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# GENETIC MECHANISMS FOR ADAPTING TO A CHANGING ENVIRONMENT

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## INTRODUCTION

Darwin's *The Origin of Species* demonstrated the value of systematic and biogeographic studies and, as a result, the analysis of genetic variation within and between species has continued to be a major research focus of evolutionary biologists for more than a century. Population biologists focused their efforts on morphological characters until the 1960s, when the application of protein electrophoresis uncovered a wealth of unexpected genetic variation. Subsequent biogeographical studies sometimes revealed directional changes in gene frequency (i.e. clines; 44) correlated with one or more environmental parameters. Although these authors touted such studies as evidence for natural selection, other researchers suggested alternate mechanisms.

Over the next two decades, the extent of the genetic variation uncovered by protein electrophoresis was so great that questions arose concerning the significance, or lack thereof, of these polymorphic loci. In fact, few subjects in biology have been more strongly debated than the evolutionary significance of protein polymorphisms. Most of the debate centered around two contrasting views: the "selectionist" and the "neutralist." Proponents of the selectionists' school asserted that natural selection maintains protein polymorphisms, whereas those of the neutralists' persuasion argued that the vast majority of such variation is selectively neutral, and others favored intermediate positions.

Some biologists addressed the conflict between these two extreme views by developing theoretical models in the hope that such models might be tested by estimates of evolutionary rates, mutation rates, genetic load, effective population sizes, genome sizes, and other parameters. Stebbins & Lewontin (78), however, showed that a purely mathematical treatment could not resolve the conflict. Clarke (11) pointed out that estimates of evolutionary rates and other parameters are so variable that values can be found to favor almost any of the models. Thus the same biogeographic data could be used to support diametrically opposed theories. In 1974, Lewontin (48) summarized the failure of evolutionary biologists to resolve this important controversy by biogeographical and mathematical approaches alone.

In the following years, a few evolutionary biologists developed an experimental approach that allowed one to evaluate the theoretical basis of the neutralist/selectionist controversy. This effort was led by Clarke (12) who suggested that the neutralist hypothesis could be rejected for specific loci whenever functional nonequivalence was established between genetic alternatives. He proposed the following four-step strategy for addressing problems of genetic variation at enzyme synthesizing loci:

1. Make a detailed biochemical and physiological study of the allelic isozymes;

2. based on the nature of the differences delineated in step one, the function of the enzyme, and the ecology of the organism, postulate one or more selective factors and generate a hypothesis that establishes a mechanistic link between the selective factor and the gene product;
3. test the hypothesis by experimentally manipulating the environment to produce a predictable response;
4. based on the experimental results, reexamine the natural populations and develop a comprehensive theory for the observed gene frequency patterns.

Clarke's (12) strategy assumes that one has already established the existence of allelic variation, and that gene frequency patterns have been observed for these loci. Thus, the above approach begins where conventional biogeographical approaches end. The major advantage of this strategy is the ability to generate and experimentally test hypotheses.

The full power of Clarke's strategy is evident only when carried through completely. For example, if biochemical analysis of allelic isozymes leads to predictable differences in cell physiology, organism response, etc, that can be substantiated by experimentation, then the neutralist hypothesis can be rejected for that locus. Experiments designed to test these cellular predictions should yield results that allow the researcher to make other testable predictions at higher levels of biological organization. As each new cycle of predictions is followed by experimental validation, one can ultimately be led to accepting either the selectionist or the neutralist paradigm. If predictions can be followed by experimental validation, then the selectionist viewpoint would be supported; otherwise, the neutralist position would be favored. It is this cycle of a priori predictions, coupled with testing those predictions, that provides the power of the experimental approach.

A few examples that reflect this general approach are:

1. Alcohol dehydrogenase from *Drosophila*: kinetic differences between allelic isozymes were associated with differences in survivorship and developmental time (21, 29, 81).
2. Alpha-glycerophosphate dehydrogenase allelic isozymes from *Drosophila*: kinetic differences were correlated with environmental temperature, flight metabolism, and power output (20, 55, 57, 72).
3. Esterase-6 in *Drosophila*: allelic isozyme activity and other kinetic parameters were associated with reproduction (68).
4. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from *Drosophila*: metabolic flux and partitioning difference (4, 10) were reflected in fitness differences (43);
5. Aminopeptidase from *Mytilus edulis*: activity differences between allelic isozymes were associated with improved osmoregulation and phenotypic fitness (40, 45).

6. Glucose phosphate isomerase in *Colias* butterflies (82, 83) and sea anemones (87): kinetically different allelic isozyme variation was correlated with survivorship and mating success (82, 83) and differential modulation of pentose-shunt metabolism (87).
7. Glutamate-pyruvate transaminase in the copepod *Tigriopus californicus*: allelic isozyme activities were associated with different rates of alanine accumulation and differential responses to hyperosmotic stress (8).
8. Several enzymes involved in glycolysis, the pentose shunt, and the malate/isocitrate shuttles from the teleost, *Fundulus heteroclitus*: differences in the kinetic and other biochemical properties of allelic isozymes (60–62, 70, 71, 80) were used to predict and subsequently establish significant differences in metabolism, oxygen transport, swimming performance, developmental rate, and relative fitness (22–27, 58).

Not all of these elegant studies have taken advantage of the full power of the experimental approach, but each has revealed insights into the neutralist/selectionist controversy.

### ANALYSIS OF GENETIC VARIATION IN *FUNDULUS HETEROCLITUS*: A MULTIDISCIPLINARY EXAMPLE OF CLARKE'S SUGGESTED STRATEGY

Although several of the studies cited could illustrate the experimental approach, we shall use our work because it more completely couples the cycle of predictions and experimental validation alluded to above. We are studying allelic variants of several enzyme-synthesizing loci in the model teleost *Fundulus heteroclitus*—an abundant Atlantic Coast fish ranging from the Mantanzas River in Florida to Port au Port Bay in Newfoundland, Canada. *F. heteroclitus* has been a popular experimental organism for over 100 years and a symposium on this biological model illustrates both its historic and present role for a wide array of scientific studies (1).

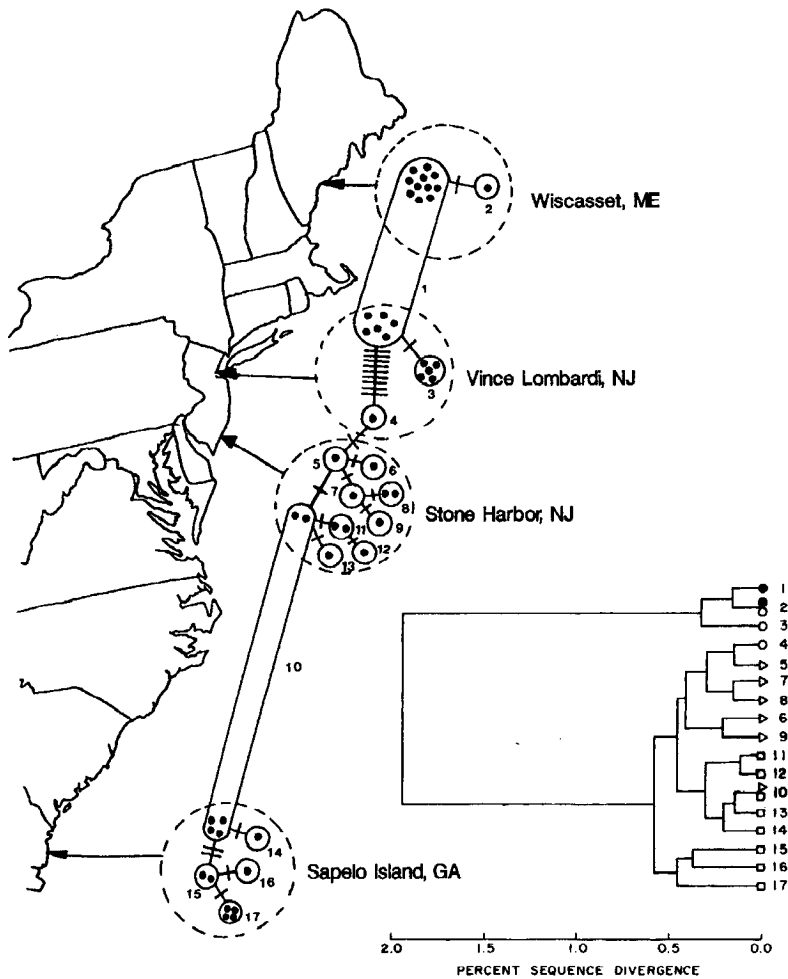
#### *Genetic Variability in Relation to Latitude*

*F. heteroclitus* maintain a high level of protein polymorphism (7, 59, 67, 79). Examination of these polymorphic loci has uncovered significant directional changes with latitude in gene frequency (i.e. clines) and in degree of genetic diversity (9, 27, 66, 69). These protein polymorphisms have been divided into four general classes (9, 67). Class I loci are clinal and have two predominant alleles—one fixed in northern populations and the other fixed in southern populations. Class II loci are fixed for one allele at one end of the species range but are variable over the remainder. Class III loci are fixed for an allele having the same electrophoretic mobility at both the northern and

southern extremes, but they show variability at middle latitudes. Class IV loci show no significant change in gene frequency with change in latitude.

Directional changes in genetic characters with geography (i.e. clines, 44) have classically been described by two general models: primary and secondary intergradation (30). In the primary intergradation model, adaptation to local conditions along an environmental gradient and/or genetic drift may lead to genetic differences along the gradient. Gene flow may not eliminate these differences, either because it is too small or because of nonrandom dispersal along the gradient. In the secondary intergradation model, populations are first separated by some barrier that prevents gene flow, and then adaptation to local conditions and/or genetic drift produces genetic differences between these disjunct populations. Finally, when the barrier is removed, the formerly disjunct populations interbreed, producing a cline in gene frequencies between them. The main difference between these two models, therefore, is the need for the temporary existence of isolating barriers to gene flow in the latter.

One cannot distinguish between these on the basis of classical zoogeographical data unless it is available within a few hundred generations of an alleged secondary contact (30). For example, the present day spatial patterns of *F. heteroclitus* gene frequencies could have arisen by either type of intergradation (67). However, analysis of mitochondrial DNA (mtDNA) can provide insight concerning primary and secondary intergradation long after a secondary contact. *F. heteroclitus* populations were analyzed by studying mtDNA fragments, obtained by digestion, with each of 17 restriction endonucleases (35). Analysis of the mtDNA restriction fragment data indicated an intergrade zone at or near 41°N latitude (35). This conclusion was based on the fact that the mtDNA restriction patterns of fish at specific localities could be interrelated by a network of single nucleotide base changes (Figure 1). However, populations on each side of 41°N latitude required many nucleotide changes. Previously, Cashon et al (9) had suggested that the last glacial event (approximately 20,000 years ago) might have helped shape the present allelic isozyme clines because several showed sharp gene frequency changes near the Hudson River that are associated with the edge of the last major glacial advance. Although the mtDNA data (35) showed a sharp disjunction consistent with that hypothesis (Figure 1), the nucleotide differences between the "northern" and "southern" mtDNA haplotypes (see phenogram in Figure 1) suggested a divergence time prior to the Wisconsin glaciation (35). Since the Chesapeake and Delaware bays were only rivers during the last glacial advance, Smith (76) examined the mtDNA haplotypes of approximately 700 individual *F. heteroclitus* from 20 populations within these bays to determine if remnant "northern" mtDNA haplotypes could be detected. Not only were the "northern" mtDNA haplotypes detected, they were distributed in a clinal



*Figure 1* Phylogenetic network and phenogram of mtDNA genotypes. The phylogenetic network is a composite of 17 mtDNA restriction phenotypes. Each circle indicates a different mtDNA clone. Dots inside circles identify the number of individual fish sharing the mtDNA phenotype. Clones are interconnected by branches with solid lines crossing the branches of the network indicating the minimum number of base substitutions required to account for the different mtDNA clones. Arrows indicate the collection areas. Clone 1 is shared by the populations of Maine and Vince Lombardi, New Jersey, while clone 10 is shared by Stone Harbor, New Jersey, and Georgia populations. The phenogram of mtDNA genotypes illustrated in the phylogenetic network was generated by a UPGMA analysis of nucleotide sequence divergence (p) estimates. The numbers and symbols (circles, triangles, and squares) correspond to the mtDNA clones illustrated in the network (from Reference 35).

fashion up the Bays and tributaries. The "northern" mtDNA haplotypes were common in the head waters of the bays and tributaries while the "southern" mtDNA haplotypes dominated in the lower bays and rivers. Smith's (76) data clearly indicate that the intergrade zone has been temporally unstable for many thousands of years; prior to the last glaciation, it had existed at least as far south as the mouth of the Chesapeake Bay.

While the above findings are useful for placing these clines in a historical context, they do not provide insight concerning the relative contributions of chance (e.g. genetic drift) and adaptive (e.g. natural selection) forces that shaped the genetic divergence between allelic alternatives prior to, during, and/or after an isolation event. Yet, it is the relative roles of chance and adaptive forces that strike at the very heart of the neutralist/selectionist controversy. Thus, additional approaches must be undertaken to determine the role of natural selection, if any, as a driving force for generating and/or maintaining gene diversity. In an attempt to address these issues, we have taken an approach similar to that suggested by Clarke (12).

*F. heteroclitus* is found in one of the steepest thermal gradients in the world and, being a poikilotherm, must be profoundly influenced by this environmental parameter. The fish's natural distribution is dominated by a mean water temperature change of approximately 1°C per degree latitude (Figure 2C). In the southern marshes, summer water temperatures in excess of 40°C are commonly recorded, whereas summer temperatures of the northern most marshes are relatively cold. Although northern marshes are often covered by ice in the winter, southern marshes are generally free of such severe low water temperatures. South of 41°N latitude, the minimum water temperature increases at 1°C per degree latitude (Figure 2B). The heterozygosity of this species decreases at latitudes greater than 41°N but is unchanged at southern latitudes (Figure 2A). Powers et al (64) have previously pointed out that this phenomenon is correlated with the amount of time that each population spends at or below freezing temperatures.

Thus, if temperature affects, or has affected, the differential survival of *F. heteroclitus* with specific allelic isozymes, then natural selection could be, or may have been, acting to change the gene frequency of populations that experience different thermal regimens along the East Coast. This possibility is supported by the finding of Mitton & Koehn (56) that some loci are shifted toward "southern" phenotypes in fish inhabiting a power plant's thermal effluent. It is further supported by shifts in gene frequency in several loci at localities where temperature anomalies are found (67). While these correlations are suggestive of a causal relationship, they are not definitive because they could be explained by stochastic arguments. As mentioned previously, the problem with a classical natural history and biogeography approach is that it often leads to two or more diametrically opposed hypotheses that can

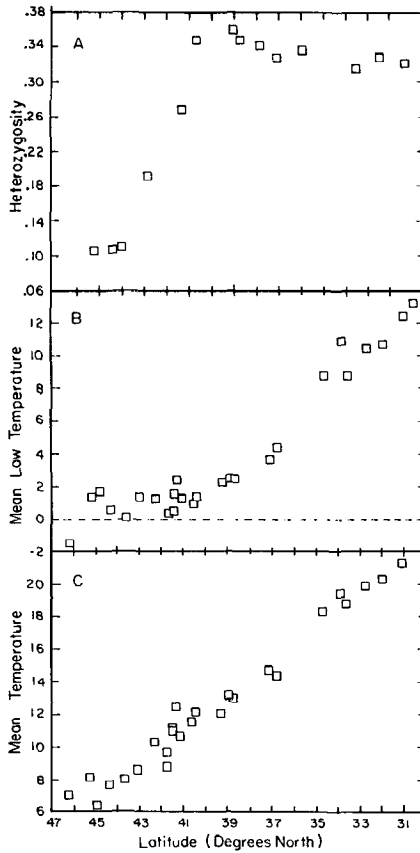


Figure 2 (A) Heterozygosity within populations versus latitude ( $^{\circ}$ N); (B) lowest average monthly surface water temperature ( $^{\circ}$ C) versus latitude; (C) mean surface water temperature versus latitude.

explain the same data. For that reason, such studies often raise more questions than they answer.

### Biochemical Analysis of Allelic Isozymes

We have employed an experimentally based strategy similar to that suggested by Clarke (12) in an attempt to falsify the hypothesis that natural selection was (or is) a major driving force responsible for the observed gene diversity in *F. heteroclitus*. As stated earlier, the strategy is to begin with a detailed biochemical study of the allelic isozymes and progress through higher levels of biological organization by a linked series of predictions followed by ex-

perimental validation. So far, we have found evidence consistent with functional differences between allelic isozymes of seven loci, including the lactate dehydrogenase "heart" locus (*Ldh-B*), a cytoplasmic malate dehydrogenase locus (*Mdh-A*), a cytoplasmic NADP-dependent isocitrate dehydrogenase (*Idh-B*), hexose-6-phosphate dehydrogenase (*H6pdh*), a glucose phosphate isomerase locus (*Gpi-B*), a phosphoglucomutase locus (*Pgm-B*), and a cytoplasmic aspartate amino transferase locus (*Aat-A*). There are seven different kinds of functional evidence: (a) steady-state kinetic analyses, (b) thermal stability differences, (c) enzyme concentration differences, (d) differences in cellular metabolism, (e) genotype specific swimming ability, (f) differential developmental rates and hatching times, and (g) laboratory and field selection experiments. Although all of these enzymes have been studied for several of these areas, only the *Ldh-B* locus has been studied by all seven functional approaches. Since the work on *Ldh-B* has been most extensive to date, we present a summary of those findings to illustrate our multidisciplinary approach to the neutralist/selectionist controversy.

## A BRIEF REVIEW OF STEADY-STATE KINETIC PARAMETERS

Functional differences between the allelic isozymes are usually characterized by classical "steady-state" kinetic parameters. In brief, the velocity ( $v$ ) at which an enzymatic reaction occurs is proportional to the concentrations of the substrates and enzyme. In the absence of products, when substrates are in excess, a reaction will proceed at its maximum velocity ( $V_{\max}$ ). The  $V_{\max}$  reflects the enzyme's catalytic efficiency ( $k_{\text{cat}}$ ) and the enzyme concentration ( $[E_t]$ ) at saturating substrate (i.e.  $V_{\max} = k_{\text{cat}} [E_t]$ ). Therefore, apparent differences in  $V_{\max}$  may be attributed either to different apparent first order rate constants ( $k_{\text{cat}}$ ), or to different enzyme concentrations. When substrate concentrations are limiting, the velocity is less than  $V_{\max}$ . The substrate concentration at which the velocity ( $v$ ) is 50% of  $V_{\max}$  is the  $K_m$  (Michaelis-Menten constant), which often approximates the dissociation constant for the enzyme-substrate complex. While a single substrate reaction has one  $K_m$ , multisubstrate reactions have a  $K_m$  for each substrate.

Substrate concentrations *in vivo* are normally too low to saturate enzymes; therefore, at very low substrate concentrations,  $V_{\max}/K_m$  is a pseudo-first-order rate constant. However,  $k_{\text{cat}}/K_m$ , the second order rate constant, is a more appropriate parameter because at very low substrate concentrations the enzyme must be considered a reactant. Also, *in vivo* enzyme concentration can vary and  $V_{\max}/K_m$  is enzyme concentration dependent while  $k_{\text{cat}}/K_m$  is not. Because  $k_{\text{cat}}/K_m$  cannot be larger than any of the second-order rate constants of the enzyme's reaction pathway, its value sets a lower limit on the rate of

enzyme-substrate association. When  $k_{\text{cat}}/K_m$  is very large, the reaction is maximized and becomes diffusion limited.

Considerable discussion has centered on the evolutionary importance of  $V_{\text{max}}/K_m$  (5, 6, 14, 19, 31). If the prime evolutionary objective is to attain the highest possible catalytic rates, then  $V_{\text{max}}$  (i.e.  $k_{\text{cat}}$  and/or  $[E_t]$ ) and  $K_m$  should increase within the evolutionary constraint imposed by diffusion. According to Fersht (31) and Crowley (19),  $k_{\text{cat}}[E_t]$  should be as large as possible and  $K_m$  should be large compared with the in vivo physiological substrate concentrations.

Since living cells contain substrates, reaction products, and other metabolites that can inhibit enzyme reactions, it is also important to determine the inhibition constants ( $K_I$ ) for products, substrates, and/or metabolic inhibitors and to determine how these can affect the reactions in vivo.

## LACTATE DEHYDROGENASE: A MODEL FOR ALLELIC ISOZYME ANALYSIS

The enzyme lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate and is thus involved in both the catabolism and anabolism of carbohydrates. During anaerobic glycolysis, the conversion of pyruvate to lactate by LDH is essential for continued ATP production. LDH also may convert lactate to pyruvate, which in turn may be used in gluconeogenesis or in the generation of ATP by aerobic metabolism.

In addition to metabolic roles in cells, LDH has been implicated in a number of other important biological processes. For example, several reports have identified LDH-A<sub>4</sub> as a single-stranded DNA-binding protein that may be involved in DNA replication (73, 84), and Gosti et al (36) found that the LDH commonly found in heart tissue (LDH-B<sub>4</sub>) and centrosomal proteins shared a common epitope, suggesting an additional function. An interesting LDH has been found to be associated with cells transformed by Kirsten murine sarcoma virus (2, 3). Li and his colleagues (49) have shown that this LDH is a tyrosylphosphorylated form of the LDH common to vertebrate muscle (LDH-A<sub>4</sub>) that appears to be complexed with the *ras* P21 protein. Phosphorylated forms of LDH-A<sub>4</sub> have been found in Rous sarcoma virus transformed chicken fibroblasts (13), and rat fibroblasts induced with epidermal growth factor (EGF), which has tyrosine kinase activity, increased the synthesis of LDH-A<sub>4</sub> (50). Finally, LDH also appears to perform structural roles because the epsilon-crystalline protein from duck eye lens is the LDH-B<sub>4</sub> isozyme (39, 86).

Another LDH locus is expressed in the testis of mammals and in a variety of tissues in teleosts. This locus is referred to as *Ldh-C*, but there is no evidence that it is the same in mammals and fish. The products of the three

*Ldh* genes are designated LDH-A<sub>4</sub>, LDH-B<sub>4</sub>, and LDH-C<sub>4</sub>, respectively. The three vertebrate *Ldh* genes appear to be independently regulated and show tissue specificity. Although *Ldh-A* and *Ldh-B* are simultaneously expressed in the same tissues of many vertebrates, the specificity and exclusivity of LDH expression in the tissues of many marine fishes is remarkable. White skeletal muscle, whose metabolism is predominantly anaerobic, expresses the *Ldh-A* locus. Red muscle and liver that have significant aerobic metabolism express almost exclusively *Ldh-B*. *F. heteroclitus* erythrocytes, which have some aerobic capability (about 5 to 10%), also express *Ldh-B* exclusively. The suggested functional significance of the difference in LDH isozymes is that LDH-A<sub>4</sub> is principally involved in the conversion of pyruvate to lactate (i.e. anaerobic glycolysis), whereas LDH-B<sub>4</sub> is principally involved in the conversion of lactate to pyruvate (i.e. gluconeogenesis and aerobic metabolism).

In *F. heteroclitus*, *Ldh-B* has two codominant alleles: *Ldh-B<sup>a</sup>* and *Ldh-B<sup>b</sup>*. As illustrated in Figure 3, the relative proportions of these alleles vary with latitude. *Ldh-B<sup>b</sup>* is predominant in the northern (i.e. colder) portions of the range, and *Ldh-B<sup>a</sup>* is predominant in the southern (i.e. warmer) portion of the range. According to Clarke's (12) strategy, the first step in the analysis of this pattern should be to establish any functional differences between the products of these two alleles. We have used various biochemical and physiological techniques to accomplish this step. Our conclusions are that the LDH-B<sub>4</sub> allelic isozymes of *F. heteroclitus* differ in: (a) catalytic efficiency at low substrate concentrations, (b) degree of inhibition by lactate, (c) enzyme stability, and (d) enzyme concentration. We elaborate on these points below.

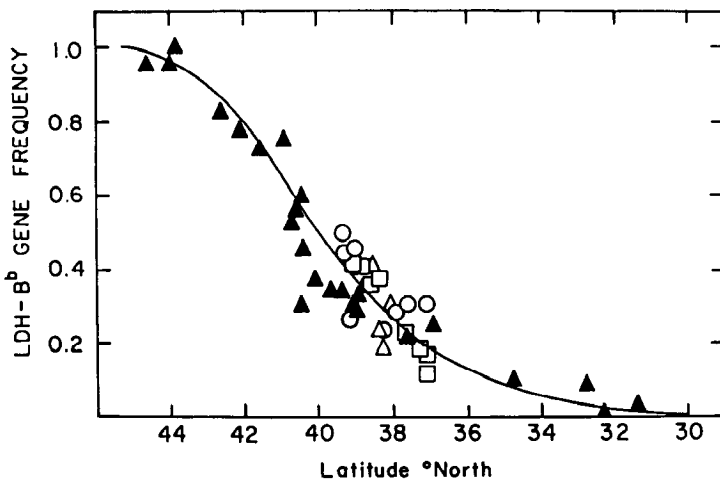


Figure 3 Gene frequency of *Ldh-B<sup>b</sup>* versus latitude °N (from Reference 60).

*Differences in catalytic efficiency at low substrate concentrations* At all temperatures and pH values, the  $k_{\text{cat}}$  values of the two allelic isozymes are identical (62). At low temperatures, the LDH-B<sub>4</sub><sup>b</sup> allelic isozyme, whose gene frequency is greatest in the northern colder waters (Figure 3), has a greater apparent catalytic efficiency at low substrate concentrations than LDH-B<sub>4</sub><sup>a</sup> (60, 62). At higher temperatures the situation appears to be reversed. LDH-B<sub>4</sub><sup>a</sup>, whose gene frequency is greatest in the southern warm waters, has a greater catalytic efficiency. Since the  $k_{\text{cat}}$  values of the allelic isozymes are equivalent at all temperatures and the enzyme concentrations were held constant, these differences in catalytic efficiency are a function of different  $K_m$  values. A possible confounding factor is that the pH of cells decreases as their temperature increases. However, even after pH changes are taken into account, catalytic efficiency differences between LDH-B allelic isozymes are still evident (Figure 4).

*Substrate and product inhibition* Very high concentrations of reaction products or substrates may inhibit an enzyme's function. For example, during the conversion of pyruvate to lactate, the LDH-B<sub>4</sub><sup>a</sup> allelic isozyme is much less susceptible to product (i.e. lactate) inhibition than the LDH-B<sub>4</sub><sup>b</sup> isozyme. For the conversion of lactate to pyruvate, the LDH-B<sub>4</sub><sup>b</sup> allelic isozyme is more susceptible to substrate (i.e., lactate) inhibition than the LDH-B<sub>4</sub><sup>a</sup> isozyme. Moreover, the magnitude of inhibition is greater at cool temperatures than at

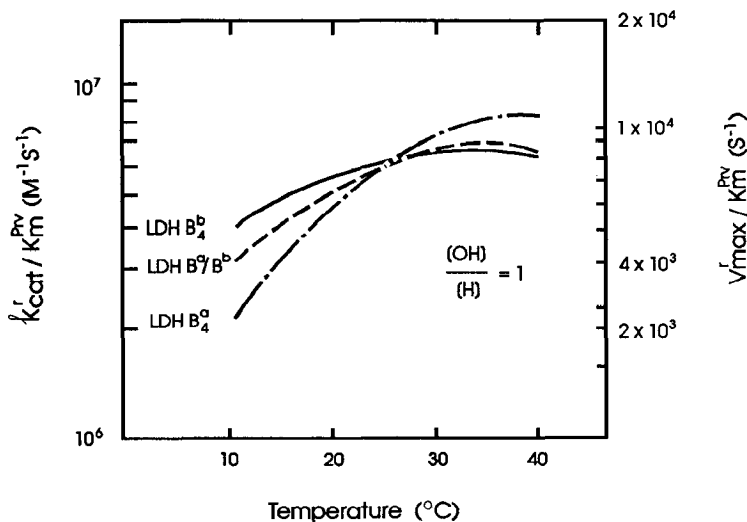


Figure 4 Effect of temperature on pyruvate reduction at a constant  $[\text{OH}^-]/[\text{H}^+]$  ratio of unity. Both  $k_{\text{cat}}/K_m$  and  $V_{\text{max}}/K_m$  are plotted against temperature ( $^{\circ}\text{C}$ ) where enzyme concentration,  $[\text{E}_t]$ , is considered constant for the two LDH-B allelic isozymes.

warm ones. The putative selective significance of this difference may relate to the accumulation of lactate, which can exceed 20 mM, during extreme swimming activity in fish (24, 62, 65), or during early embryonic development when lactate concentrations range between 40 and 50 mM (58).

*Differences in stability of LDH-B<sub>4</sub> allelic isozymes* Place & Powers (61) used heat, urea, and proteolytic digestion to study the structural stability of LDH-B<sub>4</sub> allelic isozymes. Heat denaturation studies (Figure 5A) revealed that LDH-B<sub>4</sub><sup>b</sup> was more stable than LDH-B<sub>4</sub><sup>a</sup>. Similar differences were found in a variety of other stability studies (61). Since the least stable allelic isozyme is most common in the warmer southern waters of the species range, these results are inconsistent with the expectation that the most thermally stable enzyme should be most common in the warmer environments. However, Figure 5A shows that each of the LDH-B allelic isozymes is completely stable at 50°C, which is well above the fish's lethal temperature. Therefore, while

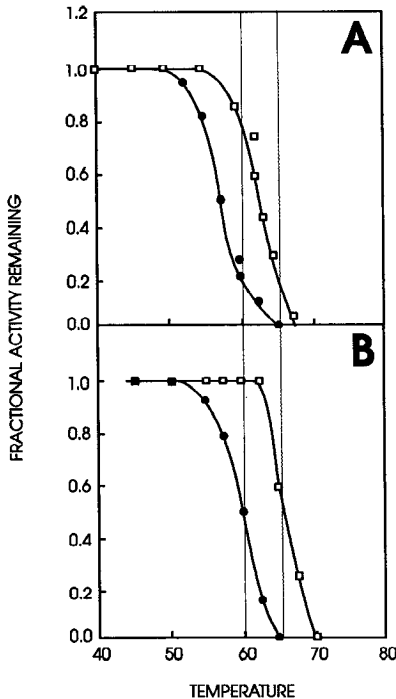


Figure 5 (A) Heat denaturation profiles of LDH-B<sub>4</sub> allelic isozymes purified from several hundred individual livers from Maine and Georgia populations of *Fundulus heteroclitus*. The symbols are LDH-B<sub>4</sub><sup>b</sup> = open squares, LDH-B<sub>4</sub><sup>a</sup> = closed circles. (B) Heat denaturation profiles of two recombinant LDH-B<sub>4</sub> proteins generated from cloned cDNA. Symbols: Ser 185 = open squares and Ala 185 = closed circles.

heat and other denaturants are useful for probing the structural flexibility and integrity of these enzymes, they do not necessarily reflect a direct response to an elevated environmental temperature. On the other hand, allelic isozymes encoded by several other loci in this teleost (*Idh-B*, *H6pdh*, *Gpi-B*, and *Aat-A*) have thermal stabilities that are below the animal's lethal temperature, and their order of thermal denaturation is correlated with the environmental temperature in which the allelic isozyme is most abundant (34, 71, 80; L. I. Gonzalez-Villasenor and D. A. Powers, submitted).

*Differences in enzyme concentrations* Near the extremes of its natural distribution in the United States (e.g. Maine and Georgia), *F. heteroclitus* experiences a mean annual temperature difference of 12.6°C. In addition, Maine fish spend a significant fraction of the year at temperatures that approach freezing while Georgia fish never experience such cold extremes. In order for Maine fish to maintain the same reaction velocities as their Georgia counterparts, approximately twice as much enzyme is needed for every 10°C decrease in environmental temperature. Consistent with that adaptive strategy, we found that the specific activity of some liver enzymes in *F. heteroclitus* from Maine were greater than in those of Georgia fish (16, 18). For example, the LDH-B<sub>4</sub> of fish liver collected from Maine had a specific activity approximately twice that of liver collected from Georgia (Figure 6A). Even after extensive temperature acclimation, the differences in specific activity remained (18). Similar analyses of populations from intermediate latitudes indicate LDH-B<sub>4</sub> specific activities intermediate to Maine and Georgia fish (17).

Maine fish are essentially only *Ldh-B<sup>b</sup>* genotype, and Georgia fish are *Ldh-B<sup>a</sup>* genotype. Since the LDH-B<sub>4</sub> allelic isozymes have the same first order rate constant ( $k_{\text{cat}}$ ) at the same temperatures, the observed differences in enzyme specific activity are most likely a function of different enzyme concentrations because  $V_{\text{max}} = k_{\text{cat}} [E_i]$ . Evidence to support this possibility comes from immunoprecipitation studies (18) wherein enzyme activity differences were directly correlated with differences in LDH-B<sub>4</sub> protein concentrations (Figure 6B). Dissimilar LDH-B<sub>4</sub> concentrations between populations appeared to be primarily genetic because the differences were present even after long-term acclimation (18); inheritance studies are consistent with that hypothesis.

The differences in LDH-B<sub>4</sub> concentrations could be the result of different *Ldh-B* gene copies, transcriptional factors, LDH-B mRNA concentrations, rates of mRNA degradation, ratios of active versus inactive enzyme, or rates of LDH-B<sub>4</sub> synthesis (or degradation). As an initial test of these possibilities, the *Ldh-B<sup>b</sup>* cDNA was cloned, sequenced (15), and used as a probe to determine if numbers of *Ldh-B* gene copies and LDH-B mRNA con-

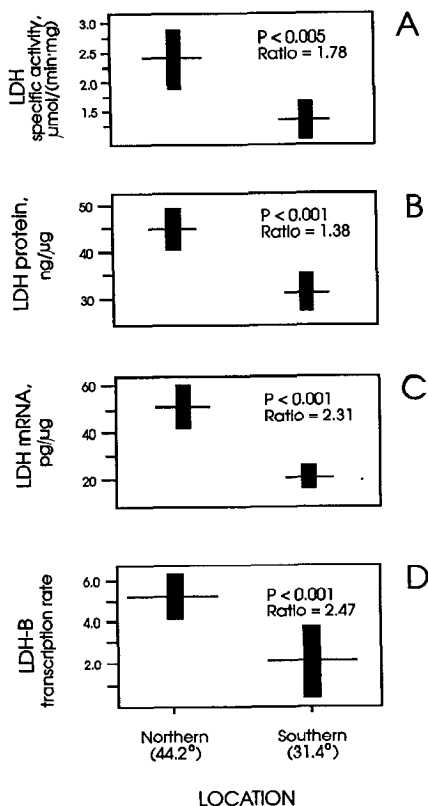


Figure 6 Livers from Maine and Georgia populations collected at 44.2°N and 31.4°N, respectively: (A) LDH-specific activity; (B) LDH protein concentrations; (C) LDH-specific mRNA concentrations; and (D) relative LDH-B transcription rate.

centrations differed between Maine and Georgia populations (18). While the number of gene copies did not vary between populations, the LDH-B mRNA concentrations were found to be different (Figure 6C). The LDH-B mRNA concentrations were approximately twofold higher in Maine fish than in their Georgia counterparts.

Because the differences in LDH-B mRNA could be due to transcriptional or posttranscriptional regulation, the *Ldh-B* transcriptional rates for Maine and Georgia fish were determined (D. L. Crawford and D. A. Powers, submitted). Northern *F. heteroclitus*, acclimated to 20°C, had a significantly higher transcription rate from the *Ldh-B* locus than acclimated fish from the southern population (Figure 6D). However, there was no difference in the total rate of RNA synthesis, nor was there a difference in the rate of actin or

$\beta$ -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. Thus, populations of *F. heteroclitus* that live in different thermal regimes appear to compensate by the transcriptional regulation of the *Ldh-B* locus. Working out the detailed molecular mechanisms responsible for this difference in gene regulation could provide insight concerning the adaptive significance of such regulatory events to natural populations in a changing thermal environment.

Studies on enzymes from distantly related and congeneric species have shown that variations in kinetic rate constants and/or enzyme concentration are important for environmental adaptation (37, 41, 74, 75, 77). In some cases, allelic variants within a species have evolved different kinetic constants that affect important physiological parameters (21, 24, 25, 40, 58, 87). In other cases, enzyme concentrations have been altered by differential gene regulation (18, 52, 51; D. L. Crawford and D. A. Powers, submitted). Since gene regulation allows enzyme activity to vary independent of amino acid changes in the gene product (52, 85), it is important to establish the relative role of differential gene expression as an evolutionary mechanism for population adaptation to changing environments, and the potential role, *if any*, of regulatory changes in the genesis of new species. This is one of the most exciting and important areas of research for population and evolutionary biologists for the coming decade.

*Some structural differences between two of the LDH-B<sub>4</sub> allelic isozymes* As mentioned, Crawford et al (15) cloned and sequenced an *Ldh-B<sup>b</sup>* cDNA of two fish from a Maine population. Recently, Lauerman et al (47) cloned and sequenced the *Ldh-B<sup>a</sup>* cDNA from one fish from a Georgia population. Nucleotide substitution in the noncoding regions and silent substitutions in the coding region varied between *Ldh-B<sup>a</sup>* and *Ldh-B<sup>b</sup>* by approximately two percent. In the absence of evolutionary constraints (i.e. selection), fourteen amino acid replacements were expected but only two were found—suggesting an evolutionarily constrained structure. Kreitman (46) found an even greater divergence from expectation in the alcohol dehydrogenase from *Drosophila*.

The amino acid sequences derived from the two *F. heteroclitus* cDNA clones indicated variation at amino acid residues 185 (Ser for Ala) and 311 (Asp for Ala). Since the lactate dehydrogenase structure has been highly conserved throughout evolution, structural comparisons can be made across taxa (42). Residue 311 is located at the exterior of the molecule pointing toward the solvent (Figure 7). While substitution of Asp by Ala at residue 311 is responsible for the charge difference between the allelic isozymes, there is no obvious functional significance to this substitution. On the other hand, residue 185 is located at the interface between subunits (Figure 7). Residues

182 to 185 form a hairpin loop that extends deep into the crevice of the other subunit. This structure may well be stabilized by hydrogen bonding between the hydroxy hydrogen of Ser 185 and the imidazolium nitrogen of His 182. The possible involvement of other residues is presented elsewhere (47). The substitution at residue 185 of Ser in LDH-B<sub>4</sub><sup>b</sup> by Ala in LDH-B<sub>4</sub><sup>a</sup> should result in a loss of hydrogen bonding between subunits. We reasoned that this substitution might account for the increased susceptibility to heat denaturation of the latter. Site-directed mutagenesis studies were done (T. Lauerman and D. A. Powers, submitted) to ascertain the role, if any, that amino acid substitutions at positions 185 and/or 311 might play in the heat denaturation and/or other functional or structural differences. As predicted, residue 185 was found to affect the thermal stability of the LDH-B<sub>4</sub> isozymes (Figure 5B). Although substituting Asp for Ala at residue 311 affected the charge of the LDH-B<sub>4</sub> electromorphs, there was little if any effect on its thermal stability. Each of the four recombinant proteins (i.e. LDH-B<sub>4</sub><sup>Ala185/Asp311</sup>; LDH-B<sub>4</sub><sup>Ser185/Asp311</sup>; LDH-B<sub>4</sub><sup>Ala185/Ala311</sup>; LDH-B<sub>4</sub><sup>Ser185/Ala311</sup>) had  $K_m^{prv}$  and  $K_I^{Lact}$  values and rates of proteolysis (T. Lauerman and D. A. Powers, submitted) that were essentially identical to those for the LDH-B<sub>4</sub><sup>b</sup> allelic isozyme isolated from a Maine population (61). These results suggest several possibilities: (a) Kinetically different but electrophoretically cryptic polymorphisms exist at the *Ldh-B* locus; (b) a high affinity inhibitor was co-purified during the purification of enzyme preparations from southern populations but not during purification of the enzyme from northern localities; or (c) there is differential postranslational modification (e.g. phosphorylation) of the LDH-B<sub>4</sub> allelic isozymes.

Since only one cDNA from one individual was sequenced with an LDH-B<sub>4</sub><sup>a</sup> electrophoretic phenotype, it cannot be said for certain that these two amino acids are the only differences between the LDH-B<sub>4</sub> allelic isozymes from Maine and Georgia populations. The fact that one cryptic amino acid substitution (position 185; Ser for Ala) was found suggests that there may be others. A variety of data support this hypothesis. For example, while substituting Ala for Ser at residue 185 affected the thermal stability of LDH-B<sub>4</sub>, the thermal profiles obtained for the recombinant proteins did not completely match the profiles obtained from enzyme purified from several hundred individuals from Maine and Georgia, respectively (Figure 5). These data suggest more than one allelic isozyme in the preparations purified from natural populations. Other support comes from detailed zoogeographic studies of enzyme polymorphisms (9, 66, 69), mtDNA restriction length polymorphisms (35, 76), and DNA fingerprinting at the *Ldh-B* locus (unpublished); all of which indicate that the Georgia population of *F. heteroclitus* has a much greater genetic diversity than the Maine population. Because genetic diversity is greatest in the southern population, cryptic amino acid substitutions will most

likely be found in southern localities. Sequencing a larger number of individual *Ldh-B* cDNA clones from Maine and Georgia populations, coupled with site-directed mutagenesis and functional studies of the recombinant proteins, would not only help resolve the functional role of specific amino acid substitutions in the LDH-B<sub>4</sub> allelic isozymes but would also provide a much deeper understanding of the extent and significance of electrophoretically cryptic genetic variation in natural populations. Those studies, which are currently underway, have already revealed a significant number of cryptic allelic variants that involve isopolar amino acid substitutions (G. Bernardi and D. A. Powers, in preparation).

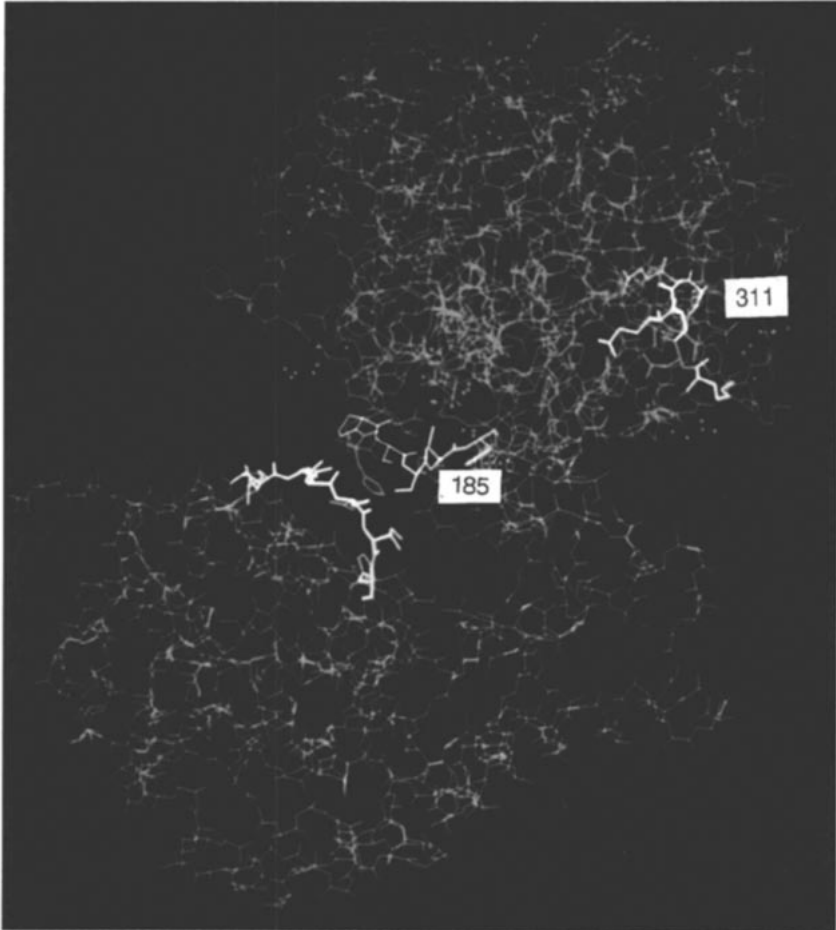
Differences between the LDH-B<sub>4</sub> allelic isozymes as measured by amino acid sequence, thermal stability, enzyme concentration, and other parameters indicate that these isozymes are structurally and functionally nonequivalent both within and between populations. Moreover, the nonequivalence can be correlated with the temperatures at which each allelic isozyme is most common. The question now arises, "Are these and other structural and functional differences reflected at higher levels of biological organization?"

### *Functional Differences at the Cellular Level*

Ideally, one would like to predict physiological differences at the cellular and organismal level that should result from observations at the molecular level. However, such predictions assume that in vitro differences are large enough to produce significant variations in vivo. In other words, physiological differences predicted on the basis of in vitro molecular studies must be proved experimentally at the cellular and organismal level, which is where selection takes place.

Because the LDH-B<sub>4</sub><sup>b</sup> enzyme had a greater catalytic efficiency at low substrate concentrations than the LDH-B<sub>4</sub><sup>a</sup> enzyme, we reasoned that, in the absence of high lactate levels, the metabolic flux or initial metabolic rates of cells that rely heavily on glycolysis should be greater for *Ldh-B<sup>b</sup>* genotypes at temperatures less than 20°C. These differences should, in turn, result in higher steady-state ATP concentrations.

In order to test that hypothesis, fish erythrocytes of each *Ldh-B* homozygous genotype were examined for differential glucose and lactate utilization and for intraerythrocyte ATP concentration. Figure 8A illustrates the rates of CO<sub>2</sub> released from cultures of *F. heteroclitus* erythrocytes when glucose is labeled at carbon one (closed symbols) and carbon six (open symbols), respectively. In both cases, cells from homozygous *Ldh-B<sup>b</sup>* fish (triangles; Figure 8A) had a greater rate than cells from *Ldh-B<sup>a</sup>* homozygotes. The phenomenon was even more pronounced when uniformly labeled lactate was utilized (Figure 8B; unlike mammals, fish erythrocytes have active mitochondria). Consistent with our expectations, the *F. heteroclitus* erythro-



**Figure 7** Computer-simulated structure of LDH-B<sub>4</sub>. Two subunits of the tetramer are shown; the subunit interaction involving residue 185, and residue 311 on the outside of the molecule.

cytes with LDH-B<sub>4</sub><sup>b</sup> also had  $2.11 \pm 0.22$  ATP's/Hb, while cells with LDH-B<sub>4</sub><sup>a</sup> only had  $1.65 \pm 0.12$  ATP's/Hb (65).

The finding of differences between *Ldh-B* genotypes in intraerythrocyte ATP concentrations was particularly important because it allowed the prediction of hemoglobin-oxygen (Hb-O<sub>2</sub>) affinity differences between adult fish (65), differences in swimming performance (24), and differential developmental rates and hatching of embryos with different *Ldh-B* genotypes (25). All of these predictions revolve around the fact that ATP is an allosteric effector of fish hemoglobin, i.e. it affects hemoglobin-oxygen affinity.

The preferential allosteric binding of organic phosphates to deoxyhemoglobin results in a decrease in hemoglobin-oxygen affinity. The major organic phosphate in fish erythrocytes is either adenosine triphosphate, (ATP) (33) or guanosine triphosphate (GTP) (32), for example, both of which decrease the affinity of hemoglobin for oxygen.

Homozygous *F. heteroclitus* with the *Ldh-B*<sup>a</sup> genotype have erythrocyte ATP levels that are significantly less than those with the *Ldh-B*<sup>b</sup> genotype, while heterozygotes have intermediate concentrations. We predicted that fish with higher levels of intraerythrocyte ATP (i.e., *Ldh-B*<sup>b</sup>) should have blood with a lower oxygen affinity at pH values favoring ionic interactions between ATP and hemoglobin (63). As predicted, *Ldh-B*<sup>a</sup> erythrocytes had a higher

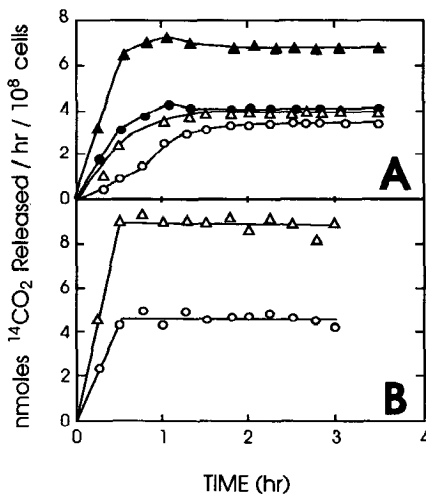


Figure 8 Nanomoles of <sup>14</sup>CO<sub>2</sub> released when *Fundulus* erythrocytes with different LDH-B<sub>4</sub> allelic isozymes (LDH-B<sub>4</sub><sup>b</sup> = triangles; LDH-B<sub>4</sub><sup>a</sup> = circles) are incubated in <sup>14</sup>C-labeled substrates. (A) Closed symbols = [1-<sup>14</sup>C]glucose and open symbols = [6-<sup>14</sup>C]glucose; (B) erythrocytes with different LDH-B<sub>4</sub> allelic isozymes (LDH-B<sub>4</sub><sup>a</sup> = triangles; LDH-B<sub>4</sub><sup>b</sup> = circles) incubated in uniformly <sup>14</sup>C-labeled lactate.

oxygen affinity than *Ldh-B<sup>b</sup>* genotypes. From these differences in oxygen loading and unloading, we can make testable predictions about whole organism responses. We shall illustrate this point with two examples: (a) differential swimming ability at low temperature (24), and (b) differential developmental rates and hatching times (25–27).

### *Physiological Basis for Swimming Endurance Differences Between Ldh-B Genotypes*

Our analyses of purified LDH-B<sub>4</sub> allelic isozymes indicate that the greatest catalytic differences between LDH-B<sub>4</sub><sup>a</sup> and LDH-B<sub>4</sub><sup>b</sup> exist at low temperature (10°C), whereas no significant difference exists at 25°C (see Figure 4). We reasoned that if the LDH-B<sub>4</sub> enzyme has a direct influence on erythrocyte ATP concentration, then differences in ATP and blood oxygen affinity should only exist at body temperatures below 25°C. Furthermore, since organic phosphate amplifies the Bohr effect of *F. heteroclitus* hemoglobins (54), these phenomena should be exaggerated at low pH values like those produced during swimming performance. To test these predictions, after an acclimation period, fish of each of the two homozygous LDH-B<sub>4</sub> phenotypes were swum to exhaustion in a closed water tunnel. As predicted, swimming performance was highly correlated with genetic variation at the *Ldh-B* locus for *F. heteroclitus* acclimated to, and swum at, 10°C while no such difference existed for the 25°C treatment (24).

Among resting fish acclimated to 10°C, hematocrit, blood pH, blood oxygen affinity, serum lactate, liver lactate, and muscle lactate did not differ significantly between the two *Ldh-B* homozygous genotypes (Table 1). Fish exercised to fatigue at 10°C showed a significant change in all of these parameters. The LDH-B<sub>4</sub><sup>b</sup> phenotype fish were able to sustain a swimming speed 20% higher than that of LDH-B<sub>4</sub><sup>a</sup> fish. Blood oxygen affinity, serum lactate, and muscle lactate also differed between the phenotypes. Since the rate of lactate accumulation was the same for the LDH-B<sub>4</sub> phenotypes, fish with LDH-B<sub>4</sub><sup>b</sup> accumulated more lactate in the blood and muscle simply because they swam longer.

In an extensive analysis of the binding of ATP to carp deoxyhemoglobin, Greaney et al (38) have shown that the organophosphate-hemoglobin affinity constants change by two orders of magnitude between pH 8 and pH 7. The same general phenomenon appears to be true for *F. heteroclitus* hemoglobins (63). In resting *F. heteroclitus* at 10°C, the blood pH was about 7.9. At this pH, the difference in erythrocyte ATP between LDH-B<sub>4</sub> phenotypes (ATP/Hb were  $1.65 \pm 0.12$  and  $2.11 \pm 0.22$  for LDH-B<sub>4</sub><sup>a</sup> and LDH-B<sub>4</sub><sup>b</sup>, respectively) is not reflected as a significant difference in blood oxygen affinity. However, as blood pH falls with increasing exercise, the organophosphate-hemoglobin affinity constant increases, and differences in blood oxygen affinity between

**Table 1** Response to swimming stress by *Fundulus heteroclitus* LDH-B phenotypes acclimated to 10° and 25°C

Parameter	10°C		25°C		p
	LDH-B <sub>4</sub> <sup>a</sup>	LDH-B <sub>4</sub> <sup>b</sup>	LDH-B <sub>4</sub> <sup>a</sup>	LDH-B <sub>4</sub> <sup>b</sup>	
<b>Resting fish</b>					
Hematocrit	23 ± 1	24 ± 1	24 ± 1	25 ± 2	NS
Blood pH	7.87 ± 0.05	7.84 ± 0.04	7.40 ± 0.05	7.48 ± 0.04	NS
P <sub>50</sub> (mm Hg)	4.2 ± 0.2	3.8 ± 0.2	5.0 ± 0.3	4.7 ± 0.2	NS
Serum lactate (mM)	1.82 ± 0.28	1.37 ± 0.15	2.6 ± 0.69	4.5 ± 0.86	NS
Liver lactate (μM/g)	0.390 ± 0.055	0.383 ± 0.044	1.8 ± 0.42	1.53 ± 0.39	NS
Muscle lactate (μM/g)	7.93 ± 0.75	8.17 ± 1.02	11.8 ± 2.2	12.5 ± 1.6	NS
<b>Exercised fish</b>					
Critical swim speed (body lengths/sec)	3.6 ± 0.12	4.3 ± 0.1	5.6 ± 0.3	5.8 ± 0.3	NS
<b>Resting fish</b>					
Hematocrit	30 ± 3	35 ± 2	36 ± 1	38 ± 2	NS
Blood pH	7.24 ± 0.04	7.15 ± 0.05	7.12 ± 0.03	7.09 ± 0.09	NS
P <sub>50</sub> (mm Hg)	6.57 ± 0.5	9.1 ± 0.5	7.4 ± 0.6	7.2 ± 0.5	NS
Serum lactate (mM)	12.19 ± 1.21	16.29 ± 0.79	23.4 ± 2.5	17.5 ± 1.6	NS
Liver lactate (μM/g)	1.39 ± 0.12	1.56 ± 0.17	6.6 ± 1.2	5.0 ± 0.6	NS
Muscle lactate (μM/g)	17.08 ± 1.86	24.01 ± 1.46	23.2 ± 4.8	20.7 ± 1.6	NS

NS = No significant difference.

\*Significant at p < 0.05

homozygous *Ldh-B* genotypes become apparent (Figure 9). As blood pH is lowered, ATP amplifies the dissociation of oxygen from *F. heteroclitus* hemoglobin; the more ATP, the greater the effect. This difference is translated into a differential ability to deliver oxygen to muscle tissue, which in turn affects swimming performance (24).

Fish acclimated to 25°C did not differ significantly in erythrocyte ATP concentrations. The ATP/Hb ratios were  $1.45 \pm 0.24$  and  $1.65 \pm 0.31$  for the *Ldh-B<sup>a</sup>* and *Ldh-B<sup>b</sup>* genotypes, respectively. In addition, there were no significant differences between LDH-B<sub>4</sub> phenotypes in any of the other parameters (Table 1). Since there were swimming performance differences between *Ldh-B* genotypes at 10°C but none at 25°C, our prediction was validated.

### Differential Hatching Times

DiMichele & Taylor (28) have shown that respiratory stress triggers the hatching mechanism in *F. heteroclitus*. In view of this, we reasoned that the hatching rates of *Ldh-B* genotypes should differ because of differences in hemoglobin-oxygen affinity (25). We predicted that the *Ldh-B<sup>a</sup>* genotype embryos should feel oxygen stress before *Ldh-B<sup>b</sup>* genotypes and thus should hatch first. Consistent with that expectation, we found that *F. heteroclitus* embryos hatched at rates that were highly correlated with *Ldh-B* genotype. *Ldh-B<sup>a</sup>* genotypes hatched before *Ldh-B<sup>b</sup>* genotypes, and the heterozygotes had an intermediate hatching distribution.

Mostly *Ldh-B<sup>a</sup>* genotype eggs emerged during the first three days of the

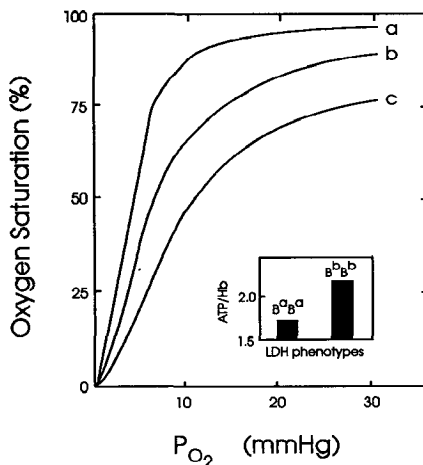


Figure 9 Oxygen equilibrium whole blood of *Fundulus heteroclitus* acclimated to 10°C: (a) resting fish of both homozygous *Ldh-B* genotypes; (b) *Ldh-B<sup>a</sup>* genotype fish; and (c) *Ldh-B<sup>b</sup>* genotype fish swum to exhaustion (from Reference 24).

hatching period, and mostly *Ldh-B<sup>b</sup>* eggs hatched in the last half. The heterozygote eggs hatched over the entire time span. The overall mean hatching times for offspring were 11.9 days for the *Ldh-B<sup>a</sup>* genotype, 12.4 days for the heterozygotes, and 12.8 days for the *Ldh-B<sup>b</sup>* homozygotes (25). In a much larger experiment that included four loci (*Ldh-B*, *Mdh-A*, *Gpi-B*, and *Pgm-A*) and slightly different physical conditions, the hatching order remained the same and the hatching time differences were even more pronounced (22, 27).

The time of hatching is very important to *F. heteroclitus* populations because of their reproductive strategy. The eggs are laid in empty mussel shells or between the leaves of the marsh grass *Spartina alterniflora*. Under these conditions, the eggs incubate in air for most of their developmental period. Hatching occurs when eggs laid on one spring tide are immersed in water by the following spring tide. As water covers the eggs, environmental oxygen decreases at the egg surface, which is the hatching cue for the embryo. Hatching at the correct time is important for survival of the fry. Therefore, overall plasticity in hatching times may be important in protecting *F. heteroclitus* populations that live under variable environmental conditions. Our data suggest that premature hatching cues (e.g. rainstorms) would select mostly against *Ldh-B<sup>a</sup>* homozygotes, while late hatching (i.e. after the tide has retreated) would primarily select against the *Ldh-B<sup>b</sup>* homozygous genotypes. In a variable environment, therefore, the heterozygote would have a selective advantage.

Since erythrocyte ATP concentrations are correlated with *Ldh-B* genotype and ATP regulates hemoglobin-oxygen affinity, the simplest interpretation is that hypoxia-induced hatching of *Ldh-B* variants results from functional differences between LDH-B<sub>4</sub> allelic isozymes that affect ATP levels.

### *Differential Developmental Rates*

Although differential hatching may be regulated by the effect of intraerythrocyte ATP concentration on hemoglobin-oxygen affinity, it should have no obvious effect on early developmental rate, i.e. prior to the development of a circulatory system. However, DiMichele & Powers (26) have presented evidence of differences between *Ldh-B* genotypes in oxygen consumption and developmental rates such that oxygen stress-induced hatching is achieved earlier in the *Ldh-B<sup>a</sup>* homozygotes. In other words, *Ldh-B<sup>a</sup>* homozygotes develop faster than *Ldh-B<sup>b</sup>* homozygotes even during the first few days of embryonic development.

In an attempt to better understand the metabolic basis for these and other developmental rate differences, Paynter et al (58) examined the metabolic properties of early developing *F. heteroclitus* embryos as a function of their *Ldh-B* genotype. During the first two days of development, the LDH-B<sub>4</sub>—

provided by the female parent—is the only LDH isozyme present in the egg. For that reason, Paynter et al (58) focused their studies during the first 24 hours of development. They showed that the unfertilized eggs had very high lactate concentrations and were different for the *Ldh-B* genotypes ( $Ldh-B^a = 48$  mM;  $Ldh-B^b = 40$  mM). They showed that the lactate was utilized at a rate 100 times higher than glucose and, within the first 24 hours, half of the lactate in the oocyte was consumed, suggesting that lactate was a major carbon source during early development. After ten hours of development, the  $Ldh-B^a$  homozygous embryo began to consume more oxygen (Figure 10A) and produce more metabolic heat (Figure 10B) than its  $Ldh-B^b$  counterpart, and the difference became even more pronounced during the remainder of the experiment. Although the  $Ldh-B^a$  homozygote developed faster and hatched sooner than the  $Ldh-B^b$  homozygote, both phenotypes took the same amount of oxygen and energy (i.e. calories) to complete the developmental process (26 and references therein). The data in Figure 10, therefore, represent an increased developmental rate for the  $Ldh-B^a$  homozygote during a time when maternal LDH-B<sub>4</sub> is the only LDH isozyme present in the egg. Since lactate is apparently a major carbon source during early development and LDH-B<sub>4</sub> is the only LDH isozyme available to metabolize lactate, it is reasonable to assume that embryos with different LDH-B<sub>4</sub> allelic isozymes are utilizing the lactate in a manner that may account for their differential developmental rates and hatching times.

Collectively, the molecular, cellular, developmental, hatching time, and swimming performance data strongly suggest that the *Ldh-B* genotypes are differentially affected by natural selection. However, those data do not absolutely rule out the possibility that other genes, tightly linked to the *Ldh-B* locus, may be responsible for the observed cellular and whole organism responses alluded to above. In fact, one could invoke a linked “mystery” locus for almost any of the published genetic and physiological studies of allelic isozymes. While it is difficult to rule out this possibility even with the most sophisticated genetic analysis, recent studies (23) clearly indicate that developmental rate can be altered by microinjection of purified LDH enzyme. In fact, the developmental rates of the two *Ldh-B* homozygous genotypes can be reversed by microinjection of the purified alternative allelic isozyme (23) (Table 2). These studies show a specific effect of LDH on developmental rate that is independent of genetic background. These enzyme transfer experiments clearly demonstrate that the LDH-B<sub>4</sub> allelic isozyme directly affects the developmental rate of early embryos. However, the metabolic and genetic mechanisms by which this is accomplished remain to be resolved.

### *Selection Experiments*

Field selection experiments have substantiated the laboratory studies alluded to above (22). DiMichele & Powers (27) predicted developmental hetero-

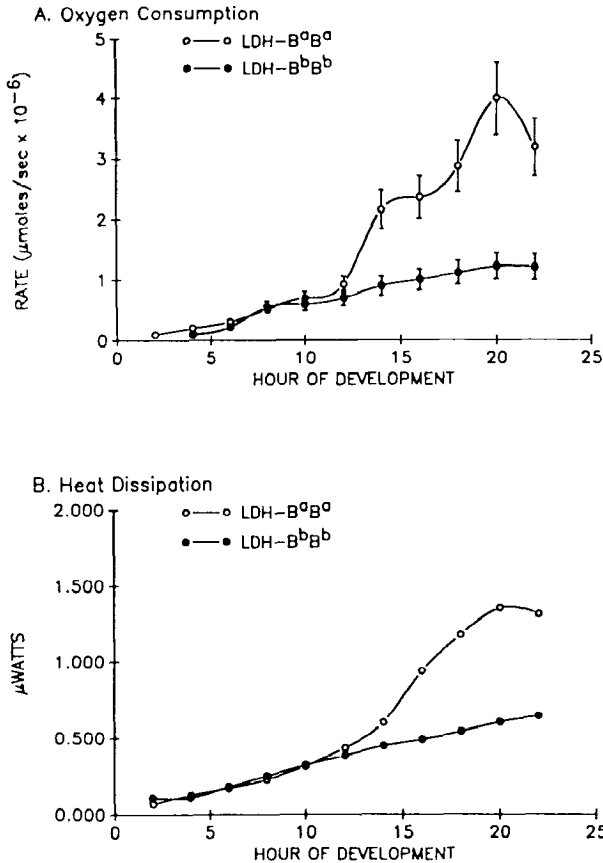


Figure 10 (A) Oxygen consumption rate per egg. Data are means from nine manometric measurements. Error bars are standard errors. (B) Heat dissipation per egg in microJoules per second (from Reference 58).

chryon and mortality differences between isozyme phenotypes as a function of temperature and salinity. Those predictions were realized as hatching times and mortality differences at high temperatures for both single and multilocus phenotypes. The fish that survived the highest temperature regime were also the most common phenotypes at the warm southern extreme of the species' natural distribution. This is particularly interesting in light of the finding of Meredith & Lotrich (53) that the mortality of *F. heteroclitus* in age class zero (eggs to fry of 59 mm) is greater than 99.5%.

If extremes in hatching time are selected against, there would be a net heterozygote advantage in a variable or uncertain environment. Such an advantage would result in the maintenance of genetic variability at the *Ldh-B* locus, as well as a stability in gene frequency at those localities where such

**Table 2** Glucose utilization rates ( $\text{mM} \times 10^{-8} \text{ sec}^{-1} \text{ egg}^{-1}$ ) of eggs injected with purified LDH-B<sub>4</sub>\*

Homozygous genotype of embryo	Injected enzyme		Significance†
	LDH-B <sub>4</sub> <sup>a</sup>	LDH-B <sub>4</sub> <sup>b</sup>	
<i>Ldh-B<sup>a</sup></i>	7.1 ± 0.9	5.15 ± 0.4	$P < 0.05$
<i>Ldh-B<sup>b</sup></i>	6.2 ± 0.8	4.4 ± 0.5	$P < 0.05$
Significance‡	NS	NS	

\*The data are means (± SE).

†Anova shows no significant (NS) differences between eggs injected with the same enzyme as that of the native homozygote but significant differences ( $P < 0.05$ ) when injected with the purified enzyme of the opposite homozygous genotype (from Ref. 23).

selection operates. Although this is consistent with the temporal stability of *Ldh-B* gene frequencies at several localities, spatial changes in the gene frequencies for a number of loci are probably due to other physical, biological, ecological, and stochastic factors (67).

The approach illustrated by our analysis of the *Ldh-B* locus of *F. heteroclitus* may lead one to conclude that natural selection is acting on that locus, at least to some extent. On the other hand, demonstrating functional nonequivalence of allelic products and even selection for a single locus will not resolve the neutralist/selectionist controversy. The major question is not "Does selection operate at the molecular level?" rather, it is "What fraction of the observed polymorphic loci are a function of natural selection?" A sufficient number of enzyme loci must be evaluated within a given species in order to determine what portion of the genome can actually be affected by natural selection and how specific gene combinations interact at the functional or regulatory levels to increase or reduce relative fitness. Moreover, this must be done in a series of model organisms with different life-history strategies. Since such an effort requires a long-term dedication to the problem and a multidisciplinary approach, we should not expect a large fraction of researchers to adopt this approach.

## CONCLUSION AND PROSPECTS FOR THE FUTURE

We have reviewed how a multidisciplinary approach to problems of intraspecies biochemical variation can provide a better understanding of complex biodiversity problems that cannot be addressed by more monolithic approaches. We have emphasized the importance of starting with simple molecular systems and making predictions that can be tested by experimentation at a higher level of biological complexity—leading from molecules to cells to organ systems to organisms and eventually laboratory and field selection experiments. While this strategy has proven fruitful in the past, the

use of molecular techniques in the future may help resolve evolutionary questions that have been previously unapproachable.

As DNA sequences of allelic alternatives from a variety of loci become available, it will be possible to determine (a) the presence and extent of silent (cryptic) allelic variants in natural populations, (b) the functional significance of isopolar amino acid differences between cryptic allelic alternatives, (c) the extent and number of genomic copies within and between populations, (d) the quantity of allele specific mRNA and the molecular mechanisms controlling them, and (e) the molecular mechanisms that regulate the expression of genes encoding allelic isozymes and their evolutionary significance.

Development of new molecular "tools" for population genetic studies will open the door to the investigation of evolutionary aspects of tissue specificity, evolutionary rates for coding and noncoding regions, the detailed mechanisms that regulate gene expression, and perhaps the role that regulatory molecules and events play in the creation and maintenance of genetic diversity within and between taxa. These molecular technologies, in turn, will provide biologists with new tools to study the genetic architecture of populations and communities as well as the ability to delineate difficult life-history stages of species with little or no morphological differences.

In addition to molecular genetic techniques, new and exciting biochemical and biophysical technologies will allow population biologists to address the physiological significance of allelic isozymes within living tissues, embryos, and even adults. For example, the use of noninvasive methods like NMR will allow one to study the dynamic change of critical metabolites during the development of living embryos. These and other technologies will usher in a new frontier that will allow population biologists to resolve fundamental evolutionary questions that have been unapproachable in the past.

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