

# Molecular basis of evolutionary adaptation at the lactate dehydrogenase-B locus in the fish *Fundulus heteroclitus*

(reaction rate/specific activity/thermal adaptation/acclimation)

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**ABSTRACT** At the extremes of its natural distribution, populations of the common killifish *Fundulus heteroclitus* experience a difference of more than 15°C in mean annual temperature. These populations are virtually fixed for two different codominant alleles at the heart-type lactate dehydrogenase locus (*Ldh-B*) which code for allozymes with different and adaptive kinetic responses to temperature. Two populations near the extremes of the species range (i.e., Maine and Georgia) were further studied for thermal adaptation at this locus. In the absence of any kinetic differences one would predict that to maintain a constant reaction velocity, 2 to 3 times as much enzyme would be required for each 10°C decrease in environmental temperature. Consistent with this adaptive strategy and in addition to the adaptive kinetic characteristics, the LDH-B<sub>4</sub> enzyme (EC 1.1.1.27) concentration and its mRNA concentration were approximately twice as great in the northern population as in the southern population. Acclimation experiments allow us to conclude that these differences are due to a combination of fixed genetic traits (evolutionary adaptation) and plastic responses to temperature (physiological acclimation). Furthermore, our calculations show that the LDH-B<sub>4</sub> reaction velocities are essentially equivalent for these two populations, even though they live in significantly different thermal environments.

Populations of the common killifish *Fundulus heteroclitus* are distributed along the eastern coastal waters of North America. This region has one of the steepest thermal gradients in the world, with a 1°C change per degree latitude or a difference of more than 15°C in the mean annual temperature ( $t_M$ ) between the latitudinal extremes of the species' natural distribution (1). A number of enzyme-encoding loci have clinal variation in allelic frequency that are concordant with latitudinal changes in  $t_M$  (2-4). The most extensively studied is the *Ldh-B* locus, which encodes the heart-type lactate dehydrogenase (LDH-B<sub>4</sub>; L-lactate:NAD<sup>+</sup> oxidoreductase, EC 1.1.1.27). Populations at the latitudinal extremes are virtually fixed for two different codominant alleles (*Ldh-B<sup>b</sup>* in northern populations and *Ldh-B<sup>a</sup>* in southern populations).

These LDH-B<sub>4</sub> allozymes are kinetically different in several characteristics such as substrate affinities, reaction rates, heat stabilities, and inhibition constants (5-7). For example, at 10°C the LDH-B<sub>4</sub><sup>b</sup> has a greater second-order rate constant (i.e.,  $k_{cat}/K_m$ ) than that of LDH-B<sub>4</sub><sup>a</sup>; the opposite is true above 27°C (5, 7). Fish with the northern allozyme, LDH-B<sub>4</sub><sup>b</sup>, swim faster than their southern counterparts at 10°C, suggesting that the kinetic differences affect swimming performance (8, 9). These kinetic differences also appear to affect both the timing of developmental events and the metabolic rates of developing embryos (9, 10). Preliminary data suggest that metabolic rate is altered in a predictable

manner, depending upon the type of LDH-B<sub>4</sub> microinjected into fertilized eggs (L. DiMichele, Texas A & M; personal communication). Moreover, embryos show differential mortality at high temperatures consistent with the *Ldh-B* allelic frequency and environmental  $t_M$  at southern latitudes (L. DiMichele and D.A.P., unpublished data). Taken together, these data strongly suggest that this locus is affected by environmental temperature in an adaptationally important manner.

At lower environmental temperatures in the absence of changes in isozyme types, more enzyme is required to maintain a constant reaction velocity; for each decrease of 10°C 2 or 3 times as much enzyme is required (11, 12). Consistent with this expectation, our preliminary results showed that killifish from Maine have a greater LDH-B<sub>4</sub> concentration than those from Georgia (13). However, given our prior studies of the differences in kinetic properties of the LDH-B<sub>4</sub> allozymes, these results were surprising, and they suggested that the temperature-adaptation response at this locus was more complex than had been previously expected. In addition to different genes encoding the LDH-B<sub>4</sub> primary structures, evolutionary adaptation (i.e., genetic adaptation), physiological acclimation, or both could be the source of the variation in enzyme concentration. To distinguish among these possibilities, we further analyzed the amount of LDH-B<sub>4</sub> protein and LDH-B mRNA in acclimated fish from populations near the extremes of the species natural distribution (i.e., Maine and Georgia, whose  $t_M$  values differ by 12.6°C). This study indicates that the amounts of both LDH-B<sub>4</sub> protein and LDH-B mRNA are significantly different between populations, and these differences are due to both genetic adaptation and physiological acclimation.

## MATERIAL AND METHODS

*F. heteroclitus* were collected from Bar Harbor, Maine (44.2° N; frequency of *Ldh-B<sup>b</sup>* = 0.96) and Sapelo Island, Georgia (31.4° N; frequency of *Ldh-B<sup>b</sup>* = 0.03). Fish were collected from mid-July to early August [annual monthly temperature 14.1°C and 31.7°C for the northern and southern populations, respectively (1)]. These fish were acclimated for 6 weeks to 20°C, 15 ppt seawater, and a 14:10 light:dark cycle. Similar regimes were employed when acclimating fish from Stone Harbor, New Jersey (39.06° N; frequency of *Ldh-B<sup>b</sup>* = 0.30) to 10°C and 20°C. Sidell *et al.* (14) demonstrated that 4 weeks are adequate for temperature acclimation in fish. Additionally, compensatory changes in LDH-B<sub>4</sub> activity in *F. heteroclitus* were completed within a 4-week period when these fish were subjected to hypoxia (15) or temperature acclimation (D.A.P., unpublished results). Therefore, a 6-week period should be more than adequate for complete acclimation.

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Abbreviations: LDH, lactate dehydrogenase;  $t_M$ , mean annual temperature;  $k_{cat}$ , catalytic rate constant; ANOVA, analysis of variance. \*Present address: Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950-3094.

After acclimation, livers were removed, weighed, and frozen in liquid N<sub>2</sub>. Since the only LDH expressed in *F. heteroclitus* liver is LDH-B<sub>4</sub> (2), this tissue was used for measurement of LDH-B<sub>4</sub> activity, as well as LDH-B protein and mRNA concentrations. These three assays were determined on each liver sample from the Maine and Georgia populations ( $n = 24$ , 12 from each population).

Enzyme activity was determined from livers homogenized in 50 mM sodium phosphate buffer, pH 7.0/1 mM EDTA/5 mM 2-mercaptoethanol, then centrifuged at 16,000 × *g* for 15 min. LDH activity was determined at 25°C by measuring the oxidation of NADH spectrophotometrically at 340 nm as a function of time (Beckman DU-8 with a water-jacketed cuvette holder). Saturating conditions at 25°C, pH 7.5, were empirically determined (0.1 M sodium phosphate, 2.64 mM pyruvate, and 0.34 mM NADH), and these conditions were used for all assays. The LDH-B<sub>4</sub> allelic isozymes have the same  $k_{cat}$  under these conditions (5, 7) and, thus, the measure of enzyme activity should be proportional to enzyme concentration. Enzyme activity determinations were alternated between populations to avoid sample bias. LDH-B<sub>4</sub> activity was stable during the course of enzyme assays as determined by repeated measurements on the same samples. Liver homogenates were diluted so that the initial rate of oxidation was less than 0.1 A<sub>340</sub>/min.

Immunoassay dot blots were performed with affinity-purified antibody to *F. heteroclitus* LDH-B<sub>4</sub> and an <sup>125</sup>I-labeled antibody to rabbit immunoglobulin as secondary antibody (16). Protein-antibody complexes were quantified with a  $\gamma$  counter. The <sup>125</sup>I  $\gamma$  counts from the serial dilution of purified LDH-B<sub>4</sub> (6) were used to transform sample counts into ng of LDH. Protein concentration was determined by the BCA (bicinchoninic acid) protein method (Pierce). Bovine  $\alpha$ -globulin was used as a standard.

Total RNA was purified from livers stored in liquid N<sub>2</sub> by protocols similar to those described by Chirgwin *et al.* (17) and Turpen and Griffith (18). LDH-B mRNA was synthesized *in vitro* by using an SP6 vector (SP64, Promega Biotec). The synthetic LDH-B mRNA resolved as a single band on both a 1.5% agarose and a 4% acrylamide gel and was used as a standard to determine the exact amount of LDH-B mRNA in tissue samples.

All nucleic acid hybridizations were carried out by UV crosslinking RNA and DNA to nylon membranes (Amersham; Hybond-N). Filters were hybridized in a solution containing 5 × SSPE (5 × SSPE = 0.8 M NaCl/10 mM NaPO<sub>4</sub>, pH 7.5/1 mM EDTA), 5 × Denhardt's solution (19), 0.1% NaDodSO<sub>4</sub>, 50% (vol/vol) deionized formamide, calf thymus DNA at 100  $\mu$ g/ml, and yeast RNA at 50  $\mu$ g/ml at 37°C.

The amount of LDH-B mRNA was determined by dot blotting. Blotted onto each filter were 20  $\mu$ g of total RNA, a serial dilution of synthetic *F. heteroclitus* LDH-B mRNA, a serial dilution of a *Drosophila melanogaster* actin clone, and yeast RNA (as a negative control). The LDH-B-specific probe was a 440-base-pair (bp) *Pst* I fragment isolated from the coding region of an *Ldh-B* cDNA (20); there are only two nucleotide substitutions between the northern and southern *Ldh-B* alleles (unpublished data). The LDH-B-specific probe was labeled by random priming using [<sup>32</sup>P]dCTP (21, 22). In addition to the LDH-B-specific probes, the dot blots were simultaneously probed with a deoxycytidine 5'-[<sup>35</sup>S]thio]triphosphate-labeled *D. melanogaster* actin clone (23).

The amount of <sup>32</sup>P- and <sup>35</sup>S-labeled probe which hybridized to the RNA was quantified by a liquid scintillation counter employing windows specific for <sup>35</sup>S and <sup>32</sup>P. Spillover was corrected according to Segel (24).

The integrity of all mRNA samples (whether degraded or not) was verified by Northern analysis using LDH-B as a probe (20). There was no noticeable size polymorphism for LDH-B mRNA.

The genomic copy number of *Ldh-B* was determined by dot-blot analysis. Samples (60 and 30  $\mu$ g) of genomic DNA were dot-blotted onto nylon filters along with a serial dilution of a twice gel-purified *Pst* I 440-bp fragment of an *Ldh-B* cDNA, pLDHBb (20), using calf thymus DNA as a carrier. Genomic DNA dot blots were hybridized at 42°C in the presence of the <sup>32</sup>P-labeled (21, 22) *Pst* I 440-bp fragment of pLDHBb. Filters were washed extensively at 65°C in 2 × SSPE once, in 2 × SSPE and 0.2% NaDodSO<sub>4</sub> twice, then at 20°C in 0.2 × SSPE once. Under these conditions the *Ldh-B* cDNA fragment did not cross-react with calf thymus DNA.

## RESULTS

The LDH-B<sub>4</sub> specific activities of acclimated fish from northern and southern populations were significantly different [analysis of variance (ANOVA)  $P < 0.005$ ; Fig. 1A]. These results could be due to a number of factors, including differences in enzyme inhibitors, enzyme degradation,  $k_{cat}$ , LDH concentration, etc. Since saturating, but not inhibitory, levels of cofactor and substrate were used, differential inhibition did not seem likely. As demonstrated by repeated determinations on the same samples, there was no apparent enzyme degradation during the course of the specific activity measurements. Because the  $k_{cat}$  values are identical for the allozymes (5, 7), we propose that the major parameter responsible for the observed differences between populations in specific activity was the result of variation in LDH-B<sub>4</sub> enzyme concentration. As a test of this hypothesis, the concentration of LDH-B<sub>4</sub> from each liver was determined by an immunoassay.

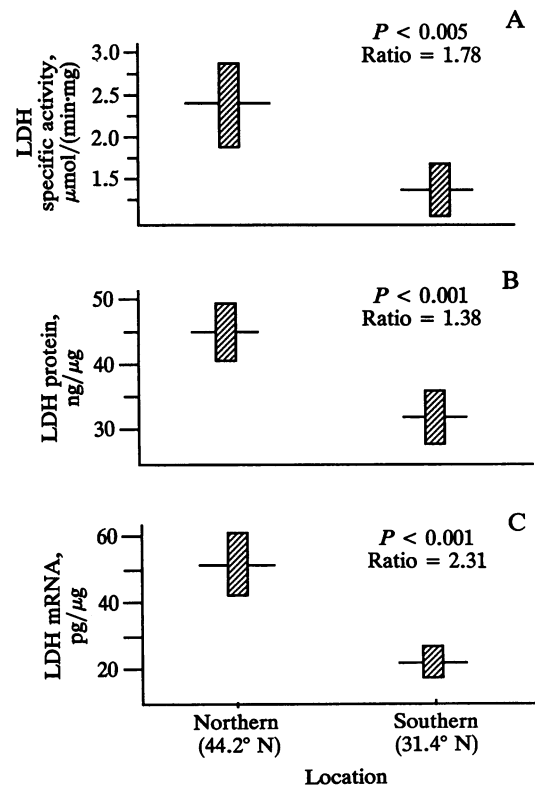


FIG. 1. LDH-B<sub>4</sub> enzyme and mRNA concentrations in two populations acclimated to 20°C. (A) Concentration of LDH-B<sub>4</sub> enzyme ( $\mu$ mol per min per mg of total protein) determined spectrophotometrically. (B) Concentration of enzyme (ng of LDH-B<sub>4</sub> per  $\mu$ g of total protein) determined by immunoassay. (C) Concentration of LDH-B mRNA (pg of LDH-B mRNA per  $\mu$ g of total RNA) determined by employing a liquid scintillation counter. Boxes represent  $\pm$  2 SEM; the horizontal lines are the means.

The immunoassay yielded results essentially identical to those obtained from the specific activity measurements (Fig. 1B). The  $\gamma$  counts from the immunoassay were linearly related to the amount of LDH-B<sub>4</sub> as indicated by the relationship between these counts and the amount of purified LDH-B<sub>4</sub> standard. Both the analysis of covariance (ANCOVA;  $\gamma$  counts as the dependent variable and amount of protein loaded as the covariate) and the ANOVA, using only the amount of LDH-B<sub>4</sub> in 20  $\mu$ g of total protein, indicated a significant difference between populations (ANCOVA  $P < 0.001$  or ANOVA  $P < 0.001$ ).

The concentration of LDH-B mRNA was determined by hybridizing with a probe that has less than 0.5% sequence difference from either of the two *Ldh-B* alleles. The liquid scintillation counts were linearly related to the amount of LDH-B mRNA as indicated by the relationship between these counts and the synthetic LDH-B mRNA. The concentrations of LDH-B mRNA were significantly different between populations (ANOVA  $P < 0.001$ ; Fig. 1C). However, the amounts of actin mRNA were not significantly different (ANOVA  $P > 0.05$ ). When the concentrations of LDH-B mRNA and LDH-B<sub>4</sub> enzyme were compared a significant correlation was found (Fig. 2;  $r = 0.75$ ,  $P < 0.01$ ).

To determine whether the differences presented above were also affected by physiological acclimation, fish from an intermediate latitude were subjected to two acclimation regimes: 10°C and 20°C ( $n = 60$ , 10 for each genotype and acclimation regime). The concentration of LDH-B<sub>4</sub> from these acclimated fish was determined spectrophotometrically. Acclimation, but not genotype, had a significant effect on the LDH-B<sub>4</sub> concentration (two-way ANOVA  $P < 0.005$  and  $P > 0.40$  for acclimation temperature and genotype, respectively). There was approximately 1.4 times as much LDH-B<sub>4</sub> at 10°C as at 20°C.

The amount of *Ldh-B* genomic DNA is not significantly different between populations ( $t$  test,  $P > 0.20$ ). The hybridization appears to have been specific to *Ldh-B* because the probe hybridized to only a single band of *EcoRI*-digested genomic DNA and there was no detectable hybridization to the calf thymus DNA. Since the *Ldh* probe did not cross-react with the calf thymus DNA, it is unlikely that it hybridized to the other *Ldh* isozymic loci in *F. heteroclitus* (i.e., *Ldh-A* and *Ldh-C*). It is more likely for the probe to hybridize with the calf *Ldh-B* DNA than with the other isozyme DNAs because there is greater sequence similarity between the same isozymes in different species than between different isozymes within a species (20).

One trivial explanation for the observed differences in LDH concentrations is the difference in body size in the two

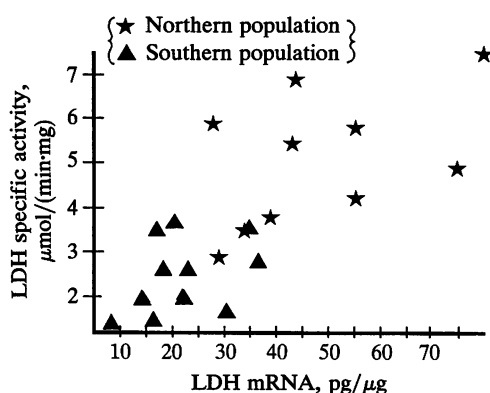


FIG. 2. Relationship between concentration of LDH-B<sub>4</sub> mRNA and LDH-B<sub>4</sub> specific activity. These parameters are significantly correlated ( $r = 0.75$ ,  $P < 0.01$ ). A single point was excluded from analysis because it was more than two standard deviations below the line that fits the relationship between the mRNA and enzyme.

populations. The effect of size-scaling does not present a problem in this study because there is no significant relationship between body weight and the amount of LDH-B mRNA or enzyme activity within a population ( $r < 0.35$ ,  $P > 0.2$ ).

## DISCUSSION

Temperature is one of the predominant environmental parameters that influences the physiology, distribution, and survival of organisms (11, 12). Shifting enzyme activity is a common adaptive strategy in response to a change in environmental temperature (for reviews see refs. 11, 12, 25, and 26). Organisms adapt to variable thermal environments by utilizing two general mechanisms: (i) evolutionary adaptation (i.e., genetic), which is heritable and achieved by the selection of alternative genes, and (ii) physiological acclimation, which is achieved by metabolic adjustments within an individual's lifespan.

The velocity of a bisubstrate enzyme reaction, such as that catalyzed by LDH, can be described by

$$v = k_{\text{cat}}[E][S][N]/\{K_m^s(K_m^n + [N]) + [S](K_m^n + [N])\},$$

where  $k_{\text{cat}}$  is the catalytic rate constant, [E] is the enzyme concentration, [S] is the substrate concentration, [N] is the concentration of cofactor (NAD<sup>+</sup> or NADH), and  $K_m^s$  and  $K_m^n$  are the Michaelis-Menten constants for substrate and cofactor, respectively. If one assumes that cofactor is saturating, as suggested by Tischler *et al.* (27), then Eq. 1 reduces to

$$v = k_{\text{cat}}[E][S]/(K_m^s + [S]).$$

Some of the parameters shown in this equation can be altered only by evolutionary adaptation, while others can be altered by both evolutionary and physiological mechanisms. Although allosteric modifiers of enzymes can be affected by physiological acclimation, the intrinsic  $k_{\text{cat}}$  and  $K_m$  of an enzyme are genetically determined by its amino acid sequence and therefore can be altered only by evolutionary change (for reviews see refs. 9, 11, 12, 28, and 29). Graves and Somero (30) have demonstrated that in congeneric species of barracudas living in different thermal environments the  $K_m$  for pyruvate of LDH-A<sub>4</sub> isozymes shows temperature-compensatory changes. Place and Powers (5, 7) demonstrated that the LDH-B<sub>4</sub> allelic isozymes of *F. heteroclitus* vary in their  $K_m$  values as a function of temperature. The inhibition constants and heat denaturation kinetics also differed between these allozymes. Moreover, the LDH-B<sub>4</sub> phenotypes are associated with differential development, swimming performance, and survivorship (8–10). Allozyme variations in other species have also been shown to be associated with selectively important traits and, in some cases, differential survival (28, 29, 31–33).

If the existing enzyme's catalytic properties are not sufficient to compensate for temperature changes, organisms may adjust enzyme concentration. However, unlike  $k_{\text{cat}}$  and  $K_m$ , enzyme concentration, [E], can be a function of both evolutionary adaptation and physiological acclimation. For example, genetically based changes in enzyme concentration have been demonstrated in salmonids, where variation in enzyme activity is due to the additional expression of a phosphoglucose isomerase isozyme in one case (34) and the lack of expression of an LDH-A<sub>4</sub> allozyme in another case (35). Our results suggest that the LDH-B<sub>4</sub> protein and LDH-B mRNA concentration differences between *F. heteroclitus* populations are also genetically based, because these differences remain even after extensive acclimation to a common temperature (Fig. 1). Preliminary data on the LDH-B<sub>4</sub> activity from progeny of crosses between two geographically extreme populations tend to support this hypothesis (16).

LDH-B<sub>4</sub> concentration is highly correlated with LDH-B mRNA concentration (Fig. 2), which strongly suggests that the differences in enzyme concentration are due to variation in *Ldh-B* gene regulation. Evolutionary modification in a regulatory element is well documented for *D. melanogaster* alcohol dehydrogenase (ADH), where enzyme activity varies when flies are selected for ethanol tolerance (36–39). This increase in ADH activity is the result of a trans-acting regulatory element. The changes in ADH concentration are controlled by differential mRNA concentration and post-translational regulation (40–42). Such regulatory changes in enzyme activity are thought to be very important evolutionarily because they allow activity to change independent of changes in protein primary structure (37, 38, 43).

Although the variation in LDH-B mRNA concentration could be the result of several different factors, it is not due to a gene duplication, because there is no significant difference between the populations in the number of *Ldh-B* gene copies. The increased expression of LDH-B mRNA in the northern population could be the result of transcriptional or post-transcriptional regulation. Alternatively, structural differences in the LDH-B mRNA could alter their relative stabilities, causing the northern form to accumulate more LDH-B mRNA and thus more LDH-B<sub>4</sub> protein than in its southern counterpart. One could distinguish between these hypotheses by determining the amount of LDH-B mRNA and the transcription rate for each *Ldh-B* genotype.

In addition to the presumed genetic differences in LDH-B<sub>4</sub> concentration, physiological acclimation can also alter enzyme levels. For example, when fish are acclimated to 10°C and 20°C (near the  $t_M$  values of the northern and southern populations, respectively) there is approximately 1.4 times more LDH-B<sub>4</sub> in fish acclimated to the lower temperature. Thus, the enzyme concentration at a given  $t_M$  ( $[E]_{t_M}$ ) is a function of the genetically based intrinsic enzyme concentration ( $[E]_i$ ) and the amount due to physiological acclimation. Knowing the  $k_{cat}$  and  $K_m$  for each allelic isozyme as a function of temperature (5, 7), one can calculate the reaction velocity at a particular  $t_M$  and determine the potential compensatory contribution of each parameter.

If one assumes that (i) the substrate concentration of a given *Ldh-B* genotype is equal to its  $K_m$  at its  $t_M$  and (ii) our experimentally determined acclimation results are similar to those occurring in natural populations, then at a given  $t_M$  Eq. 2 reduces to

$$v = k_{cat}[E]_{t_M}/2,$$

where  $v$  and  $k_{cat}$  are at the  $t_M$ .

The first assumption (i.e.,  $K_m = [S]$ ) is supported by empirical evidence. DiMichele and Powers (8) have shown that at 10°C the lactate concentration in *F. heteroclitus* is most similar to the  $K_m$  of the northern LDH-B<sub>4</sub> and at 20°C most similar to the  $K_m$  of the southern LDH-B<sub>4</sub>. Substrate and  $K_m$  are also similar for a variety of enzymes in other organisms (11, 44). Thus, the assumption that the  $K_m$  is equal to the substrate concentration appears reasonable.

The second assumption, concerning the role of acclimation, also appears valid. The ratio of the amount of LDH-B<sub>4</sub> in natural populations from Maine to that in populations from Georgia is 2.50 (D.L.C., unpublished data), which is essentially identical to the ratio for experimentally determined values from these populations (i.e., ratio of  $[E]_{t_M} = 2.49$ ; Table 1).

Table 1 illustrates the compensatory effect of the kinetic differences between the allelic isozymes and the effect of an increase in enzyme concentration due to both evolutionary adaptation and physiological acclimation. If only the kinetic differences of the allelic isozymes are considered, then the reaction rate in the northern population would be 30–40%

Table 1. Summary of parameters and predicted LDH-B<sub>4</sub> reaction rates

Parameter	Northern	Southern	Ratio
Specific activity, $\mu\text{mol}/(\text{min}\cdot\text{mg})$	2.37	1.33	1.78
$[E]_i$ , $\mu\text{M}$	0.669	0.376	1.79
$[E]_{t_M}$ , $\mu\text{M}$	0.937	0.376	2.49
$t_M$ , °C	7.8	20.4	
Blood pH at $t_M$	7.8	7.6	
Lactate to pyruvate			
$k_{cat}$ at $t_M$ , $\text{s}^{-1}$	9.9	29.6	0.33
$v$ at $t_M$ and $[E]_i$ , $\mu\text{M}\cdot\text{s}^{-1}$	3.31	5.57	0.60
$v$ at $t_M$ and $[E]_{t_M}$ , $\mu\text{M}\cdot\text{s}^{-1}$	4.64	5.57	0.83
Pyruvate to lactate			
$k_{cat}$ at $t_M$ , $\text{s}^{-1}$	117	294	0.40
$v$ at $t_M$ and $[E]_i$ , $\mu\text{M}\cdot\text{s}^{-1}$	39.1	55.2	0.71
$v$ at $t_M$ and $[E]_{t_M}$ , $\mu\text{M}\cdot\text{s}^{-1}$	54.8	55.2	0.99

Kinetic parameters, determined previously (6, 7), are at constant alkalinity ( $[\text{OH}]/[\text{H}] = 1$ ) at  $t_M$ .  $v$  is the enzyme reaction rate (Eq. 3).  $[E]_{t_M}$  is the enzyme concentration, which is a function of the intrinsic enzyme concentration  $[E]_i$  and the increase in  $[E]_i$  due to physiological acclimation. Blood pH was determined previously (8).

that in the southern population. This is indicated in Table 1 by the ratio of the  $k_{cat}$  values. Additional compensation is achieved when one also considers the effect of the variation in the intrinsic enzyme concentration. Due to the higher LDH-B<sub>4</sub> concentration in the northern population the reaction rate is 60–70% of that in the southern population. If one considers the contribution of thermal acclimation in addition to the intrinsic differences alluded to above, the calculated enzyme velocities are essentially equivalent for the populations at their respective  $t_M$  values (Table 1).

These calculated enzyme velocities suggest that both physiological and genetic mechanisms are necessary to achieve complete thermal compensation. It is the blending of this suite of characters that provide the necessary evolutionary plasticity for adapting to a varying thermal environment.

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