A multidisciplinary approach to the selectionist/neutralist controversy using the model teleost, *Fundulus heteroclitus*

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1. INTRODUCTION

Few subjects in biology have been more strongly debated than the evolutionary significance, or lack thereof, of protein polymorphisms. Most of the debate has centered around the 'selectionist/neutralist' controversy. Proponents of the selectionist's school have asserted that natural selection maintains protein polymorphisms, whereas those of the neutralist's persuasion have argued that the vast majority of such variation is selectively neutral; others have favored intermediate positions.

Some biologists have 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 and Lewontin (1972), however, showed that a purely mathematical treatment could not resolve this conflict. Clarke (1975) 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. It eventually became clear that the same biogeographic data could be used to support diametrically opposed theories and, almost two decades ago, Lewontin (1974) summarized the failure of evolutionary biologists to resolve this important controversy by conventional biogeographical and mathematical approaches alone.

Implicit in the neutralist hypothesis is that most structural differences between allelic products of a given enzyme synthesizing locus are, in essence, functionally equivalent (Harris 1976), or in Darwin's words '... of no service or disservice to the species and which consequently have
not been seized on and rendered definite by natural selection (Darwin 1859). With this as the operating hypothesis, a few evolutionary biologists have developed experimental approaches that allow one to evaluate the theoretical basis of the neutralist/sele

onist controversy with the assumption that the neutralist hypothesis could be rejected for specific loci whenever functional nonequivalence could be established between genetic alternatives. Clarke (1975) was the first to summarize the general approach that experimental biologists were using to address problems of genetic variation at enzyme synthesizing loci. First, he suggested that investigators make a detailed biochemical and physiological study of the allelic isozymes. Based on the nature of the observed biochemical and physiological differences, the function of the enzyme being studied, and the ecology of the organism, he suggested that investigators postulate one or more selective factors that might be operating on the allelic isozymes and then generate a hypothesis that establishes a potential mechanistic link between the selective factors and the gene product in question. The hypothesis could then be tested by experimental manipulation to produce a predictable response. Based on the experimental results, Clarke (1975) recommended that investigators re-examine the natural populations and develop a comprehensive theory for the observed gene frequency patterns. Koehn (1978) summarized a strategy similar to that of Clarke (1975) for establishing the direct effects of allelic isozymes on natural populations.

During the past two decades, several research groups have used the general approach summarized by Clarke (1975) and Koehn (1978) to study allelic isozymes of model organisms. For example, a number of enzyme encoding loci have been studied in Drosophila, including alcohol dehydrogenase (e.g., Daly and Clarke 1981; Vigue et al. 1982; Dorado and Barbancho 1984), alpha-glycerophosphate dehydrogenase (e.g., O'Brien and MacIntyre 1972; Miller et al. 1975; Sacktor 1975; Curtsinger and Laurie-Ahlberg 1981), esterase-6 (Richmond et al. 1980), and glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (e.g., Hughes and Lucchesi 1977; Bijlsma 1978; Cavener and Clegg 1981). Koehn and his colleagues have used this approach to study aminopeptidase from Mytilus edulis (Koehn and Immermann 1981; Hilbish and Koehn 1985). Watt and his colleagues have studied the evolutionary significance of glucose phosphate dehydrogenase in Colias butterflies (Watt 1992; Watt et al. 1983, 1985), and Hoffman has studied the same enzyme in sea anemones (Zamer and Hoffman 1989). Burton and Feldman (1983) have studied the physiological effects of allelic isozyme variation at the glutamate-pyruvate transaminase in the copepod Tigriopus californicus. While not all of these important studies take advantage of the full power of the experimental approach, each has contributed important information.
about the adaptive significance of allelic isozymes, and each has revealed insights into the selectionist/neutralist controversy.

Ever since 1972 when we began our studies on the model teleost Fundulus heteroclitus, we have been using a strategy similar to that later delineated by Clarke (1975) and Koehn (1978). We have emphasized an experimental approach with a progressive analysis starting with molecules and leading to higher and higher levels of biological organization. We have reasoned that 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 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 real power of the experimental approach. In addition, we have also developed experiments to address multilocus effects and, in some cases, definitive experiments that eliminate the possibility of 'hitchhiker' effects of linked loci.

2. THE MODEL ORGANISM

Studies on the genus Fundulus have probably contributed more to the advancement of various branches of biological science in North America than any other group of fishes. Fundulus heteroclitus (Linnaeus 1866; common name: mummichog) in particular has played a key role in such diverse fields as physiology, environmental biology, genetics, parasitology, comparative histology, comparative endocrinology, and developmental biology. A symposium on the biology of Fundulus, published in American Zoologist (1986), is a testament to the continued use of this excellent model in a variety of scientific disciplines.

Fundulus heteroclitus is typically found in bays, inlets, and tidal estuaries. Adults are generally 5–10 cm in length, and they are polygamous and oviparous. Spawning occurs in the spring along the Atlantic Coast of North America with the date varying as a function of latitude starting in the south and moving northward as the water temperature increases.

This species ranges from the Matanzas River in Florida to Port au Port Bay in Newfoundland, Canada. This region features a 1°C change in annual mean water temperature per degree change in latitude (Fig.)
1C) making it one of the steepest thermal gradients in the world. Fish in the southern marshes can experience summer temperatures in excess of 40°C, while northern populations are seldom exposed to such temperatures. Winter temperatures in northern regions result in extensive ice formation, while southern marshes do not even approach freezing temperatures (Fig. 1B).

Fig. 1. (A) heterozygosity within populations versus latitude (°N); (B) lowest average monthly surface water temperature (°C) versus latitude; (C) mean surface water temperature versus latitude (adapted from Ropson et al. 1990).
Although many environmental fluctuations exist in this fish's habitat, most are secondary effects brought about by changes in temperature, oxygen, and salinity. Since *F. heteroclitus* is a poikilotherm, temperature has a dramatic effect on its metabolism. The temperature difference of approximately 15°C in annual mean water temperature between the northern and southern extremes of the species range (Fig. 1C) will result in a significant difference in the metabolic activity of these cold-blooded animals unless adequate thermal compensation could be achieved. There are two fundamental levels at which such thermal compensatory readjustments could take place: (1) at the individual level through either behavioral or physiological acclimation; and/or (2) at the population level by differential selection of allelic alternatives. In order for the second level of compensatory adjustments to take place, there has to be adequate genetic variation within the species.

3. GENETIC VARIATIONS OF NUCLEAR AND MITOCHONDRIAL LOCI

3.1 Variations in protein-encoding loci

Several studies have shown *F. heteroclitus* to be highly polymorphic for a number of enzyme encoding loci (Whitt 1969, 1970a,b; Holmes and Whitt 1970; Massaro and Brooke 1972; Avise and Kitto 1973; Kempf and Underhill 1974; Mitton and Koehn 1975; Place and Powers 1978; Van Beneden et al. 1981; Brown et al. 1988). Mitton and Koehn (1975) found that *F. heteroclitus* from Long Island Sound were polymorphic for approximately half of the 25 loci scored, with an average heterozygosity of 18 per cent per locus per individual. We have examined 50 loci from several localities along the Atlantic Coast and found that approximately half (45 per cent) of the loci were polymorphic. Using inheritance studies, we have shown that 16 of the 17 polymorphic loci studied in F1 generations of *F. heteroclitus* segregate as autosomally inherited codominate alleles (Place and Powers 1978; Van Beneden et al. 1981; Brown et al. 1988), and we have established two new linkage groups that have not been previously observed in any species (*Fum-A* with *Pgm-A* and *H6pdh-A* with *Pgm-B*). * Allele frequencies of all of these polymorphic loci have

*We have used the following abbreviations to represent enzyme-encoding loci: *Ldh-B*, the 'heart type' lactate dehydrogenase; *Mdh-A*, the cytoplasmic form of malate dehydrogenase; *Gpi-B*, glucosephosphate isomerase-B; *H6pdh-A*, hexose-6-phosphate dehydrogenase; *Idh-A* and *Idh-B*, the A and B loci of the NADP-dependent isocitrate dehydrogenase; *Mpi-A*, mannosephosphate isomerase; *Pgm-A* and *Pgm-B*, the A and B loci of phosphoglucomutase; *Fum-A*, fumarase; *Est-S*, the serum esterase; *Est-B* and *Est-D*, two liver and muscle esterases; *Aat-A* and *Aat-B*, two aspartate aminotransferase loci common to liver and other tissue;

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been determined for a large number of populations along the Atlantic Coast of North America and from the Chesapeake and Delaware Bays and their tributaries (Powers and Place 1978; Cashon et al. 1981; Powers et al. 1986; Ropson et al. 1990; Smith et al. 1992). Some of these loci have allelic isozymes that change in relation to latitude, while others do not. Two different types of aquatic environments have been studied: saltwater coastal environments, and brackish water bays and their tributaries. Some allelic isozymes have patterns of variation along the Atlantic Coast and within bays and rivers that are similar, while other loci have allelic isozymes that change within one type of environment but either do not change or change in a different way in the other environments.

Examination of the polymorphic enzyme encoding loci in *F. heteroclitus* along the Atlantic Coast has uncovered significant directional changes in gene frequency (i.e., clines) and in degree of genetic diversity in relation to latitude (Powers and Place, 1978; Cashon et al. 1981; Ropson et al. 1990). Variable loci usually have between two and eight alleles. The gene frequency patterns of coastal populations, in relation to latitude, can be grouped into four general types. The first type is represented by those loci that have two or three predominant alleles—one that dominates in northern populations, while an alternate allele (or alleles) dominates at southern latitudes. The six loci that have been identified with this gene frequency pattern are: *Ldh-B*, *Mdh-A*, *Gpi-B*, *Pgm-B*, *Mpi-A*, and *H6pdh-A* (e.g., see Fig. 2). The second pattern involves loci that are multi-allelic with one allele fixed at the northern end of the species range, but whose gene frequency drops to an intermediate value in southern populations with several other alleles being well represented (e.g., see Fig. 3). This pattern is represented by the following loci: *Idh-A*, *Idh-B*, *Pgm-A*, *Fum-A*, *Est-S*, and *Est-B*. The third gene frequency pattern that has been observed involves the fixation of an allele with the same electrophoretic mobility at both the northern and southern extremes of the species range, but with genetic variability at middle latitudes (e.g., see Fig. 4). This pattern is represented by *6Pgdh-A* and *Aat-A*. The fourth gene frequency pattern does not show a directional change in

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Ap-A, acid phosphatase-A; Pk-B, pyruvate kinase-B; Adk-A, adenylate kinase-A; 6Pgdh-A, 6-phosphogluconate dehydrogenase. Alleles of these loci are identified by superscript lower case letters (e.g., *Ldh-B*\textsuperscript{a}, *Mdh-A*\textsuperscript{b}, *6Pgdh-A*\textsuperscript{b}, etc.).

When enzymes are referred to in the text, capital letters are used and, when appropriate, the number of protein subunits are denoted by a subscript (e.g., LDH-B\textsubscript{4}, IDH-A\textsubscript{2}, and PGM-A for 4, 2, and 1 subunit proteins, respectively). The different allelic isozymes are identified by different superscript letters (e.g., LDH-B\textsuperscript{1}, MDH-A\textsuperscript{3}, 6PGDH-A\textsuperscript{a}, etc.).
Fig. 2. Frequency of Mdh-A^a allele versus latitude °N. Solid triangles are from coastal populations whereas the open symbols are from populations from the Chesapeake Bay and its tributaries (from Powers et al. 1986).

Fig. 3. Frequency of Idh-B^a allele versus latitude °N. Solid triangles are from coastal populations whereas the open symbols are from populations from the Chesapeake Bay and its tributaries (from Powers et al. 1986).

gene frequency with latitude (e.g., see Fig. 5). This pattern is represented by Aat-B, Ap-A, Adk-A, and Est-D.

In addition to genetic variations studies of coastal populations, populations have been studied from the Chesapeake and Delaware Bays
Fig. 4. Frequency of $6Pgdh-A^b$ allele versus latitude °N. Solid triangles are from coastal populations whereas the open symbols are from populations from the Chesapeake Bay and its tributaries.

Fig. 5. Frequency of $Ap-A^b$ allele versus latitude °N. Solid symbols are from coastal populations (from Powers et al. 1986).

and their tributaries. For example, the gene frequencies of 12 protein-encoding loci from *F. heteroclitus* populations from 30 localities within the Chesapeake Bay and its tributaries were studied in the late 1970s and early 1980s (Powers et al. 1986, 1991, unpublished). Of these 12 loci, nine were also studied from Delaware Bay populations, and three
additional loci (Pk-B, Fum-A, and Mpi-A) were analyzed for a number of Delaware Bay populations (Powers et al. 1986). Six of the loci previously studied from Chesapeake Bay populations and seven loci studied in Delaware Bay populations were assayed again in 1988 from 19 Chesapeake and Delaware Bay populations (Smith et al. 1992). Of the 14 enzyme loci studied, four (Aat-A, Idh-B, Fum-A, and Mpi-A) showed little or no significant change in gene frequency either up the bays or within the tributaries. The other ten loci, Ldh-B, Est-S, Pk-B, Gpi-B, Mdh-A, 6Pgdh-A, Aat-B, Pgm-A, Idh-A, and Pgm-B showed some clinal variations in one or both of the bays – some changed modestly (e.g., Aat-B, Pgm-A), others changed substantially (e.g., Mdh-A, 6Pgdh-A; Figs 2 and 4), and still others changed in an intermediate fashion (e.g., Ldh-B, Est-S, Pk-B, Gpi-B, Idh-A, and Pgm-B; Fig. 6). In general, the allele that increased in gene frequency up the Chesapeake Bay was also the allele that increased up major tributaries like the Potomac River. With the exception of 6Pgdh-A, the regional clines within the Chesapeake Bay (i.e., eastern shore, western shore, and up tributaries) were quite similar to frequency changes associated with latitude up the bay. The 6Pgdh-A\textsuperscript{b} gene frequency increased more rapidly within the major tributaries like the Potomac River than along either shore of the Chesapeake Bay. However, when the 6Pgdh-A\textsuperscript{b} gene frequency was compared to the average salinity at the sampling sites, the cline for the river more closely approximated the bay cline. This correlation between salinity and gene frequency for 6Pgdh-A\textsuperscript{b} within the Chesapeake Bay.

![Image](image-url)

**Fig. 6.** Frequency of the Ldh-B\textsuperscript{b} allele versus latitude °N. Solid triangles are from coastal populations whereas the open symbols are from populations from the Chesapeake Bay and its tributaries (from Powers et al. 1991).
and Potomac River (Fig. 7) suggests that salinity, either directly or indirectly, may be a selective environmental factor. This possibility is reinforced by the fact that there is little, if any, directional gene frequency variation for this locus along the Atlantic Coast of North America.

When the gene frequency patterns of populations from coastal saltwater environments were compared with those of brackish water bay/tributary environments, some populations reflected similarities, while others differed dramatically. A comparison of coastal and bay populations by regression and Mantel analyses identified several patterns (Smith et al. 1992). The first pattern had gene frequency changes that vary with latitude in the same manner and magnitude as their coastal counterparts—that is to say, populations within the bays have gene frequencies that are not significantly different than populations at equivalent latitudes along the Atlantic Coast. Within this pattern, there are loci that are clinal at equivalent latitudes (e.g., Ldh-B; Fig. 6), and there are loci that do not have directional changes at equivalent latitudes (e.g., Idh-B; Fig. 3). A second pattern that emerges is one where populations within the bays have directional gene frequency changes that are similar to

![Diagram](image-url)

**Fig. 7.** (A) Surface salinity versus latitude °N; solid circles are for the Chesapeake Bay while the open circles represent data for the Potomac River. (B) Frequency of the δPgdh-A allele versus latitude °N; solid circles are for the Chesapeake Bay while the open circles represent data for the Potomac River (from Powers et al. 1986).
those found along the Atlantic Coast, but the rapid change that takes place in coastal populations at latitudes north of 40°N are reflected within bay populations at much more southern latitudes (i.e., between 37°N and approximately 39°N). In other words, the clines within the bay are similar to the clines along the coast, but the latitude at which the rapid gene frequency change occurs is shifted south by approximately 200 miles (e.g., Mdh-A; Fig. 2). The third pattern indicates directional gene frequency changes along the Atlantic Coast, but none within the bays at equivalent latitudes (e.g., Mpi-A; see Smith et al. 1992). The fourth pattern, represented only by 6Pgdh-A, shows no directional change along the coast, but significant clinal changes within the bays (Fig. 4).

3.2 Mitochondrial DNA analysis of coastal and bay populations

In addition to nuclear protein-encoding loci, significant clines in mitochondrial restriction fragment length polymorphisms (RFLPs) of *F. heteroclitus* have been observed. Populations from coastal (Gonzalez-Villaseñor and Powers 1990) and bay populations (Smith 1989; Smith et al. submitted) have been studied for variation in RFLP of mitochondrial DNA (mtDNA). Analyses of these mtDNA RFLP patterns have provided insight about the previous distribution and evolutionary history of this species. Brown and Chapman (1991) have used RFLP analyses of mtDNA to study gene flow in *Fundulus heteroclitus*.

Directional changes in genetic characters with geography (i.e., clines) have classically been described by two general models: primary and secondary intergradation. In the primary intergradation model, adaptation to local conditions along an environmental gradient 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 either adaptation to local conditions or genetic drift produces genetic differences between these disjunct populations (see Fig. 8). 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 previous existence of isolating barriers to gene flow in the latter. Although 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 (Endler 1977), an analysis of mitochondrial DNA (mtDNA) can sometimes provide insight concerning primary and secondary intergradation long after a secondary contact.

We have analyzed *F. heteroclitus* populations along the Atlantic Coast by studying mtDNA fragments obtained by digestion with several
restriction endonucleases (Gonzalez-Villasenor and Powers 1990). Analysis of the mtDNA restriction fragment data indicated an intergrade zone at or near 41°N latitude. This conclusion was based on the fact the mtDNA restriction patterns of fish at specific localities could be interrelated by a network of single nucleotide base changes (Fig. 9). However, populations on each side of 41°N latitude required many nucleotide changes. The distribution of these haplotype assemblages could be explained either by primary intergradation coupled with selection on nuclear genes (Takahata and Slatkin 1984), or secondary intergradation of previous isolates that had diverged as a result of genetic drift and/or natural selection. Of
Fig. 9. 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 between the populations of Maine and Vince Lombardi, NJ, while clone 10 is shared between Stone Harbor, NJ, and Georgia. The phenogram of mtDNA genotypes illustrated as an insert in the figure, 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 Gonzalez and Powers 1990).
these two possibilities, Gonzalez-Villasenor and Powers (1990) concluded that the mtDNA RFLP data tended to support the secondary intergradation model, but primary intergradation could not be ruled out because of the possibility that intermediate mtDNA haplotypes could have been eliminated by periodic population crashes and expansions into newly available habitat by founder mtDNA haplotypes.

Previously, Cashon et al. (1981) 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 which is associated with the edge of the last major glacial advance. Although the mtDNA data showed a sharp disjunction consistent with that hypothesis, the nucleotide differences between the 'northern' and 'southern' mtDNA haplotypes (see inserted phenogram in Fig. 9) suggested a divergence time significantly before the last glacial advance. The Chesapeake and Delaware Bays were only rivers during the last glacial advance. Smith et al. (submitted) used five diagnostic restriction enzymes to examine the mtDNA haplotypes of several hundred individuals from populations within these bays to determine if 'northern' mtDNA haplotypes could be detected as remnants of a preglacial distribution. Fifty mtDNA haplotypes were found in 740 specimens from some 29 localities. These mtDNA haplotypes were easily divided into three major groupings which were referred to as 'northern,' 'southern,' and 'intermediate' forms. Eleven of the remaining 47 haplotypes were rare but present in at least two individuals, and 19 were found in two or more populations. A diagram showing the relationships among the various mtDNA haplotypes is illustrated in Fig. 10. In addition to the relationship between haplotypes illustrated in Fig. 10, there were significant variations in the zoogeographic distribution of these haplotypes. Not only were northern mtDNA haplotypes detected within the Chesapeake and Delaware Bays, but they were distributed in a cinal fashion up the bays and their tributaries (Fig. 11). The northern mtDNA haplotypes were common in the headwaters of the bays and tributaries, while the southern mtDNA haplotypes dominated in the lower bays and rivers. The Wagner parsimony network and bootstrap analysis (Fig. 10) identified at least two haplotypes that were considered transitional between the 'northern' and 'southern' forms. These intermediate forms were found in the Chesapeake Bay, but they were predominantly found in rivers and estuaries several hundred miles south of the present day mouth of the Chesapeake Bay (Smith et al. submitted). The presence of intermediate mtDNA haplotypes is consistent with the primary intergradation model with selection as a major driving force, but the secondary intergradation model cannot be ruled out because of the possibility of evolutionary convergence and the low frequency of the intermediate mtDNA haplotypes. On the other hand, the low frequency
Fig. 10. Wagner parsimony network minimizing mutation steps between mitochondrial DNA haplotypes observed in this study. Each dot represents one individual except for the three major mtDNA haplotypes where the number observed is reported. A dash corresponds to one detected restriction site change between haplotypes. The number of times that a bootstrap analysis of 100 replicates supports this network is shown between major haplotype groups.

of intermediate forms is also consistent with differential selection of the mtDNA haplotypes. Further studies need to be done to better distinguish between these alternatives.
Fig. 11. Frequencies of 'northern' and 'southern' mtDNA haplotypes superimposed as pie diagrams on a map of the Atlantic Coast and insert of the Mid-Atlantic region. Symbols: solid regions represent the fraction of a population represented by a 'northern' mtDNA haplotype, open regions represent the fraction of a population represented by a 'southern' mtDNA haplotype.

3.3 Glacial cycles and genetic divergence of *Fundulus heteroclitus* populations

There are at least three different hypotheses that could account for the presence of northern phenotypes (i.e., northern mtDNA haplotypes, allelic isozymes, and morphological characters) of *Fundulus heteroclitus* in the upper Chesapeake and Delaware Bays and their tributaries. The first hypothesis can be described as the headwater or *inoculation hypothesis*. In this case, the Champlain–Hudson glacial lobe (20,000 years ago; Mickelson *et al.* 1983) would have to have depressed the landmass sufficiently to generate a temporary glacial watershed connecting the Atlantic Coast with the headwaters of the Delaware and Susquehanna Rivers. Fish with the northern phenotype could then have theoretically been able to invade the tributary headwaters and estuaries of the Delaware and Susquehanna Rivers via an inland route. Northern phenotypes of *Fundulus heteroclitus* are known to be more freshwater tolerant and can thrive in freshwater habitats (Garside 1969; Dennoncourt *et al.* 1978; Able and Palmer 1988). Fish inoculated from northern populations (around 41°N) would have had to disperse into coastal habitats, successfully displacing southern phenotypes at the mouths of
both the Delaware and Susquehanna Rivers from about 18,000–11,000 years ago (Bloom 1983). Afterward, the inoculated northern phenotypes would have been displaced in the warmer, more saline, portions of Chesapeake and Delaware Bays by southern phenotype fish as a result of rising sea levels and oceanic warming over the past 10,000 years. Although there is evidence of landmass depression by the glacier, there is no geological evidence of any previous connection between headwaters of the Delaware and Susquehanna Rivers and the northern Atlantic Coast waters near the present day Hudson River. Therefore, the inoculation hypothesis is not compelling.

A second hypothesis, coastal flooding, that could explain the presence of northern phenotypes in the upper Chesapeake and Delaware Bays is a derivative of the first and depends, not upon depression of the landmass, but rather on coastal flooding during periodic sea level changes. Sea levels 72,000, 95,000, 120,000, 188,000, and 440,000 years ago were well above present day heights (Cronin et al. 1981). This could have created water course connections from the northern Atlantic Coast areas to the Susquehanna and Delaware Rivers. However, those connections would probably have had to have arisen during significantly warmer climatic conditions when southern phenotypes would have been at a selective advantage and more likely to occupy available habitat in the mid-Atlantic region. Unless cooler and less saline areas were available within the flooded coastal zone for colonization by the northern phenotypes during these periods, this second hypothesis seems as unlikely as the first.

The third and most plausible possibility is that of the coastal invasion hypothesis which assumes that both Fundulus heteroclitus genetic assemblages were of coastal origin and that the coastal zone of intergradation was south of the present mouth of the Chesapeake Bay prior to the last sea level rise. Similar coastal invasion hypotheses have been suggested previously (Cashon et al. 1981; Morin and Able 1983; Able and Felley 1986; Powers et al. 1986; Ropson et al. 1990), and each postulates a different species range, 10,000–20,000 years ago, than exists today. The presence of northern phenotypes in the Chesapeake Bay and its tributaries supports the hypothesis that the southern extreme of the northern phenotype’s range was at or below the present mouth of the Chesapeake Bay prior to coastal flooding 11,000 years ago. At that time, the northern phenotype would have occupied a range from north of Cape Hatteras, which included the future mouth of the Chesapeake Bay, to limits of their cold and freshwater tolerance near the glacial ice sheet (around 41°N latitude). The southern phenotype would then have been distributed from Cape Hatteras south to Florida. Since the Pamlico estuary (b) contains both common southern mtDNA haplotypes, the Cape Hatteras area most likely served as a source population for the southern race that invaded the mid-Atlantic Coast and bays. Once coastal
flooded 11,000 years ago (Bloom 1983), the northern nuclear and mitochondrial DNA phenotype would have been in position to colonize the new marsh habitat being formed as a result of the flooding of Chesapeake and Delaware Bays, as well as the new habitat generated north of New Jersey as a result of the retreating ice sheet. Concurrently and subsequently, the southern phenotype would have been in a position to invade the warming waters of the mid-Atlantic Coast and the lower parts of the Chesapeake and Delaware Bays. The freshwater and cold tolerant northern phenotype would then be pushed into freshwater refugia at the northern extremes of the bays and upper parts of their tributaries. These northern phenotypes would also dominate in the colder northern waters liberated by the retreating ice sheet. As a result, the present distribution of Fundulus heteroclitus phenotypes include intergrade zones within the Chesapeake and Delaware Bays and along the Atlantic Coast.

When one considers all the zoogeographical data of Fundulus heteroclitus (gene frequencies of allelic isozymes, mitochondrial DNA haplotype assemblages, and morphological characters), the most parsimonious explanation is that the separate coastal and bay intergrade zones once shared a common ancestral origin (100,000–1,000,000 years ago) somewhere south of the present mouth of the Chesapeake Bay. Therefore, of the three possibilities, the coastal invasion hypothesis seems to be the most plausible.

Barton and Hewitt (1989) suggested that movement of contact (tension) zones, such as those described above, provided an important test of Wright’s shifting-balance theory of evolution. The data presented in this paper and elsewhere supplies an example of a contact zone which has changed since the Wisconsinian glaciation. Since there have been several glaciations and sea level changes during the past million years or more that Fundulus heteroclitus has occupied habitats along the Atlantic Coast, it is reasonable to conclude that the contact zones between northern and southern phenotypes have changed many times during the last several hundred thousand years.

Periodic oscillations in temperature, causing glaciations, and sea level changes are governed by cyclic alterations in the tilt and orbit of the earth which occur with a 20,000–100,000 year cycle, i.e., Milankovitch cycles (Bennett 1990). These periodic changes have occurred many times during the Pleistocene since the presumed divergence of the two mitochondrial DNA haplotype assemblages of Fundulus heteroclitus (Gonzalez-Villasenor and Powers 1990). Given the past sea level changes and geological history, any primary or secondary intergradation predates flooding of these bays following the retreat of the last glacier. The significantly lower frequency of northern haplotype variants suggests that northern populations have either undergone a bottleneck more recently than their southern counterparts, or a founder northern phenotype rapidly expanded into the unoccupied habitat liberated by the retreating glacial ice sheet and
flooded plains of the Chesapeake and Delaware Bays. Whichever the case, the instability in population sizes and fluctuating geographical locations of intergradation zones must be incorporated into general models of hybridization and cline formation in order to provide a comprehensive understanding of the problem (Barton and Hewitt 1983, 1985).

A stepping-stone model represents a simplification of the Fundulus heteroclitus population structure. Traditionally, colonies (populations) are considered static in their location with migration occurring between them. The Pleistocene environmental oscillations may have caused Fundulus heteroclitus to move north and south along the Atlantic Coast many times, resulting in increased interactions between populations. Thus, the ever-changing habitat effectively increased the migration rate. Clearly, the opening of the new habitats created by the retreat of the glacial ice sheet and the flooding of the Chesapeake and Delaware Bays would favor directional migration into these new unoccupied habitats by phenotypes that were at the northern extreme of the species range and thus ideally poised to take advantage of the newly available habitat as the range was extended. The Pleistocene environment most likely also resulted in a greater migration between populations, which makes the continued presence of genetic and morphological clines surprising. Barton and Hewitt (1989) suggest that, given tension zone movement, these intact genomes are evidence for cohesive coadapted gene combinations.

Combining previous work on morphology and allelic isozymes with an analysis of the maternally inherited mitochondrial genome achieved a new synthesis and understanding of Fundulus heteroclitus history. The ever-changing environment has been paced by astronomical cycles related to the earth’s tilt and orbit which oscillates over a 20,000–100,000 year time frame (Bennett 1990). Paleontological data for many flora and fauna indicate that local populations have frequently tracked environmental changes related to glacialiations and sea level changes of these Milankovich cycles (Bennett 1990). Morphological, physiological, and genetic patterns characteristic of Fundulus heteroclitus populations demonstrate the impact of frequent glacialiations and sea level changes in shaping present-day zoogeographic patterns.

4. THE MULTIDISCIPLINARY EXPERIMENTAL APPROACH TO THE SELECTIONIST/NEUTRALIST CONTROVERSY

While zoogeographical studies, like those described above, are useful for placing genetic variations into 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 the allelic alternatives of specific loci prior to, during, and/or after a geographic isolation event (see Fig. 8). 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 within and between populations of Fundulus heteroclitus.

As pointed out earlier, our model organism, F. heteroclitus, is found in one of the steepest thermal gradients in the world (Fig. 1) and, being a poikilotherm, must be profoundly influenced by this environmental parameter. In the southern marshes, summer water temperatures in excess of 40°C are commonly recorded, whereas summer temperatures of the northernmost marshes are relatively cold. Alternatively, northern marshes are often covered by ice in the winter, while southern marshes are generally free of ice (see Fig. 1B). The gene diversity of this species decreases at latitudes greater than 41°N, but is unchanged at southern latitudes (Fig. 1A). Powers et al. (1983) 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 and Koehn (1975) 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 (Powers et al. 1986). While these correlations are suggestive of a causal relationship, they are not definitive because they could be explained by stochastic arguments.

We have employed an experimentally based strategy similar to that suggested by Clarke (1975) in an attempt to falsify the hypothesis that natural selection was (or is) a major driving force responsible for the observed gene diversity in Fundulus heteroclitus. The strategy begins with a detailed biochemical study of the allelic isozymes and progresses through higher levels of biological organization by a linked series of predictions followed by experimental validation.

5. THE BIOCHEMICAL LEVEL

Biochemical differences between the allelic isozymes are usually characterized by classical ‘steady-state’ kinetic parameters. Because a broad
array of researchers with different types of expertise will presumably read this paper, it seems appropriate to briefly review the important parameters in classical steady-state kinetics. 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 and when substrates are in excess, a reaction will proceed at its maximum velocity \( (V_{\text{max}}) \). The \( V_{\text{max}} \) reflects the enzyme's catalytic efficiency \( (k_{\text{cat}}) \) and the enzyme concentration \( ([E_i]) \) at saturating substrate concentration \( (i.e., V_{\text{max}} = k_{\text{cat}} [E_i]) \). Therefore, apparent differences in \( V_{\text{max}} \) may be attributed to either different apparent first-order rate constants \( (k_{\text{cat}}) \) or to different enzyme concentrations. When substrate concentrations are limiting, the reaction is less than \( V_{\text{max}} \). The substrate concentration at which the velocity \( (v) \) is 50 per cent of \( V_{\text{max}} \) is the \( K_{\text{m}} \) (Michaelis–Menten constant) which often approximates the dissociation constant for the enzyme–substrate complex. While a single substrate reaction has one \( K_{\text{m}} \), multisubstrate reactions have a \( K_{\text{m}} \) for each substrate.

Substrate concentrations \textit{in vivo} are normally too low to saturate enzymes; therefore, at very low substrate concentrations, \( V_{\text{max}}/K_{\text{m}} \) is a pseudo first-order rate constant. However, \( k_{\text{cat}}/K_{\text{m}} \), the second-order rate constant, is a more appropriate parameter because at very low substrate concentrations, the enzyme may be considered a reactant. Also, \textit{in vivo} enzyme concentration can vary and \( V_{\text{max}}/K_{\text{m}} \) is concentration dependent, while \( k_{\text{cat}}/K_{\text{m}} \) is not. Because \( k_{\text{cat}}/K_{\text{m}} \) 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 \( k_{\text{cat}}/K_{\text{m}} \) is very large, the reaction is maximized and becomes diffusion limited.

Considerable discussion has centered on the evolutionary importance of \( V_{\text{max}}/K_{\text{m}} \) (Crowley 1975; Brocklehurst and Cornish-Bowden 1976; Cornish-Bowden 1976; Brocklehurst 1977, and Fersht 1977). If the prime evolutionary objective is to attain the highest possible catalytic rates, then \( V_{\text{max}} \) (i.e., \( k_{\text{cat}} \) and/or \( [E_i] \) and \( K_{\text{m}} \) should increase within the evolutionary constraint imposed by diffusion. According to Fersht (1977) and Crowley (1975), \( k_{\text{cat}} [E_i] \) should be as large as possible, and \( K_{\text{m}} \) should be large compared with the \textit{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 determine how these can affect the reactions \textit{in vivo}.

We have found biochemical differences between the allelic isozymes of six loci \((Gpi-B, H0pdh-A, Idh-B, Aat-A, Mdh-A, and Ldh-B)\). These
differences range from large variations in steady-state kinetic parameters and thermal stability to little or no differences in kinetic parameters, and only small differences in thermal stabilities.

5.1 Glucose phosphate isomerase

Glucose phosphate isomerase (GPI) catalyzes the isomerization of glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P) in the glycolytic pathway. Under standard conditions, the reaction is reversible with an equilibrium constant ($K_{eq}$) of 2.3 and a free reaction energy ($\Delta G$) of approximately 0.4 kcal/mole.

The fate of G6P is determined at a major branchpoint of carbohydrate metabolism (Fig. 12). At this branchpoint, G6P can be directed toward:

*Fig. 12. Schematic representation of the glucose-6-phosphate (G6P) branchpoint and the different metabolic pathways competing for this metabolite. Fructose-6-phosphate (F6P) is ultimately converted into lactate through the glycolytic pathway, whereas 6-phosphogluconate (6PG) is oxidized through the pentose shunt. Symbols: Glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), 6-phosphogluconate (6PG), mannose-6-phosphate (M6P), nicotinamide adenine dinucleotide phosphate (NADP), phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6PDH), glucosephosphate isomerase (GPI or GPI-B$^2$ for the liver form), hexokinase (HK), glucose-6-phosphatase (G6Pase), 6-phosphogluconate dehydrogenase (6PGDH), mannose-6-phosphate isomerase (MPI). Allosteric and inhibitory effects are represented by negative and positive signs inside triangles, representing negative and positive effects, respectively.*
(1) glycogen synthesis by phosphoglucomutase (PGM); (2) the production of free glucose through glucose-6-phosphatase (G6Pase); (3) the catabolic pathway of the hexose monophosphate shunt by glucose-6-phosphate dehydrogenase (G6PDH); or (4) glycolysis through GPI catalysis. In addition, G6P can also be metabolized by hexose-6-phosphate dehydrogenase (H6PDH) to provide reducing equivalents for microsomal functions (see section on H6PDH below). Because temperature and other variables shift the relative metabolic needs of a cold-blooded organism, one might expect the various enzymes that compete for G6P to change as a function of temperature, which would be reflected in changes in the partitioning of carbon toward the pathways illustrated in Fig. 12. Hazel and Prosser (1974) showed that major metabolic shifts toward biosynthesis and away from glycolysis occur in cold acclimated fish. This type of metabolic reorganization enables fish to direct more energy toward growth processes (Somero and Hochachaka 1976). This is more extensively reviewed in Prosser (1986).

Glucose phosphate isomerase is generally classified as an equilibrium enzyme and, therefore, its role as a metabolic regulator has been minimized by some researchers (Ozand and Narahara 1964; Minakami and Yoshikawa 1966; Wilson et al. 1967); but some investigators point out that the relatively low inhibition constant for 6PG and the shift from equilibrium values under some conditions, suggests a subtle regulatory role (Landau and Katz 1964; Johnson 1974). In addition, studies on glucose phosphate isomerase deficiency disease in humans support this contention (e.g., Baughan et al. 1968; Blume et al. 1972). Gracy (1975) has pointed out that those studies indicate that a decrease in GPI activity can have severe metabolic consequences, even though enzyme levels based on in vitro assays should not be rate limiting. Several nuclear magnetic resonance (NMR) studies on living tissues have shown that bound versus free substrates and cofactors are tremendously different than those determined by classical in vitro metabolite and enzyme assays. It is time that biologists re-evaluate the notion that classical metabolite and enzyme assays of cell extracts reflect the true internal state of living cells and tissues. Our kinetic studies are done on purified enzymes rather than cell extracts and our metabolism studies are done on living cells, tissues, and intact organisms.

While many vertebrates and invertebrates possess a single GPI enzyme, teleosts have at least two loci. Fundulus heteroclitus has two Gpi loci: Gpi-A and Gpi-B (Avise and Kitto 1973). The enzyme encoded by Gpi-A is expressed in white skeletal muscle, while the isozymes encoded by Gpi-B are found in liver and other internal organs. The two major electrophoretic allelic variants were first described by Mitton and Koehn (1975). Place and Powers (1978) reported the existence of two additional alleles and evidence that all the allelic isozymes were inherited in a
Mendelian manner. Powers and Place (1978) showed that the major alleles had gene frequency changes that were clinal in nature along the Atlantic Coast of North America.

The isozymes of both glucose phosphate isomerase loci have been purified and their steady-state kinetics parameters determined (Palumbi et al. 1980; Van Beneden and Powers 1985). In addition, the major allelic isozymes of the Gpi-B locus have been purified and characterized (Van Beneden and Powers 1989). A kinetic analysis of these allelic products, in relation to both temperature and pH, revealed several interesting differences. While all the steady-state kinetic parameters showed strong temperature dependence, the allelic isozymes only differed in their inhibition constants for 6-phosphogluconate ($K_i$) at high temperatures. The GPI-$B_2^k$ allelic isozyme had a higher $K_i$ than its GPI-$B_2^s$ counterpart. Therefore, the allelic isozyme that predominates in populations at southern latitudes is less sensitive to 6-phosphogluconate inhibition at high temperatures than the allelic isozyme that predominates in populations from the northern extreme of the species range. *Fundulus heteroclitus* acclimated to 30°C have significantly higher concentrations of 6-phosphogluconate than do fish acclimated to 10°C. Van Beneden and Powers (1989) suggested that the higher $K_i$ for the GPI-$B_2^s$ allelic isozyme may provide an adaptive advantage to compensate for the increased concentration of 6-phosphogluconate at elevated environmental temperatures. However, metabolic flux studies need to be done to test that hypothesis.

At 25°C there was a significant difference in the apparent $V_{\text{max}}$ between pH 7.5 and 8.5, but no significant difference at higher and low pH values. In temperature stability studies, it was found that the GPI-$B_2^s$ allelic isozyme was more stable than the GPI-$B_2^s$ allelic isozyme (Fig. 13) at temperatures within the temperature range experienced by this fish in its natural habitat — especially at southern latitudes. The more stable isozyme was also the one that is most common among populations at southern latitudes. On the basis of these biochemical studies, Van Beneden and Powers (1989) rejected the hypothesis that the allelic isozymes were functionally equivalent. Later studies at higher levels of biological organization were consistent with that conclusion (e.g., DiMichele et al. 1986; DiMichele and Powers 1991).

### 5.2 Hexose-6-phosphate dehydrogenase

Hexose-6-phosphate dehydrogenase (H6PDH) catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate with the aid of NADP$^+$ as a cofactor. While this is the same reaction catalyzed by glucose-6-phosphate dehydrogenase, H6PDH also reacts with several other sugar
Fig. 13. Thermal denaturation of *Fundulus heteroclitus* GPI-B<sub>2</sub> allelic isozymes. (A) Fraction of remaining activity in relation to incubation temperature. (B) Fraction of remaining activity as a function of time at 42°C. The GPI-B<sub>2</sub> and GPI-B<sub>5</sub> are represented by squares and triangles, respectively (from Van Beneden and Powers 1989).

substrates, including: galactose-6-phosphate, 2-deoxyglucose-6-phosphate, glucose-6-sulfate, and glucose itself. In addition to the use of NADP<sup>+</sup>, the H<sub>6</sub>PDH of *Fundulus heteroclitus* also uses a number of other cofactors and has a novel reaction mechanism (Ropson and Powers 1988). The enzyme's apparent function is to provide reducing equivalents in the form of NADPH to the microsomal fraction for steroid metabolism and detoxification within the liver.

The three major alleles of *H<sub>6</sub>pdh-A* change as a function of latitude, with the *H<sub>6</sub>pdh-A<sup>c</sup>* allele being fixed at the northern extreme of the species range and being replaced by the *H<sub>6</sub>pdh-A<sup>b</sup>* and *H<sub>6</sub>pdh-A<sup>a</sup>* alleles in more southerly populations. Biochemical analysis of the purified allelic isozymes encoded by these alleles reveals a number of interesting
functional differences in both steady-state kinetic parameters and thermal stability. For example, significant differences were found among the allelic isozymes for the $K_m$ of NADP$^+$, the $K_m$ of glucose-6-phosphate, and the $K_i$ of glucose-6-phosphate. For example, when compared to the allelic isozyme that predominates in northern populations (H6PDH-A$^+$), the homozygous enzymes common in southern populations (H6PDH-A$^+$ and H6PDH-A$^-$) had smaller $K_m$ values. These differences in $K_m$ and $K_i$ may be important relative to the microsomal substrate concentrations for NADP$^+$ and sugar phosphates, but those values have yet to be determined.

There were also differences in $V_{max}$ found among the allelic isozymes, but as was the case for the glucose-6-phosphate isomerase allelic isozymes, the biological significance of these $V_{max}$ differences must remain questionable until active enzyme concentrations can be determined. The value of $V_{max}$ depends on the intrinsic turnover rate ($k_{cat}$) and the active-enzyme concentration. The active-enzyme concentration is not necessarily equivalent to the protein concentration. If the purified enzyme has some inactive enzyme (e.g., because of denaturation or the result of inhibition), then the protein concentration of the purified enzyme does not reflect the actual active-enzyme concentration. This is a very important point that is often misunderstood or misinterpreted by some researchers studying the biochemistry of allelic isozymes. To measure active-enzyme concentration, one must use purification methods that require activity for the final step (substrate affinity) or, better yet, active-site titration to quantify the concentration of active-enzyme.

The most striking difference between the allelic isozymes was their relative stability to thermal denaturation. The isozyme common to northern waters was the least stable. For example, Fig. 14 illustrates the rates of heat inactivation of the three major homozygous allelic isozymes. The relative thermal stabilities of these enzymes are consistent with the gene frequencies of the alleles encoding them in relation to latitude and thus environmental temperature.

Rennis (1978) recorded temperatures of marsh pools at Lewes, Delaware in excess of 39°C that were inhabited by immature Fundulus heteroclitus, and we have recorded temperatures within marsh pools in Georgia and Florida that are as high as 50°C. Clearly, if the thermal stability of the isozyme common to northern waters is as unstable in vivo as it is in vitro, then the H6PDH-A$^+$ allelic isozyme might be disadvantageous in the warmer waters common to southern latitudes compared with the more heat-stable allelic isozymes (H6PDH-A$^+$ and H6PDH-A$^-$) that are commonly found at the warmer, southern latitudes. However, rate studies of enzyme synthesis and degradation would have to be done to test this hypothesis properly.
Fig. 14. Thermal denaturation of Fundulus heteroclitus H6PDH-A allelic isozymes. (A) Fraction of remaining activity in relation to incubation temperature. (B) Fraction of remaining activity as a function of time at 46°C. The allelic isozymes encoded by H6pdh-A*, H6pdh-Ab*, H6pdh-A', are represented by squares, triangles, and circles, respectively (from Ropson and Powers 1989).

5.2.1 The malate–aspartate–isocitrate shuttle
The oxidation of isocitrate to alpha-ketoglutarate is catalyzed by isocitrate dehydrogenase. Most higher plants and animals contain at least two different types of isocitrate dehydrogenases. One type requires NAD⁺ as an electron acceptor, while the other uses NADP⁺. Both the NAD-linked and NADP-linked isocitrate dehydrogenases occur in the mitochondria of animal tissue, but the NAD-linked form is only found in the mitochondria, while the NADP-linked form is found in both cytoplasm and mitochondria. In liver, heart, and other aerobic tissues, the NADP-linked forms participate in the shuttling of reducing equivalents across the mitochondrial membrane in a manner similar to that of the
malate–aspartate shuttle, except the cofactors are NADP$^+$ and NADPH instead of NAD$^+$ and NADH. Since one of the products of the malate–aspartate shuttle is alpha-ketoglutarate, we have taken the liberty of combining the malate and isocitrate shuttles into a single illustration that we shall term the malate–aspartate–isocitrate shuttle (Fig. 15).

We have studied the kinetic, physical, and chemical characteristics of several of the isozymes and allelic isozymes from _Fundulus heteroclitus_ that are represented in this shuttle system. We have analyzed the three major multilocus isozymes of malate dehydrogenase (MDH-A$_2$, MDH-B$_2$, and MDH-C$_2$), as well as the allelic isozymes of the cytoplasmic malate dehydrogenase (MDH-A$_2$) (Cashon _et al._ 1981; Cashon and Powers, submitted). We have also studied the kinetics and thermal stability of the isozymes expressed by the three isocitrate dehydrogenase loci (Gonzalez-Villasenor and Powers 1985, 1986) and the allelic isozymes of the cytoplasmic NADP-dependent locus (Gonzalez-Villasenor and Powers, submitted). We have also purified the cytoplasmic and mitochondrial form of aspartate amino transferase, and have studied the kinetics and thermal stability of the allelic isozymes of the mitochondrial form. Those results will be summarized below.

![Diagram](image)

*Fig. 15. Malate–aspartate–isocitrate shuttle illustrating the role of the NADP-dependent IDH isozymes, the NAD-dependent MDH isozymes and the mitochondrial and cytoplasmic forms of AAT. Enzymes involved are numbered as follows: 1 = IDH-B$_2$; 2 = IDH-A$_2$; 3 = AAT-B$_2$; 4 = AAT-A$_2$; 5 = MDH-A$_2$; and 6 = MDH-C$_2$. All the isozymes are polymorphic except MDH-C$_2$.)*
5.3 Malate dehydrogenase

Malate dehydrogenase catalyzes the reversible conversion of malate to oxaloacetate with the aid of NAD$^+$ as a cofactor for the forward direction, and NADH for the reverse direction. In animals there are at least two, and usually more, NAD-dependent malate dehydrogenases. In many tissues there are two or more loci that are expressed, with at least one being found in the cytosolic compartment and another in the mitochondrial compartment. These cytosolic and mitochondrial malate dehydrogenases participate in the malate–aspartate shuttle which is an important mechanism for transporting NADH-type reducing equivalents into mitochondria.

In Fundulus heteroclitus there are three loci: Mdh-A, Mdh-B, and Mdh-C. The isozymes encoded by Mdh-A and Mdh-B are found in cytosolic compartments, while the isozyme of Mdh-C is found in the mitochondrial fraction. The Mdh-A locus is expressed in all tissues, but Mdh-B is only expressed in the cytosolic fraction of skeletal muscle, eye, brain, and gill. Since Mdh-C encodes the only mitochondrial isozyme, it is expressed in essentially every tissue that has a subcellular compartment.

The gene products of each of the isozymes from these three loci have been purified and their steady-state kinetics and physical properties determined (Cashon et al. 1981; Cashon and Powers, submitted). Kinetic properties were determined for all three multilocus isozymes as a function of pH and temperature. While the $K_m$ values for NADH were relatively insensitive to changes in temperature or pH for the cytoplasmic isozymes, the mitochondrial form was sensitive to both temperature and pH changes. The $K_m$ values for oxaloacetate increased as a function of both temperature and pH for all three multilocus isozymes. Their $V_{max}$ values increased as a function of temperature, as would be expected; but their $V_{max}$ values were relatively insensitive to temperature. The apparent $V_{max}$ was also sensitive to increasing concentrations of the oxaloacetate substrate with rapid increases to about 300 micromolar with a rapid inhibition at higher concentrations.

Of the three loci expressed in Fundulus heteroclitus, only Mdh-A is polymorphic. The two major homozygous allelic isozymes, MDH-A$_3^1$ and MDH-A$_3^2$, expressed by this locus have been purified and their kinetics and physical characteristics determined at two temperatures (10°C and 25°C) and three pH values (Cashon et al. 1981; Cashon and Powers, submitted). Thermal stability studies indicated that the two major allelic isozymes were indistinguishable in the presence or absence of substrate and cofactor. Kinetic analyses indicated that their $K_m$ values for both substrate and cofactor were essentially identical for the two major homozygous allelic isozymes. Although the allelic isozyme that dominates
in northern populations (MDH-A2, see Fig. 2) appeared to have a greater $V_{\text{max}}$ at low temperatures, it cannot be said for certain that it was the result of a greater $k_{\text{cat}}$ rather than different amounts of active MDH-A2 enzyme in the preparation. Clearly higher $V_{\text{max}}$ values at lower temperatures would provide an advantage since each drop in $10^\circ$C reduces the catalytic efficiency by a factor of two. Although the 'northern' allelic isozyme may or may not have a greater $k_{\text{cat}}$ at lower temperatures than its 'southern' counterpart, there are significantly greater amounts of MDH-A2 enzyme present in northern populations than in southern populations. Populations from the northern portion of the species range have approximately twice as much MDH enzyme activity as their southern counterparts. Even if the $k_{\text{cat}}$ values are identical between the allelic isozymes, a two-fold greater enzyme level in colder northern climates would provide those fish with a $V_{\text{max}}$ value that is twice as large as their southern relatives (Crawford et al. 1985). Since northern populations experience temperatures 10–15$^\circ$C colder than their southern counterparts, a two-fold higher enzyme level would provide a significant selective advantage at colder temperatures. This issue will be addressed to a greater extent later in this paper when a similar situation is raised relative to the biological basis and advantage of different concentrations of LDH-B4 allelic isozymes in northern and southern populations of Fundulus heteroclitus.

5.4 Aspartate aminotransferase

Aspartate aminotransferase, which is sometimes referred to as aspartate transferase, catalyzes the reversible transfer of an amino group from aspartate to alpha-ketoglutarate to form oxaloacetate and glutamate, or the reverse (see Fig. 15). Although the enzyme is most active with aspartate as an amino-group donor in the forward direction, it will accept a number of other amino acids as donors. This reaction is freely reversible and has an equilibrium constant that is essentially unity.

In animals, aspartate aminotransferase (AAT) is present as two or three isozymes that are found in cytosolic and mitochondrial compartments. In Fundulus heteroclitus there are two polymorphic loci in liver – one is expressed in the cytosol (Aat-B), and the other in the mitochondria (Aat-A). Both of these multilocus isozymes have been purified and their kinetic and physical characteristics studied for both catalytic directions (Van Elkan et al. 1987; Ropson et al., submitted). The cytosolic form has a $K_m$ for aspartate that is an order of magnitude greater than that of the mitochondrial form, but the cytosolic AAT has a $K_m$ for alpha-ketoglutarate that is one-fourth as large as that of the mitochondrial AAT. Since the $V_{\text{max}}$ values of the two aspartate aminotransferases are not significantly different, these $K_m$ values may be useful in predicting
the direction of the malate-aspartate-isocitrate shuttle, and may reflect
the relative substrate concentrations in the cytosolic and mitochondrial
compartments of Fundulus heteroclitus liver cells. As might be expected,
the cytoplasmic AAT is more stable to temperature and other denaturants
than the mitochondrial isozyme, but both isozymes are stable at
temperatures as high as 45°C.

In addition to the functional comparison of the cytosolic and mitochon-
drial AAT isozymes, two of the major allelic isozymes of the mitochondrial
AAT have been purified and their kinetics and thermal stabilities studied
(Van Elkan et al. 1987; Ropson et al., submitted). There were no kinetic
differences observed between the allelic isozymes as a function of
temperature, i.e., the \( K_m \) and \( V_{\text{max}} \) values were not different between
allelic isozymes. There was a small but significant difference in the
thermal stabilities of the two homozygous allelic isozymes, but this
difference could only be observed at temperatures well above those
experienced by the fish in its natural environment. On the basis of these
studies, we concluded the two major allelic isozymes encoded by the
Aat-A locus were essentially functionally equivalent and thus may reflect
selectively neutral alleles.

5.5 NADP-dependent isocitrate dehydrogenase

Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation
of isocitrate to alpha-ketoglutarate and requires a divalent metal ion like
\( \text{Mn}^{2+} \) or \( \text{Mg}^{2+} \) for activity. The NAD\(^+\) and the NADP\(^+\)-dependent
forms of IDH are found in the mitochondria, but only the NADP-linked
IDHs are expressed in the cytoplasm. The NAD-linked enzyme is
allosteric and requires ADP as a specific activating modulator, but there
is no evidence that the NADP-linked form is allosteric. The NAD-linked
form functions primarily in respiration (i.e., the transfer of electrons
from substrates toward oxygen), while the NADP-dependent IDHs are
primarily involved in the transfer of electrons from intermediates of
catabolism to intermediates of biosynthesis and only secondarily involved
in respiration. For example, IDH is the major source of NADPH-
reducing equivalents for the synthesis of fatty acids. Fig. 15 is a schematic
diagram representing the role of the NADP-dependent IDH isozymes in
the shuttling of NADPH-reducing equivalents across the mitochondrial
membrane.

Fundulus heteroclitus has three NADP-dependent isocitrate dehydrogen-
ase loci: \( \text{Idh}-A \), \( \text{Idh}-B \), and \( \text{Idh}-C \). The first two loci are polymorphic,
having 3–5 alleles each which are expressed in the liver and several other
aerobic tissues (Cashon et al. 1981). The \( \text{Idh}-C \) locus is electrophoretically
monomorphic and is the only \( \text{Idh} \) locus found to be expressed in relatively
anaerobic white skeletal muscle. It is not expressed in aerobic tissues.
like liver, red muscle, and other organs. Of the two *Idh* loci expressed in the liver, the allelic isozymes expressed by *Idh-A* are found only in the mitochondria, while the products of *Idh-B* are located in the cytoplasm. Since the liver is a major organ for biosynthesis, the allelic isozymes of *Idh-A* and *Idh-B* serve the typical NADP-dependent IDH biosynthetic functions in the organ, and they participate in the malate–isocitrate–aspartate shuttle (Fig. 15) by providing a mechanism for transferring NADPH-reducing equivalents across the mitochondrial membrane. Because NAD-dependent IDH activity could not be detected in some fish liver mitochondria, Moon and Ouellet (1979) suggested that the NADP-linked IDH may couple with a NADPH:NADH transhydrogenase system to serve the normal NAD-dependent IDH function in the TCA cycle. However, McCormack and Denton (1981) showed that trout heart had a NAD-linked IDH that is activated and regulated by Ca²⁺, suggesting that other researchers may have missed the presence of the NAD-linked IDH enzyme because appropriate activators were not present in their assay.

The NADP-dependent IDH isozymes encoded by the three *Idh* loci in *Fundulus heteroclitus* have been purified and characterized (Gonzalez-Villasenor and Powers 1985, 1986). The three isozymes were found to have different thermal stabilities, with the mitochondrial forms (IDH-A₂ and IDH-C₂) being the least stable. All the enzymes were found to have increased stability in the presence of substrates, cofactors, and appropriate metal ions. The liver IDH isozymes (IDH-A₂ and IDH-B₂) have identical *Kₘ* values for isocitrate, but the cytoplasmic form (IDH-B₂) has a higher affinity for NADP than the mitochondrial form (IDH-A₂). The white muscle mitochondrial form (IDH-C₂) had a much larger *Kₘ* for isocitrate than either of the other IDHs, but the *Kₘ* for NADP was essentially the same as that of the liver mitochondrial form (IDH-A₂) which probably reflects the intramitochondrial NADP concentration. The *Kₘ* differences determined for the various NADP-dependent IDH isozymes are consistent with cytoplasmic-mitochondrial shuttling of NADPH-reducing equivalents into the cytoplasm (Gonzalez-Villasenor and Powers 1985). Studies done at other temperatures and pH values confirmed those findings and indicated that the major NADP-dependent IDH isozymes have unique physiological and metabolic functions that are adapted to the tissues and cellular compartments in which they are expressed (Gonzalez-Villasenor and Powers 1986).

In addition to the kinetic and thermal stability studies cited above, the allelic isozymes of the cytoplasmic NADP-dependent IDH (IDH-B₂) have been purified and their kinetic parameters determined at a number of temperatures (Gonzalez-Villasenor and Powers, submitted). During those studies, we found that there were two different allelic isozymes with the same electrophoretic mobility. When the enzyme encoded by
the \textit{Idh-B} allele was isolated from northern and southern populations, it was found that the kinetic parameters differed. Extensive functional studies and sequential electrophoretic analysis confirmed that conclusion. The northern and southern IDH-B\textsubscript{N} homozygous isozymes had identical \(V_{\text{max}}\) values at all temperatures, and their \(K_m\) values for both NADP\textsuperscript{+} and isocitrate were indistinguishable at temperatures between 15 and 25°C. However, at lower temperatures (e.g., 10°C), the northern enzyme had a lower \(K_m\) value for NADP\textsuperscript{+} and isocitrate, while at higher temperatures (e.g., 40°C) the southern enzyme had a lower \(K_m\) value for NADP\textsuperscript{+} but not for isocitrate. Using the assumption that low \(K_m\) values reflect higher substrate affinity, the northern fish have higher NADP\textsuperscript{+} affinity and thus a higher pseudo first-order rate constant (\(V_{\text{max}}/K_m\)) at cold temperatures (e.g., 10°C) than fish from the more southerly population. At higher temperatures (40°C), the opposite was true for NADP\textsuperscript{+} but not for isocitrate. Those data are consistent with functional differences that reflect the thermal environment in which the allelic isozyme is most abundant. A similar case for selection against the allelic isozyme encoded by the \textit{Idh-B} allele could be made at cold temperatures, but there was no obvious advantage at higher temperatures. This may explain why the southern \textit{Idh-B} allele is the predominant allele at southern latitudes (Fig. 3), while the \textit{Idh-B} is minor and the northern \textit{Idh-B} allele is apparently absent from southern populations (Gonzalez-Villasenor and Powers, unpublished).

5.6 Lactate dehydrogenase

Although all five of the enzymes discussed above have been studied at various levels of biological organization, the \textit{Ldh-B} locus and its products have been studied most extensively. Since the work on \textit{Ldh-B} has been most extensive to date, we shall present a much more comprehensive summary of findings at the biochemical level and higher levels of biological organization for the \textit{Ldh-B} locus than for the other loci in order to properly illustrate our multidisciplinary experimentally based approach to the selectionist/neutralist controversy.

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 processes. For example, several reports have identified LDH-A\textsubscript{4} as a single-stranded DNA-binding protein that may be involved in DNA replication (Sharief \textit{et al.} 1986; Williams \textit{et al.} 1985),
and Gosti et al. (1987) found that the LDH commonly found in heart tissue (LDH-B4) and centrosomal proteins shared a common epitope, (i.e., the area of an antigen molecule that determines the specific antibody to which the antibody binds). An interesting LDH has been found to be associated with cells transformed by Kirsten murine sarcoma virus (Anderson and Kovacik 1981; Anderson et al. 1981). Li and his colleagues (1988) have shown that this LDH is a tyrosylphosphorylated form of the LDH common to vertebrate muscle (LDH-A4) which appears to be complexed with the ras P21 protein. Phosphorylated forms of LDH-A4 have been found in Rous sarcoma virus transformed chicken fibroblasts (Cooper et al. 1984), and rat fibroblasts induced with EFG, which has tyrosine kinase activity, increased the synthesis of LDH-A4 (Matrisian et al. 1985). Finally, LDH also appears to perform structural roles as well as metabolic and potential DNA replication roles because the epsilon-crystalline protein from duck eye lens is the LDH-B4 isozyme (Hendriks et al. 1988; Wistow et al. 1987).

Another LDH locus is expressed in the testis of mammals and in a variety of tissues in teleosts. While this locus is generally referred to as Ldh-C in mammals, birds, and fish, Quattro et al. (1993) have demonstrated that the Ldh-C of mammals and fish are derived independently. The products of the three Ldh genes are designated LDH-A4, LDH-B4, and LDH-C4, respectively. The three vertebrate Ldh genes appear to be independently regulated and show tissue specificity. While Ldh-A and Ldh-B are simultaneously expressed in the same tissues of many vertebrates, there is a remarkable tissue specificity and exclusivity of LDH expression in the tissues of many marine fishes. White skeletal muscle, whose metabolism is predominantly anaerobic, expresses the Ldh-A locus. Red muscle and liver that have significant aerobic metabolisms express almost exclusively Ldh-B. F. heteroclitus erythrocytes, which have some aerobic capability (about 5–10 per cent), also express Ldh-B exclusively. The suggested functional significance of the difference in LDH isozymes is that LDH-A4 is principally involved in the conversion of pyruvate to lactate (i.e., anaerobic glycolysis), while LDH-B4 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>B</sup> and Ldh-B<sup>b</sup>. As illustrated in Fig. 6, 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>B</sup> is predominant in the southern (i.e., warmer) portion of the range. According to Clarke's (1975) suggested 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 task. Our conclusions are that the LDH-B4 allelic isozymes of F.
heteroclitus differ in: (1) catalytic efficiency at low substrate concentrations; (2) degree of inhibition by lactate; (3) enzyme stability; and (4) enzyme concentration between northern and southern populations.

5.6.1 Kinetic and thermal stability studies of LDH-B₄ allelic isozymes

At all temperatures and pH values, the \( k_{cat} \) values of the two LDH-B₄ allelic isozymes are identical, but the pseudo first-order rate constant \( (V_{max}/K_m) \) and the second-order rate constant \( (k_{cat}/K_m) \) are significantly different (Place and Powers 1979, 1984b). At low temperatures, the LDH-B₄ isozyme, whose gene frequency is greatest in the northern colder waters (Fig. 6), has a greater apparent catalytic efficiency at low substrate concentrations \( (V_{max}/K_m) \) than LDH-B₄ (Place and Powers 1979, 1984b). At higher temperatures the situation appears to be reversed, with LDH-B₄, whose gene frequency is greatest in the southern warm waters, having a greater catalytic efficiency (Fig. 16). The most dramatic differences occur at low temperatures and low pH values. For example, at 10°C and pH 6.5, the allelic isozyme that dominates in colder northern waters has a second-order rate constant that is twice that of the allelic isozyme that dominates at southern latitudes. These differences become less pronounced at higher pH values. At 25°C the two homozygous allelic isozymes are essentially equivalent, but at higher temperatures (e.g., 40°C), the allelic isozyme that predominates in populations from warmer southern latitudes has a greater catalytic efficiency than its northern cold water counterpart. Since the \( k_{cat} \) 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 decrease as their temperature increases. Since there is a physical relationship between temperature and pH of fluids, it is possible to transform the three-dimensional data represented in Fig. 16 into a two-dimensional representation that reflects the maintenance of internal relative constant alkalinity (Fig. 17). After these pH changes are taken into account, the catalytic efficiency differences between LDH-B₄ allelic isozymes that were described above are still evident and even more obvious (Fig. 17).

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-B₄ allelic isozyme is much less susceptible to product (i.e., lactate) inhibition than the LDH-B₄ isozyme. For the conversion of lactate to pyruvate, the LDH-B₄ allelic isozyme is more susceptible to substrate (i.e., lactate) inhibition than the LDH-B₄ isozyme. Moreover, the magnitude of inhibition is greater at cool temperatures than at warm ones. The putative selective significance of this difference may relate to the accumulation of lactate during extreme swimming activity in fish which can exceed 20 mM (Powers et al. 1979; DiMichele and Powers
Fig. 16. The effect of temperature and pH on the pseudo first-order rate constant ($V_{\text{max}}/K_m$) at low pyruvate concentrations and second-order rate constant ($k_{\text{cat}}/K_m$) are plotted against temperature where enzyme concentration, $[E]$, is considered constant for the various LDH-B$_4$ isozymes.
Fig. 17. The three-dimensional data illustrated in Fig. 16 is reduced to two dimensions using the Nernst equation to maintain a constant relative alkalinity. Both $k_{cat}/K_m$ and $V_{max}/K_m$ are plotted against temperature where enzyme concentration, $[E]$, is considered constant for the various LDH-B$_4$ isozymes. The effect of temperature on pyruvate reduction at a constant [OH$^-$/H$^+$] ratio of unity (adapted from Place and Powers 1979).

1982a; Place and Powers 1984b), or during early embryonic development where lactate concentrations start somewhere between 40 and 50 mM (Paynter et al. 1991).

Place and Powers (1984a) used heat, urea, and proteolytic digestion to study the structural stability of LDH-B$_4$ allelic isozymes. Heat denaturation studies (Fig. 18A) revealed that LDH-B$_1^+$ was more stable than LDH-B$_4$. Similar differences were found in a variety of other stability studies (Place and Powers 1984a). Since the least stable allelic isozyme is most common in the warmer southern waters of the species range, it is inconsistent with the expectation that the most thermally stable enzyme should be most common in the warmer environments. However, Fig. 18A shows that each of the LDH-B$_4$ allelic isozymes are completely stable at 50°C, which is well above the fish’s lethal temperature. Therefore, while 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, as pointed out above, several of the allelic isozymes
Fig. 18. (A) Heat denaturation profiles of LDH-B₄ allelic isozymes purified from several hundred individual livers from Maine and Georgia populations of *F. heteroclitus*. The symbols are: LDH-B₂ = open squares; LDH-B₄ = closed circles. (B) Heat denaturation profiles of two recombinant LDH-B₄ proteins generated from cloned cDNA. Symbols: Ser 185 = open squares; and Ala 185 = closed circles (from Powers *et al.* 1991).

from this teleost 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 (Gonzalez-Villasenor and Powers 1985, submitted; Ropson and Powers 1989; Van Beneden and Powers 1989; Ropson *et al.*, unpublished).

5.6.2 Enzyme concentration differences between populations
Near the extremes of its natural distribution in the USA (e.g., Maine and Georgia), *F. heteroclitus* experience 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 was greater than in those of Georgia fish (Crawford *et al*. 1985, 1990). For example, the LDH-B$_4$ of fish liver collected from Maine had a specific activity approximately twice that of liver collected from Georgia (Fig. 19A). Even after extensive temperature acclimation, the differences in specific activity remained (Crawford and Powers, 1989). Similar analyses of populations from intermediate latitudes indicate LDH-B$_4$ specific activities intermediate to Maine and Georgia fish (Crawford *et al*. 1990).

Maine fish are essentially only *Ldh-B*$_b$ genotype, and Georgia fish *Ldh-B*$_a$ genotype. Since the LDH-B$_a$ allelic isozymes have the same first-order rate constant (*k*_cat) at the same temperatures, the observed differences in enzyme specific activity are most likely a function of different enzyme concentrations because *V*_max* = *k*_cat [E$_i$]. Evidence to support this possibility comes from immunoprecipitation studies (Crawford and Powers 1989, 1990) wherein enzyme activity differences were directly correlated with differences in LDH-B$_a$ protein concentrations (Fig. 19B). Dissimilar LDH-B$_4$ concentrations between populations were apparently primarily genetic because the differences were present even after long-term acclimation (Crawford and Powers 1990). Preliminary genetic transmission analyses are also consistent with that hypothesis.

The differences in LDH-B$_4$ 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$_4$ synthesis (or degradation). As an initial test of these possibilities, the *Ldh-B*$_b$ cDNA was cloned, sequenced (Crawford *et al*. 1989), and used as a probe to determine if there were different numbers of *Ldh-B* gene copies and LDH-B mRNA concentrations between Maine and Georgia populations (Crawford and Powers 1989). While the number of gene copies did not vary between populations, the LDH-B mRNA concentrations were found to be different (Fig. 19C). The LDH-B mRNA concentrations were approximately two-fold higher in Maine fish than in their Georgia counterparts.

Because the differences in LDH-B mRNA could be due to transcriptional or post-transcriptional regulation, the *Ldh-B* transcriptional rates for Maine and Georgia fish were determined (Crawford and Powers 1992). 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 (Fig. 19D). However, there was no difference in the total rate of RNA synthesis, nor was there a difference 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*
Fig. 19. (A) LDH specific activity of livers collected from Maine and Georgia populations collected from 44.2°N and 31.4°N, respectively; (B) LDH protein concentrations of the livers referred to previously; (C) LDH-specific mRNA concentrations of the liver samples; and (D) LDH-B transcriptional rate for the same samples as the other measurements illustrated in this figure (from Powers et al. 1991).

...se, but appears to be specific for Ldh-B gene 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 environment and at the extremes of a species natural range.

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 (Siebenaller and Somero 1978, 1982; Graves and Somero 1982; Hochachka and Somero 1984; Somero and Hand 1990). In some cases, allelic variants within a species have evolved different kinetic constants that affect important physiological parameters (Daly and Clarke 1981; DiMichele and Powers 1982a,b; Hilbish and Koehn 1985; Zamer and Hoffman 1989; Paynter et al. 1991). In other cases, enzyme concentrations have been altered by differential gene regulation (McDonald et al. 1977; McDonald and Ayala 1978; Crawford and Powers 1989, 1992). Since gene regulation allows enzyme activity to vary independent of amino acid changes in the gene product (Wilson 1976; McDonald and Ayala 1978), 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.

5.6.3 Structural differences between LDH-B<sub>4</sub> allelic isozymes

As mentioned, Crawford et al. (1989) cloned and sequenced an Ldh-B<sup>b</sup> cDNA of two fish from a Maine population. Lauerman et al. (submitted) 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 2 per cent. In the absence of evolutionary constraints (i.e., selection), 14 amino acid replacements were expected but only two were found, suggesting an evolutionary constraint structure. Kreitman (1983) found an even greater divergence from expectation in the alcohol dehydrogenase from Drosophila.

The amino acid sequences derived from the two cDNA clones indicated variation at amino acid residues 185 (Ser in Ldh-B<sup>b</sup> and Ala in Ldh-B<sup>a</sup>) and 311 (Ala in Ldh-B<sup>b</sup> and Asp in Ldh-B<sup>a</sup>). Since the lactate dehydrogenase structure has been highly conserved throughout evolution, structural comparisons can be made across taxa (Holbrook et al. 1975). Residue 311 is located at the exterior of the molecule pointing toward the solvent (Fig. 20). 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 (Fig. 20). 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 (Lauerman et al., submitted). The substitution at residue 185 of Ser in LDH-B2 by Ala in LDH-B4 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 (Lauerman and 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-B4 isozymes (Fig. 18B). Although substituting Asp for Ala at residue 311 affected the charge of the LDH-B4 electromorphs, there was little, if any, effect on its thermal stability. Each of the four recombinant proteins (i.e., LDH-B4(Ala185/Asp311), LDH-B4(Ser185/Asp311), LDH-B4(Ala185/Ala311), LDH-B4(Ser185/Ala311)) had $K_m^{prv}$ and $K_i^{Lact}$ values and rates of proteolysis (Lauerman and Powers, submitted) that were essentially identical to those for the LDH-B2 allelic isozyme isolated from a Maine population (Place and Powers 1984b). These results suggest several possibilities, including: (1) kinetically different but electrophoretically cryptic polymorphisms exist at the Ldh-B locus; (2) a high affinity inhibitor was copurified during the purification of enzyme preparations from southern populations but not during purification of the enzyme from northern localities; or (3) there is differential posttranslational modification (e.g., phosphorylation) of the LDH-B4 allelic isozymes.

Since only one cDNA from one individual was sequenced with an LDH-B2 electrophoretic phenotype, it cannot be said for certain that these two amino acids are the only differences between the LDH-B4 allelic isozymes from Maine and Georgia populations. The fact that one cryptic amino acid substitution (position 185; Ser for Ala) was found suggested that there may have been others. There are a variety of data to support this hypothesis. For example, while substituting Ala for Ser at residue 185 affected the thermal stability of LDH-B4, 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 (Fig. 18). These data suggested more than one allelic isozyme in the preparations purified from natural populations. Other support comes from detailed zoogeographic studies of enzyme polymorphisms (Powers and Place 1978; Cashon et al. 1981; Ropson et al. 1990), mtDNA restriction length polymorphisms (Smith 1989; Gonzalez-Villasenor and Powers 1990: Smith et al., submitted), and DNA fingerprinting at the Ldh-B locus (unpublished), all of which indicates that the Georgia population of F. heteroclitus has a much greater genetic diversity than the Maine population. Because
Fig. 20. Computer simulated structure of LDH-B₄. Two subunits of the tetramer are shown with the subunit interaction involving residue 185 illustrated, and with residue 311 on the outside of the molecule (from Powers et al. 1991).
Addressing the 'selectionist/neutralist' controversy

Genetic diversity is greatest in the southern population, we expected that a greater number of cryptic amino acid substitutions would be found in southern localities than in northern ones. Sequencing a larger number of individual Ldh-B cDNA clones from Maine, Georgia, and intermediate 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-B4 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 significant variation in the third codon position and identified several cryptic amino acid substitutions in a Georgia population, but only one in a Maine population (Bernardi et al., in press). Of the 20 Ldh-B cDNAs sequenced so far from these two populations, the southern population always had Ala at position 185 and Asp at position 311, while the Ldh-B cDNAs from the northern group always had Ser and Ala at positions 185 and 311, respectively. All six of the Ldh-B cDNA sequences obtained from a New Jersey population had slightly different amino acid sequences. For example, some had 'northern' phenotypes at positions 185 and 311, others had 'southern' phenotypes, and still others had a mixture at these amino acid positions (e.g., Ala at 185 and Ala at 311, or Ser at 185 and Asp at 311). In addition, half of the amino acid sequences also had cryptic amino acid substitutions (Bernardi and Powers, unpublished). Further Ldh-B cDNA sequencing studies within and between populations should significantly clarify the extent of 'cryptic' genetic variation at the Ldh-B locus in this species, but it will take site-directed mutagenesis studies of these variants in order to elucidate whether these substitutions affect the catalytic function or thermal stability of the active enzyme.

Differences in amino acid sequence, thermal stability, enzyme concentration, and other parameters that have been uncovered between the LDH-B4 allelic isozymes indicate that they are structurally and functionally non-equivalent, both within and between populations. Moreover, the non-equivalence 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?'

6. 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$_4$ enzyme had a greater catalytic efficiency at low substrate concentrations than the LDH-B$_4$ 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$^e$ 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. When erythrocytes with different LDH-B$_4$ allelic isozymes are incubated in uniformly labeled $^{14}$C-glucose, the rate of CO$_2$ released is significantly different (Fig. 21). Cells that are homozygous for Ldh-B$^e$ metabolize much faster than cells homozygous for Ldh-B$^o$. In order to determine relative partitioning between pathways (e.g., see Fig. 12), erythrocyte cultures with different LDH-B$_4$ allelic isozymes were incubated in $^{14}$C-glucose labeled at carbon one (closed symbols) and carbon six (open symbols), respectively (Fig. 21). In both cases, cells from homozygous Ldh-B$^o$ fish (triangles; Fig. 22A) had a greater rate than cells from Ldh-B$^e$ homozygotes. The phenomenon was even more pronounced when uniformly labeled lactate was utilized (Fig. 22B; unlike mammals, fish erythrocytes have active

![Graph](image)

Fig. 21. Nanomoles of $^{14}$CO$_2$ released when F. heteroclitus erythrocytes with different LDH-B$_4$ allelic isozymes (LDH-B$^e$ = open circles; LDH-B$^o$ = solid circles) are incubated in uniformly labeled $^{14}$C-glucose.
Fig. 22. Nanomoles of $^{14}$CO$_2$ released when F. heteroclitus erythrocytes with different LDH-B$_4$ allelic isoforms (LDH-B$_4^a$ = triangles; LDH-B$_4^b$ = circles) are incubated in $^{14}$C-labeled substrates. (A) Closed symbols = $^{14}$C-l-glucose, and open symbols = $^{14}$C-6-glucose; (B) erythrocytes with different LDH-B$_4$ allelic isoforms (LDH-B$_4^a$ = triangles; LDH-B$_4^b$ = circles) incubated in uniformly $^{14}$C-labeled lactate (from Powers et al. 1991).

mitochondria). Consistent with our expectations, the F. heteroclitus erythrocytes with LDH-B$_4^b$ also had 2.11 ± 0.22 ATPs/Hb, while cells with LDH-B$_4^a$ only had 1.65 ± 0.12 ATPs/Hb (Powers et al. 1979).

The finding of differences between Ldh-B genotypes in intraerythrocyte ATP concentrations was particularly important because it allowed us to make predictions at higher levels of biological organization, including: (1) hemoglobin–oxygen (Hb–O$_2$) affinity differences between adult fish (Powers et al. 1979); (2) differences in swimming performance (DiMichele and Powers 1982a); and (3) differential developmental rates and hatching of embryos with different Ldh-B genotypes (DiMichele and Powers 1982b). 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) (Gillen and Riggs 1971), or guanosine triphosphate (GTP) (Geohagan and Poluhowich 1974), e.g., both of which decrease the affinity of hemoglobin for oxygen.

Homozgyous F. heteroclitus with the Ldh-B$^a$ genotype have erythrocyte
ATP levels that are significantly less than those with the *Ldh-B^b* genotype, while heterozygotes have intermediate concentrations. We predicted that fish with higher levels of intraerythrocyte ATP (i.e., *Ldh-B^a*) should have blood with a lower oxygen affinity at pH values favoring ionic interactions between ATP and hemoglobin (Powers 1980). As predicted, *Ldh-B^a* erythrocytes had a higher oxygen affinity than *Ldh-B^b* 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: differential swimming ability at low temperature (DiMichele and Powers 1982a), and differential developmental rates and hatching times (DiMichele and Powers 1982b, 1984, 1991; DiMichele et al. 1986, 1991).

7. THE ORGANISMAL LEVEL

7.1 Physiological basis for swimming endurance differences

Our analyses of purified LDH-B\_4 alleric isozymes indicate that the greatest catalytic differences between LDH-B\_4 and LDH-B\_5 exist at low temperature (10°C), while no significant difference exists at 25°C (see Figs 16 and 17). We reasoned that if the LDH-B\_4 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 (Mied and Powers 1978), these phenomena should be exaggerated at low pH values like those produced during swimming performance. To test those predictions, after an acclimation period, fish of each of the two homozygous LDH-B\_4 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 (DiMichele and Powers 1982a).

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\_5 phenotype fish were able to sustain a swimming speed 20 per cent higher than that of LDH-B\_4 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\_4 phenotypes, fish with LDH-B\_5 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,
Table 1
Response to swimming stress by Fundulus heteroclitus LDH-B phenotypes acclimated to 10°C and 25°C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10°C</th>
<th></th>
<th>25°C</th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDH-B₁</td>
<td>LDH-B₂</td>
<td>P</td>
<td>LDH-B₁</td>
<td>LDH-B₂</td>
</tr>
<tr>
<td>Resting fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td>23 ± 1</td>
<td>24 ± 1</td>
<td>NS</td>
<td>24 ± 1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.87 ± 0.05</td>
<td>7.84 ± 0.04</td>
<td>NS</td>
<td>7.40 ± 0.05</td>
<td>7.48 ± 0.04</td>
</tr>
<tr>
<td>$P_{SO_2}$ (mmHg)</td>
<td>4.2 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>NS</td>
<td>5.0 ± 0.3</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Serum lactate (mM)</td>
<td>1.82 ± 0.28</td>
<td>1.37 ± 0.15</td>
<td>NS</td>
<td>2.6 ± 0.69</td>
<td>4.5 ± 0.86</td>
</tr>
<tr>
<td>Liver lactate (µM/g)</td>
<td>0.390 ± 0.055</td>
<td>0.383 ± 0.044</td>
<td>NS</td>
<td>1.8 ± 0.42</td>
<td>1.53 ± 0.39</td>
</tr>
<tr>
<td>Muscle lactate (µM/g)</td>
<td>7.93 ± 0.75</td>
<td>8.17 ± 1.02</td>
<td>NS</td>
<td>11.8 ± 2.2</td>
<td>12.5 ± 1.6</td>
</tr>
<tr>
<td>Exercised fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical swim speed (body lengths/s)</td>
<td>3.6 ± 0.12</td>
<td>4.3 ± 0.1</td>
<td>&lt;0.05</td>
<td>5.6 ± 0.3</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>30 ± 3</td>
<td>35 ± 2</td>
<td>NS</td>
<td>36 ± 1</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.24 ± 0.04</td>
<td>7.15 ± 0.05</td>
<td>NS</td>
<td>7.12 ± 0.03</td>
<td>7.09 ± 0.09</td>
</tr>
<tr>
<td>$P_{SO_2}$ (mmHg)</td>
<td>6.57 ± 0.5</td>
<td>9.1 ± 0.5</td>
<td>&lt;0.05</td>
<td>7.4 ± 0.6</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td>Serum lactate (mM)</td>
<td>12.19 ± 1.21</td>
<td>16.29 ± 0.79</td>
<td>&lt;0.05</td>
<td>23.4 ± 2.5</td>
<td>17.5 ± 1.6</td>
</tr>
<tr>
<td>Liver lactate (µM/g)</td>
<td>1.39 ± 0.12</td>
<td>1.56 ± 0.17</td>
<td>NS</td>
<td>6.6 ± 1.2</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>Muscle lactate (µM/g)</td>
<td>17.08 ± 1.86</td>
<td>24.01 ± 1.46</td>
<td>&lt;0.05</td>
<td>23.2 ± 4.8</td>
<td>20.7 ± 1.6</td>
</tr>
</tbody>
</table>

NS = not significant.
Greaney et al. (1980) 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 (Powers 1980). In resting F. heteroclitus at 10°C, the blood pH was about 7.9. At this pH, the difference in erythrocyte ATP between LDH-B₄ phenotypes (ATP/Hb were 1.65 ± 0.12 and 2.11 ± 0.22 for LDH-B₄ and LDH-B₂, 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 homozygous Ldh-B genotypes become apparent (Fig. 23). 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 (DiMichele and Powers 1982a).

Fish acclimated to 25°C did not differ significantly in erythrocyte ATP concentrations. The ATP/Hb ratios were 1.45 ± 0.24 and 1.65 ± 0.31 for the Ldh-B₄ and Ldh-B₂ genotypes, respectively. In addition, there

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Fig. 23. Oxygen equilibrium whole blood of F. heteroclitus acclimated to 10°C: (a) resting fish of both homozygous Ldh-B genotypes; (b) Ldh-B₄ genotype fish; and (c) Ldh-B₂ genotype fish swum to exhaustion, respectively (from DiMichele and Powers 1982b).
were no significant differences between LDH-B4 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.

7.2 Differential hatching times of developing embryos

DiMichele and Taylor (1980) 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 (DiMichele and Powers 1982b). We predicted that the Ldh-Ba genotype embryos should feel oxygen stress before Ldh-Bb 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-Ba genotypes hatched before Ldh-Bb genotypes, and the heterozygotes had an intermediate hatching distribution. When incubated at 20°C, mostly Ldh-Ba genotype eggs emerged during the first 3 days of the hatching period, and mostly Ldh-Bb 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-Ba genotype, 12.4 days for the heterozygotes, and 12.8 days for the Ldh-Bb homozygotes (DiMichele and Powers 1982b). In a much larger experiment that included four loci (Ldh-B, Mdh-A, Gpi-A, and Pgm-A) and slightly different physical conditions, the hatching order remained the same and the hatching time differences were even more pronounced (DiMichele et al. 1986; DiMichele and Powers 1991).

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, during the highest tide. Under these conditions, the eggs incubate in air for most of their developmental period until the next highest tide comes in again. 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. If they hatch after the tide has gone out, they will be stranded. If they do not hatch in time, they will be exposed to predation for an additional 2 weeks until the next highest tide. If eggs hatch too early as a result of a rainstorm, they will die. 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-Ba 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.

### 7.3 Differential developmental rates

Though 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 and Powers (1984) have presented evidence that at 20°C there are 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, and the heterozygotes have intermediate developmental rates. It is interesting to note that the morphological variation in the *Ldh-B* heterozygotes is lower than that of the homozygotes (Mitton 1978).

In an attempt to better understand the metabolic basis for these and other developmental rate differences, Paynter *et al.* (1991) examined the metabolic properties of early-developing *F. heteroclitus* embryos as a function of their *Ldh-B* genotype. During the first 2 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.* (1991) focused their studies during the first 24 hours of development. The unfertilized eggs had very high lactate concentrations and were different for the *Ldh-B* genotypes (*Ldh-B*<sup>a</sup> = 48 mM; *Ldh-B*<sup>b</sup> = 40 mM). 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 10 hours of development, the *Ldh-B*<sup>a</sup> homozygous embryo began to consume more oxygen than its *Ldh-B*<sup>b</sup> counterpart, and the difference became even more pronounced during the remainder of the experiment (Fig. 24A). Moreover, the *Ldh-B*<sup>a</sup> homozygote embryo developed faster – as indicated by the difference in the metabolic heat generated during development (Fig. 24B). These studies suggest that lactate is a major carbon source during early development, and the different *Ldh-B* homozygous embryo genotypes utilize it differently.
Fig. 24. (A) Oxygen consumption rate/egg. Data are means from nine manometric measurements. Error bars are standard errors. (B) Heat dissipation per egg in μJ/s (Paynter et al. 1991).

7.4 Ruling out the ‘hitchhiker’ effect

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.

The ideal way to determine whether genetic variation at a particular locus directly affects a biochemical or metabolic function would be to study organisms that only vary at that locus. The construction of such strains can take years or decades and is not always feasible. In addition, even with the best of genetic manipulations, the elimination of effects of tightly linked loci is essentially not possible. An alternate experiment
would be to change an organism's enzyme phenotype by exchanging its allelic isozyme with that of a different genotype.

Klyachko et al. (1982) successfully inserted exogenous LDH in loach blastomeres by microinjection into the yolk shortly after fertilization. These experiments showed that LDH was disproportionately accumulated in the blastomeres through binding of the enzyme to some cellular component, and that the foreign protein competes with the native enzyme for binding sites. We hypothesized that it is possible to quantitatively replace an embryo's LDH-B₄ (the only LDH isozyme present in eggs) with exogenous LDH by injecting an excess of the foreign protein into the fertilized egg. The hypothesis was tested by creating blastulas whose native LDH content was replaced by either the LDH-B₄ allelic isozyme of a different genotype or one of two porcine LDH isozymes. Our results generally confirmed those of Klyachko et al. (1982). The blastulas from injected eggs contain about 62 per cent of the LDH originally present in oocytes – the same amount as in uninjected or saline injected blastulas. Electrophoretic analysis showed that the LDH in the blastulas was of the type injected, while no detectable amounts of the native enzyme remained. Since it was possible to quantitatively replace an embryo's native LDH-B with foreign enzymes of different kinetic and/or structural properties, we used these preparations to test whether it is the physical LDH type present in the blastula that influences glucose utilization or some other factor.

Blastulas were prepared as above, but ¹⁴C-6-glucose was added to the washing buffer. Ten embryos were sealed in 1.2 ml tubes without additional buffer. The data in Table 2 show that the actual LDH-B₄ homotetramer incorporated into the blastula affected glucose utilization rates. Genotypes injected with their own enzyme utilized glucose in a pattern similar to whole eggs; i.e., the Ldh-B⁴ homozygote utilized

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose utilization rates (µmol x 10⁻⁸ s⁻¹ per egg) of injected eggs*</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Material injected</td>
</tr>
<tr>
<td>Homozygous genotype</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>LDH-B⁴</td>
</tr>
<tr>
<td>LDH-B⁻¹</td>
</tr>
</tbody>
</table>

*Data are means (± SD) of three pools of ten eggs each. Saline-injected controls were not significantly different from phenotypes injected with their own allozyme; Ldh-B⁴ and Ldh-B⁻¹ homozygotes injected with the same allozyme were not different from each other; phenotypes injected with LDH-B⁻¹ were significantly different from those injected with LDH-B⁴ (ANOVA, P < 0.005).
glucose at a higher rate (Paynter et al. 1991). However, when a genotype's allelic isozyme was replaced by the alternate allelic isozyme, it utilized glucose at a rate determined by the injected allelic isozyme rather than the genotype.

The results above indicate that the genetic differences between LDH-B allelic isozymes can alter glycolytic flux by about 30 per cent (Table 2). We hypothesized that the substitution of other genetically more divergent LDH isozymes might have an even greater influence. Therefore, we replaced the native enzyme of Ldh-B* homozygotes with two porcine LDH isozymes. Porcine heart type LDH is physiologically optimized to oxidize lactate to pyruvate. In this regard it is more like the southern F. heteroclitus LDH (Place and Powers 1979, 1984b), and its effect on the Ldh-B* homozygote was in the same direction and of a greater magnitude than the fish phenotype (Table 3). Conversely, the physiological function of the porcine muscle type LDH is the reduction of pyruvate to lactate. Its effect on the Ldh-B* homozygote was to increase glucose utilization. Thus, the results of the porcine LDH replacement studies are consistent with and reinforce the native allelic isozyme replacement studies.

The mechanism by which LDH influences glucose metabolism in mummichog embryos is not clear. Since both the LDH reaction and glycolysis are functioning to produce pyruvate and the phenotype that is utilizing glucose faster is also utilizing lactate faster (Paynter et al. 1991), it is unlikely that feedback inhibition, competition for NAD, or some similar mechanism is responsible for the observed differences. Srivastra and Bernhard and their colleagues (1984, 1985) have shown that enzyme–enzyme interactions, including those between LDH and other glycolytic enzymes, have significant effects on the kinetics of enzyme reactions and consequently metabolic flux. They suggested that an important aspect of enzyme evolution may be related to changes in protein structure that alter metabolite transfer within a pathway. In addition to the kinetic differences between the allelic isozymes, there

<table>
<thead>
<tr>
<th>Injected enzyme</th>
<th>Porcine (heart)</th>
<th>Fundulus (heart)</th>
<th>Porcine (muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original homozygous phenotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH-B*</td>
<td>3.2 ± 0.4</td>
<td>7.1 ± 0.9</td>
<td>14.7 ± 0.5</td>
</tr>
</tbody>
</table>

*Data are means (± SD) of three pools of ten eggs each. All means are significantly LDH-B* (ANOVA, P < 0.005), different from each other (ANOVA, P < 0.001).
may also be differential associations with other glycolytic enzymes that affect the rate of glucose utilization.

Evidence from a variety of other animals and enzyme systems has also indicated that allelic isozyme variation is associated with metabolic and/or organismal level differences that may affect fitness. However, our experiments suggest that the organismal differences correlated with the Ldh-B polymorphism in the mummichog are causally linked directly to the Ldh-B locus.

While it is difficult to rule out this possibility, even with the most sophisticated genetic analysis, our studies (DiMichele et al. 1991) clearly indicate that developmental rate can be altered by microinjection of purified LDH enzyme. Moreover, the developmental rates of the two Ldh-B homozygous genotypes can be reversed by microinjection of the purified alternative allelic isozyme (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, allelic isozyme directly affects the developmental rate of early embryos, but the mechanisms by which this is accomplished remain to be resolved.

7.5 Selection experiments: differential mortality

Based on the biochemical, cellular, and organismic studies reviewed above, DiMichele and Powers (1991) predicted developmental heterochrony and mortality differences between phenotypes as a function of temperature. Those predictions were realized as hatching times and high temperature mortality differences (30°C) that were observed for both single and multilocus phenotypes of the four enzyme-encoding loci studied (i.e., Ldh-B, Pgm-A, Mdh-A, and Gpi-B).

Fish that survived the highest temperature regime were also the most common phenotypes at the warm southern extreme of the species’ natural distribution. Two of three ‘southern’ single locus phenotypes showed a greater fitness at elevated temperatures than their ‘northern’ counterparts (Table 4). The heterozygotes appeared to be more fit, or almost as fit, as the best homozygote. More interestingly, the fitnesses at 30°C of the common ‘southern’ dilocus and trilocus phenotypes were near unity (between 0.84 and unity), while the fitnesses of the ‘northern’ dilocus and trilocus phenotypes varied between zero and 0.50. The double and triple heterozygotes had fitnesses that were closer to that of the more heat-resistant ‘southern’ phenotype than the temperature sensitive ‘northern’ phenotypes (Table 4). These selection coefficients (Table 4), especially the multilocus coefficients, suggest that selection at high temperatures is enormous. These results are particularly interesting in light of the finding of Meredith and Lotrich (1979) that the mortality of
Table 4
Relative fitnesses of embryos continuously exposed to 30°C heat stress during embryonic development
Single locus fitness values

<table>
<thead>
<tr>
<th>Single locus</th>
<th>Northern phenotype</th>
<th>Heterozygote phenotypes</th>
<th>Southern phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpi-B</td>
<td>0.53</td>
<td>0.93</td>
<td>1</td>
</tr>
<tr>
<td>Mdh-A</td>
<td>0.95</td>
<td>1</td>
<td>0.66</td>
</tr>
<tr>
<td>Ldh-B</td>
<td>0.25</td>
<td>1</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Gene combination fitness values

<table>
<thead>
<tr>
<th>Locus combinations</th>
<th>Northern phenotype</th>
<th>All loci</th>
<th>One or two loci</th>
<th>Southern phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mdh-A/Gpi-B</td>
<td>0.50</td>
<td>0.92</td>
<td>0.67</td>
<td>1</td>
</tr>
<tr>
<td>Ldh-B/Gpi-B</td>
<td>0.05</td>
<td>0.67</td>
<td>0.62</td>
<td>1</td>
</tr>
<tr>
<td>Ldh-B/Mdh-A</td>
<td>0 (0.32)*</td>
<td>1</td>
<td>0.92</td>
<td>0.84</td>
</tr>
<tr>
<td>Ldh-B/Mdh-A/Gpi-B</td>
<td>0</td>
<td>0.95</td>
<td>0.83</td>
<td>1</td>
</tr>
</tbody>
</table>

*Values were calculated as portion of observed survivors at 30°C to the survivors expected if random mortality had occurred which was estimated from the 20°C control group. The relative fitness was then calculated by dividing the fraction of survivors within a phenotypic class by the largest fraction of survivors within the locus in question. The parenthetical value for the Ldh-B/Mdh-A dilocus northern phenotype is the expected fraction of survivors based on single locus fitnesses — there is clearly a synergistic interaction for selection against the dilocus phenotype.

Values for the intermediate classes of multilocus phenotypes were combined into a single class.

F. heteroclitus in age class zero (eggs to fry of 59 mm) is greater than 99.5 per cent. Therefore, selection coefficients like those reflected in Table 4 would have a tremendous impact on the gene frequencies of populations exposed to temperatures that approach 30°C, which includes most of the southeast coast of the United States.

DiMichele and Powers (1991) found that, when each locus was considered separately, all loci showed significant differences in hatching times between their respective allelic isozyme phenotypes. For example, the mean hatching times for phenotypes of the Ldh-B locus were LDHB_B1 > LDHB_B2. And hatching for two of the dilocus and three of the trilocus phenotypic interactions were also significantly different (DiMichele and Powers 1991). 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, as well as gene frequency stability at those localities where such selection operates. Although this is consistent with the temporal stability of gene frequencies of several loci, spatial changes in the gene frequencies for some of loci are probably due to other physical, biological, ecological, and stochastic factors (Powers et al. 1986).

8. SUMMARY AND CONCLUSIONS

We have reviewed how an experimental strategy using 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.

We have examined the biochemical parameters of the allelic isozymes of six enzyme encoding loci (Gpi-B, H6pdh-A, Mdh-A, Aat-A, Idh-B, and Ldh-B) and found significant kinetic and/or thermal stability differences for the allelic isozymes of five of the six loci. On this basis we can reject the hypothesis that the isozymes of at least five loci are functionally equivalent. In addition, we have done studies at higher levels of biological organization (i.e., cellular and organismic levels) for three of the six loci and again found significant differences. Using temperature as a selective force, we have shown enormous survival differences between single and multilocus genotypes. On the basis of this limited data set, we cautiously conclude that selection appears to be acting on the three loci we have examined to date.

The experimental strategy that we have used to address the selectionist/neutralist controversy has led us to conclude that natural selection is acting on the allelic alternatives of the Ldh-B locus and, perhaps, two to four other enzyme encoding loci. However, demonstrating functional non-equivalence of allelic products and even selection for a single locus, or even three loci, will not in itself 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 rigorous multidisciplinary approach, we should not expect a large fraction of the scientific community to adopt this approach in the near future.

9. PROSPECTS FOR THE FUTURE

While our 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: (1) the presence and extent of silent (cryptic) allelic variants in natural populations; (2) the functional significance of isopolar amino acid differences between cryptic allelic alternatives; (3) the extent and number of genomic copies within and between populations; (4) the quantity of allele-specific mRNA and the molecular mechanisms controlling them; and (5) 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 non-coding 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 ecologists 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 non-invasive 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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