

**EVOLUTION OF METABOLISM:
COMBINING PHYLOGENETIC PHYSIOLOGICAL
AND BIOCHEMICAL INFORMATION
TO STUDY METABOLIC ADAPTATION IN KILLIFISH.**

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Introduction

The organizers of this symposium have challenged the participants to combine “morphological, physiological and biochemical traits and fish phylogenies” to understand the evolution of physiological and biochemical traits. My research has focused on identifying physiological and biochemical traits that have evolved in response to selection. The rationale for this has been not only to understand the evolution of these traits themselves, but to use evidence of adaptation to evaluate models of physiological and biochemical function. My interests have been specifically focused on how and which enzymes in metabolic pathways are adjusted in response to environmental challenges, notably temperature.

Temperature has a profound effect on metabolism and enzyme function, with enzyme activities halving for every 10°C drop in temperature. (Hochachka and Somero, 1984; Schmidt-Nielsen 1983). As organisms colonize colder environments, one would expect adaptations that compensate for this reduced activity to evolve. My work has focused on adaptations in cardiac glycolytic enzymes in killifish belonging to the North American genus *Fundulus*. Four species in this genus have independently colonized the steep thermal cline of the North American Atlantic Coast and must cope with over 10°C difference in mean annual temperature over their range (NDOC, 1982).

These fish may compensate for reduced activity at lower temperatures by several different mechanisms. They may increase their cardiac mass or allometric scaling of enzyme activity to maintain power output. At the biochemical level, they may express isozymes with different thermal properties

or they may increase enzyme expression to compensate for the reduced activity of each enzyme molecule (Lin and Somero, 1995; Crawford and Powers, 1992; Pierce and Crawford, 1994; 1997).

My primary question is how many and which enzymes should be adjusted by these various mechanisms during temperature adaptation. Classical biochemical theories predict that there is one “master” regulatory (usually non-equilibrium) enzyme per pathway (Crabtree and Newsholme, 1987) while metabolic control theories argue that all enzymes can modulate flux (Kacser and Burns, 1973; Cornish-Bowden and Cardenas, 1990). Laboratory studies tend to support the classical theories, yet field studies suggest that near-equilibrium enzymes can be important (e.g. Watt *et al.* 1986, Zamer and Hoffman, 1989). I have taken an evolutionary approach, which defines important enzymes as those enzymes whose variation in activity produces physiological consequences that are selectively advantageous or disadvantageous. By identifying enzymes that show evidence of selection and adaptive variation, we can infer that variation in these enzymes has functional consequences. That is, if the variation in enzyme activity is convincingly demonstrated to be due to natural selection, then that variation must affect a biologically important phenotype. However, drift and phylogenetic inertia also affect patterns of physiological and genetic differentiation (Garland and Adolph, 1994). Ideally, studies of physiology and metabolism should attempt to assess the influence of a variety of evolutionary factors on observed patterns of variation (Burggren and Bemis, 1990; Garland and Adolph, 1994). Two of the most common methods for taking into account evolutionary patterns in continuous data are phylogenetic autocorrelation (Cheverud and Dow, 1985; Cheverud *et al.* 1985) and independent contrasts (Felsenstein, 1985; Garland, 1992). These methods incorporate phylogenetic information into analysis of traits of multiple taxa to distinguish between adaptation and other evolutionary forces.

In this paper, I will examine whether the four Atlantic coast species show adaptive changes in heart mass, allometric scaling of enzymes or changes in enzyme levels using both intraspecific and interspecific analyses. Intraspecific analyses will determine population differences in these traits between northern and southern populations within each Atlantic species. These patterns will be compared to the results of interspecific analyses on mean trait values across among fifteen taxa (populations and species) within the genus. Interspecific analyses will be performed with and without phylogenetic information to assess the effect of ignoring phylogeny on the outcome.

Methods

Phylogeny of Fundulus

The genus *Fundulus* is a group of small, North American killifish found in both freshwater and saltwater environments. Most species are distributed along the Gulf of Mexico or throughout the southeastern United States (Wiley, 1986; Cashner *et al.* 1992). Its sister genus, *Plancterus*, ranges from Texas to Kansas. The relationships of the taxa used in this study are based on a combination of morphological and sequence data (Fig. 1; Wiley, 1986; Bernardi and Powers 1995; Pierce and Crawford, 1997).

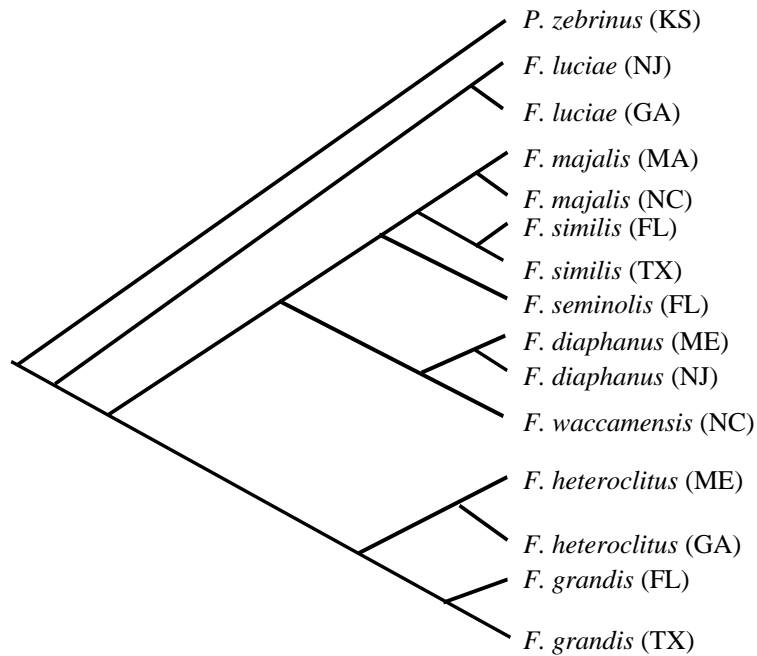


Figure 1. Phylogenetic Relationships of *Fundulus* species. Only species included in this study are shown. Phylogeny modified after Wiley (1986), Cashner *et al.* (1992), and Bernardi and Powers (1995). States of origin for each

Four species are found along the Atlantic Coast: *F. heteroclitus* from the *Fundulus* subgenus, *F. luciae* from the *Zygonectes* subgenus, and *F. majalis* and *F. diaphanus* from the *Fontinus* subgenus. The four Atlantic species each have a sister species in the Gulf of Mexico or in the southeastern United States and thus have independently colonized the Atlantic Coast. Each Atlantic species serve as replicates to identify changes in morphology and enzyme expression that correlate with changes in environmental temperature independently of phylogenetic history (Pierce and Crawford, 1997). Animal collection and handling were described previously (Pierce and Crawford, 1997).

Enzyme Assays

In order to test the competing models of metabolic regulation, all the enzymes in a pathway need to be tested systematically. Choosing only the one or few enzymes that one believes are important introduces an *a priori* bias to the data set generated. Maximal activity assays for all ten glycolytic enzymes plus lactate dehydrogenase were performed as described previously (Pierce and Crawford, 1994; Pierce and Crawford, 1997). Activities are expressed as micromoles of pyridine nucleotide metabolized per minute per milligram total heart protein. These measurements quantify enzyme concentrations, which is one measure of enzyme expression (Pierce and Crawford, 1994). Abbreviations for glycolytic enzymes are given in Pierce and Crawford (1997).

Statistical Analyses

Intraspecific analyses focused on the four Atlantic coast species. Analyses of variance and covariance were used to examine the allometry of body mass, heart mass and enzyme activities (units/mg protein) within and between northern and southern populations in each species.

Interspecific analyses used standard regression analysis to examine the effect of body mass and environmental temperature on mean population enzyme levels. Phylogenetic analyses used two methods, phylogenetic autocorrelation and independent contrasts, as described previously (Pierce and Crawford, 1997). Interspecific analyses included both Atlantic and Gulf coast species, for a total of fifteen taxa (Fig. 1). Phylogenetic autocorrelation is analogous to scaling on body mass and decomposes the measured variance in a trait into a phylogenetic value (the amount of variance explained by genetic distance) and residual, or “phylogeny-independent”, values for each taxon (Cheverud *et al.* 1985).

Results and Discussion

Variation in Physical Characteristics

Body mass varied 2-4 fold among individuals within a species and ten-fold among *Fundulus* species. Heart and body mass did not vary consistently between northern and southern populations within species (Table 1). Interspecific analysis of mean body showed no significant correlation with temperature using either standard regression analysis or phylogenetic methods. Body mass did have a significant phylogenetic component, with 47% of the variation in body mass among taxa explained by phylogenetic distance and more closely related taxa tending to have more similar body masses (Pierce and Crawford, 1997). The lack of a consistent north-south mass difference may indicate that size *per se* does not confer an advantage with respect to temperature in *Fundulus* or that other factors may have greater influence on size.

In all but one species, population differences in heart mass could be explained by differences in body mass. Heart mass was proportionately larger in the northern populations of *F. majalis*, which may increase performance at lower temperature. However, because this response evolved in only one species out of four studied, it is not possible to distinguish between adaptiveness and chance differentiation of this trait (Garland and Adolph, 1995).

Table 1. North-South Differences in Body and Heart Mass. ANOVAs identified significant mass differences between northern and southern populations within each species. ANCOVA with body mass as a covariate was used to test whether population differences in heart mass remained after accounting for body size differences.

Species	Body Mass	Heart Mass	Body- Corrected Heart Mass
<i>F. diaphanus</i>	N > S	N > S	none
<i>F. heteroclitus</i>	none	none	N/A
<i>F. majalis</i>	N > S	N > S	N > S
<i>F. luciae</i>	S > N	S > N	none

Scaling of Enzymes with Mass

Mass-specific metabolic traits usually scale negatively with body mass. Recent papers have proposed that this is the result of the physics of branching networks and thus should apply to all biological systems (West *et al.* 1999). However, positive scaling has been reported in fish white muscle for three glycolytic enzymes, PFK, LDH and PYK, even though an aerobic enzyme in the same muscle scaled negatively (Somero and Childress, 1980; Burness *et al.*, 1999). Somero and Childress (1980) noted that fish white muscle contraction is powered primarily by anaerobic glycolysis and thus should function independently of transport during burst activity. They proposed that because the power requirement increases exponentially with size due to the increased drag in water, anaerobic power would also have to increase at a higher exponential rate. It is not clear whether this positive relationship will extend to other muscle types.

In *Fundulus* hearts, we find the scaling relationship between mass and specific activity (per unit total protein) of the different glycolytic enzymes to be complex and not very consistent among species (Table 2). No enzymes scale with body mass or heart mass in *F. luciae* or *F. grandis*. Ten out of eleven glycolytic enzymes show scaling with mass, but never in more than three species. We find the same positive scaling of PFK and LDH with body mass that others have reported, but PYK in two species shows a negative relationship. The Gulf species, *F. similis*, shows negative scaling of seven enzymes. The Atlantic species tend to show positive scaling (with two exceptions, ALD and PYK in *F. diaphanus*), suggesting that perhaps positive scaling of enzyme activity with mass is advantageous along a thermal cline. Alternatively, the lack of consistency among species may indicate that scaling of glycolytic enzymes in this tissue is not very important and may vary due to drift.

Table 2. Intraspecific Scaling of Enzymes with Body Mass[†]

Enzyme	Scales with body mass in	Relationship
HK	<i>F. heteroclitus</i> (A)	+
	<i>F. majalis</i> (A)	+
	<i>F. similis</i> (G) [‡]	-
PGI	<i>F. similis</i> (G) [‡]	-
PFK	<i>F. majalis</i> (A)	+
	<i>F. heteroclitus</i> (p=0.06)(A)	+
ALD	<i>F. majalis</i> (A)	+
	<i>F. heteroclitus</i> (p=0.06) (A)	+
	<i>F. diaphanus</i> (p=0.054)(A)	-
TPI	<i>F. majalis</i> (A)	+
	<i>F. similis</i> (G)	-
GAPDH	<i>F. similis</i> (G) [‡]	-
PGK	none	
PGM	<i>F. heteroclitus</i> (A)	+
	<i>F. similis</i> (G) [‡]	-
ENO	<i>F. diaphanus</i> (A)	+
	<i>F. similis</i> (G)	-
PYK	<i>F. diaphanus</i> (A) [‡]	-
	<i>F. similis</i> (G)	-
LDH	<i>F. majalis</i> (A)	+

[†] no enzymes scaled with mass in *F. luciae* or *F. grandis*

[‡] scales with heart mass only

A= Atlantic G= Gulf

The interspecific analyses show substantially different results. Only one enzyme, HK showed a positive relationship with body and heart mass, and three others, PGM, ENO and LDH, showed a negative relationship with body mass (Table 3). In all four cases, the scaling relationship became non-significant after correcting for phylogenetic effects on body mass and enzyme levels (Table 3; $p > 0.26$ in all cases). HK, PGM, LDH and body mass have significant positive autocorrelation coefficients, indicating that phylogenetic distance among the taxa explain some of the variation in these traits. Thus apparent mass scaling

across species is confounded with phylogeny and may reflect correlated evolution of variation in body mass and enzyme levels.

Table 3. Interspecific scaling of enzyme activities with body mass before and after phylogenetic correction. Mean population enzyme activities and mean population body sizes were used for all

Trait	Mass scaling relationship	
	Before phylogenetic correction	After phylogenetic correction
HK	+	none
PGM	-	none
ENO	-	none
LDH	-	none

Population Differences in Enzyme Levels

If increasing enzyme levels is a way to compensate for reduced enzyme activity at lower temperatures, then northern populations should have higher levels than their southern counterparts. However, most of the significant intraspecific comparisons show that southern populations have higher enzyme levels. As with the scaling data, the population differences are not always consistent across all four replicate species. PGI is higher in the south in 3 species but higher in the north in the fourth (Table 4). Other enzymes show significant population differences in only one or two species (Table 4).

Again, the interspecific analyses show a very different picture from the intraspecific comparisons. When mean enzyme levels are regressed on mean annual temperature for fifteen taxa, only two enzymes, GAPDH and LDH, show significant or nearly significant relationships with temperature (Table 4; $p=0.033$ and $p=0.074$, respectively). These relationships are both negative, indicating that levels of these enzymes increase as environmental temperature decreases, as predicted.

Table 4. Effect of Mean Annual Temperature on Enzyme Activities.
 Results of significant ANOVAs and ANCOVAs performed on populations within species are given as intraspecific differences. Interspecific relationships are based on regressions across fifteen

Trait	Intraspecific differences	Interspecific temperature correlations	
		Before phylogenetic correction	After phylogenetic correction
HK**	S>N: <i>F. diaphanus</i>	none	none
PGI	S>N: <i>F. diaphanus</i> <i>F. majalis</i> <i>F. luciae</i> N>S: <i>F. heteroclitus</i>	none	none
PFK	S>N: <i>F. diaphanus</i> <i>F. majalis</i>	none	none
ALD	S>N: <i>F. luciae</i> N>S: <i>F. heteroclitus</i>	none	none
TPI**	none	none	none
GAPDH	none	negative	negative
PGK**	none	none	none
PGM*	S>N: <i>F. diaphanus</i> <i>F. luciae</i>	none	none
ENO	S>N: <i>F. diaphanus</i>	none	none
PYK**	S>N: <i>F. majalis</i>	none	negative
LDH**	none	negative (p=.074)	negative

* significant phylogenetic effect, p<0.05

** significant phylogenetic effect, p<0.01

Phylogenetic analyses do not substantially change these results, except to increase the significance of the GAPDH and LDH regressions, and phylogenetic autocorrelation indicates that PYK also correlates negatively with temperature (Table 4). Seven enzymes, including LDH and PYK, have a significant phylogenetic component, indicating that 59-79% of the variance in these enzymes can be explained by variation in the genetic distance of the taxa studied. Thus, in this system, natural selection has increased GAPDH, LDH, and possibly PYK, activities in northern taxa, independently of phylogeny.

What are the implications of these results for the regulation of metabolism? Two of the three enzymes, LDH and GAPDH, are considered near-equilibrium enzymes that should not affect flux; yet they appear to have undergone natural selection. If they have undergone selection, then their variation must have functional consequences that affect the survival or reproduction of the fish. The simplest explanation is that they affect metabolic flux, thus supporting metabolic control theories that say any enzyme can be important. This finding highlights the importance of sampling more as many enzymes in a pathway as is feasible - choosing only one or a few enzymes may miss other enzymes that are important.

This argument is based on evolutionary data about enzymes, but recent physiological work by Podrabsky *et al.* (2000) supports these findings. They found that 87% of the variation in oxygen consumption in *F. heteroclitus* hearts could be explained by variation in just three enzymes: GAPDH, PYK and LDH. Thus laboratory physiological measurements agree with the interspecific findings of evolutionary analyses that take into account multiple evolutionary factors.

What has this combination of morphology, physiology, biochemistry and phylogenetics told us about the evolution of physiological and biochemical traits? There are several conclusions that come from this work:

- 1) A lot of variation in enzyme activity is genetically based and thus subject to the forces that lead to differentiation of species, including neutral drift.
- 2) Near-equilibrium enzymes can also be important in adaptation and the “marker enzyme” approach may miss important adaptive responses.
- 3) Where phylogenetic information is available, it allows for a more powerful and informative analysis.

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