

**EVOLUTIONARY PHYSIOLOGY OF CLOSELY RELATED TAXA:  
ANALYSES OF ENZYME EXPRESSION**

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*I have always wondered why comparative biologists choose their study organisms from a single population. Is it to avoid biological variation and thus make it easier to statistically distinguish between species? This variation, the variation within a species, is the stuff of evolution.*

James Hamrick, 1983 pers. comm.

#### **ABSTRACT**

Comparative biochemistry and physiology offer the advantage of specifically defining the functional parameters or traits that affect an organism's performance (e.g., amino acids that affect  $K_m$ , enzymes that affect metabolism). By combining these functional determinations with both intraspecific and phylogenetically appropriate analyses, comparative biologists should be able to indicate that a trait is biologically important, that it is evolving by natural selection. An evolutionary approach may benefit from the analysis of variation within and among closely related species. The advantages of analyzing closely related species are that they allow one to identify more definitively the derived conditions and suggest why differences arose. Importantly, there is substantial variation in physiological and biochemical traits within and among closely related species. For example, among species within a single genus of teleost, *Fundulus*, the variation in enzyme expression is similar to the variation seen among most superorders of teleost. However, most of the variation within the genus *Fundulus* is most readily explained by evolutionary distance, and thus there is no compelling reason for further adaptive hypotheses. Extending this observation, the greater the phylogenetic distance between taxa in a comparative study, the more likely there will be a statistically significant difference that may only represent evolutionary time. The molecular mechanisms affecting this variation in enzyme expression appear to be readily altered and may vary within a species or between acclimation conditions. Thus, studies among closely related organisms are more likely to identify the specific molecular or biochemical changes responsible for adaptive variation.

## INTRODUCTION

What constitutes a biochemical adaptation ( a derived biochemical trait that benefits an organism by increasing its longevity, reproductive fitness or probability of survival)? We present an argument that examining variation among closely related taxa (e.g., within species or congeners)<sup>1</sup> is more likely to provide answers to this question than comparing distantly related taxa. In order for this to be the case, sufficient variation must exist among closely related animals and there must be experimental and analytical techniques available that can distinguish between adaptive and nonadaptive variation. We present a brief overview of literature that demonstrates that within or among closely related species there is substantial variation in biochemical traits. This is followed by more detailed analyses of the variation in glycolytic enzyme concentration within and among species of the teleost fish *Fundulus*, how thermal acclimation affects this trait and the molecular mechanisms affecting the expression of one of these enzymes, lactate dehydrogenase (*Ldh-B*). These data suggest that evolutionary change occurs frequently and that many of these changes are not obviously adaptive. Thus, comparisons among distantly related species may include changes unrelated to the environmental adaptation that is being hypothesized.

The determination that a biochemical trait has evolved by natural selection is evidence that it is biologically important, because a trait can only be selected for if it effects phenotypic change that affects the fitness of an organism (Endler 1986). If a biochemical trait is evolving by natural selection, comparative biochemists can use this evidence to demonstrate to a broader scientific audience that these traits are likely to affect the health or performance of humans and the animals that we depend on. The problem, and one of the focuses of the symposium on Evolutionary Physiology (Society of Integrative and Comparative Biology, January 1998), is: what constitutes evidence for adaptive evolution and how should we study it?

Comparative biochemistry provides information concerning the variation in an enzyme or biochemical trait and deduces from this information the functional importance of this trait. A common

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<sup>1</sup> What constitutes “closely related taxa” should in fact be defined by the probability of random changes in the trait being examined. Practically, closely related taxa should be the phylogenetic closest relatives where measurable variation in a trait exist.

approach is to choose organisms from different environments and look for differences in a biochemical trait that should be affected by these environmental differences, or that effects a homeostatic change. Often these organisms are distantly related, belonging to different classes or phyla. If there is a compensatory change in the biochemical trait (e.g., more enzyme or greater enzyme activity in colder environments), this is taken as evidence that this variation is an adaptation -- evolved by natural selection.

For evolutionary biologists, the functional differences between two enzymes, variation in oxygen consumption or any other functionally defined trait is not evidence that these differences represent adaptations. Even if there is a good correlation between a biochemical trait and an environmental gradient (a compensatory change), there are evolutionary mechanisms other than natural selection that can create this correlation. The explicit goal is to reject the null hypothesis that neutral or random genetic variations are responsible for changes within or between species. This, to a large measure, encompasses two of the major differences between current evolutionary biology and comparative biology: (A) evolutionary biologists use neutral evolution as a null hypothesis when considering evolutionary changes and (B) comparative biologists often seek patterns of change among distantly related organisms, ignoring the variation within species and the neutral processes that introduce nonadaptive variation that may explain these patterns.

The advantage comparative biologists have is the ability to more explicitly test the neutral model by defining functionally important parameters, examining how they affect an organism's life and determining the variation in these parameters within and among species. Specifically, there should be an *a priori* prediction concerning the amounts or patterns of variations for traits whose functions are evolving by natural selection versus traits that are changing due to neutral processes. Thus, with functional information, comparative biologists can, in the context of recent theoretical frameworks and statistical methods, more specifically test whether patterns of change among taxa are most likely due to non-random evolutionary changes (e.g., see Graves and Somero 1982, Garland and Janis, 1993). The perspective provided here is that these types of evolutionary analyses are best suited for comparisons

within and among closely related species, and that they are likely to yield insight not available from analyses that neglect intraspecific variation.

One of the assumed problems with an intraspecific approach is the lack of measurable differences. Yet, there are several excellent examples of intraspecific variation. Tsuji (1988a; 1988b) provided data showing that acclimation affects on metabolic rates are different among populations of the lizard *Sceloporus occidentalis*. When acclimated to cold temperatures, colder northern populations of these lizards tended to reduce metabolism (inverse acclimation), while warmer southern populations have a compensatory increase in metabolism. Analyses of familial differences in lipid composition (Gibbs et al., 1991; Gibbs and Mousseau, 1994) clearly demonstrate that there are measurable differences within a species, and that this difference is heritable. Examining enzyme kinetic constants, Place and Powers (1979, 1984) have demonstrated that there are significant differences between populations for the Michaelis-Menten constant ( $K_M$ ), inhibition constant and catalytic rate constants for the two alleles of heart-type lactate dehydrogenase (LDH-B). Importantly, these allelic variations correlate with variation in swimming speed, developmental rate, metabolism and survival (DiMichele and Powers 1982a, 1982b; DiMichele et al., 1991a 1991b; Paynter et al., 1991). Similarly, Watt et al. (1986) demonstrated that kinetic differences among phosphoglucose isomerase (PGI) alleles in *Colias* butterflies affect flight capability and ultimately affect reproductive fitness. In sea anemones, variation in glucose metabolism is affected by allelic variation in PGI (Zamer and Hoffmann 1989). Variation in the *Pgi* genotype also affects carbon fixation in primroses (Kruckeberg et al. 1989). These studies demonstrate how the combination of functional analyses with population studies provides data on the importance of a biochemical trait. However, these data do not address how often these types of adaptive changes occur nor do they address how these traits arose. That is, one could argue that these types of biochemical changes are rare occurrences. Additionally, without knowledge of the ancestral state, we are left wondering if these changes are a recent adaptation (occurring within a species in response to new environmental challenges) or represent a more ancestral polymorphism.

Data on LDH-A (muscle-specific) kinetic variation in vertebrates suggest these changes are common. Graves and Somero's work on LDH-A (muscle specific lactate dehydrogenase) kinetic

variation included both closely related fish (Graves *et al.* 1983; Graves and Somero 1982) and a wide diversity of vertebrates (Hochachka and Somero 1984), providing information about the frequency of adaptive change. This work has demonstrated that vertebrates living in different thermal habitats have similar LDH-A  $K_m$ s when measured at the biologically relevant temperatures and pHs. Of these examples, the most definitive is the pattern of kinetic variation among closely related barracudas (Graves and Somero 1982). When measured at standard conditions, barracuda LDH-A have different  $K_m$ s that compensate for the differences in environmental temperature and thus when measured at the biologically appropriate temperature and pH, barracuda LDH-A  $K_m$ s are conserved. Similar results were found in studies on a wider diversity of vertebrates. These data on  $K_m$ s indicate that a difference of a few degrees in mean annual temperature is responsible for significant differences in LDH-A kinetic constants. It is difficult to explain the pattern variation of LDH-A  $K_m$ s among barracudas by random or neutral evolutionary processes, and thus this pattern is likely due to evolution by natural selection (Graves and Somero 1982). Importantly, variations in barracuda  $K_m$ s are due to few amino acid replacements and, in at least one case, a single amino acid change (Holland *et al.*, 1997). Interestingly, these amino acid substitutions are not near the active site or other regions directly involved in catalysis (Holland *et al.*, 1997). These data suggest that there may be many more sites (in comparison to the limited number of amino acids that are directly involved in catalysis) that can effect a kinetic change and thus, because so few changes are required, adaptive kinetic change could occur readily. Support for this hypothesis is provided by the data above and other data by Somero and colleagues (Hochachka and Somero 1984, Somero 1978, 1995) indicating that there is substantial variation in  $K_m$ s even among closely related taxa.

The variation in these enzymes and species illustrate two important points: (1) comparisons among closely related species, or within a species, can more definitively demonstrate that functional differences evolve by natural selection, and (2) because natural selection has resulted in  $K_m$  variation, the variation in  $K_m$ s must be biologically important even though the differences in  $K_m$  appear to be relatively small. We extend this analysis below to the topic of glycolytic enzyme expression:

evolutionary difference among *Fundulus* species, the evolution of acclimation responses as measured by changes in enzyme concentration and the molecular mechanisms affecting enzyme expression.

#### EVOLUTIONARY VARIATION IN ENZYME EXPRESSION

Variation in enzyme concentration (most frequently determined by measuring the maximal activity) is a parameter often measured by comparative biologists. Many studies examine one or a few enzymes as indices of a metabolic pathway among a diversity of species. Differences in enzyme concentration among species are often interpreted as important, but what is the null expectation? As pointed out by Felsenstein (1985) and amplified by Garland and colleagues (Garland and Adolph, 1994; Garland and Carter, 1994; Garland et al., 1992; Garland and Janis, 1993), there are two common oversights: (1) between two taxa, one should expect differences that are unrelated to a specific adaptive hypothesis and (2) comparisons among many taxa that ignore phylogenetic relationships are statistically biased toward finding significant differences .

Is it reasonable to expect that some variation in enzyme concentration will have no important phenotypic effect and thus differences should exist between taxa that are statistically significant but biologically unimportant? Pierce and Crawford (1997a) examined the concentration of all the glycolytic enzymes within and among species of *Fundulus* acclimated to one temperature. The analysis of all the glycolytic enzymes raised questions concerning the importance of statistically significant differences within and between species. For example, among a phylogenetically diverse group of teleosts (Fig. 1), there is 2.5 to 5.6 fold difference for hexokinase (HK), pyruvate kinase (PK) and LDH {Fig. 1B; (Driedzic and Gesser 1994)}. This appears to be a meaningful amount of variation among these evolutionarily distant species. However, measurement of these three enzyme concentrations in 14 taxa (8 species, with 2 populations from 6 species) of *Fundulus* reveals as much or more variation (Fig. 2, Pierce and Crawford 1997a). The exception to this pattern is seen in the fourth glycolytic enzyme, phosphofructokinase (PFK), which has 8 to 28 fold variation among superorders of teleost versus only 2.9 fold variation within *Fundulus*. Importantly, within *Fundulus*, most of the variation in 7 of the 11 glycolytic enzymes is explained by random change or phylogenetic

distance (Pierce and Crawford 1997a). The amount of variation that can be explained by phylogenetic distance exceeds 70% for some enzymes! Therefore, with greater evolutionary distances, there are greater differences between taxa and much of these differences are most parsimoniously explained by random changes among lineages. Thus, there is no overwhelming reason to believe that these changes are adaptive. To generalize, this means that if one compares any single enzyme between a pair of taxa, one is likely to find a significant difference in enzyme concentration; but this difference, even if correlated with environmental change, may not be an evolutionary adaptation. Extending this observation, the greater the phylogenetic distance between taxa the more likely there will be neutral changes and thus the more likely there will be a statistically significant difference in enzyme concentration that is not necessarily due to evolutionary adaptation.

We suggest that the pattern or amount of variation for a trait will be different if this trait is evolving by natural selection versus neutral processes. Phylogeny can affect this pattern and thus analytical methods that use phylogenetic relationships should be considered (Garland and Carter, 1994; Garland et al. , 1992, 1993). An example of this approach is provided by further analyses of glycolytic enzyme expression in *Fundulus* species (Pierce and Crawford 1997a). Among the glycolytic enzymes within and among *Fundulus* species, there are significant variations in three enzymes (glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and LDH) that cannot be explained by phylogenetic distance (Pierce and Crawford 1997a). These three enzymes have a pattern of variation in which species distributed along the steep thermal cline of the North American Atlantic coast have greater intraspecific variation than their sister taxa that exist in the Gulf of Mexico, where no clinal variation in temperature exists. Not only is there a difference in the amount of variation but there is a specific pattern, a pattern that most comparative biochemists would predict: more enzyme in colder environments. The concentrations of these three enzymes are greater in taxa subjected to colder environmental temperatures and this trend, more enzyme in colder environments, is significant among *Fundulus* species after accounting for the phylogenetic relationships (Pierce and Crawford 1997a). It is very unlikely that random or neutral evolutionary processes could create this pattern of variation

among all the species and populations tested. The most likely, and the most parsimonious, explanation for the variation in these three enzymes is that they have evolved by natural selection.

What phenotypic change could this variation in enzyme concentration affect? The most likely change that could be selected for is a change in metabolism. This is not predicted by the standard model of metabolic regulation because two of these enzymes are classified as equilibrium enzymes and thus are thought to be unable to effect a change in metabolic flux (Newsholme and Crabtree, 1973, 1986; Newsholme et al., 1979). Yet, these results, indicating an evolutionarily significant pattern of enzyme concentration, suggest that (1) variation in enzyme concentration is biologically important and (2) that the enzymes that are important in a metabolic pathway are not limited to a few non-equilibrium enzymes (e.g., PFK).

There is an additional message in this phylogenetic analysis: that significant variation in enzyme concentration arose independently in several taxa. Multiple independently derived states imply that changes in enzyme concentration are not rare, but are a common mechanism for adaptation.

Identifying the derived conditions is useful because they can indicate which biochemical parameters have changed and how much change has occurred. For example, in two *F. heteroclitus* populations the proximal promoter (a region of DNA that initiates mRNA transcription) of *Ldh-B* has substantial sequence variation (Fig. 3, Crawford *et al.* 1998). Some of these variable DNA regions are functionally important because they bind proteins and affect transcriptional processes (Crawford *et al.* 1998; Segal *et al.* 1996). Other regions are defined as non-functional because they have no measurable effect on transcription and are not known to bind transcription factors (Crawford *et al.* 1998; Segal *et al.* 1996).

In agreement with neutral evolutionary theory, the functional regions should have fewer nucleotide substitutions than non-functional regions because of greater constraints on protein:DNA binding sequences. With fewer substitutions, these functional regions should have fewer fixed differences and also fewer polymorphisms than non-functional regions (fixed difference: where one taxon has an invariant nucleotide at a position that is different from the nucleotide in the same position in another taxon; McDonald, 1998; McDonald and Kreitman, 1991). Thus, the ratio of shared

polymorphisms among taxa relative to fixed differences between taxa in these two regions should be similar if they are subject to the same evolutionary processes. One prediction under this neutral model, then, is that an analysis of sequence variation in both functional and non-functional regions would yield similar phylogenetic relationships. That is, even though the amount of polymorphism in these regions might be different (because, for example of greater constraint on protein:DNA binding sequences), both functional and non-functional sequences would yield similar phylogenetic relationships. However, separate analyses of the functional and nonfunctional regions of the *Ldh-B* proximal promoter yield very different relationships between *F. heteroclitus* populations and with its sister taxa *F. grandis* (Fig. 3, Crawford *et al.*, 1998). Analysis of the non-functional region suggests that there are few differences between the two *F. heteroclitus* populations and that these populations are distinctly different from *F. grandis*, in accordance with current phylogenies of these species (Wiley, 1986; Fig. 3B, Crawford *et al.*, 1998). If functional regions are used, the northern and southern populations no longer form a single clade and there is no phylogenetically significant difference between the southern *F. heteroclitus* and *F. grandis* (Fig. 3C, Crawford *et al.*, 1998). Among all the nucleotide polymorphisms (approx. 10% of sites are polymorphic), only eight are fixed differences between populations. All eight are found in the functional regions. All eight are unique to northern population and are not found in the southern population or in *F. grandis*. No other nucleotides have these uncommon features. Thus, northern populations have unique derived nucleotide sequences in functional regions, while the southern population has the same nucleotides as the sister species, *F. grandis*. In contrast, in the non-functional regions, there are no derived sequence in the northern that are not also found in southern populations (Crawford *et al.*, 1998). The hypothesis one could draw from this is that these eight nucleotides or a subset of these eight nucleotides are responsible for the two-fold difference in transcription rates between populations.

Importantly, the presence of all fixed difference only in functional regions with a considerable amount of sequence polymorphism is a non-random pattern of sequence variation (Crawford *et al.* 1998) and there are no reasonable neutral genetic models that would explain why unique sequences (all fixed differences) would only occur among the interspersed functional regions. For example, isolation

of a small population in the north should have enhanced the probability for fixed differences in both functional and non-functional regions, but this is not observed. Alternatively, natural selection could rapidly drive these eight characters to fixation, and thus explain the pattern of nucleotide changes.

Notice that these analyses are possible because molecular-biochemical studies were used to define functional regions and this allows one to focus on the variation within these regions. The determination of a derived state within the functionally important regions suggests that a few nucleotide substitutions can result in changes in gene expression and these changes can evolve readily within a species.

#### EVOLUTION OF ACCLIMATION

In addition to fixed genetic differences between taxa in enzyme expression, the amount of many enzymes varies when an organism is acclimated to a different thermal environment (Hazel and Prosser 1974). In general, enzyme catalysis halves for every ten degree 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 phenomenon, 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.

Although the effect of thermal acclimation on enzyme concentration has a long and well published history, little is known about its phylogenetic distribution and the variation between closely related taxa. Examining all the glycolytic enzymes in 5 species of *Fundulus* demonstrated that an acclimation response has arisen independently in several species (Pierce and Crawford 1997b). This was unexpected; we assumed that the mechanisms that are responsible for acclimation responses would

be complex {e.g., Sidell (1977)} and thus unlikely to be evolutionarily labile (readily changed within or among species). But acclimation (as measure by change in enzyme concentration) appears to be labile: among 5 species, (1) there were no equivalent acclimatory responses among all or most taxa; (2) acclimation responses were not shared among sister taxa, and (3) those taxa that did have an acclimation response, responded with different enzymes. The lack of a shared acclimation response among species, suggested that the responses were not present in their common ancestors and thus acclimation responses have evolved independently in different *Fundulus* species (Pierce and Crawford 1997b). This concept, that acclimation response is readily changed, is support by acclimation studies examining metabolic rates in different population of *Sceloporus occidentalis* (Tsuji 1988a; Tsuji 1988b).

Among the species examined there is one pattern: species distributed along a thermal cline of the North Atlantic Coast were more likely to have an acclimatory response than species distributed along the Gulf of Mexico. There are two explanations for this pattern: (1) evolutionary pressure for an acclimation effect is stronger among species exposed to large geographic variations in temperature or (2) this pattern represents random variation, that is, the acclimation response is not beneficial. The fact that an acclimation response only occurs in phylogenetically independent taxa that are distributed along a thermal cline is unusual but not totally unlikely. Thus, although we argue that the pattern of acclimation response is most readily explained by natural selection, with only 5 taxa in this analysis, there can be no firm evidence for this conclusion. It is important to note that acclimation responses (evolutionarily significant or not) among these taxa were (1) not limited to a few "rate-limiting" enzymes, (2) may reflect geographical variation in the thermal environment among populations as well as seasonal variation within a population and (3) may be affected by the evolutionary history of a species. Although a phylogenetic analysis did not yield easily interpretable results, it indicated that acclimation responses are evolutionarily labile.

Is it reasonable that an acclimation response can be evolutionarily labile? Are there molecular mechanisms that can readily change due to one or a few mutations and give rise to heritable variation in an acclimation response? Although we do not have direct evidence addressing this question, there are data for *Ldh-B* to suggest that yes, there are variations within a species in the molecular mechanisms

affecting an acclimation response. The original observation concerning the molecular mechanisms affecting *Ldh-B* acclimation responses in *F. heteroclitus* livers examined only one population {New Jersey; (Crawford and Powers 1989a)}. These data suggested that in livers, the expression of both *Ldh-B* protein and its mRNA were similarly increased at low acclimation temperatures. These data, in combination with other results (Crawford and Powers 1989b; Crawford and Powers 1992), suggested that the acclimation response was due to a change in *Ldh-B* transcription rate.

Studies on the *Ldh-B* acclimation response in northern and southern populations of *F. heteroclitus* indicated that the molecular mechanisms responding to acclimation temperature are different in the northern population (Fig. 4; Segal and Crawford, 1994). At both the low and high acclimation temperatures there were approximately two fold differences in *Ldh-B* enzyme concentrations between populations, and these populations had similar acclimation responses when enzyme concentration was measured. However, equivalent acclimation responses are not seen for *Ldh-B* mRNA. Similar to protein concentration, at 20°C there is an approximately two fold difference in mRNA concentration between populations. However, at 10°C there is no difference between populations in liver *Ldh-B* mRNA because (1) in the northern population, acclimation to 10°C reduces *Ldh-B* mRNA concentration and (2) in the southern population, there was no significant effect of acclimation on mRNA concentration. Thus, populations differ in the *Ldh-B* mRNA acclimation response. Importantly, the relationship between *Ldh-B* enzyme concentration and its mRNA is different at low acclimation temperatures for the northern population versus all other population-temperature combinations (Fig. 4). Specifically, at 20°C in all populations and in the southern population at 10°C there is a significant or nearly significant correlation between *Ldh-B* mRNA and protein concentration ( $r = 0.81$   $p < 0.05$  for 20°C;  $r = 0.57$   $p = 0.086$  for southern population at 10°C). This relationship between mRNA and protein at different acclimation temperatures is similar to the results for the population at an intermediate latitude {NJ, (Crawford and Powers 1989a)}. Unlike these data, in the northern population at low temperature there is little, if any, correlation between *Ldh-B* mRNA and protein concentration ( $r = 0.22$ ,  $p > 0.25$ ). Thus, at 20°C and possibly in the southern population at 10°C, the amount of *Ldh-B* enzyme is regulated by its mRNA expression. However, at

10°C in the northern population, the increase in *Ldh-B* enzyme concentration is independent of its mRNA expression. These data suggest that the molecular mechanisms regulating an acclimation response vary between populations. This supposition and the observation that a few nucleotide changes in the *Ldh-B* proximal promoter can affect gene expression (Crawford *et al.* 1998) suggest that changes in enzyme expression can occur readily.

### CONCLUSION

Our data, summarized here, indicate that (1) glycolytic enzyme expression changes readily among taxa (there are multiple independently derived states), (2) acclimation responses independently evolved in several taxa, (3) unique derived sequences within protein:DNA binding regions of the proximal promoter occurs within a species and these changes affect transcriptional processes and (4) the molecular mechanisms that effect an acclimation response may vary within a species. These results suggest that determination of an adapted state will be more problematic the greater the phylogenetic distance because with greater evolutionary distances between study organisms, there are more likely to be many changes, some that are adaptive, others that are not. A more rigorous analysis thus may require closely related species to be able to identify the derived state and define the evolutionary mechanism effecting change.

Integrating biochemical characterizations with evolutionary analyses provides for a more specific test of the biological importance of variation in biochemical traits. In applying this approach, it is important to determine how much of the variation between taxa is due to phylogenetic distance. This is most readily accomplished using techniques similar to those proposed by Garland and others (Cheverud and Dow 1985; Cheverud *et al.* 1985; Garland *et al.* 1993; Garland *et al.* 1992; Gittleman and Luh, 1994). The null hypothesis in such an analysis is that the variation in a biochemical trait is explained by phylogenetic relationship, and thus there is no need to evoke an adaptive hypothesis. Notice however, if speciation and adaptive change are concurrent, it may be difficult to distinguish between phylogenetic affects and adaptive change. In *Fundulus*, colonization of the colder Atlantic waters has occurred independently in several taxa (Pierce and Crawford, 1997a), and thus this potential problem is avoided; allowing one to investigate independently evolved derived states. These studies

exemplify how evolutionary analysis of physiological and biochemical traits not only provides one with information concerning the biological importance of these traits but also suggests how often a trait may evolve and how many molecular changes may be required.

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**Figure 1. VARIATION IN ENZYME EXPRESSION AMONG TELEOSTS.**

**A: PHYLOGENETIC RELATIONSHIPS AMONG TELEOSTS.** A simplified relationship among teleosts used in comparison of enzyme expression. Listed are superorders or infradivisions based on:

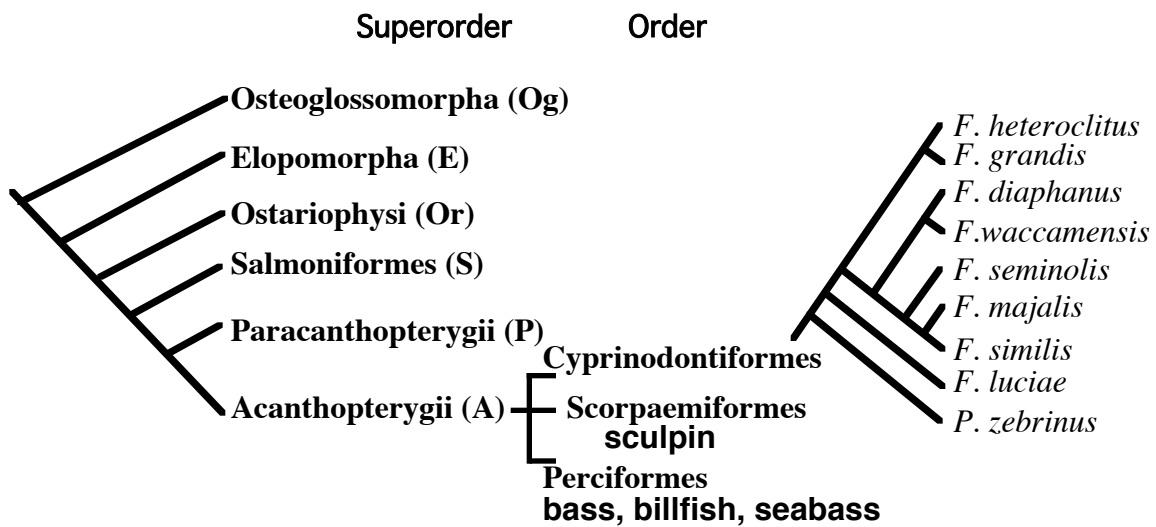
Maddison, D.R. and W. P. Maddison. 1996. The Tree of Life: A distributed internet project containing information about phylogeny and biodiversity. Internet address:

<http://phylogeny.arizona.edu/tree/phylogeny.html>, and on Gilbert (1992) and Nelson (1984).

Relationship among *Fundulus* are derived from Bernardi and Powers (1995), Cashner *et al.* (1992), Pierce and Crawford (1997a), and Wiley (1986).

**B: VARIATION IN FOUR GLYCOLYTIC ENZYMES AMONG TELEOSTS** Enzyme activities are from Driedzic and Gesser's review of cardiac function (Driedzic and Gesser 1994) and include cardiac enzyme activities from 22 teleost species among the superorders shown in 1A. Species with greatest and least amounts of enzyme are listed and the initials of superorders or infradivisions (see Fig. 1A) are included. Two ratios of the highest/lowest activities are given in the column labeled "Ratios". These ratios represent the highest or next highest levels of activity divided by the lowest level or next lowest level of activity (respectively). HK- hexokinase, PFK- phosphofructokinase, PK-pyruvate kinase and LDH-lactate dehydrogenase-B.

**A. Simplified Teleost Phylogeny**



**B. ENZYME ACTIVITIES AMONG TELEOST FISH  
RATIO OF THE TWO MOST DIFFERENT ACTIVITIES**

ENZYME	SPECIES & ORDERS USED IN COMPARISON	ENZYME ACTIVITY	RATIO
<b>HK</b>	<i>Esox niger</i>	S	4.8
	<i>Salmo salar</i>	S	27.0
	<i>Macrozoarces americanus</i>	A	7.0
	<i>Morone saxatilis</i>	A	23.9
<b>PFK</b>	<i>Esox niger</i>	S	1.5
	<i>Onchorhynchus mykiss</i>	S	41.9
	<i>Hemitripterus americanus</i>	A	3.7
	<i>Salmon salar</i>	S	30.8
<b>PK</b>	<i>Lophius piscatorius</i>	P	36.5
	<i>Morone americanus</i>	A	138.0
	<i>Morone saxatilis</i>	A	59.7
	<i>Gadus morhua</i>	P	117
<b>LDH</b>	<i>Lophius piscatorius</i>	P	224
	<i>Makaira nigricans</i>	A	708
	<i>Gaidropsaurs vulgaris</i>	P	264
	<i>Gadus morhua</i>	P	660



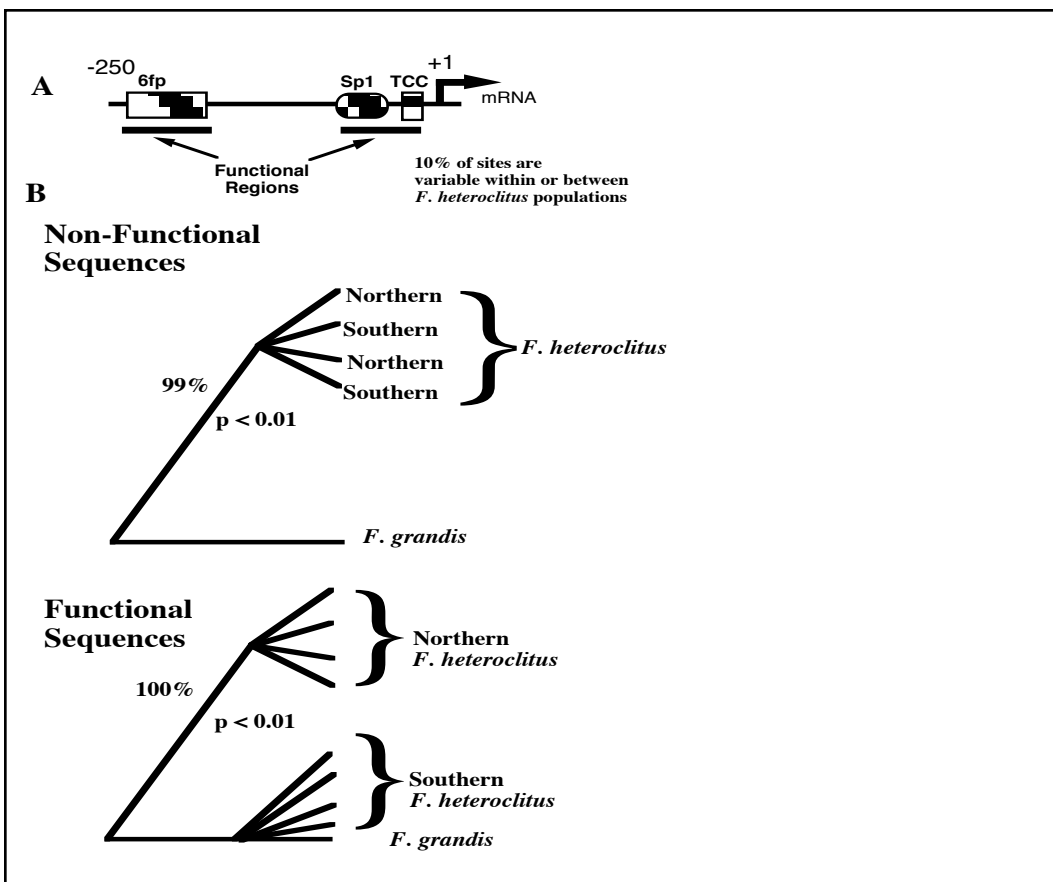
**Figure 2.** RANGE OF MAXIMAL GLYCOLYTIC ENZYME ACTIVITIES WITHIN *FUNDULUS*. Maximal enzyme activities for all ten glycolytic enzymes and LDH from heart ventricles within the genus *Fundulus* (Pierce and Crawford 1997a). The column "Fold Difference" represents the ratio of the highest maximal activities over the lowest among all *Fundulus* examined. The \* in the first column, "Enzyme", indicates which enzymes have a significant correlation between enzyme concentrations and environmental temperatures that is independent of phylogenetic distance among the taxa examined. Enzymes are in order of glycolytic catabolism.

**TABLE 1. RANGE OF MAXIMAL GLYCOLYTIC ENZYME ACTIVITIES WITHIN *FUNDULUS*.**

ENZYME	<i>Fundulus</i> species with largest difference		Mean Annual Temp.	Range of Max. Act.	Fold Difference
<b>HK</b>	<i>waccamensis</i>		17.6°C	0.615	<b>3.8</b>
	<i>heteroclitus</i>	N	8.5°C	2.345	
<b>PGI</b>	<i>waccamensis</i>		17.6°C	7.06	<b>6.4</b>
	<i>heteroclitus</i>	N	8.5°C	45.14	
<b>PFK</b>	<i>seminolis</i>		22.9°C	1.935	<b>2.9</b>
	<i>majalis</i>	S	17.6°C	5.615	
<b>ALD</b>	<i>similis</i>	W	22.9°C	1.777	<b>3.1</b>
	<i>heteroclitus</i>	N	8.5°C	5.450	
<b>TPI</b>	<i>diaphanus</i>	N	8.5°C	127.7	<b>3.9</b>
	<i>luciae</i>	S	12.3°C	492.5	
* <b>GAPDH</b>	<i>seminolis</i>		22.9°C	7.25	<b>4.5</b>
	<i>heteroclitus</i>	N	8.5°C	32.27	
<b>PGK</b>	<i>seminolis</i>		22.9°C	3.35	<b>8.4</b>
	<i>luciae</i>	S	20.4°C	28.23	
<b>PGM</b>	<i>seminolis</i>		22.9°C	11.78	<b>3.0</b>
	<i>luciae</i>	S	20.4°C	35.00	
<b>ENO</b>	<i>similis</i>	W	23.1°C	1.567	<b>1.7</b>
	<i>luciae</i>	S	20.4°C	2.711	
* <b>PYK</b>	<i>seminolis</i>		22.9°C	10.83	<b>3.4</b>
	<i>luciae</i>	N	12.3°C	36.93	
* <b>LDH</b>	<i>grandis</i>	E	22.9	38.37	<b>3.4</b>
	<i>luciae</i>	N	12.3°C	129.30	

Maximal enzyme activities for all ten glycolytic enzymes and LDH from heart ventricles within the genus *Fundulus* (Pierce and Crawford 1997a). The column "Fold Difference" represents the ratio of the highest maximal activities over the lowest among all *Fundulus* examined. The \* in the first column, "Enzyme", indicates which enzymes have a significant correlation between enzyme concentrations and environmental temperatures that is independent of phylogenetic distance among the taxa examined. Enzymes are in order of glycolytic catabolism.

**Figure 3.** PROXIMAL PROMOTER SEQUENCE VARIATION IN FUNCTIONAL AND NON-FUNCTIONAL REGIONS. (A) A diagram of the *Ldh-B* proximal promoter. Three functional regions (underlined): 6fp, Sp1 and TCC repeat. Functional regions are defined as those regions that bind transcription factors *in vivo* and affect transcription in cell culture (Crawford *et al.* 1998; Segal *et al.* 1996). (B) Two diagrammatic phylogenetic trees using non-functional and functional sequences based upon Crawford *et al.* (1998). Bootstrap values (% values above branch) for all branches with significant maximum likelihood values (p value below branches) are listed. These trees are illustrative of the major phylogenetic differences (branches with large bootstrap values and significant maximum likelihood values) between these two sequence types. Both trees have significant phylogenetic signals: g-test  $p < 0.01$  (Huelsenbeck and Hillis 1992); PTP test  $p < 0.01$  (Faith and Cranston 1991). The g-test measures the skewness of tree-lengths: tree lengths with a significant left skewness indicate that relatively few solutions exist. PTP tests are randomization tests for cladistic structure, comparing the observed tree length to the frequency of trees that have equal, or shorter lengths from a randomization of the original data. The original data is randomized (100 times) among taxa and the shortest tree for each randomization is determined. The relative frequency that a random tree is as short or shorter is the probability. A more detailed analysis and topology are provided in Crawford *et al.*, (1998).



**Figure 4.** VARIATION IN THE MOLECULAR MECHANISMS AFFECTING LIVER *LDH-B* ACCLIMATION RESPONSE. *Fundulus heteroclitus* individuals from northern (ME) and southern (GA) populations were acclimated to either 10° or 20°C, and both *Ldh-B* mRNA and enzyme levels were determined (Segal and Crawford 1994). The line drawn through both 20°C acclimated populations was fitted by eye to attract attention to the significant relationship between mRNA and enzyme concentration at this acclimation temperature. Notice there is no relationship between mRNA and enzyme concentration among northern fish acclimated to 10°C. *Ldh-B* enzyme levels were determined by measuring maximal activity which is highly correlated to enzyme concentration determined immunologically (Crawford and Powers 1989b). Enzyme levels are significantly correlated with body weight and are significantly different between populations and acclimation temperatures {ANCOVA  $p < 0.005$  with body weight as a covariate; (Segal and Crawford 1994)}. At both acclimation temperatures, the northern population has approximately 2 fold more *Ldh-B* protein than the southern population. For *Ldh-B* mRNA, population, acclimation temperature and the interaction terms are significant { $p < 0.001$  (Segal and Crawford 1994)}. Separate analyses of these factors indicate that at 20°C the mRNA concentration in the northern population is significantly different than mRNA concentration in the southern population ( $p < 0.001$ ) but there is no significant difference at 10°C. Acclimation had a significant affect on mRNA levels only in the northern population ( $p < 0.001$ ).

