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Cold Acclimation Strategy Is Highly Variable among the Sunfishes (Centrarchidae)

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ABSTRACT

We tested the hypothesis that the physiological strategy for acclimating to low body temperature is similar among closely related fish. Largemouth bass (*Micropterus salmoides*), green sunfish (*Lepomis cyanellus*), bluegill sunfish (*Lepomis macrochirus*), black crappie (*Pomonix nigromaculatus*), and white crappie (*Pomonix annularis*), all members of the family Centrarchidae, were acclimated to 5° and 25°C. Morphometric variables (total mass, total length, organ masses) and enzyme activities (hexokinase; lactate dehydrogenase; and cytochrome oxidase in heart, liver, and muscle) were measured in 5°C- and 25°C-acclimated fish at 5° and 25°C assay temperatures. Each species displayed a distinct physiological response to cold acclimation that differed among tissues. These data suggest that the response to cold acclimation is highly variable within families. Our findings are consistent with other studies suggesting that acclimation responses are labile and may evolve independently even among closely related species.

Introduction

Temperate-zone fishes undergo wide seasonal fluctuations in habitat temperature and thus body temperature (4° to 30°C); as such, they make an excellent group for thermal acclimation studies. Numerous studies have described dramatic changes in morphology and physiology of fish as they acclimate to low body temperatures. Typical responses to cold acclimation include increased heart size (Kent et al. 1988; Sephton and Driedzic 1991; Rodnick and Sidell 1997), increased mitochondrial density (Eggington and Sidell 1989; Guderley 1990; Rodnick and Sidell 1994), increased enzymatic indicators of aerobic and anaerobic metabolism (Shaklee et al. 1977; Kent et al. 1988; Rodnick and Sidell 1994; Pierce and Crawford 1997; Podrabsky et al. 2000), increased lipid oxidation in red muscle (Rodnick and Sidell 1994), increased red muscle mass (Jones and Sidell 1982; Eggington and Sidell 1989; Rodnick and Sidell 1994), and increased calcium ATPase activity (Johnston et al. 1990).

From the rich literature of temperature acclimation in fish comes the general assertion/observation that response to cold is similar among many different species. Following this is a second assertion that the generalized suite of physiological responses to cold are adaptive (e.g., increased heart size compensates for increased blood viscosity at low temperature and allows the fish to remain active). However, one cannot definitively characterize these changes as adaptive unless they increase the fitness of the organism (Gould and Lewontin 1979). For example, fitness does not always increase in *E. coli* as a result of temperature acclimation (Leroi et al. 1994). We wish to test the assertion that cold-acclimation response is similar among different species, as an initial approach to our long-term goal of testing the beneficial acclimation assumption in fishes (e.g., What variation, if any, is available for selection to act on in this group?).

To investigate the question of how acclimation response varies among species, we chose the Centrarchidae (sunfishes). Sunfishes are well suited for acclimation studies. They are easily maintained and abundant, they have an established phylogeny (Mabee 1993; Fig. 1), and all have a similar habitat and range. In addition, species within Centrarchidae apparently exhibit a variety of responses to seasonal fluctuations in environmental temperature. It has been reported that largemouth bass do not show any of the indicators of cold acclimation (Kolok 1992); however, smallmouth bass do (doubling of heart ventricle mass; Sephton and Driedzic 1991). Green sunfish may or may not cold acclimate depending on the variables measured; Kent et al. (1988) documented an increase in ventricular heart mass and protein content on cold acclimation, Sidell (1977) demonstrated reduced cytochrome C turnover rates in cold-acclimated green sunfish, and Shaklee et al. (1977) documented tissue-specific responses to cold acclimation. Kolok (1991), however, reported no increase in ventricular mass or citrate synthase activity in this species.

Data for largemouth bass, green sunfish, and smallmouth bass currently exist; however, expanding the study to other

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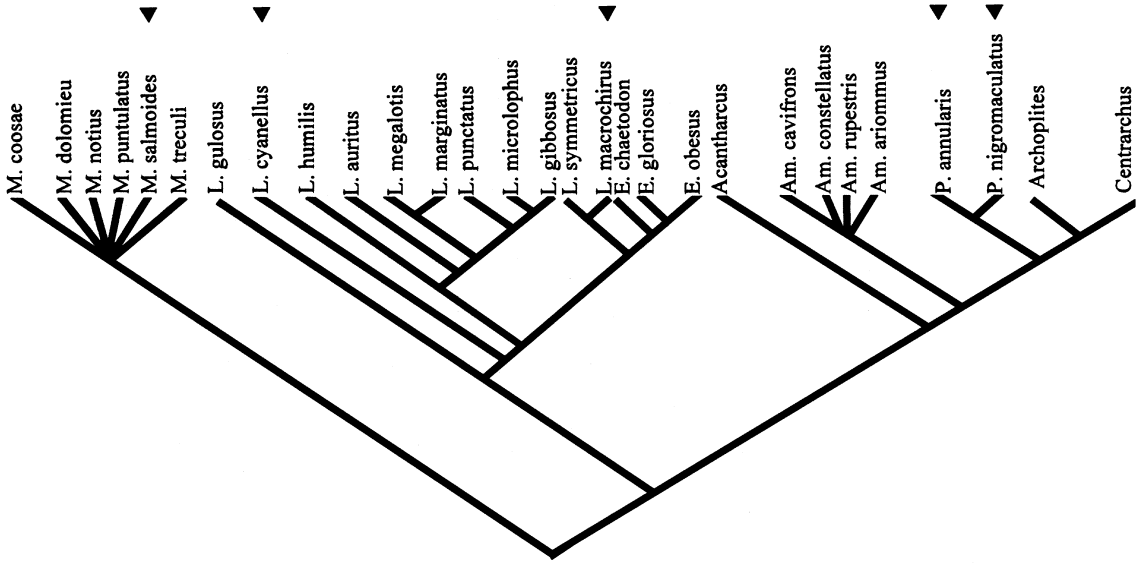

Figure 1. Phylogeny of the Centrarchidae, redrawn from Mabee (1993; with permission of author and publisher). Triangles indicate species in this study.

closely related species within the sunfish family allowed us to ask the question: Is there similarity of acclimation response among species in the Centrarchidae? We chose congeneric species within three separate clades of Centrarchidae so we could compare response within and among clades (Fig. 1). As a first approach to the general problem of how acclimation response affects fitness, we measured various indicators of acclimation in these five species to determine the following: (1) Is acclimation response variable among species? (2) If acclimation response is variable, is that variation correlated with phylogeny? Our results indicate that cold acclimation response is highly

Table 1: Morphometric variables

Species and Acclimation Temperature (°C)	TW (g)	TL (cm)	Heart (g)	Liver (g)	Brain (g)	Carcass (g)
Green sunfish:						
5	57.62 ± 4.83	14.4 ± .43	.06 ± .004	1.16 ± .06	.06 ± .01	53.27 ± 4.50
25	68.59 ± 10.52	15.1 ± .74	.07 ± .01	1.88 ± .32	.04 ± .006	63.88 ± 9.90
Bluegill:						
5	37.13 ± 4.94	13.29 ± .47	.03 ± .006	.40 ± .09	.06 ± .01	34.33 ± 4.48
25	54.84 ± 5.77	14.94 ± .33	.04 ± .004	1.20 ± .33	.07 ± .007	51.96 ± 5.89
Black crappie:						
5	73.26 ± 5.25	17.33 ± .27	.06 ± .009	.83 ± .17	.07 ± .02	69.47 ± 5.02
25	107.34 ± 5.17	19.00 ± .32	.05 ± .004	.64 ± .10	.07 ± .01	101.90 ± 5.22
White crappie:						
5	161.21 ± 12.77	23.5 ± .47	.12 ± .009	1.21 ± .29	.12 ± .007	153.89 ± 12.18
25	128.79 ± 20.41	21.8 ± 1.53	.08 ± .01	.66 ± .12	.06 ± .005	121.04 ± 19.42
Largemouth bass:						
5	107.25 ± 12.45	20.2 ± .52	.09 ± .006	2.60 ± .61	.06 ± .02	103.27 ± 12.17
25	135.57 ± 17.20	20.79 ± 1.12	.09 ± .01	4.20 ± .83	.14 ± .04	127.30 ± 16.07

Note. TW = total weight; TL = total length. Green sunfish, $N = 5$ per acclimation group; bluegill, $N = 7$ at 5°C, $N = 5$ at 25°C; black crappie, $N = 3$ per acclimation group; white crappie, $N = 4$ per acclimation group; largemouth bass, $N = 3$ per acclimation group. Values are means ± SERR.

Table 2: Two-way ANCOVA testing for effects of species and acclimation in heart weight and liver weight

Source	df		Sum of		
	Numerator	Denominator	Squares	*F* ratio	Prob > *F*
Heart weight:[a]					
Acclimation	1	1	.00155846	7.7609	.0084
Species	4	4	.00110680	1.3779	.2604
Acclimation × species	4	4	.00225788	2.8110	.0392
Covariate	1	1	.01260750	62.7839	<.0001
Liver weight:[b]					
Acclimation	1	1	.635970	.8095	.3741
Species	4	4	11.593489	3.6891	.0126
Acclimation × species	4	4	3.323705	1.0576	.3911
Covariate	1	1	8.197924	10.4346	.0026

[a] $R^2 = 0.82$, overall $F = 21.8846$, prob > $F < 0.0001$. Covariate is total mass minus organ mass.
[b] $R^2 = 0.56$, overall $F = 6.8648$, prob > $F < 0.0001$. Covariate is total mass minus organ mass.

variable among the five species we tested, with no clear pattern related to phylogeny.

Material and Methods

Animal Husbandry

Largemouth bass (*Micropterus salmoides*), black crappie (*Pomonix nigromaculatus*), and green sunfish (*Lepomis cyanellus*) were purchased from Fender's Fish Hatchery (Baltic, Ohio). Fender's is an extensive versus intensive hatchery (fish are raised in ponds with full exposure to natural temperature and photoperiod variation). Bluegill sunfish (*Lepomis macrochirus*) were collected at Bath Nature Preserve, and white crappie (*Pomonix annularis*) were caught locally (Portage Lakes), both with hook and line. Each species was housed in rectangular, temperature-controlled, 80-gal recirculating aquaria (one species per tank). Tap water was treated with Stresscoat (Aquarium Pharmaceuticals, Chalfont, Pa.) to neutralize chlorine. Fish were fed commercial trout pellets daily, with the exception of white and black crappie, which were fed fathead minnows ad lib. Tanks were checked daily for water temperature, ammonia levels, animal health, and equipment condition. All fish were >2 yr in age and were considered adults.

Fish were acclimated over 8 wk, two species at a time (largemouth bass and green sunfish, then bluegill and white crappie, then black crappie). Water temperature was raised or lowered 1.5°C per day until temperatures of 5° and 25°C were reached for cold- and warm-acclimated fish, respectively. Once the appropriate acclimation temperatures were achieved, the fish were maintained at these temperatures for 6 wk. At the end of the 8-wk (total) acclimation period, the animals were killed by an overdose of 3-aminobenzoic acid ethyl ester (MS-222), and their tissues were harvested and either assayed immediately (hexokinase and cytochrome oxidase; all tissues assayed within hours of dissection) or frozen in liquid nitrogen for later analysis (lactate dehydrogenase). Total weight and length of each fish were recorded. Hearts were removed, and the ventricle was rinsed, blotted, and weighed. White glycolytic muscle was removed from the left body wall just below the dorsal fin. Total liver was removed, rinsed, blotted, weighed, and diced to randomize the portion of liver used for enzyme assays.

Enzyme Assays

Enzyme activities were measured with a Spectronic Genesys 2 spectrophotometer (Spectronic Instruments, Rochester, N.Y.) fitted with a water-jacketed, multisample cuvette holder. Six cuvettes were run simultaneously (duplicates plus one control from two animals). Assay temperature was controlled by circulating an antifreeze-water mixture through the cuvette holder and an external recirculating bath. Homogenates (10%, w/v) of heart ventricle, glycolytic muscle, and liver were prepared in an extraction medium containing 40 mM HEPES, 1 mM EDTA, and 2 mM $MgCl_2$ (pH 7.6 at 15°C). Each assay was initially optimized by varying homogenate concentration at the given substrate concentration to achieve a linear change in absorbance over time. Enzyme activity was measured at both 5° and 25°C to determine whether enzyme activity was affected by acclimation (e.g., lactate dehydrogenase from bluegill sunfish acclimated to 5°C was measured at 5° and 25°C). Enzyme activity was expressed as units (micromoles per minute per gram). q2

Hexokinase, HK (*EC 2.7.1.1*). This assay was performed essentially as described by Zammit and Newsholme (1976). The assay was initiated by the addition of glucose; formation of product was monitored by following the reduction of NADP at 340 nm over 5 min. Background activity (subtracted from total activity) was monitored without addition of glucose.

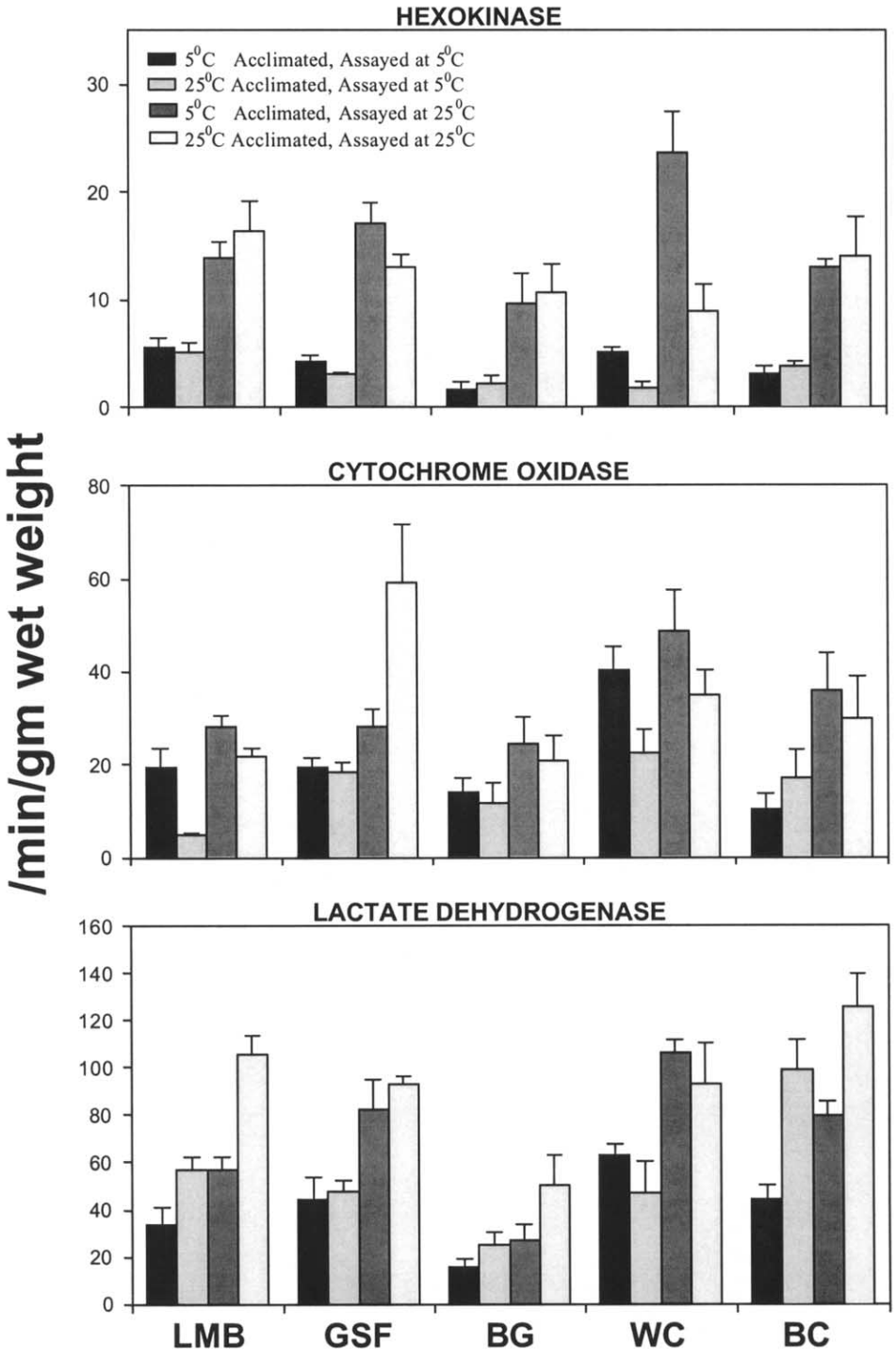

Figure 2. Enzyme activities from heart tissue of acclimated sunfish. LMB = largemouth bass (*Micropterus salmoides*), GSF = green sunfish (*Lepomis cyanellus*), BG = bluegill (*Lepomis macrochirus*), WC = white crappie (*Pomonix annularis*), BC = black crappie (*Pomonix nigromaculatus*).

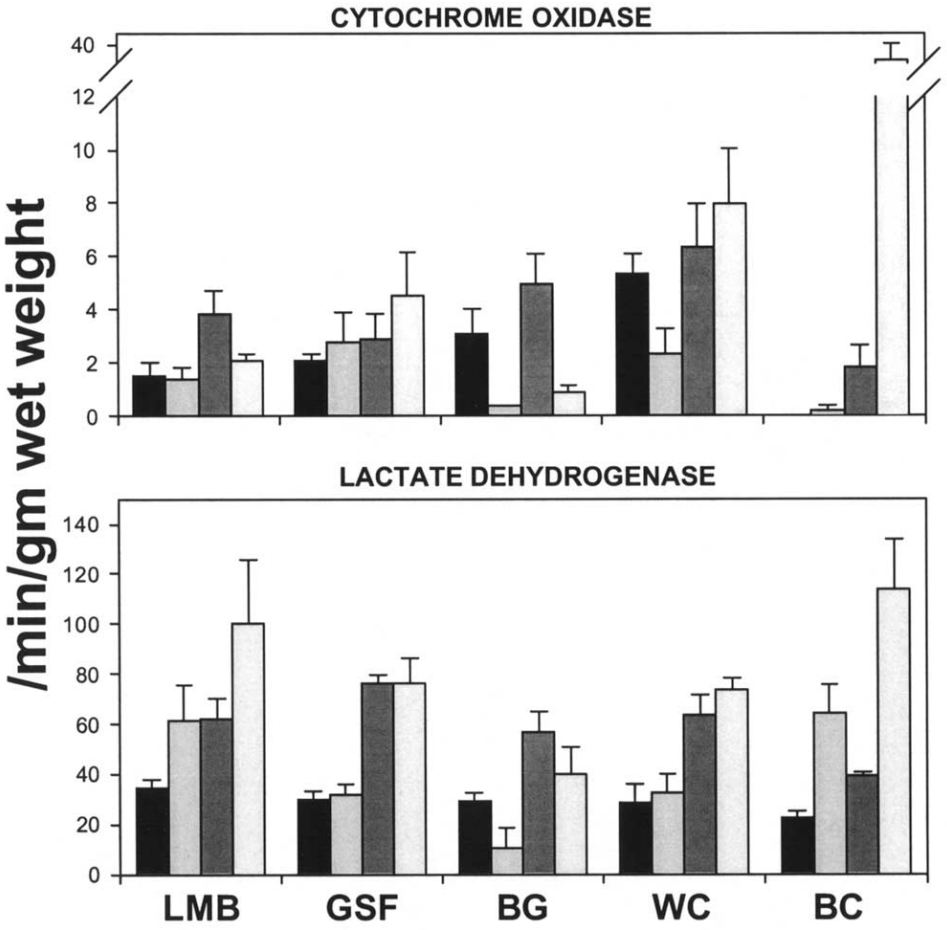

Figure 3. Enzyme activities from skeletal muscle tissue of acclimated sunfish. LMB = largemouth bass (*Micropterus salmoides*), GSF = green sunfish (*Lepomis cyanellus*), BG = bluegill (*Lepomis macrochirus*), WC = white crappie (*Pomonix annularis*), BC = black crappie (*Pomonix nigromaculatus*).

Lactate Dehydrogenase, LDH (*EC 1.1.1.27*). This assay was initiated with the addition of sodium pyruvate and monitored by following the oxidation of NADH at 340 nm over 5 min, as described in Hanson and Sidell (1983). Background activity (subtracted from total activity) was monitored without addition of sodium pyruvate.

Cytochrome Oxidase, CYTOX (*EC 1.9.3.1*). The assay was initiated by adding homogenate and monitoring the oxidation of cytochrome C (reduced with ascorbate) at 550 nm over 3 min, as described by Wharton and Tzagoloff (1967) and Hanson and Sidell (1983). Background was measured as oxidation of cytochrome C without homogenate.

Swimming Activity

Swimming activity was measured three times per week during each of the final 3 wk of acclimation (for all species except white crappie, total observations/acclimation group = 9; for white crappie, total observations/acclimation group = 6). Fish were viewed via overhead mirrors to prevent the fish from seeing the observer. Before feeding, chillers were turned off (so that fish could be seen clearly), and fish were given 10 min to adjust to the change in water flow and presence of the observer in the room. Activity was defined as the number of times any individual crossed the center line of the tank in a 5-min interval. Activity was expressed per fish to correct for the number of fish in a tank (five to 13).

Statistical Analysis

Statistical analyses were performed using JMP statistical software (SAS Institute, N.C.). Our goal was to test whether response to acclimation differed significantly among species.

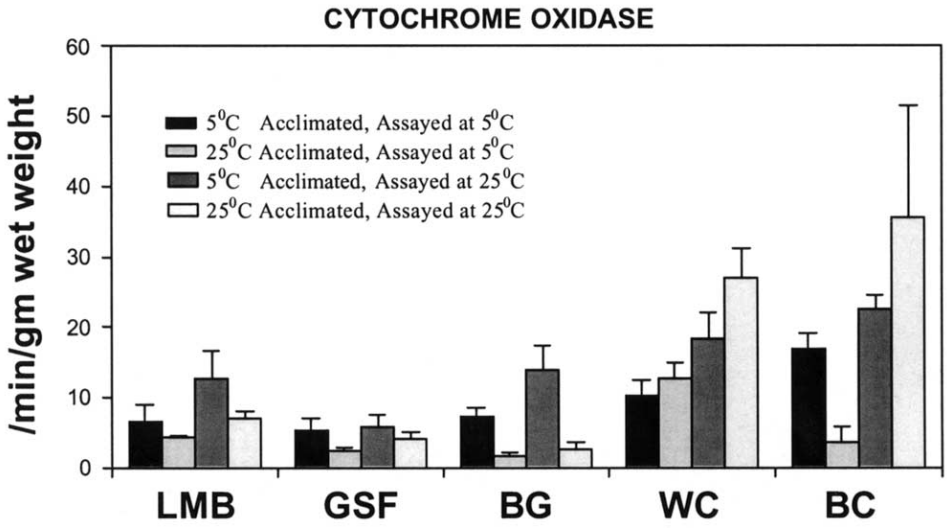

Figure 4. Cytochrome oxidase activity from liver tissue of acclimated sunfish. LMB = largemouth bass (*Micropterus salmoides*), GSF = green sunfish (*Lepomis cyanellus*), BG = bluegill (*Lepomis macrochirus*), WC = white crappie (*Pomonix annularis*), BC = black crappie (*Pomonix nigromaculatus*).

Table 3: Repeated measures MANOVA on heart enzyme activity by source of variation

		df		
	F	Numerator	Denominator	Prob > *F*
F-test[a]	3.321	9	30	.0063
Source of variation:				
Enzyme	6.0513	18	58	<.0001
Species	7.6093	8	58	<.0001
Acclimation	10.0270	2	29	.0005
Species × acclimation	4.3803	8	58	.0004
Assay temperature	3.3210	9	30	.0063
Species	3.8519	4	30	.0121
Acclimation	4.4400	1	30	.0436
Species × acclimation	2.4579	4	30	.0669
Assay temperature × enzyme	3.8664	8	58	<.0001
Species	3.3371	8	58	.0033
Acclimation	6.3497	2	29	.0052
Species × acclimation	3.3912	8	58	.0029

Note. Probability level for each source of variation indicates whether or not the factor had a significant effect on enzyme activity. In this analysis, assay temperature and the assay temperature × enzyme interaction were treated as repeated measures, and enzyme was treated as a nonrepeated multivariate measure. Sources of variation below the main headings (assay temperature, enzyme, assay temperature × enzyme) indicate the effect of factors when averaged across the effect identified by the heading; df = degrees of freedom.

[a] Test of the significance of the full factorial model incorporating the effects listed below "Source of variation").

Table 4: Repeated measures MANOVA on heart hexokinase activity

Source of Variation	*F*	df		Prob > *F*
		Numerator	Denominator	
Between subjects:				
Species	.7790	4	30	.5477
Acclimation	4.9502	1	30	.0338
Species × acclimation	3.5315	4	30	.0178
Within subjects:				
Assay temperature	97.2117	1	30	<.0001
Assay temperature × species	1.9616	4	30	.1260
Assay temperature × acclimation	3.4637	1	30	.0726
Assay temperature × species × acclimation	3.4204	1	30	.0203

Note. Overall model (between subjects) significance $P < 0.0157$; df = degrees of freedom.

Test of Variation in Organ Mass. For analysis of heart and liver mass, we used a two-way ANCOVA to test for the effects of species and acclimation temperature on organ mass while controlling for body-size variation. We did this by using total mass minus organ mass as a covariate (Hayes and Shonkwiler 1996).

Overall Test of Sources of Variation in Enzyme Activity. For each tissue (heart, liver, muscle), we used a repeated measures MANOVA to test whether species, acclimation temperature, assay temperature, and their statistical interactions were significant sources of variation in enzyme activity. We chose this over the univariate approach because it makes fewer assumptions about the structure of the covariance matrices (Keselman et al. 2001). If the overall MANOVA was significant, we followed up with simpler repeated measures ANOVAs to test the importance of specific independent variables in explaining observed variation in enzyme activities. This approach is analogous to following up ANOVA with individual *t*-tests to test the significance of specific comparisons (e.g., levels of a factor in one-way ANOVA) subsequent to ANOVA (Sokal and Rolhf 1981). In theory, we could have added tissue as a repeated measure in the overall analysis described above. However, given small sample sizes and missing cells for some enzymes and species, we would not have had enough degrees of freedom for the tests of interest; treating tissues in separate analyses is a reasonable approach to take given the limitation of our dataset. For example, we analyzed heart tissue as follows: (1) dependent variables: (*a*) HK assayed at 5° and 25°C, (*b*) CYTOX assayed at 5° and 25°C, and (*c*) LDH assayed at 5° and 25°C; (2) independent variables: (*a*) acclimation temperature and (*b*) species.

The model is a compound repeated measures MANOVA; enzyme activity is measured at assay temperatures of 5° and 25°C, and each individual fish was sampled for all three enzymes. Assay temperature is treated as a repeated measure and enzyme as a nonrepeated multivariate measure.

Results

Heart and Liver Mass

Body size varied considerably among species and across acclimations (Table 1), confounding the direct comparison of heart and liver mass as a result of acclimation. When accounting for variation in body mass by ANCOVA, none of the interactions

Table 5: Repeated measures MANOVA on heart cytochrome oxidase activity

Source of Variation	*F*	df		Prob > *F*
		Numerator	Denominator	
Between subjects:				
Species	6.1425	4	30	.0010
Acclimation	.5395	1	30	.4683
Species × acclimation	3.2713	4	30	.0244
Within subjects:				
Assay temperature	8.1574	1	30	.0077
Assay temperature × species	2.8910	4	30	.0389
Assay temperature × acclimation	3.1682	1	30	.0852
Assay temperature × species × acclimation	3.8951	1	30	.0115

Note. Overall model (between subjects) significance $P < 0.0013$; df = degrees of freedom.

Table 6: Repeated measures MANOVA on heart lactate dehydrogenase activity

Source of Variation	*F*	df		Prob > *F*
		Numerator	Denominator	
Between subjects:				
Species	9.1759	4	30	<.0001
Acclimation	11.6854	1	30	.0018
Species × acclimation	4.1546	4	30	.0085
Within subjects:				
Assay temperature	46.9055	1	30	<.0001
Assay temperature × species	4.5524	4	30	.0054
Assay temperature × acclimation	6.9668	1	30	.0130
Assay temperature × species × acclimation	2.3319	1	30	.0785

Note. Overall model (between subjects) significance $P < 0.0001$; df = degrees of freedom.

involving the covariate was significant (for heart or liver), and thus they were dropped from the full model. The reduced model for heart was highly significant, explaining ~82% of the variance in heart mass ($P < 0.0001$; Table 2). Acclimation did result in significantly larger hearts in 5°C fish (all species included). Acclimation had no significant effect on liver size (Table 2).

Enzyme Activity

Mean (±SE) enzyme activities for heart, muscle, and liver of each species are presented in Figures 2–4. One can do any pairwise comparison visually by comparing overlap of 2 SEs. However, although multiple *t*-tests have been used for acclimation studies in the past (Shaklee et al. 1977), the field now recognizes that this approach will likely lead to a significant Type I error. In addition, the question we are addressing (cold acclimation response in the Centrarchidae) is less concerned with comparisons between species than with total response across species. This question is most appropriately analyzed by MANOVA, and therefore all enzymes were analyzed collectively in a MANOVA by tissue (e.g., separate MANOVAs for heart, muscle, liver). The full factorial model, using dependent variables (hexokinase assayed at 5° and 25°C, cytochrome oxidase assayed at 5° and 25°C, and lactate dehydrogenase assayed at 5° and 25°C) and independent variables (acclimation temperature, species, and all possible interaction terms), was strongly significant (Table 3). Therefore, we are justified in conducting more specific tests at the tissue level.

Heart. The overall model for heart is highly significant (prob > $F < 0.0001$; Table 3). Of particular import to the question of how species respond to acclimation, the species × acclimation interaction is significant (prob > $F = 0.0005$), indicating that considering all heart enzymes, species do not respond equivalently to acclimation. Since the whole model is significant, we were justified in doing more specific tests by enzyme. The general structure of these enzyme-level models was activity at 5°, activity at 25°C = acclimation + species + species × acclimation. Species and species × acclimation are "between" subject factors, and assay temperature is a "within" subject factor. The former measures effects across individuals (on average), and the latter asks whether individuals had a homogeneous response to assay temperature as a function of the other factors. For hexokinase (Table 4) and cytochrome oxidase (Table 5) activities in heart, species × acclimation interactions are significant, as are the assay temperature × species × acclimation interactions within subjects. Put another way, to predict the activities of these two enzymes in heart, one must know assay temperature, species, and acclimation history. For lactate dehydrogenase (Table 6), the species × acclimation interaction is significant, but the more complex assay temperature × species × acclimation interaction is marginal (prob > $F = 0.0785$). Therefore, for LDH in heart, not all species respond equally to acclimation, but in a way that perhaps does not depend on their response to assay temperature.

Muscle. The whole-model, full factorial MANOVA is not significant (Table 7). Therefore, further, more specific tests (e.g., each enzyme) are not warranted. However, because our sample sizes are small and therefore Type II error (low power) is a concern, we extended the analyses to further explain the whole-model result. Enzyme is marginally insignificant (Table 8), perhaps because of a lack of statistical power. However, for the individual enzymes measured in muscle (cytochrome oxidase [Table 9] and lactate dehydrogenase [Table 10]), only species

Table 7: Whole-model (full factorial) repeated measures MANOVA for muscle

Test	Value	Exact *F*	df		Prob > *F*
			Numerator	Denominator	
F-test	.662951	2.1214	5	16	.1155

Note. A test of the significance of the full factorial model incorporating the effects listed in Table 3.

Table 8: Repeated measures MANOVA on muscle enzyme activity by source of variation

Source of Variation	*F*	df		Prob > *F*
		Numerator	Denominator	
Assay temperature	2.1214	5	16	.1155
Enzyme	2.3475	5	16	.0886
Assay temperature × enzyme	1.7137	2	16	.1265

Note. Probability level for each source of variation indicates whether or not the factor had a significant effect on enzyme activity. In this analysis, assay temperature and the assay temperature × enzyme interaction were treated as repeated measures, and enzyme was treated as a nonrepeated multivariate measure. Sources of variation below the main headings (assay temperature, enzyme, assay temperature × enzyme) indicate the effect of factors when averaged across the effect identified by the heading.

effects are significant (the absolute value of these enzymes is different in muscle tissue of different species). The species × acclimation interaction (indicating that species respond differently to acclimation) is not significant, supporting the result of the full model.

Liver. Only cytochrome oxidase was measured in liver, and therefore the structure of the MANOVA model is the same as in the more specific tests in other tissues (e.g., cytochrome oxidase in heart; Table 5). There is a significant species × acclimation interaction in liver ($\text{prob} > F = 0.0004$; Table 11) and a marginally significant assay temperature × species × acclimation interaction ($\text{prob} > F = 0.048$). Not all species' livers respond equivalently to acclimation, and the magnitude of this effect depends also on assay temperature.

Swimming Activity

Largemouth bass, green sunfish, and bluegill sunfish all decreased average swimming movements per 5-min period by more than an order of magnitude on cold acclimation (Fig. 5). In black crappie, however, the reduction in activity is only fourfold, and in white crappie there is no difference in mean activity between cold- and warm-acclimated fish.

Discussion

The study of cold acclimation in fishes has a rich history that reaches back to the pioneers of modern comparative physiology (Das and Prosser 1967). Many of the responses to cold that fish exhibit are unmistakable, including lipid deposition in aerobic muscle (Eggington and Sidell 1989), increased cardiac output (Bailey and Driedzic 1990), and temperature-dependent expression of myofibrillar protein isoforms (Crockford and Johnston 1990). These studies leave little question that fish do respond to cold and in a manner that is similar among species. To what extent is response to cold (cold acclimation strategy) variable in the Centrarchidae? Does that variability covary with phylogeny? Our results document that cold acclimation strategy is highly variable among the Centrarchidae, with no clear pattern related to phylogeny.

Heart and Liver Hypertrophy

A general response to cold acclimation is hypertrophy at the organ level. This response has been documented most frequently in three tissues: heart (green sunfish and channel catfish, Kent et al. 1988; smallmouth bass, Sephton and Driedzic 1991; striped bass, Rodnick and Sidell 1997), liver (channel catfish, Kent et al. 1988), and red muscle (striped bass, Jones

Table 9: Repeated measures MANOVA on muscle cytochrome oxidase activity

Source of Variation	*F*	df		Prob > *F*
		Numerator	Denominator	
Between subjects:				
Species	11.5384	2	16	.0008
Acclimation	1.9369	1	16	.1830
Species × acclimation	.1557	2	16	.8571
Within subjects	2.1286	5	16	.1145

Note. Overall model (between subjects) significance $P < 0.0058$; df = degrees of freedom.

Table 10: Repeated measures MANOVA on muscle lactate dehydrogenase activity

Source of Variation	*F*	df Numerator	df Denominator	Prob > *F*
Between subjects:				
Species	4.7926	2	16	.0234
Acclimation	2.1142	1	16	.1653
Species × acclimation	2.4013	2	16	.1225
Within subjects	2.1286	5	16	.1436

Note. Overall model (between subjects) significance $P < 0.0396$; df = degrees of freedom.

and Sidell 1982; Eggington and Sidell 1989; Guderley 1990; Rodnick and Sidell 1994; goldfish, Johnston and Lucking 1978; Eggington and Sidell 1989; Guderley 1990). Our analyses demonstrate that there is a significant effect of acclimation on heart size (after correcting for body size, heart size is larger in 5°C-acclimated animals). This effect was largely driven by black crappie and white crappie (other species did not have a large increase in heart mass on acclimation; Table 1). This is the one case where we observed a similar strategy (increase in organ mass) among congeners. However, even within *Pomonix*, other dimensions of response to acclimation differ between the congeners.

Cold Acclimation's Effects on Enzyme Activity

One way to determine a metabolic response to temperature is to assess the relative capacity of metabolic pathways via representative enzyme assays (e.g., Crockett and Sidell 1990; Rodnick and Sidell 1994; Pierce and Crawford 1997). We chose three enzymes that are typically measured in acclimation studies and also represent three major metabolic pathways: aerobic glycolysis (hexokinase), anaerobic carbohydrate metabolism (lactate dehydrogenase), and oxidative phosphorylation (cytochrome oxidase). Our absolute values for activity are within the range reported for teleosts by Sidell et al. (1987), and we saw similar patterns of enzyme response in green sunfish to those reported by Shaklee et al. (1977). Specifically, responses to cold acclimation of lactate dehydrogenase in muscle, heart, and cytochrome oxidase activity in liver were identical between the two studies. Shaklee et al. did demonstrate an increase in cytochrome oxidase activity in muscle on cold acclimation, whereas we did not. We assume this is due to muscle being sampled from a different part of the fish (not specified by Shaklee et al.) or a size- or age-specific phenomenon (Shaklee et al. had a larger size range than we did).

We did not attempt to cover any one pathway completely (e.g., Pierce and Crawford 1997) but rather to estimate capacity in several pathways (e.g., Sidell et al. 1987; Crockett and Sidell 1990). We also recognize that some tissues in this study are sampled more extensively than others (e.g., heart vs. liver) and that sample sizes are low for some species. We chose to compromise between a question that is large in scale and practical data collection (collecting and acclimating multiple species, dissecting multiple organs, >1,000 individual assays at multiple temperatures). Our goal was to gather enough data to accept or reject the hypothesis that response to acclimation is uniform within a family of fishes. Our analyses resoundingly reject that hypothesis.

Previous acclimation studies have shown that cold acclimation generally increases enzyme capacities in fish (Guderley q9

Table 11: Repeated measures MANOVA on liver cytochrome oxidase activity

Source of Variation	*F*	df Numerator	df Denominator	Prob > *F*
Between subjects:				
Species	15.9715	4	30	<.0001
Acclimation	8.7378	1	30	.0060
Species × acclimation	7.1129	4	30	.0004
Within subjects:	4.9561	9	30	.0004
Assay temperature	64.4309	1	30	<.0001
Assay temperature × species	8.3120	4	30	<.0001
Assay temperature × acclimation	.7069	1	30	.4071
Assay temperature × species × acclimation	2.7083	4	30	.048

Note. Overall model (between subjects) significance $P < 0.0001$; df = degrees of freedom.

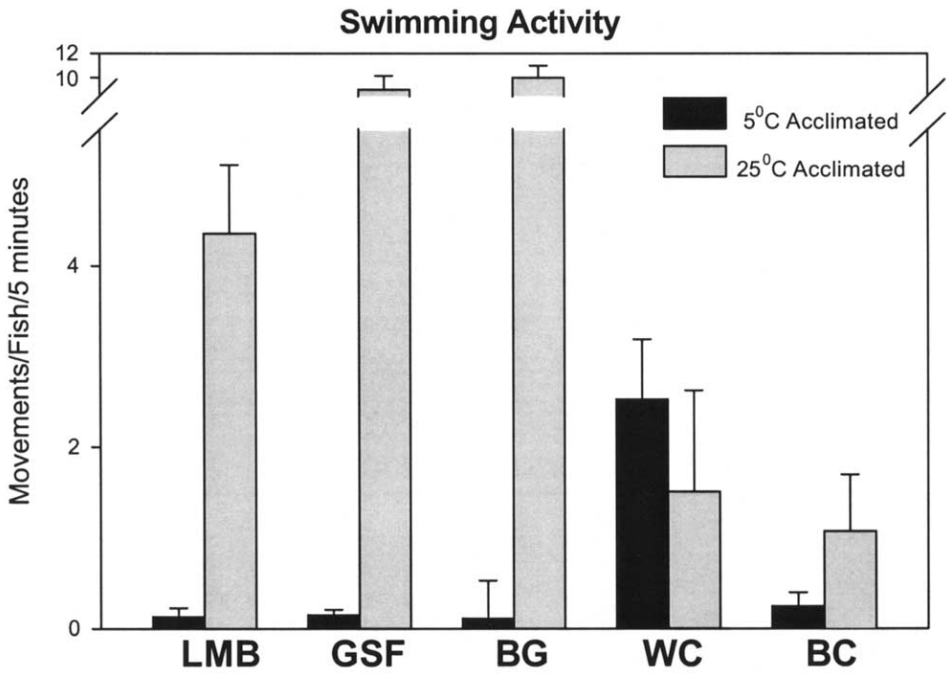

Figure 5. Swimming activity was measured as the number of times a fish crossed the center line of the tank during a 5-min observation period (per fish). LMB = largemouth bass (*Micropterus salmoides*), GSF = green sunfish (*Lepomis cyanellus*), BG = bluegill (*Lepomis macrochirus*), WC = white crappie (*Pomonix annularis*), BC = black crappie (*Pomonix nigromaculatus*).

1990; Sephton and Driedzic 1991; Rodnick and Sidell 1994, 1997). In these studies, univariate analyses were appropriate for the question addressed (e.g., Does variable *X* in tissue *Y* respond to cold acclimation in species *Z*?). We also observed increases in enzyme activity; however, we specifically addressed the multivariate nature of cold acclimation to determine whether cold-acclimation strategy was similar across species within a family. This approach does not negate the validity of differences for univariate-type questions. For example, hexokinase activity, assayed at 5°C, is significantly greater (by *t*-test) in cold-acclimated versus warm-acclimated white crappie heart (Fig. 2). If one wished to test a hypothesis related to carbohydrate metabolism in white crappie heart, this result, derived by univariate analysis, would support an acclimation effect.

We hypothesized a priori that many of our variables would covary, and the analyses bear that out (Tables 3–11). Univariate analyses, by their nature, cannot account for complex covariance matrices. Also, when variables covary in a complex manner, MANOVA has increased statistical power (decreased Type I error) versus univariate analyses (Keselman et al. 2001). Given low sample sizes, one may be concerned about low power (increased Type II error). However, we demonstrated significant effects in all cases other than muscle; therefore, by definition we have a sufficient sample size to demonstrate an effect. Simply put, our question and design were more appropriately analyzed by MANOVA, whereas other questions and designs in cold acclimation (perhaps a majority) are better analyzed by various univariate analyses. It is notable, however, that the univariate repeated measures gave qualitatively identical results (data not shown).

Do enzymes of sunfish species respond equivalently to temperature acclimation? Our data suggest the answer is a resounding no. The whole model, which includes effects of acclimation, species, and all possible interactions on enzyme activity, is highly significant (Table 3). Parsing out what is driving that significance, we find that all enzymes in heart (Tables 3–6) and liver (Table 11) have a significant species × acclimation interaction (indicating that species do not respond equivalently to acclimation). Response to acclimation in muscle is not significant, raising the possibility that we do not have sufficient power to estimate muscle response. However, species × acclimation interaction effects are $\text{prob} > F = 0.8571$ and 0.1225 for cytochrome oxidase and lactate dehydrogenase, respectively, suggesting the effects are strongly not significant. These results suggest that cold-acclimation response is complex; tissues, species, and tissues among species will not respond equivalently. Shaklee et al. (1977) also saw no effect of acclimation on LDH activity but did see an effect on cytochrome oxidase, cytochrome C, pyruvate kinase, and alcohol dehydrogenase. It may be that the difference in cytochrome

oxidase response is due to the population of muscle fibers sampled or that this response falls within the plasticity of responses seen within species. It is also possible that if we assayed different enzymes in all species (such as pyruvate kinase or alcohol dehydrogenase), we would have detected a significant species × acclimation interaction (i.e., we may have reduced power in this situation).

Swimming Activity

Recognizing that response to cold acclimation is multidimensional, we attempted to estimate some aspect of behavioral response. Although admittedly crude, our behavior measurements did show a dramatic effect of cold acclimation on "routine" swimming behavior. All but one species (white crappie) dramatically decrease activity on cold acclimation. Between the *Pomonix* congeners, white and black crappie are both less severe in their response to cold acclimation than the other sunfishes, but only white crappie has equivalent activity in the cold. It is difficult to translate these measurements to behavior on acclimatization (field response); however, white crappie are among the first fish to bite in the spring (anecdotal information from anglers), perhaps because they maintain higher activity in the cold. Reduction of routine activity should impact fuel reserves and may be an important component of a fish's multidimensional overwintering strategy.

Phylogeny

There is no obvious correlation of acclimation response to phylogenetic relationship. Within *Pomonix*, swimming activity is qualitatively similar, and cardiac hypertrophy is similar, but individual enzyme response is strikingly different between white and black crappie (e.g., compare response among enzymes in heart; Fig. 2). For the *Lepomis* congeners, there is no similarity of response except for swimming activity.

Acclimation Strategies

Our results clearly demonstrate that the pattern of acclimation response is different among the five species studied. What is the source of this variation? We expected that congenerics would show common responses to acclimation, given that they are closely related. However, Pierce and Crawford (1997) examined the response of all glycolytic enzymes to temperature acclimation in five species of *Fundulus* and found that each had a distinct acclimation strategy. Further, enzyme profiles of *Fundulus heteroclitus* are different between northern and southern populations, suggesting that acclimation (acclimatization) strategies may be different even within a species (Podrabsky et al. 2000). These studies (including ours) suggest that acclimation response is flexible among closely related species and even within species. If cold acclimation is adaptive, plasticity of response (depending on age, sex, predation pressure, social hierarchy, nutritional status, etc.) may be the selective trait. Fish express an acclimation strategy that maximizes their fitness in a specific situation. To that end, Seddon and Prosser (1996) found that channel catfish collected during different seasons had different responses to acclimation. Another possibility is that cold acclimation is not adaptive but rather a historical remnant or correlate of another adaptive response. Now that we have established variation among the sunfish, we plan to test variation within a species (among sibs under different initiating conditions). q10

Conclusions

Our results demonstrate that members of the sunfish family differ significantly in their responses to cold acclimation. Significant species × acclimation variation was demonstrated in heart mass, heart enzyme activity, liver enzyme activity, and swimming behavior (qualitatively) but not muscle-enzyme activity. Variability in acclimation responses even among closely related taxa does not appear to be unique to sunfishes (Crawford et al. 1999). By exploiting this interfamily variation and considering multiple variables simultaneously, we can test plasticity of response within a species.

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