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Rapid Enzyme Assays Investigating the Variation in the Glycolytic Pathway in Field-Caught Populations of *Fundulus heteroclitus*

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Variation in enzyme expression may be important in evolutionary adaptation, yet is seldom studied. Furthermore, no studies have examined the expression of all enzymes in a defined metabolic pathway. Enzyme concentration is a measure of enzyme expression and was ascertained by assaying maximal activity. Presented here is an analysis of variation of maximal enzyme activity for all the enzymes in a single metabolic pathway, glycolysis, from three clinically distributed populations of the fish, Fundulus heteroclitus. *Techniques for rapidly analyzing maximal enzyme activity for all the enzymes of an entire metabolic pathway from many individuals are described. The high degree of repeatability (mean coefficient of variation for replicates, 4.4%) and sensitivity (less than 3 mg of tissue is required to measure all 10 enzymes) of these assays demonstrate the utility of such an approach for analyzing variation among populations for a large numbers of enzymes. Results from these studies indicate that (1) the average coefficient of variation for all enzyme determinations within a population is 45.3% and (2) between populations, the activity of 5 of the 10 glycolytic enzymes are significantly different. This considerable variation occurs even in populations where there is little allelic variation. These data demonstrating substantial variation in enzyme expression support the idea that changes in gene regulation may be as important as, or even more important than, changes in biochemical kinetic parameters in evolutionary processes.*

KEY WORDS: glycolysis; enzyme expression; maximal enzyme activity; *Fundulus heteroclitus;* microplate reader.

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INTRODUCTION

Describing variation between and within species has been a major research focus in seeking to understand speciation and adaptive change in organisms. Much of this research involved studies of protein polymorphism within a species, establishing qualitative differences between allelic isozymes by examining electrophoretic mobility. On average in vertebrates, the percentage of polymorphic loci is 23%, with the heterozygosity near 0.054 (Gillespie, 1991). The neutralist hypothesis (Lewontin, 1974; Harris, 1976; Kimura, 1983) argued that variations between allelic isozymes were not adaptively significant, prompting numerous investigators to establish that many allelic isozymes are functionally nonequivalent, possessing different catalytic properties (Place and Powers, 1979, 1984a,b). In a few cases, differences between allelic isozymes have been associated with changes in physiological performance. For example, in the teleost fish *Fundulus heteroclitus,* the two kinetically distinct lactate dehydrogenase-B (LDH-B_4) allelic isozymes are associated with differences in swimming performance, developmental rates, metabolic flux, and survival (DiMichele and Powers, 1982a,b, 1991; Paynter *et al.,* 1991). The direct involvement of this locus in developmental rate was recently demonstrated by replacing one LDH-B_4 allele with the other in eggs (DiMichele *et al.,* 1991). Other examples involve the *Pgi* locus in the butterfly *Colias* (Watt *et al.,* 1986), the sea anemone *Metridium senile* (Zamer and Hoffmann, 1989), the mussel *Mytilus edilus* (Silva *et al.,* 1989), and the evening-primrose *Clarkia* (Kruckeberg *et al.,* 1989). Variation in kinetic constants of allelic isozymes at other loci also affect important physiological traits: leucine aminopeptidase (*Lap*) in mussels (Hillibish and Koehn, 1985), glutamate–pyruvate transaminase (*Gpt*) in copepods (Burton and Feldman, 1983), and glucose-6-phosphate dehydrogenase (*G6pdh*) in *Drosophila melanogaster* (Cavener and Clegg, 1981). These studies of biochemical evolutionary adaptation focused primarily on variation in the intrinsic kinetic rate constants (e.g., K_m and k_{cat}), seldom addressing the possibility of changes in enzyme expression and resulting changes in enzyme concentration.

Modifications of both the intrinsic kinetic rate constants and the level of expression affect enzyme activity. Thus variation in enzyme expression should be as adaptively important as, or more than, changes in the intrinsic rate constants (Crawford and Powers, 1992; Laurie-Ahlberg, 1985; McDonald and Ayala, 1978; Wilson, 1976). For example, the transcription rate at the *Ldh-B* locus in *F. heteroclitus* varies between populations in a manner that does more to compensate for the changes in the thermal environment than the changes in LDH-B_4 kinetics (Crawford and Powers, 1989, 1992). Additionally, work on ADH polymorphism in *Drosophila melanogaster* has indicated that the variation in ADH concentration is selectively important

(Chambers, 1988; Laurie-Ahlberg, 1985). The question that naturally comes to mind is how much natural variation exists in the expression of any one enzyme and how many enzymes exhibit significant variation in expression (i.e., how prevalent this variation is).

To investigate these two questions we choose a system where temperature varies across a species' distribution in a consistent manner, because of the influence environmental temperature has on enzyme activity in ectotherms. In general, for every 10°C drop in body temperature, enzyme reaction rates decrease by one-half (Hochachka and Somero, 1984). This loss of activity can be compensated for by changes in the amount of active enzyme. Thus, the *a priori* prediction is that ectothermic organisms in a colder environment will increase the expression of functionally important enzymes, resulting in greater enzyme concentration. The Atlantic Coast of North America has a steep thermal gradient, changing 1°C per degree latitude. Populations of the teleost fish *Fundulus heteroclitus* are naturally distributed along this gradient, making this species an ideal system to examine variation in enzyme expression. Populations near the northern and southern extremes of the distribution experience approximately 13°C difference in mean annual temperature. This difference in ambient temperature among populations should affect enzymatic function in a predictable manner: fish in the colder northern environment should have higher enzyme activities than those in the warmer southern populations. Thus, if enzyme expression is adaptively important, there should be variation in enzyme concentration among these populations.

Selecting only a few enzymes based on a criterion that these enzymes are more likely to affect biological processes may bias the estimation of the prevalence of altered enzyme expression. To avoid this problem, we have chosen to examine all the enzymes in a single metabolic pathway—glycolysis. Choosing all the enzymes in a single pathway has the additional advantage of allowing us to look at enzyme variation in one functional unit and to compare our results with the predictions of various metabolic control theories (Kacser and Burns, 1973; Newsholme and Crabtree, 1986). We are focusing on the enzymes in the glycolytic pathway in the ventricle of these fish because glycolysis is a well-characterized pathway and reduction of flux through it may impair energy production necessary for function. These experiments were performed with fish heart ventricles because (1) fish hearts are more dependent than other vertebrate taxa on glycolysis (Sidell *et al.,* 1987), (2) cardiac glycolysis functions primarily in the forward direction, with little diversion into the pentose shunt (a pathway branching off the glycolytic pathway), and (3) it is a discrete tissue with a few unique cell types that is easily isolated. The use of a single organ, versus a whole organism (which is common in most invertebrate studies), is superior because a

metabolic pathway may serve different functions depending on the tissue, resulting in variation between tissues in enzyme expression.

Examining all the enzymes of a single metabolic pathway requires a large number of enzyme assays, which can be time- and tissue-consuming when using traditional techniques. These problems can be addressed with the use of a temperature-regulated 96-well microplate spectrophotometer that allows the simultaneous measurement of all 10 glycolytic enzymes in triplicate and requires only a few milligrams of tissue. Although similar spectrophotometers have been available for several years, recent improvements (including the ability to regulate temperature and the ability to measure absorbance at 340 nm) and the use of an octopipettor make the technique sufficiently precise for wider applications. The feasibility of enzyme kinetic assays on a microplate spectrophotometer is demonstrated here. For the first time all enzymes from a single metabolic pathway in a single organ are examined to determine the level of variation within and between natural populations. We report the maximal activity of all 10 glycolytic enzymes in field-caught individuals from three *Fundulus heteroclitus* populations distributed along the Atlantic coast, including data on the variation within and between populations.

METHODS

Fundulus heteroclitus were collected from Sapelo Island, Georgia (31.39°N), Stone Harbor, New Jersey (39.06°N), and Wiscasset, Maine (44.23°N), in September 1992. They were immediately weighed, killed, and dissected; the hearts were flash-frozen in liquid nitrogen and stored at −80°C until enzyme assays were performed.

Hearts from 10 fish of each population were individually weighed and homogenized with a glass–glass homogenizer (homogenization buffer: 100 m*M* Hepes, pH 7.5, 10 m*M* KCl, 5 m*M* EGTA, 0.1 m*M* DTT) to make a 1% homogenate. EGTA was used in place of the more common EDTA because some of the linking enzymes (see below) are inhibited by calcium. Dilutions were made for each enzyme such that its activity yielded a change of absorbance between 10 and 50 mOD/min, a range where activity is proportional to the amount of homogenate added. All assays were performed immediately after homogenization; the remaining homogenate was stored at −20°C for protein assays.

Enzyme initial velocity is described by the equation

$$V_o = [E]k_{cat}[S]/K_m + [S]$$

where $[E]$ is the enzyme concentration, $[S]$ is the substrate concentration,

K_m is the Michaelis–Menten constant, and k_{cat} is the catalytic constant. When $[S] \gg K_m$, the maximal initial velocity (V_{max}) can be described more simply as

$$V_{max} = [E]k_{cat}$$

At saturating conditions the maximal initial activity is a function of only two parameters: enzyme concentration and the catalytic rate constant. If there is no variation in the k_{cat}, then the difference in maximal activity is due to a difference in enzyme concentration. Thus, maximal enzyme activity for the 10 glycolytic enzymes was measured as an index of enzyme concentration with the assumption that there is little variation due to changes in the k_{cat} (see Discussion).

The activities of all 10 glycolytic enzymes were assayed simultaneously (in triplicate with their controls) at 25°C for 3 min using a temperature-controlled 96-well microplate spectrophotometer (Molecular Devices, Menlo Park, CA). Enzyme activity was measured at 25°C because we are using maximal activity solely as an index of enzyme concentration. Thus it is necessary only that all enzymes be measured at the same nondenaturing temperature. At 25°C, all these enzymes have a high activity that is maintained for several hours.

Each assay is linked to the oxidation or reduction of a pyridine molecule and rates are measured as change in absorbance at 340 nm. Linking enzymes were added in vast excess. Each assay was empirically determined to be at saturating conditions by varying the concentrations of substrates, cofactors, and allosteric modifiers. Concentrations used in our assays were 5- to 10-fold greater than the minimal concentration that produced maximal activity but below concentrations producing substrate inhibition. The specificity of each assay was tested by omitting each component (substrate, cofactor, homogenate, and linking enzyme) systematically and verifying that activity was essentially zero. All known allosteric modifiers were tested for effects and those that had an effect were added to maximize velocity. Ammonium sulfate suspensions of linking enzymes were dialyzed overnight, brought up in 100 m*M* Hepes, pH 7.5, 10 m*M* KCl, 50% glycerol, and stored at −20°C. Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and linking enzymes were purchased from Boehringer–Mannheim (Indianapolis, IN). Controls contained the reaction mixture and homogenate but not the specific substrate. Reactions were initiated by the addition of specific substrates simultaneously with an Eppendorf octopipettor. The protocols for the assays are modified from Misset and Opperdoes (1984), Sidell *et al.* (1987), and White and Fothergill-Gilmore (1992). Individual assays are listed below in glycolytic order.

Hexokinase (HK; EC 2.7.1.1.): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 3.1 m*M* ATP, 1 m*M* NADP, 7.5 m*M* $MgCl_2$, 10 m*M* creatine phosphate, 2 U/ml creatine kinase, 1 U/ml glucose-6-phosphate dehydrogenase, 7.5 m*M* glucose.

Phosphoglucoisomerase (PGI; EC 5.3.1.9): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 2.5 m*M* NADP, 1 U/ml glucose-6-phosphate dehydrogenase, 4 m*M* fructose-6-phosphate (glucose-6-phosphate free).

Phosphofructokinase (PFK; EC 2.7.1.11): 100 m*M* Hepes, pH 8.2, 10 m*M* KCl, 7.5 m*M* $MgCl_2$, 1 m*M* KCN, 1.25 m*M* ATP, 5 m*M* AMP, 0.2 m*M* NADH, 1 U/ml aldolase, 20 U/ml triose phosphate isomerase, 10 U/ml glycerol-3-phosphate dehydrogenase, 5 m*M* fructose-6-phosphate.

Aldolase (ALD; EC 4.1.2.13): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 0.2 m*M* NADH, 20 U/ml triose phosphate isomerase, 10 U/ml glycerol-3-phosphate dehydrogenase, 1.5 m*M* fructose 1,6-bisphosphate.

Triose phosphate isomerase (TPI; EC 5.3.1.1): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 0.4 m*M* NADH, 10 U/ml glycerol-3-phosphate dehydrogenase, 4.8 m*M* glyceraldehyde-3-phosphate.

Glyceraldehyde-3-phosphate dehydrogenase (GAP; EC 1.2.1.12): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 2 m*M* $MgCl_2$, 3.1 m*M* ATP, 0.4 m*M* NADH, 23 U/ml phosphoglycerokinase, 5.6 m*M* 3-phosphoglycerate.

Phosphoglycerokinase (PGK; EC 2.7.2.3): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 2 m*M* $MgCl_2$, 3.1 m*M* ATP, 0.2 m*M* NADH, 8 U/ml glyceraldehyde-3-phosphate dehydrogenase, 28 m*M* 3-phosphoglycerate.

Phosphoglyceromutase (PGM; EC 2.7.5.3): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 5 m*M* $MgCl_2$, 0.125 m*M* 2,3-bisphosphoglycerate, 1.25 m*M* ADP, 0.22 m*M* NADH, 9 m*M* glucose, 0.1 U/ml enolase, 0.5 U/ml pyruvate kinase, 0.75 U/ml L-lactate dehydrogenase, 1.6 U/ml hexokinase, 2.8 m*M* 3-phosphoglycerate. Hexokinase was added as an ATPase to prevent the PGK–GAP reverse reactions from occurring.

Enolase (ENO; EC 4.2.1.11): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 5 m*M* $MgCl_2$, 1.3 m*M* ADP, 0.2 m*M* NADH, 9 m*M* glucose, 0.6 U/ml pyruvate kinase, 0.75 U/ml L-lactate dehydrogenase, 1.6 U/ml hexokinase, 5 m*M* 2-phosphoglycerate.

Pyruvate kinase (PYK; EC 2.7.1.40): 100 m*M* Hepes, pH 7.4, 10 m*M* KCl, 5 m*M* $MgCl_2$, 3.8 m*M* ADP, 0.4 m*M* NADH, 0.75 U/ml L-lactate dehydrogenase.

All rates are reported as net change (i.e., minus control rates) in activity, units per minute, where units are micromoles of pyridine nucleotide catalyzed per milligram of protein. Protein concentrations were determined using the Pierce Micro BCA protein assay kit with BSA as a standard.

We genotyped 20 individuals each from Maine and Georgia for PGI and ALD and 8 individuals each for GAP and PGM using cellulose acetate

electrophoresis according to the methods of Hebert and Beaton (1989). PGI was run on a Tris–glycine buffer system and ALD on a citrate–morpholine buffer system. GAP was on a Tris–citrate buffer system, and PGM on a citrate–phosphate buffer (Richardson *et al.,* 1986). Staining buffers were at pH 8.6 for PGI and ALD, pH 7.0 for GAP and PGM, and pH 8.0 for ENO and the protocols were modified from Harris and Hopkinson (1976). PGI, ALD, and GAP gels were stained using a 2% agar overlay, while PGM was stained using a filter paper overlay. After incubation, the filter paper was removed and the PGM gel was counterstained with MTT and PMS (Richardson *et al.,* 1986).

To test the repeatability of the enzyme assays, the coefficient of variation, referred to as the CV_{rep}, was calculated among the triplicate assays for each individual ($N = 30$) and these values were pooled to calculate the mean CV_{rep} for each enzyme.

To look at activity variation within populations, the coefficient of variation (standard deviation/mean), called the CV_{pop}, was calculated from the 10 individuals in each population sample.

Analyses of variance on heart mass and body mass were performed to look for differences between populations and sexes in these physical characteristics. Analyses of covariance were used to test if either body or heart mass was a significant covariate of any enzyme's activity. Analyses of variance were used to calculate the mean squares of error for each enzyme and these were used to test for significant differences among the means of the Maine, Georgia, and New Jersey populations using least-significant difference (LSD) multiple comparisons for each enzyme. Since making all possible pairwise comparisons (ME vs GA, ME vs NJ, and GA vs NJ) results in a nonorthogonal design, the experimentwise error rate was set at $\alpha = 0.03$, yielding an α level of 0.01 for each comparison (Sokal and Rohlf, 1981). All statistical analyses used Systat software for MS-DOS.

RESULTS

Repeatability of Method

We calculated the mean CV_{rep} (Table I) to measure the repeatability of the assay technique. For 6 of the 10 enzyme assays, the CV_{rep} ranged from 1.9 to 5.4%; none of the 10 were greater than 11%. This variation is smaller than commonly found using traditional methods [mean CV_{rep}: HK, 14.8%; PGI, 4.1%; and PFK, 8.6% (unpublished data)]. Thirty-five of the 900 assays were judged outliers, but the values reported in Table I include all data. The CV_{rep} for ALD is 9%, which includes four individuals who each had one outlier that increased their coefficient of variation by an order of magnitude

Table I. Coefficients of Variation[a]

Enzyme	Replicates	Maine	New Jersey	Georgia
HK	7.71%	34.8%	35.8%	55.0%
PGI	2.87%	39.9%	39.7%	60.3%
PFK	2.73%	37.6%	45.4%	50.6%
ALD	9.01%	41.3%	40.3%	51.9%
TPI	3.38%	32.8%	44.1%	45.4%
GAP	7.25%	57.2%	37.4%	47.9%
PGK	10.50%	39.7%	50.2%	63.6%
PGM	3.09%	31.0%	38.3%	43.6%
ENO	1.88%	34.0%	41.7%	42.5%
PYK	5.40%	49.8%	39.0%	44.6%
Avg.	4.44%	39.8%	41.2%	50.5%

[a]The repeatability of each enzyme assay is indicated by the entries under "Replicates." This mean coefficient of variation for each enzyme was calculated from CVs of triplicate assays for each individual [90 assays per enzyme (10 individuals from three populations, in triplicate)]. The coefficient of variation for the maximal activities within a population (CV_{pop}) was calculated for each enzyme in the cardiac glycolytic pathway from 10 individuals in each population; values are listed under the appropriate population heading.

or more above the mean for the other individuals. Without these four individuals, the mean CV_{rep} for ALD was 2.2%. GAP, PGK, and HK also had several individuals where one of the three replicates was higher than the others, increasing the CV_{rep} for those enzymes.

Flash-freezing did not alter the activity of most enzymes, although the activities of HK, PFK, and TPI were affected. The change in activity in these enzymes versus fresh tissue did not vary with population.

Intrapopulation Variation

The mean specific activity (Table II) and coefficient of variation (CV_{pop}; Table I) were calculated for each enzyme in each population to look at the variability within these populations. The CV_{pop}'s range from 31.9 to 63.6%. The Georgia population had higher coefficients of variation for 8 of the 10 enzymes than the New Jersey and Maine populations. The CV_{pop} for each enzyme was similar among the New Jersey and Maine populations.

Interpopulation Variation

Body mass was compared among the three populations. The means for the Maine and Georgia fish did not differ, while the New Jersey fish had a greater mean for body mass [10.31 g (NJ) vs 6.85 g (GA) and 6.22 g (ME);

Table II. Mean Specific Activity (U/min) in Three Poplations[a]

Enzyme	Maine		New Jersey		Georgia
HK	3.444		3.721		2.781
PGI	**40.35***	=	**41.51***	>	**27.87***
PFK	3.474		4.361		3.693
ALD	**4.169***	=	**3.959***	>	**2.709***
TPI	367.3		**445.1***	>	**297.5***
GAP	27.17		**35.52***	>	**18.87***
PGK	15.37		17.41		14.49
PGM	2.910		**3.450***	>	**2.485***
ENO	3.343		3.498		2.882
PYK	21.11		15.31		17.86

[a]All rates are reported as net change in activity as units per minute, where units are micromoles of pyridine nucleotide catalyzed per milligram of protein. Boldfaced values are significantly different among the populations. An equal sign (=) indicates that two populations had similar values (not statistically different) and both were significantly different from the remaining population; > indicates the direction of the difference. If only two of the three population values are boldfaced, the remaining population is not significantly different from either of these populations.

$P < 0.03$]. Body mass did not significantly affect enzyme activity (ANCOVA, all P's > 0.05) and was ignored in further analyses. There was no significant difference between populations or sexes in heart mass [5.77 mg (ME) vs 6.99 mg (NJ) vs 6.34 mg (GA); $P > 0.05$]. There was a significant or nearly significant sex × heart mass interaction for all 10 enzymes (P values ranged from 0.004 for ENO to 0.076 for ALD), so analyses of covariance could not be performed using heart mass as a covariate. While some variation in enzyme activity may be explained by variation in heart mass within populations, this should not affect among-population comparisons since there is no significant difference in heart mass among populations.

Multiple comparisons found statistically significant differences among populations' means for five enzymes (means are reported in Table II). Excluding outliers did not alter these results (data not shown). For those enzymes that differ significantly between populations, there are two general patterns. In the first pattern, found in two enzymes, PGI and ALD, the Georgia population had a lower mean than either the Maine or the New Jersey populations, while the latter two did not differ significantly from each other. In the second pattern, found in TPI, PGM, and GAP, New Jersey fish always had a greater mean activity than Georgia fish; the Maine fish had means intermediate between the New Jersey and the Georgia fish and were not significantly different from either.

Allelic Variation

Genotyping indicates that there is only one GAP locus, one PGI locus, PGI-B, and one ALD locus, ALD-A, expressed in the heart ventricles of these fish. Both PGM-A and PGM-B as well as diphosphoglyceromutase are expressed the heart ventricle. We found no electrophoretic variation in GAP, DPGM, or either PGM locus. There are two predominant PGI-B alleles, one in the north, designated *c*, and one in the south designated *b* (Ropson *et al.,* 1990). We detected a total of four PGI-B alleles, although only three were present in a given population. In the Maine population, the *c* allele frequency was 0.85, while the *b* and *d* alleles each had a frequency of 0.075. In the Georgia population, the *b* allele had a frequency of 0.90, while the *a* and *c* alleles had frequencies of 0.025 and 0.075, respectively. We detected only two ALD-A alleles in these populations. The *b* allele predominated in the south (frequency = 0.75), while the *c* allele was more common in the north (frequency = 0.67).

DISCUSSION

These experiments demonstrate that it is now feasible to conduct studies on the variation of enzyme concentration in a complete metabolic pathway in a single organ or an individual animal because of increased sensitivity (reducing the amount of tissue required) and precision. Use of a temperature-controlled microplate spectrophotometer allows a large number of enzymes (10) to be accurately measured, even in tissues weighing as little as 2.6 mg, the smallest heart in our survey. The precision is indicated by the coefficient of variation (CV_{rep}), which has an overall mean of 4.4% and is less than 11% for all enzymes. Previous research (Clark and Keith, 1987) using microplate assays had a mean CV_{rep} of 10.7% for 10 enzymes, ranging from 3.89 to 19.49%, and this variation was larger than they found using a traditional spectrophotometer. The reduction in the experimental variation found in our study is most likely due to the regulation of internal temperature, use of an octopipettor, and direct measurement of the oxidation or reduction of a pyridine nucleotide at 340 nm. The CV_{rep} values found in this study include all assays, even those which could be justifiably labeled outliers. Thirty-five of the 300 sets of triplicates (900 assays) had a single outlier which increased the CV_{rep} for that set above 10%. Excluding these outliers greatly reduces the mean CV_{rep} (e.g., CV_{rep} for ALD is 2.2% rather than 9%).

The variation in enzyme maximal activities within a population (CV_{pop}) is considerable, ranging from 31.9 to 63.6%. The variation reported here is similar to levels of variation in expression reported for β-glucuronidase in wild mouse populations (Bush and Paigen, 1993) and for aminopeptidase in

Mytilus edulis (Koehn and Immermann, 1981). This variation in enzyme activities is much greater than the variation due to protein polymorphism: the average heterozygosity for vertebrates is 5.4%, with about 23% of all loci being polymorphic (Gillespie, 1991).

For *Fundulus heteroclitus,* 14 of the 18 polymorphic loci examined in the same populations have one predominant allele in the northern population [frequency ranging from 0.85 to 1.00, with 12 of these 14 loci having a frequency greater than 0.95 (Cashon *et al.,* 1981; Powers and Place, 1978; Ropson *et al.,* 1990)]. The only glycolytic locus examined in their study, *Pgi-B* (expressed in cardiac tissue), is virtually fixed for a single allele in Maine (major allele frequency of 0.94), while another allele is found at a high frequency in Georgia [major allele frequency of 0.75 (Powers and Place, 1978)]. We found similar allele frequencies in our survey; the *c* allele had a frequency of 0.85 in Maine, while the *b* allele had a frequency of 0.90 in the Georgia population. The heterozygosity for *Pgi-B* in the Maine, New Jersey, and Georgia populations is 11, 50, and 38%, respectively (Cashon *et al.,* 1981), compared to a CV_{pop} of 40, 40, and 60%. It is clear that there is no correlation between the level of heterozygosity and variation in enzyme activity for this locus. All individuals surveyed for PGM and GAP were electrophoretically identical, yet the CV_{pop} values for all the populations for these enzymes were comparable to those of PGI and ALD. If the remaining glycolytic loci show a pattern similar to that of PGI, PGM, or GAP, then there appears to be little relationship between CV_{pop} and protein polymorphism.

In this study, all the fish were caught during a single tidal influx from a single tidal creek. Tidal creeks experience episodic influx of well-mixed waters (Pickard and Emery, 1990); thus the within-population variation in enzyme activity is not due to any obvious difference in their thermal environment at the time of capture. The large activity variation could be due to variation in k_{cat} or to variation in the level of enzyme expression due to genetic or physiological differences. Differences due to k_{cat} may result from different alleles present in the population, but this seems unlikely given the lack of protein polymorphism in the northern populations and the incongruence between CV_{pop} and heterozygosity. For at least PGI, it is unlikely that variation in k_{cat} is responsible because (1) there is no difference in the k_{cat} under these assay conditions [pH and temperature (Van Beneden and Powers, 1989)] and (2) the New Jersey population has greater allelic variation than the Maine population, yet it has nearly identical CV_{pop} values for PGI. This contradicts the idea that kinetic differences can explain the variation in PGI's maximal activity. For PGM and GAP only one electrophoretic allele was identified per locus, thus k_{cat} variation is unlikely to explain the observed variation in maximal activity. For the other glycolytic enzymes,

the allelic variation in k_{cat} is not known. In general, variations in k_{cat} in *Fundulus heteroclitus* are small. For LDH-B_4 the largest difference in k_{cat} is at 10°C and ranges from 3 to 15%, depending upon the pH. For the IDH-B_2 and PGI-B allozymes there is no significant difference in k_{cat} (Powers *et al.*, 1993; Van Beneden and Powers, 1989). In this study variation in maximal activity between populations is approximately three times greater (45%) than the largest reported difference in k_{cat}. Thus, variation within and between populations in maximal enzyme activity may be due partially to protein polymorphisms that affect k_{cat}, but it is unlikely to be a major source of the variation.

Because these animals were field-caught, it cannot be determined whether the intrapopulational variation is due solely to genetic polymorphisms among individuals or is also affected by differences in physiological status, such as nutritional, hormonal, or reproductive state. If at least some of this variation is genetically based, as is likely (see Crawford and Powers, 1989), natural selection could potentially act on this variation. Some of the physiologically based variation can be removed by acclimating fish to common environmental conditions. The genetic component can be estimated by conducting breeding experiments to measure heritability.

The most surprising result is the prevalence of significant interpopulational variation in enzyme expression as measured by maximal enzyme activity. Five of the ten enzymes were significantly different between two or more populations. The biological basis for the interpopulational variation in the *F. heteroclitus* glycolytic enzymes is currently unknown. It may be due either to genetic differences or to physiological factors such as those discussed above. In addition to these physiological factors, there is an obvious difference among populations in the environmental temperature. To compensate for temperature differences it is expected that for enzymes with significant interpopulation differences, the Maine population would have greater activity than the Georgia population. The New Jersey population may be intermediate or similar to one of the other two populations, depending on the underlying genetic mechanisms. PGI and ALD activities are higher in the more northern populations than in the southern population and thus vary in the manner predicted if increasing activity is a mechanism for compensating for colder temperatures. The differences in GAP, TPI, and PGM are not consistent with the compensation by enzyme expression for the effect of temperature on reaction rate. For these three enzymes, activity in New Jersey fish is significantly greater than that in Georgia fish, and the activity in the Maine population appears to be intermediate between these two populations. This may be the result of physiological factors apart from temperature. The New Jersey fish are larger than the other two populations, suggesting that the Maine and Georgia populations may be

more food-limited than the New Jersey fish. Thus, limited food supplies may depress metabolism, resulting in the lower activity seen in the Maine and Georgia populations. Laboratory experiments need to be carried out to minimize physiological and environmental differences between populations and thus determine the effect of reversible acclimation.

By examining all the enzymes in glycolysis, we have avoided making any *a priori* assumptions about which enzymes will be important. This allows us to compare our results with the predictions of various metabolic control theories. Most theories classify enzymes as either equilibrium or nonequilibrium (and thus rate limiting). Classically, nonequilibrium enzymes were thought to be the most important for control of flux through a pathway (Crabtree and Newsholme, 1972a,b, 1987; Newsholme and Crabtree, 1986; Newsholme and Start, 1973). This paradigm has dominated many investigations; e.g., most researchers seeking to explore the biochemical basis for changes in a physiological process examine only a few nonequilibrium enzymes, ignoring the possible contribution from other enzymes in a metabolic pathway. However, there are alternative metabolic control theories which suggest the control of flux may rest equally in all enzymes (Kacser and Burns, 1973, 1979; Cornish-Bowden and Cardenas, 1990) or that equilibrium enzymes may regulate flux by determining the capacity of the pathway (Watt, 1985; Hochachka and Somero, 1984). Thus it is unclear which, if any, enzymes are more important.

In mammals, variation in the expression of HK, PFK, and PYK does occur and is thought to be an important physiological response (Granner and Pilkis, 1990). In our study, the expression of these three "rate-limiting enzymes" does not vary significantly among populations. This implies either that changes in these enzymes are less important for physiological compensation or that their catalytic rates are altered by mechanisms other than changes in enzyme concentration (Somero and Hand, 1990; Storey, 1988). The enzymes that do have significant interpopulational differences are equilibrium enzymes. These differences between populations, especially the compensatory changes in PGI and ALD, suggest that equilibrium enzymes may affect metabolic processes. Other research supports our findings by demonstrating that equilibrium enzymes may be important for metabolic control (Pettersson and Ryde-Pettersson, 1989; Watt, 1985; Watt *et al.,* 1986; Zamer and Hoffmann, 1989). The functional importance of these equilibrium enzymes requires further research.

These results demonstrate (1) that there is considerable variation in enzyme expression and (2) that several equilibrium enzymes have significant differences in expression, suggesting that variation in enzyme expression may be important in evolutionary adaptation. The genetic and molecular bases, as well as the physiological consequences of this variation in enzyme

expression, need to be established to ascertain their role in evolutionary processes.

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