ALLOMETRIC SCALING IN CENTRARCHID FISH

Origins of Intra- and Inter-specific Variation in Oxidative and Glycolytic Enzyme Levels in Muscles

by

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Abstract

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. Two pairs of sister species of centrarchid fishes were studied: black bass (largemouth bass, *Micropterus salmoides* and smallmouth bass, *Micropterus dolomieui*), and sunfish (pumpkinseed, *Lepomis gibbosus* and bluegill, *Lepomis macrochirus*). The goal was to assess the regulatory basis of both intraspecific and interspecific variation in relation to body size, as well as 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.
Co-Authorship

Chapter 2, “Allometric scaling in centrarchid fish: Origins of intra- and inter-specific variation in oxidative and glycolytic enzyme levels in muscle,” was co-authored with Dr. C. D. Moyes. The thesis was edited by Dr. C. D. Moyes and Dr. Y. X. Wang.
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<tbody>
<tr>
<td>BMR</td>
<td>Basal Metabolic Rate</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome Oxidase</td>
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<tr>
<td>CS</td>
<td>Citrate Synthase</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
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<tr>
<td>FMR</td>
<td>Field Metabolic Rate</td>
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<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MMR</td>
<td>Maximal Metabolic Rate</td>
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<tr>
<td>NRF</td>
<td>Nuclear Respiratory Factor</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenol Pyruvate</td>
</tr>
<tr>
<td>PGC1alpha</td>
<td>PPAR Gamma Coactivator-1 alpha</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate Kinase</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activated Receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SMR</td>
<td>Standard Metabolic Rate</td>
</tr>
<tr>
<td>Sp</td>
<td>Specificity Factor</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Chapter 1: Introduction and General Literature Review

1.1 Overview

It has been recognized for more than 100 years that the rate of physiological processes is affected by body mass. Several theories have been put forth to explain the scaling of whole animal metabolic rate, ranging from single-cause explanations of supply system limitations (e.g., West et al. 2002), to multi-cause assessments of the cellular pathways that determine the metabolic phenotype (e.g., Darveau et al. 2002). These model-oriented approaches address ultimate causes of scaling patterns across diverse models. An alternative approach to understanding metabolic scaling is to address more proximate relationships. Muscle, for example, shows the same patterns of scaling of oxidative enzymes. Regardless of the underlying basis for metabolic phenotype in whole animals, the molecular mechanism regulating the reciprocal change in muscle phenotype is still unknown. Muscles also exhibit phenotypic plasticity in bioenergetic enzymes as a result of differences between muscle fiber types, in response to ecological and behavioral changes and throughout ontogeny. In my thesis, I use fish experimental models to explore the mechanisms regulating muscle phenotype, an approach that integrates molecular genetics, muscle physiology, fish ecology and metabolic gene expression.

I investigated the relationship between the scaling of oxygen consumption and oxidative enzymes, and the regulatory basis of the reciprocal relationship between oxidative and glycolytic enzymes. Fish were chosen as model for this study for a variety of reasons. Firstly, fish muscle is organized in ways that facilitate studies of muscle cell biology. The trunk musculature is composed two muscle fiber types: oxidative red fibers
(slow oxidative or type I) and glycolytic white fibers (fast glycolytic or type IIb). The two fiber types are organized in homogeneous regions that are easily separated. The white muscle accounts for greater than 70% of fish mass (see Sanger and Stoiber 2001). Secondly, many fish exhibit indeterminate growth (see Mommsen and Moon 2001), unlike mammals, allowing me to study adult animals of a wide size range but similar body geometry.

Two pairs of closely related species: largemouth bass (*Micropterus salmoides*), smallmouth bass (*M. dolomieui*), pumpkinseed sunfish (*Lepomis gibbosus*) and bluegill sunfish (*L. macrochirus*), were studied. While these species are closely related, they are diverse in ecology enabling investigation of other factors that might influence metabolic profile, like activity level and hypoxia tolerance. Furthermore, the use of closely related species means the influences of both ontogeny and phylogeny on metabolic phenotype can be investigated.

### 1.2 Allometric Scaling of Whole Animal Metabolism

The relationship between body mass and a metabolic process, such as metabolic rate, is typically shown as a log-log plot. The linear regression is described by the equation $Y = a M^b$, where $Y$ is the metabolic process of interest, $a$ is the normalization coefficient, $M$ is the body mass, $b$ is the scaling coefficient. Scaling is termed allometric when the value for $b$ is less than 1. Scaling relationships are sometimes expressed in mass specific terms; a whole animal metabolic rate that scales with a slope of 0.75 shows a mass-specific scaling slope of $-0.25$. The effect of body size on metabolic processes has
been investigated since the 1800’s, Rubner (1883) found metabolic rate to scale with surface area in dogs (see Hulbert and Else 2004). It was not until the twentieth century when common themes in scaling relationships of whole animal metabolic rates emerged. In 1932, Kleiber analyzed the metabolic rate of several species of birds and mammals and found metabolic rate to scale with an exponent of $b = 0.75$, meaning larger animals have lower mass-specific metabolic rates. In the subsequent years, both the value of $b$ and the reason for the decline of mass-specific metabolic rate with body size have been debated.

The scaling of whole animal metabolic rate is influenced by an animal’s activity level, state of digestion and body temperature. Scaling studies traditionally examine the relationship between body mass and basal metabolic rate (BMR), defined as “the lowest energy demands of an animal at rest in a post-prandial state under thermoneutral conditions” (Weibel et al. 2004). In ectotherms this is sometimes referred to as standard metabolic rate (SMR) when environmental temperature is controlled (White and Seymour 2005). Whether there is a single $b$ value that is applicable to all animals is controversial, as numerous studies have found variation in scaling exponents across taxa.

Though basal metabolic rate in terrestrial endotherms displays scaling coefficients between +0.7 to +0.8 (as reviewed by Glazier 2005, White and Seymour 2005), aquatic ectotherms, show more variable $b$ values. In fish, metanalysis of resting whole animal metabolic rate shows mean scaling coefficients near +0.79, with a range of +0.65 to +0.95 (Clarke and Johnson 1999). Centrarchids, my taxa of interest, show little or no allometric scaling of SMR, with $b$ values ranging from +0.80 to +1.06 (Wohlschlag and Juliano 1959, Moss and Scott 1961). Bluegill sunfish have shown seasonal variation in
scaling coefficients (Wohlschlag and Juliano 1959), and pumpkinseed sunfish have demonstrated seasonal variation in basal metabolic rate, independent of acclimation temperature (Evans 1984). The wide variation observed in teleost scaling exponents suggests whole animal metabolic rate differs across species and season.

The activity state of an animal affects the degree to which metabolism scales with body size. Maximal metabolic rate (MMR) allometrically scales with body mass with greater $b$ values than basal metabolic rate. MMR is defined as “the energy needs of locomotor activity at its sustainable maximum during a run” (Weibel et al. 2004). Weibel and colleagues (2004) investigated MMR in 34 mammals and found scaling coefficients of +0.872, well above the range found for BMR. Furthermore, they found MMR varied amongst species and individuals not only with body size, but also the degree of athleticism. In teleost, scaling of MMR, like BMR varies across species with $b$ values ranging from 0.87 to 0.93 in marine teleost (Killen et al. 2006) to 1 (Goolish 1991b).

What remains unclear are the underlying mechanisms that are responsible for scaling of metabolic rate in resting and active animals. At rest, the majority of oxygen is consumed in all cells for basic cellular processes, like protein synthesis and maintaining membrane potential. During maximal exercise, the majority of oxygen demands are made by skeletal muscle. In mammals, maximal metabolic rate scales with the same scaling coefficient as skeletal muscle mitochondrial volume and capillary volume (Weibel et al. 2004). This suggests the aerobic capacity of the muscle, that is the ability of mitochondrial enzymatic pathways to convert oxygen to energy, may limit the maximal metabolic rate. The factorial aerobic scope (the ratio of MMR: BMR) is greater in both
larger animals and more athletic animals, suggesting both body size and activity levels increase the degree to which an organism can elevate metabolic rate from resting levels (Weibel et al. 2004, Killen et al. 2006). In teleost, aerobic scope increases throughout their lifespan (Killen et al. 2006). The bioenergetic pathways of skeletal muscle cells may influence whole animal maximal metabolic rate, as changes in mitochondrial enzyme activities may impact the aerobic scope of the animal.

Understanding the processes that regulate the scaling of energy metabolism in muscle is an important step in explaining why higher processes scale. Muscle tissue is a large contributor to whole animal metabolic rate, both at rest and during exercise. During maximal exercise, 90% of metabolic demands come from skeletal muscle (Weibel et al. 2004). Changes in the metabolic phenotype of a muscle fiber, such as the reciprocal decrease in oxidative enzymes and increase in glycolytic enzymes with body mass, may influence whole animal metabolic rate. Knowledge of the mechanism regulating this reciprocal change may help elucidate factors influencing the scaling of whole animal metabolic rate.

1.3 Muscle Phenotype

Skeletal muscle accounts for a large percentage of vertebrate body mass and metabolic activity. In mammals, there are four predominant muscle fiber types, type I, IIA, IIB, and IIX/d. Type I fibers have slow oxidative phenotypes and high myoglobin and mitochondria densities. These muscle fibers generally support long term, low intensity activity. Type II fibers are predominately glycolytic, but differ in mitochondrial densities. In general, these fibers support high intensity, short duration activity. Type IIA fibers have
greater mitochondria densities and are thus more aerobic than Type IIb fibers. The
distinction between fiber types, made on the basis of myosin heavy chain isoform
eexpression, is valid for all vertebrate locomotor muscles. However, fish and mammals
differ in the way fiber types are combined to produce a muscle, with profound
implications for studies of scaling.

In fish, skeletal muscle accounts for 60% of body mass and has three distinct fiber
types (Sanger and Stoiber 2001). In teleost fish, more than 70% of the muscle mass is
white muscle. White muscle is composed of type IIb fibers (fast-glycolytic). It is used in
burst swimming related to predator avoidance and prey capture (Sullivan and Somero
1980). Red muscle tissue is composed of Type I fibers (slow oxidative) and constitutes 5-
30% of overall skeletal muscle mass, depending on the species (Sanger and Stoiber
2001). This muscle tissue is highly aerobic, with greater vascularization and
mitochondrial densities of 25-44% compared to white muscles, which have mitochondrial
densities of only 2-4% (Johnson and Moon 1981). Some species have pink muscle in
regions localized between red and white muscle, composed of a mixture of fiber types. In
the Centrarchids I am studying, there is negligible pink muscle and, with the exception of
smallmouth bass, little red muscle in the trunk.

Mammals use combinations of different fiber types to produce each muscle.
Though a few specific muscles are dominated by a single fiber type, most mammalian
muscles are mixed fibers. One complication for scaling studies in mammals is that the
fiber type profile for a specific muscle (e.g., gastrocnemius) differs between large and
small animals as a result of different locomotory demands (Wang and Kernell 2001). In
fish, the muscles are composed of homogenous fiber types and the fiber type profile does not change with age, though its metabolic profile does change. Second, it is feasible to study a single muscle in mammals, but even the largest muscles contribute relatively little to body mass; thus, specific skeletal muscles in mammals are unlikely to play a large role in determining whole animal metabolic rate. In fish, muscle is a greater proportion of body mass and thus skeletal muscles play a greater role in whole animal metabolism.

Muscle cells, like all cells, have a metabolic phenotype that is the result of the enzymatic machinery, anatomical specializations (e.g., vascularization), and regulatory pathways. Muscle metabolic phenotypes differ not only between fiber types, but within a specific tissue, the phenotype may change as the animal grows and/or ages. Muscle growth can lead to irreversible changes in muscle phenotype. Post-embryonic muscle growth occurs through either hyperplasia (an increase in muscle fiber number) or hypertrophy (an increase in fiber size) (see Rowlerson and Veggetti 2001). In fish, there are two forms of hyperplasia. Stratified hyperplasia continues from the embryonic stage into the larval stage and is the process that increases slow muscle fibers (see Johnson 2006). Mosaic hyperplasia increases the number of fast muscle fibers in juveniles and young adults. As younger muscle fibers have smaller diameters, the collection of old and young muscle fibers creates a mosaic of fiber diameters, hence the name mosaic hyperplasia (see Johnson 2006). Hypertrophy can occur throughout ontogeny and in adults is the only mechanism for muscle growth (see Rowlerson and Veggetti 2001). Reversible changes in muscle phenotype can occur as a result of behavioral, environmental, and allometric changes related to body size. All these factors, as
elaborated below, can cause the muscle phenotype to respond in unique ways to meet the changing demands.

In this study, I am focusing on teleost white muscle. Teleost white muscle is a distinguishable glycolytic tissue that constitutes a large proportion of body mass in teleost and thus contributes largely to whole animal metabolism at rest and during exercise. White muscle phenotype is also responsive to changes in behavioral responses of the fish to the environment. The above characteristics make it the perfect candidate to investigate the molecular mechanisms responsible for the reciprocal change in muscle metabolic enzymes with body size.

1.4 Locomotory Behaviour

One of the factors that complicate interspecies studies of scaling is the influence of the ecological niche on metabolic properties. The ecological niche of an animal can influence locomotory behaviour, which influences the phenotype of skeletal muscle (Somero and Childress 1990). White muscle, the tissue of interest in this study, is recruited for high intensity burst exercise. Independent of size, animals may differ in the importance of high intensity exercise in their life style. For example, sit-and-wait predators, such as northern pike, undertake short, intense bursts to capture prey. Though pelagic fish engage red muscle for cruising, they also recruit white muscle frequently to capture prey or evade predators. Sullivan and Somero (1980) found skeletal muscle from pelagic fish species, such as the half moon *Medialuna californiensis*, and northern anchovy *Engraulis mordax*, to have 2-4 times the glycolytic activity of lactate dehydrogenase (LDH), and nearly twice the activity of pyruvate kinase (PK) than the
more sedentary benthic species the white croaker *Genyoenemus lineatus* and the ocean whitefish *Caulolatilus princeps*. Locomotory behaviour also influences aerobic capabilities of muscle cells. In white muscle, mitochondria support basal metabolic demands, but also fuel recovery from burst exercise (Moyes et al. 1992). Mitochondrial enzyme activity shows similar trends as glycolytic enzyme activity when comparing pelagic and benthic species (Somero and Childress 1990). Thus, when comparing species, differences related to lifestyle are superimposed on differences related to body size. It is not clear whether all of the interspecies differences are evolutionary adaptations or if an element of the interspecies differences reflects phenotypic plasticity, essentially a training response. Long term low intensity exercise training in rainbow trout has led to increased mitochondrial density and mitochondrial enzyme activity (Farrell et al. 1991) and increased gene expression of citrate synthase (McClelland et al. 2006). Thus, the evolution and ecology of fish can influence the muscle metabolic phenotype in complex ways.

### 1.5 Environmental influences

Temporal and spatial changes in habitat impacts whole animal metabolic rate and can lead to compensatory changes in skeletal muscle phenotype. Seasonal temperature fluctuations can result in changes in the metabolic phenotype between species and individuals. Temperate teleost fish face the challenge of maintaining energy production while dealing with large seasonal variations in temperature ranging from 0-4°C in winter to 25-30°C in summer (Sanger and Stoiber 2001). In many species, exposure to cold temperatures leads to an increased aerobic phenotype (Guderley 1990, Battersby and
The magnitude of phenotypic change between cold- and warm-acclimated teleost is comparable to differences resulting from exercise training (see Moyes and Battersby 1998, McClelland et al. 2006). The degree of the phenotypic change is variable amongst species and unrelated to phylogeny (Tschantz et al. 2002). The regulatory basis for the changes in muscle metabolic phenotype with temperature is not yet clear. Though the fish in this study were collected when summer water temperatures had stabilized, the centrarchids do not typically change their muscle metabolic phenotype seasonally (Tschantz et al. 2002).

Like temperature, oxygen availability affects the metabolic rate of animals. Survival in hypoxia requires a redistribution of metabolic energy demands to allow ATP demand to meet ATP supply. Generally, increased tolerance to hypoxia is marked by a reduction of energy expenditure in cellular processes such as protein synthesis and increased energy production by anaerobic pathways, leading to an increase in glycolytic efficacy and a down-regulation of aerobic metabolism (Hochachka and Lutz 2001). Thus, environmental hypoxia may cause an increase in glycolytic phenotype in skeletal muscles.

Hypoxia tolerance varies between species, even those that are closely related, and increased hypoxia tolerance in teleost can help define ecological niches. Fish living in the Amazon River deal with severe variations in oxygen availability on a seasonal and daily basis. The Amazonian cichlid, *Astronotus ocellatus*, acclimates to the fluctuations in oxygen availability by activating glycolysis and down-regulating aerobic metabolism in white muscle (Richards et al. 2007). In temperate teleost, low oxygen concentrations
during the winter months are a huge challenge, and may limit the range of certain species. Amongst centrarchids, pumpkinseed sunfish have been found to have greater glycolytic enzyme activity than bluegill sunfish, suggesting an increased tolerance for hypoxia (Farwell et al. 2007). Pumpkinseed sunfish are frequently found in water bodies that experience temporal or spatial hypoxia and which lack bluegills, suggesting that an increased tolerance for hypoxia has aided in defining their ecological niche (Keast and Fox 1990).

These bioenergetic responses to environmental fluctuation, in the form of temperature and oxygen availability, further demonstrate the plasticity of skeletal muscle phenotype. The ability of an organism to alter glycolytic and mitochondrial enzyme levels in response to environmental change can help define their ecological niche. While much is known about the regulation of these phenotypic changes, many questions remain about the molecular mechanisms.

1.6 Allometric Scaling of Muscle Metabolism

Many studies have proposed that whole animal metabolic rate should scale with body size to a universal exponent. However, whole animal metabolic rate scaling coefficients show great variability across species, especially amongst teleosts. Similar to the controversy surrounding the idea of a universal scaling coefficient, over the years a multitude of mechanistic explanations for why metabolic scaling occurs has been proposed.

Some studies have attempted to understand the basis of allometric scaling based on single-cause biophysical constraints. Banavar and colleagues (2002) proposed a flow
theory that suggests the 0.75 allometric scaling is a result of supply constraints. In larger animals the constraint of delivering materials to tissues is the limiting factor that causes the observed allometric scaling. West and colleagues (1997, 2002) also suggest supply is the biophysical constraint that generates the observed 0.75 scaling exponent, but propose that a fractal-like branching network supplies materials to the organism, of which the final branches are size invariant and the energy required to distribute materials is minimized, assuming that as a result of natural selection organisms have evolved to maximize metabolic rate (West et al. 1999). These theories both suggest that larger animals have decreased metabolic rate because they are constrained by supply networks. In reality many organisms metabolic systems exist at a level to meet demands greater than basal metabolism (Weibel 2002).

Darveau and colleagues (2002) proposed a multi-cause explanation for metabolic scaling that shows how multiple cellular processes regulate whole animal metabolic scaling and provides an explanation for the difference in $b$ values observed between basal and maximal metabolic rate. While at rest basal metabolic rate is predominately determined by cellular processes such as ion transport and protein synthesis. However, when an organism exercises, the metabolic demands change from basic maintenance to high ATP demand in skeletal muscles.

The supply and demand models put forth by Banavar et al. (2002) and West et al. (1997, 1999, 2002) suggest supply networks such as the vascular system in animals are the limiting constraint responsible for the 0.75 allometric scaling. If this is the case, then the scaling coefficient of cells grown in culture should be close to 1. However, Porter and
Brand (1993) found that liver hepatocytes grown in vivo show allometric metabolic scaling, suggesting metabolic scaling occurs on a cellular level. Cellular processes demonstrate allometric scaling as well. In skeletal muscle, oxidative enzyme activities have been found to decrease with body mass in mammals (Emmett and Hochachka 1981) and fish (Somero and Childress 1980). Reciprocally, glycolytic enzymes have been found to increase with body mass, in both mammals (Emmett and Hochachka 1981) and fish (Somero and Childress 1980, Burness et al. 1999, Norton et al. 2000). This allometric scaling has led larger animals to have a different muscle phenotype that is less oxidative and more glycolytic than smaller animals. Whether a single or multiple factors regulate the molecular mechanism responsible for this phenotypic plasticity is unknown.

1.7 Glycolytic Metabolism

White muscle relies on glycolysis to produce high rates of ATP to support burst exercise. The basic glycolytic pathway, conserved for 4 billion years of evolution (Webster 2003), converts one glucose or glucosyl molecule into 2 molecules of pyruvate. For each molecule of pyruvate produced, 1 molecule of NADH and 1-1.5 molecules of ATP (depending on starting substrate) are produced. Under anaerobic conditions lactate dehydrogenase catalyzes the reduction of pyruvate to lactate and NADH to NAD⁺, enabling maintenance of redox state without oxidative phosphorylation. If oxygen is present, pyruvate is converted to acetyl CoA, the entry substrate for the Krebs cycle. Glycolysis has multiple roles in the cell in addition to generating pyruvate for oxidative phosphorylation, maintaining the cellular redox balance through the generation of
NADH, and energy production under anaerobic conditions of high intensity locomotion and environmental hypoxia, such as production of nucleotides and muscle glycogen.

Glycolytic enzymes may be regulated at all stages of protein synthesis from transcriptional regulation to post-translational modifications (see Moyes and LeMoine 2005). Several glycolytic enzymes are allosterically regulated by their products. An example of this being hexokinase, a regulatory enzyme that converts glucose to glucose-6-phosphate. The regulatory enzyme pyruvate kinase (PK) is also allosterically regulated. PK catalyzes the transfer of the phosphoryl group of phosphoenol pyruvate (PEP) to ADP to form pyruvate and ATP (Valentini et al. 2000). PK has been found to be allosterically inhibited by the amino acid Phe (Yu et al. 2003), and excess concentrations of the product ATP has been found to reduce the affinity of PK for the substrate PEP. PK is of interest to me, as it is the final regulatory enzyme of glycolysis, so changes in PK activity can represent changes in overall glycolytic metabolism.

As the main energy pathway for anaerobic energy production during both environmental hypoxia and high intensity exercise, the regulation of glycolysis is tightly linked to oxygen availability. Two transcription factors are known to play a role in the regulation of glycolysis, hypoxia-inducible factor-1 alpha (HIF-1 alpha), and the specificity protein (Sp) family group of factors. Under normoxic conditions, the HIF-1 alpha protein is targeted for ubiquitin degradation by hydroxylation of a conserved proline residue (Dann et al. 2002). In hypoxic conditions, this hydroxylation does not occur and HIF-1 alpha activates transcription of target genes by binding hypoxia response elements in their promoters (Semenza 2006). HIF-1 alpha up-regulates pyruvate kinase
M, lactate dehydrogenase a, and many other genes involved in glycolysis and the hypoxia response.

The Sp family of transcription factors consists of four proteins (Sp1, Sp2, Sp3 and Sp4) that are involved in glycolytic regulation. These proteins bind GC-box promoter elements and all have similar domain structure consisting of 3 zinc fingers near the C-terminus and a glutamine rich area near the N-terminus (Suske 1999). The ratio of Sp1 to Sp3 has been shown to regulate glycolytic transcription (Li et al. 2003). When Sp1 binds a promoter element, transcription is activated. The actions of Sp3 depend on the promoter region. If a promoter has one binding site, then Sp3 acts as a transcriptional activator, but if a promoter has multiple binding sites the binding of Sp3 prevents the transcriptional activation by Sp1 (Suske 1999). These transcription factors regulate the expression of several genes involved in glycolysis, including glucose-6-phosphate isomerase, phosphofructokinase, aldolase A, beta-enolase, pyruvate kinase and glucose transporter 1 (as reviewed by Moyes and LeMoine 2005). Like hypoxia inducible factor, Sp1/Sp3 glycolytic regulation is sensitive to cellular oxygen levels. Under hypoxic conditions, Sp3 is down-regulated, preventing transcriptional repression and therefore leading to an increase in Sp1 activation of pyruvate kinase M and beta-enolase transcription (Disher et al. 1998).

1.8 Mitochondrial Metabolism

Under aerobic conditions, vertebrates meet energy demands predominately by mitochondrial metabolism. The mitochondria plays a crucial role in the production of energy to meet metabolic needs during rest, steady state exercise and recovery from burst
exercise. In aerobic conditions, oxidative metabolism converts 1 molecule of glucose and 6 molecules of oxygen to 36 molecules of ATP, 6 molecules of carbon dioxide and 42 molecules of molecular water (Hochachka and Somero 2002). In addition to energy production the mitochondria plays a role in redox balance, generates heat and produces reactive oxygen species.

The regulation of mitochondrial enzyme profiles is challenging, as it requires the coordination of both nuclear and mitochondrial gene expression (Goffart and Wiesner 2003, Moyes et al. 1998). Several transcription factors have been identified to be involved in this process. The nuclear respiratory factor 1 (NRF1) is a homodimeric DNA binding protein that is involved in the activation of both nuclear and mitochondrial genes, such as cytochrome c (Kelly and Scarpulla 2004). The peroxisome proliferator activated receptors (PPARs) are a subfamily of nuclear hormone receptors involved in the regulation of lipid metabolism (see Moyes and LeMoine 2005). The PPAR gamma coactivator-1α (PGC-1 alpha) regulates gene expression of slow muscle contractile proteins and has been suggested to be the master controller of mitochondrial regulation (Lin et al. 2005, Moyes and LeMoine 2005, Lehman and Kelly 2002).

In teleost, the transcriptional regulator for differences in aerobic phenotypes is still unknown. Differences between red and white fiber type phenotypes appear to be regulated by nuclear encoded genes (Battersby and Moyes 1998). An increase in aerobic capacity and the transcription factor NRF-1 in zebrafish skeletal muscle in response to both cold acclimation and exercise training suggests a conserved response for changing aerobic phenotypes exist (McClelland et al. 2006).
1.9 Gene Expression and Allometric Scaling

Whether the regulation of the reciprocal change in muscle enzymes with body size is under translational or transcriptional control is still unknown. Previous studies examining the relationship between enzyme activity and mRNA levels have found differing results. In barred sand bass, LDH activity increased with body mass, but LDH mRNA levels did not parallel this change (Yang and Somero 1996). In rainbow trout, PK activity and PK mRNA increased in parallel in fish less than 1000g, but in larger fish PK mRNA levels were lower than PK enzyme activities (Burness et al. 1999). The lack of correlation between enzyme activities and mRNA levels suggests transcriptional regulation does not explain glycolytic scaling patterns. Instead, increased rates of translation or decreased rates of enzyme degradation in larger fish may explain the observed scaling patterns. While under hypoxic conditions the transcription factors HIF-1 alpha and Sp1 / Sp3 have been shown to regulate the expression of many glycolytic enzymes, the regulation of glycolytic genes such as PK under normoxic conditions is not fully understood (see Moyes and LeMoine 2005).

Like the glycolytic scaling patterns, the regulation of the changes in mitochondrial enzymes is still unknown. Burness et al. (1999) also examined mitochondrial enzymes in rainbow trout and found neither CS activity nor CS mRNA transcripts scaled with body size. The regulation of mitochondrial metabolism is complex and several transcription factors are involved. The transcription factor Sp1 has been found to play a role both in glycolytic regulation and the regulation of several mitochondrial genes (see Kelly and
Scarpulla 2004). If the reciprocal scaling of muscle metabolic enzymes was regulated by the same factor, Sp1 could be a potential candidate.

1.10 Fish Ecology

In previous sections I outlined the main reasons that fish in general are both useful models (e.g., muscle fiber homogeneity) and interesting variants (e.g., environmental responsiveness). In this study, two pairs of sister species from the family Centrarchidae were used as model organisms. The goal of this approach was to provide an opportunity to investigate the genetic basis of patterns in metabolism and metabolic enzymes arising with both ontogenetic and phylogenetic differences in body mass. The species used were the black basses largemouth (*Micropterus salmoides*), smallmouth (*M. dolomieui*) and the sunfish bluegill (*Lepomis macrochirus*), and pumpkinseed (*L. gibbosus*). All species are local to the region and while they have unique ecological niches, may inhabit the same waterways, facilitating capture and controlling for water quality differences.

The sunfish are slower swimmers than the black basses. As juveniles, both bluegill and pumpkinseed sunfish share similar diet and habitat, living in the littoral zone feeding on non-gastropod vegetation dwelling prey (Mittelbach 1984). As adults, once predation pressures are reduced and adult pharyngeal morphology develops, these species experience ontogenetic niche shifts and both diet and habitat diversifies (Werner and Hall 1988). Bluegills feed on open water zooplankton, such as *Daphnia*, a soft-bodied prey suitable for their fine needle like pharyngeal teeth (Mittelbach 1984). Adult pumpkinseed sunfish have strong molariform pharyngeal teeth, morphologically different than bluegills
that are used to crush gastropods living in the vegetated littoral zone. While adult pumpkinseed sunfish are usually benthic in the littoral zone, a planktivorous limnetic form has also been found in the pelagic zone (Robinson and Wilson 1996). This trophic polymorphism results from predominately phenotypic plasticity, although genetic variation may be a contributing factor.

Of the black basses, the smallmouth bass tend to be found deeper in the water column of lakes and in faster flowing rivers (Peake and Farrell 2004). Smallmouth bass are also more active predators in comparison to the sit-and-wait predation style of largemouth basses. Largemouth bass feed on littoral invertebrates during their first year, and once size advantage is established over the young of the year sunfish, largemouth become piscivorous (Olson 1996). Largemouth can be found in most inshore habitats (Keast 1978). Largemouth are typically the top predator in eastern North American water bodies.

While these four species are closely related, ecological differences may be reflected by metabolic phenotype differences. Metabolic differences have already been shown in centrarchids in species-specific acclimation to cold (Tschantz et al. 2002). Pumpkinseed sunfish exhibit a more glycolytic phenotype than bluegill sunfish in response to hypoxia (Farwell et al. 2007). The plasticity of metabolic phenotypic these species demonstrate makes them excellent models to study the molecular mechanism for the reciprocal change in glycolytic and oxidative enzymes observed in scaling patterns.
1.11 Hypotheses

The goals of this study are to investigate (1) the relationship between metabolic rate and oxidative enzymes in the same animals and (2) the regulatory basis of the reciprocal relationship between oxidative and glycolytic enzymes in relation to scaling. I expect to find (1) metabolic rate and oxidative enzymes will scale to the same degree and (2) the scaling of glycolytic and oxidative enzymes will be transcriptionally regulated, suggesting the reciprocal relationship is controlled by a single factor.

As muscle mitochondrial enzymes are indicative of aerobic capacity, whole animal metabolic rate and muscle mitochondrial enzymes should display similar scaling with respect to body size. Previous studies investigating mitochondrial metabolism in muscle found it to decrease with body mass, similar to previously published values in mammals (Emmett and Hochachka 1981) and fish (Somero and Childress 1980), but did not compare enzyme activities and whole animal metabolism in the same animals. I expect to observe a parallel decrease in mass-specific whole animal metabolic rate and oxidative enzyme activity with body size in this study.

If the reciprocal scaling of oxidative and glycolytic enzymes with body mass is regulated by a single factor, then it is likely that changes in both enzymes are transcriptionally regulated. While there is not a transcription factor that is known to regulate both glycolytic and oxidative metabolism, it is possible that the change in enzymes with body size is regulated by one factor. It is also possible that these two processes are independent and a result of different physiological demands. It has been shown that glycolytic phenotype is related to burst exercise in teleost and therefore is
likely that increased demands for burst exercise in larger fish are responsible for the increase in glycolytic phenotype. Likewise, the decreased mitochondrial enzyme activity seen in larger fish may be to a necessity to decrease reactive oxygen species (ROS) (see Moyes and LeMoine 2005). ROS are produced as a result of mitochondrial metabolism and are harmful to the cell in large numbers. Thus a decrease in mitochondrial metabolism in larger fish decreases the amount of ROS produced and may lead to a longer life span of the organism. Investigating the molecular mechanisms regulating this phenotypic change can clarify whether a single factor is responsible for the allometric scaling of muscle metabolism.
Chapter 2: Allometric Scaling in Centrarchid Fish: Origins of Intra- and Inter-specific Variation in Oxidative and Glycolytic Enzyme Levels in Muscle

2.1 Introduction

Many metabolic processes depend on body size, and are usually described by the equation $Y = a M^b$, 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 (1932) compared the respiratory rate of birds and mammals of differing sizes and described a scaling coefficient of $+0.75$. 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 Johnson 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). Emmett and Hochachka (1981) studied gastrocnemius muscle of selected mammals, showing that 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). 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). Somero and Childress (1980) showed that 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).

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 Hochachka 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 (1981), the gastrocnemius, 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 in 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 greater than 70% of the total muscle mass (Sanger and Stoiber 2001), more than 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 exhibit indeterminate growth (Mommsen and Moon 2001), such as centrarchids, 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 independent 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 salmoides*), smallmouth bass (*M. dolomieui*), 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.

### 2.2 Methods

#### 2.2.1 Animal and tissue collection

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

#### 2.2.2 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 (Ocean Optics foxy R probe) until dissolved oxygen concentration 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).

2.2.3 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, masses were recorded and white muscle samples were 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 Molecular Devices SpectroMax plate spectrometer in 96-well format at 25°C.
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.

CS was assayed on the tissue extracts that had been frozen at −80°C. Freeze-thawing the homogenate typically improving specific activity by about 10%. It 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.

**2.2.4 DNA extraction**

Tissue samples were suspended in buffer (200 mM 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.
2.2.5 RNA Analysis

Powdered tissue was diluted and homogenized using a Polytron homogenizer 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) 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: BC067143), created using the primers:

PK-F 5’-TGTGTCTGCTGGACATCGACT-3’
PK-R 5’-TCATGGTTCTCCAGCTTGCT-3’.

The CS probe was homologous to swordfish (GenBank: AY461851) with primers:

CS-F 5’-GGATCAAGARCTTCAAACAGCAG-3’
CS-R 5’-GTTGGYGAAATTAKSGGACCAGTC-3’.

Membranes were prehybridized for 3 h in Church’s solution, composed of 1 mol l⁻¹ Na₂PO₄, 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 NaCl, 0.015 mol l⁻¹ sodium citrate, 0.1% SDS, pH 7 (i.e., 1x 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., 0.1x 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).

### 2.2.6 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.

### 2.3 Results

#### 2.3.1 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 (Figure 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) (Figure 1b) which were not significant.
Figure 1. Whole animal metabolic rate.
Panel A. Estimates of metabolic rate as calculated by O\textsubscript{2} consumption (mg O\textsubscript{2} h\textsuperscript{-1}) performed at 21°C. Equations were: for bluegill sunfish (BG), log\textit{y} = 0.93 log\textit{x} – 0.72, \(n = 10\), \(R^2 = 0.81\); pumpkinseed sunfish (PS), log\textit{y} = 0.87 log\textit{x} – 0.50, \(n = 18\), \(R^2 = 0.62\); largemouth bass (LM), log\textit{y} = 0.94 log\textit{x} – 0.54, \(n = 14\), \(R^2 = 0.93\); smallmouth bass (SM), log\textit{y} = 0.96 log\textit{x} – 0.51, \(n = 9\), \(R^2 = 0.97\). Panel B. Log-log relationships were used to predict metabolic rate (mg O\textsubscript{2} (kg h\textsuperscript{-1}) of a standardized 15 g, 50 g and 150 g fish (+/- 95% confidence interval).

2.3.2 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) (Figure 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), with scaling coefficients not significantly different between species (Figure 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.

Mass-specific enzyme activity for CS scaled negatively with body mass (Figure 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) (Figure 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 $b = 0.41$, $R^2 = 0.58$).
Figure 2. Enzyme activities.
Enzyme activities are shown relative to mass (U / g mass) in panels A (PK) and B (CS), and relative to DNA (U / g DNA) in panels C (PK) and D (CS). Symbols for species are the same as Figure 1. Equations for linear regressions of data are as follows: Panel A: bluegill sunfish, logy = 0.19logx + 1.89, n = 15, R² = 0.47; pumpkinseed sunfish, logy = 0.22logx + 2.02, n = 19, R² = 0.39; largemouth bass, logy = 0.12logx + 1.94, n = 11, R² = 0.56; smallmouth bass, logy = 0.08logx + 2.58, n = 11, R² = 0.17. Panel B: bluegill sunfish, logy = 0.34logx + 2.54, n = 15, R² = 0.62; pumpkinseed sunfish, logy = 0.36logx + 2.70, n = 19, R² = 0.58; largemouth bass, logy = 0.37logx + 2.71, n = 11, R² = 0.61; smallmouth bass, logy = 0.37logx + 2.97, n = 11, R² = 0.54. Panel C: bluegill sunfish, logy = -0.17logx + 0.25, n = 15, R² = 0.48; pumpkinseed sunfish, logy = -0.19logx + 0.32, n = 19, R² = 0.39; largemouth bass, logy = -0.17logx + 0.55, n = 11, R² = 0.42; smallmouth bass, logy = -0.16logx + 0.75, n = 11, R² = 0.77. Panel D: As the data is not significant, regressions are provided for illustrative purposes. bluegill sunfish, logy = -0.02logx + 0.91, n = 15, R² = 0.01; pumpkinseed sunfish, logy = -0.05logx + 0.99, n = 19, R² = 0.04; largemouth bass, logy = -0.07logx + 1.33, n = 11, R² = 0.08; smallmouth bass, logy = -0.13logx + 1.14, n = 11, R² = 0.32.
2.3.3 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 (Figure 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 activities of PK/ PK mRNA were statistically indistinguishable between species or within species, as a function of body size (Figure 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 (Figure 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 (largemouth bass) to 0.14 mg DNA / g (pumpkinseed sunfish), suggesting differences in fiber geometry. RNA concentrations ranged from 0.45 mg RNA / g (largemouth bass) to 0.55 mg RNA / g (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 $b = +0.09$ (Figure 4).
2.4 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.

2.4.1 Scaling of metabolic rate

Metabolic rates were measured in fish that had recovered at least 12h 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 50g fish at 20°C, we found the following rates (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 h)$^{-1}$): smallmouth bass, 259; largemouth bass, 226; pumpkinseed sunfish, 192; bluegill sunfish, 143. For comparison, Clarke and Johnson (1999) reported that 50g perciform fish have a predicted metabolic rate of 8.7 mg O$_2$ h$^{-1}$ (at 20°C, assuming Q$_{10}$ = 2). Dent and Lutterschmidt (2003) found that rates for bluegill sunfish and longear sunfish (Lepomis megalotis) fell within the range of 123 – 192 mg O$_2$ (kg h)$^{-1}$. Some studies show lower metabolic rates; Moss and Scott (1961) reported rates of 118 mg O$_2$ (kg h)$^{-1}$ for bluegill sunfish and 126 mg O$_2$ (kg h)$^{-1}$ for largemouth bass, though the fish were held in the lab at least 72h without feeding. The metabolic rate of largemouth bass, for example, declines by 50% by 48h post-feeding (Glass 1968). By reducing the time from angling to oxygen consumption measurements to about 12h, 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, Clarke and Johnston (1999) found that whole animal rates of oxygen consumption scaled with a mean slope of $+0.79$, and range of $+0.65$ to $+0.95$. The scaling coefficients we found for centrarchids ($b = +0.87$ to $+0.96$) are within the normal range seen in fish.
Previous studies have found centrarchids 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 15g (Moss and Scott 1961). In general, our scaling coefficients are similar to 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 Johnson 1999, Glazier 2005).

2.4.2 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 (Figure 2a) and glycolytic enzyme (PK) specific activities scaled positively with body mass (Figure 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 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 is 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. 2007). Water bodies with seasonal or diurnal hypoxia often support pumpkinseed sunfish populations, but lack bluegill sunfish (Keast and Fox 1990).

2.4.3 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 this 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. Burness et al. (1999) found that body size led to parallel increases in PK catalytic activity and PK mRNA in trout up to about 1kg in mass. Larger trout showed the same scaling of PK catalytic activity, but with considerably lower PK mRNA levels.

Conversely, Yang and Somero (1996) examined the underlying basis of LDH activities in another teleost species and found that LDH mRNA levels did not parallel LDH catalytic activities. 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. 2003). It remains unclear 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). 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.

### 2.4.4 Post-transcriptional determinants of CS gene expression

Mitochondrial oxidative capacity of a tissue is probably best indicated by the levels of cytochrome 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 10 nuclear-encoded gene products and 3 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 is related 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, 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 than 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 (Figure 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 lesser 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 through post-transcriptional regulation. These results suggest that no single factor controls the reciprocal scaling of oxidative and glycolytic enzyme activities, but several factors that may influence the maximal enzyme activity by either increased gene expression or alteration of the enzyme either at the mRNA transcript or protein level.
Chapter 3: General Discussion

This study investigated the regulation of metabolism and metabolic enzymes in centrarchids. Two pairs of sister species, the largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieui*), and the pumpkinseed sunfish (*Lepomis gibbosus*) and bluegill sunfish (*Lepomis macrochirus*), were used as models to examine the influence of both ontogeny and phylogeny on metabolic scaling. This study was the first to investigate (1) the relationship between metabolic rate and oxidative enzymes in the same animals and (2) the regulatory basis of the reciprocal relationship between oxidative and glycolytic enzymes in relation to scaling.

3.1 Scaling of Metabolic Rate

In centrarchids, whole animal metabolic rate was not found to scale with body mass, but muscle enzymes scaled as expected. Oxidative enzyme activity (citrate synthase, CS), decreased with body mass and glycolytic enzyme activity (pyruvate kinase, PK) increased. The increase in PK activity per g tissue correlated with increased PK mRNA per g tissue, suggesting regulation occurs at the transcriptional level. Conversely, the decrease in CS activity per g tissue did not correlate with transcript levels, suggesting post-transcriptional regulation. These results suggest multiple factors are involved in regulating the reciprocal scaling of glycolytic and oxidative metabolism.

One surprising outcome from the current study was the lack of allometric scaling seen in whole animal metabolic rate. Although I believe that the results are consistent
with previous studies, it is possible that the study was complicated by artificially elevated metabolic rate determinations. Studies in mammals suggest that as activity levels increase scaling coefficients increased from +0.686 at BMR, to +0.73 at field metabolic rate (FMR), to finally +0.87 at maximal metabolic rate (MMR) (see White and Seymour 2005). If our metabolic rate measurements taken were not truly SMR, this might have influenced our scaling relationships.

Due to logistic constraints, it was not possible to allow the animals long periods of time to become accustomed to the respirometry chamber. Though fish were allowed to recover from the stress of angling for 12h prior to analysis, respiration measurements began only 20 min after transfer to the respirometer by dip net. Since metabolic rate measurements took several hours, during which no change in metabolic rate was observed, I believe that the 20 min acclimation time to the respirometer was sufficient. The short acclimation time was chosen to decrease the effects of holding on the fish and reduce the potential impacts of starvation on metabolic rate (Glass 1968). Improvements to this study could be made by extending the acclimation time to verify that true basal measurements were taken. However, since the metabolic rates determined matched previously published metabolic rates for these species no change in results would be expected with a longer holding time.

### 3.2 Scaling of Metabolic Enzymes

The positive scaling of glycolytic muscle enzyme activity provides increased power for burst exercise in larger fish (Somero and Childress 1990). Larger fish require
more power than smaller fish to maintain the same burst exercise speed (Goolish 1989).
In this study, PK mRNA levels paralleled increased PK activity in white muscle of larger
fish, suggesting transcriptional regulation. The factor regulating this increase in
transcription is not known and could be a potential area for future exploration. In
mammals under hypoxia, the transcription factor HIF-1 up-regulates genes involved in
glucose transport and anaerobic metabolism, such as muscle PK (Semenza 2000,
Semenza et al. 1994). In fish, similar patterns of gene regulation have been observed in
the goby fish (*Gillichthys mirabilis*) during hypoxia, suggesting a conserved role of HIF-
1 (Gracey et al. 2001). The role of HIF-1 during conditions other than environmental
hypoxia is not clear, but recent studies have found evidence of exercise induced HIF-1
transcriptional regulation in mammals (Ameln et al. 2005). Acute burst exercise
increased HIF-1 alpha protein levels, DNA binding activity and gene expression of the
HIF-1 target genes VEGF and EPO in human skeletal muscle. Whether HIF-1 is
responsible for the increased PK expression to meet the demands of burst exercise in fish
is not known. On one hand, it is unlikely that HIF-1 affects constitutive expression
because it has a prominent role in initiating a hypoxic response. However, HIF-1
regulation of genes is proving to be very complex, with multiple isoforms of the alpha
subunit (1,2,3), which exhibit different degrees of inducibility and may exert repressor
effects under some conditions.

While CS activity, a marker of oxidative capacity, decreased with body mass, CS
mRNA transcript levels did not parallel this change suggesting post-transcriptional
regulation of this enzyme. CS enzyme activity did not display negative scaling relative to
DNA. This suggests that myonuclear domains are constant throughout ontogeny. That CS activity relative to DNA increased interspecific differences, suggests increased levels of CS activity in bass species versus sunfish is a result of phylogeny. The mechanism of CS post-transcriptional regulation is not clear, and could be an area for potential research. Several studies have found a lack of correlation between CS mRNA and CS enzyme activity and elucidation on the regulation of CS could be of great interest.

3.3 Interspecific Trends

The centrarchid model used in this study was ideal to investigate the gene regulatory basis of allometric scaling of metabolism. The use of closely related species of similar body geometry enabled a comparison of metabolic scaling with respect to differences in ontogeny and phylogeny. From the data, several interesting interspecific trends emerge that suggest future areas for investigation in the regulation of muscle phenotype with regards to both environmental hypoxia and locomotory behaviour. Firstly, the increased PK activity levels in pumpkinseed sunfish compared to bluegill sunfish suggest a potential model for examining the regulation of hypoxia tolerance. Secondly, the increased CS activity levels in the bass species suggest a useful model for investigating changes in muscle phenotype resulting from locomotory behaviour.

A significant interspecific difference was observed between the sunfish pair, where pumpkinseed sunfish were observed to have 50% greater PK activity than bluegill sunfish, suggesting pumpkinseeds may have increased glycolytic potential than bluegills. As increased glycolytic potential is associated with increased hypoxia tolerance.
(Hochachka and Lutz 2001), this suggests that of the sunfish, pumpkinseeds have a greater tolerance to hypoxia. Previous studies have found pumpkinseed sunfish to be more tolerant to lower oxygen levels than bluegill sunfish (Farwell et al. 2007). In addition, pumpkinseed sunfish are often found in water bodies lacking bluegills that are prone to variable seasonal oxygen fluctuations (Fox 1994). An increased hypoxia tolerance may enable pumpkinseeds to survive in these lakes that experience chronic or seasonal hypoxia, such as winterkill (Keast and Fox 1990). The pumpkinseed sunfish in this study were from the same lake as the bluegill sunfish and therefore would be expected to experience the same seasonal variations in oxygen concentrations. However, adult pumpkinseed and bluegill sunfish have distinct ecological niches and therefore habitat differences may result in pumpkinseed sunfish experiencing hypoxia resulting from spatial oxygen concentration differences in the lake. Since the pumpkinseeds in this study were found to have increased glycolytic activity, suggesting increased hypoxia tolerance, the question arises whether this difference in muscle phenotype between species is a result phenotypic plasticity to the environment or an evolutionary adaptation.

In hypoxic conditions glycolytic genes, including muscle PK, are upregulated by HIF-1 alpha (see Semenza 2000). In the presence of oxygen, the hydroxylation of a conserved proline residue tags HIF-1 alpha for degradation by ubiquitin (Dann et al. 2002). In hypoxia this hydroxylation does not occur and HIF-1 alpha is able to act as a transcription factor increasing the expression of genes associated with hypoxia tolerance, such as muscle PK. Thus HIF-1 transcriptional regulation, resulting from environmental hypoxia may be one mechanism increasing glycolytic activity in pumpkinseeds.
Increased hypoxia tolerance may have evolved through mutations to HIF-1 alpha that would prevent degradation under normoxic conditions. A mutation on the conserved proline residue of HIF-1 alpha could prevent degradation under normoxic conditions and lead to constant upregulation of glycolytic gene expression. Further research is needed to determine the regulatory basis of increased hypoxia tolerance in pumpkinseed sunfish, and whether it is a result of acclimation to environmental hypoxia or an evolutionary adaptation.

As maintaining increased glycolytic metabolism is costly, increased hypoxia tolerance must confer ecological advantages to pumpkinseed sunfish. One possible advantage could be decreased competition by an extended habitat range. Pumpkinseed and bluegill sunfish share ecological niches as juveniles and the resulting competition from bluegills negatively impacts pumpkinseed growth (Mittelbach 1988). An increased hypoxia tolerance would enable pumpkinseeds to inhabit a wider area of the lake, decreasing competition pressures. Pumpkinseeds may also be able to use the hypoxic regions of the lake for predator avoidance. This behaviour has been observed in Lake Victoria; African cichlids use hypoxic regions of the lake as refuge from predation from the hypoxia intolerant Nile perch (Chapman et al. 2002). In Canadian lakes, juvenile pumpkinseed sunfish are susceptible to predation by largemouth bass (see Keast 1978). In laboratory settings, adult largemouth bass have been shown to avoid hypoxic regions of water (Burleson et al. 2001), suggesting the use of hypoxic regions to avoid predation by largemouth bass could be an effective strategy.
Another interesting interspecific difference was increased oxidative enzyme activity in the bass species in comparison to the sunfish species. Both bass species are more athletic than the sunfish, frequently engaging in burst exercise for predation. The increased oxidative enzyme activity likely is a result of increased demands for recovery from burst exercise. Teleost white muscle uses energy from mitochondrial metabolism to metabolize lactate produced by burst exercise (Moyes et al. 1992). The differences in muscle phenotype between the bass and sunfish are of interest for future investigation for the regulation of phenotype changes associated with locomotory behaviour.

### 3.4 Conclusions

In conclusion the centrarchid model used was effective for the investigation of the regulation of metabolic scaling. Changes in muscle enzyme regulation with growth and between species was determined and future research for this model system came to light. No allometric scaling of whole animal metabolic rate was observed, although muscle oxidative enzymes showed negative scaling. While the scaling of muscle oxidative enzymes did not reflect the scaling of metabolic rate, it is possible that they do reflect a decrease metabolism in larger fish, as artificially elevated metabolic rates may have affected these results. When oxidative enzymes were expressed per gram of DNA, no scaling was observed, similar to metabolic rate. No single factor was found to regulate the reciprocal scaling of muscle oxidative and glycolytic enzymes. Citrate synthase appears to be post-transcriptionally regulated, while the intra and interspecific variation in pyruvate kinase seemed to be mediated through transcriptional mechanisms.
Literature Cited


