

Evolutionary Adaptation to Different Thermal Environments via Transcriptional Regulation¹

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Populations of the teleost fish *Fundulus heteroclitus* are subjected to the clinal variation in environmental temperatures that occurs along the eastern seacoast of North America. In concordance with this change in temperature is the clinal variation in the enzyme concentration of the heart-type lactate dehydrogenase (LDH-B; E.C.1.1.1.27). Previously we have shown that the compensating change in the LDH-B enzyme concentration is due to a change in the amount of LDH-B mRNA, but we did not define whether this was due to differences in mRNA stability or to differences in rate of transcription. The results presented here help clarify the molecular mechanism responsible for the variation in *Ldh-B* gene expression: the rate of transcription from the *Ldh-B* locus is significantly different between populations, and this difference is responsible for the compensatory change in LDH-B enzyme concentration.

Introduction

The fish *Fundulus heteroclitus* is distributed along one of the steepest thermal gradients in the world, the eastern seaboard of North America, which has a 1°C change/degree latitude. Near the extremes of its natural distribution in the United States (Maine and Georgia), *F. heteroclitus* experiences a difference of ~12.6°C in mean annual temperatures. Adapting to these environmental conditions could involve mechanisms that maintain individual homeostasis (physiological acclimation) and mechanisms that act on heritable differences within or between species (evolutionary adaptation). At the molecular level, this would include altering the activity of specific enzymes via changes in kinetic rate constants or active enzyme concentrations (Hochachka and Somero 1984). This can be accomplished by one or more of several physiological mechanisms: allosteric modifiers, protein-protein interactions, phosphorylations, and altering enzyme concentrations (Storey 1988; Somero and Hand 1990; Granner and Pilkis 1990). Alternatively, compensation to different environmental temperatures could be accomplished evolutionarily by altering intrinsic enzymatic rate constants (e.g., K_m or k_{cat}) or by a heritable change in the enzyme concentration.

Several evolutionary adaptations have been described in *F. heteroclitus* (Powers et al. 1991). Populations of *F. heteroclitus* in Maine and Georgia are essentially fixed for two different codominant alleles at the heart-type lactate dehydrogenase (LDH-B) locus (*Ldh-B* locus). The allelic isozymes encoded by this locus have different temperature-dependent kinetic characteristics (Place and Powers 1979, 1984) due to a few amino acid replacements (unpublished data). These kinetic differences appear

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to be evolutionarily important because they are associated with differences in swimming performance, developmental rates, metabolic flux, and survival at high temperatures (DiMichele and Powers 1982a, 1982b, 1991; Powers et al. 1983; Paynter et al. 1991). Recent evidence confirms that the early-developmental variation associated with the *Ldh-B* locus is due directly to differences between the allelic isozymes (DiMichele et al. 1991).

In addition to differences in kinetic parameters, fish in the colder Maine population have approximately twice as much of the LDH-B enzyme as their southern counterpart in Georgia (Crawford and Powers 1989b). This difference is due to a higher concentration of the LDH-B mRNA in the northern population (Crawford and Powers 1989b). The greater concentration of LDH-B mRNA and the resulting enzyme concentration in the northern population could be due to an increased stability of its mRNA or to a higher rate of transcription from the *Ldh-B* locus. To distinguish between these alternatives, the transcription rates were determined from individuals in each population, after long-term acclimation. Long-term acclimation to a common temperature removes the effect of physiological acclimation, and thus any remaining differences should be due to heritable evolutionary adaptation. The LDH-B mRNA and enzyme activity were also determined from the same individuals, to be consistent with our previous studies (Crawford and Powers 1989a, 1989b).

Methods

Fundulus heteroclitus from Bar Harbor, Maine, and Sapelo Island, Ga., were acclimated to 20°C, 15 parts/1,000 parts of seawater, 14:10 light:dark cycle for a minimum of 24 wk. All assays were done in pairs, one member from each population.

All assays used liver tissue. Liver only expresses the *Ldh-B* locus in *F. heteroclitus*. The LDH-B enzyme concentration was assessed by determining the specific activity [μmol of reduced NADH/(min/mg of total protein)] spectrophotometrically under saturating concentration for both pyruvate and NADH at 25°C ($N = 9$, for each population). All the LDH-B allozymes have the same k_{cat} under these conditions, and thus the specific activities should be solely dependent on enzyme concentrations. This assertion was verified by immunoassay: LDH-B specific activities are highly correlated with LDH-B enzyme concentrations, as determined by immunoassays ($r = 0.86$; $P < 0.01$; Crawford and Powers 1989b).

LDH-B mRNA concentrations were determined by liquid scintillation counting of RNA dot blots ($N \geq 10$, for each population). Twenty micrograms of total RNA from each individual were vacuum blotted onto a nylon membrane. The concentration of LDH-B mRNA was assayed using a *Pst*I 440-bp LDH-B^b fragment as the ³²P-labeled hybridization probe (Crawford et al. 1989). This restriction fragment contains <0.5% (2/440) difference between the two alleles (authors' unpublished data). The hybridization conditions were the same as those described by Crawford and Powers (1989b) and were chosen because only a single-molecular-weight mRNA species is identified under these conditions. Prior to the RNA dot-blot analysis, the quality of all RNAs was determined by northern analysis. Only those RNAs that yielded a discreet band were used for quantitative analysis. In addition to the RNA samples from *F. heteroclitus*, three samples of yeast RNA and two samples of tRNA were added as negative controls [the mean numbers of disintegrations per min (dpm) from these negative controls were subtracted from all other samples].

Transcription rates were determined by nuclear run-on assays ($n = 9$, for each population). Nuclei from *F. heteroclitus* livers were isolated via centrifugation on a

sucrose step gradient and were frozen in a glycerol buffer (Love et al. 1985). To perform the transcriptional assay, nuclei were thawed on ice, whereupon ATP, GTP, and CTP (final concentration 0.5 mM each), 0.5 of mCi ^{32}P UTP (final concentration 0.5 μM), and reaction buffer were added. The reaction was incubated for 30 min at 20°C. Under these standard conditions, no new initiation should occur and only elongated RNAs are labeled. Newly transcribed RNAs were isolated according to a modification of Chomczynski and Sacchi's (1987) procedures. Modifications included acid precipitating all of the RNA after the last ethanol precipitation. The newly transcribed labeled RNAs were selectively hybridized to membranes containing 20 μg of one of the following cDNAs: *F. heteroclitus* LDH-B cDNA (10 μg of each allele), *F. heteroclitus* actin, or mouse β -tubulin. Negative controls included the plasmid pBSKS and nitrate reductase cDNA (provided by R. Albertes and J. Smith, Stanford University). These filters were hybridized in a small volume (3 ml) in petite glass vials. The amount of labeled RNA used for hybridization was determined by acid precipitation and was routinely 5×10^6 dpm. This amount of newly transcribed RNA did not saturate the filters, as was determined by varying the amount of labeled RNA added to the hybridization (10^6 – 2×10^7 dpm) or by spiking the samples with labeled LDH-B cRNA (LDH-B RNA produced from an SP6 vector). Filters were hybridized for 36 h (hybridization efficiency, as assayed with ^{32}P labeled LDH-B cRNA, did not increase after 24 h). The amount of RNA hybridized was determined by placing filters in scintillation vials containing 0.4 ml of 0.1 N NaOH at 55°C for 15 min and then adding scintillation cocktail and counting for 10 min. Counting efficiencies were determined, and counts per minute were converted to dpm. The dpm's from the negative controls were subtracted from all dpm's reported.

Results

The northern population of *Fundulus heteroclitus*, acclimated to 20°C, had significantly greater concentrations of both LDH-B enzyme and its mRNA than did the southern population (table 1). To determine whether this compensatory change in LDH-B was due to altered gene regulation, the transcription rate was ascertained in these same acclimated individuals. The results from these experiments demonstrated that fish from the northern population had a greater transcription rate from the *Ldh-*

Table 1
Between-Population Variation in LDH-B Expression

LDH-B EXPRESSION	MEAN (\pm SE)		RATIO (Northern/ Southern)	ANOVA (<i>P</i>)
	Northern	Southern		
Specific activity (μmol reduced NADH/min/mg total protein)	2.48 (\pm 0.30)	1.31 (\pm 0.08)	1.89	<0.01
mRNA (dpm/20 μg RNA) . . .	248.9 (\pm 46.7) ^a	121.6 (\pm 18.5) ^b	2.04	<0.028
Transcription rate (dpm/10 ⁶ dpm hybridized) ^c	5.47 (\pm 0.54)	2.21 (\pm 0.45)	2.47	<0.001

^a *N* = 11.

^b *N* = 10.

^c 3–7 million dpm's from the nuclear runoff were hybridized.

B locus than did acclimated fish from the southern population (table 1). Determination of the transcription rate utilized cDNAs for both LDH-B alleles and thus represents the total synthesis from this locus. Unlike the data for the *Ldh-B* locus, there were no differences between populations, either in the total rate of RNA synthesis (dpm of total RNA synthesized/ μ g of nuclear DNA; ANOVA, $P > 0.5$) or in the transcription rates of actin or b-tubulin (ANOVA, $P > 0.25$ and $P > 0.12$, respectively; $N = 9$ for each population). For actin, the net dpm/million counts hybridized were 5.1 [standard error (SE) = 1.64] and 4.6 (SE = 1.05) for the northern and southern populations, respectively. For β -tubulin, the net dpm/million counts hybridized were 5.64 (SE = 0.91) and 3.81 (SE = 0.67) for the northern and southern population, respectively. Therefore, the greater LDH-B transcription rate appears to be specific for the *Ldh-B* locus and, perhaps, for other loci not assayed.

In the northern population the increase in LDH-B enzyme and its mRNA is similar to the increase in transcription rate from this locus (table 1). Thus, it would appear that the between-population variation in LDH-B enzyme concentration is the result of increased transcription. This supposition is supported by the significant correlation between the compensatory change in the LDH-B concentration with transcription rate (fig. 1; $r = 0.74$, $P < 0.01$).

Difference in body size has been demonstrated to affect enzyme concentration (Somero and Childress 1980). Typically, larger organisms have lower specific activities (units of enzyme activity/unit of body mass), but the opposite has also been demonstrated (Somero and Childress 1980). Thus, because southern fish tend to be larger

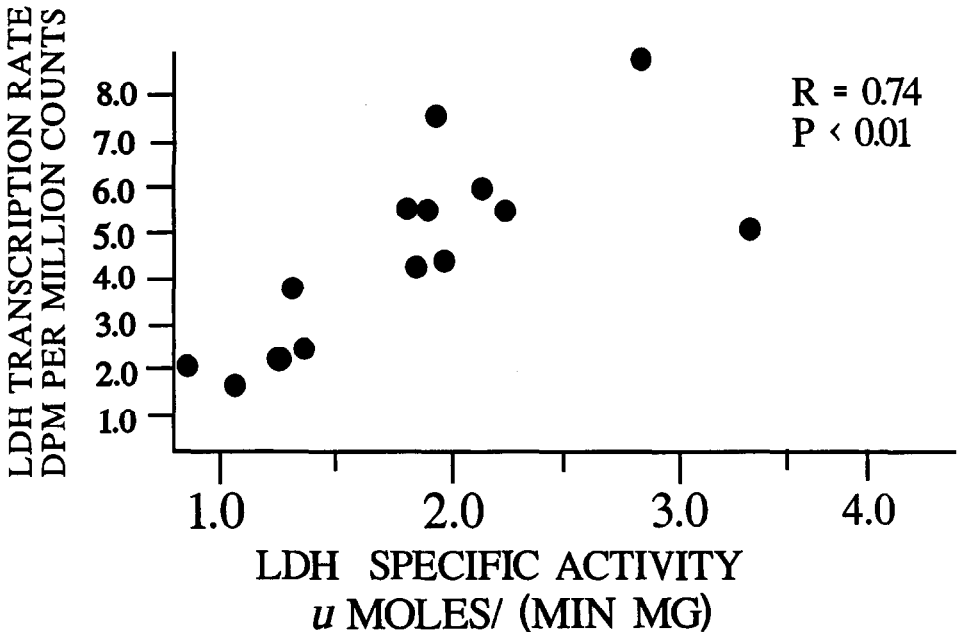


FIG. 1.—Relationship between the transcription rate from the *Ldh-B* locus and the amount of LDH-B enzyme. Transcription rates (net dpm/million dpm hybridized) for both populations are significantly correlated ($r = 0.74$, $P < 0.01$; no. of paired samples = 14) to the amount of LDH-B enzyme determined spectrophotometrically [μ mol of reduced NADH/(minute/mg of total protein)]. The correlation between these two parameters is not explained by metabolic scaling: body mass is not related to either the transcription rate or the amount of LDH-B enzyme (ANCOVA; $P > 0.25$).

than their northern counterparts, and because enzyme concentration may vary with body mass, one might expect that the northern fish would have more LDH-B, per milligram of total protein or per unit of wet weight, independent of the effect of temperature. This difference in size does *not* appear to contribute to the differences in the amount of product from the *Ldh-B* locus, because there is no significant relationship between body mass and these parameters (regression of body mass vs. the net dpm/million counts hybridizes for both populations or within either population have $R^2 < 10\%$; $P > 0.5$). In addition, as demonstrated elsewhere (Crawford and Powers 1989b), the difference in either the LDH-B activity or LDH-B mRNA concentration is not due to body-mass differences between the populations [ANCOVA, $P > 0.25$ (with body mass the covariate)]. Thus, populations of *F. heteroclitus* appear to achieve thermal compensation by the transcriptional regulation of the *Ldh-B* locus.

Discussion

Fundulus heteroclitus from the two populations used for this study experience a difference of 12.6°C in their mean annual temperature. In order for the colder, northern fish to maintain the same reaction velocities as do their southern counterparts, twice as much enzyme is needed for each 10°C decrease in environmental temperature. Consistent with that adaptive strategy, we found that the amounts of LDH-B mRNA and enzyme in livers of *F. heteroclitus* from Maine were approximately twice those in livers of *F. heteroclitus* collected from Georgia (Crawford and Powers 1989a, 1989b; Crawford et al. 1990). The demonstration of an increased transcription rate from the *Ldh-B* locus in the northern population helps clarify the molecular mechanism responsible for the differences in the amount of both LDH-B mRNA and enzyme found in our previous studies.

The between-population differences in *Ldh-B* transcription rates, LDH-B mRNA, and enzyme concentrations are described as an evolutionary adaptation because they persist after 24 wk of acclimation and therefore are not due to physiological acclimation. Sidell et al. (1973) demonstrated that 4 wk were adequate for temperature acclimation in fish. In similar studies, compensatory changes in LDH-B activities in *F. heteroclitus* were completed within a 4-wk period when these fish were subjected to either hypoxia (Greaney et al. 1980) or temperature acclimation (D.A.P., unpublished). Therefore, 24 wk of acclimation is more than adequate to remove any physiological acclimatory effects. Thus, the between-population differences in *Ldh-B* gene expression are thought to be genetically based. These experiments, however, do not rule out the possibility that environmental effects on development cause the changes in *Ldh-B* gene expression. Genetic studies are currently underway to address that possibility.

Northern *F. heteroclitus*, acclimated to 20°C, had a significantly higher transcription rate from the *Ldh-B* locus [5.5 dpm/10⁶ (SE = 0.58 dpm/10⁶)] than did acclimated fish from the southern population [2.2 dpm/10⁶ (SE = 0.44 dpm/10⁶); table 1]. The greater transcription rate in northern populations results in a greater concentration of LDH-B enzyme (fig. 1). This difference does not appear to be due to a between-population difference in the number of gene copies, since both populations have quantitatively similar amounts of LDH-B genomic DNA (Crawford and Powers 1989b). Nor is the variation in transcription rate due to an overall increase in transcription, because there are no significant differences either in the total rate of RNA synthesis or in the rate of actin or β -tubulin synthesis. Therefore, the greater *Ldh-B* transcription rate was not due to a general increase in the rate of RNA synthesis per se but appears to be specific for *Ldh-B* and, perhaps, a few other loci. To our knowledge,

this is the first example of between-population variation in the transcription rate, and it is important that this variation acts to compensate for the differences in environmental temperatures.

The transcription-rate variation between populations of *F. heteroclitus* could be due to differences in DNA regulatory sequences linked to *Ldh-B* alleles, to the *trans*-acting factors that interact with these sequences, or both. This is being investigated.

Between-population variation in enzyme concentrations that is due to differential gene regulation has also been demonstrated to occur at the alcohol dehydrogenase (ADH) locus (*Adh* locus) in *Drosophila melanogaster* (reviewed in Laurie-Ahlberg 1985; Chambers 1988). The differences in ADH concentration have been attributed to *trans*-acting elements modifying the concentration of mRNA and protein (McDonald and Ayala 1978; Anderson and McDonald 1983; King and McDonald 1987), to *cis*-acting elements acting on both the mRNA and protein (Schoot et al. 1988), or to both the translatability of the mRNA and protein stability (Laurie and Stam 1988). The variation in *Adh* gene expression is apparently selectively important. The concentration of ADH varies between natural populations of flies that are exposed to different amounts of ethanol (Ward and Herbert 1972; Anderson and Gibson 1985), and survival of *D. melanogaster* in an ethanol-enriched environment is dependent on the level of ADH expression (McDonald et al. 1977; McDonald and Ayala 1978).

Although the variation in *Ldh-B* gene expression has not yet been shown to effect fitness or physiological processes, it does have a major affect on enzyme reaction rates. That is, the LDH-B reaction rates are essentially equal for the northern and southern populations at their appropriate environmental temperature, substrate concentration, and pH (Crawford and Powers 1989*b*). It is important that the variation in enzyme expression contributes more to this compensation than does the variation in the kinetic parameters. The enzyme-activity variation due to the differences in LDH-B kinetic parameters is apparently an important adaptational mechanism affecting several physiological or developmental processes (DiMichele and Powers 1982*a*, 1982*b*; Powers et al. 1983; Paynter et al. 1991). Thus, the evolutionary modification of *Ldh-B* gene expression may also be an important adaptational mechanism.

The variation in *Ldh-B* gene expression may be indicative of the importance that altered gene expression has for evolutionary adaptation. Gene regulation allows enzyme activity to vary independently of changes in the amino acids of a protein. Replacements of the amino acids in an enzyme are necessarily constrained because of the limited number of residues that will both fulfill the necessary catalytic function and maintain secondary and tertiary structure. In addition, some enzymes approach their maximum catalytic efficiency (Fersht 1977), and thus the only avenue open for increasing enzyme reaction rates is via a change in enzyme concentration. Altering enzymatic activity by varying its expression may therefore be a more prevalent mechanism for evolutionary adaptation than has previously been envisioned (Wilson 1976). It is important to establish the relative role of differential gene expression as an evolutionary mechanism for population adaptation. Delineating the molecular mechanisms responsible for this difference in gene regulation will not only provide insight concerning the adaptive nature of such regulatory events but will also enrich our understanding of how gene expression is controlled.

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