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Differential expression of duplicated LDH-A genes during temperature acclimation of weatherfish *Misgurnus fossilis*

Functional consequences for the enzyme

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Temperature acclimation in poikilotherms entails metabolic rearrangements provided by variations in enzyme properties. However, in most cases the underlying molecular mechanisms that result in structural changes in the enzymes are obscure. This study reports that acclimation to low (5 °C) and high (18 °C) temperatures leads to differential expression of alternative forms of the LDH-A gene in white skeletal muscle of weatherfish, *Misgurnus fossilis*. Two isoforms of LDH-A mRNA were isolated and characterized: a short isoform ($mRNA^{\alpha}_{ldh-a} = 1332$ bp) and a long isoform ($mRNA^{\beta}_{ldh-a} = 1550$ bp), which both have 5′-UTRs and ORFs of the same length (333 amino acid residues), but differ in the length of the 3′-UTR. In addition, these two mRNAs have 44 nucleotide point mismatches of an irregular pattern along the complete sequence, resulting in three amino acid mismatches (Gly214Val; Val304Ile and Asp312Glu) between protein products from the short and long mRNA forms, correspondingly LDH-A^{α} and LDH-A^{β} subunits. It is expected that the β-subunit is more aliphatic due to the properties of the mismatched amino acids and therefore sterically more restricted. According to molecular modelling of *M. fossilis* LDH-A, the Val304Ile mismatch is located in the subunit contact area of the tetramer, whereas the remaining two mismatches surround the contact area; this is expected to manifest in the kinetic and thermodynamic properties of the assembled tetramer. In warm-acclimated fish the relative expression between α and β isoforms of the LDH-A mRNA is around 5 : 1, whereas in cold-acclimated fish expression of $mRNA^{\beta}_{ldh-a}$ is reduced almost to zero. This indicates that at low temperature the pool of total tetrameric LDH-A is more homogeneous in terms of α/β-subunit composition. The temperature acclimation pattern of proportional pooling of subunits with different kinetic and thermodynamic properties of the tetrameric enzyme may result in fine-tuning of the properties of skeletal LDH-A, which is in line with previously observed kinetic and thermodynamic differences between 'cold' and 'warm' LDH-A purified from weatherfish. Also, an irregular pattern of nucleotide mismatches indicates that these mRNAs are the products of two independently evolving genes, i.e. paralogues. Karyotype analysis has confirmed that the experimental population of *M. fossilis* is tetraploid ($2n = 100$), therefore gene duplication, possibly through tetraploidy, may contribute to the adaptability towards temperature variation.

Abbreviations
AT, acclimation temperature; LDH-A, lactate dehydrogenase type A; PDB, protein data bank.

Temperature adaptation (both long- and short-term) in poikilotherms results in significant metabolic rearrangements, in which functional and structural enzyme properties become variables to achieve adaptation [1–9]. Seasonal adaptation or acclimation (short-term) of poikilotherms to temperature very often leads to changes in two main traits of some metabolic enzymes: quantitative and qualitative.

The quantitative properties (concentration and, as a consequence, total activity) of an enzyme can be changed by affecting rates of transcription and/or translation and/or mRNA and protein degradation. This represents a quantitative strategy to offset the lack or excess of kinetic energy (as a measure of temperature) and its effect on enzyme activity.

Alternatively, we know of a number of examples of qualitative strategies, for example, when expression of distinctly different isoenzymes contributes to seasonal temperature adaptation by adjusting a particular metabolic node to new environmental conditions. Known examples are the isoforms of acetylcholine esterase, choline esterase [10,11], ferritin H [12], ependymin [13], β-subunits of protein kinase CK2 [14,15], F_0F_1-ATPase [16], etc. Temperature-dependent gene expression of these enzymes may result from: (a) the temperature-dependent expression profile of transcription factors like Pit-1 [17]; (b) changes in the ratio of isoenzymes that are expressed simultaneously [6,9]; or (c) changes in the kinetic and thermodynamic properties of an enzyme through post-translational modifications under invariant isoenzyme expression profiles. In fact, variations in lactate dehydrogenases (LDHs; EC 1.1.1.27) in fish over the course of seasonal temperature adaptations satisfy all these three qualitative criteria, because LDH is a tetrameric enzyme that is present over a wide isoenzyme spectrum (A, B and C that play different metabolic roles) and is tissue specific. Also, some LDH isoenzymes have allelic variants. It has been shown that, at an evolutionary scale, some amino acid substitutions result in modified LDH properties [18,19]. However, in some cases, the observed kinetic and structural differences among LDH from related species cannot be attributed to the amino acid sequence because they are identical [7]. Moreover, there is evidence that some fish LDHs can undergo structural modifications in the course of temperature acclimation that lead to different functional (kinetic and thermodynamic) properties of the enzymes [3,8,11,20–22].

In Table 1 we summarize all previous observations of the effects of seasonal temperature acclimation on the properties of purified LDH-A from skeletal muscle of weatherfish *Misgurnus fossilis* acclimatized to either 5 °C ('cold' enzyme) or 18 °C ('warm' enzyme). 'Cold' LDH-A reveals greater stability to heat-, pH- and urea-induced inactivation [3]. Although the denaturation temperature (T_d) of 'cold' and 'warm' enzymes was the same, the specific heat capacity (C_p) was higher in 'cold' LDH-A [21]. This indicates a higher degree of freedom of the native enzyme, i.e. higher structural flexibility, which is reflected in higher specific activity [3]. The calorimetric enthalpy of denaturation of the 'cold' enzyme was lower than that of the 'warm' LDH-A at all pH values studied [20], which indicates a difference in the number of hydrogen bonds between native and denaturated states. Three stages of heat denaturation were observed in LDH-A and the difference between 'cold' and 'warm' enzymes was attributed to the first stage of heat denaturation, i.e. tetramer → monomer [20]. Electrophoretically and chromatographically, 'cold' and 'warm' LDH-As cannot be distinguished. Thus, in sum, 'cold' LDH-A is more resistant to inactivation (pH, temperature and urea), displays a higher specific activity, a higher specific heat capacity and a lower calorimetric enthalpy but the same denaturation temperature. All of these observations point to differences between 'cold' and 'warm' LDH-As that originate in the molecular structure but have not previously been identified.

Molecular mechanisms causing this phenomenon on an acclimation scale are unknown. It is obvious that variation in enzyme properties under acclimation to seasonal temperature variation can be defined by genetic processes. It is known that acclimation to low temperatures, or seasonal temperature variation, modulates gene expression in some enzymes and structural proteins, as well as transcriptional factors [12–14,17,23]. All the observed dynamic changes in enzyme properties under acclimation should be considered in the context of the relevance for the performance of metabolic networks, because the theory of metabolic control analysis states that enzyme properties (concentrations, kinetics and thermodynamics) become variables to achieve adaptation of the metabolic system, such that some global system parameters (e.g. flux control coefficients) are maintained or adjusted to new functional states [24].

To obtain a better understanding of the mechanisms of temperature adaptation in enzymes we studied LDH-A mRNA from the skeletal muscle of weatherfish *M. fossilis* acclimated to low and high temperatures.

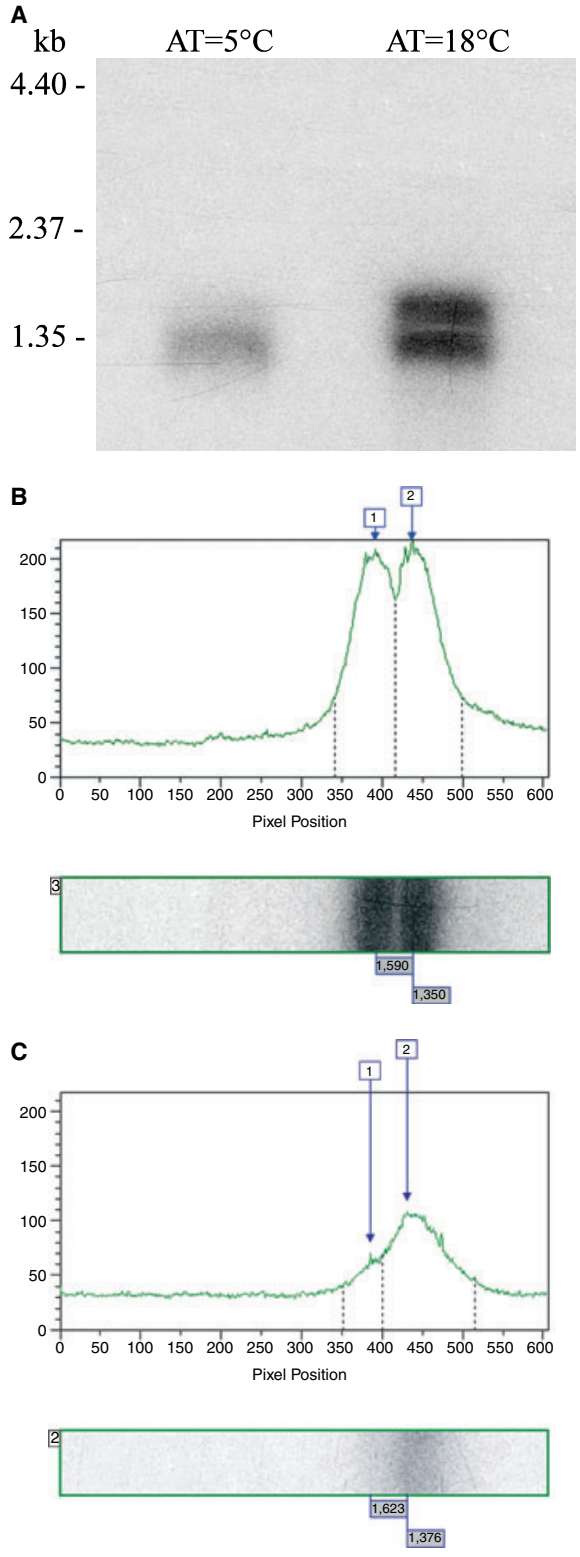
Results and Discussions

Our initial hypothesis about the qualitative differences between 'cold' and 'warm' LDH-A from *M. fossilis*

Table 1. Differences identified between 'cold' (AT = 5 °C) and 'warm' (AT = 18 °C) LDH-A purified from white skeletal muscle of weatherfish *Misgurnus fossilis* acclimated to different temperatures (ATs) for 20–25 days.

Property	Differences Cold (AT = 5 °C)	Warm (AT = 18 °C)	Ref
Specific activity	176 ± 24 U·mg^{-1} protein	141 ± 14 U·mg^{-1} protein	[22]
pH dependence	Similar in the normal pH range (6.2–9.0) with pH optimum at 7.2, but below pH 6.2 the cold enzyme is more resistant (~ +20%)		[3,37]
Thermal inactivation (after 30 min of incubation between 60 and 72 °C)	T_{50} = 70.2 °C more stable > 66 °C Similar between 60 and 66 °C, but above 66 °C the cold enzyme is more stable	T_{50} = 67.0 °C	[3]
Rate constant of thermal inactivation (80 min at 70 °C)	0.0110 ± 0.0004 min^{-1}	0.0272 ± 0.0011 min^{-1}	[22]
Urea inactivation (10 min at 20 °C)	At all urea concentrations (1.5–6 M) the cold enzyme is more stable/resistant (~ +10%)		[3]
Heat capacity at 25 °C	1.39 ± 0.03 J (g·K)$^{-1}$	1.14 ± 0.05 J (g·K)$^{-1}$	[21]
Calorimetric enthalpy of denaturation (scanning rate 2 °C·min^{-1} at pH 7.0)	2856 kJ·mol^{-1}	3272 kJ·mol^{-1}	[20,21]
Pattern of heat denaturation (scanning rate = 2 °C·min^{-1} between 10 and 110 °C)	There are three independent transition states during denaturation: tetramer → monomer → domain 1 → domain 2 Dynamics of the second and third transitions are similar, whereas the first one is different		[20]
Number of cooperative units (scanning rate = 2 °C·min^{-1} at pH 7.0)	No significant differences, values are between 1.76 and 1.86		[20,21]
Denaturation temperature (scanning rate = 1 °C·min^{-1})	No difference, identical at any scan rates and pH (e.g. 77.3 °C at 2 °C·min^{-1} and pH 7.0)		[20,21]
Chromatographic/electrophoretic mobility	No detectable differences		[3,20–22,37,38]
Energy domains	No difference, three domains in both		[20]
Phosphorylation	No phosphorylation in both		[21]
Ca^{2+} content	No content		[21]

considered the possibility of alternative splicing of LDH-A mRNA, resulting in subunits with different functional properties, therefore detailed analysis of LDH-A mRNA ($mRNA_{ldh-a}$) was carried out. Northern blot analysis of $mRNA_{ldh-a}$ in white muscle of *M. fossilis* which had been acclimated to 18 °C revealed two strong hybridization signals (~ 1400 and ~ 1600 bp); samples from 5 °C also yielded two hybridization signals, but they were less profound, and the ~ 1600 bp band was almost lacking (Fig. 1). Correspondingly, two isoforms of $mRNA_{ldh-a}$ with a temperature-dependent expression profile were expected.



Determination of the entire transcripts using RACE techniques confirmed the existence of two LDH-A mRNAs of different lengths: short (α-isoform; $mRNA^{\alpha}_{ldh-a}$ = 1332 bp) and long (β-isoform; $mRNA^{\beta}_{ldh-a}$ = 1550 bp). Sequence analysis of these mRNAs has shown that these two forms have equal length 5′-UTRs (105 bp) and ORFs (1002 bp), but the 3′-UTRs differ significantly in length (225 bp in $mRNA^{\alpha}_{ldh-a}$ and 443 bp in $mRNA^{\beta}_{ldh-a}$). In addition to 3′-UTR length differences ($\Delta = 218$ bp), 44 nucleotide mismatches have been found along homologous parts of the mRNAs: 1 in the 5′-UTRs, 19 in the ORFs and the remaining 25 occur in the 3′-UTRs (Fig. 2). All the nucleotide differences are point-mismatches with an irregular pattern, except for a five-nucleotide insert in the 3′-UTR of $mRNA^{\beta}_{ldh-a}$ (Fig. 2), this fact excludes that these are products of alternative splicing of the same transcript. In contrast, it points directly to the existence of two independently evolving genes with a common origin possibly through duplication, i.e. paralogs. This raises questions about the origin of these paralogs.

Some species of the genus *Misgurnus* can be found as either diploid ($2n = 50$) or tetraploid species ($2n = 100$) [25], for example, populations of *M. fossilis* from Eastern Europe [26]. Therefore, we performed karyotypic analysis of gill tissue from experimental *M. fossilis* and found 100 chromosomes, i.e. tetraploidy. This may explain the origin of highly homologous gene paralogs of skeletal LDH-A in the weatherfish.

Many bony fish (*Teleostei*) are polyploidy, for example salmonids (*Salmonidae*) and cyprinids (*Cyprinidae*), and the loach family (*Cobitidae*) is closely related to the latter. Genome duplication preceded the extensive radiation of bony fish [27,28], and many genes found in teleost fish are present in two copies (paralogues), located on different chromosomes. For example, in the

Fig. 1. (A) Northern hybridization of LDH-A mRNA from weatherfish *Misgurnus fossilis* indicates presence of two forms of LDH-A mRNA as (B) two strong signals (~1.4 kb and ~1.6 kb) at 18°C acclimation (AT=18°C), whereas (C) at 5°C acclimation (AT=5°C) the signals are weaker and moreover ~1.6 kb mRNA is almost missing.

whole genome of zebrafish 49 genes have been shown to be paralogues, while being a single-copy gene in human [28]. Also, it has been shown that paralogues originating from preteleost genome duplication can achieve different function. For example, in several teleosts, including weatherfish, zebrafish and others, tissue-specific light myosine chain forms are encoded by paralogous genes *mlc1* and *mlc3*, whereas in amphibians, birds and mammals these proteins are encoded by alternative splicing [29,30]. However, the two forms of LDH-A in weatherfish should be much younger than preteleost genome duplication. For all teleosts for which a complete genome sequence is available (zebrafish, tetraodon and fugu), only one copy of the LDH gene has been found. Sequence divergence between pairs of isoforms, known in diploid teleosts is ~20–25% (for ORFs of *mlc1* and *mlc2* genes), while divergence between the two forms of LDH-A in weatherfish is 1.9%. This high sequence similarity of LDH-A paralogs may indicate their recent origin.

Translation of the ORFs from both mRNAs reveals amino acid sequences of 333 residues in both cases, however, they display three amino acid mismatches: Gly214Val; Val304Ile and Asp312Glu (Figs 2 and 3). Therefore, we denoted the LDH-A subunit translated from $mRNA^{\alpha}_{ldh-a}$ as the α-subunit (or LDH-A^{α}) and, correspondingly, the β-subunit (or LDH-A^{β}), which is translated from $mRNA^{\beta}_{ldh-a}$. All observed amino acid mismatches increase the aliphatic properties of the β-subunit and therefore should restrict it sterically within the context of a tetramer. Also, such subtle amino acid differences between α- and β-subunits would not be distinguished electrophoretically or chromatographically (Table 1).

Insertion of five nucleotides in the 3′-UTR of $mRNA^{\beta}_{ldh-a}$, together with the difference in 3′-UTR length (Fig. 2), allowed unique detection (see primers in Table 2) and relative quantification of both LDH-A mRNA isoforms using real-time PCR (Table 3). Taking the $mRNA^{\alpha}_{ldh-a}$ content at AT = 18 °C (as the most abundant) to be 100 arbitrary units (a.u.), the relative content of each $mRNA_{ldh-a}$ isoform per 5 ng total RNA at each acclimation temperature was quantified (Table 3). At AT = 18 °C the ratio between $mRNA^{\alpha}_{ldh-a}$ and $mRNA^{\beta}_{ldh-a}$ is almost 5 : 1. However, at AT = 5 °C the specific total $mRNA_{ldh-a}$ content decreases and $mRNA^{\beta}_{ldh-a}$ almost disappears, i.e. exhibiting a temperature-dependent expression profile. This observation is in line with results from the northern blot hybridization (Fig. 1). Thus, $mRNA^{\alpha}_{ldh-a}$ forms the major constituent of the total $mRNA_{ldh-a}$ pool, and its amount is affected slightly by acclimation temperature (Table 3). By contrast, the minor constituent ($mRNA^{\beta}_{ldh-a}$) displays a strong temperature-dependent expression profile (Table 3). Hence, we observe temperature-dependent fractional pooling of $mRNA^{\alpha}_{ldh-a}$ and $mRNA^{\beta}_{ldh-a}$, i.e. at AT = 5 °C the overall $mRNA_{ldh-a}$ pool is almost homogeneous, whereas at AT = 18 °C it is substantially heterogeneous.

Alignment analysis (SWISS-MODEL) of LDH-A^{α} and LDH-A^{β} subunits has revealed that the amino acid sequence of LDH-A^{α} displays 93.7% identity with LDH-A from the skeletal muscle of common carp *Cyprinus carpio* (1v6a.pdb; K. Watanabe & H. Motoshima, unpublished results), whereas LDH-A^{β} shares 92.8% identity with the same protein. 1v6a.pdb describes secondary, tertiary and quaternary structures of LDH-A from the skeletal muscle of common carp including subunit and ligand interactions. Therefore, the structure of weatherfish skeletal LDH-A has been predicted using SWISS-MODEL and visualized with PDB Viewer (Fig. 3). This approach revealed that the Val304Ile mismatch is located in the contact area between the subunits of the tetramer, whereas the remaining two mismatches Gly214Val and Asp312Glu flank the contact area (Fig. 3). This should be manifest

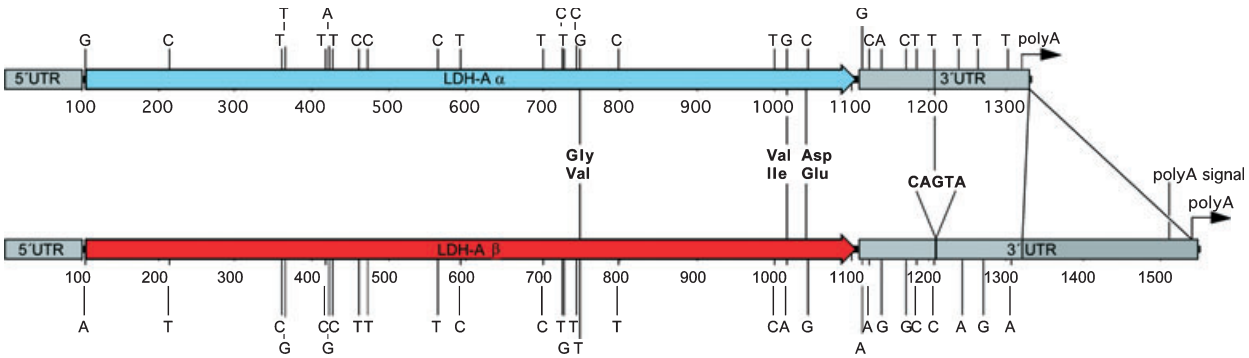


Fig. 2. Structure of the short ($mRNA^{\alpha}_{ldh-a}$ = 1332 bp) and long ($mRNA^{\beta}_{ldh-a}$ = 1550 bp) forms of LDH-A mRNAs from skeletal muscle of weatherfish *Misgurnus fossilis*. Nucleotide mismatches are indicated outwards, whereas amino acid mismatches are indicated inwards.

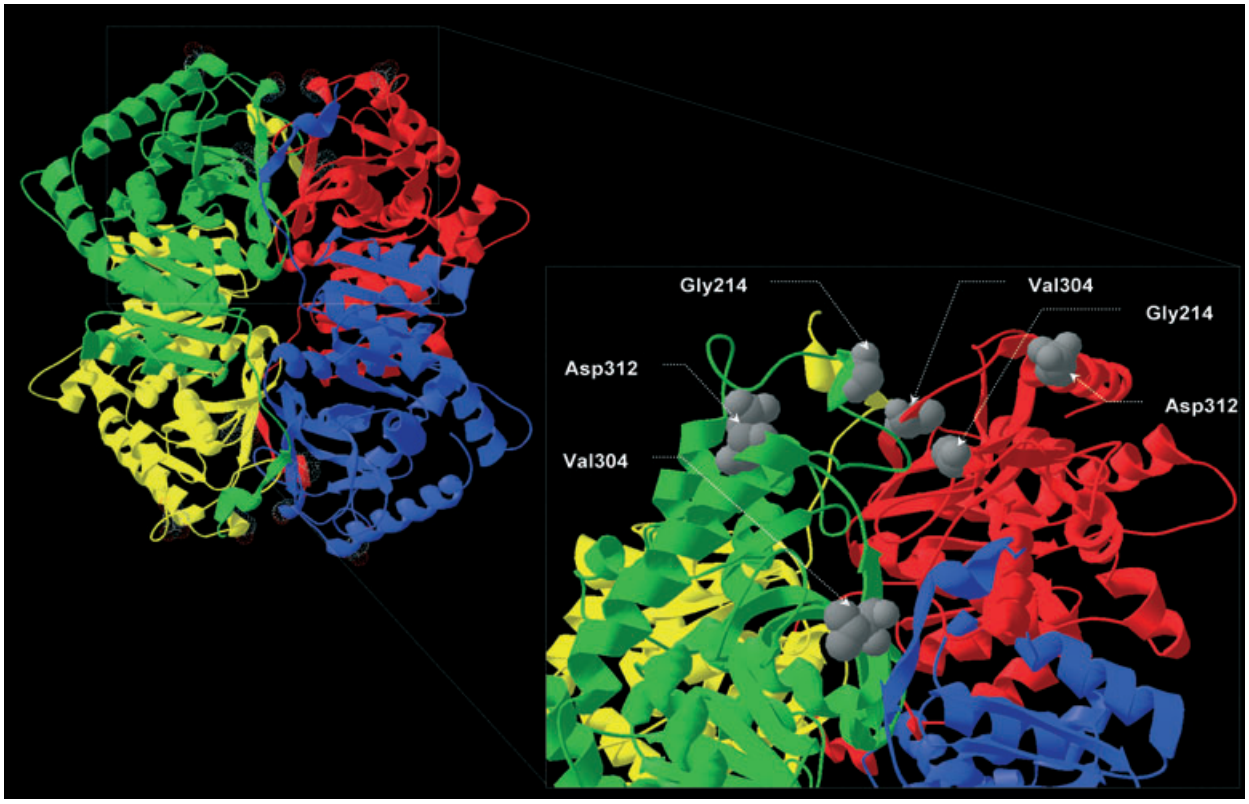


Fig. 3. Predicted quaternary structure of skeletal muscle LDH-A α_4-homotetramer (LDH-A$^{\alpha 4}$) from weatherfish *Misgurnus fossilis* and close up view on the contact area between two neighbouring subunits. Each subunit is coloured and the corresponding mismatched amino acids are indicated.

Table 2. Primers used in the research of mRNA of LDH-A from weatherfish *Misgurnus fossilis*.

Method	No.	Primer to detect mRNA		Primer sequence (5′- to 3′)	Name	PCR product length (bp)
Northern blot & PCR	1	both	forward	GTGGACGTGATGGAGGATAAG	A1F	728 (with A1F and A1R)
	2		reverse	GAAGGCACGCTGAGGAAGAC	A1R	
5′-RACE	3	both	outer reverse	GGATGAATGCCCAACTTCTCCC	B13R	
	4	both	outer reverse	ACGAAACCTGGCAGAGTCCAAG	B6R	
	5	long	inner reverse	GACTACTTTGGAGTTTGCGGTCAC	B1R	
3′-RACE	6	both	forward	AGTTGGGCATTCATCCATCC	F13R	
	7	both	forward	CAGAAAAAGACAAGGAGGAC	F19R	
Isoform-specific PCR	8	both	forward	ACAACACCACTGCTGCGGAGTTA	J1F	
	9	short	reverse	ACATCAAGGAGCGTTAGAATCTAA	J2R	1201 (with J1F and J2R)
	10	long	reverse	GATTTAAGTGGAGCGGAATGCTA	J3R	1385 (with J1F and J3R)
Real time PCR	11	short	forward	TGTGAAACGCAGTCTCTTCC	H1F	122 (with H1F and H1R)
	12		reverse	CAAGGAGCGTTAGAATCTAAAG	H1R	
	13	long	forward	TCTCCAAACCAGATCTCTACAG	H2F	224 (with H2F and H2R)
	14		reverse	GATTTAAGTGGAGCGGAATGCTA	H2R	

in the functional properties of tetrameric LDH-A via long-range structural effects due to expected differences in the aliphatic and steric properties of α- and β-subunits. Earlier, molecular dynamic simulation of LDH has revealed that the tetrameric nature of LDH plays a crucial role in maintaining the geometry of the active site through the contact among subunits [31]. Neighbouring subunits are necessary to prevent water penetration into the active site and provide rigidity to the helix that neighbours the active site. This also

Table 3. Content of α/β-isoforms o $mRNA_{ldh-a}$ in total RNA samples (1 ng total RNA per μL) from weatherfish *Misgurnus fossilis* acclimated to 5 °C or 18 °C for 20 days. AT, acclimation temperature (°C); C_t, number of real time PCR cycles at fluorescence threshold of 0.0314 provided with 95% confidence interval.

$mRNA_{ldh-a}$	AT = 5 °C C_t	relative content (au)[a]	AT = 18 °C C_t	relative content (au)[a]
α	19.98 ± 0.20	90.8	19.84 ± 0.17	100.0
β	24.75 ± 0.19	3.3	22.13 ± 0.19	20.4
Sum:		94.1		120.4

[a] Relative content of mRNA in total RNA sample (1 ng·μL^{-1}) if content of $mRNA^{\alpha}_{ldh-a}$ at AT = 18 °C is accepted as 100 arbitrary units (au).

explains why LDH monomers are not biologically active.

Because none of the physical–chemical detection methods was able to distinguish LDH-A^{α} and LDH-A^{β}, we have computed the probabilities (frequencies) of particular LDH-A *iso*-tetramers assembled from α- and β-subunits under different acclimation conditions (actually different α/β $mRNA_{ldh-a}$ ratio; Table 3) using combinatorics (Fig. 4) based on following assumptions: (i) similar translational activity of both (α/β) mRNA isoforms, which is expected to be equal due to the identity of the 5′-UTRs; and (ii) random assembly of LDH-A tetramers from different subunits. In accordance with general knowledge about eukaryotic translation control, many mRNAs carry in their 3′-UTR sequence binding sites for specific proteins that increase/decrease the rate of poly(A) shortening, i.e. affect the lifetime of the mRNA [32], therefore it is likely that the lifetime of $mRNA^{\alpha}_{ldh-a}$ and $mRNA^{\beta}_{ldh-a}$ differ. Nevertheless, using real-time PCR we assessed steady-state mRNA levels. Under the first assumption, the probability of α/β $mRNA_{ldh-a}$ isoform translation is proportional to their concentration. Thus, the above-mentioned assumptions are a reasonable compromise to estimate LDH-A subunit composition.

Computation shows that, in terms of α/β-subunit composition, the overall pool of tetrameric LDH-A at AT = 18 °C should be significantly heterogeneous, whereas at AT = 5 °C it should be almost homogeneous (Fig. 4), which must inevitably manifest in differentiation of the overall properties of pooled LDH-A iso-tetramers from warm and cold acclimations. This is in line with most of the observations summarized in Table 1. In particular, differences in the first denaturation transition state of LDH-A (tetramer → monomer) [20] and different levels of specific heat capacity and calorimetric enthalpy of denaturation between 'cold' and 'warm' LDH-As [21] directly prove this conclusion. Also, because of the expected steric constraints, it is obvious that LDH-A tetramers that accommodate LDH-A^{β} subunits have a lower specific activity and are less resistant to low pH, high temperature and high urea concentrations (Table 1). Therefore, more homogeneous composition of the 'cold' enzyme with LDH-A^{α} subunits may explain its higher specific activity and resistance to environment stressors.

LDH-A is allocated in the pyruvate node, which is the terminal step in the glycolytic pathway, consequently, it is a very important enzyme for muscle activity. Obviously, the proposed mechanism adds more plasticity to this node in the face of temperature acclimation. Therefore, we think that the described mechanism maintains either (a) the kinetic/thermodynamic properties of this metabolic node by 'dilution' of the major mRNA with the minor one; or (b) the steady-state enzyme concentration (meaning overall activity) by means of translational control of α/β-$mRNA_{ldh-a}$, in accordance with the requirements of a metabolic flux at a new temperature conditions.

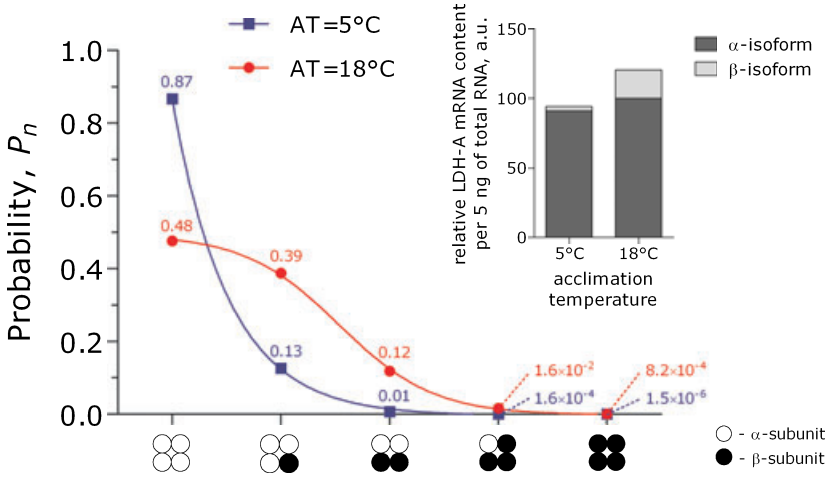


Fig. 4. Expected probabilities of *iso*-tetramers in overall LDH-A pool (LDH-A^{α_4}, LDH-$A^{\alpha_3\beta}$, LDH-$A^{\alpha_2\beta_2}$, LDH-$A^{\alpha\beta_3}$ and LDH-A^{β_4}) in skeletal muscle of weatherfish *M.fossilis* under AT = 5°C and AT = 18°C due to fractional mixing of α- and β-subunits correspondingly translated from $mRNA^{\alpha}_{ldh-a}$ and $mRNA^{\beta}_{ldh-a}$ isoforms (LDH-A mRNA ratios shown in the embedded histogram), assuming similar translational activity of both mRNA isoforms and a random assembly of the tetrameric enzyme.

Differences in the expression of paralogues can be considered as an adaptive mechanism during temperature acclimation. Therefore, gene duplication, which an important evolutionary factor [27,28,33,34], may also play a significant role in seasonal acclimation to temperature. Therefore, the structures of paralogue genes (e.g. promoters, enhancers) which lead to temperature-dependent mRNA levels have to be identified. Also, for a more detailed understanding of the functional and metabolic consequences, further study needs to identify the kinetic, thermodynamic and regulatory properties of recombinant LDH-A^{α_4} and LDH-A^{β_4} homotetramers and reconstituted LDH-$A^{\alpha_2\beta_2}$ tetramer.

Experimental procedures

Animals and acclimation

All experiments were carried out on adult and sexually mature weatherfish *Misgurnus fossilis* (Linnaeus 1758) family Cobitidae (loaches), order Cypriniformes (carps), class Actinopterygii (ray-finned fishes). Fish were acclimatized to either low (5 °C) or high (18 °C) temperatures for 20 days in flow-through aquaria. All fish were treated according to guidelines set down in [35].

Karyotyping and chromosome preparation technique

Fish were injected i.p. with 10 μL of 0.01% colchicine solution per gram of fresh body weight. After 5 h of exposure to 25 °C, fish was killed by cold anaesthesia. Gill tissue was homogenized in a hypotonic solution (75 mM KCl) and kept at 32 °C for 20 min. Air-dried preparations were made after repeating the routine aceto-alcohol fixation procedure three times and the chromosomes were stained with Giemsa.

Extraction of total RNA

Total RNA was extracted using TRIzol® reagent (Cat.No.15596-026, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol applying 50–100 mg of fresh muscle tissue per 1 mL TRIzol® reagent.

Northern hybridization of mRNA

mRNAs were fractioned in 1.5% agarose–formaldehyde gel (10 $V \cdot cm^{-1}$, 40 min), blotted onto Nitran® nylon membrane [Schleicher and Schuell, (New Hampshire, VE, USA) Cat. No.77413 N, with pore size 0.45 μm] and were cross-linked in UV light (254 nm) according to the manufacturer's instructions. The PCR fragments obtained from cDNAs and labeled with [^{32}P]dATP[αP] (3000 $mBq \cdot mM^{-1}$) by random priming (BRL kit) were used as a hybridization probe. The specific activity of the probe was 1×10^8 $c.p.m. \cdot \mu g^{-1}$ DNA. Hybridization was carried out in formaldehyde mixture (Quik and Hyb mix, Stratagene, LA Jolla, CA) at 68 °C, while the washing was carried out at 60 °C.

Determination of the LDH-A mRNA sequences

The following DNA/protein sequence analysis software has been used throughout the molecular biology work: DNASTAR LASERGENE (DNASTAR, Inc., Madison, WI, USA); VECTOR NTI 10.0 (Invitrogen); MACVECTOR 7.2 program package (Accelrys, Cambridge, UK); and CLONE MANAGER PROFESSIONAL SUITE (Scientific & Educational Software, Cary, NC, USA).

Fragments of the fish LDH-A gene were isolated by means of reverse transcription followed by PCR. Primers (nos 1–2, Table 2) were designed using conservative parts of the published cDNA sequences of the open reading frames of LDH-As from relative fish species (BLAST) as references. Reverse transcription was performed with Superscript RT (Invitrogen, Karlsruhe, Germany) and gene specific primers (A1F and A1R; Table 2) according to the manufacturer's instructions with mRNA as templates. In the following PCR, primer pair A1F/A1R has resulted in an ~720-nucleotide fragment. The cDNA was amplified with *Taq* DNA polymerase (Invitrogen) in the presence of 1.5 mM $MgCl_2$ (PCR conditions: 1 min denaturation at 94 °C, 1 min annealing at 59 °C and 1 min elongation at 72 °C, 30 cycles followed by a final amplification step of 8 min at 72 °C). The sequences from the gel-purified PCR products were determined by MWG-Biotech (Martinsried, Germany). The obtained conservative part of the LDH-A ORF was further used as a gene specific area for the RACE sequencing.

The full-length cDNA was determined using RACE, with the RLM-RACE kit (Ambion, Austin, TX) according to the manual. Isolated cDNA fragments were used to design 3′-RACE forward primers and 5′-RACE reverse primers with sequences, giving access to RACE fragments with a sufficient overlap to the first set of cDNA clones. RACE gene-specific primers were designed and their sequences are listed in Table 2 (nos 3–7). Purification, cloning and sequencing of the PCR fragments, isolation of plasmids were done as described earlier [36]. Assemblage of the clones yield the full-length cDNA sequences of at least two distinct LDH-A isoforms, which differ substantially in the coding sequence and length of the 3′-UTR. Therefore, isoform-specific PCR was carried out. RT-PCR and sequence determination were performed as described above.

Additional 'verifying' PCR was carried out to double check the RACE sequences. Primers were designed to get unique PCR products from each mRNA isoform containing the entire coding sequence, the forward universal primer was allocated in 5′-UTR, whereas reverse primers were sequence specific and allocated in 3′-UTRs, around the deletion in short mRNA and in the mismatched tail of long mRNA

(J1F, J2R and J3R; Table 2). The primers were designed to get unique PCR products from each mRNA isoform (1201 and 1385 bp; Table 2). cDNAs were synthesized from total RNAs using MuLV reverse transcriptase (New England BioLabs, Frankfurt am Main, Germany) according to the manufacturer's instructions. The reaction mixture was subjected to amplification wit h *Taq* DNA polymerase (PCR in temperature gradient: 1 cycle of 4 min at 95 °C; 30 cycles of 1 min at 95 °C/1.5 min at 54.5–65.5 °C /3 min at 72 °C; and the last cycle for 10 min at 72 °C and keep at 4 °C). Sequences from the gel-purified PCR products were determined by MWG-Biotech (Martinsried, Germany).

cDNA sequences of both isoforms of LDH-A mRNA can be obtained from GenBank under following accession numbers: DQ991254 for LDH-A^S and DQ991253 for LDH-A^L.

Quantification of LDH-A transcripts

For RT-PCR 100 μL of total RNA extracts (500–600 ng RNA·mL^{-1}) has been treated with DNase I (New England BioLabs, cat No MO303S; 2000 U·mL^{-1}) according to manufacturer's instructions and then RNA was purified using purification kit (PureLink™ Micro-to-Midi™ total RNA purification system, Invitrogen, cat. No. 12183–018) and standard procedures according to the manufacturer's protocol. Treatment resulted in complete elimination of the genomic DNA from the total RNA extracts. RNA samples without reverse transcriptase were used as a control.

Primers were designed to obtain unique PCR products from short or long forms of LDH-A mRNA (Table 2). Again, five-nucleotide insert and length differences in the 3′-UTR between mRNA isoforms were exploited to design isoform-specific primer pairs (H1F/H1R and H2F/H2R;). For control purposes, each total RNA sample was diluted to 1 and 0.1 ng·mL^{-1} and then 5 μL of the diluted sample was mixed with 18 μL Qiagen QPCR SybrGreen Master-Mix Kit (50 μL Qiagen-Master Mix 2×; 0.5 μL 100 μM forward primer; 0.5 μL 100 μM reverse primer; 1 μL reverse transcriptase; and 28 μL H_2O) for the real-time PCR (M × 3000P real-time PCR system, Stratagene): reverse transcription step, 30 min at 50 °C; initial denaturation, 15 min at 95 °C; 40 cycles, 15 s at 95 °C/30 s at 55 °C/30 s at 72 °C; and the final cycle, 1 min at 95 °C/30 s at 55 °C. The kinetics of real-time PCR were compared at $C_t = 0.0314$ dRn (Table 3) using values fitted to five-parameter asymmetric logistic equation with variable slope and corresponding 95% confidence intervals. For final confirmation, products of real-time PCR were separated in 1% agarose gel and were quantified by IMAGEQUANT TL v2005 using GeneRuler™ (#SM0331, Fermentas) as DNA standard.

Molecular analysis and modelling

SWISS-MODEL (http://swissmodel.expasy.org/SWISS-MODEL.html) was used for the homology search for translated weatherfish amino acid sequences among proteins of known structure based on running a pair-wise algorithm. High similarity between target amino acid sequences and skeletal muscle LDH-A from common carp *Cyprinus carpio* [1v6a.pdb; PDB (http://www.rcsb.org/pdb/Welcome.do) and PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum)] allowed SWISS-MODEL to predict the structure of weatherfish LDH-A, which was visualized using PDB Viewer (http://www.expasy.org/spdbv/) (Fig. 3).

Probabilities of tetramers

Assuming random assembly of LDH-A tetramers and direct proportionality between mRNA and protein contents, the probability of a particular LDH-A tetramer being assembled from two distinctive subunits (LDH-A^α and LDH-A^β) each with its own unique probability was calculated according to Bernoulli's binominal distribution:

$$P_n(m) = C_m^n p^m (1-p)^{n-m}$$

where

$$C_m^n = \frac{n!}{m!(n-m)!}$$

Where: n, total number of subunits in LDH-A (here $n = 4$, meaning tetrameric enzyme); m, number of α-subunits in a tetramer (e.g. LDH-$A^{\alpha}{}_3{}^{\beta}$ for $m = 3$); $P_n(m)$, probability of a tetramer possessing m α-subunits; C_m^n , combinatorial binominal coefficient for m-th tetramer (e.g. $C_m^n = 4$ for LDH-$A^{\alpha}{}_3{}^{\beta}$); p, probability of α-subunit (e.g. 100/120.4 at AT = 18 °C); $(1 - p)$, probability of β-subunit (e.g. 20.4/120.4 at AT = 18 °C).

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