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Intraspecific variation in aerobic metabolism and glycolytic enzyme expression in heart ventricles

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Podrabsky, Jason E., Chris Javillonar, Steven C. Hand, and Douglas L. Crawford. Intraspecific variation in aerobic metabolism and glycolytic enzyme expression in heart ventricles. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R2344–R2348, 2000.—A previous phylogenetic analysis among 15 taxa of the teleost fish *Fundulus* suggested that there should be thermal-adaptive differences in heart metabolism among populations. To test this hypothesis, the rate of oxygen consumption and the activities of all 11 glycolytic enzymes were measured in isolated heart ventricle from two populations of *Fundulus heteroclitus*. Heart ventricular metabolism is greater in a northern population versus a southern population of these fish. Analysis of the amount of glycolytic enzymes indicates that 87% of the variation in cardiac metabolism within and between populations is explained by the variation in three enzymes (pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase). These enzymes are the same three enzymes that were predicted to be important based on previously determined phylogenetic patterns of expression. Our data indicate that near-equilibrium enzymes, as well as classically defined rate-limiting enzymes, can also influence metabolism.

evolution; cardiac adaptation

ADAPTATION IS DEFINED as a derived trait evolved by natural selection (2, 8). Natural selection acting on intraspecific variation for physiological traits could be evolutionarily important for adaptation to different environments. However, there are few data on the evolutionary variation in physiological traits within a single species. Recent work examining evolutionary patterns of glycolytic enzyme expression in heart tissue of teleost fishes within the genus *Fundulus* sug-

gested that three enzymes [pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and lactate dehydrogenase (LDH)] are important for temperature adaptation and thus should affect myocardial metabolism (15). Here we provide data on the variation in aerobic respiration and glycolytic enzyme levels among populations of the teleost fish *F. heteroclitus* that support the original hypothesis that evolutionary variation in these enzymes affects quantitative changes in heart metabolism.

Cardiac metabolism in teleosts has been extensively studied (for review, see Ref. 5). Most of this work is concerned with either differences among species or the effect of physiological acclimation on cardiac function. Among 16 species of teleost fish, studies examining a select subset of enzymes suggest that glucose metabolism and fatty acid utilization both increase with increased energy demand (19). Among three species of Amazonian teleosts, the data suggest a greater reliance on glucose utilization versus teleosts from higher latitudes (3). These data agree with the tendency for lipids to be metabolically more important in species of fish from cold or polar environments (4, 18). Additionally, these data (summarized in Ref. 5) demonstrate a large interspecific difference in enzymes important for metabolism. However, these studies do not provide information on the variation within a species or among closely related species. Additionally, because only a few select glycolytic enzymes were investigated, we know little about the physiological and evolutionary importance of most enzymes in the glycolytic pathway. Metabolic control theory suggests that many enzymes may affect metabolism under the appropriate physiological condition (1). These theoretical findings are supported

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by several examples where near-equilibrium enzymes (classically considered to be "non-rate limiting") influence metabolic flux (1, 6, 11–13, 21). Thus determining the variation of all enzymes in a pathway is important if we are to understand how adaptations arise. To begin to understand the biochemical basis for adaptive differences, we investigated intraspecific variation in heart metabolism by examining a single metabolic pathway, glycolysis.

METHODS

Experimental organism. Specimens of *F. heteroclitus* were collected from two populations. The southern population was sampled at Sapelo Island Ferry Dock, GA, and the northern population was sampled at Wiscasset, ME. These fish were maintained in the laboratory for ~1.5 years. During this period, they were subjected to two pseudowinters [6°C, light-dark cycle (L:D), 6:18 h]. After subjection to pseudowinters, fish were maintained at 20°C, 15 parts per thousand seawater, with ~12:12-h L:D cycles. The second pseudowinter ended at least 8 wk before the respiration measurements.

Oxygen consumption measurements. Respirometry was used to determine metabolic rates of isolated heart ventricles. Fish were weighed and then killed by quickly severing the spinal cord. The ventricle, with the bulbus arteriosus attached, was carefully removed and placed in a glucose Ringer solution with 10 U/ml heparin where the ventricle continued to contract and expel blood. Oxygen consumption of individual ventricles was measured in Strathkelvin water-jacketed RC 300 respiration cells (25°C). The rate of spontaneous contraction, without any pre- or afterload, was determined immediately after placing the ventricles in the respiration cell and twice during the determination of oxygen consumption. Strathkelvin model 1302 oxygen electrodes were coupled to model 781 oxygen meters. The oxygen electrodes were calibrated before each experiment with air- and nitrogen-saturated solutions. Each ventricle was loosely attached to a nylon mesh using a single suture over the bulbus arteriosus and suspended above a glass-covered magnetic stir bar in the 1.5-ml respiration cell filled with glucose Ringer. The decline in partial pressure of O₂ (P_{O₂}) in the chambers was digitally recorded for 3–4 min with the Datacan V data-acquisition program (Sable Systems, Las Vegas, NV). After the measurement was completed, each ventricle was removed, covered with aluminum foil, quickly frozen by placing on a –80°C aluminum block, and stored at –80°C.

Respiration data were analyzed with DatGraf software (Oroboros, Innsbruck, Austria) following methods described by Mendez and Gnaiger (14). Oxygen concentration in the chamber (c_{O₂}, nmol O₂/ml) was calculated from P_{O₂} measurements based on O₂ solubility in the respiration medium (osmotic pressure = 282 mosM) at 25°C and ambient barometric pressure. Oxygen consumption (pmol O₂·s⁻¹·ml⁻¹) was calculated as the time derivative of c_{O₂}; typically, this value was determined from the interval between 30 and 90 s. Corrections were made for 1) consumption of oxygen by the electrode and back diffusion of oxygen into the chamber ($J_{O_2} = -1.27 + 0.0126 c_{O_2}$) and 2) the exponential time constant of the oxygen sensor (half-life = 3.2 s), as determined by injection of anoxic water into the chamber and analysis of the exponential approach to steady state. Respiration rates of isolated ventricles were stable during the recording period as indicated by analyses of the oxygen consumption rates for different intervals across the total recording period (2.5 min). The change in rate over time was

not significantly different from zero (i.e., regression of pmol O₂·s⁻¹·ml⁻¹ vs. time, $P = 0.26$).

Enzyme assays. Frozen ventricles were homogenized using an ultrasonic cell disrupter (Kontes, Vineland, NJ) in 100 mM HEPES buffer, pH 7.4, with 10 mM KCl, 0.5 mM dithiothreitol, and 0.2% Triton X-100. HEPES buffer was used because it is a nonphosphate buffer whose pH is relatively stable to the effects of dilution and has a smaller temperature sensitivity than many other buffers (10).

Maximal activities of all glycolytic enzymes: HK (E.C. 2.7.1.1), phosphoglucose isomerase (PGI) (E.C. 5.3.1.9), phosphofructokinase (PFK) (E.C. 2.7.1.11), aldolase (Ald) (E.C. 4.1.2.13), triosephosphate isomerase (E.C. 5.3.1.1), GAPDH (E.C. 1.2.1.12), (E.C. 2.7.2.3), phosphoglyceromutase (E.C. 2.7.5.3), enolase (Eno) (E.C. 4.2.1.11), PK (E.C. 2.7.1.40), and LDH (E.C. 1.1.1.27) were determined by assays linked to the oxidation or reduction of pyridine nucleotides. These rates were measured spectrophotometrically at 340 nm (16, 17). Initial activities (0.02–0.03 absorbance units/min) were recorded for 3 min at 25°C with a temperature-regulated 96-well spectrophotometer (Molecular Devices, Menlo Park, CA; see Ref. 16 and below). All eleven enzymes from two individuals (in triplicate, plus a negative control without substrate) were assayed simultaneously on one plate. 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. Concentrations of substrates were three to ten times the minimal amounts required for maximal activity, but they were kept below the levels promoting substrate inhibition. Thus substrate concentrations were at least one order of magnitude greater than the apparent Michaelis-Menten constant determined empirically from crude homogenates. Ammonium sulfate suspensions of linking enzymes were dialyzed overnight and diluted in 100 mM HEPES (pH 7.4) with 10 mM KCl and 50% glycerol. Reaction rates without ventricle homogenates (i.e., only substrates, cofactors, and linking enzymes) were investigated to determine if any spurious activities occurred. Chemicals (substrates, cofactors, etc.) were obtained from Sigma (St. Louis, MO; fructose-6-phosphate from certain other manufacturers is often contaminated with glucose-6-phosphate), and linking enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN) and Sigma. Reactions were initiated by addition of substrate using an Eppendorf octopipetter. Protein concentrations were determined using the Pierce BCA microassay kit (Pierce Biochemical). Activity units are micromoles of pyridine nucleotide catalyzed per minute per milligram of total protein.

Maximal activity is used solely as an index of enzyme concentration and not as an indicator of in vivo activity. Thus it is important only that all enzymes be measured at the same nondenaturing temperature. Maximal activities, although determined from crude homogenates, approach the apparent maximal initial velocity (V_{max}). V_{max} is measured from purified enzyme and is described as $V_{max} = [E] k_{cat}$, where [E] is the enzyme concentration and k_{cat} is the catalytic rate constant. The k_{cat} can vary due to changes in enzyme primary structure or, for a few enzymes, changes in covalent modification. If there is no variation in k_{cat} , then differences in maximal activity are due to changes in enzyme concentration. In *F. heteroclitus*, it is unlikely that variation in k_{cat} is responsible for large differences in maximal activity variation (17).

Statistical analyses. The two populations of *F. heteroclitus* had different mean body masses (Table 1; range was 4.97–

Table 1. Heart ventricle aerobic respiration and enzyme determinations from populations of *F. heteroclitus*

	North	South	<i>P</i>
<i>Physiological properties</i>			
Respiration per heart, pmol O ₂ ·s ⁻¹	28.79 (2.01)	35.76 (3.89)	
Body mass, g	7.29 (0.620)	16.20 (2.07)	†
Respiration/body mass, pmol O ₂ ·s ⁻¹ ·g ⁻¹	4.09 (0.320)	2.31 (0.229)	‡
Spontaneous contractions, beats/min	34.4 (3.46)	22.1 (5.82)	
<i>n</i>	10	8	
<i>Enzyme levels, μmol·min⁻¹·mg protein⁻¹</i>			
Hexokinase	1.756 (0.178)	2.011 (0.243)	
Phosphoglucose isomerase	59.67 (6.14)	49.12 (2.79)	
Phosphofructokinase	2.681 (0.393)	2.025 (0.332)	*
Aldolase	5.362 (0.708)	3.913 (0.583)	†
Triose phosphate isomerase	482.6 (45.5)	537.3 (88.3)	
GAPDH	51.53 (7.08)	45.30 (7.03)	
Phosphoglycerate kinase	24.26 (3.11)	17.72 (1.36)	
Phosphoglyceromutase	21.66 (1.50)	17.64 (2.85)	
Enolase	2.670 (0.178)	3.055 (0.195)	*
Pyruvate kinase	32.58 (2.28)	27.43 (4.63)	
Lactate dehydrogenase	93.54 (8.38)	107.0 (18.03)	*
<i>n</i>	7	5	

Values are means with SE in parentheses. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Significance from ANCOVA, **P* < 0.05, †*P* < 0.01, ‡*P* < 0.001.

10.20 g for fish from Maine and 8.89 to 27.08 g for fish from Georgia). Thus all statistical analyses used analysis of covariance (ANCOVA) with log body mass as the covariate. Regressions for both populations were inspected graphically, and the differences in regression coefficient were tested vis-à-vis interaction terms in ANCOVA. If regression coefficients were not different, the interaction term was not included in the analysis. Statistical analyses were performed using both Minitab (10.0) and Statview (5.0).

RESULTS AND DISCUSSION

Isolated heart ventricles from teleosts are a robust system for the analysis of physiological function. Heart ventricles continue to contract at fairly constant rates during the experimental period, and the change in the rate of oxygen consumption was not significantly different from zero (regression of rates vs. time, *P* = 0.26). Both these results suggest that the isolated heart ventricles are maintaining constant metabolism during the experimental procedures.

Table 1 lists means and SEs for the measurements made on individual heart ventricles. The levels of sta-

tistical significance are based on ANCOVA using log body mass as a covariate. Thus, for example, respiration rates are reported as picomoles of O₂ consumed per second per gram body mass, but the statistical significance is based on an ANCOVA of log (picomoles per second) with log body mass as the covariate.

Oxygen consumption in heart ventricles from northern populations was significantly greater than that from southern populations when corrected for body mass (ANCOVA, *P* < 0.01; Fig. 1). These differences exist after both populations were subjected to the same environmental conditions for >1.5 years. Thus these differences are not due to physiological acclimation and most likely represent evolved differences between populations. This variation in cardiac metabolism could compensate for the ~12°C colder temperature that individuals in the northern populations experience relative to their southern counterparts. These data add to a growing body of information that indicates thermal adaptive differences exist between populations of *F. heteroclitus*. Yet, based solely on the data from two populations, it is difficult to claim that the observed difference in metabolism is a derived condition that has evolved by natural selection, and thus it is difficult to claim that the difference represents an adaptation. That is, differences between two taxa may not be derived or may only represent random chance (9) and thus are not adaptive. Investigations of other closely related taxa are required to resolve this issue (2, 9). However, the difference in metabolism reported here is within a species and thus does not suffer from all the criticisms concerning two-species comparisons (9). Ad-

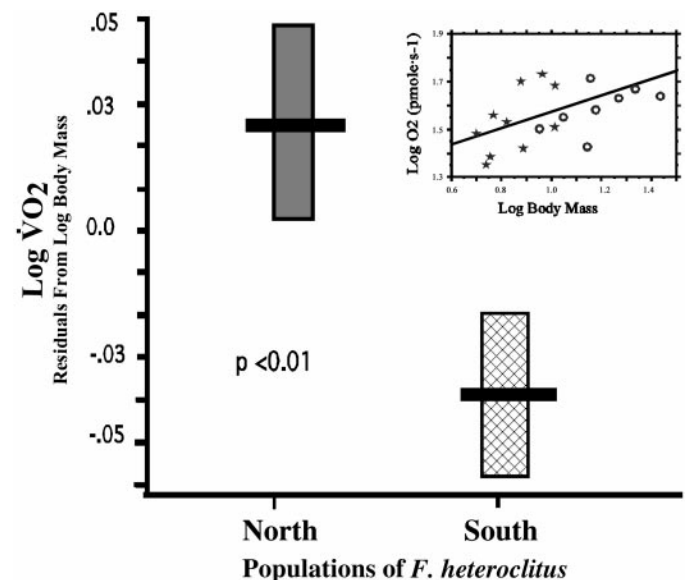


Fig. 1. Intraspecific variation in heart metabolism. Metabolism measured as oxygen consumption ($\dot{V}O_2$) in a glucose-only medium was determined in 2 populations of *Fundulus heteroclitus* acclimated to a common temperature. Solid horizontal bars represent the means, and shaded boxes depict \pm SE. Probability for rejecting the null hypothesis (north = south) is from analysis of covariance (ANCOVA) of log $\dot{V}O_2$ versus log body mass. Inset is the plot of the relationship between $\dot{V}O_2$ and body mass. ★, Individuals from the northern population; ○, southern individuals.

ditionally, our application of a two-tail statistical test was conservative because there was an a priori prediction that fish from colder thermal environments would have a greater metabolic rate when compared at the same temperature. This prediction is based on a general understanding of evolutionary physiology and more specifically on phylogenetic analyses of 15 *Fundulus* taxa (15). Finally, further analysis (presented below) of the covariation of enzyme levels with ventricular oxygen consumption strongly suggests that the difference between populations is due to the variation in expression of three independent loci. When these factors are taken into consideration, the data presented here support the hypothesis that the differences in heart metabolism between northern and southern populations of *F. heteroclitus* are not random and are likely to represent an adaptation.

Heart metabolism was measured in a glucose-only medium. To determine if variation in the level of glycolytic enzymes is responsible for the differences between populations, the maximal activity of all 10 glycolytic enzymes and LDH was measured. These determinations are used as an index of enzyme concentration (2, 15–17). They are not meant to represent in vivo activity, which is a function of substrate, product, and allosteric modifier concentrations. Four enzymes (Ald, Eno, PFK, and LDH) were significantly different between populations (ANCOVA, Table 1). Surprisingly, except for LDH, these are not the enzymes that explain most of the variation in oxygen consumption. To determine which enzymes covary with ventricular oxygen consumption, the effect of body mass has to be removed. Thus the variation in oxygen consumption not explained by body mass was used for the regression analysis, or, more specifically, the residuals of log oxygen consumption versus log body mass were used. A stepwise linear regression of all enzymes indicates that PK, GAPDH, and LDH explain 87% of the variation in oxygen consumption among isolated heart ventricles (Fig. 2). No other enzyme was a significant factor. This relationship is depicted in Fig. 2, in which the rate of oxygen consumption is plotted against the predicted value from the three-enzyme regression equation.

The data presented here suggest that the difference between populations for heart ventricular metabolism is due to variations in the amount of three enzymes (PK, GAPDH, and LDH). These are the same three enzymes identified by evolutionary analyses as being important for temperature adaptation (15). Specifically, populations among all 15 taxa of *Fundulus* have significantly more of these three enzymes in colder environments after accounting for the phylogenetic relationships. These phylogenetic analyses show that significant variation in enzyme concentration arose independently in several taxa, indicating that variation in enzyme expression is a derived state and is a common adaptive mechanism. It is improbable that random or neutral evolutionary processes are responsible for the pattern of variation among all the species and populations tested. Thus the expression of these enzymes is most likely the product of natural selection

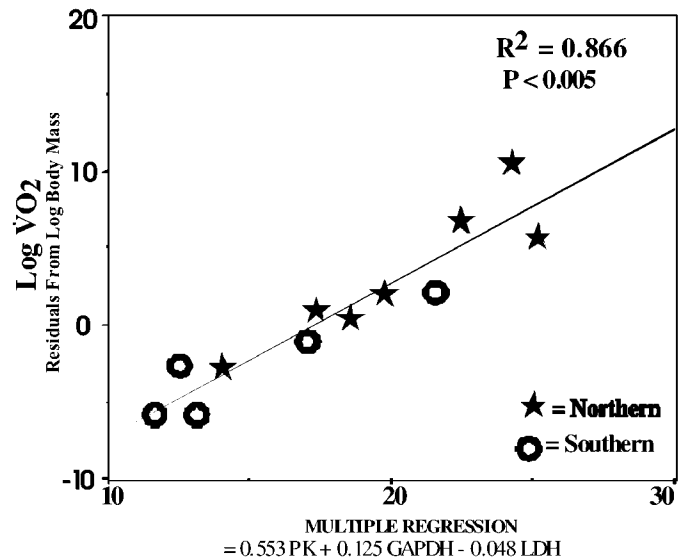


Fig. 2. Relationship between heart metabolism and enzyme activity. Residuals of log $\dot{V}O_2$ versus log body mass are plotted against the predicted value based on the result of stepwise multiple regression using the amount of 3 enzymes [pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and lactate dehydrogenase (LDH)] in each individual. Although all 11 enzymes were used for the stepwise regression, only these 3 enzymes had a significant covariation with heart metabolism. These 3 enzymes are not significantly affected by body mass among organisms used in this study.

and the variation in expression is biologically important; i.e., this variation produces phenotypic changes that affect the longevity, reproductive fitness, or probability of survival. Together, the phylogenetic studies and the data presented here suggest that changes in both near-equilibrium and far from equilibrium enzymes have evolved to produce an adaptive change in cardiac metabolism. The hypothesis that metabolism is affected by near-equilibrium enzymes is supported by other studies. For example, the control of glucose metabolism in rat hearts is not exerted by a single enzyme but variably distributed among several enzymes (including near-equilibrium enzymes), depending on substrate availability, hormonal stimulation, or other changes of conditions (12). Similarly, variation in the activity of the near-equilibrium enzyme PGI affects flight performance in butterflies (20), modulates glucose metabolism in the sea anemone (21), and affects carbon flux in the evening primrose (13). Additionally, yeast selected for growth on a limited glucose medium alters the expression of many different glycolytic enzymes, not just a few rate-limiting enzymes (7). These data suggest that analyses of a few selective enzymes may miss important enzyme differences that could influence our understanding of biochemical and physiological adaptation.

Perspectives

Our data suggest that there is substantial variation among individuals in both physiological and biochemical traits. In an era when complete genomes are being characterized from a cell line, single strain, or few

individuals, it is appropriate to note that such data do not include the variation among individuals, and this variation may be important biologically.

The biologically important variation identified here is quantitative. It is the level of gene expression that is consequential, not an identifiable variation in protein primary structure. Implicit in this statement is that differences in the amount of a protein are likely to be important for health and disease. Some of this variation is subtle (a 2-fold or less difference). Methods to measure these differences among both classically rate-limiting proteins and those proteins thought to be non-rate limiting need to be employed if we are to understand how developmental, physiological, and molecular variations influence health and adaptation among humans or the organisms we depend on.

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