and tree B, for each cluster of A, the value of the distortion coefficient between A and C is added to the value of the distortion coefficient between A and B. This mathematical procedure removes shifts among chemical groups that are also shifts among plant clades. With this modification the coefficient remained statistically significant for the comparison between *Blepharida*'s phylogeny and the chemogram (0.84, $P < 0.05$). However, for the comparison of *Blepharida* and *Bursera* phylogenies, the modified distortion coefficient increased to 0.94 ($P = 0.25$). This suggests that the relationship between the two phylogenies is due in large part to the correlation between plant phylogeny and plant chemistry, whereas the relationship between *Blepharida*'s phylogeny and the chemogram of *Bursera* does not depend on the correlation between plant phylogeny and plant chemistry. Thus, comparisons ignoring the correlation between plant phylogeny and plant chemical variation, as well as comparisons controlling for this correlation, indicated a greater influence of host plant chemistry than host plant phylogeny in the evolution of host use in *Blepharida* and *Bursera*.

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14. The internal transcribed spacers (ITS1 and ITS2) and the 5.8*S* cistron of nuclear ribosomal DNA were sequenced for 57 *Bursera* species and five outgroups following [B. G. Baldwin, *Mol. Phyl. Evol.* **1**, 3 (1992)]. The outgroups were three species of *Commiphora*, one species of *Boswellia* (Burseraceae), and one Anacardiaceae species of the genus *Spondias*. The sequences were aligned with the University of Wisconsin Genetics Computer Group software package (GCG) and Sequencher [Gene Codes Corporation Incorporated, *Sequencher 3.0 User Manual* (Ann Arbor, MI, 1995)], producing a matrix of 831 characters, of which 53.6% were potentially informative phylogenetically. Phylogenies were inferred by using parsimony analysis with PAUP 3.1.1 [D. L. Swofford, *Phylogenetic Analysis Using Parsimony (PAUP) Version 3.1.1* (Smithsonian Institution, Washington, DC, 1993)] [100 heuristic searches, random addition, Tree Bisection Reconnection (TBR) branch swapping]. A bootstrap analysis (500 bootstrap searches, 40 random additions, TBR branch swapping) was performed to estimate the relative internal support for different elements of the trees. The phylogeny of Fig. 1 includes only species on which *Blepharida* was found.
15. The ITS2 region was sequenced for one individual of each *Blepharida* species found feeding on each *Bursera* species in the field and one species of its sister genus *Podontia*. Alignment resulted in a matrix of 662 characters, of which 41.7% were potentially informative. Alignment and analyses of sequences followed the same strategy as with *Bursera*.
16. Leaves of 38 *Bursera* species were collected in the field at the same time that *Blepharida* beetles were collected, and their chemical constituents were immediately extracted in ethyl acetate. Extracts were analyzed by gas chromatography, which distinguished between 10 and 15 main compounds in each species. A matrix of Euclidean distances between these species was constructed on the basis of the presence or absence of each compound. The robustness of the clusters produced was determined by looking at the consensus of three clustering techniques {Complete linkage, UPGMA, and Ward's method [P. H. Sneath and R. R. Sokal, *Numerical Taxonomy* (Freeman, San Francisco, CA, 1973); SAS Institute Incorporated, *SAS/STAT User's Guide, Version 6* (Cary, NC, ed. 4, 1989), vol. 4]}. Two of these methods agreed in dividing species into four main clusters, whereas the other (complete linkage) divided them into five by splitting cluster 4. Hewlett-Packard 5890 gas chromatograph with flame ionization detector and a 15-m column of 0.32-mm internal diameter fused silica capillary column (J & W Scientific) coated with 0.25-μm DB-5 were used for chemical analyses. Nitrogen served as the carrier gas with a linear velocity of 20.8 cm/s at a pressure of 20 kPa. Injections were made in the splitless mode with the injector at 200°C and the detector at 220°C. The oven temperature was programmed at 60°C for 1 min, then an increase of 10°C/min to 220°C, holding at 220°C for 3 min.
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Phylogenetic Analysis of Glycolytic Enzyme Expression

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Although differences among species in enzyme maximal activity or concentration are often interpreted as adaptive and important for regulating metabolism, these differences may simply reflect phylogenetic divergence. Phylogenetic analysis of the expression of the glycolytic enzymes among 15 taxa of a North American fish genus (*Fundulus*) indicated that most variation in enzyme concentration is due to evolutionary distance and may be nonadaptive. However, three enzymes' maximal activities covary with environmental temperature and have adaptive value. Additionally, two pairs of enzymes covary, indicating coevolution. Thus, metabolic flux may be modulated by many different enzymes rather than by a single rate-limiting enzyme.

Phylogenetic analyses can test for the adaptive importance of enzyme variation and address the debate concerning the control of metabolism. Many models concerning metabolic regulation have been proposed: from classical biochemical theories that predict one master regulatory enzyme per pathway (*1*), to metabolic control theories that argue that many enzymes can modulate flux (*2*, *3*). Experimental evidence suggests that the control of flux shifts among enzymes depending on laboratory conditions (*4*, *5*). In contrast, a phylogenetic perspective can reveal changes in enzyme amounts or activity produced by natural selection and thus are indicative of an enzyme's importance over evolutionary time. If variation in an enzyme's concentration is selectively important, then that variation must have functional consequences, such as changes in metabolic flux. Thus, phylogenetic analyses that identify patterns of adaptive variation in particular glycolytic enzymes suggest that variations in these enzymes are functionally important. Results from phylogenetic analyses can be compared to the predictions of different theories on metabolic control. Specifically, if there are a few master regulatory enzymes per pathway, and other equilibrium (*6*) enzymes

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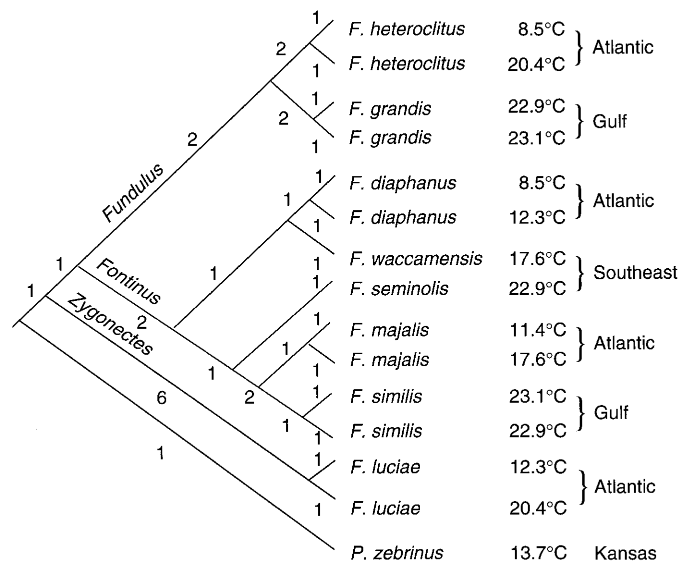

Fig. 1. Phylogenetic relationships and locations of *Fundulus* species used in this study. Coastal species in the subgenera *Fundulus*, *Fontinus*, and *Zygonectes* were examined (*9*). Mean annual temperatures for each taxa are listed next to species name. Atlantic species are subjected to a steep thermal cline of 1°C per degree latitude (*24*). Gulf coast species are exposed to little geographic variation in temperature. Subgeneric names are indicated along the basal branch for each subgenus. Internode distances, used for the phylogenetic analyses, are calculated as the number of known speciation events, including those involving species not examined in this study. Although these nodal distances assume a punctuational evolutionary model, the statistical consequences are not expected to be serious (*25*). The phylogeny shown is based on molecular, morphological, and allozyme data (*7–9*). Bernardi and Powers (*7*) resolved relationships among the three subgenera *Fundulus*, *Zygonectes*, and *Xenisma*. Because Wiley (*9*) united *Fontinus* with a fourth subgenus, *Xenisma*, we infer that *Zygonectes* is the sister clade to the *Fundulus*–(*Fontinus-Xenisma*) grouping. The species in two of these subgenera, *Fontinus* and *Fundulus*, and one species in *Zygonectes* are distributed along the Atlantic and Gulf of Mexico coasts. The species in the other two *Fundulus* subgenera, *Xenisma* and *Zygonectes*, are distributed primarily along the Gulf of Mexico or throughout the southeastern United States (*8*, *9*). For species that had a wide geographic distribution, one population near each end of the distribution was included. If only two populations with little or no gene flow between them are used, they can be treated as distinct taxa in phylogenetic analyses (*16*). Because these fish occur in small streams and coastal marshes and have low migration rates (*26*), geographically distant populations are treated as separate taxa. *Plancterus zebrinus* from Kansas was included as an outgroup for the genus *Fundulus* (*7*, *27*), but only represents an additional contrast in these analyses. Mean annual temperatures of shallow coastal waters are used as the environmental temperature (*24*). For two inland species (*F. waccamensis*, *F. seminolis*), temperature was assumed to be equivalent to the nearest coastal measurement. For *P. zebrinus*, which occurs in shallow streams, mean annual air temperatures were used (*28*).

are in vast excess, then variation in equilibrium enzymes should be unrelated to environmental temperature, and variation in regulatory enzymes should be negatively correlated with environmental temperature to compensate for reduced catalysis at lower temperatures. If metabolic control theories are more accurate, then both equilibrium and regulatory enzymes can be subjected to selection because both affect flux, and both types of enzymes may be significantly and negatively correlated with environmental temperature.

Phylogenetic analyses were used to test the influence of genetic distance and temperature adaptation on enzyme concentration among 15 closely related taxa in the North American teleost fish genus *Fundulus* (Fig. 1). The phylogeny of *Fundulus* is known (*7–9*), and different species and populations experience different environmental temperatures. This temperature variation is a potentially important selective factor because temperature has a large effect on enzyme function (*10*). Additionally, this study examines coevolution among five pairs of enzymes that are thought to interact in vivo (*11*).

Glycolysis in teleost ventricles proceeds primarily in the forward direction with little shunting to the pentose pathway (*12*). Concentrations of all 10 glycolytic enzymes (*13*) and lactate dehydrogenase (LDH) were determined by measuring maximal initial activity in heart ventricles (*12*). Substantial variation in maximal activity among these species is likely due to a change in enzyme concentration (*12*). Variations in putative glycolytic enzyme concentrations were analyzed with the use of phylogenetic autocorrelation methods (*14*) and standardized independent contrasts (*15–17*). These methods were originally developed for morphological characters but are applicable to any quantitative trait.

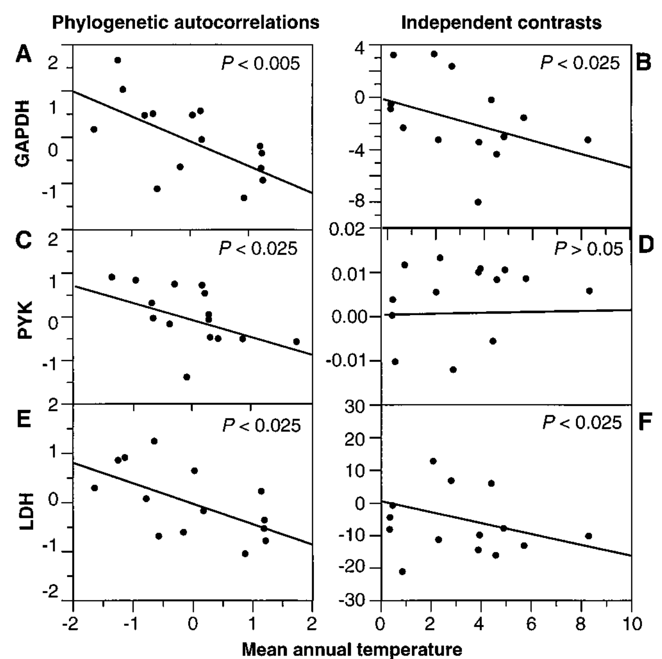

Fig. 2. Phylogenetically independent relationships between the concentrations of three enzymes and mean annual temperature. No other enzymes correlated significantly with environmental temperature. Autocorrelation residuals represent taxon-specific variation not explained by phylogenetic distance. Independent contrasts represent rates of evolutionary change (*17*). Body mass and all other enzymes did not correlate significantly with temperature by either phylogenetic method. All regressions and correlations with contrasts were through the origin because the direction of subtraction for the contrasts is arbitrary (*16*). One-tailed probabilities were calculated with standard probability tables. (**A**) Correlation between GAPDH and temperature autocorrelation residuals is significant ($r = -0.624$, $P < 0.005$). (**B**) Correlation between GAPDH and temperature-standardized independent contrasts is significant ($r = -0.576$, $P < 0.025$). (**C**) Correlation between ln PYK and temperature autocorrelation residuals is significant ($r = -0.565$, $P < 0.025$). (**D**) Correlation between reciprocally transformed PYK and temperature-standardized independent contrasts is not significant ($r = 0.408$, $P > 0.05$). (**E**) Correlation between LDH and temperature autocorrelation residuals is significant ($r = -0.601$, $P < 0.025$). (**F**) Correlation between LDH and temperature-standardized independent contrasts is significant ($r = -0.541$, $P < 0.025$).

Seven enzymes—HK, PGI, TPI, PGK, PGM, PYK, and LDH—had significant positive autocorrelation coefficients, indicating that more closely related taxa tend to have more similar enzyme concentrations (Table 1). The percentage of variation ex-

plained by the phylogenetic component ranged from 43% for PGM to 74% for TPI. No adaptive hypothesis is required to explain this variation among species. Thus, these results indicate that conventional comparisons among taxa will yield differences that are due primarily to evolutionary history, and any adaptive explanation should be considered cautiously.

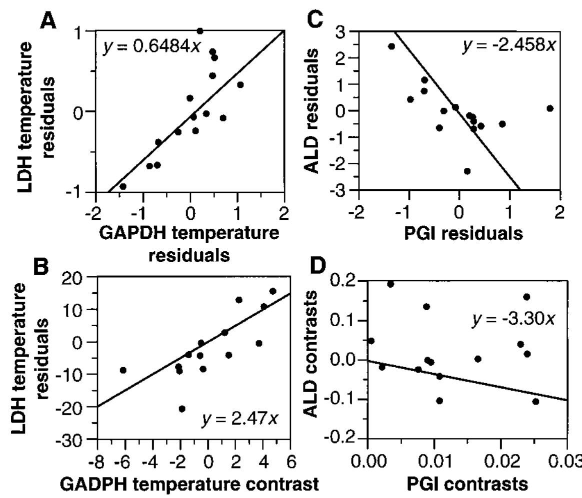

Fig. 3. Coevolution of phylogenetically independent enzyme concentrations: GAPDH-LDH and PGI-ALD. None of the other three enzyme pairs tested showed significant correlation. Analyses were performed on residuals of LDH and GAPDH regressed on mean annual temperature. ALD was log-transformed and PGI was reciprocally transformed before analysis. Because PGI was reciprocally transformed, the negative correlation indicates positive coevolution of PGI and ALD. Least-squares regression slopes are shown on each plot. (**A**) Correlation between GAPDH and LDH autocorrelation residuals is significant ($r = 0.775$, $P < 0.01$). (**B**) Correlation between GAPDH and LDH standardized independent contrasts is significant ($r = 0.576$, $P < 0.05$). (**C**) Correlation between reciprocally transformed PGI and ln ALD autocorrelation residuals is significant ($r = -0.541$, $P < 0.05$). (**D**) Correlation between reciprocally transformed PGI and ln ALD standardized independent contrasts is significant ($r = -0.552$, $P < 0.05$).

Table 1. Autocorrelation coefficients, ρ, for body mass and enzyme concentration (*$P < 0.05$, **$P < 0.001$) (*14*). Enzymes that have significant ρ values are in bold. True r^2 values indicate the percentage variance in a trait explained by phylogenetic distances. Enzyme concentrations were determined in cardiac tissue from fish acclimated at their preferred salinity in the laboratory to 20°C for 3 to 6 weeks before experimentation. Fish were fed ad libidum once daily in the late afternoon. During the laboratory acclimation period, all species went through a brief reproductive period. Eight to 10 individuals were assayed from each species, except for *F. seminolis* and *P. zebrinus*. Three and four individuals were assayed from these species, respectively. Heart ventricles were weighed and homogenized with an ultrasonic cell disrupter in a 100 mM Hepes buffer (pH 7.4), 10 mM KCl, 0.5 mM dithiothreitol, and 0.2% Triton X-100. Maximal activities of all glycolytic enzymes were determined by assays linked to the oxidation or reduction of pyridine nucleotides. Rates were measured spectrophotometrically at 340 nm for 3 min at 25°C. Assays were similar to those published previously for *F. heteroclitus* (*12*) with modifications of substrate concentrations to ensure saturating conditions for each species. Chemicals were from Sigma, and linking enzymes were from Boehringer Mannheim and Sigma. Protein concentrations were determined with the Pierce BCA microassay kit. Mean maximal activities of all enzymes except four were normally distributed among taxa. Log-transformed means of ALD and PGM and reciprocally transformed means of PGI were normally distributed. Log-transformed PVK means were used for autocorrelation analysis and reciprocally transformed means were used to generate standardized independent contrasts (to minimize the covariance between the contrast and its variance) (*16*). Phylogenetic autocorrelation was performed with the program MRHO3 (*14*). Independent contrasts were calculated with the phenotypic diversity analysis program PDAP (*23*). Standardized contrasts and their standard deviations were tested for significant correlations to verify the independence of the contrasts (*16*). All resulting autocorrelation residuals were normally distributed, and all resulting contrasts were adequately randomized with respect to their standard deviations. No enzyme scaled interspecifically with body mass ($P > 0.205$ in all instances).

Traits		ρ	r_2
Body mass		0.62*	0.47
Hexokinase	(HK)	0.68**	0.56
Phosphoglucoisomerase	(PGI)	0.59*	0.44
Phosphofructokinase	(PFK)	0.07	0.01
Aldolase	(ALD)	0.01	0.01
Triose-phosphate isomerase	(TPI)	0.79**	0.74
Glyceraldehyde-3-P DH	(GAPDH)	0.46	0.32
Phosphoglycerate kinase	(PGK)	0.67**	0.55
Phosphoglyceromutase	(PGM)	0.59*	0.43
Enolase	(ENO)	0.41	0.21
Pyruvate kinase	(PYK)	0.69**	0.58
Lactate dehydrogenase	(LDH)	0.67**	0.55
Mean annual temperature		0.24	0.07

After using phylogenetic methods to remove the effect of phylogenetic history on enzyme concentration, we tested the relation between enzyme concentration and temperature for each of the 11 enzymes. Although we realize that these tests may not be completely independent, all comparisons were planned before analyses, and thus Bonferroni correction would be extreme. The concentrations of two enzymes, GAPDH and LDH, correlated negatively and significantly with environmental temperature as determined by either phylogenetic method (Fig. 2). The autocorrelation residuals of PYK correlated with temperature, but its independent contrasts did not (Fig. 2). Because independent contrasts measure rates of evolutionary change, PYK changes as temperature changes, but its rate of change is unrelated to the rate of temperature change (*17*).

As mean annual temperature decreases, the concentrations of cardiac LDH, GAPDH, and PYK increase and should compensate for the reduced activity of each enzyme at colder temperatures. The significant correlations between temperature and enzyme concentrations suggest that (i) temperature is an important selective factor influencing these enzyme concentrations; (ii) these enzymes affect metabolic flux; and (iii) the interspecific variation in the other enzymes is not a direct adaptation to temperature.

In vivo interactions between some glycolytic enzymes alter their activity (*11*, *18*). If two enzymes form functional complexes in vivo, then selection may favor their coevolution. Five such pairs were chosen before analyses: (i) PGI and PFK (*18*), (ii) PGK and GAPDH (*19*), (iii) PFK and ALD (*20*), (iv) GAPDH and LDH (*20*), and (v) PGI and ALD (*12*). Only two of the five pairs, LDH-GAPDH and PGI-ALD, covaried significantly when analyzed by either phylogenetic method.

Activities of LDH and GAPDH are significantly positively correlated, even after accounting for their similar correlations with mean annual temperature (Fig. 3, A and B). These two enzymes are thought to form a heteromeric complex in vivo that may facilitate the rapid transfer of their common cofactor, NAD^+/NADH (nicotinamide adenine dinucleotide/reduced form of NAD^+), and increase flux through these steps (*19*). Thus, the coordinate evolution of these enzymes suggests this complex is physiologically important. Alternatively, these two enzymes may be responding similarly to an additional, unidentified environmental factor.

PGI and ALD correlate significantly

with each other (Fig. 3, C and D). PGI and ALD are thought to be in vast excess relative to PFK (*21*), but some experimental data suggest that the relative in vivo activities of PGI and ALD are low enough for variations in these enzymes to be functionally relevant (*2*, *4*, *22*). These two enzymes flank PFK in the glycolytic pathway and thus their activities relative to that of PFK may affect flux. If PFK activities in *Fundulus* taxa are altered evolutionarily by allosteric modulation, then the correlation between PGI and ALD concentrations may reflect coordinate changes in PFK concentration or activity.

Which theory of metabolic flux best fits the observed evolutionary patterns? Adaptive variation in PYK concentration is expected under classical biochemical models because PYK catalyzes an irreversible adenosine 5′-triphosphate–producing reaction and experiences allosteric modulation. However, PFK, a regulatory enzyme, does not vary adaptively with temperature. Both LDH and GAPDH are equilibrium enzymes that are thought to be unable to affect flux. Our data indicate that these two equilibrium enzymes are adaptively important and thus affect metabolic flux. The coevolution of two pairs of equilibrium enzymes suggests that enzyme interactions (that is, epistasis between enzyme steps) may be as important as single enzymes for flux modulation. Thus, the data presented here suggest that many different enzymes modulate flux, supporting metabolic control theories.

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Synaptic Vesicle Endocytosis Impaired by Disruption of Dynamin–SH3 Domain Interactions

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The proline-rich COOH-terminal region of dynamin binds various Src homology 3 (SH3) domain–containing proteins, but the physiological role of these interactions is unknown. In living nerve terminals, the function of the interaction with SH3 domains was examined. Amphiphysin contains an SH3 domain and is a major dynamin binding partner at the synapse. Microinjection of amphiphysin's SH3 domain or of a dynamin peptide containing the SH3 binding site inhibited synaptic vesicle endocytosis at the stage of invaginated clathrin-coated pits, which resulted in an activity-dependent distortion of the synaptic architecture and a depression of transmitter release. These findings demonstrate that SH3-mediated interactions are required for dynamin function and support an essential role of clathrin-mediated endocytosis in synaptic vesicle recycling.

The guanosine triphosphatase (GTPase) dynamin has an essential role in endocytosis (*1*, *2*). It forms a collar at the neck of endocytic pits and participates in the fission reaction that generates a free vesicle (*3*). Block of dynamin GTPase function in nerve terminals