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Allometric scaling in centrarchid fish: origins of intra- and inter-specific variation in oxidative and glycolytic enzyme levels in muscle

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Summary

The influence of body size on metabolic rate, muscle enzyme activities and the underlying patterns of mRNA for these enzymes were explored in an effort to explain the genetic basis of allometric variation in metabolic enzymes. We studied two pairs of sister species of centrarchid fish: black bass (largemouth bass *Micropterus salmoides* and smallmouth bass *Micropterus dolomieui*) and sunfish (pumpkinseed *Lepomis gibbosus* and bluegill *Lepomis macrochirus*). Our goal was to assess the regulatory basis of both intraspecific and interspecific variation relative to body size, as well as to gain insights into the evolutionary constraints within lineages. Whole animal routine metabolic rate showed scaling coefficients not significantly different from 1, ranging from (+0.87 to +0.96). However, there were significant effects of body size on the specific activities of oxidative and glycolytic enzymes. Mass-specific activity of the oxidative enzyme citrate synthase (CS) scaled negatively with body size in each species, with scaling coefficients ranging from –0.15 to –0.19, whereas the glycolytic enzyme pyruvate kinase (PK) showed positive scaling, with scaling coefficients ranging from +0.08 to +0.23. The ratio of mass-specific enzyme activity in PK to CS increased with body size, whereas the ratio of mRNA transcripts of PK to CS was unaffected, suggesting the enzyme relationships were not due simply to transcriptional regulation of both genes. The mass-dependent differences in PK activities were best explained by transcriptional regulation of the muscle PK gene; PK mRNA was a good predictor of PK specific enzyme activity within species and between species. Conversely, CS mRNA did not correlate with CS specific enzyme activities, suggesting post-transcriptional mechanisms may explain the observed inter-specific and intraspecific differences in oxidative enzymes.

Key words: allometry, metabolism, white muscle energetics, citrate synthase, pyruvate kinase.

Introduction

Many metabolic processes depend on body size, and are usually described by the equation $y=aM^b$, where y is the parameter of interest, a is a normalization coefficient, M is body mass, and b is the scaling coefficient. Experimentally, the value for b is the slope obtained from a linear regression of log$y$ *versus* log$M$. Kleiber compared the respiratory rate of birds and mammals of differing sizes and described a scaling coefficient of +0.75 (Kleiber, 1932). Despite many years of investigation on other species and metabolic parameters, there remain many unanswered questions about how body size influences metabolic processes. Some studies search for underlying biophysical constraint(s) that might explain why metabolic processes scale as they do (e.g. West et al., 1999). Some consider the influence of environment and phylogeny on b values (Clarke and Johnston, 1999). Others consider the more proximal explanations for observed relationships by assessing the cellular pathways that determine the metabolic phenotype (e.g. Darveau et al., 2002). Regardless of the ultimate reason for scaling patterns, cells have the responsibility of ensuring that they create an appropriate enzymatic and regulatory environment to obtain a suitable metabolic phenotype. Assessing the regulatory origins of the metabolic phenotype requires making connections between molecular genetics, signal transduction, enzymology and metabolic regulation.

Muscle tissue is a metabolically active tissue that constitutes a large proportion of body mass and largely contributes to the whole animal metabolic phenotype of most vertebrates. In terrestrial endotherms (birds and mammals), whole body metabolic rates show scaling coefficients between +0.7 and +0.8 (or mass-specific scaling coefficients of –0.2 to –0.3). In the gastrocnemius muscle of selected mammals, the oxidative enzyme citrate synthase (CS) also scales negatively, though with a shallower slope (b=–0.11) than whole animal metabolic rate (–0.2 to –0.3) (Emmett and Hochachka, 1981). Likewise in fish, metanalysis of whole animal metabolic rate shows mean scaling coefficients near +0.79, with a range of +0.65 to +0.95 (Clarke and Johnston, 1999), and white muscle mass-specific oxidative enzyme activities of 13 fish species scaled negatively with body mass with a scaling coefficient of –0.26 (similar to mass-specific metabolic rates seen in metanalyses) (Somero and Childress, 1980). Understanding these relationships is

complicated by the fact that no study on vertebrates has assessed both muscle oxidative enzymes and whole animal metabolic rate in the same animals. Another general observation in scaling studies is the reciprocal relationship between oxidative and glycolytic enzymes, which generally show positive scaling in muscles of fish (e.g. Somero and Childress, 1980; Burness et al., 1999; Norton et al., 2000) and mammals (e.g. Emmett and Hochacka, 1981). In reconciling the patterns in metabolic rate and metabolic enzymes, two questions remain unanswered. (1) Do muscle oxidative enzymes scale with oxygen consumption in the same animals and (2) what is the regulatory basis of the reciprocal relationship between oxidative and glycolytic enzymes?

For vertebrate researchers interested in either the relationship between metabolic enzymes and metabolism, or the origins of the reciprocal relationship between oxidative and glycolytic enzymes, studies on tetrapods face numerous challenges. First, it is unlikely that the metabolic phenotype of a single leg muscle would dominate whole animal metabolic rate. Second, homologous muscles perform different types of activity in large and small animals and thus size is not the only factor affecting the phenotype. For example, the muscle studied by Emmett and Hochachka, the gastrocnemius (Emmett and Hochachka, 1981), constitutes only about 0.5% of the body mass of a mammal and, although it performs the same locomotor role in each species (foot flexion), the muscles from large and small mammals differ in fiber type profiles (Wang and Kernell, 2001).

Fish offer numerous advantages for studying how body size affects muscle metabolic enzymes (see Somero and Childress, 1980). Most of the body mass of a fish is trunk muscle, composed primarily of two homogeneous regions: slow-oxidative red muscle and fast-twitch white muscle. In most fish, white muscle constitutes >70% of the total muscle mass (Sanger and Stoiber, 2001), >90% in centrarchids. Thus, the metabolic conditions within white muscle dominate whole animal metabolic rate, even at rest (see Moyes et al., 1992). Unlike mammalian models, many species of fish, such as centrarchids, exhibit indeterminate growth (Mommsen and Moon, 2001), enabling studies of intra-specific scaling of animals with similar geometries. The combination of intra-specific growth and differences in size of closely related species may enable the separation of the effect of size from growth. It is also possible to draw upon closely related fish species that exhibit striking diversity in lifestyle (e.g. activity levels) and environmental sensitivity (e.g. hypoxia tolerance), factors that would be expected to influence metabolic profiles independently of body size.

In this study, we examine the metabolic phenotype in relation to body size in two pairs of closely related species: largemouth bass *Micropterus salmoide* and smallmouth bass *M. dolomieui*, and pumpkinseed *Lepomis gibbosus* and bluegill *L. macrochirus*. This approach provides an opportunity to investigate the genetic basis of patterns in metabolism and metabolic enzymes arising with both ontogenetic and phylogenetic differences in body mass.

Materials and methods

Animal and tissue collection

All fish were collected using angling or seine nets. Smallmouth bass *Micropterus dolomieui* Lacepède 1802 were collected from Lake Ontario (44°15′, 76°31′). Largemouth bass *Micropterus salmoides* Lacepède 1802, pumpkinseed *Lepomis gibbosus* L. and bluegill *Lepomis macrochirus* Rafinesque 1819 were collected from Lake Opinicon, Ontario (44°35′, 76°20′). All fish were allowed to recover in flow-though tanks (420 l) for at least 12 h prior to experimental procedures.

Oxygen consumption measurements

Respiration measurements were performed in standard glass aquaria, with removable, sealable tops constructed of Plexiglass™. Fish were captured by dipnet and placed in respirometry chambers (50–100 ml g^{-1} fish) held at 20°C. The containers were closed with water flowing into the chamber for approximately 20 min. At this point the containers were sealed, air bubbles removed with a syringe and respiration measurements commenced. Oxygen levels were measured continuously using a fluorescent fiber optic probe (foxy R probe, Ocean Optics, Dunedin, FL, USA) until oxygen levels had declined by 10%. Oxygen consumption rates were calculated using linear regression and expressed relative to fish mass. Our goal was to measure respiration in animals freshly captured from the natural environment, and thus we chose to minimize the effects of holding time and food deprivation (Glass, 1968). Though the animals recovered overnight after capture, we cannot demonstrate that the duration of the adjustment period following transfer to the respirometry chamber (20 min) was sufficient to ensure that the fish exhibited a true routine metabolic rate. However, the respiration measurements obtained from these fish are in close agreement with other studies on these same species (see Discussion).

Enzyme assays and DNA extraction

Fish were anaesthetized in a solution of tricaine methane sulphonate (0.4 g l^{-1}) and sodium bicarbonate (0.8 g l^{-1}). After fish were killed, their masses were recorded and white muscle samples taken from the epaxial region near the dorsal fin. Muscle samples were rapidly frozen in liquid nitrogen and stored at –80°C. Tissues were powdered in liquid nitrogen and stored at –80°C.

Enzyme extracts were prepared by homogenizing powdered tissue in 20 volumes of homogenization buffer (20 mmol l^{-1} Hepes, 1 mmol l^{-1} EDTA, 0.1% Triton X-100, pH 7.2) using a ground-glass homogenizer. Homogenates were used directly without centrifugation. Enzyme activities were assayed using a Spectromax plate spectrometer (Molecular Devices, Sunnyvale, CA, USA) in 96-well format at 25°C.

Pyruvate kinase (PK) activity, measured within 2 h of homogenization, was assayed in 50 mmol l^{-1} Hepes (pH 7.4), 5 mmol l^{-1} ADP, 100 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 0.15 mmol l^{-1} NADH, 0.01 mmol l^{-1} fructose 1,6-biphosphate, 5 mmol l^{-1} phosphoenolpyruvate and excess lactate dehydrogenase (10 units ml^{-1}). All substrate levels were saturating.

Citrate synthase (CS) was assayed on the tissue extracts that had been frozen at –80°C. Freeze–thawing the homogenate typically improved specific activity by about 10%. Activity was assayed in 50 mmol l^{-1} Tris (pH 8.1), 0.1 mmol l^{-1} 5,5′-dithiobis(2-nitro-benzoic acid), 0.15 mmol l^{-1} acetyl CoA, and

0.5 mmol l^{-1} oxaloacetate (omitted for the control). All substrate levels were saturating.

DNA extraction

Tissue samples were suspended in buffer (200 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, 50 mmol l^{-1} EDTA, 0.10% SDS, pH 8.0) with proteinase K (0.2 mg ml^{-1}) and digested overnight. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the sample was mixed thoroughly and centrifuged for 10 min at 1700 ***g***. The aqueous phase was retained and DNA precipitated by the addition of 0.1 volume ammonium acetate (7.5 mol l^{-1}) and 2 volumes of 100% ethanol. The solution was centrifuged for 3 min at 1700 ***g*** and washed with 70% ethanol. The pellet was air-dried and resuspended in 250 μl double distilled water. DNA purity was assessed using absorbance at 260 nm and 280 nm, then quantified based on the 260 nm reading.

RNA analysis

Powdered tissue was diluted and homogenized using a homogenizer (Polytron, Lucerne, Switzerland) in 10 volumes of RNA extraction buffer containing guanidine thiocyanate, purified and analyzed as previously described (Moyes et al., 1997). RNA was separated on a 1% agarose-formaldehyde gel. The gels were blotted overnight onto a nylon membrane (Duralon, Strategene, La Jolla, CA, USA) and RNA was fixed to the membrane using UV cross-linking.

To negate the effects of minor sequence differences between our experimental species, we used heterologous probes to assess mRNA levels. The probe for PK was based on zebra fish sequence (GenBank accession no. BC067143), created using the primers: PK-F 5′-TGTGTCTGCTGGACATCGACT-3′ and PK-R 5′-TCATGGTTCTCCAGCTTGCT-3′. The CS probe was homologous to swordfish (GenBank accession no. AY461851) with primers: CS-F 5′-GGATCAAGARCTTC-AAACAGCAG-3′ and CS-R 5′-GTTGGYGAAATTAKSG-GACCAGTC-3′. Membranes were prehybridized for 3 h in Church's solution, composed of 1 mol l^{-1} Na_2PO_4, 0.5 mol l^{-1} EDTA, 20% sodium dodecyl sulphate (SDS). After prehybridization, the membrane was incubated in hybridization medium, containing radiolabelled cDNA probe (boiled and cooled rapidly on ice). After hybridization overnight, the membrane was washed twice at 42°C in a solution of 0.15 mol l^{-1} NaC1, 0.015 mol l^{-1} sodium citrate, 0.1% SDS, pH 7 (i.e. 1× SSC/0.1% SDS), then twice at 65°C in 0.015 mol l^{-1} NaCl, 0.0015 mol l^{-1} sodium citrate, 0.1% SDS, pH 7 (i.e. 0.1× SSC/0.1% SDS). The membrane was exposed to a Kodak phosphor imager screen and bands were quantified using a Molecular Dynamics Typhoon System and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

Rates were examined by transforming data to a log–log plot. Linear regression determined the relationship between the two variables. The slope of the log–log plot was the scaling coefficient. Analysis of covariance (ANCOVA) was used to test whether scaling coefficients were significantly different between species ($P \leq 0.05$). All statistical calculations were completed using JMP 6.0 software.

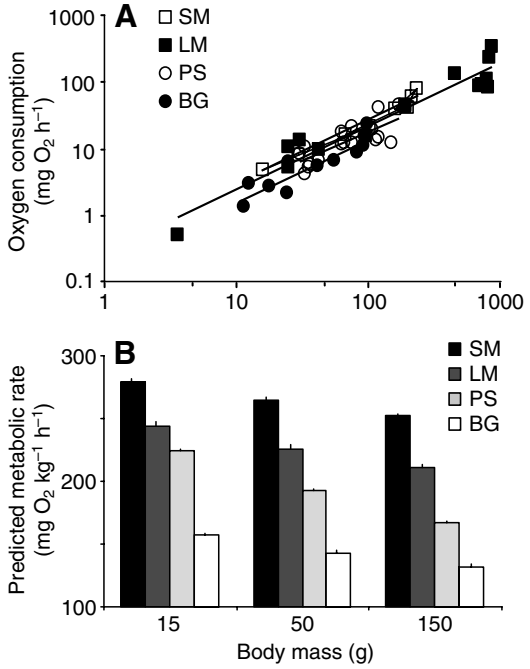

Fig. 1. Whole animal metabolic rate. (A) Estimates of metabolic rate as calculated by O_2 consumption (mg O_2 h^{-1}) at 21°C. BG, bluegill sunfish: y=0.93x–0.72, N=10, R^2=0.81; PS, pumpkinseed sunfish: y=0.87x–0.50, N=18, R^2=0.62; LM, largemouth bass: y=0.94x–0.54, N=14, R^2=0.93; SM, smallmouth bass: y=0.96x–0.51, N=9, R^2=0.97. (B) Log–log relationships were used to predict metabolic rate (mg O_2 kg^{-1} h^{-1}) of a standardized 15 g, 50 g and 150 g fish (±95% confidence interval).

Results

Whole animal metabolic rate

No allometric scaling was observed for whole animal metabolic rate. Whether all four species were analyzed together or individually, the scaling coefficients were not statistically distinguishable from 1 (Fig. 1A). Scaling coefficients ranged from +0.96 (smallmouth bass) to +0.87 (pumpkinseed sunfish). When these relationships were used to compare species at a given size, there were only modest differences in metabolic rates between species for a standardized small fish (15 g), medium fish (50 g) and larger fish (150 g) (Fig. 1B), and these were not significant.

Enzyme specific activities

The specific activity of PK in white muscle increased with body size, with values for b ranging from +0.08 (smallmouth bass) to +0.23 (pumpkinseed sunfish) (Fig. 2A). Scaling coefficients were not significantly different between species. When expressed per gram of DNA (to account for differences in myonuclear content), increased positive scaling was observed with values for b ranging from +0.34 (bluegill sunfish) to +0.37 (smallmouth bass), and scaling coefficients not significantly different between species (Fig. 2B). Based on these equations, for a standardized 150 g fish, the bass species (largemouth and smallmouth) had a similar PK activity, as did the sunfish species (pumpkinseed and bluegill), though the PK activity in the bass pair was about 40% greater than in the sunfish.

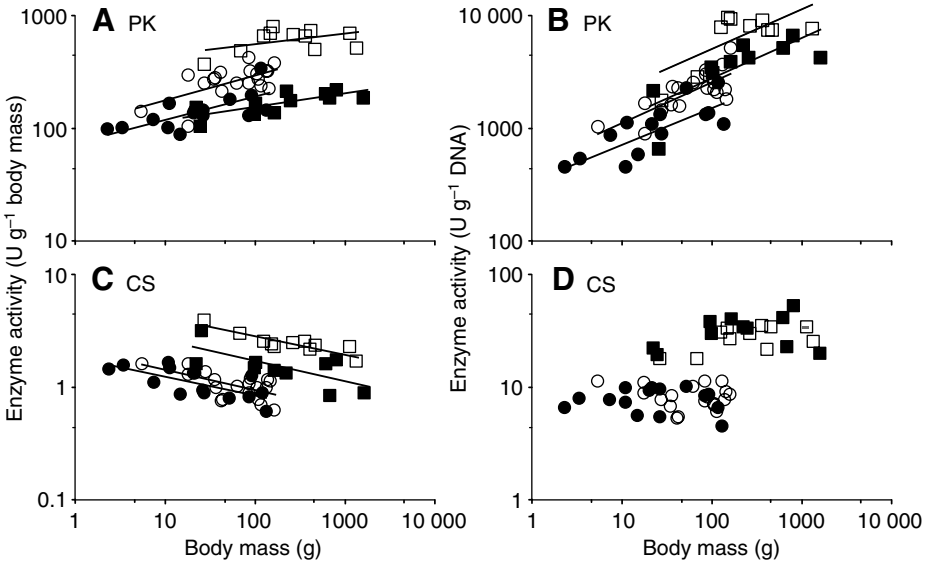

Fig. 2. Activities of (A,C) pyruvate kinase (PK) and (B,D) citrate synthase (CS), relative to (A,B) mass (U g^{-1} body mass) and (C,D) DNA (U g^{-1} DNA). Symbols for species are the same as in Fig. 1. Linear regression equations: (A) bluegill sunfish, y=0.19x+1.89, N=15, R^2=0.47; pumpkinseed sunfish, y=0.22x+2.02, N=19, R^2=0.39; largemouth bass, y=0.12x+1.94, N=11, R^2=0.56; smallmouth bass, y=0.08x+2.58, N=11, R^2=0.17; (B) bluegill sunfish, y=0.34x+2.54, N=15, R^2=0.62; pumpkinseed sunfish, y=0.36x+2.70, N=19, R^2=0.58; largemouth bass, y=0.37x+2.71, N=11, R^2=0.61; smallmouth bass, y=0.37x+2.97, N=11, R^2=0.54; (C) bluegill sunfish, y=–0.17x+0.25, N=15, R^2=0.48; pumpkinseed sunfish, y=–0.19x+0.32, N=19, R^2=0.39; largemouth bass, y=–0.17x+0.55, N=11, R^2=0.42; smallmouth bass, y=–016x+0.75, N=11, R^2=0.77. (D) Bluegill sunfish, y=–0.02x+0.91, N=15, R^2=0.01; pumpkinseed sunfish, y=–0.05x+0.99, N=19, R^2=0.04; largemouth bass, y=–0.07x+1.33, N=11, R^2=0.08; smallmouth bass, y=–0.13x+1.14, N=11, R^2=0.32.

Mass-specific enzyme activity for CS scaled negatively with body mass (Fig. 2C). The b values range from –0.15 (smallmouth bass) to –0.19 (pumpkinseed sunfish) and were not significantly different between species. When expressed per gram of DNA, CS demonstrated an insignificant linear regression, with scaling coefficients ranging from –0.02 (bluegill sunfish) to –0.13 (smallmouth bass) (Fig. 2D). Based on these equations, for a standardized 150 g fish, the bass species showed approximately a 2.5-fold higher CS activity than did the sunfish species.

The ratio of mass-specific enzyme activity of PK to CS increased with body size, yielding positive scaling coefficients ranging from +0.24 to +0.41 (smallmouth bass, b=0.24, R^2=0.51; largemouth bass b=0.30, R^2=0.64; bluegill sunfish b=0.36, R^2=0.75; pumpkinseed sunfish b=0.41, R^2=0.58).

Gene expression

If the observed specific enzyme activities were due to transcriptional regulation of their respective genes, then the ratio of PK/CS enzyme activities should parallel the ratio of PK mRNA/CS mRNA (Fig. 3). While the PK/CS enzyme ratio increased with body mass, the PK/CS mRNA ratio was largely unaffected by body size. This suggests that simple transcriptional regulation cannot explain the size-dependent differences in both PK and CS.

The activity of PK/PK mRNA was statistically indistinguishable between species or within species, as a function of body size (Fig. 3C). Thus, the interspecific and intraspecific differences in PK activities can best be explained by differences in PK gene expression.

The ratio of CS/ CS mRNA significantly decreased with an increase in body size (Fig. 3D). Thus, the negative allometric scaling of CS enzyme is not due to CS gene expression but rather due to a post-transcriptional mechanism.

For nucleic acids, the species were found to have concentrations of DNA ranging from 0.05 mg DNA g^{-1} fish mass (largemouth bass) to 0.14 mg DNA g^{-1} fish mass (pumpkinseed sunfish), suggesting differences in fiber geometry. RNA

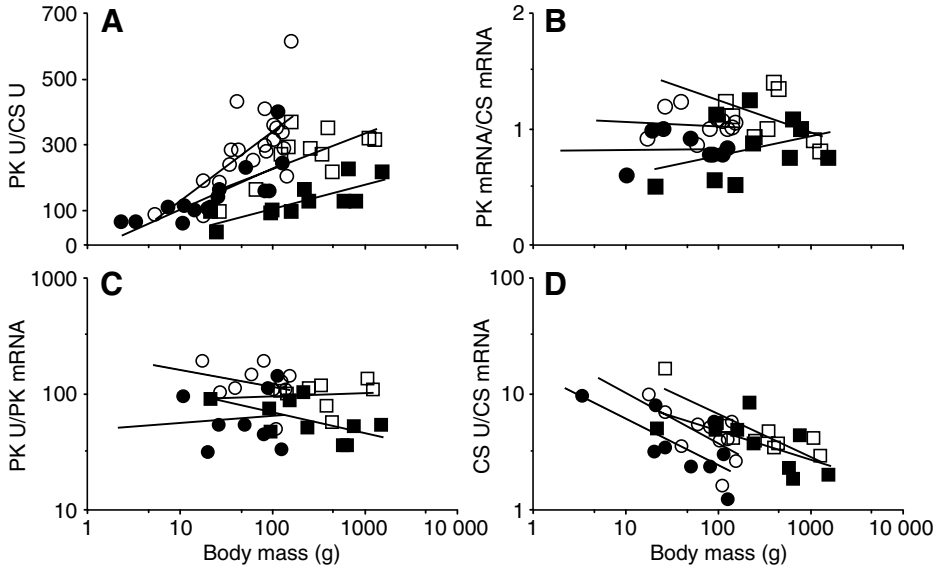

Fig. 3. (A,B) Relative PK and CS catalytic activities (U) and mRNA levels and (C,D) PK and CS catalytic activities relative to mRNA levels for each enzyme. Symbols for species are the same as in Fig. 1. The regression lines for panel C are for illustrative purposes.

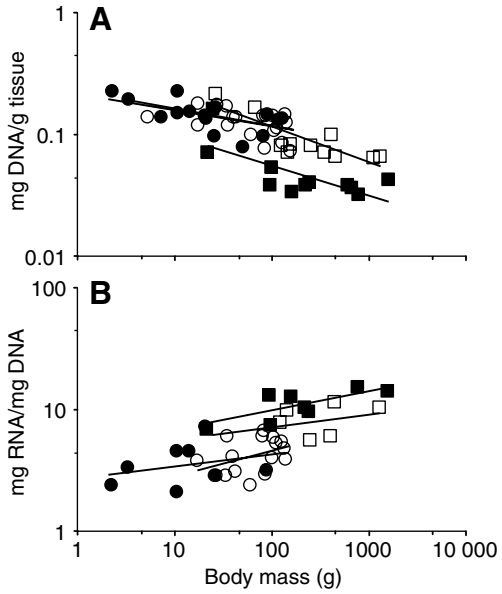

Fig. 4. mg DNA:g tissue (A) and RNA:DNA ratios (B) for each centrarchid species. Symbols for species are the same as in Fig. 1. Linear regression equations: (A) bluegill sunfish, y=–0.15x–0.66, N=15, R^2=0.39; pumpkinseed sunfish, y=–0.14x–0.68, N=19, R^2=0.22; largemouth bass, y=–0.24x–0.78, N=11, R^2=0.52; smallmouth bass, y=–0.28x–0.39, N=11, R^2=0.67; (B) bluegill sunfish y=0.09x+0.44, N=15, R^2=0.06; pumpkinseed sunfish, y=0.21x+0.24, N=17, R^2=0.16; largemouth bass, y=0.18x+0.62, N=11, R^2=0.59; smallmouth bass, y=0.10x+0.65, N=11, R^2=0.08.

concentrations ranged from 0.45 mg RNA g^{-1} fish mass (largemouth bass) to 0.55 mg RNA g^{-1} fish mass (pumpkinseed sunfish). The ratio of RNA/DNA showed positive scaling in each species: largemouth bass, b=+0.18; smallmouth bass, b=+0.05; pumpkinseed sunfish, b=+0.36; and bluegill sunfish, b=+0.09 (Fig. 4).

Discussion

We investigated the relationship between body size and metabolic phenotype both intraspecifically and interspecifically among two bass species and two sunfish. These local species were chosen as they are frequently found in the same waterways, but despite their geographic overlap they differ in microhabitat selection and behaviour. For example, the sunfish are relatively slow swimmers, living in shallow water often among weeds. Juveniles of both species feed on invertebrates in the littoral zone, but as adults they have separate niches; adult bluegill feed on zooplankton in open water and adult pumpkinseeds feed on snails in the littoral zone (Mittelbach, 1984). Within each sunfish species there are polymorphisms in morphology associated with feeding strategies, possibly resulting from phenotypic plasticity and genetic variation (e.g. Ehlinger and Wilson, 1988). Sunfish morphs can be specialized as littoral/benthic forms feeding on macrophytes, or pelagic/limnetic forms feeding on zooplankton (Mittelbach, 1984). Among the bass, smallmouth bass tend to be found deeper in the water column of lakes and in faster flowing rivers (Peake and Farrell, 2004). Largemouth bass are sit-and-wait predators, whereas smallmouth bass are more active predators. The bass species grow faster and larger than the sunfish pair. Thus, though these are closely related fish, they have differences that might be expected to manifest as variation in metabolic phenotype. There are also differences in the way individual centrarchid species acclimate to temperature (Tschantz et al., 2002). Nonetheless, it is a model system that presents opportunities to study potential molecular mechanisms underlying phylogenetic and ontogenetic scaling patterns.

Scaling of metabolic rate

Metabolic rates were measured in fish that had recovered at least 12 h after capture from the wild then transferred to a respirometry chamber where they were left for 20 min prior to respirometry measurements. This approach reflected a strategy that was the best compromise between fish availability (freshly caught), a need for short holding times (minimizing food deprivation) and logistic constraints (respirometry chambers). Though there is a potential concern that the fish might not have been at their absolute routine metabolic rates, our data are in close agreement with other studies on perciforms in general and sunfish and black bass specifically. Based on a typical 50 g fish at 20°C, we found the following rates of oxygen consumption (mg O_2 h^{-1}): smallmouth bass, 12.9; largemouth bass, 11.3; pumpkinseed sunfish, 9.6; bluegill sunfish, 7.2. Expressed per kg body mass, we found the following rates (mg O_2 kg^{-1} h^{-1}): smallmouth bass, 259; largemouth bass, 226; pumpkinseed sunfish, 192; bluegill sunfish, 143. For comparison, 50 g perciform fish have a predicted metabolic rate of 8.7 mg O_2 h^{-1} (at 20°C, assuming Q_{10}=2) (Clarke and Johnston, 1999). Rates for bluegill sunfish and longear sunfish (*Lepomis megalotis*) fell within the range of 123–192 mg O_2 kg^{-1} h^{-1} (Dent and Lutterschmidt, 2003). Some studies show lower metabolic rates; rates of 118 mg O_2 kg^{-1} h^{-1} for bluegill sunfish and 126 mg O_2 kg^{-1} h^{-1} for largemouth bass were reported (Moss and Scott, 1961), though in these studies the fish were held in the laboratory for at least 72 h without feeding. The metabolic rate of largemouth bass, for example, declines by 50% by 48 h post-feeding (Glass, 1968). By reducing the time from angling to oxygen consumption measurements to about 12 h, the effect of starvation can be kept to a minimum. While we have no evidence that our approach elevated respiration above routine metabolic rate, there is little reason to believe that it would differentially affect size classes of fish or the allometric relationships.

Previous studies on fish have shown that scaling coefficients are similar in most respects to those shown in tetrapods. In a meta-analysis of 138 studies of 69 species, whole animal rates of oxygen consumption scaled with a mean slope of +0.79, and range of +0.65 to +0.95 (Clarke and Johnston, 1999). The scaling coefficients for centrarchids found in the present study (b=+0.87 to +0.96) are within the normal range seen in fish. Previous studies have found centrachids to show little or no scaling, with b values ranging from +0.80 to +1.06 in bluegill sunfish (Wohlschlag and Juliano, 1959), and no scaling was observed in largemouth bass and bluegill sunfish larger than 15 g (Moss and Scott, 1961). In general, our scaling coefficients are similar to the results of previous studies on these fish and their relatives, though the collection of studies shows centrarchids to be on the higher end of the range seen in fish. Scaling coefficients in fish tend to be closer to 1 in less active

species (Morris and North, 1984) and those with low metabolic rates (see Clarke and Johnston, 1999; Glazier, 2005).

Scaling of metabolic enzymes

In contrast to the lack of scaling of mass-specific metabolic rate, the scaling of metabolic enzyme specific activities was more pronounced. Specific activities of our oxidative enzyme (CS) scaled negatively with body mass (Fig. 2A) and glycolytic enzyme (PK) specific activities scaled positively with body mass (Fig. 2B). These enzyme patterns seen within species (i.e. ontogenetic variation) are similar to those published previously on other species (Norton et al., 2000; Burness et al., 1999; Yang and Somero, 1996). Previous studies have attributed differences in metabolic enzymes to locomotor strategy (Somero and Childress, 1980), and explained how this is affected by factors such as predator–prey interactions (Goolish, 1991a) and hydrodynamic constraints (Goolish, 1991b; Norton et al., 2000). In white muscle, glycolytic enzyme levels likely reflect the requirement for ATP production to support high intensity (burst) exercise, whereas oxidative enzymes reflect the demands for both resting and recovery metabolism.

In addition to the size-dependent variation, we also saw differences between species that are consistent with previous studies and known differences in lifestyle. For example, smallmouth bass are considered the most athletic of the species we studied; they showed the highest specific activities of both CS and PK, and mass-specific metabolic rate. When comparing between species, controlling for body size, differences in white muscle glycolytic enzyme activity are most clearly related to locomotion and activity levels (Somero and Childress, 1980). Thus, largemouth and smallmouth bass, not surprisingly, have higher glycolytic enzyme activities than do sunfish. However, environmental factors may also play a role. Within the sunfish pair of species, pumpkinseeds had higher glycolytic enzyme activity than bluegill, which may be part of a strategy for hypoxia tolerance (Farwell et al., 2006). Water bodies with seasonal or diurnal hypoxia often support pumpkinseed sunfish populations, but lack bluegill sunfish (Keast and Fox, 1990).

Transcriptional determinants of PK gene expression

It is challenging to establish definitely the genetic mechanisms responsible for particular patterns of metabolic enzyme levels seen between animals or physiological states. If a difference/change in protein is correlated with a difference/change in mRNA, then the most parsimonious explanation is that the patterns are due to transcriptional regulation. In the present study, differences in PK activities seen with size were paralleled by differences in PK mRNA in each of the four species examined. Furthermore, the interspecific differences in PK activities were also paralleled by mRNA patterns. Thus, the differences seen in PK activities in both phylogenetic and ontogenetic comparisons are consistent with transcriptional regulation. At this point, we cannot comment on the nature of the transcriptional regulators that dominate the control of expression of the PK gene. A similar finding was made when studying the allometric scaling of PK in rainbow trout. Body size led to parallel increases in PK catalytic activity and PK mRNA in trout up to about 1 kg in mass (Burness et al., 1999). Larger trout showed the same scaling of PK catalytic activity, but with considerably lower PK mRNA levels. Conversely, Yang and Somero examined the underlying basis of LDH activities in another teleost species and found that LDH mRNA levels did not parallel LDH catalytic activities (Yang and Somero, 1996). Thus, it is unlikely that simple transcriptional regulation explains the patterns for all glycolytic enzymes in relation to phylogenetic and ontogenetic scaling.

The transcription factors that control PK gene expression under normoxic conditions are not yet known (reviewed by Moyes and LeMoine, 2005). As with many genes that encode housekeeping enzymes, transcription factors of the specificity protein 1 (Sp1) family likely play an important role in control of expression of PK genes in most tissues (e.g. Li et al., 2004). The nature of the transcriptional regulators that drive the greater expression of the PK genes in large fish (of each species) and bass (relative to sunfish) remains unclear. Under hypoxic conditions, the *PKM* gene, as well as other glycolytic genes, is induced though the HIF-1 pathway (Semenza, 2000; Semenza et al., 1994). Hypoxia leads to stabilization of the transcription factor hypoxia-inducible factor (HIF1α), activating genes with HIF-responsive elements. We cannot directly rule out the possibility that the higher levels of PK in pumpkinseed are part of a physiological response to environmental hypoxia. Pumpkinseed and bluegill overlap in their biogeographic distribution. Though our fish were captured in the same region of the lake, it remains possible that movements within different microhabitats could contribute to differences in PK between sunfish species.

Post-transcriptional determinants of CS gene expression

Mitochondrial oxidative capacity of a tissue is probably best indicated by the levels of cytochome oxidase. Under most conditions, a stoichiometry is preserved between COX and other proteins of the electron transport system and thus COX is a good proxy for oxidative capacity in a tissue. COX is a complex multimeric protein composed of ten nuclear-encoded gene products and three mitochondrial-encoded gene products. It remains unclear which (if any) of the specific COX genes is ‘rate-limiting’ to COX synthesis and thus we would have a difficult time showing that changes in mRNA for a specific COX subunit affect COX holoenzyme levels. Since our goal was to link gene expression to enzyme levels, we chose to measure CS rather than COX. In contrast, CS is a homodimer and thus a clearer linkage between gene expression and enzyme activity can be assessed.

One potential explanation for differences in mitochondrial gene expression relates to differences in myonuclear domain (see Moyes and LeMoine, 2005). Smaller myofibers typically have higher levels of both nuclei and mitochondria per g tissue. Thus, higher levels of nuclear gene transcripts (per g tissue) can arise even if there are no differences in gene expression *per se.* In this study, the negative scaling in CS enzyme activities diminished when enzymes were expressed relative to DNA. Thus, as an animal grows, the myonuclear domain remained constant, as did the quantitative relationship between nuclear content and mitochondrial content. There were obvious clade-dependent differences in myonuclear domain, with the bass species showing about twice the mitochondrial content of the sunfish species.

Based on the lack of scaling seen when CS levels were

reported relative to DNA, the most parsimonious explanation for ontogenetic patterns in CS patterns is that changes in myonuclear domain drive the changes in mitochondrial content. Thus, we initially predicted that CS enzyme levels would reflect CS mRNA levels, which would in turn reflect CS gene levels (i.e. nuclear content). However, this simple model of transcriptional determination of CS levels was not supported; CS mRNA per nucleus was not constant, and CS enzyme levels were not reflected in CS mRNA levels (Fig. 3). This suggests that in each of our species the declines in CS levels with size are due to changes in post-transcriptional pathways; larger animals have a decreased level of CS enzyme with higher CS mRNA. Muscle of larger fish could have lower translation efficiency of the CS transcripts, shorter CS protein half-life, or faster organelle turnover. The negative scaling in CS activity could be related to a reduced metabolic rate in larger fish, despite our observation of isometric scaling of whole-animal oxygen consumption. The small size range (1–2 orders of magnitude) and potential methodological artifacts discussed above for oxygen consumption reduces our confidence in such scaling coefficients. Though the nature of post-transcriptional regulation of CS levels is unknown, we have found similar results in other contexts. There is a poor relationship between CS mRNA and CS activity in rainbow trout of differing size (Burness et al., 1999). The differences in CS activities of homologous muscles of tuna and billfish do not appear to be explained by transcriptional regulation (Dalziel et al., 2004).

In conclusion, metabolic rate was largely independent of body mass in each of the species we studied. However, the specific activities of glycolytic enzymes (PK) showed positive scaling and oxidative enzymes (CS) showed negative scaling. From analysis of mRNA levels, the observed positive scaling of PK is likely due to transcriptional regulation. In contrast, the negative scaling of CS likely arises though post-transcriptional regulation. These results suggest that no single factor controls the reciprocal scaling of oxidative and glycolytic enzyme activities, but several factors may influence the maximal enzyme activity by either increased gene expression or alteration of the enzyme at the mRNA transcript or protein level.

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