

Phylogenetic Analysis of Thermal Acclimation of the Glycolytic Enzymes in the Genus *Fundulus*

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ABSTRACT

Physiological acclimation that alters enzyme activity can compensate for the effect of temperature on function and may be achieved by altering enzyme concentration. This study uses phylogenetic analyses to investigate the evolutionary history of and to test several hypotheses about acclimation responses among all the glycolytic enzymes. These hypotheses are that (1) acclimation increases enzyme concentration at lower temperatures to compensate for reduced activity; (2) equilibrium enzymes tend to show acclimation responses; and (3) acclimation responses are more common in species whose populations experience either large temporal or geographical temperature variations. Using maximal activities as indices of enzyme concentration, the presence of acclimation responses in all the glycolytic enzymes in the heart ventricle was determined for five species in the teleost genus *Fundulus*. Three of these species are distributed along the steep thermal cline of the North American Atlantic coast, and thus these species experience both seasonal and geographical variation in temperature. The other two species are found in the Gulf of Mexico and experience seasonal variation similar to the Atlantic species but no geographical variation in temperature. Two Atlantic coast species, *Fundulus heteroclitus* and *Fundulus majalis*, have unique derived acclimation responses. No derived acclimation responses occur in the Gulf species. A conserved response in hexokinase was observed within one subgenus comprising both Atlantic and Gulf species. In *F. heteroclitus*, enolase responded to acclimation, and in *F. majalis*, aldolase, triphosphate isomerase, and lactate dehydrogenase had acclimation responses. These en-

zymes are equilibrium enzymes, and the concentrations of all of them increase at lower temperatures, which would compensate for the effect of temperature on enzyme activity. The compensatory changes all occur in the Atlantic species and may be a mechanism for species to expand their ranges. These data suggest that physiological acclimation is evolutionarily labile.

Introduction

Enzyme functions are sensitive to temperature (Hazel and Prosser 1974; Hochachka and Somero 1984). In general, enzyme catalysis halves for every 10°C drop in temperature, and this reduced activity should affect metabolic processes. Ectothermic organisms experience variation in body temperature daily, seasonally, or over their habitat range. These variations in temperature can affect metabolism, with potentially detrimental consequences. One way of ameliorating the effect of temperature changes on enzyme function is physiological acclimatization. Physiological acclimatization can be defined as a reversible change in metabolism or other physiological traits in response to natural environmental cues (Garland and Adolph 1991). Acclimation refers to the same pattern, but as it occurs in the laboratory (Garland and Adolph 1991). That is, acclimation is measured under controlled laboratory conditions and is indicative of a physiological response that occurs in an organism's natural setting.

Acclimation responses alter overall metabolic flux. For example, respiration rates in a variety of fish tissues increase in cold-acclimated individuals compared with tissues acutely exposed to lower temperatures (striped bass liver slices [Stone and Sidell 1981]; goldfish liver homogenates [Kanungo and Prosser 1959]; brook trout liver homogenates [Hochachka and Hayes 1962]; rainbow trout hepatocytes [Hazel and Prosser 1979]). This change in respiration may be due to the effect of temperature on the relative contributions of lipid and carbohydrate pathways to metabolism. Generally, glycolysis is found to be more important at lower temperatures, because glycolytic rate is relatively independent of the acclimation temperature, while lipid catabolism is temperature-dependent and thus contributes less to overall metabolism in cold-acclimated individuals (Hochachka and Hayes 1962; Moerland and Sidell 1981; Stone and Sidell 1981). The relative temperature independence of glycolysis indicates that there are physiological mechanisms, such as increased enzyme activity, maintaining a constant metabolic flux.

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Which enzymes are involved in affecting metabolism, and thus are important in acclimation responses, is the subject of much debate (e.g., Cornish-Bowden and Cardenas 1990). Enzymes in a metabolic pathway can be divided into two categories. One class is near-equilibrium enzymes, enzymes that catalyze a reaction in which the drop in free energy between the substrate and product is small and thus the reaction is reversible. The other category is nonequilibrium enzymes, enzymes that catalyze a reaction in which the drop in free energy between substrate and product is large enough that the reaction is considered irreversible. In glycolysis, hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) are typically considered nonequilibrium, rate-limiting enzymes (Stryer 1981). Classical biochemical theories indicate that only nonequilibrium enzymes can regulate flux, suggesting that all other enzymes are superfluous with regard to modulating flux (Crabtree and Newsholme 1972a, 1972b, 1987; Rolleston 1972; Newsholme and Start 1973). Alternatively, metabolic control theories suggest that many different enzymes can affect flux (Cornish-Bowden and Cardenas 1990).

In the glycolytic pathway, one of the more potent regulatory enzymes is PFK. PFK has allosteric kinetics and is modulated by a large number of activators and inhibitors (Hochachka and Somero 1984). The evolution of this kinetic complexity complements the experimental data (Rolleston 1972; Carpenter and Hand 1986; Galazzo and Bailey 1990; Somero and Hand 1990; Van Der Veen et al. 1995; Wegener 1996) indicating the regulatory importance of the nonequilibrium enzyme PFK. However, the presence of nonequilibrium regulatory enzymes does not exclude the possibility that equilibrium enzymes influence flux. This may be especially true for long-term physiological changes like thermal acclimation, where there is enough time to reestablish a different enzyme concentration.

For most acclimation responses, it is uncertain which enzymes are involved. In some cases, but not all, an acclimation response has been demonstrated for a select few enzymes within a pathway (Yaumuchi et al. 1975; Campbell and Davies 1978; Moerland and Sidell 1981; Kleckner and Sidell 1985). It cannot be determined from these data whether all the enzymes in a pathway acclimate or whether only certain classically defined rate-limiting enzymes respond to temperature changes. Walesby and Johnston (1980) found that in muscles of brook trout, the adenylate charge and phosphorylation state ratio were lower in cold-acclimated fish, and they suggested that this would activate glycolysis (implicitly through the activation of PFK). Thus, the concentrations of classical rate-limiting enzymes might not be limiting with respect to temperature, and acclimation responses that alter enzyme concentrations may occur predominantly in enzymes that do not have allosteric regulation. Moerland and Sidell (1981) did not find an acclimation response in PFK or PK in *Fundulus heteroclitus* livers, despite changes in glycolytic rate. It is possible that changes in the activities of

these enzymes were mediated by allosteric modulators in vivo and that acclimation responses occurred in other enzymes that were not examined.

Two important issues need to be addressed: the prevalence of acclimation responses among all the enzymes in a single pathway and the evolutionary history of acclimation responses (that is, the evolution of the natural physiological response, acclimatization, as measured in the laboratory, i.e., acclimation). Which enzymes in a single metabolic pathway have an acclimation response has implications for the function of the response and the issue of the regulation of flux through a pathway.

No study to date has incorporated fully both evolutionary data and acclimation data. This study examines the effect of thermal acclimation on the concentrations of all 10 glycolytic enzymes and lactate dehydrogenase (LDH), and the phylogenetic distributions of acclimation response among the species of the teleost fish genus *Fundulus*. Three specific hypotheses are examined with phylogenetic analyses. First, if physiological acclimation is a mechanism for increasing or maintaining a constant rate of glycolysis, then enzyme concentrations should increase at lower temperatures. Second, species whose populations are subject to large variation in temperature, either temporally or geographically, are more likely to evolve an acclimation response than species not subjected to variation in temperature (Pierce and Crawford 1996). In particular, the Atlantic coast species, because of their distribution along the steep thermal cline, will more likely demonstrate an acclimation response than species in the Gulf of Mexico, where there is little geographic variation in temperature. Finally, if one or a few enzymes are always important for modulating metabolic flux, then in those species that have an acclimation response, the same enzymes should always acclimate. Which enzymes have an acclimation response will depend on whether classical biochemical theories or metabolic control theories are more accurate. There is an expectation that if variation in enzyme concentration is a mechanism for acclimation then either only nonequilibrium enzymes will have an evolutionarily significant pattern of acclimation response (Crabtree and Newsholme 1972a) or many different enzymes will show an acclimation response.

Material and Methods

Design of Experiments: Using Phylogenetic Information

The genus *Fundulus* consists of small North American killifish whose phylogenetic relationships have been examined using both morphological characters (Wiley 1986) and allozyme and molecular data (Cashner et al. 1992; Bernardi and Powers 1995). Thus, there are almost no polytomies in this phylogeny, making it ideal for inferring the evolution of physiological characters (Pagel 1993). Most of the species are grouped into

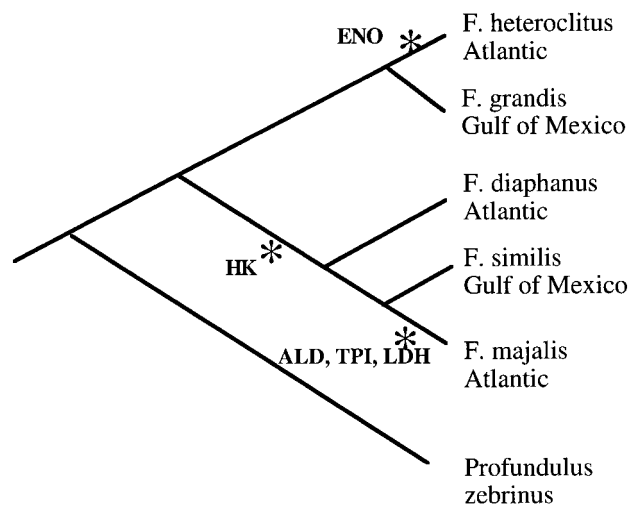


Figure 1. Phylogenetic relationships among *Fundulus* species and hypothesized origins of effects of acclimation. Phylogenetic relationships are adapted from Wiley (1986). Asterisks indicate hypothesized origin of acclimation effect. Enzyme abbreviations are listed in Material and Methods.

four subgenera. The species in two of these subgenera, *Fontinus* and *Fundulus*, are distributed along the Atlantic and Gulf of Mexico coasts. Three species have independently colonized the Atlantic coast, and each has a sister species in the Gulf of Mexico or in the southeastern United States. The evolutionary history of acclimation responses can be inferred in this genus because the relationships among these species are known.

Study Animals and Acclimation

Three Atlantic coast species, *Fundulus heteroclitus*, *Fundulus diaphanus*, and *Fundulus majalis*, and two Gulf coast species, *Fundulus grandis* and *Fundulus similis*, were examined (Fig. 1). *F. grandis* is the sister species of *F. heteroclitus*, and both are in the subgenus *Fundulus* (Wiley 1986). *F. similis* is the sister species of *F. majalis*, and both, with *F. diaphanus*, are in the subgenus *Fontinus* (Wiley 1986). *F. grandis* and *F. similis* were collected from Panacea, Florida, *F. majalis* from Woods Hole, Massachusetts, *F. heteroclitus* from Wiscasset, Maine, and *F. diaphanus* from Green Banks, New Jersey. Individuals from a population of each species were obtained in the late summer of 1995 and maintained at 20°C for approximately 2 wk before acclimation at different temperatures. Light cycle was 14L:10D. Each species was kept at its preferred salinity: *F. diaphanus* in freshwater, *F. heteroclitus* and *F. grandis* at half-strength seawater, and *F. similis* and *F. majalis* in full seawater, using Instant Ocean and dechlorinated tap water. Equal numbers of each species were then acclimated to 10°C or continuously maintained at 20°C. *F. heteroclitus*, *F. diaphanus*, and *F. grandis* were acclimated for 6–8 wk. *F. similis* and *F. majalis* were acclimated for 3.5–6 wk. Fish were fed frozen

brine shrimp and Tetramin Food Flakes ad lib. once daily in the late afternoon. During acclimation, all species came into reproductive condition and spawned. The reproductive tissues were in regression in all species when assayed. Acclimation responses occur by 3–4 wk of treatment (Johnston and Dunn 1987), and several species in this study did show acclimation responses, which suggests enough time had occurred for acclimation (see Results).

Homogenization Procedure

Fish were weighed and then killed by quickly severing the spinal cord from skull. Hearts were removed and placed in a Ringer's solution, where they continued to contract and expel blood. Heart ventricles were weighed and homogenized with an ultrasonic cell disrupter (Kontes, Vineland, N.J.) in a 100 m mol L⁻¹ Hepes buffer, pH 7.4, 10 m mol L⁻¹ KCl, 0.5 m mol L⁻¹ dithiothreitol, and 0.2% Triton X-100. Hepes buffer was used because it is a nonphosphate buffer whose pH is relatively stable to dilution effects and has a smaller temperature sensitivity than many other buffers (Good and Isawa 1972). Eight to 10 individuals from each acclimation temperature were assayed for each species.

Enzyme Assays

Initial activities (20–30 mOD min⁻¹) of all 10 glycolytic enzymes and LDH were assayed for 3 min at 25°C with a temperature-regulated 96-well spectrophotometer (Molecular Devices, Menlo Park, Calif.; see Pierce and Crawford [1994] and below). All enzymes from two individuals (in triplicate plus control) were assayed at one time. Controls contained no substrate. Since assay concentrations varied slightly from species to species, two individuals from same species, one acclimated to 10°C and the other to 20°C, were always assayed together on a plate. All assays were performed at 25°C because we are using maximal activity solely as an index of enzyme concentration. It is important only that all enzymes be measured at the same nondenaturing temperature. All enzymes in all species examined have high activities that are maintained for several hours at 25°C.

We examined all glycolytic enzymes: HK (EC 2.7.1.1); phosphoglucose isomerase (PGI, EC 5.3.1.9); PFK (EC 2.7.1.11); aldolase (ALD, EC 4.1.2.13); triosephosphate isomerase (TPI, EC 5.3.1.1); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12); phosphoglycerokinase (PGK, EC 2.7.2.3); phosphoglyceromutase (PGM, EC 2.7.5.3); enolase (ENO, EC 4.2.1.11); PK (EC 2.7.1.40), and LDH (EC 1.1.1.27). Maximal activities were determined by assays linked to the oxidation or reduction of pyridine nucleotides, and rates were measured spectrophotometrically at 340 nm. All rates were initial activities (less than 0.090 change of absorbance during the 3-min time course). Linking enzymes were added in vast excess, as

determined by measuring their activities in the presence of their specific substrates. Concentrations of substrates, cofactors, and all known allosteric modifiers were varied to empirically determine saturating conditions for each enzyme for each species. Concentrations of substrates used were 3–10 times the minimal amounts that produce maximal activity but do not cause substrate inhibition. Thus, substrate concentrations were at least an order of magnitude greater than the apparent Michaelis-Menten constants (K_m), which were determined empirically from crude homogenates. Ammonium sulfate suspensions of linking enzymes were dialyzed overnight, brought up in 100 m mol L⁻¹ Hepes (pH 7.4), 10 m mol L⁻¹ KCl, and 50% glycerol. Reaction rates without homogenates (i.e., substrates, cofactor, and linking enzymes) were investigated to determine if any spurious activities occurred. Chemicals (substrates, cofactors, etc.) were obtained from Sigma Chemical Co. (St. Louis; note: fructose-6-phosphate from other manufacturers is often contaminated with glucose-6-phosphate), and linking enzymes were purchased from Boehringer-Mannheim (Indianapolis) and Sigma. Reactions were initiated by addition of substrate using an Eppendorf octopipettor. Assays were similar to those published previously for *F. heteroclitus* (Pierce and Crawford 1994, 1996), with appropriate modifications of concentrations to ensure saturating conditions for each species. Protein concentrations were determined with the Pierce BCA microassay kit (Pierce Biochemical), and activity was determined in units of micromoles of pyridine nucleotide catalyzed per minute per milligram of total protein.

The maximal initial velocity (V_{max}) can be described as $V_{max} = [E]k_{cat}$, where $[E]$ is the enzyme concentration and k_{cat} is the catalytic rate constant. At saturating conditions, the maximal initial velocity is a function of only two parameters: enzyme concentration and the catalytic rate constant. If there is no variation in catalytic rate constant, then differences in maximal activity are due to changes in enzyme concentration. In *F. heteroclitus*, variation in catalytic rate constants are not responsible for maximal activity variation (Pierce and Crawford 1996).

Statistical Analyses and Phylogenetic Inferences

The presence of an effect of acclimation on each enzyme was determined separately in each species through ANOVA in Minitab (version 10). For some species, it was not possible to sample equal numbers of both sexes. Homoscedasticity between groups was tested using Bartlett's test and Levene's nonparametric test. The homogeneity of slopes was verified before using ANCOVA to test for an effect of body mass on enzyme maximal activity. If body mass had no effect on enzyme activity, then an ANOVA was performed with acclimation temperature, sex, and the temperature-by-sex interaction as factors. If there was a significant temperature-by-sex interaction term, only one sex was used in the analysis.

The goal of this study was to examine the evolutionary history of a physiological trait, acclimation response of enzyme concentration. To do this, the acclimation data were coded (as presence/absence data), and then acclimation responses were mapped on an existing phylogeny. Parsimony, which postulates the fewest number of evolutionary changes required to create the observed pattern, was used to infer whether an observed effect of acclimation in a species is the derived or primitive condition. These analyses used an existing phylogeny of the species studied, rather than creating one. To avoid circularity when mapping traits onto a phylogeny, it is essential to use phylogenetic relationships determined from traits different from the ones under study. In this case, a physiological trait, acclimation response of enzyme concentration in the heart ventricle, was mapped on a phylogeny based on morphological characters (Wiley 1986), with which there is likely to be little relationship.

The phylogenetic analysis gives more information than purely comparative studies and allows the generation of a priori orthogonal hypotheses. In this study, the mean for each temperature group was used in only one comparison with the other temperature group for that species. Thus, five comparisons among 10 groups are made for each enzyme rather than all possible comparisons. When a priori orthogonal hypotheses are tested, no multiple comparison correction is needed (Steel and Torrie 1980; Sokal and Rohlf 1981). The second stage of the analysis used the data as presence/absence binary data and directly incorporated phylogenetic information to look for concordance among species. Each enzyme was treated independently within a species. There may be coordinated regulation of the enzymes in a pathway, such that the evolution of a response in one enzyme increases the chance of a response in another enzyme. There are also common allosteric regulators for a few enzymes, and correlations among a few pairs of enzymes have been noted (Eanes 1984; Wang and Clark 1995). However, it is more parsimonious to treat the enzymes as independent of each other because there is little evidence of coordination of all, or even most, of the enzymes in a pathway.

Results

Mean and standard errors of enzyme maximal activities at both acclimation temperatures for all species are listed in Tables 1 and 2. Results of the initial statistical analyses are presented below for each species separately. They are then combined on the phylogeny and discussed in an evolutionary context (Fig. 1). Analysis of the duration of acclimation is provided last.

Fundulus heteroclitus (Atlantic)

Previously published data for this species were used in the phylogenetic analysis (Pierce and Crawford 1996; Table 1), with the exception of two enzymes, PGM and LDH. LDH

Table 1: Mean maximal activity for acclimation groups in three Atlantic coast species

Enzyme	<i>Fundulus diaphanus</i>		<i>Fundulus heteroclitus</i>		<i>Fundulus majalis</i>	
	10°C	20°C	10°C	20°C	10°C	20°C
HK080 ^a (.023)	1.03 ^a (.021)	2.909 (.335)	2.631 (.348)	1.921 ^a (.023)	1.579 ^a (.031)
PGI	10.61 (.177)	11.79 (.180)	49.01 (3.24)	45.14 (6.48)	9.667 (.185)	9.908 (.143)
PFK	4.895 (.102)	5.774 (.122)	3.97 (.338)	3.78 (.481)	3.873 (.097)	4.409 (.076)
ALD	2.706 (.056)	2.643 (.062)	6.232 (.462)	5.324 (.740)	3.21 ^a (.055)	2.017 ^a (.075)
TPI	161.5 (5.65)	193.1 (6.15)	416.9 (42.1)	356.5 (45.6)	361.3 ^a (4.74)	303.1 ^a (6.70)
GAPDH	21.62 (.472)	21.99 (.580)	26.89 (1.42)	33.15 (7.21)	16.13 (.322)	14.32 (.249)
PGK	12.10 (.224)	13.76 (.255)	11.65 (1.27)	10.12 (1.23)	18.59 (.397)	14.57 (.561)
PGM	21.01 (.495)	23.01 (.477)	26.29 (.500)	23.96 (.524)	18.97 (.318)	19.15 (.246)
ENO	2.43 (.065)	2.53 (.067)	3.376 ^a (.230)	2.295 ^a (.308)	2.035 (.060)	1.492 (.085)
PK	19.35 (.461)	20.14 (.413)	32.89 (3.04)	29.9 (3.57)	18.75 ^a (.284)	16.43 ^a (.220)
LDH	77.07 (1.84)	84.43 (1.71)	46.46 (1.67)	42.13 (1.84)	69.46 ^a (.873)	61.28 ^a (.691)

Note. Maximal activity is in micromoles pyridine nucleotide catalyzed per minute per milligram total protein. Standard errors are listed beneath each mean in parentheses. Enzyme abbreviations are listed in Material and Methods. ALD, HK, and ENO covaried with body mass in *Fundulus heteroclitus*, and ALD, HK, and LDH covaried with body mass in *Fundulus majalis*. Means are adjusted accordingly.

^a Activity differed significantly between acclimation groups.

activity was not measured in the previous study, and PGM determinations from the previous study (Pierce and Crawford 1996) were much lower. The increase in *F. heteroclitus* PGM activity reported here may be due to changes in the homogenization procedure and revised assay protocols for this enzyme. The data shown here indicate that there was no effect of acclimation on PGM maximal activity. There was also no effect of acclimation on LDH in the heart ventricle. These LDH results are similar to results determined in a pilot study of 10 fish from New Jersey (1993; D. L. Crawford, unpublished data). The absence of an LDH acclimation response in heart ventricles contrasts with the acclimation response of the same LDH locus in liver tissue (Segal and Crawford 1994).

Fundulus diaphanus (Atlantic)

Body mass, heart mass, and the maximal activities of all enzymes were homoscedastic ($P > 0.05$ in all cases). Body mass and heart mass did not differ significantly between acclimation

groups or sexes. ALD and ENO activities were analyzed in females only because they had significant heterogeneity of slopes for the body-mass-by-sex term and could not be analyzed by the full ANCOVA design. No enzyme in *F. diaphanus* was significantly affected by body mass. Only HK activities showed a significant difference between acclimation groups ($P = 0.023$; Table 1).

Fundulus majalis (Atlantic)

Body mass, heart mass, and all enzyme activities were homoscedastic ($P > 0.06$ in all cases). Body mass and heart mass did not differ significantly between acclimation groups but did differ between sexes ($P = 0.046$). PFK activity had heterogeneous slopes for the body-mass-by-sex term and was analyzed in females only. There was a significant effect of body mass on ALD ($P = 0.042$), HK ($P = 0.005$), LDH ($P = 0.006$), and PFK ($P = 0.027$) activities. ANCOVAs were used to analyze these four enzymes, and ANOVAs were used to analyze the

Table 2: Mean maximal activity for acclimation groups in two Gulf coast species

Enzyme	<i>Fundulus grandis</i>		<i>Fundulus similis</i>	
	10°C	20°C	10°C	20°C
HK	1.351 (.056)	1.511 (.046)	.887 ^a (.037)	1.238 ^a (.024)
PGI	12.97 (.400)	12.46 (.320)	8.802 (.393)	10.84 (.250)
PFK	2.879 (.087)	2.752 (.071)	3.322 (.928)	3.632 (.059)
ALD	2.123 (.072)	1.934 (.058)	1.735 (.054)	1.735 (.034)
TPI	284.3 (9.19)	287.0 (7.51)	199.4 (3.70)	260.3 (4.97)
GAPDH	10.67 (.450)	12.29 (.368)	10.09 (.419)	10.97 (.267)
PGK	12.54 (.489)	13.64 (.399)	12.00 (.392)	13.35 (.250)
PGM	18.30 (.619)	17.94 (.506)	13.67 (.440)	16.52 (.280)
ENO	1.937 (.075)	2.037 (.061)	1.492 (.360)	2.027 (.343)
PK	17.00 (.580)	16.80 (.476)	11.35 ^a (.320)	13.93 ^a (.205)
LDH	35.33 (1.183)	38.40 (.966)	45.76 (1.64)	57.42 (1.04)

Note. Maximal activity is in micromoles pyridine nucleotide catalyzed per minute per milligram total protein. Standard errors are listed beneath each mean in parentheses. Abbreviations for enzymes are given in Material and Methods.

^aActivity differed significantly between acclimation groups.

other seven enzymes. Although the effect of acclimation temperature was not significant, there was a significant sex-by-acclimation-group term for GAPDH ($P = 0.010$), PGI ($P = 0.007$), PGM ($P = 0.006$), and PK ($P < 0.05$) activities. These enzymes also were analyzed only in females. There was a significant effect of acclimation on ALD ($P = 0.001$), HK ($P = 0.010$), LDH ($P = 0.050$), and TPI ($P = 0.038$) activities. There was a nearly significant effect of acclimation on PK activity ($P = 0.055$).

Fundulus similis (Gulf of Mexico)

Body mass, heart mass, and all enzymes activities except ENO activity were homoscedastic among temperature groups and did not differ between them. Log-transformed ENO activity data were homoscedastic. There was significant heterogeneity of slopes for body mass for ALD, ENO, GAPDH, PFK, PGM, PK, and TPI activities. These enzyme activities were regressed

on body mass independently in each temperature group, and the equations and residuals were used to calculate the predicted activity values as if the fish all had the same body mass. Two-way ANOVAs with acclimation temperature and sex were used to analyze these adjusted data. PK and HK activities differed significantly between temperature groups ($P = 0.050$ and $P = 0.023$, respectively).

Fundulus grandis (Gulf of Mexico)

Body mass, heart mass, and all enzyme activities were homoscedastic among temperature groups. No enzyme scaled significantly with body mass, and no enzyme activity differed between acclimation temperatures ($P > 0.39$ in all cases).

Phylogenetic Mapping

Acclimation in *F. heteroclitus* affected ENO maximal activity. Since this is not observed in any other species, it is most parsimonious to assume that this effect arose along the lineage to *F. heteroclitus* after it diverged from all other species (Fig. 1). Similarly, *F. majalis* showed effects of acclimation on ALD, LDH, and TPI that are unique to this species. PK had a marginally significant effect of acclimation in *F. similis* and was nearly significant in *F. majalis*. In contrast, three species, *F. majalis*, *F. similis*, and *F. diaphanus*, all in the subgenus *Fontinus*, showed an effect of acclimation on HK. The most parsimonious interpretation is that the acclimation response for HK either arose before the divergence of the three species but after the divergence between the two subgenera or was present in the common ancestor and subsequently lost in the *F. heteroclitus*-*F. grandis* clade (Fig. 1).

Acclimation

Enzyme assays on a single species were conducted over an approximately 10-d period. There was no significant relationship between maximal enzyme activities and assay date (duration of acclimation); regressions between enzyme activity and duration of acclimation were nonsignificant ($P > 0.1$), and assay date was not a significant factor in ANOVA ($P > 0.1$). In addition, plots of enzyme activity versus date displayed no obvious trend.

Discussion

The evolutionary history of acclimation responses can provide information on whether physiological acclimation is evolutionarily conserved or if these responses are evolutionarily labile. The use of cladistic analyses and the increasing availability of both molecular and morphological data have refined our understanding of the phylogenetic relationships among species (Mortitz and Hillis 1996). This information can be incorpo-

rated into the design of comparative physiological studies to allow hypothesis testing and a better understanding of the evolutionary factors likely to influence the traits under investigation (Garland and Adolph 1994; Garland and Carter 1994). In addition, comparisons of closely related species minimize the number of stochastic differences between study taxa. Knowing phylogenetic relationships allows the identification of taxa that may act as replicates for a particular hypothesis. Mapping the character state of the trait on the phylogeny permits the inference of its history and can distinguish similarity due to shared evolutionary history (synapomorphy) and similarity due to convergent evolution.

In this study, we used species in the *Fundulus* clade. The phylogeny of this clade has been constructed using molecular, allozymic, and morphological data, so the relationships among the species are known with a high degree of confidence (Wiley 1986; Cashner et al. 1992; Bernardi and Powers 1995; Fig. 1). It is important to note that a number of species in this clade are distributed along the North American Atlantic coast, which has a steep thermal cline as well as a pronounced seasonal variation. The most parsimonious explanation for the distribution of Atlantic coast species is that they have colonized the coast independently, because each one has a sister species located in the Gulf of Mexico or the southeastern United States (Fig. 1) and more basal taxa in this clade are located in the Gulf of Mexico. Thus, any traits shared by the Atlantic species, but absent in their sister species, should represent convergent evolution, indicative of natural selection.

The evolutionary history of acclimation responses was investigated by quantifying the maximal activity of all the glycolytic enzymes among *Fundulus* species. That is, this study examined the evolutionary history of the natural physiological response by quantifying acclimation responses in the laboratory. Acclimation is indicative of the physiological response, acclimatization, that occurs in an organism's natural setting. Glycolysis was chosen to examine the effect of acclimation on enzyme maximal activity because it is a well-characterized pathway containing classically defined rate-limiting enzymes (typically HK, PFK, and PK) and near-equilibrium enzymes (PGI, ALD, TPI, GAPDH, PGK, PGM, ENO, and LDH; Rolleston 1972; Stryer 1981). In fish hearts, glycolysis may supply a greater proportion of metabolic fuel than in the hearts of tetrapods (Sidell et al. 1987), and the increased contribution of glycolysis to respiration at lower temperatures has been documented in several teleost fish tissues, including *Fundulus heteroclitus* livers (Johnston and Dunn 1987). Thus, acclimation responses in glycolytic enzymes may be advantageous.

The interpretation of these results depends on meaningful acclimation conditions and enzyme assays that measure differences in enzyme concentrations resulting from quantitative changes in gene expression. These criteria are discussed below.

Acclimation

In our study, fish were kept at 20°C for approximately 2 wk and then either maintained at this temperature or maintained

at 10°C for a minimum of 3–6 wk before assays were initiated. Both acclimation groups were assayed together during a 10-d period. Four of the five *Fundulus* species had an acclimation response (a significant difference in enzyme activity between acclimation temperatures). These changes in enzyme maximal activities were observed after 25 d (e.g., *Fundulus majalis* and *Fundulus similis*). Within a species there was no relationship (statistically significant or obvious trend) between the length of acclimation and maximal glycolytic enzyme activity. In the species that did not have an acclimation response (*Fundulus grandis*), more than 6 wk did not produce significant differences in enzyme activity between temperatures. In *F. heteroclitus*, acclimation periods greater than 6 wk did not alter the pattern of enzyme expression (data not shown). Lin and Somero (1995) and Johnston and Dunn (1987) noted that most acclimation responses have been observed to occur within 2 d to 4 wk of exposure to the new temperature. Freed (1971) observed that new steady state levels of sugar phosphates in goldfish are attained after 7 h of acclimation. These observations suggest there was sufficient time for acclimation responses to occur.

An important question is whether the acclimation temperatures used are biologically meaningful. For example, the lowest mean monthly temperature for species from Florida is only 13.3°C (Table 3), and thus the low acclimation temperature used in this study (10°C) may be biologically irrelevant. Fish at the Florida location do experience these temperatures; two months have mean minimum monthly temperatures of less than 10°C (30-yr average of the low temperatures that occur in any month), and minimum temperatures below 10°C occur in five different months (U.S. Department of Commerce 1955). In comparison, *F. heteroclitus* populations in southern Georgia and northern Florida are less likely to experience these low temperatures, yet they have an acclimation response (Crawford 1988; Segal and Crawford 1994). The results presented here are limited; there was no attempt to discern the complete range of acclimation responses for all the species tested nor the effect of season on acclimation. However, these data do address the evolutionary history of acclimation responses among groups of organisms subjected to the same biologically relevant acclimation temperatures during the same season.

Assays

Maximal activity measurements are used to quantify enzyme concentration and are not intended to approximate *in vivo* activities (see Material and Methods). Maximal activities are good indices of enzyme concentrations if initial reaction rates approach the theoretical maximum (e.g., an order of magnitude greater than the activity at K_m) and all allosteric modulators are taken into account. The maximal activities reported here had activities 10-fold greater than the apparent K_m determined from crude extracts, were insensitive to a threefold de-

Table 3: Mean monthly temperature near collection sites

<i>Fundulus</i> Species	Location	Lowest Mean	Highest Mean	Mean Temperature Change/yr
<i>F. heteroclitus</i>	ME	2.2	17.2	15
<i>F. diaphanus</i>	NJ	1.7	22.8	21.1
<i>F. majalis</i>	MA	1.1	22.2	21
<i>F. grandis</i>	FL	13.3	30.0	16.7
<i>F. similis</i>	FL	13.3	30.0	16.7

Source. National Oceanographic Data Center (1982, 1985).

Note. Means are coastal water temperatures in degrees Celsius at a depth of 1 m. ME, Maine; NJ, New Jersey; MA, Massachusetts; FL, Florida.

crease in substrate concentration, and used all known allosteric regulators. Thus, these determinations should quantify enzyme concentration. In addition, maximal activity assays using a 96-well microplate reader are precise; the coefficients of variation among triplicates are less than 11% for all enzymes studied and average 4.4% for *F. heteroclitus* (Pierce and Crawford 1994). Using a microplate reader allows the collection of data on a large number of enzymes in a large number of individuals, an important consideration for conducting phylogenetic studies.

We have chosen to focus on enzyme concentration as one parameter of physiological acclimation. Other factors, such as phosphorylation and the concentration of allosteric regulators, can be important in vivo. The phosphorylation state of enzymes such as PFK or PK affect K_m and are correlated with changes in metabolism (Storey 1988). However, enzyme phosphorylation does not affect PFK's maximal velocity (Foe and Kemp 1982; Luther and Lee 1986; Papadopoulos et al. 1991; Benoit et al. 1994) and would not affect our maximal activity assays. Variation in allosteric regulators may also contribute to physiological acclimation. For example, acclimation to lower temperatures decreases the adenylate energy charge and adenylate ratios in trout muscles (Walesby and Johnston 1980) and therefore potentially activates PFK activity (Johnston and Dunn 1987). In our study, all known allosteric regulators are added to the reaction mixture to maximize activity, and any differences between acclimation conditions in the in vivo levels of allosteric regulators should not affect our assays. Thus, even for allosterically regulated enzymes, maximal activity reflects enzyme concentration. Finally, most of the enzymes in the glycolytic pathway are not known to be modulated by either of these mechanisms, and therefore altering concentration may be the only physiological mechanism to alter these enzyme rates.

In this study, individuals from the same population for each species were sampled at 10°C and 20°C, and differences between acclimation groups cannot be explained by allelic differences in catalytic rate constant because we assigned tempera-

ture treatment randomly. Thus, allelic variation should be distributed randomly between the groups, and systematic differences should average out. The allelic isoforms present in one species could have greater temperature sensitivity than the alleles for the same locus in other species and could explain differences among species. These differences would still be an evolutionary change in the physiological response and result in concentration changes with acclimation temperature. These changes could be achieved by differences in the transcription or translation rate, or in the stability of the mRNA or protein. Knowing the molecular mechanisms underlying the acclimation responses would help to confirm or refute conservation of the HK response and possibly identify common mechanisms for coordinated evolution of these enzymes (i.e., within *F. majalis*, or between *F. majalis* and *F. heteroclitus*).

Quantitative Change in Enzyme Expression as a Mechanism of Adaptation

Thermal acclimation of enzymes can be achieved by either induction of different genetic loci of an enzyme (isozymes) that have different kinetic characteristics or by quantitatively altering the expression of an enzyme ("expression" used in the broad sense to include RNA synthesis through protein turnover). The relative prevalence of these two mechanisms is unknown, but demonstration of thermal induction of different loci has been infrequent. Johnson and Bennett (1995) reported acclimatory changes in myosin isoform expression in goldfish. Rainbow trout undergo acclimatory induction of acetylcholinesterase isoforms (Baldwin and Hochachka 1970), but not of myosin isoforms (Johnson et al. 1996). Acclimatory induction of different cytoplasmic malate dehydrogenase was observed in the teleosts spot and blenny (Schwantes and Schwantes 1982a, 1982b) and has recently been demonstrated in a goby (Lin and Somero 1995). Differential isozyme induction was not observed for glycolytic enzymes in *F. heteroclitus* or in green sunfish (Shaklee et al. 1977; Pierce and Crawford 1996). Induction

may exist in the other *Fundulus* species examined, but on the basis of evidence from other fish and *F. heteroclitus*, it is not very likely.

A more general mechanism may be to quantitatively alter the expression of enzymes. Increasing the amount of enzyme may compensate for the reduced activity of each individual molecule at lower temperatures. Cytochrome *c* concentrations increase in sunfish muscles acclimated to lower temperatures (Sidell 1977). Increased concentration has been demonstrated for liver LDH-B (Crawford and Powers 1992), cardiac enolase (Pierce and Crawford 1996), and muscle myofibrillar ATPase (Johnson and Bennett 1995) in *F. heteroclitus* acclimated to lower temperatures. Sunfish brain, but not liver, showed increased amounts of five glycolytic enzymes at colder temperatures (Shaklee et al. 1977). Even in the case of goldfish, where induction of different myosins occur, a change in the amount of myofibrillar ATPase is also observed (Johnson and Bennett 1995). Modulation of enzyme expression is a common response to insulin/glucagon hormonal regulation (Granner and Pilkis 1990). The data provided here suggest that altered enzyme concentration is a common acclimation response, as observed in six of the 11 glycolytic enzymes.

Evolution of Acclimation

The goal of this study was to examine hypotheses about the evolution of acclimation responses in enzyme concentration among closely related species. This was accomplished by using an existing phylogeny of the species studied to infer the evolutionary history, rather than creating a phylogeny. Acclimation responses were highly variable within the *Fundulus* clade and changed readily through evolutionary time. Unique acclimation responses were seen in two Atlantic species, *F. heteroclitus* and *F. majalis*. These responses were absent in their sister species, with the possible exception of a PK response (marginally significant) that might be shared between *F. majalis* and *F. similis*. Only one response, in HK, was conserved within a subgenus (Fig. 1). This article demonstrates that physiological acclimation responses that alter enzyme concentration are evolutionarily labile (i.e., evolve independently in different lineages and involve different glycolytic enzymes in these lineages). These observations support the proposition of Burggren and Bemis (1990) that behavior and physiological adjustments such as acclimation are first-line defenses against environmental change.

Are these changes in enzyme concentration meaningful? Could these patterns arise from neutral or random changes that increase an enzyme's concentration at colder temperatures (e.g., a random amino acid substitution resulting in a greater relative protein stability at low temperatures)? This explanation does not seem reasonable. If neutral processes are responsible, there should be an equal number of enzymes that increase or decrease at low temperatures. Further, one of two expectations

arise if random changes in enzyme expression are responsible for the acclimation responses. First, if neutral changes in enzyme expression occur readily in these taxa, then most taxa should have acclimation responses and no phylogenetic signal would be observed. Second, if these neutral changes occur rarely, then acclimation should be phylogenetically restricted and occur in a few enzymes. The data do not support a neutral evolutionary hypothesis: for the equilibrium enzymes, compensatory acclimation occurs often (in four of the eight enzymes) but only in two phylogenetically unrelated species.

Evaluation of Hypotheses

Acclimation response and its relationship to enzyme concentration has a vast and complex history (e.g., see reviews by Hazel and Prosser [1974]; Shaklee et al. [1977]; Hochachka and Somero [1984]; Johnston and Dunn [1987]). Three hypotheses about acclimation responses (see Introduction) were tested by examining all the enzymes in a single metabolic pathway among a phylogenetically appropriate set of species. These data are a useful start for evaluating the evolution of acclimation response.

Hypothesis 1. The first hypothesis was that enzyme concentration will increase at lower acclimation temperatures if physiological acclimation is a mechanism for increasing or maintaining a constant rate of glycolysis. Concentrations of equilibrium enzymes that have a significant acclimation response increased at lower temperatures in the Atlantic species. Since these responses arose after their divergence from other *Fundulus* species, they are relatively recent and may be in response to modern conditions, such as present-day temperatures. Thus, for the equilibrium enzymes, the acclimation response is in a direction that would ameliorate the effect of temperature variation.

The HK response is more complex: an acclimation response only occurs within a single subgenus, yet HK concentrations decrease at the lower temperature in one Gulf coast species, *F. similis*, and in a single Atlantic coast species, *F. diaphanus*, and increase in the other Atlantic species of this subgenus, *F. majalis*. Thus, an acclimation response may have arisen in an ancestor, and, since the divergence of the species, the response has been modified within the subgenus. The observation that HK maximal activity changes with acclimation conditions in one subgenus, while the direction of the response is variable among the species in the subgenus, may be indicative of the sensitivity of this enzyme's expression to physiological conditions. Because the direction of the HK response is variable, it is unclear whether this physiological sensitivity is due to selection or random genetic drift. One solution to this issue is to determine cardiac glycolytic flux among the species of this subgenus and correlate the change in HK with change in glycolytic flux.

Ignoring the usual criteria for significant differences, there are other trends in these data. We can define a meaningful difference between acclimation temperatures as a difference between means plus or minus 1 standard error. In two species, most of the glycolytic enzymes that were different when using these liberal criteria had a larger value at 10°C than at 20°C: six of six and eight of 10 enzymes that were different were greater at 10°C than 20°C in *F. heteroclitus* and *F. majalis*, respectively. In two other species, most of the glycolytic enzymes that were different when using these liberal criteria had the opposite trend: eight of eight and nine of 10 enzymes that were different were lower at 10°C than at 20°C for *F. similis* and *F. diaphanus*, respectively. In the fifth species, *F. grandis*, there were approximately an equal number of enzymes that increased or decreased (three of eight were larger at 10°C vs. 20°C). These patterns (that most of the enzymes were greater or lesser at 10°C) were significant in three of the five species (*F. heteroclitus*, *F. diaphanus*, and *F. similis*; sign-rank test; $P < 0.05$; Siegel 1956). These trends may suggest that (1) within these species the expression of each glycolytic enzyme was not completely independent, (2) in two species most of the loci had a compensatory increase in enzyme concentration, and (3) in two other species most the glycolytic loci decreased at low acclimation temperatures. If we assume that these are meaningful trends, they suggest that acclimation response involves all enzymes in a pathway, not just a few rate-limiting enzymes. A positive or negative trend was not restricted to a specific clade, which suggests that acclimation is evolutionary labile. However, it should be stressed that most of these differences between acclimation conditions were not statistically significant and thus it is difficult to define how meaningful they are.

Hypothesis 2. The second hypothesis predicted that an acclimation response would occur most frequently in Atlantic coast species because of their distribution along a steep thermal cline. Two of the three Atlantic species showed unique acclimation responses, and neither of the Gulf of Mexico species had unique acclimation responses. In a third Atlantic species (*F. diaphanus*), there were no unique acclimation responses. The only responses observed in the Gulf species were shared with other species and thus are presumably more ancient.

Clarke (1987) predicted that the type of response (genetic differentiation vs. physiological acclimation) would depend on the time scale of the variation in temperature. Seasonal variation would result in acclimation responses, while genetic changes would evolve in response to geographical changes. These predictions are not supported by our data. Seasonal variation alone cannot account for the observed patterns; the magnitude of seasonal variation is similar for both coasts (Table 3). However, geographic variation in environmental temperatures can explain the observed pattern because the mean annual temperatures vary over the ranges of the Atlantic species, while the mean temperatures are essentially constant over

the ranges of the Gulf species (National Oceanic Data Center 1982, 1985). For species whose populations are subject to large geographic variation in temperature, the evolution of an acclimation response may be one of the mechanisms that can compensate for environmental variation in temperature (Pierce and Crawford 1996). That is, as a species expands its range, selection may favor individuals with new genetic variants (e.g., protein polymorphisms or a heritable difference in protein expression), or it may favor individuals that have a physiological response (e.g., acclimatization response that alters enzyme concentration). The presence of this physiological response (acclimatization) may mitigate the selection pressures and preclude the evolution of further genetic differentiation (Pierce and Crawford 1996). For example, if there is selection for increased enzyme activity in colder environments, acclimatization could eliminate this selective pressure by providing a physiological mechanism that increases enzyme activity. This would explain the higher prevalence of acclimation responses in Atlantic coast species.

Unlike other Atlantic coast species, *F. diaphanus* (Atlantic coast) lacked an acclimation response. If geographical variation in temperature is important, then one would also expect to find acclimation responses in *F. diaphanus*. The absence of an acclimation response could be explained by its biogeographical history. This species is found in freshwater and is distributed north of Virginia. As recently as 10,000 yr ago, the current geographic range of this species was covered by ice (Mickelson et al. 1983), and thus its distribution was greatly limited. In contrast, the geographic distribution of *F. heteroclitus*, which is found in coastal salt marshes, was much less affected by the last ice age because of how sea levels changed (Bloom 1983; Mickelson et al. 1983). The third Atlantic species, *F. majalis*, occurs in coastal marshes only as far north as Massachusetts, and thus its geographic distribution was the least affected by the last glaciation. The absence of an acclimation response in *F. diaphanus*, unlike the other Atlantic species, may reflect an insufficient time for the evolution of a detectable acclimation response.

Hypothesis 3. The third hypothesis predicted that among species that had an acclimation response, the same enzymes would be affected. Which enzymes respond to acclimation would depend on if there are a few rate-limiting enzymes or if equilibrium enzymes also influence physiological processes. ENO concentration increased at lower temperatures in *F. heteroclitus*. The concentrations of ALD, TPI, and LDH increased at lower temperatures in *F. majalis*, another coastal Atlantic species. There was a variable effect of acclimation on HK in three species, *F. majalis*, *F. similis*, and *F. diaphanus*, all belonging to the same subgenus. From these data, there is no obvious pattern of acclimation and thus no one enzyme or set of enzymes that always evolves an acclimation response.

There are, however, some trends. There was a higher fre-

quency of altered expression in equilibrium enzymes versus nonequilibrium enzymes, and PFK did not show an effect of acclimation in any species. Changes in PFK concentration may be physiologically unnecessary because its allosteric regulators can change in response to temperature (Johnston and Dunn 1987). Conversely, compensatory changes in the concentrations of equilibrium enzymes may reflect the fact that allosteric regulators and phosphorylation do not modulate the activity of these enzymes, and, thus, the only physiological mechanism to modulate activity is altered expression. The changes in equilibrium enzymes are in a compensatory direction, and, at least in *F. heteroclitus*, acclimation is associated with a change in hepatic metabolism (Moerland and Sidell 1981). Although we do not provide data demonstrating that acclimation responses affect metabolism, our results indicate that equilibrium enzymes are indicative of physiological acclimation and should not be ignored in future studies.

The observation that equilibrium enzymes change is counter to predictions of classical control theories (Crabtree and Newsolme 1972a, 1972b, 1987; Newsholme and Start 1973) but is in agreement with metabolic control theories that predict that many enzymes can modulate flux (Cornish-Bowden and Cardenas 1990). Experimental data on a variety of organisms have supported this idea. Variation in PGI activity affects fitness in *Colias* butterflies and carbohydrate metabolism in sea anemones (Watt et al. 1986; Zamer and Hoffmann 1989). In primroses, the control coefficient (a quantitative measure of how metabolic flux changes in response to variation in enzyme activity) of PGI is significant and varies with environmental conditions (Kruckeberg et al. 1989). The activity of another near-equilibrium enzyme, ENO, modulates metabolism in the bacteria *Zymomonas mobilis* and in rat hearts (Viikari 1988; Kashiwaya et al. 1994). In rat hearts, ENO flux control coefficient ranges from 0.27 to 0.55, depending on physiological conditions (Kashiwaya et al. 1994). In *F. heteroclitus*, ENO acclimated in a compensatory manner, while in *F. majalis* other enzymes have compensatory responses. These data suggest that control of metabolic flux may not reside in any one particular enzyme, but that control may be variably distributed among enzymes depending on physiological conditions and evolutionary opportunities.

Conclusions

Acclimation responses are evolutionarily labile: no response is conserved throughout the genus, and responses unique to one species are observed in four enzymes. These responses are primarily in equilibrium enzymes and are achieved by altering enzyme concentration in a potentially compensatory direction. These responses are more prevalent in Atlantic coast species, whose distributions encompass a greater range of temperatures, and may be a mechanism for coping with environmental temperature variation and expanding geographic ranges.

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