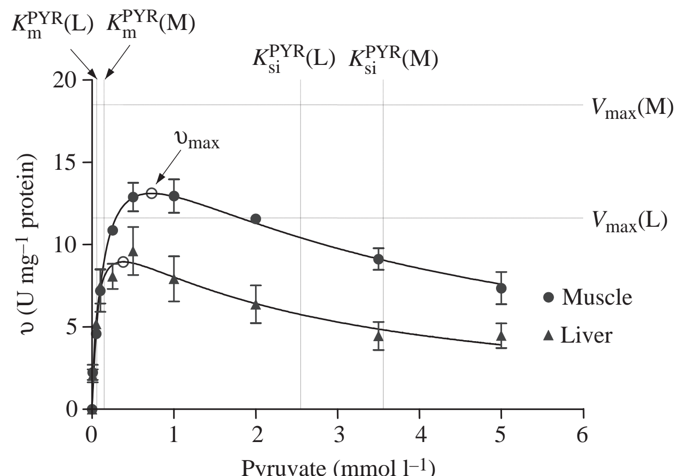

Fig. 1. Typical set of kinetic properties of the lactate dehydrogenase (LDH) isozyme mixture in crude homogenates prepared from muscle (M) and liver (L) of cod *Gadus morhua*. Values are means ± S.D.

USA) 3550-UV microplate reader set at 340 nm. The sample was shaken for 2 s before each reading. In order to keep assay conditions within the limits of linearity at the different temperatures, the dilution factor of the 1% homogenate was increased correspondingly (for muscle, from 50 at 4°C to 500 at 30°C; for liver, from 11 at 4°C to 100 at 30°C). To collect statistically sufficient data on the temperature dependence of the enzyme kinetic parameters, we designed a temperature-controlled 96-well microplate for the Bio-Rad 3550-UV microplate reader (Zakhartsev and Blust, 2002). This system allows for a highly accurate (±0.1°C) on-line control of temperature in the microplate over a broad temperature range (from 0°C to 60°C).

The reaction was started by mixing 100 µl of diluted homogenate with 200 µl of stock solution of the assay medium pre-set to the desired temperature. The reaction was started directly in the 96-well temperature-controlled microplate placed in the microplate reader. A 30 s time delay with constant shaking was used prior to the beginning of the reading to allow the reaction solution to mix and reach the assay temperature; only then was the reaction monitored for 3 min. The final volume of the assay was 300 µl containing 200 µmol l^{-1} β-NADH (this is the saturated level for LDH of marine fish; Place and Powers, 1984), variable pyruvate levels (0–5 mmol l^{-1}) and 100 mmol l^{-1} KCl in 50 mmol l^{-1} Hepes, pH 7.0 (at 20°C). Enzyme activity was always determined in triplicate at 10 pyruvate concentrations (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 3.5 and 5 mmol l^{-1}; see Fig. 1) and at seven assay temperatures (4, 8, 12, 16.5, 21, 25.5 and 30°C; see Fig. 2A). Thus, 30 enzyme assays were performed on each individual fish at one assay temperature, yielding 210 different assays for seven different assay temperatures.

It is well known that LDH activity is inhibited by high substrate concentrations (substrate inhibition kinetics; Fig. 1; Table 1); therefore, at saturating levels of the cofactor (NADH) the reaction rate is described by:

$$υ = \frac{V_{max}[S]}{K_m^{PYR} + [S] + \frac{[S]^2}{K_{si}^{PYR}}}, \quad (2)$$

where V_{max} is the maximal rate of the reverse reaction (U mg^{-1} protein); K_m^{PYR} is the Michaelis–Menten constant for pyruvate (mmol l^{-1}); K_{si}^{PYR} is the constant of substrate inhibition for pyruvate (mmol l^{-1}); [*S*] is the pyruvate concentration (mmol l^{-1}).

Nonlinear regression analysis (GraphPad Prism software) was used to calculate all kinetic parameters and fit the curve. The actual maximal reaction rate ($υ_{max}$) always falls short of

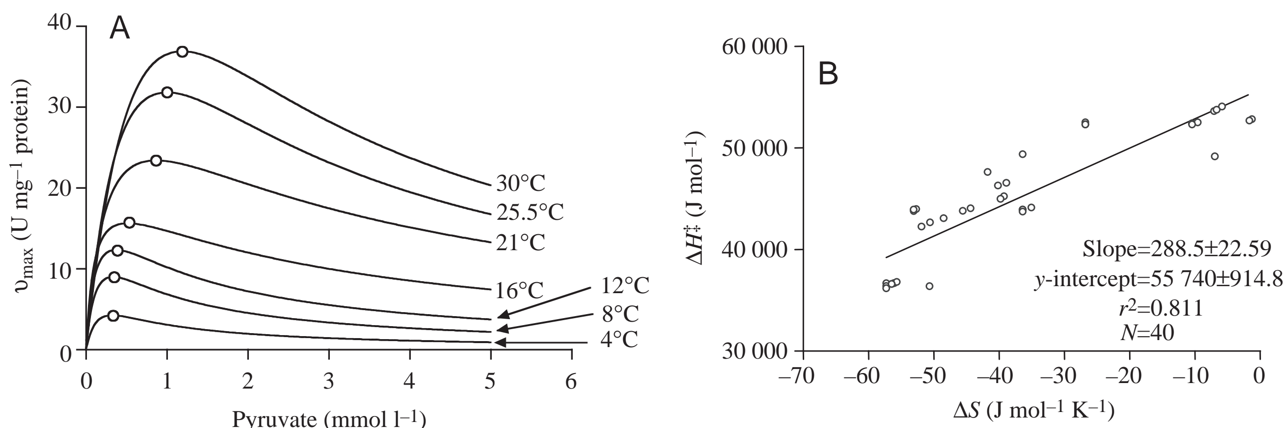

Fig. 2. (A) Temperature dependence of $υ_{max}$ (open circles) was used to calculate the Arrhenius activation energies (E_a) for lactate dehydrogenase (LDH) in crude tissue homogenates from the slope of Arrhenius plots [$10^3/T$ K against ln($υ_{max}$)]. (B) The enthalpy–entropy compensation plot was compiled from different sources for purified LDH (Hochachka and Somero, 1984; Low et al., 1973; Place and Powers, 1984; Prosser, 1986; Somero and Low, 1976; Uvarov and Archakov, 1990) and used to calculate thermodynamic parameters (Δ*H*, Δ*S*, Δ*G*). Results of linear regression are given ± S.E.M.

Table 1. *Kinetic properties of the lactate dehydrogenase (LDH) mixture in crude homogenates prepared from muscle and liver of* Gadus morhua

	Muscle	Liver
V_{max} (U mg^{-1} protein)	18.49±1.08	11.63±0.90
K_m^{PYR} (mmol l^{-1})	0.149±0.022	0.057±0.013
K_{si}^{PYR} (mmol l^{-1})	3.56±0.52	2.55±0.54
V_{max}/K_m^{PYR}	124	203
$[S]_{max}$ (mmol l^{-1})	0.729	0.382
υ_{max} (U mg^{-1} protein)	13.12	8.95
n	30	30
Temperature of assay (°C)	12	12

Detailed specifications: cod N56; *Ldh-B* phenotype *a/a*; acclimation temperature 4°C; reading time of initial reaction kinetic, 3 min); V_{max}, K_m^{PYR} and K_{si}^{PYR}, kinetic parameters were calculated according to equation 2; $[S]_{max}$ and υ_{max} were calculated according to equations 3, 4.

Values are means ± S.E.M.; *n*, total number of enzymatic reactions, which were monitored at different pyruvate concentrations to obtain kinetic parameters.

V_{max} (from equation 2; open circle in Fig. 1). Therefore, the substrate concentration at which the reaction rate is maximal can be calculated by differentiating υ with respect to $[S]$ and finding a value of $[S]$ at which the slope of the curve is zero ($[S]_{max}$) (Cornish-Bowden, 1999):

$$[S]_{max} = \sqrt{K_m^{PYR} K_{si}^{PYR}}\,. \quad (3)$$

Substitution of this result into the original equation 2 shows that at $[S]_{max}$ the actual reaction rate reaches its maximum (υ_{max}):

$$\upsilon_{max} = \frac{V_{max}}{1 + 2\sqrt{\dfrac{K_m^{PYR}}{K_{si}^{PYR}}}}\,. \quad (4)$$

The specific LDH activity was expressed as μmol of NADH oxidized per minute per mg of total protein (U mg^{-1} protein) in the reaction mixture using 6.22×10^3 O.D. mol^{-1} cm^{-1} as the extinction coefficient of NADH (Dawson et al., 1986). NanoOrange® Protein Quantitation Kit (Molecular Probes, Eugene, OR, USA) was used to determine the total protein concentration in diluted homogenates.

LDH thermodynamic studies

The calculation of the apparent Arrhenius activation energy (E_a) for enzymatic reactions is commonly based on rate measurements made at saturating substrate concentrations (i.e. V_{max}), when substrate availability will not be limiting for the reaction rate (Segel, 1976). However, in cases of high-substrate-inhibition kinetics, the calculated V_{max} never coincides with υ_{max} (Fig. 1; Table 1). Thus, we used temperature dependence of υ_{max} (from equation 4; Fig. 2A open circles) to calculate E_a, since it is closer to the real rate values at saturated conditions. The E_a (J mol^{-1}) was calculated from the slope of the Arrhenius plots [E_a=–slope×R, where R is the universal gas constant (J mol^{-1} K^{-1})].

The change in activation enthalpy (ΔH; J mol^{-1}) was calculated as follows:

$$\Delta H = E_a - RT\,, \quad (5)$$

where R is the universal gas constant (8.31434 J mol^{-1} K^{-1}), and T is temperature (K).

The change in activation entropy (ΔS; J mol^{-1} K^{-1}) was derived from 'enthalpy–entropy compensation plots' (Hochachka and Somero, 1984; Prosser, 1986). The compensation plot (Fig. 2B) was compiled from published data for purified LDH according to the concept outlined in Hochachka et al. (1976), Prosser (1986) and Cornish-Bowden (2002). Gibbs free energy change (ΔG) was computed according to the following (Prosser, 1986):

$$\Delta G = \Delta H - T\Delta S\,. \quad (6)$$

ΔH and ΔG were calculated for corresponding acclimation temperatures (T_A).

Results

Genetic variability

No significant change in allele frequencies was observed by gel electrophoresis for the six loci analysed in cod tissues (muscle and blood) after the acclimation (contingency χ^2 analysis, χ^2=9.644, d.f.=7, P=0.209; Table 2), except for locus *Gpi* in the 12°C acclimation group, which slightly deviated from the expected Hardy–Weinberg equilibrium (P_{ext}=0.042; Table 2). Contingency χ^2 analysis for the *Ldh-B* locus shows no effect of acclimation temperature on allele frequency (χ^2=0.687, d.f.=1, P=0.407).

Growth performance

Two-way analysis of variance (ANOVA) revealed a significant effect of acclimation temperature on growth performance in terms of both length and mass (P<0.001) but did not reveal an effect of *Ldh-B* phenotypes on growth performance (Table 3). Interestingly, during the summer period, growth rate was markedly different between acclimation groups. However, after November 1999, daily growth rate became similar at the two acclimation temperatures (Fig. 3). This is a common phenomenon for maturing cod. A significant decrease in growth rate in November might be caused by physiological changes in preparation for spawning in the spring of the following year. These patterns indicate that not only acclimation temperature but also probably daylight duration (season) and maturation stage have an effect on growth rate (multiple-factor effect); however, *Ldh-B* phenotypes give no advantage for the growth performance.

Further analysis did not reveal any effect of *Ldh-B* phenotypes nor acclimation temperature on *k*-factor and hepatosomatic index (HI), indicating that the mass–length

Table 2. *Allele frequencies, observed* (H_{obs}) *and expected* (H_{exp}) *heterozygosity levels and exact probabilities* (P_{ext}) *for the Hardy–Weinberg equilibrium determined in white muscle and blood of two groups of* Gadus morhua *after acclimation to 4°C and 12°C, respectively*

Locus	Allele	4°C	12°C
Gpi-1	(n)	74	26
	a	0.446	0.365
	b	0.399	0.558
	c	0.155	0.077
	H_{obs}	0.676	0.769
	H_{exp}	0.622	0.560
	P_{ext}	0.246	0.042
Ldh-B	(n)	73	26
	a	0.372	0.308
	b	0.628	0.692
	H_{obs}	0.500	0.462
	H_{exp}	0.470	0.434
	P_{ext}	0.625	1.000
Pgm	(n)	74	26
	a	0.041	0.019
	b	0.959	0.981
	H_{obs}	0.054	0.038
	H_{exp}	0.078	0.038
	P_{ext}	0.100	1.000
Idh	(n)	74	26
	a	1.000	1.000
	H_{obs}	0.000	0.000
	H_{exp}	0.000	0.000
	P_{ext}	–	–
Gpd	(n)	74	26
	a	0.014	0.038
	b	0.824	0.731
	c	0.162	0.231
	H_{obs}	0.284	0.538
	H_{exp}	0.296	0.419
	P_{ext}	0.684	0.133
Hb-I	(n)	74	26
	a	0.486	0.577
	b	0.514	0.423
	H_{obs}	0.568	0.538
	H_{exp}	0.503	0.498
	P_{ext}	0.351	0.707

Loci: *Gpi*, glucose phosphate isomerase; *Ldh-B*, lactate dehydrogenase B; *Pgm*, phosphoglucomutase; *Idh*, isocitrate dehydrogenase; *Gpd*, glucose-6-phosphate dehydrogenase; *Hb-I*, haemoglobin; *n*, number of fishes in the group.

Contingency χ^2 analysis for all loci showed χ^2=9.644, d.f.=7, P=0.209.

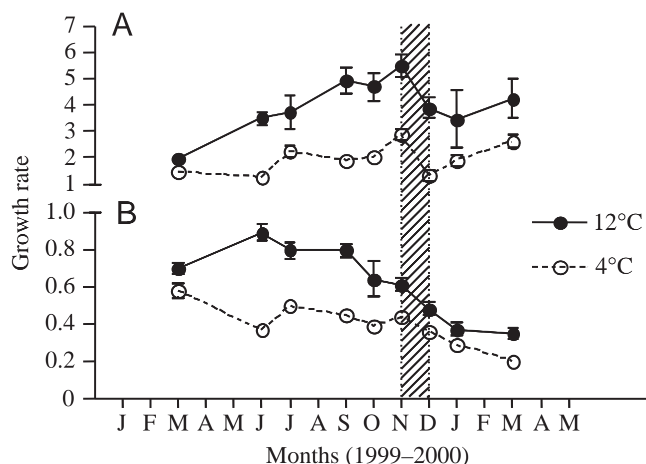

Fig. 3. The change in growth kinetics of Norwegian coastal cod (*Gadus morhua*) throughout the year in terms of daily increments of (A) body mass (g day^{-1}) and (B) length (mm day^{-1}) for two acclimation groups (4°C and 12°C). The values are means ± S.E.M. After November 1999 (shaded area), growth rate became more or less similar at both acclimation temperatures.

relationship remained unaffected by the acclimation temperature. However, the gonadosomatic index (GI) was considerably higher (4.25) after acclimation to 4°C compared with 12°C acclimation (2.68) (Table 3). Rates of oxygen consumption ($\dot{M}_{O_2}$) of cold-acclimated (4°C) cod from the North Sea were significantly higher (t-test, P<0.05) than those of warm-acclimated cod (12°C), even when the comparison was carried out at their acclimation temperatures (Table 3). However, the Arrhenius activation energy for $\dot{M}_{O_2}$ was lower in 4°C-acclimated cod (33.9 kJ mol^{-1}) than in 12°C-acclimated cod (38.4 kJ mol^{-1}) (T. Fischer et al., unpublished data on Southern North Sea cod).

Isoelectric focusing

Only the *Ldh-B* locus displayed a protein polymorphism due to two alleles, *Ldh-B(a)* and *Ldh-B(b)*, that give three corresponding phenotypes (Fig. 4). The tetrameric LDH gives the classical five-band pattern (with pI values between 5.16 and 5.45; Table 4; Fig. 4) for the *Ldh-B* heterozygote [*Ldh-B(a/b)*]. As *Ldh-B* was the only polymorphic locus among *Ldh* loci in cod, and since it was equally expressed in both tissues, we used this distinctive locus to identify LDH electrophoretic patterns (overall LDH suites). We worked on crude homogenates where several *Ldh* loci and alleles were simultaneously present. Therefore, the particular *Ldh-B* phenotype actually includes the overall LDH pattern (Fig. 4).

Detailed analysis of isoelectric focusing patterns revealed distinctive differences between the two tissues (Fig. 4). *Ldh-B* and *Ldh-A* loci were expressed in both tissues, while the *Ldh-C* locus was only expressed in liver (Fig. 4). The *Ldh-A* locus in muscle showed a complicated pattern of bands with pIs between 6.0 and 6.3. At the same time, LDH-A in liver displayed only one band with a pI equivalent to the pI of the central LDH-A band in muscle (pI 6.15; Table 4). These observations indicate that the sub-bands in muscle LDH-A represent an artefact rather than allozyme variability. In order to see more clearly the minor LDH-B bands we were forced to increase the protein concentration in applied muscle samples, which resulted in an overloading of the major LDH-A band.

Table 3. *Effect of acclimation temperature* (T_A) *and* Ldh-B *phenotype on mean growth performance and physiological condition indices of* Gadus morhua

T_A	*Ldh-B* phenotype	Mean growth rate (mm day^{-1})	Mean growth rate (g day^{-1})	*k*-factor	HI	GI	$\dot{M}_{O_2}$ (µmol min^{-1} g^{-1})	*N*
4°C	*a/a*	0.44±0.03	2.02±0.21	1.15±0.03	9.41±0.58	3.10±0.83		7
	a/b	0.39±0.02	1.78±0.15	1.19±0.02	8.10±0.43	5.30±0.44		36
	b/b	0.42±0.01	1.97±0.14	1.19±0.02	8.09±0.36	3.25±0.44		29
	Pooled	**0.41±0.01**	**1.88±0.09**	**1.19±0.01**	**8.22±0.27**	**4.25±0.32**	**0.042±0.01**	**72**
12°C	*a/a*	0.73±0.06	4.26±0.72	1.18±0.03	9.13±1.04	2.45±0.43		3
	a/b	0.65±0.04	3.59±0.45	1.10±0.04	6.77±0.84	2.54±0.38		11
	b/b	0.63±0.05	4.29±0.48	1.31±0.04	7.83±0.72	2.90±0.29		11
	Pooled	**0.65±0.03**	**3.98±0.30**	**1.20±0.03**	**7.52±0.51**	**2.68±0.21**	**0.038±0.01**	**25**

Values are means ± S.E.M.

M is fresh mass of fish (g); *l* is length of fish (mm); M_{liver} is fresh mass of liver (g); M_{gonad} is fresh mass of gonads (g); *t* is time (days).

Growth rate was calculated as $\upsilon_L=\Delta l/\Delta t$ (mm day^{-1}) and $\upsilon_M=\Delta M/\Delta t$ (g day^{-1}); HI, hepatosomatic index [HI=(100×M_{liver})/*M*]; GI, gonadosomatic index [GI=(100×M_{gonad})/*M*]; *k*-factor, condition factor [$k=(100\times M)/l^3$]; $\dot{M}_{O_2}$, rate of oxygen consumption for North Sea cod (measured for *n*=8, unpublished data by T. Fischer, AWI, Germany); *N*, number of fishes that survived.

This may have caused the development of sub-band artefacts. It is a common observation that samples from different tissues with similar enzyme expression patterns, when overloaded, exhibit differences in the formation of sub-bands during electrophoresis (Richardson et al., 1986).

The bands with pI values between 5.65 and 5.86 that were located between LDH-A and LDH-B are probably interlocus hybrid bands. Therefore, they appeared in different positions in homozygotes [for *Ldh-B(a/a)* pI=5.85; for *Ldh-B(b/b)* pI=5.65] and heterozygotes [*Ldh-B(a/b)* pI=5.65–5.85].

Locus *Ldh-C* (pI 8.3) and intermediate bands (pI 7.4 and pI 7.7) appear to be unique for the liver of cod, as shown previously (Mork et al., 1985). A monomorphic band at pI 7.15 (band No 4 in Table 4) is present in both tissues; however, this isozyme has never been described before, although it appears as a major LDH isozyme in liver. Quantitative analysis of the composition of the LDH suite showed that products of the polymorphic locus (*Ldh-B*) represent only ~14% of the total LDH suite in muscle and ~19% in liver.

Image analysis and statistical analysis of the enzyme patterns (ANOVA) obtained by IEF (Table 4) revealed distinct differences between tissues in LDH isoelectric patterns but did not reveal any effect of acclimation temperature on the appearance of new LDH isoforms and on the pI of the corresponding LDH bands (*P*>0.05). In other words, we found no evidence that acclimation to different temperatures has any effect on the composition of the LDH suite and the quaternary structure of the LDH enzymes.

LDH kinetic studies

All kinetic studies of the overall LDH suite in crude homogenates were performed at saturating levels of the cofactor (NADH). The kinetic studies of LDH in crude homogenates demonstrated the well-known substrate inhibition of LDH by pyruvate (Fig. 1; Table 1). We could not identify differences between *Ldh-B* phenotypes (overall LDH suites) in any of the kinetic parameters (V_{max}, K_m^{PYR} and K_{si}^{PYR}), which were measured at both acclimation temperatures (ANOVA, *P*>0.05; Table 5). However, there was a distinct difference between tissues (Fig. 1; Table 1). Liver LDH in crude homogenate displayed a lower K_m^{PYR} and K_{si}^{PYR} than did muscle LDH and, consequently, higher relative catalytic efficiencies of the reaction at physiological substrate concentrations (V_{max}/K_m^{PYR}; Fig. 1; Table 1).

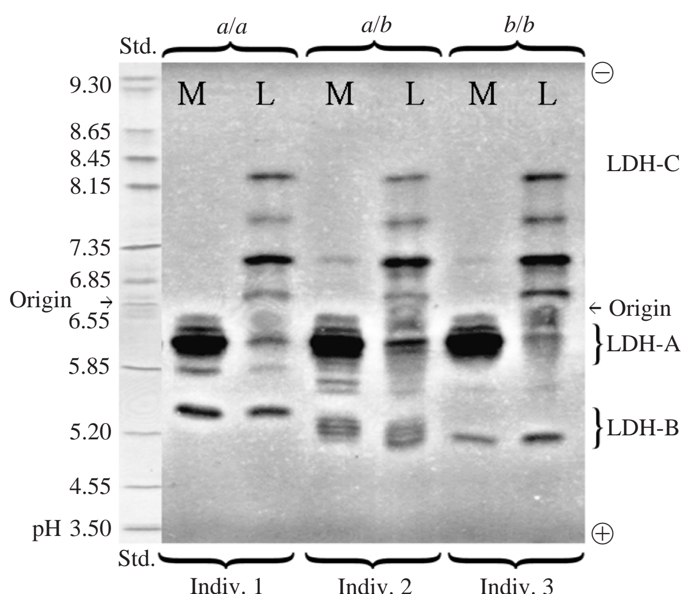

Fig. 4. Isoelectric focusing (IEF) and staining for lactate dehydrogenase (LDH) activity in crude homogenates of three individual cod (*Gadus morhua*). M, white muscle; L, liver; std, lane with pI calibration markers (IEF 3.5–9.3; Amersham Pharmacia Biotech). *Ldh* locus classification (*A*, *B* and *C*) is according to Zietara and Skorkowski (1993). *a/a*, *a/b* and *b/b* are *Ldh-B* phenotypes according to polymorphism in that locus. For more information, see Table 4.

Table 4. *Results of isoelectric focusing (pI values) of LDH in crude tissue homogenates from* Gadus morhua *acclimated to different temperatures*

Ldh locus	*Ldh-B* phenotype	Muscle 4°C *a/a*	Muscle 4°C *a/b*	Muscle 4°C *b/b*	Muscle 12°C *a/a*	Muscle 12°C *a/b*	Muscle 12°C *b/b*	Liver 4°C *a/a*	Liver 4°C *a/b*	Liver 4°C *b/b*	Liver 12°C *a/a*	Liver 12°C *a/b*	Liver 12°C *b/b*
C	1							8.19±0.07	8.36±0.13	8.28±0.13	8.36±0.13	8.27±0.11	8.33±0.10
	2							7.64±0.07	7.77±0.11	7.71±0.11	7.79±0.11	7.69±0.08	7.77±0.06
	3							7.43±0.09	7.43	7.45±0.08		7.43±0.01	7.42±0.02
	4	7.16±0.09	7.16±0.06	7.12±0.02		7.09±0.05	7.12±0.06	7.10±0.06	7.19±0.09	7.17±0.08	7.20±0.02	7.13±0.004	7.18±0.05
	5							6.66±0.07	6.66±0.06	6.66±0.06	6.69±0.02	6.64±0.003	6.68±0.07
A	6	6.26±0.04	6.27±0.08	6.26±0.05	6.23±0.02	6.28±0.07	6.26±0.07						
	7	6.14±0.04	6.15±0.08	6.15±0.06	6.14±0.04	6.15±0.06	6.15±0.05	6.16±0.06	6.13±0.04	6.17±0.04	6.11±0.07	6.12±0.004	6.15±0.07
	8	6.03±0.03	6.05±0.07	6.03±0.06	6.05±0.03	6.04±0.05	6.04±0.04						
	9	5.86±0.03	5.87±0.06		5.86±0.03	5.87±0.05		5.89±0.05	5.86±0.02		5.81±0.06	5.83±0.02	
	10		5.77±0.07			5.75±0.04			5.77±0.04			5.74±0.03	
	11		5.65±0.06	5.64±0.04		5.65±0.04	5.64±0.04		5.70±0.05	5.68±0.04		5.64±0.03	5.65±0.05
B	12	5.43±0.02	5.45±0.06		5.44±0.03	5.44±0.04		5.47±0.04	5.43±0.03		5.42±0.05	5.43±0.03	
	13		5.38±0.06			5.37±0.03			5.35±0.03			5.36±0.04	
	14		5.31±0.05			5.30±0.03			5.28±0.03			5.30±0.04	
	15		5.23±0.06			5.23±0.04			5.21±0.03			5.23±0.03	
	16		5.16±0.06	5.16±0.04		5.17±0.03	5.16±0.04		5.14±0.03	5.16±0.05		5.17±0.04	5.15±0.06
N		7	11	14	3	6	11	5	7	9	3	6	6

Ldh locus classification (*A*, *B* and *C*) is according to Zietara and Skorkowski (1993). *a/a*, *a/b* and *b/b* are *Ldh-B* phenotypes according to polymorphism in that locus (see Fig. 4); *N* is number of analysed fishes. For more information, see Fig. 4. Values are means ± S.D.

The presence of *Ldh-B* phenotypes did not cause significant differences between levels and temperature dependencies of V_{max}, K_m^{PYR} and K_{si}^{PYR} of LDH in crude homogenates (*F*-test, $P>0.05$). Thus, in terms of reaction kinetics, we could not find significant differences between overall LDH suites in crude homogenates that might be associated with different *Ldh-B* phenotypes. Therefore, the kinetic data from different phenotypes were pooled for further analysis and presentation.

The analyses of the temperature dependence of kinetic parameters were performed for values between 4°C and 30°C. An assay temperature of 30°C already results in a significant deviation of activities from the general trend. This conclusion originated from the observation that the values of r^2 for exponential curves became considerably higher once the rate values at 30°C were omitted. At the same time, the absolute sum of square (ASS) and the standard deviation of the residuals ($Sy.x$) became considerably lower. Such omission increased r^2 to up to 0.99. Additionally, the high values of r^2 indicate that the chosen exponential function adequately fits the data. Consequently, the rate values at 30°C were omitted from further calculations of temperature dependence of kinetic parameters and thermodynamic parameters (Figs 5–7).

υ_{max} and K_m^{PYR}

Comparison of the temperature dependencies of υ_{max} per mg of total tissue protein (U mg^{-1} protein; Fig. 5A) showed that muscle displayed significantly higher specific LDH activities in comparison with liver in the 12°C acclimation group (*F*-test, $P<0.01$). Unpaired *t*-tests revealed that a quantitative component of the exponential equation became significantly higher in the 12°C acclimation group. As υ_{max} is a relative measure of enzyme concentration {υ_{max} is in direct proportion to V_{max} (equation 4), but $V_{max}=k_{cat}[E]_t$}, then we can conclude that the specific concentration of LDH in muscle was reduced in response to cold (4°C), probably as the result of an increased aerobic capacity (see Introduction). However, recalculation of υ_{max} in terms of activities per g wet mass (U g^{-1} wet mass; Fig. 5B) showed almost complete compensation of the final enzyme activity.

Analysis of K_m^{PYR} at acclimation temperature (ANOVA, $P>0.05$) and its temperature dependence (*F*-test, $P>0.05$) did not reveal a significant difference among *Ldh-B* phenotypes, which is why the data were pooled. Further

Table 5. *Kinetic parameters of LDH in crude homogenates measured at acclimation temperature* (T_A)

Tissue	T_A (°C)	*Ldh-B* phenotype	V_{max} (U mg^{-1} protein)	K_m^{PYR} (mmol l^{-1})	K_{si}^{PYR} (mmol l^{-1})	V_{max}/K_m^{PYR} (U mg^{-1} protein)	[Protein] (mg g^{-1} wet mass)	V_{max} (U g^{-1} wet mass)	V_{max} (U g^{-1} wet mass)/K_m	*N*
Muscle	4	*a/a*	8.05±1.14	0.130±0.019	1.22±0.32	79±13	44.53±4.72	308±94	3374±361	5
		a/b	8.45±0.89	0.123±0.025	1.52±0.40	86±27	48.34±4.94	408±67	3783±967	7
		b/b	5.11±0.69	0.089±0.008	1.97±0.30	91±26	47.32±5.62	242±16	3864±751	9
		Pooled	**6.87±0.59**	**0.111±0.010**	**1.64±0.20**	**86±14**	**46.96±2.92**	**343±59**	**3720±445**	**21**
	12	*a/a*	20.75±3.11	0.182±0.051	2.22±0.78	132±43	32.70±4.70	678±182	4030±839	3
		a/b	25.78±1.47	0.185±0.040	2.91±0.30	176±32	34.79±3.37	896±114	5754±626	6
		b/b	19.08±0.88	0.193±0.048	3.89±0.52	137±14	40.10±1.98	765±169	5238±586	6
		Pooled	**22.64±1.39**	**0.188±0.025**	**3.20±0.32**	**151±16**	**36.62±1.84**	**829±155**	**5202±391**	**15**
Liver	4	*a/a*	5.37±0.85	0.031±0.005	1.41±0.72	177±37	28.28±2.34	151±18	4072±919	5
		a/b	5.68±0.84	0.038±0.008	1.15±0.31	142±18	30.59±3.70	173±18	5172±1562	7
		b/b	4.45±0.47	0.031±0.005	1.22±0.15	158±14	29.82±1.64	132±18	4399±904	9
		Pooled	**5.15±0.40**	**0.033±0.003**	**1.23±0.18**	**158±12**	**29.70±1.49**	**153±18**	**4597±678**	**21**
	12	*a/a*	14.56±2.92	0.058±0.011	1.57±0.80	251±18	19.36±2.46	282±58	4771±237	3
		a/b	9.44±1.03	0.050±0.011	2.50±0.60	226±35	25.69±1.99	242±10	5798±1089	6
		b/b	11.46±2.54	0.061±0.019	2.37±0.50	209±37	23.27±3.13	266±30	5233±681	6
		Pooled	**11.27±1.25**	**0.056±0.009**	**2.27±0.34**	**224±20**	**23.47±1.55**	**264±32**	**5366±496**	**15**

V_{max}/K_m^{PYR}, relative catalytic efficiency of the reaction at physiological substrate concentrations; [Protein], concentration of total protein in tissue; *N*, number of analysed fishes.
Values are means ± S.E.M.

analysis did not reveal significant differences between K_m^{PYR} values for LDH in crude homogenate at the two acclimation temperatures (*F*-test for entire curves, $P>0.05$; Fig. 6). The change in K_m^{PYR} with temperature showed an exponential pattern resulting in a lower thermal sensitivity at lower temperature. Two-phase linear regression analysis showed that at temperatures below 16±1°C, K_m^{PYR} displays low temperature dependence, with a slope of 0.003–0.008 mmol l^{-1} deg. (Fig. 6).

K_{si}^{PYR}

Analysis of K_{si}^{PYR} at corresponding acclimation temperature (ANOVA, $P>0.05$) and its temperature dependence (*F*-test, $P>0.05$) did not reveal a significant difference between *Ldh-B* phenotypes. At corresponding acclimation temperatures, K_{si}^{PYR} was higher in white muscle than in liver (Table 5). K_{si}^{PYR} of both tissues underwent a significant change over the course of acclimation to different temperature (*F*-test, $P<0.05$; Fig. 7). Acclimation to low temperature resulted in a lowering of K_{si}^{PYR} values compared with acclimation to high temperature, as well as a decrease in the temperature-dependent increment of K_{si}^{PYR} in both tissues.

V_{max}/K_m^{PYR}

Analysis of V_{max}/K_m^{PYR} (when V_{max} was expressed in U mg^{-1} total protein) at corresponding acclimation temperatures did not reveal a significant difference among *Ldh-B* phenotypes (ANOVA, $P>0.05$; Table 5). Nevertheless, the efficiency of the LDH suite is higher in liver than in muscle, when expressed in terms of V_{max} per mg protein (Table 5). However, when V_{max}/K_m^{PYR} was expressed in terms of total V_{max} per g of wet tissue (U g^{-1} wet mass), the final LDH efficiency became similar in both tissues at corresponding acclimation temperatures (Table 5; Fig. 8). This means that, despite a considerable difference between the total mass of muscle and liver, the total LDH efficiency in each tissue is similar. Moreover, acclimation to different temperatures results in partial compensation of total V_{max}/K_m^{PYR} in muscle and almost complete compensation in liver (unpaired *t*-test; Fig. 8).

Total protein concentration

The concentration of total tissue protein was not different among *Ldh-B* phenotypes (ANOVA, $P>0.05$). However, it was increased during acclimation to low temperature (Table 5) from 36.6 mg g^{-1} wet mass to 47.0 mg g^{-1} wet mass in muscle and from 23.4 mg g^{-1} wet mass to 29.7 mg g^{-1} wet mass in liver. In terms of υ_{max} (U mg^{-1} protein), the LDH-specific concentration was reduced under acclimation to low temperature, but in terms of final υ_{max} per tissue (U g^{-1} wet mass) the total LDH-specific concentration was restored by quantitative compensation after acclimation to low temperature.

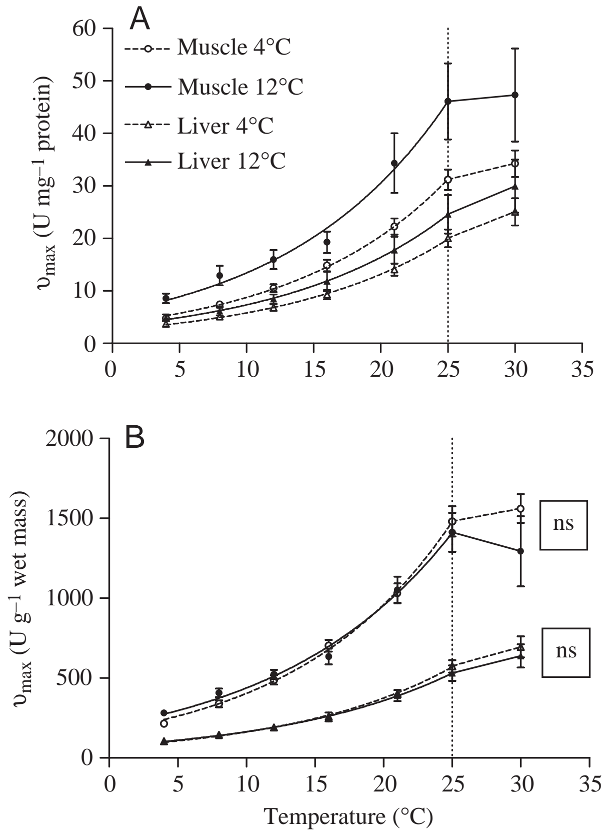

Fig. 5. Temperature dependence of υ_{max} [expressed as units per mg of total protein (A) and units per g wet mass (B)] for pyruvate reduction by lactate dehydrogenase (LDH) in crude homogenates prepared from white muscle (circles) and liver (triangles) of cod (*Gadus morhua*) acclimated to different temperatures. Values are means ± S.E.M. (N=21 for 4°C and N=15 for 12°C). ns, not significant. The dotted line indicates the upper border of data accepted for regression analysis.

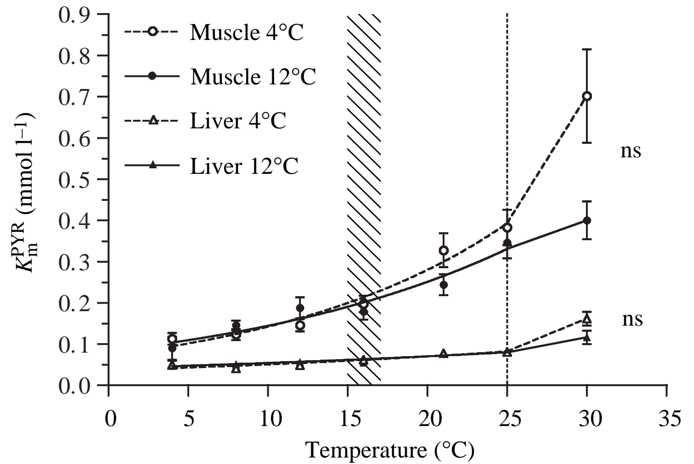

Fig. 6. Temperature dependence of K_m^{PYR} of lactate dehydrogenase (LDH) in crude homogenates prepared from muscle (circles) and liver (triangles) of cod (*Gadus morhua*) acclimated to different temperatures. Two-phase linear regression analysis of the temperature dependence of K_m^{PYR} reveals that at temperatures below 16±1°C (shaded area) the K_m^{PYR} is almost independent of temperature (the slope of the curve is ~0.003 mmol l^{-1} deg.). Values are means ± S.E.M. (N=21 for 4°C and N=15 for 12°C). ns, not significant. The dotted line indicates the upper border of data accepted for regression analysis.

LDH thermodynamic studies

The temperature dependence of υ_{max} between 4°C and 25°C was used for the calculation of E_a, since values of υ_{max} at 30°C already indicate partial inactivation of the LDH activity. There were no significant differences in E_a values for LDH suites between *Ldh-B* phenotypes (two-way ANOVA, P>0.05; Table 6). E_a values were very similar in both tissues (~47 kJ mol^{-1}) at 12°C acclimation, despite very different isozyme patterns and significant differences in kinetic properties between tissues (Figs 1, 4; Table 1). This means that pyruvate reduction in both tissues has the same thermodynamic requirements despite very distinct differences between tissues both in isozyme patterns and kinetic properties. However, at 4°C acclimation, the E_a significantly increased (to ~53 kJ mol^{-1} in liver and to ~57 kJ mol^{-1} in muscle; two-way ANOVA, P<0.05; Table 6).

Analysis of activation enthalpies (Table 6) did not reveal any effect of *Ldh-B* phenotypes. We have observed a difference (Δ in Table 6) in ΔH between acclimation groups by 5.63–8.99 kJ mol^{-1} (depending on tissue). This difference is much higher than would be expected from equation 5, indicating that the observed change in ΔH (Δ) has another cause than just temperature dependence of ΔH under the condition of constant E_a.

Statistical analysis of Gibbs free energy change values (ΔG) did not reveal any effect of acclimation temperature, tissue or *Ldh-B* phenotype on the level of ΔG, which remained more or less constant at ~55.5 kJ mol^{-1} (Table 6).

Discussion

Genetic variability: functional consequences

In the present study, we have attempted to assess the effect of acclimation temperature on growth rate in cod and combine it with the possible effect of enzyme polymorphism. It was assumed that both experimental groups had the same genetic background prior to acclimation, as both groups were randomly selected from the same year class of cod. Analysis of allele frequencies after acclimation showed that there was no difference between the genetic background of the acclimation groups after the experiment (Table 2). However, the locus *Gpi* was not in Hardy–Weinberg equilibrium after acclimation to 12°C because P_{ext} was slightly below 0.05. This is probably due to the small number of cod in this group (N=26; Hedrick, 1985), as there was no indication of selective survival in this group (T. Johansen et al., unpublished observations). Therefore, it can be concluded that none of the loci (including *Ldh-B*) were subjected to selection during thermal acclimation (Table 2).

The classification of *Ldh* loci (*A*, *B* and *C*) was taken from

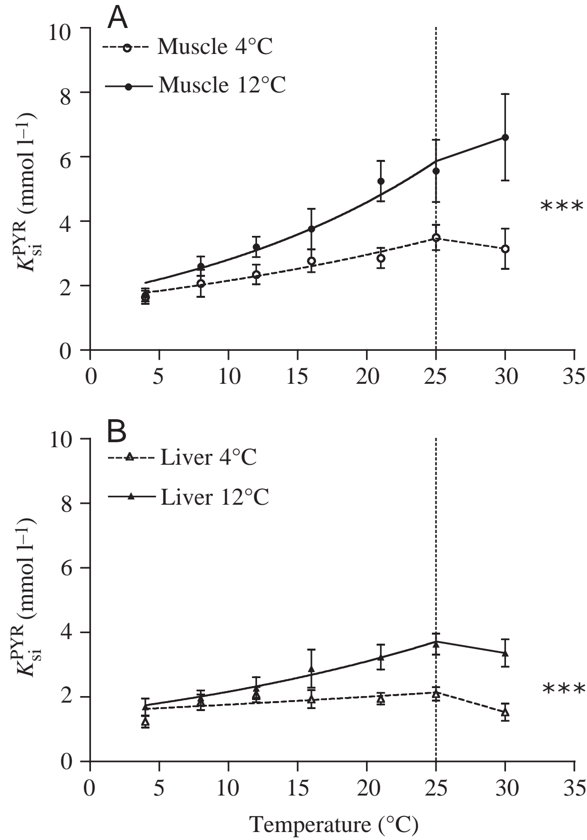

Fig. 7. Temperature dependence of K_{si}^{PYR} of lactate dehydrogenase (LDH) in crude homogenates prepared from muscle (A) and liver (B) of cod (*Gadus morhua*) acclimated to different temperatures. Values are means ± S.E.M. (N=21 for 4°C and N=15 for 12°C). Asterisks indicate that differences are significant (F-test, P<0.001). The dotted line indicates the upper border of data accepted for regression analysis.

Grant and Stahl (1988) and Zietara and Skorkowski (1993), while the *Ldh-B* phenotype classification followed the general practice in population genetics to name multiple alleles of a specific locus by lower-case letters with parentheses (Hillis et al., 1996). The *Ldh-B* locus showed the presence of two alleles, *Ldh-B(a)* and *Ldh-B(b)*, yielding three phenotypes: *Ldh-B(a/a)*, *Ldh-B(a/b)* and *Ldh-B(b/b)*. We did not use a classification such as LDH-B^a_4, LDH-B^a/B^b and LDH-B^b_4, because we refer to the overall LDH pattern under *Ldh-B* phenotypes (i.e. the mixture of all isozymes and allozymes in crude homogenates – the overall LDH suite), while the latter classification only refers to the subunit composition of purified allozymes (Place and Powers, 1984).

Initially, starch or agar electrophoresis was the main analytical tool for analysis of population genetics. This method resulted in a number-based classification that relied on the sequential appearance of isozymes on a gel (1, 2, 3, etc.) and, thus, the relative mobility of a protein on the gel (Shaklee et

Table 6. *Thermodynamic parameters for LDH in crude homogenate of* Gadus morhua*, which were calculated for the temperature dependence of* υ_{max}

Tissue	T_A (°C)	*Ldh-B* phenotype	E_a (kJ mol–1)	r^2	$\Delta H(T_A)$ (kJ mol–1)	ΔS (kJ mol–1 K–1)	$\Delta G(T_A)$ (kJ mol–1)	N	n
Muscle	4	*a*/*a*	53.76±0.74	0.972±0.008	51.45±0.74	–14.84±2.57	55.56±0.03	5	1016
		a/*b*	56.76±1.96	0.981±0.005	54.46±1.96	–4.42±6.79	55.68±0.08	7	1430
		b/*b*	58.64±1.33	0.991±0.002	56.34±1.33	2.11±4.60	55.76±0.05	9	1758
		Pooled	**56.55±0.97**	**0.983±0.003**	**54.25±0.97**	**–5.14±3.38**	**55.67±0.04**	**21**	**4204**
	12	*a*/*a*	46.55±1.26	0.892±0.049	44.18±1.26	–40.07±4.38	55.60±0.01	3	566
		a/*b*	48.94±1.63	0.947±0.013	46.57±1.63	–31.79±5.66	55.63±0.02	6	1199
		b/*b*	46.87±1.88	0.966±0.008	44.50±1.88	–38.96±6.51	55.60±0.02	6	1221
		Pooled	**47.63±1.01**	**0.944±0.012**	**45.26±1.01**	**–36.31±3.49**	**55.61±0.01**	**15**	**2986**
		Δ			8.99				
Liver	4	*a*/*a*	57.96±4.85	0.956±0.025	55.65±4.85	–16.87±3.59	55.73±0.19	5	1032
		a/*b*	47.75±1.44	0.981±0.003	45.44±1.44	–35.68±4.99	55.33±0.06	7	1435
		b/*b*	55.13±1.31	0.972±0.006	52.83±1.31	–10.06±4.55	55.62±0.05	9	1799
		Pooled	**53.34±1.57**	**0.971±0.006**	**51.04±1.57**	**–20.39±3.75**	**55.55±0.06**	**21**	**4266**
	12	*a*/*a*	49.01±4.33	0.947±0.022	46.64±4.33	–31.53±15.01	55.63±0.05	3	588
		a/*b*	47.18±1.83	0.958±0.008	44.81±1.83	–37.88±6.36	55.61±0.02	6	1181
		b/*b*	47.76±2.58	0.960±0.012	45.39±2.58	–35.88±8.97	55.61±0.03	6	1144
		Pooled	**47.78±1.42**	**0.956±0.007**	**45.41±1.42**	**–35.81±4.91**	**55.61±0.02**	**15**	**2913**
		Δ			5.63				

T_A, acclimation temperature; E_a, apparent Arrhenius activation energy calculated on the base of temperature dependence of υ_{max} (equation 4); r^2, squared Pearson correlation coefficient for the slope of the Arrhenius plot; $\Delta H(T_A)$, change of activation enthalpy calculated for corresponding acclimation temperature; ΔS, change of activation entropy; $\Delta G(T_A)$, change of Gibbs free energy calculated for corresponding acclimation temperature; N, number of analysed fishes; n, total number of enzymatic reactions, which were read at different pyruvate concentrations and different temperatures of assay to calculate mean E_a; Δ, difference between ΔH for different acclimation temperatures. Values are given as means ± S.E.M.

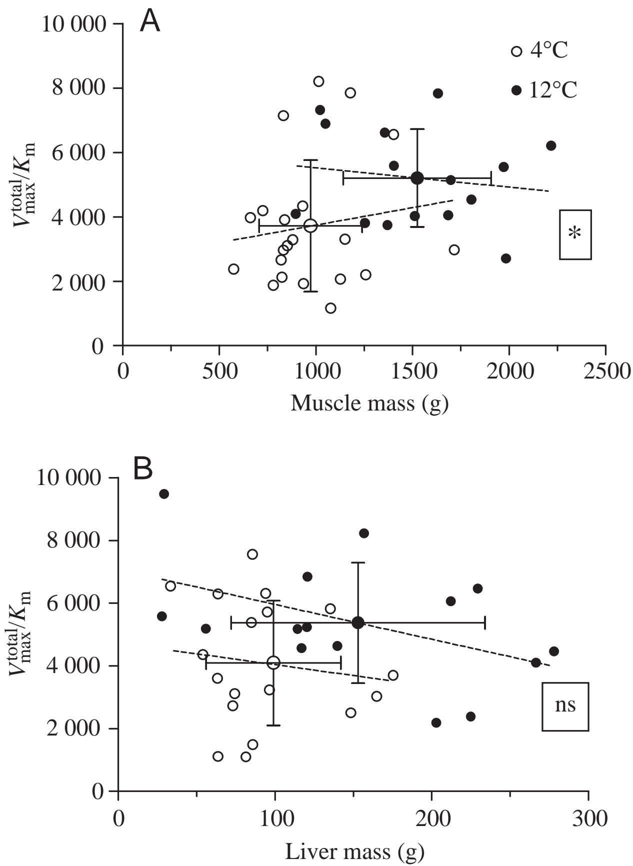

Fig. 8. Relationship between the relative catalytic efficiency of the overall lactate dehydrogenase (LDH) suite in crude homogenates and the total mass of muscle (A) and liver (B). $V_{max}^{total}/K_m^{PYR}$ is the total tissue relative catalytic efficiency; V_{max}^{total} is expressed in U g^{-1} wet mass; mass of muscle = 90% of the mass difference between fresh body mass and liver [$M_{muscle}=(M_{fish}-M_{liver})\times0.9$]. Mean data are presented as larger symbols with bars representing S.D. (N=21 for 4°C acclimation and N=15 for 12°C acclimation). Broken lines are linear regressions. (A) 4°C, $y=2670+1.079x$ (r^2=0.02; N=21); 12°C, $y=6121-0.61x$ (r^2=0.02; N=15); (B) 4°C, $y=4728-6.921x$ (r^2=0.02; N=18); 12°C, $y=7069-11.1x$ (r^2=0.08; N=15). Asterisks indicate that differences are significant (unpaired t-test, $P<0.05$); ns, differences are not significant (unpaired t-test, $P>0.05$).

al., 1990). For the sake of comparability between current and previous classifications, we have to note that locus *Ldh-A* corresponds to *Ldh-2*, *Ldh-B* to *Ldh-3*, and *Ldh-C* to *Ldh-1*. In the same way, the *Ldh-B* allele *a* corresponds to 70, and *b* to 100 units of relative mobility.

In the present study, we found that the loci *Ldh-A* and *Ldh-B* were expressed in skeletal muscle, while all three *Ldh* loci were found in liver. Grant and Stahl (1988) investigated both Atlantic and Pacific cod isozymes and observed LDH-B in eye fluids and cardiac muscle as well. The products of the polymorphic locus *Ldh-B* contribute only 14% to the total LDH in skeletal muscle and 19% in liver. Correspondingly, the products of the remaining *Ldh* loci comprised ~86% of the total LDH quantity in muscle (mostly *Ldh-A*) and ~81% of total LDH in liver (both *Ldh-A* and *Ldh-C* with some non-classified proteins).

Zietara and Skorkowski (1993) analysed cod tissue by IEF and found that in *Gadus morhua* from the Baltic Sea the products of *Ldh-A* and *Ldh-B* loci are predominant in both skeletal and heart muscle (bands with pI=6.4 and pIs from 5.1 to 5.6, respectively), while products of the *Ldh-C* locus predominate in liver (band with pI=8.2). The bands with pIs of 7.64, 7.10 and 6.66 seen in our measurements have not been reported before, however; these bands represent a large fraction of the liver's overall LDH suite (11%, 26% and 11% of total LDH, respectively; 48% in total). This pattern raises a problem for the precise classification of LDH proteins in liver of our cod, as some proteins have not been previously described. In order to avoid these complications, we preferred to operate with the overall LDH pattern (the LDH suite), qualified according to the polymorphism in locus *Ldh-B*.

Analysis of the IEF patterns did not reveal differences in the number of bands between different acclimation groups. This indicates that acclimation temperature did not cause the expression of new LDH isozymes. Earlier, Ozernyuk et al. (1994) and Vornanen (1994) reported the same phenomenon for LDH of loach (*Misgurnus fossilis*) and crucian carp (*Carassius carassius*) during seasonal temperature variations. Thus, Atlantic cod does not exploit the so-called qualitative adaptation strategy, i.e. the expression of new LDH isozymes that suit a new environmental condition better.

Quantitative analysis of pIs did not reveal an effect of acclimation temperature on enzyme mobility. This means that the quaternary structure of LDH was not affected by acclimation. Previous electrophoresis studies (Ozernyuk et al., 1994; G. Nævdal, personal communication) did not reveal any difference in LDH mobility between individuals acclimated to different temperatures. Thus, based on the unchanged frequencies of the *Ldh-B* allele after acclimation and on unchanged isozyme patterns and enzyme mobilities, we can conclude that cod pass through the seasonal temperature cycle with genetically the same LDH suite.

The cod that displayed different *Ldh-B* phenotypes did not reveal differences in growth performance or physiological condition. This is in accordance with findings by Nævdal et al. (1992), who carried out a similar growth experiment for cod at three environmental temperatures (6°C, 10°C and 12°C), where they showed that the growth rate was significantly affected only by acclimation temperature. Acclimation temperature had no effect on HI and *k*-factor, indicating that the fish just grew slower at cold temperature without any resulting anomalies (deviation from mass–length relationship). At first sight, this may simply appear as a consequence of reduced metabolic rate in the cold; however, we found that the temperature-specific rate of oxygen consumption ($\dot{M}_{O_2}$) was higher in cold- than in warm-acclimated fish, which appears typical for temperate eurythermal marine fish (Van Dijk et al., 1999; Zakhartsev et al., 2003). The observations that growth performance was reduced and aerobic metabolic rates increased with decreasing acclimation temperature indicate

metabolic reorganization during cold acclimation. Under laboratory conditions, the decrease of growth rate in the cold may be a consequence of high baseline costs of cold adaptation, in line with a hypothesis developed by Pörtner et al. (2001). An enhanced level of aerobic metabolism may be paralleled by a decreased capacity of anaerobic metabolism and modified functional properties of LDH. In the following, LDH functional properties will be discussed in this context.

Kinetic study

Hepes was used as a medium buffer because it displays a temperature-dependent pH slope of –0.015 pH deg.$^{-1}$ (Dawson et al., 1986; Yancey and Somero, 1978), which is very close to that of the pattern of intracellular pH change in some marine fish (e.g. common eelpout *Zoarces viviparus*; Van Dijk et al., 1999). At the same time, in contrast to imidazole, Hepes does not interfere with the protein quantification method we used (NanoOrange®).

Despite the extensive use of LDH in analyses of population genetics and biochemical adaptation, only a few publications deal with comprehensive kinetic studies of LDH (including iso- and allozymes) from ectothermic animals; for example, see Place and Powers (1984) and Sharpe et al. (2001). It is well documented that LDH from most ectothermic animals displays clear substrate inhibition kinetics (Almedia-Val et al., 1991; Baldwin et al., 1989; French and Hochachka, 1978; Narita and Horiuchi, 1979; Place and Powers, 1984; Tsukuda and Yamawaki, 1980; Zietara et al., 1996; Zietara and Skorkowski, 1993), caused by the formation of a covalent adduct between pyruvate and the oxidized form of the cofactor, i.e. the formation of an inhibitory enzyme–NAD^+–pyruvate complex (Eszes et al., 1996; Hewitt et al., 1999). LDH in crude homogenates of cod tissues also showed substrate inhibition. LDH is an equilibrium enzyme, but in our research we studied only the rate of pyruvate reduction, since pyruvate is the key substrate for both aerobic and anaerobic branches of the metabolism.

It has been shown several times that purified LDH allozymes differ significantly in their kinetic properties (Henry and Ferguson, 1986; Hoffmann, 1981; Place and Powers, 1984; Zera, 1987; Zietara and Skorkowski, 1993). However, since all LDH isoforms operate simultaneously in the cytoplasm (Voet and Voet, 1995), each isoform of LDH should play a particular role in the overall performance of LDH. This is the reason why we used crude homogenates for our kinetic studies. Nevertheless, the question arises of what effect the LDH-B allozymes have on the overall performance of the LDH suite in the cytoplasm. Thorough analysis of the temperature dependence of kinetic parameters (V_{max}, K_m^{PYR} and K_{si}^{PYR}) did not reveal any difference between *Ldh-B* phenotypes (overall LDH suites). As LDH-B comprises only 14–19% of all LDHs present in the crude homogenate, differences between these allozymes have no significant consequences for the final kinetic parameters of the LDH suite in the cytoplasm and, hence, for the net performance of the LDH suite.

We observed very significant tissue-specific differences in the kinetic properties of LDH suites related to different isozyme patterns. Since the different pI patterns in the two tissues were completely due to the presence of isozyme *Ldh-C* and to unclassified isozymes with pIs of 7.64, 7.10 and 6.66, the tissue-specific kinetic difference must be caused by the presence of these isozymes but not by the allozymes of LDH-B. According to Zietara and Skorkowski (1993), LDH-B allozymes are among those LDH isozymes most sensitive to high substrate concentrations. Consequently, LDH-B allozymes could serve as sensitive control elements in complex multi-isozyme LDH ensembles.

Comparison of tissue-specific kinetic parameters at different acclimation temperatures showed that muscle and liver LDH suites have distinctively different kinetic profiles (Fig. 1; Table 1). Muscle LDH displayed higher values of all kinetic parameters (V_{max}, K_m^{PYR} and K_{si}^{PYR}), which indicates that this LDH suite is designed to work at higher, and over a wider range of, pyruvate concentrations than liver LDH. This allows a higher substrate flux *via* LDH and thereby supports a higher rate of anaerobic glycolysis and, at the same time, fine control at elevated flux rates (higher pyruvate levels). The higher relative catalytic efficiency (V_{max}/K_m^{PYR}) in liver indicates that this LDH suite is designed to work faster and more efficiently under conditions of lower substrate concentrations. Possibly, these are lowered by higher tissue aerobic capacities when pyruvate is readily oxidized by mitochondria. Since LDH is an equilibrium enzyme, we can also conclude that the observed tissue peculiarities reflect different biochemical roles of LDH in the two tissues. On the one side, anaerobic muscular work relies on the glycolytic pathway under conditions of higher metabolite flux as well as elevated lactate levels. On the other side, liver is a sink for lactate, and consequently LDH operates efficiently at lower lactate and pyruvate levels in this organ (Cori cycle; Voet and Voet, 1995).

Neither acclimation temperature nor *Ldh-B* phenotype had an effect on K_m^{PYR} for the overall LDH suite. Since the affinity to the substrate (K_m^{PYR}) is a major 'fingerprint' of the enzyme, we can conclude that the functional properties of the LDH suite remained the same after acclimation to different temperatures. Although the temperature dependence of K_m^{PYR} followed an exponential curve (Fig. 6), we decided to apply a two-phase linear regression analysis to this data set, because it is obvious that K_m^{PYR} displayed a very weak temperature dependence in the low temperature range (Fig. 6). This analysis revealed that at temperatures below 16±1°C (which was found to be the breakpoint) K_m^{PYR} displayed a very low temperature dependence with a slope of 0.003–0.008 mmol l^{-1} deg.$^{-1}$. It is remarkable that both tissues at both acclimation temperatures showed the same breakpoint at ~16°C. This has important implications for the capacity of anaerobic metabolism. The loss of substrate affinity (increase of K_m^{PYR} beyond this breakpoint) results in a change of the enzyme's 'reserve capacity' to control metabolic flux (Hochachka and Somero, 1984).

According to Fig. 5, the specific concentration of LDH was reduced in both tissues during acclimation to cold temperatures. By contrast, the rise in total protein

concentration (Table 5) reflects an increase in the concentration of functional proteins to compensate for the loss in functional capacity of the tissues in the cold (Fig. 5).

The ratio V_{max}/K_m^{PYR} reflects the relative catalytic efficiency of an enzyme, which represents a rate constant at physiological substrate concentrations; therefore, to some extent, it is a measure of enzyme efficiency *in vivo* (Place and Powers, 1984). Acclimation to low temperature resulted in a significant decrease of enzyme efficiency in both tissues [V_{max}(U mg^{-1} protein)/K_m^{PYR}; Table 5]. However, due to the increase in overall tissue protein levels with acclimation to low temperature, the total tissue LDH activity [V_{max}(U g^{-1} wet mass)/K_m^{PYR}] was almost completely compensated in liver and partially compensated in muscle (Fig. 8). Interestingly, the magnitudes of total tissue LDH efficiencies ($V_{max}^{total}/K_m^{PYR}$) are similar in both tissues (Fig. 8). This finding probably reflects biochemical tasks of the tissues (e.g. in the Cori cycle, which, however, is of little relevance in fish). There are three main variables that support the accomplishment of such compensation: LDH-specific concentration, total protein concentration and total mass of the organ. Therefore, we can conclude that, at both acclimation temperatures, pyruvate reduction by LDH (when calculated per organ wet mass) displayed similar catalytic efficiencies at physiological substrate concentrations in liver, and the compensation in muscle was not completed. Total LDH efficiency was somewhat reduced in muscle, possibly as a consequence of the cold-induced metabolic reorganization towards aerobic metabolism (Lannig et al., 2003).

Place and Powers (1984) showed that a high degree of substrate inhibition is typical for both directions of the reaction catalysed by LDH. In the marine teleost *Fundulus heteroclitus*, substrate inhibition at 25°C was observed at K_{si}^{LAC}= 20 mmol l^{-1} and at K_{si}^{PYR}=2 mmol l^{-1}. The latter matches very well the level of K_{si}^{PYR} found in the present study in cod. K_{si}^{PYR} is another distinctive feature to characterize the capacity of LDH to cope with increased metabolic flux. Acclimation to low temperature significantly reduced K_{si}^{PYR} in both tissues, indicating enhanced inhibition, and K_{si}^{PYR} also became less dependent on assay temperature (Fig. 7). Narita and Horiuchi (1979) also found that K_{si}^{PYR} in muscle LDH of crayfish (*Procambarus clarki*) fell during acclimation to low temperature (from 0.24 mmol l^{-1} at 25°C to 0.09 mmol l^{-1} at 5°C). The substrate inhibition phenomenon is caused by the formation and dissociation of a covalent adduct between pyruvate and the oxidized form of the cofactor. The Ser163 amino acid residue plays a key role in this mechanism (Eszes et al., 1996; Hewitt et al., 1999), as well as the presence of several active sites and subunit interactions within the tetrameric protein. Consequently, the change in temperature dependence of K_{si}^{PYR} reflects changes in the formation and dissociation of a covalent adduct between pyruvate and the oxidized form of the cofactor, probably as a result of an altered interaction between LDH subunits. By contrast, K_m^{PYR} remained unaffected by temperature acclimation. The difference between K_{si}^{PYR} and K_m^{PYR} ($\Delta=K_{si}^{PYR}-K_m^{PYR}$) characterizes the range of pyruvate concentrations where LDH can work at maximal capacity. This range shrank significantly in both tissues with acclimation to low temperature, emphasising a reduction of anaerobic scope at low temperature (4°C). This also means that at low acclimation temperature (4°C) the capacity of LDH becomes more limited ($\Delta=K_{si}^{PYR}-K_m^{PYR}$) to support rapid turnover of high pyruvate concentrations. This is indicative of restricted capacity of anaerobic glycolysis after acclimation to low temperature (Pörtner, 2002b).

Thermodynamics

To perform the large number of enzymatic analyses in the temperature range between 4°C and 30°C, which is required for the thermodynamic characterisation of the enzyme from wild animals, we have designed a temperature-controlled 96-well microplate to employ a microplate reader for the purpose of high-throughput screening (see N and n in Table 6; Zakhartsev and Blust, 2002). Usually, studies of the temperature dependence of enzyme kinetic parameters and thermodynamics from individuals are limited in terms of group sizes (maximum, 2–3 animals), mainly because of the technical limitation of the corresponding equipment (M. Zakhartsev, H. O. Pörtner and R. Blust, unpublished). Now, this methodological limitation has been overcome and allows more robust statistical comparisons of enzymatic E_a between acclimation groups (see N and n in Table 6).

The classical view on changes in E_a with thermal adaptation or acclimation is that E_a decreases in the cold in order to compensate for the effect of decreasing temperature on reaction rates (Hochachka and Somero, 1984). Our finding that E_a of LDH can increase with a decrease in acclimation temperature is in contrast to this concept. However, our observation is unequivocal and supported by a large dataset (see n in Table 6). Moreover, a number of more recent examples has shown that a decrease of E_a in the cold is not necessarily a unifying trend (Pörtner et al., 2000). Van Dijk et al. (1999) showed that the E_a for oxygen consumption of Antarctic eelpout *Pachycara brachycephalum* was 99.4±5.9 kJ mol^{-1} *versus* 89.2±4.9 kJ mol^{-1} in congeneric temperate eelpout *Zoarces viviparus* (acclimated to 12°C). After cold acclimation (at 4°C), the rate of oxygen consumption of *Z. viviparus* was elevated, but E_a had fallen to 55±3 kJ mol^{-1} (Van Dijk et al., 1999; Zakhartsev et al., 2003). Sommer and Pörtner (2002) found that the E_a of NADP-dependent isocitrate dehydrogenase of *Arenicola marina* was higher (83.3±10.6 kJ mol^{-1}) in a cold-adapted White Sea population (sub-polar) than in a temperate North Sea population (63.7±3.6 kJ mol^{-1}). These examples suggest a variety of potential responses by E_a to cold. Since coping with temperature always involves compensatory strategies during short-term (acclimation) or long-term (adaptation) temperature variation (Clarke, 1991), the observed increase of E_a with a decrease in T_A raises the question of which mechanism of compensation was effective?

According to the literature, fish LDH at low acclimation

temperature undergoes molecular reorganization, which results in enhanced molecular stability. LDH isolated from skeletal muscle of loach acclimated to 5°C (a 'cold' enzyme) has a higher thermal stability (heat resistance) and is more resistant to urea-induced inactivation than LDH from fishes acclimated to 18°C (a 'warm' enzyme; Klyachko et al., 1995a; Ozernyuk et al., 1994). Accordingly, the enthalpy of denaturation (ΔH_d) of enzyme purified from loach acclimated to 18°C was larger (23.3±1.6 J g^{-1}) than for the enzyme purified from fish acclimated to 5°C (21.4±1.7 J g^{-1}; Danilenko et al., 1998). The enthalpy of the unfolded state (H_u) of both cold- and warm-acclimated enzymes should be the same since both 'forms' of the enzyme have identical amino acid sequences. The phenomenon of ΔH_d indicates that the enthalpy of the folded state (H_f) of 'cold' LDH increased and, as a consequence, ΔH_d was reduced ($\Delta H_d = H_u - H_f$). The specific heat capacity (C_p) measured at 25°C by differential scanning microcalorimetry of cold LDH was higher (1.39±0.03 J g^{-1} K^{-1}) than that of warm LDH (1.14±0.05 J g^{-1} K^{-1}), reflecting higher molecular stability in the cold (Danilenko et al., 1998). It is clear that these differences relate to differences in the intramolecular organization of the LDH extracted from fishes acclimated to different temperatures. These differences are probably related to the number of intramolecular interactions, which probably result in modified surface properties of the molecule (Danilenko et al., 1998). Zasedateleva et al. (1999) found molecular differences in the secondary structure of cold and warm LDH (acclimation at 5°C *versus* 18°C) of loach by means of circular dichroism spectrometry. These differences become apparent during thermal denaturation of the enzyme, which mainly relates to differences in the 'melting' of α-helical structures: cold LDH exhibited a higher cooperativity in the melting of α-helices and it occurred in a narrower temperature range (70–85°C) than in warm LDH (40–85°C). However, the temperatures of denaturation (T_d=74°C) were indistinguishable between the two acclimation forms of LDH at 5°C and 18°C (Danilenko et al., 1998; Klyachko et al., 1995b; Persikov et al., 1999). This is not in contrast to the other findings, because the determination of T_d is a relatively rough method to estimate molecular differences.

It is well known that increasing the number of intramolecular hydrogen bonds (Pace et al., 1996) as well as non-polar bonds will stabilize protein structure and cause a higher resistance to denaturation (Privalov and Tsalkova, 1979; Sowdhamini and Balaram, 1993). As a consequence, it increases the H_f and E_a of the molecule. This phenomenon is frequently observed in enzymes of microorganisms living in thermal springs (Hochachka and Somero, 1984). Therefore, one possible explanation for the observed phenomenon of an increase in E_a of LDH with acclimation to low temperature might be the introduction of additional weak interactions within the LDH molecule. This additional weak interaction causes the observed functional changes and enhanced molecular rigidity. This conclusion is supported by the effect of urea, which exerts its effect by interfering with weak bonds such as hydrogen bonds (Ozernyuk et al., 1994). It is very likely that these additional hydrogen bonds were introduced in loop regions, increasing their rigidity and consequently affecting the molecular E_a and its catalytic efficiency. Zasedateleva et al. (1999) observed a change of conformational structure of LDH during seasonal adaptation of loach and related this shift to a change of electrostatic interactions between the elements of secondary structure in LDH. Furthermore, Fields et al. (2002) studied homologous LDHs from congeneric species of the goby fish *Gillichthys seta* and *Gillichthys mirabilis* and proposed that subtle differences in conformation around residual Tyr246, which is involved in subunit interaction within the homotetramer and sits in a hinge between a static α-helix and one involved in catalytic conformational changes, probably play a role both in altered flexibility and in the potentially adaptive differences in kinetics between the two LDH forms.

In fact, the analysis of activation enthalpy change (Table 6) calculated for corresponding acclimation temperatures revealed that in the condition of cold acclimation ΔH became higher by approximately 5.63–8.99 kJ mol^{-1} (depending on the tissue). The magnitude of this energy change is roughly the same as the enthalpy change involved in the formation of one hydrogen bond (Haynie, 2001), which is 5.70 kJ mol^{-1} at 25°C. This observation suggests that an additional 1–2 hydrogen bonds could appear in the molecular structure of LDH under conditions of cold acclimation. However, it is presently unclear how much change in activation enthalpy of the enzymatic reaction is caused by the formation of one weak bond in enzyme structure.

Our measurements indicate that LDH molecules did not undergo conformational phase-transition states within the range of assay temperatures (4–25°C). The Arrhenius plot was linear over the whole temperature range with no evident breakpoint, as shown by a high value of r^2 (Table 6).

Recently, it was reported that the thermal stability of some enzymes can be increased by calcium binding (Declerck et al., 2000; Harris and Davidson, 1994). Danilenko et al. (1998) investigated this possibility in cold and warm variants of LDH using x-ray fluorescence and did not find any difference in the calcium contents of purified enzymes. Danilenko et al. (1998) also found no phosphorus in both forms of LDH (cold and warm) from loach, indicating that phosphorylation was not involved in the modification of thermostability. Some low-molecular-mass osmolytes (free amino acids, glycine-based osmolytes) have been found to increase the thermostability of enzymes (Taneja and Ahmad, 1994). Such effects can be excluded for our enzyme preparations because of the use of diluted homogenates. At the highest assay temperatures applied (30°C), the final dilution factor of the tissue was 5×10^4 for muscle and 10^4 for liver. Therefore, it is very unlikely that low-molecular-mass osmolytes were effective in the diluted solution.

All arguments mentioned above indicate a very high probability that additional weak bonds were introduced into the molecular structure of LDH adjusted to cold. Hochachka and Somero (1984) pointed out that the introduction of additional

or enhanced weak interactions can considerably increase the thermostability of a protein by stabilization of its conformational state. This strategy may occur at the expense of a decrease in catalytic efficiency. This argument is supported by the observed increase in apparent E_a (Table 6) and the drop in K_{si}. This indicates that an increased enthalpy of the cold-adjusted enzyme is in line with a loss in structural flexibility.

In summary, additional weak interactions within the quaternary structure of the LDH may be one of the causes of the observed difference between LDHs from cold- and warm-acclimated animals. A non-genetic post-translational modification of the enzyme molecule probably leads to enhanced LDH folding depending on acclimation temperature (temperature-driven protein folding). It has been suggested previously that identical amino acid sequences (identical primary structures) of LDHs from congeneric species of goby fish (Gobiidae) can fold in different conformations (Fields et al., 2002; Fields and Somero, 1997), yielding conformational variants. Therefore, our data indicate that this mechanism is not only involved on evolutionary time scales but probably contributes to adaptation to seasonal temperature variation (acclimation).

Metabolic compensation

Comprehensive studies of thermal limitation indicate that the limits of thermal tolerance are, in the first place, set at the level of complex organismic processes and only secondarily at the molecular level (molecular or structure phase transition, protein denaturation, etc.). Whole-animal aerobic scope appears to be the first process limited at both low and high temperatures (Frederich and Pörtner, 2000; Pörtner et al., 1998; Pörtner, 2002a; Prosser, 1986).

Aerobic and anaerobic pathways of metabolism are competing for pyruvate. Therefore, the efficiency of energy production in particular tissues depends on adequate channelling of this metabolite (Somero, 1973). As was shown earlier by Pörtner and Grieshaber (Grieshaber et al., 1994; Pörtner and Grieshaber, 1993) and later by Boutilier and St-Pierre (2000), the contributions of aerobic and anaerobic pathways to total energy production depend on oxygen availability to mitochondria. Below the critical oxygen tension (P_c) the contribution of anaerobic pathways rises. Thus, survival in constantly fluctuating environments depends on the relationship between aerobic and anaerobic pathways and their capacity for ATP production.

The net increase in the apparent E_as for pyruvate reduction by LDH during acclimation to cold temperatures, combined with an increase in aerobic capacity (Lannig et al., 2003) and the associated increase of mitochondrial density, may be one mechanism of metabolic control and indicates a downregulation of anaerobic capacity when the relative fraction of aerobic metabolism increases. This might help to avoid pyruvate limitation for aerobic oxidation (maintenance of aerobic scope) and thereby increases the efficiency of aerobic metabolism at low temperature. Other features involved in the change of LDH functional properties include a reduced range of pyruvate concentrations required for the maximal capacity of LDH ($\Delta = K_{si}^{PYR} - K_m^{PYR}$). Although the adjustment of mitochondrial densities and their kinetic properties are one of the key mechanisms to regulate metabolic capacities during acclimation to low temperature, the outlined mechanism (temperature-driven enzyme folding) contributes to create favourable conditions for the aerobic functioning of mitochondria at low temperature.

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