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LDH-B enzyme expression: the mechanisms of altered gene expression in acclimation and evolutionary adaptation

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Segal, Jeff A., and Douglas L. Crawford. LDH-B enzyme expression: the mechanisms of altered gene expression in acclimation and evolutionary adaptation. *Am. J. Physiol.* 267 (*Regulatory Integrative Comp. Physiol.* 36): R1150–R1153, 1994.—The temperature-dependent expression of lactate dehydrogenase-B (LDH-B) was compared between two environmentally distinct populations of *Fundulus heteroclitus* acclimated to 10°C and 20°C. The variability in LDH-B protein expression both within and between populations is consistent with a model of thermal compensation. The northern population from the colder environment expresses a twofold greater amount of LDH-B protein than the warmer southern population at both acclimation temperatures. Correspondingly, both populations have 1.3-fold greater levels of the enzyme at an acclimation temperature of 10°C in comparison to 20°C. In 20°C-acclimated individuals there is a similar twofold difference between populations for LDH-B mRNA concentrations, and LDH-B protein and mRNA are highly correlated ($r = 0.81$). After acclimation to 10°C, this difference between populations is not seen and in the northern population there is no relationship between LDH-B mRNA and protein levels. Thus the molecular mechanism regulating LDH-B enzyme expression changes in response to temperature acclimation and is different between populations.

Fundulus heteroclitus; lactate dehydrogenase; enzyme concentration; messenger ribonucleic acid

TEMPERATURE IS PERHAPS the most critical environmental factor affecting an organism's physiology and biochemistry (9). A decrease in temperature causes an exponential decline in the rate of a chemical reaction: rates decrease by approximately one-half per 10°C (16). Ectothermic animals, whose body temperatures equilibrate with their thermal environments, often alter their physiological processes to compensate for the effects of temperature on metabolic function. One means of achieving compensatory changes in physiological processes is to alter the activity of specific enzymes by changing their concentration (for a review see Refs. 8 and 9). The mechanism responsible for these changes in gene expression can operate at any of several levels: transcription, mRNA stability or processing, translation, posttranslational modification, and protein stability or transport (11). Such changes in enzyme expression can be achieved by two general modes of compensation: physiological acclimation and evolutionary adaptation. Physiological acclimation occurs within an individual's lifetime and is typically reversible (7, 9, 15). Evolutionary adaptation

acts via selection on the genetic differences within or between populations. While the presence of acclimation responses via altered gene expression and the mechanistic bases of these responses are well documented (8, 10, 12, 17, 18), seldom are these processes compared between populations. By comparing different populations subjected to various acclimation regimens, we can ascertain whether the populations have similar acclimation responses for 1) the concentration of enzyme and 2) the molecular mechanisms regulating this enzyme expression.

The different thermal environments of northern and southern populations of the teleost fish *Fundulus heteroclitus* make it an ideal organism to study the role of altered enzyme expression in physiological and evolutionary adaptation to temperature. Although water temperatures vary on a seasonal basis in each environment, northern populations experience consistently cooler waters at all times than their southern counterparts (Ref. 6 and supported by unpublished data from the Environmental Monitoring Project, Maine Department of Marine Resources and Marine Institute Hydrological Monitoring Project, University of Georgia). To compensate for these differences in aquatic temperature, hepatocytes of fish from a northern Maine population have twice as much lactate dehydrogenase-B (LDH-B) protein and LDH-B mRNA as hepatocytes of fish from a southern Georgia population (3) due to a change in the *Ldh-B* transcription rate (5). This compensatory change in *Ldh-B* expression remains after long-term acclimation to a common temperature of 20°C, indicating that the variation in expression is not due to physiological acclimation and therefore is most likely an evolved difference between the two populations (3). The observed twofold difference between populations in *Ldh-B* expression and the molecular bases underlying this difference are thought to be maintained at all acclimation temperatures; however, this is an untested hypothesis. The present study was undertaken to ascertain 1) if hepatocyte LDH-B enzyme expression is modulated in response to temperature acclimation in a consistent manner between the two populations, thus maintaining the twofold difference and, if so, 2) if the molecular mechanisms regulating this modulated expression are similarly consistent between the two populations. To address these issues we have quantified *Ldh-B* expression (protein and mRNA levels) in the two populations acclimated to both 10°C and 20°C.

METHODS

Animals. *F. heteroclitus* were collected from Wiscasset, Maine (44.2°N latitude) and Sapelo Island, Georgia (31.4°N latitude) in September 1992. They were maintained in the laboratory in 15% seawater and fed ad libitum. Both populations were held in the laboratory for several weeks at 20°C on a 12:12-h light-dark (LD) cycle and then subjected to a pseudo-winter at 4°C with a 8:16-h LD cycle for 6 wk. Fish were then acclimated to 10°C or 20°C with a 14:10-h LD cycle for 8 to 12 wk. This protocol induced a reproductive phase concurrently in all study groups; thus potential seasonal effects in the field-caught animals were minimized. Fish were no longer in a reproductive state at the time of experimentation. Fish were always killed early in the morning before the daily feeding.

Allelic isozymes. Populations in Maine and Georgia are virtually fixed for different LDH-B allelic isozymes (LDH-Ba in the South and LDH-Bb in the North; Ref. 13). These alleles have different biochemical kinetic parameters (14) as well as different DNA sequences (1). The methods used in this study minimized the effects this allelic variation could have on the quantitative assays (see below).

Enzyme assays. Fish (10 Maine and 10 Georgia from both acclimation temperatures; $n = 40$) were weighed, killed, and their livers dissected and immediately frozen in liquid nitrogen. A portion of each liver was weighed and then homogenized using a glass-glass homogenizer to make a 5% solution in H-buffer [100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 7.5, 10 mM KCl, 5 mM EDTA, and 1 mM dithiothreitol (DTT)]. This crude homogenate was centrifuged at 16,000 *g* at 4°C for 10 min to separate solid material and fat. The resulting homogenate was diluted 1/100 with H buffer for the enzyme assays. Homogenates were kept on ice at all times.

LDH-B maximal activity (μmol of NADH reduced $\cdot \text{min}^{-1} \cdot \text{mg}$ protein $^{-1}$) was determined spectrophotometrically at 340 nm by measuring the initial reaction rate at 25°C at saturating conditions for substrate and cofactor. The total reaction volume was 100 μl (100 mM HEPES, pH 7.5, 10 mM KCl, 5 mM EDTA, 1 mM DTT, 0.32 mM NADH, 2.62 mM pyruvate, and 0.005% liver homogenate). Enzyme assays were performed on a Molecular Devices Thermo-Max (Menlo Park, CA) microplate reader. Each sample was assayed in triplicate with a control that lacked pyruvate. Four individuals were assayed at a time, consisting of two pairs of Maine and Georgia fish.

The mean enzyme activity for each individual was divided by the total protein concentration per reaction to standardize for biological and experimental variation in the liver homogenates. Protein assays were performed on the same diluted homogenate that was the source for the enzyme assay using the Pierce micro BCA protein assay kit with bovine serum albumin as a standard.

mRNA assays. Total RNA was isolated from livers used in enzyme assays ($n = 34$). The livers were first homogenized in 9 ml of Chaos buffer (4.5 M guanidinium thiocyanate, 2% sarcosyl, 50 mM EDTA, 25 mM Tris-HCl pH 7.5, 0.1 M β -mercaptoethanol, 0.2% antifoam A). This homogenate was then centrifuged at 12,000 *g* to remove insoluble material. CsCl was added to the supernatant (0.2 g/ml), which was then layered on top of 3.5 ml of 5.7 M CsCl and centrifuged at 50,000 *g* for 5.5 h. Typical yields were between 0.5 and 2.0 mg of RNA.

Northern blots were performed on all samples to verify that the RNAs were not degraded. Total RNA was electrophoresed on a 1.2% denaturing gel and transferred by capillary action onto a nylon membrane (Hybond Membrane). Negative (yeast RNA) and positive (a linearized clone of LDH-B cDNA) controls were included on each blot. The membrane was

hybridized at 65°C for 24 h in the presence of an excess of a ^{32}P -labeled LDH-Bb cDNA probe [a 440-base pair (bp) fragment generated with the DNA restriction enzyme *Pst*1; Ref. 2]. This 440-bp fragment contains at the most 4 bp mismatches between allelic forms of LDH-B (0.9% difference; Ref. 1) and thus hybridizes equally to both allelic mRNAs. The membrane was exposed to autoradiograph film with an intensifier for 24 h.

A dot-blot analysis was done to quantify the amount of LDH-B mRNA. The concentration of each sample of total RNA was determined in triplicate by spectrophotometric analysis. Exactly 20 μg of total RNA from each individual was loaded and vacuum blotted onto a nylon membrane. Three dots of 20 μg of yeast RNA were also blotted as negative controls. Synthetic RNA copies of a DNA template (cRNA) were made by *in vitro* transcription of linearized LDH-Ba and Bb clones [according to the Ambion Maxiscript (Austin, TX) *in vitro* transcription kit] in the presence of [^3H]-UTP with the use of SP6 DNA polymerase. These cRNA are used as standards, and the exact amount of this cRNA was determined by quantifying the amount of [^3H]-UTP incorporated. Equal concentrations of the two LDH-B allele cRNA types were mixed and various dilutions (10, 5, 2.5, 1, 0.5, 0.25, and 0.1 ng) were prepared to serve as positive controls and standards for quantification of the samples. Each of these standards was brought up in 20 μg of yeast RNA and then treated and loaded in the same way as the other samples. The dot-blot membrane was hybridized with an excess of probe and visualized in the same manner as the Northern membrane discussed above. Quantification of LDH-B mRNA was accomplished by measuring the amount of ^{32}P hybridized to each dot using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA). All ^{32}P quantifications had the mean negative control subtracted and the standards were used to convert these data into picograms of LDH-B mRNA. These data are expressed in units of picograms of LDH-B mRNA per micrograms of total RNA.

Statistics. Analysis of variance (ANOVA), two-way ANOVAs, analysis of covariance (ANCOVA), two-way ANCOVAs, and linear regression were used when appropriate (see RESULTS AND DISCUSSION). All statistical analyses were carried out in the MINITAB statistics program.

RESULTS AND DISCUSSION

LDH-B protein concentration. Maximal enzyme activities were determined under conditions that exclude the effect of allelic differences in kinetic constants (saturating conditions in which both alleles have the same catalytic rate constant; Refs. 13, 14); these maximal activities were shown previously to be highly correlated with the absolute concentration of LDH-B protein as determined by immunoassays ($r = 0.86$, $P < 0.01$; Ref. 3). Thus these assays are a direct measure of enzyme concentration.

Body weight is a significant covariate ($P < 0.01$) for LDH-B maximal activity; thus a two-way ANCOVA with body weight as the covariate was used to analyze the effect of acclimation temperature and population on LDH-B maximal activity. LDH-B maximal activity is significantly different between populations and temperatures (population: $P < 0.001$; temperature: $P = 0.005$) but the interaction between these two parameters is not significant (population \times temperature: $P > 0.5$). These results indicate 1) that the response to temperature is not population dependent [i.e., the 1.3-fold difference in LDH-B₄ concentration between acclimation tempera-

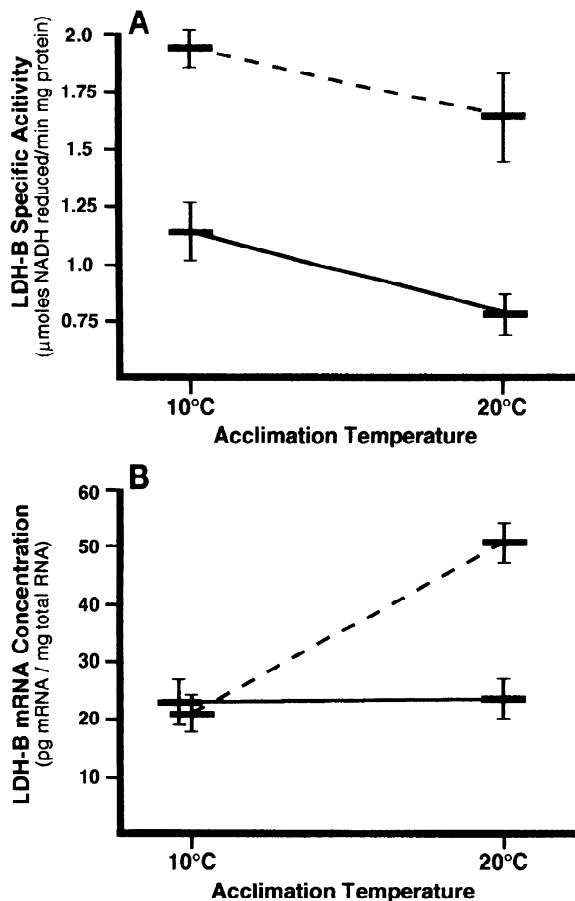


Fig. 1. LDH-B expression. A: plot of LDH-B specific activity vs. acclimation temperature for both populations. There are similar (not statistically different) patterns of acclimation for both populations whereby LDH-B specific activity increases by ~1.3-fold after long-term acclimation to 10°C. B: plot of LDH-B mRNA concentration vs. acclimation temperature for both populations. The Georgia population has a similar concentration of LDH-B mRNA at both acclimation temperatures, whereas the Maine population experiences a 0.5-fold decrease in LDH-B mRNA after acclimation to 10°C. Points are means \pm SE. Means and variance at 10°C are offset for clarity. For A, SEs were derived from the residuals obtained from the ANCOVA. Dashed lines, Maine population; solid lines, Georgia population.

tures is the same in both populations (Fig. 1A)] and 2) the difference between populations is not temperature dependent [i.e., the 1.8-fold difference between populations is the same at both temperatures (Fig. 1A)]. This effect of acclimation on LDH-B₄ concentration is the same as was previously determined for a population of *F. heteroclitus* from an intermediate latitude (New Jersey; Refs. 3 and 4). Thus populations of *F. heteroclitus* are capable of responding similarly to temperature fluctuations via physiological acclimation and therefore fish from the northern environments achieve consistently higher LDH-B₄ levels at both temperatures measured. This is presumably an evolved response to the colder environments they live in.

LDH-B mRNA concentrations. We quantified LDH-B mRNA from the same individuals used for the enzyme assays. These assays were used to determine if the effect of temperature acclimation observed at the protein level (Fig. 1A) would be similarly reflected in the molecular mechanisms used to achieve these responses. There was

no effect of body weight on mRNA concentration (ANCOVA: body weight $P > 0.45$) and thus a two-way ANOVA was used to test for significant differences between groups. Both population and temperature had a significant effect on mRNA concentration (population: $P < 0.001$; temperature: $P < 0.001$); however, there is also a strong interaction between these two parameters (population \times temperature: $P < 0.001$) and consequently the effect of population and temperature was analyzed separately. At 20°C the Maine population has twofold higher LDH-B mRNA concentrations than the Georgia population ($P < 0.001$), but at 10°C the two populations do not differ significantly ($P > 0.7$; Fig. 1B). There is no significant difference in LDH-B mRNA concentration between acclimation temperatures for the Georgia population ($P > 0.8$), while the Maine population at 10°C has significantly less LDH-B mRNA (~50%) than at 20°C ($P < 0.001$; Fig. 1B).

There is a disparity between the protein level response and the molecular mechanisms that regulate this response in the two populations. This disparity arises due to the drop in LDH-B mRNA concentration in Maine fish after acclimation to 10°C, while LDH-B protein concentration is elevated. The Georgia fish, however, maintain a constant LDH-B mRNA concentration after acclimation to 10°C. To address this distinction, we analyzed the relationship between the concentration of LDH-B protein and mRNA. In 20°C-acclimated fish from both populations, LDH-B protein and mRNA concentrations are highly correlated ($r = 0.81$, $P < 0.01$), as reported previously (3) and as is the case for transcription rate with LDH-B₄ concentration (5). Yet in 10°C-acclimated fish there is no correlation between LDH-B mRNA and protein concentrations ($r = 0.18$, $P > 0.5$). Separate analyses of the 10°C-acclimated fish reveal that the correlation between LDH-B protein and mRNA concentrations is much stronger for Georgia fish ($r = 0.57$, $P = 0.086$) than for Maine fish ($r = 0.22$, $P > 0.25$) although neither is significant. It is evident that in 10°C-acclimated Maine fish the LDH-B₄ protein concentration is no longer regulated at the mRNA level as it is in 20°C-acclimated fish. Instead it must be regulated by either translational or posttranslational mechanisms. The situation for 10°C-acclimated Georgia fish is less certain. It appears that LDH-B protein concentration is dependent on LDH-B mRNA, although perhaps not as strictly as in 20°C-acclimated fish. Thus at least for the Maine population the molecular mechanism regulating LDH-B enzyme expression changes in response to temperature acclimation.

It could be argued that the differences observed in 10°C fish simply reflect a temperature-dependent difference in protein stability between the northern and southern LDH-B allozymes. While differences in protein stability may occur (14), these data indicate that they must be accompanied by additional regulatory processes that act to reduce the level of LDH-B mRNA in 10°C Maine fish but not in 10°C Georgia fish. Thus there are populational differences in the regulatory mechanisms governing enzyme expression that exist in response to temperature acclimation.

The variation in *Ldh-B* transcription rate (5) is thought to be an evolved (i.e., genetically based) adaptation because the difference between populations was maintained after long-term acclimation to 20°C and thus is not due to physiological acclimation (3, 4, 5). This supposition is supported by recent work demonstrating that the DNA sequence differences between populations in the 5' regulatory region of the *Ldh-B* gene are functionally important because they affect the binding of *trans*-acting proteins (P. Schulte, J. A. Segal, D. A. Powers, and D. L. Crawford, unpublished observations). These observed differences in the genome indicate that the increased transcription rate between populations at 20°C is genetically based. Yet in this study, although the amount of LDH-B₄ is consistently higher in the northern population, the amount of LDH-B mRNA (and therefore transcription rate) is not always responsible for maintaining this difference. These seemingly disparate results suggest that the role of regulatory elements affecting transcription rate is modulated by environmental and physiological conditions. Thus in *F. heteroclitus* the evolved mechanisms regulating LDH-B₄ expression only operate under certain environmental conditions.

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