2006

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Recommended Citation

Martinez, M.L., Landry, C.A., Boehm, R.D., Manning, S., Cheek, A.O., and Rees, B.B. 2006. Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, Fundulus grandis. Journal of Experimental Biology, 209: 3851-3861.

The Journal of Experimental Biology 209, 3851-3861
Published by The Company of Biologists 2006
doi:10.1242/jeb.02437

Effects of long-term hypoxia on enzymes of carbohydrate metabolism in the Gulf killifish, *Fundulus grandis*

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Accepted 11 July 2006

Summary

The goal of the current study was to generate a comprehensive, multi-tissue perspective of the effects of chronic hypoxic exposure on carbohydrate metabolism in the Gulf killifish *Fundulus grandis*. Fish were held at approximately 1.3 mg l^{-1} dissolved oxygen (~3.6 kPa) for 4 weeks, after which maximal activities were measured for all glycolytic enzymes in four tissues (white skeletal muscle, liver, heart and brain), as well as for enzymes of glycogen metabolism (in muscle and liver) and gluconeogenesis (in liver). The specific activities of enzymes of glycolysis and glycogen metabolism were strongly suppressed by hypoxia in white skeletal muscle, which may reflect decreased energy demand in this tissue during chronic hypoxia. In contrast, several enzyme specific activities were higher in liver tissue after hypoxic exposure, suggesting increased capacity for carbohydrate metabolism. Hypoxic exposure affected fewer enzymes in heart and brain than in skeletal muscle and liver, and the changes were smaller in magnitude, perhaps due to preferential perfusion of heart and brain during hypoxia. The specific activities of some gluconeogenic enzymes increased in liver during long-term hypoxic exposure, which may be coupled to increased protein catabolism in skeletal muscle. These results demonstrate that when intact fish are subjected to prolonged hypoxia, enzyme activities respond in a tissue-specific fashion reflecting the balance of energetic demands, metabolic role and oxygen supply of particular tissues. Furthermore, within glycolysis, the effects of hypoxia varied among enzymes, rather than being uniformly distributed among pathway enzymes.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/209/19/3851/DC1

Key words: anaerobic metabolism, gluconeogenesis, glycolysis, gene regulation, low oxygen.

Introduction

Because of the rich evolutionary history and current ecological diversity of teleost fish, the study of this group has been particularly informative in elucidating the responses of animals to hypoxia (Nikinmaa and Rees, 2005). These responses include behaviors to avoid hypoxic areas, utilize well-aerated microenvironments, or reduce activity (Kramer, 1987; Van den Thillart et al., 1994; Dalla Via et al., 1998; Wannamaker and Rice, 2000); physiological and morphological adjustments that improve the oxygen extraction and delivery to tissues (Jensen et al., 1993; Sollid et al., 2003); and biochemical changes that increase the capacity of tissues to function and survive at low oxygen (Hochachka, 1980; Van den Thillart and Van Waarde, 1985; Hochachka and Somero, 2002). In general, this suite of responses serves to compensate for the decrease in environmental oxygen availability, and results in hypoxia tolerance that, in some species, is quite marked.

One biochemical response to hypoxia is an increase in anaerobic ATP production, typically *via* glycolysis (Van den Thillart and Van Waarde, 1985; Dalla Via et al., 1994; Virani and Rees, 2000). A number of studies indicate that hypoxic exposure increases the activities of glycolytic enzymes that presumably augment the capacity of fish tissues for anaerobic energy production (Greaney et al., 1980; Johnston and Bernard, 1982; Van den Thillart and Smit, 1984; Dickson and Graham, 1986; Lushchak et al., 1998; Zhou et al., 2000; Kraemer and Schulte, 2004). However, these increases were not uniformly observed among glycolytic enzymes, across tissues, or among species. For example, hypoxic exposure of killifish, tench, and goldfish led to increased activities of some glycolytic enzymes in liver, but not in white skeletal muscle (Greaney et al., 1980; Johnston and Bernard, 1982; Van den Thillart and Smit, 1984). The opposite trend, increased enzyme activities in muscle and no changes in liver, was observed in *Hoplias microlepis* (Dickson and Graham, 1986). In tissues of other species, enzyme activities stayed the same (Shaklee et al., 1977; Driedzic et al., 1985) or decreased during hypoxic exposure (Almeida-Val et al., 1995). In addition, all but one of the above studies report data on only a subset of the enzymes of glycolysis (as few as one or two). The single study that measured all glycolytic enzymes did so in only one tissue, liver, and found increased activities of enzymes catalyzing reactions close to equilibrium but not for those catalyzing reactions far from equilibrium (Kraemer and Schulte, 2004).

The goal of the current study was to generate a comprehensive, multi-tissue perspective of the effects of chronic hypoxic exposure on carbohydrate metabolism in the Gulf killifish *Fundulus grandis*. *F. grandis* is a common inhabitant of estuaries along the Gulf of Mexico, areas which may become hypoxic on a daily or seasonal basis. In acute laboratory exposures, aerobic metabolism of this fish decreases below a critical oxygen tension of approximately 4.5 kPa (Virani and Rees, 2000). Below the critical oxygen tension, these fish rely upon anaerobic glycolysis to compensate, at least in part, for the reduced energy provision by aerobic metabolism (Virani and Rees, 2000). In the closely related *F. heteroclitus*, hypoxic exposure of several days to several weeks leads to increased activities of selected glycolytic enzymes in liver (Greaney et al., 1980; Kraemer and Schulte, 2004). We have measured the maximal activities of all glycolytic enzymes in four tissues (white skeletal muscle, liver, heart and brain) of *F. grandis* after being held under hypoxic or normoxic conditions for 4 weeks. The hypoxic concentration of dissolved oxygen used (1.3 mg l^{-1}, ~3.6 kPa) is below the critical oxygen tension for this species, but is ecologically relevant and well tolerated by this species. To get a more complete picture of carbohydrate metabolism, we also measured the activities of enzymes of glycogen metabolism (in muscle and liver) and gluconeogenesis (in liver).

These measurements allowed us to address the following questions about the effects of chronic hypoxia on the metabolic potential of various tissues in *F. grandis*. Do different tissues respond similarly to hypoxic exposure? Do all enzymes within a given pathway (glycolysis) change in concert (i.e. in the same direction and by the same magnitude)? Finally, in a single tissue (liver) do the capacities for catabolic and anabolic processes respond similarly or differently to chronic hypoxia? The results demonstrate that when intact fish are acclimated to prolonged hypoxia, enzyme activities respond in a tissue-specific fashion and that within a tissue changes in enzyme activities are not uniformly distributed across a metabolic pathway.

Materials and methods

Animals

Fundulus grandis Baird and Girard 1853 were purchased in late May 2003 at a commercial bait shop in Pascagoula, MS, USA and transported to the Gulf Coast Research Laboratory in Ocean Springs, MS. All fish were habituated to laboratory conditions for 2 weeks, after which fish were randomly allocated to normoxic (N=24) or hypoxic (N=24) treatments. Exposures were carried out in four tanks, each measuring 48 cm×48 cm (length×width) with an overflow drain at 17.5 cm depth (~40 l). Two normoxic tanks received water from an aerated reservoir and two hypoxic tanks received water from a mixing box with input from aerated and nitrogen-sparged reservoirs. Each tank was divided into four quadrants (each 24 cm×24 cm) by mesh, and each quadrant held one male and two female *F. grandis*. The 40 l tanks were covered and the partial pressure of oxygen in the air-space above the water level (~2.5 cm) was essentially in equilibrium with the water. Treatments lasted 4 weeks, during which time dissolved oxygen (DO) was measured twice daily with a Yellow Spring Instruments (Yellow Springs, OH, USA) oxygen electrode. DO averaged 6.68±2.1 mg l^{-1} in the normoxic tanks (mean ± 1 s.d.; N=159 measurements) and 1.34±0.45 mg l^{-1} in the hypoxic tanks (N=182 measurements). Temperature was 27±0.3°C, the salinity was 15.0±0.5 ppt (Fritz Super Salt Concentrate, Mesquite, TX, USA), and the photoperiod was 16 h:8 h light:dark.

Fish were fed to satiation with frozen brine shrimp (*Artemia* spp., San Francisco Bay Brand, Newark, CA, USA), Prime Tropical Flakes (Ziegler Brothers, Inc., Gardners, PA, USA), and brine shrimp nauplii (O.S.I., Snowville, UT, USA) twice daily. The diet was changed to live grass shrimp (*Palaemonetes* spp.) after 2 weeks. At the beginning of the exposure, fish in the two treatments were equivalent in standard length (normoxia, 80.7±6.7 mm; hypoxia 79.9±4.6 mm) and mass (normoxia, 10.8±3.2 g; hypoxia 10.0±2.3 g). Both groups grew over the 4 week exposure, although normoxic fish grew more than hypoxic fish (C. A. Landry, S. L. Steele, S. Manning and A. O. Cheek, manuscript submitted for publication). Consequently, normoxic fish were longer (normoxia, 84.8±6.9 mm; hypoxia, 80.7±5.3 mm; $P\leqslant 0.05$) and heavier (normoxia, 15.2±3.6 g; hypoxia, 11.7±2.3 g; $P\leqslant 0.01$) than hypoxic fish at the end of the experiment.

Extract preparation

After 4 weeks of exposure to normoxia or hypoxia, fish were netted and killed with an overdose of buffered MS-222 (1 g MS-222 and 4 g $NaHCO_3$ per liter of water). Liver, brain, heart and white skeletal muscle were dissected, frozen in liquid nitrogen, and stored at –80°C until analysis. Skeletal muscle samples were taken dorsal to the lateral line, to avoid red muscle, and between the head and dorsal fin, to avoid longitudinal variation in enzyme activity (Martínez et al., 2000).

For glycolytic and gluconeogenic enzyme assays, tissues were weighed and homogenized in ice-cold buffer consisting of 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 0.1 mmol l^{-1} DTT and 0.2% Triton X-100 (Pierce and Crawford, 1997) with a PRO 200 homogenizer (PRO Scientific Inc., Connecticut, USA) for two 20 s periods. The samples were maintained on ice during and between periods of homogenization. Muscle, liver and brain samples were homogenized in nine volumes of buffer; hearts were homogenized in 49 volumes of buffer. Homogenates were centrifuged at 2400 ***g*** for 15 min at 4°C, and supernatant solutions were kept on ice until enzyme activity was assayed.

Separate homogenates were made for assays of glycogen synthase and glycogen phosphorylase. For these, liver and white muscle were weighed and homogenized in four volumes of ice-cold buffer containing 50 mmol l^{-1} imidazole, pH 7.5, 100 mmol l^{-1} NaF, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA, 15 mmol l^{-1} β-mercaptoethanol and 0.1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF) (Milligan, 2003). Samples were homogenized with a PRO 200 homogenizer for two 20 s periods while kept cold. These homogenates were centrifuged at 16 000 ***g*** for 2 min at 4°C, and supernatants were kept on ice until enzyme activity was assayed.

Enzyme assays

Reaction conditions for the determination of glycolytic enzyme activities were modified from Pierce and Crawford (Pierce and Crawford, 1994). For each enzyme in each tissue, the concentrations of substrates, cofactors and linking enzymes were optimized to give maximal activities. Reactions were initiated by adding the substrate specific for that enzyme (shown last for each reaction). The final reaction conditions were as follows.

Hexokinase (HK; EC 2.7.1.1): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 7.5 mmol l^{-1} $MgCl_2$, 3.1 mmol l^{-1} ATP, 1 mmol l^{-1} NADP, 10 mmol l^{-1} creatine phosphate, 2 i.u. ml^{-1} creatine kinase, 1 i.u. ml^{-1} glucose-6-phosphate dehydrogenase and 7.5 mmol l^{-1} glucose. Under these conditions, glucokinase (hexokinase type IV) contributes to the rates measured in liver tissue.

Phosphoglucoisomerase (PGI; EC 5.3.1.9): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 1.25 mmol l^{-1} NADP, 0.5 i.u. ml^{-1} glucose-6-phosphate dehydrogenase, and 2 mmol l^{-1} fructose 6-phosphate.

Phosphofructokinase (PFK; EC 2.7.1.11): 100 mmol l^{-1} Hepes (pH 8.2), 10 mmol l^{-1} KCl, 7.5 mmol l^{-1} $MgCl_2$, 1.25 mmol l^{-1} ATP (liver, brain, heart) or 2.5 mmol l^{-1} ATP (muscle), 5 mmol l^{-1} AMP, 0.2 mmol l^{-1} NADH, 1 i.u. ml^{-1} aldolase, 10 i.u. ml^{-1} glycerol-3-phosphate dehydrogenase, 29 i.u. ml^{-1} triose phosphate isomerase and 5 mmol l^{-1} fructose 6-phosphate (liver, brain, heart) or 10 mmol l^{-1} fructose 6-phosphate (muscle).

Aldolase (ALD; EC 4.1.2.13): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 0.2 mmol l^{-1} NADH, 5 i.u. ml^{-1} glycerol-3-phosphate dehydrogenase, 14.5 i.u. ml^{-1} triose phosphate isomerase and 0.75 mmol l^{-1} fructose 1,6-bisphosphate.

Triose phosphate isomerase (TPI; EC 5.3.1.1): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 0.2 mmol l^{-1} NADH, 10 i.u. ml^{-1} glycerol-3-phosphate dehydrogenase (muscle, liver, heart) or 20 i.u. ml^{-1} glycerol-3-phosphate dehydrogenase (brain) and 2.9 mmol l^{-1} glyceraldehyde 3-phosphate (muscle, liver, brain) or 5.8 mmol l^{-1} glyceraldehyde 3-phosphate (heart).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$ (muscle, brain, heart) or 1 mmol l^{-1} $MgCl_2$ (liver), 3.1 mmol l^{-1} ATP (muscle, brain, heart) or 1.55 mmol l^{-1} ATP (liver), 0.2 mmol l^{-1} NADH, 8 i.u. ml^{-1} phosphoglycerokinase and 2.8 mmol l^{-1} 3-phosphoglycerate.

Phosphoglycerokinase (PGK; EC 2.7.2.3): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 3.1 mmol l^{-1} ATP (liver, brain, heart) or 6.2 mmol l^{-1} ATP (muscle), 0.2 mmol l^{-1} NADH, 8 i.u. ml^{-1} glyceraldehyde-3-phosphate dehydrogenase, and 2.8 mmol l^{-1} 3-phosphoglycerate.

Phosphoglyceromutase (PGM; EC 2.7.5.3): For liver, brain and heart, the assay included 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 0.65 mmol l^{-1} ADP, 0.125 mmol l^{-1} 2,3-bisphosphoglycerate, 0.22 mmol l^{-1} NADH, 9 mmol l^{-1} glucose, 0.1 i.u. ml^{-1} enolase, 0.5 i.u. ml^{-1} pyruvate kinase, 0.75 i.u. ml^{-1} L-lactate dehydrogenase, 3.2 i.u. ml^{-1} hexokinase and 1.25 mmol l^{-1} 3-phosphoglycerate. For muscle, the above conditions were used except $MgCl_2$ was 2.5 mmol l^{-1}, ADP was 1.25 mmol l^{-1}, 2,3-bisphosphoglycerate was 62.5 μmol l^{-1} and glucose was 5 mmol l^{-1}.

Enolase (ENO; EC 4.2.1.11): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 1.3 mmol l^{-1} ADP (muscle, liver, brain) or 0.65 mmol l^{-1} ADP (heart), 0.2 mmol l^{-1} NADH, 4.5 mmol l^{-1} glucose, 0.6 i.u. ml^{-1} pyruvate kinase, 0.75 i.u. ml^{-1} L-lactate dehydrogenase, 1.6 i.u. ml^{-1} hexokinase (muscle, liver, brain) or 3.2 i.u. ml^{-1} (heart) and 1.25 mmol l^{-1} 2-phosphoglycerate.

Pyruvate kinase (PYK; EC 2.7.1.40): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$ (muscle and liver) or 5 mmol l^{-1} $MgCl_2$ (brain and heart), 7.6 mmol l^{-1} ADP (muscle and liver) or 3.8 mmol l^{-1} ADP (brain and heart), 0.2 mmol l^{-1} NADH, 1.5 i.u. ml^{-1} L-lactate dehydrogenase (muscle), 0.375 i.u. ml^{-1} L-lactate dehydrogenase (liver and heart), or 0.75 i.u. ml^{-1} L-lactate dehydrogenase (brain), and 1 mmol l^{-1} phosphoenolpyruvate (muscle, liver, brain) or 2 mmol l^{-1} phosphoenolpyruvate (heart).

Lactate dehydrogenase (LDH; EC 1.1.1.27): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 0.17 mmol l^{-1} NADH, 1 mmol l^{-1} pyruvate.

The assay conditions for enzymes of glycogen metabolism were modified from Milligan (Milligan, 2003).

Glycogen synthase (GSase; EC 2.4.1.11): 50 mmol l^{-1} Tris (pH 7.8), 70 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1}

phosphoenolpyruvate, 0.2 mmol l^{-1} NADH, 5 i.u. ml^{-1} L-lactate dehydrogenase, 5 i.u. ml^{-1} pyruvate kinase, 2 mg ml^{-1} glycogen (oyster muscle, dialyzed) and 2 mmol l^{-1} UDP-glucose. Total GSase activity was assayed in the presence of 5 mmol l^{-1} glucose 6-phosphate. Active GSase was measured without glucose 6-phosphate.

Glycogen phosphorylase (GPase; EC 2.4.1.1): 50 mmol l^{-1} potassium phosphate (pH 7.3), 15 mmol l^{-1} $MgSO_4$, 0.5 mmol l^{-1} DTT, 0.5 mmol l^{-1} NADP, 0.25 mmol l^{-1} EDTA, 1 i.u. ml^{-1} glucose-6-phosphate dehydrogenase, 1 i.u. ml^{-1} phosphoglucomutase, 0.01 mmol l^{-1} glucose 1,6-bisphosphate and 2 mg ml^{-1} glycogen (oyster muscle, dialyzed). Total GPase activity was measured in the presence of 2 mmol l^{-1} AMP. Active GPase was measured without AMP.

The assay conditions for gluconeogenic enzymes were modified from standard protocols.

Malate dehydrogenase (MDH; EC 1.1.1.37): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 0.175 mmol l^{-1} NADH and 0.1 mmol l^{-1} oxaloacetate (Mommsen et al., 1980).

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 10 mmol l^{-1} phosphoenolpyruvate, 0.5 mmol l^{-1} inosine diphosphate, 5 mmol l^{-1} $MnCl_2$, 0.15 mmol l^{-1} NADH, 0.3 i.u. ml^{-1} malate dehydrogenase, and 20 mmol l^{-1} $NaHCO_3$ (Opie and Newsholme, 1967). In optimizing the PEPCK assay, inosine diphosphate (IDP) and 2-deoxy-guanosine-5′-phosphate (2-dGDP) (Foster and Moon, 1990) were compared as phosphoryl group acceptors. With IDP, background rates (without $NaHCO_3$) were lower and specific rates (with $NaHCO_3$) were higher than with 2-dGDP. The resulting PEPCK activities were as much as 50% greater with IDP as the phosphoryl group acceptor. The greater activity cannot be due to competing pyruvate kinase activity, because the PEPCK assay is initiated with $NaHCO_3$.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 0.2 mmol l^{-1} NADP, 1.6 i.u. ml^{-1} phosphoglucose isomerase, 0.36 i.u. ml^{-1} glucose-6-phosphate dehydrogenase, and 0.05 mmol l^{-1} fructose 1,6-bisphosphate (Opie and Newsholme, 1967).

Glucose-6-phosphatase (G6Pase; EC3.1.3.9): 100 mmol l^{-1} Hepes (pH 6.5), 10 mmol l^{-1} KCl, 26.5 mmol l^{-1} glucose 6-phosphate, 1.8 mmol l^{-1} EDTA, 2 mmol l^{-1} NAD, 1 i.u. ml^{-1} mutarotase, 20 i.u. ml^{-1} glucose dehydrogenase (Alegre et al., 1988). This assay was initiated by the addition of extract.

Maximal enzyme activities were measured in quadruplicate in a 96-well microplate reading spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) at 27±1°C. Reaction rates were linear for ⩾3 min. Rates from blank reactions (without substrate) were subtracted for all determinations of enzyme activities. Units (i.u.) of enzyme activity were defined as the amount of enzyme needed to convert 1 μmol of substrate to product in 1 min under these conditions. The value of 6.22 was used as the millimolar extinction coefficient for NAD(P)H. All enzyme activities were measured within 5 h of tissue homogenization.

Biochemicals and coupling enzymes were purchased from Sigma Chemical Co. (St Louis, MO, USA), Roche Diagnostics Corporation (Indianapolis, IN, USA) or Calzyme Laboratories, Inc. (San Luis Obispo, CA, USA). When necessary to remove excess ammonium sulfate, coupling enzymes were centrifuged at 12,000 ***g*** for 10 min and redissolved in assay buffer [100 mmol l^{-1} Hepes (pH 7.4), 10 mmol l^{-1} KCl].

Protein assay

The protein contents in the supernatant fractions of tissue homogenates were determined by the bicinchoninic acid assay (Smith et al., 1985; Brown et al., 1989), modified for use in a 96-well microplate reading spectrophotometer. Samples were diluted in water to a concentration of approximately 0.5 mg ml^{-1}. Quadruplicate 10 μl samples were added to 200 μl of the bicinchoninic acid working reagent (Pierce Biochemicals, Rockford, IL, USA) in individual wells of a 96-well microplate. Wells were sealed and the plate was incubated at 60°C for 30 min. After cooling to room temperature, the plate was read at 562 nm. Standards of 0–1 mg ml^{-1} bovine serum albumin were included with every plate.

Calculations and statistical analyses

Enzyme activities were calculated on the basis of tissue mass (i.u. g^{-1} tissue) and on the basis of soluble protein content in tissue extracts (i.u. mg^{-1} protein). Because the same fish were used to measure the effects of low oxygen on reproduction (C. A. Landry, S. L. Steele, S. Manning and A. O. Cheek, manuscript submitted for publication), the effects of DO treatment on enzyme activities were evaluated with 2-way analyses of variance which included the sex of the fish and the interaction between DO and sex. In this model, a significant effect of sex (i.e. enzyme activities in females differ from males) or a significant interaction (i.e. the effect of DO depended upon sex of the fish) would be taken as evidence that reproductive status affects enzyme activity. Throughout, enzyme activities are presented as least-squared means for normoxic and hypoxic treatments (corrected for the effects of sex and the interaction between sex and DO) with one standard deviation (s.d.). We present statistical results at two levels of probability: one that assumes independence among variables ($P \leqslant 0.05$); and one that allows for enzyme responses within a pathway and across tissues to be linked ($P \leqslant 0.001$). The latter approach corrects P values for multiple comparisons (Sokal and Rohlf, 1981). All statistical analyses were performed with SYSTAT 10.

Results

Tissue enzyme activities

Maximal activities of enzymes of glycolysis, glycogen metabolism and gluconeogenesis were measured in tissues of *F. grandis* after 4 weeks of exposure to normoxia or hypoxia. Enzyme activities expressed per gram of tissue are presented in Tables S1–S3 in supplementary material. Expressed in this way, differences in enzyme activity between normoxic and hypoxic fish might be explained by treatment-related changes in tissue protein content. For example, the soluble protein concentration in skeletal muscle extracts from hypoxic fish (40.7±5.2 mg g^{-1} tissue) was significantly lower than in skeletal muscle extracts from normoxic fish (48.4±5.8 mg g^{-1} tissue; $P\leqslant 0.01$). Similarly, the soluble protein concentration was lower in heart from hypoxic fish (71.7±8.9 mg g^{-1} tissue) than from normoxic fish (81.6±13.6 mg g^{-1} tissue; $P\leqslant 0.05$). [The soluble protein concentrations did not differ between normoxic and hypoxic treatments for extracts prepared from liver (normoxia= 108.7±11.1 mg g^{-1} tissue; hypoxia=109.2±14.9 mg g^{-1} tissue) or brain (normoxia=60.6±6.9 mg g^{-1} tissue; hypoxia= 60.5±6.6 mg g^{-1} tissue).] If enzyme activities in the two treatment groups simply paralleled the concentration of soluble protein, then activities per gram of tissue mass would be 10% (heart) to 20% (skeletal muscle) lower in hypoxia than in normoxia. To account for treatment effects on tissue protein concentration, enzyme specific activities (in i.u. mg^{-1} protein) were calculated. These values reflect variation beyond that due to changes in bulk protein levels, and these values were used for the remainder of the analyses.

Effects of hypoxia on enzyme specific activities

Hypoxia altered glycolytic enzyme activities in white skeletal muscle, liver, heart, and brain; however, the enzymes affected and the direction of the hypoxia response were tissue-specific (Table 1; Fig. 1). Hypoxic exposure led to significantly lower specific activities of eight of ten glycolytic enzymes measured in white skeletal muscle ($P\leqslant 0.05$; Fig. 1A). Activities were reduced by 17% (PGM) to 55% (ENO). The two other enzymes (GAPDH and PGK) were lower in hypoxia than in normoxia, although these differences were not statistically significant. The enzyme HK was below the limit of detection in skeletal muscle. In contrast to the effect of hypoxia in skeletal muscle, hypoxic exposure enhanced activities of five of 11 glycolytic enzymes measured in liver ($P\leqslant 0.05$; Fig. 1B). Activities were 19% (PGI) to 74% (PGK) greater in hypoxia. In heart, three glycolytic enzymes had higher specific activities in hypoxia-exposed fish ($P\leqslant 0.05$; Fig. 1C), ranging from 18% (TPI) to 28% (HK) increases. In brain, the effects of hypoxia on maximal enzyme activities were smaller in magnitude and mixed in direction: three glycolytic enzymes had higher activities in brains from hypoxia-exposed fish, whereas one had lower activity ($P\leqslant 0.05$; Fig. 1D). The percentage changes ranged from 12% lower (PGM) to 16% higher (HK) in hypoxia.

White skeletal muscle and liver had undetectable or low levels of hexokinase, suggesting that free glucose is less important than stored glycogen as a substrate for glycolysis. Accordingly, to get a more complete picture of overall carbohydrate metabolism, the total and active levels of the enzymes of glycogen metabolism, GPase and GSase, were determined in skeletal muscle and liver (Table 2; Fig. 2). Both total and active GPase activities were lower in skeletal muscle from hypoxia-exposed fish ($P\leqslant 0.05$; Fig. 2A). This reduction in enzyme activity of glycogenolysis is consistent with lower activities of glycolytic enzymes in hypoxic muscle (see above). In skeletal muscle, the percentage of GPase in the active form was about 15% of the total GPase activity and did not differ between normoxia and hypoxia. Dissolved oxygen treatment had no effect on the specific activity of GPase (either total or active) in liver (Fig. 2B). However, small, non-statistically significant changes in liver GPase specific activity led to a modest, but significant decrease in the percentage of active GPase in liver in hypoxic fish (73±11%) compared to normoxic fish (83±10%) ($P\leqslant 0.05$). Total GSase activity in muscle was

Table 1. *Glycolytic enzyme specific activities (i.u. mg^{-1} protein) in tissues of Fundulus grandis held under normal and reduced oxygen levels for 4 weeks at 27°C*

	Muscle		Liver		Heart		Brain	
Enzyme	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
HK	n.d.	n.d.	0.003±0.001	0.002±0.002	0.11±0.02	0.14±0.02**	0.11±0.02	0.13±0.02**
PGI	2.00±0.39	1.47±0.43**	0.59±0.14	0.71±0.16*	2.20±0.34	2.24±0.38	1.31±0.16	1.38±0.17
PFK	0.20±0.07	0.15±0.08*	0.008±0.003	0.013±0.003**	0.15±0.07	0.13±0.07	0.11±0.01	0.11±0.02
ALD	1.82±0.32	1.12±0.36**	0.13±0.03	0.11±0.04	0.27±0.06	0.27±0.07	0.28±0.03	0.29±0.03
TPI	39.5±11.5	31.8±12.7*	15.4±4.9	16.6±5.5	13.9±2.5	16.4±2.8*	7.95±1.5	9.12±1.6*
GAPDH	2.47±1.28	1.64±1.42	1.30±0.30	1.68±0.33**	1.79±0.60	1.78±0.67	1.54±0.18	1.61±0.20
PGK	1.88±0.88	1.73±0.98	0.92±0.26	1.61±0.28**	1.46±0.24	1.54±0.27	1.11±0.14	1.22±0.16*
PGM	4.12±0.62	3.44±0.69*	0.63±0.12	0.68±0.13	1.78±0.35	1.98±0.39	1.16±0.18	1.03±0.20*
ENO	0.83±0.51	0.37±0.56*	0.45±0.12	0.46±0.13	0.25±0.06	0.23±0.06	0.36±0.04	0.37±0.04
PYK	2.11±0.37	1.63±0.41**	0.14±0.03	0.15±0.04	0.98±0.22	1.22±0.24*	1.48±0.19	1.49±0.21
LDH	6.91±1.32	4.84±1.47**	2.63±0.66	3.42±0.73**	4.15±0.84	4.38±0.93	2.65±0.35	2.85±0.39

HK, hexokinase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase; ALD, aldolase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; PGM, phosphoglyceromutase; ENO, enolase; PYK, pyruvate kinase; LDH, lactate dehydrogenase.

Values are least-squared means (±1 s.d.) from two-way ANOVA testing the effects of dissolved oxygen (DO) treatment and sex of fish. Sample sizes were 19 for normoxia and 22 for hypoxia. n.d., not determined.

Significantly different between normoxia and hypoxia, *$P\leqslant 0.05$; **$P\leqslant 0.001$.

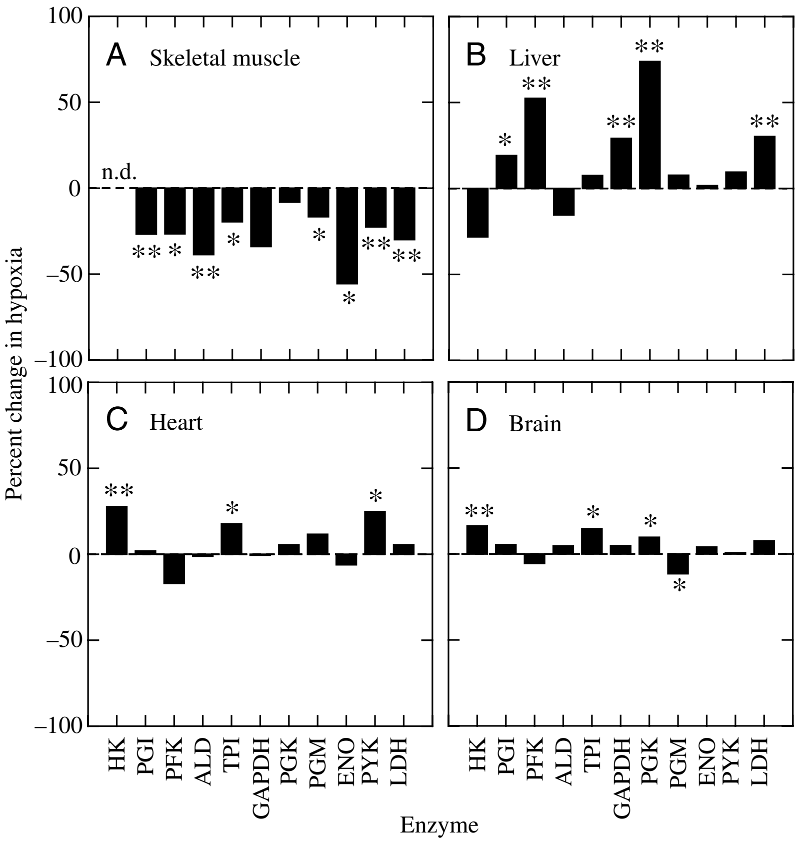

Fig. 1. Changes in glycolytic enzyme specific activities (i.u. mg^{-1} protein) in tissues of *Fundulus grandis* held under normal and reduced oxygen levels for 4 weeks at 27°C. The y axis represents the percentage change in the mean value for each enzyme measured from hypoxic fish relative to the normoxic value for (A) skeletal muscle, (B) liver, (C) heart and (D) brain. Asterisks indicate significant differences between mean specific activity values from normoxic and hypoxic groups (*$P\leqslant0.05$; **$P\leqslant0.001$; see Table 1). HK, hexokinase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase; ALD, aldolase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; PGM, phosphoglyceromutase; ENO, enolase; PYK, pyruvate kinase; LDH, lactate dehydrogenase.

lower in hypoxia ($P\leqslant0.05$; Fig. 2A). The same enzyme was significantly higher in liver tissue from hypoxia-exposed fish (Fig. 2B), although this was due to an effect of hypoxia on males but not females (see below). Active GSase was not significantly altered by hypoxia in either tissue, nor was the percentage of GSase in the active form (12–15% in both tissues).

The specific activities of enzymes that are involved in gluconeogenesis were measured only in liver (Table 3; Fig. 3). The citric acid cycle enzyme MDH catalyzes the reversible conversion of oxaloacetate to malate, which may be important during gluconeogenesis as a mechanism to shuttle reducing equivalents and carbon skeletons from the mitochondrion to the cytosol. This enzyme was significantly higher in hypoxia-exposed fish ($P\leqslant0.05$). The enzyme FBPase catalyzes the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate, bypassing the glycolytic reaction catalyzed by PFK, and it was also higher in hypoxic fish ($P\leqslant0.05$). Both enzymes were about 25% higher in hypoxia. Two other enzymes of gluconeogenesis, PEPCK and G6Pase, did not differ between normoxic and hypoxic fish.

Effects of sex on enzyme specific activities

The specific activities of six enzymes differed between male and female fish ($P\leqslant0.05$). Of these, five [ALD in heart, and MDH, FBPase, GPase (total), and GPase (active) in liver] were

Table 2. *Specific activities of enzymes of glycogen metabolism (i.u. mg^{-1} protein) in tissues of Fundulus grandis held under normal and reduced oxygen levels for 4 weeks at 27°C*

	Muscle		Liver	
Enzyme	Normoxia	Hypoxia	Normoxia	Hypoxia
Total GPase	0.328±0.075	0.240±0.083**	0.052±0.026	0.056±0.030
Active GPase	0.060±0.031	0.034±0.034*	0.044±0.024	0.043±0.027
Total GSase	0.052±0.012	0.044±0.013*	0.018±0.010	0.029±0.011*
Active GSase	0.007±0.005	0.007±0.005	0.003±0.002	0.004±0.002

GPase, glycogen phosphorylase; GSase, glycogen synthase.

Values are least-squared means (±1 s.d.) from two-way ANOVA testing the effects of dissolved oxygen (DO) treatment and sex of fish. Sample sizes were 19 for normoxia and 19 to 22 for hypoxia.

Significantly different between normoxia and hypoxia, *$P\leqslant0.05$; **$P\leqslant0.001$.

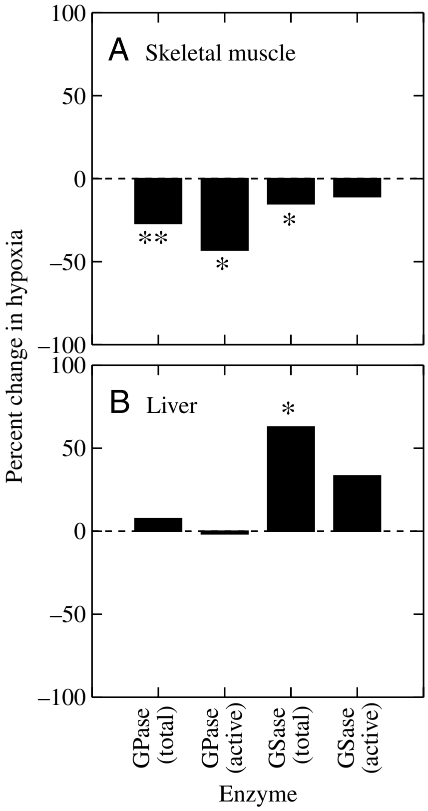

Fig. 2. Changes in specific activities (i.u. mg^{-1} protein) of enzymes of glycogen metabolism in tissues of *Fundulus grandis* held under normal and reduced oxygen levels for 4 weeks at 27°C. The y axis represents the percentage change in the mean value for each enzyme measured from hypoxic fish relative to the normoxic value for (A) skeletal muscle and (B) liver. Asterisks indicate significant differences between mean specific activity values from normoxic and hypoxic groups (* $P \leqslant 0.05$; ** $P \leqslant 0.001$; see Table 2). GPase, glycogen phosphorylase; GSase, glycogen synthase.

higher in males. These differences were relatively modest, generally being less than 25%. By contrast, the only enzyme which was greater in females (liver PEPCK) was nearly 80% greater than in males. For one enzyme (total liver GSase), there was a significant DO by sex interaction ($P \leqslant 0.05$). Males and females had equivalent activities under normoxia; hypoxic exposure led to higher levels in males, but not in females.

Summary of enzyme changes

While a P value of less than 0.05 is typically accepted as demonstrating significant differences between treatment groups, using this value assumes that the measured variables are independent of one another. It is possible that changes in enzyme activities in a given metabolic pathway occur in a coordinated fashion (i.e. they are not independent of one another), therefore the P value must take into account the total number of enzyme activities measured (approximately 50 different enzyme–tissue combinations). Even using this more stringent criterion ($P \leqslant 0.001$), a number of enzyme activities were found to differ between normoxic and hypoxic fish. Moreover, this approach clearly demonstrates that the response to hypoxia differed among the four tissues (Table 4). The only effect of sex that was significant at $P \leqslant 0.001$ was the higher value of liver PEPCK in females.

Table 3. *Gluconeogenic enzyme specific activities (i.u. mg^{-1} protein) in liver of Fundulus grandis held under normal and reduced oxygen levels for 4 weeks at 27°C*

Enzyme	Normoxia	Hypoxia
MDH	4.37±0.93	5.50±1.04**
PEPCK	0.026±0.012	0.024±0.013
FBPase	0.046±0.008	0.056±0.008**
G6Pase	0.046±0.010	0.044±0.011

MDH, malate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase.

Values are least-squared means (±1 s.d.) from two-way ANOVA testing the effects of dissolved oxygen (DO) treatment and sex of fish.

Sample sizes were 16 to 19 for normoxia and 22 for hypoxia. Significantly different between normoxia and hypoxia, * $P \leqslant 0.05$; ** $P \leqslant 0.001$.

Discussion

We undertook this study to develop a comprehensive picture of carbohydrate metabolism in multiple tissues from a single fish species subjected to prolonged hypoxia. We measured maximal activities of enzymes of glycolysis, glycogen metabolism, and gluconeogenesis in white skeletal muscle, liver, heart and brain of hypoxic and normoxic *F. grandis*. These measurements allowed us to address the following questions: do different tissues respond similarly to hypoxic exposure?; do all enzymes within a given pathway change in concert? And, do enzymes in catabolic and anabolic processes respond similarly or differently to chronic hypoxia? In addressing these questions, we concentrate on those results that satisfy the criterion of being statistically significant after accounting for multiple comparisons (Table 4). Moreover, the statistical analyses included sex of the fish, so the observed effects of DO are independent of any sex-dependent effects (e.g. changes in reproductive status) that might have occurred during the acclimation period.

White skeletal muscle

In *F. grandis*, we found that the specific activities of enzymes of glycolysis and glycogen metabolism were consistently depressed after long-term exposure to low oxygen, suggesting a decreased capacity for carbohydrate metabolism in this tissue. The lower enzyme activities may be related to a reduction in growth or activity during chronic hypoxia. Hypoxia leads to lower growth rates in this and other teleost species (Chabot and Dutil, 1999; Stierhoff et al., 2003; C. A.

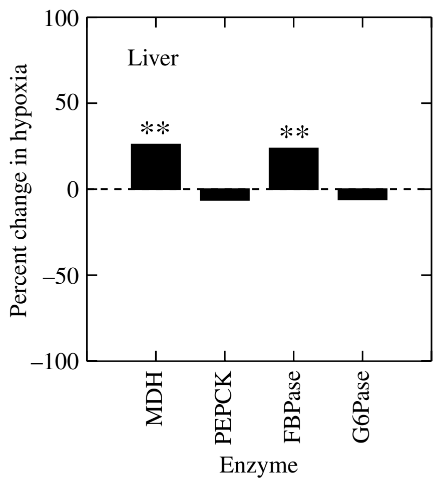

Fig. 3. Changes in gluconeogenic enzyme specific activities (i.u. mg^{-1} protein) in liver of *Fundulus grandis* held under normal and reduced oxygen levels for 4 weeks at 27°C. The y axis represents the percent change in the mean value for each enzyme measured from hypoxic fish relative to the normoxic value. Asterisks indicate significant differences between mean specific activity values from normoxic and hypoxic groups (**P*⩽0.05; ***P*⩽0.001; see Table 3). MDH, malate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase.

Landry, S. L. Steele, S. Manning and A. O. Cheek, manuscript submitted for publication), and fish experiencing low or negative growth frequently lose skeletal muscle protein, presumably due to catabolism to meet energy demands (Sullivan and Somero, 1983; Loughna and Goldspink, 1984; Pelletier et al., 1993; Pelletier et al., 1995; Martínez et al., 2003). This is consistent with our observation that soluble protein concentrations (mg g^{-1} tissue mass) were about 20% lower in skeletal muscle extracts from hypoxic fish compared to normoxic fish. If glycolytic enzymes were catabolized at the same rate as bulk proteins, then enzyme activities would decrease by a similar amount (20%) when expressed on the basis of tissue mass. Instead, skeletal muscle enzyme activities per gram tissue decreased by more than this (see Tables S1–S3 in supplementary material). Consequently, enzyme-specific activities (which account for changes in protein content) were also lower in hypoxia relative to normoxia (Tables 1, 2), demonstrating that the changes we noted were above those due to loss of bulk protein associated with decreased growth. Furthermore, we included growth rate of individual fish in preliminary statistical analyses of enzyme activity data (not shown). In general, the effects of growth rate were small and not consistent in direction: in skeletal muscle, one enzyme specific activity was positively related to growth rate and one was negatively related to growth rate. More importantly, the conclusion that skeletal muscle enzyme activities were significantly affected by hypoxia was not changed even when growth rate was included in the analyses.

Therefore, a second explanation for the reduced muscle enzyme activities relates to decreased locomotion during hypoxia. Behavioral observations in this and other studies have shown hypoxic fish to be less active than normoxic fish (Bushnell et al., 1984; Dalla Via et al., 1998; Wannamaker and Rice, 2000), and, over the long term, the lower energy demands associated with decreased locomotion might result in lower levels of glycolytic enzymes. A similar link between muscle metabolic and locomotory capacities has been forwarded to account for the observed inter- and intraspecific variation in glycolytic enzyme activities in fish of differing size, lifestyle or condition (Somero and Childress, 1980; Childress and Somero, 1990; Martínez et al., 2003).

Liver

In contrast to skeletal muscle, hypoxic acclimation of *F. grandis* led to increased specific activities of enzymes of carbohydrate metabolism in liver. These results suggest that chronic hypoxia leads to an increase in the capacity for carbohydrate metabolism in this tissue. Perhaps surprisingly, we measured higher activities for enzymes of carbohydrate catabolism (glycolysis) and carbohydrate anabolism (glycogen synthesis and gluconeogenesis). It seems paradoxical that

Table 4. *Summary of differences in enzyme specific activities in tissues of Fundulus grandis held under normal and reduced oxygen levels for 4 weeks at 27°C*

	Pathway		
	Glycolysis	Glycogen metabolism	Gluconeogenesis
Tissue			
Muscle	↓ PGI, ALD, PYK, LDH	↓ GPase (Total)	n.d.
Liver	↑ PFK, GAPDH, PGK, LDH	none	↑ MDH, FBPase
Heart	↑ HK	n.d.	n.d.
Brain	↑ HK	n.d.	n.d.

PGI, phosphoglucoisomerase; ALD, aldolase; PYK, pyruvate kinase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerokinase; MDH, malate dehydrogenase; FBPase, fructose-1,6-bisphosphatase; HK, hexokinase.

The enzymes shown are those that were significantly different between treatment groups at *P*⩽0.001. n.d. not determined.

↑ Higher in hypoxia than in normoxia; ↓ Lower in hypoxia than in normoxia.

enzymes of both catabolic and anabolic pathways were higher under hypoxia. Although certain enzymes are shared between glycolysis and gluconeogenesis (GAPDH, PGK, LDH), other enzymes are specific to catabolic or anabolic reactions (i.e. PFK in glycolysis *versus* FBPase in gluconeogenesis). Increased activities of the latter enzymes suggest a futile cycle whose net result is ATP turnover. However, there is evidence that teleost liver contains distinct cell populations that differ from one another in their glycolytic and gluconeogenic capacities (Mommsen et al., 1991), a functional separation that would have been lost during homogenization. Therefore, it is possible that glycolysis could be upregulated in one population of cells to enhance ATP production to meet the energetic demands of those cells while gluconeogenesis could be upregulated in another cell population to enhance endogenous glycogen synthesis or export of glucose to extra-hepatic tissues. Amino acids coming from the catabolism of skeletal muscle protein during hypoxia could serve as precursors for this increased gluconeogenic flux.

Heart and brain

In heart and brain, fewer enzyme activities differed between normoxic and hypoxic *F. grandis* than in other tissues, and these differences were generally smaller in magnitude. One enzyme that did change in both tissues was hexokinase, which was higher in heart and brain from fish subjected to chronic hypoxia. Because the rate of glucose utilization by teleost brain is thought to be limited by hexokinase activity (Soengas and Aldegunde, 2002), the greater hexokinase activity could result in higher rates of glycolysis in this tissue during hypoxia. Otherwise, the relatively subtle changes in heart and brain might be explained by these tissues receiving preferential blood flow, and hence oxygen delivery, during exposure of fish to low oxygen (Soengas and Aldegunde, 2002). In accord with our results, heart and brain showed fewer signs of metabolic imbalance than skeletal muscle and liver during acute exposure of rainbow trout to hypoxia (Dunn and Hochachka, 1986). Of course, all of the tissues studied may respond to low oxygen by other mechanisms that would not be reflected as changes in enzyme specific activities. In this regard, goldfish exposed to anoxia respond with large increases in the concentration of fructose 2,6-bisphosphate, a potent allosteric activator of PFK, in heart and brain tissues (Storey, 1987).

Variation within the glycolytic pathway

In no tissue did all enzymes of the glycolytic pathway change, at least in a statistically significant fashion. Among the tissues examined, skeletal muscle was characterized by the most consistent changes: eight of 10 enzymes were significantly lower in hypoxia at $P\leqslant 0.05$; four were significant at $P\leqslant 0.001$. In liver, heart and brain, the number of significant changes depended upon the P value and the tissue, but ranged from a minimum of one enzyme to a maximum of five (fewer than half of the 11 glycolytic enzymes measured in these tissues). In all tissues, hypoxic exposure affected one enzyme typically considered to be 'rate-limiting' for glycolysis: PYK in muscle, PFK in liver and HK in heart and brain. However, hypoxia also affected the activities of several 'near-equilibrium' enzymes in muscle and liver. These results suggest that the designation 'rate-limiting' or 'near-equilibrium' is not a reliable predictor of which enzymes might be affected by a particular experimental treatment. Our results support the conclusions that biologically meaningful variation in enzyme activity occurs in reactions not usually thought to be rate-determining for the glycolytic pathway (Pierce and Crawford, 1997; Kraemer and Schulte, 2004).

It has been suggested that glycolytic enzymes in a variety of organisms are coordinately upregulated during hypoxic stress (Webster, 2003). Our data do not support a simple interpretation of this hypothesis, which predicts increased levels of all glycolytic enzymes in a given tissue during hypoxia. Indeed, for the tissue with the most consistent changes (skeletal muscle), the changes were in a direction opposite of that predicted. Two possible explanations of why our conclusions differ from those predicted are that the efficiency of oxygen extraction by fish increased, or demands for energy production by tissues decreased, during long-term hypoxic exposure. In other species of fish, the capacity to extract oxygen from hypoxic waters increases during chronic hypoxic exposure, through adjustments in ventilation, oxygen transport or gill surface area (Jensen et al., 1993; Sollid et al., 2003). The result is higher rates of oxygen consumption at low oxygen (Johnston and Bernard, 1982) or a decrease in critical oxygen tension (Timmerman and Chapman, 2004). An increase in oxygen extraction by *F. grandis* would presumably lessen the need for 'compensatory' changes in anaerobic capacity as the duration of hypoxia is extended. With respect to energy demands, quantitative estimates of metabolism in fish held under hypoxia suggest that the increase in anaerobic metabolism is smaller than that expected from the decrease in aerobic metabolism (Dalla Via et al., 1994; Virani and Rees, 2000). This appears to be true in *F. grandis*, where overall metabolism (aerobic plus anaerobic components) is lower during exposure to hypoxia than normoxia (Virani and Rees, 2000). Indeed, metabolic rate reduction has been proposed as a key feature enabling hypoxic survival in fish and other hypoxia-tolerant animals (Hochachka, 1980; Hochachka and Somero, 2002). The overall result is that the tissue response to chronic hypoxia is heterogeneous, and it reflects the interplay among energetic demands, metabolic role, and oxygen supply of specific tissues. Thus, the paradigm of uniformly increased glycolytic enzyme potential might describe the response of a particular tissue at a specific duration of hypoxia, but it may not be the appropriate solution for the long-term response of fish to low oxygen.

List of abbreviations

ALD	aldolase
DO	dissolved oxygen
2-dGDP	2-deoxy-guanosine-5′-phosphate
ENO	enolase

FBPase	fructose-1-6-bisphosphatase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPase	glycogen phosphorylase
G6Pase	glucose-6-phosphatase
GSase	glycogen synthase
Hepes	*N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid]
HK	hexokinase
IDP	inosine diphosphate
LDH	lactate dehydrogenase
MDH	malate dehydrogenase
MS-222	ethyl 3-aminobenzoate methane-sulfonate salt
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
PGI	phosphoglucoisomerase
PGK	phosphoglycerokinase
PGM	phosphoglyceromutase
PYK	pyruvate kinase
TPI	triose phosphate isomerase

We are grateful to Stacy Steele for assistance during sampling and to Deb Vivian for advice on fish culture. This project was funded by NSF grant No. IBN 0236494 to B.B.R. and US-EPA STAR Program grant No. R829458 to A.O.C.

References

Alegre, M., Ciudad, C. J., Fillat, C. and Guinovart, J. J. (1988). Determination of glucose-6-phosphatase activity using the glucose dehydrogenase-coupled reaction. *Anal. Biochem.* **173**, 185-189.

Almeida-Val, V. M. F., Farias, I. P., Silva, M. N. P., Duncan, W. P. and Val, A. L. (1995). Biochemical adjustments to hypoxia by Amazon cichlids. *Braz. J. Med. Biol. Res.* **28**, 1257-1263.

Brown, R. E., Jarvis, K. L. and Hyland, K. J. (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* **180**, 136-139.

Bushnell, P. G., Steffensen, J. F. and Johansen, K. (1984). Oxygen consumption and swimming performance in hypoxia-acclimated rainbow trout *Salmo gairneri*. *J. Exp. Biol.* **113**, 225-235.

Chabot, D. and Dutil, J.-D. (1999). Reduce growth of Atlantic cod in non-lethal hypoxic conditions. *J. Fish Biol.* **55**, 472-491.

Childress, J. J. and Somero, G. N. (1990). Metabolic scaling: a new perspective based on scaling of glycolytic enzyme activities. *Am. Zool.* **30**, 161-173.

Dalla Via, J., Van den Thillart, G., Cattani, O. and de Zwaan, A. (1994). Influence of long-term hypoxia exposure on the energy metabolism of *Solea solea*. II. Intermediary metabolism in blood, liver and muscle. *Mar. Ecol. Prog. Ser.* **111**, 17-27.

Dalla Via, J., Van den Thillart, G., Cattani, O. and Cortesi, P. (1998). Behavioural responses and biochemical correlates in *Solea solea* to gradual hypoxic exposure. *Can. J. Zool.* **76**, 2108-2113.

Dickson, K. A. and Graham, J. B. (1986). Adaptations to hypoxic environments in the erythrinid fish *Hoplias microlepis*. *Environ. Biol. Fishes* **15**, 301-308.

Driedzic, W. R., Gesser, H. and Johansen, K. (1985). Effects of hypoxic adaptation on myocardial performance and metabolism in *Zoarces viviparous*. *Can. J. Zool.* **63**, 821-823.

Dunn, J. F. and Hochachka, P. W. (1986). Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. *J. Exp. Biol.* **123**, 229-242.

Foster, G. D. and Moon, T. W. (1990). Control of key carbohydrate-metabolizing enzymes by insulin and gucagon in freshly isolated hepatocytes of the marine teleost *Hemitripterus americanus*. *J. Exp. Zool.* **254**, 55-62.

Greaney, G. S., Place, A. R., Cashon, R. E., Smith, G. and Powers, D. A. (1980). Time course of changes in enzyme activities and blood respiratory properties of killifish during long-term acclimation to hypoxia. *Physiol. Zool.* **53**, 136-144.

Hochachka, P. W. (1980). *Living Without Oxygen: Closed and Open Systems in Hypoxia Tolerance*. Cambridge, MA: Harvard University Press.

Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation*. Princeton: Princeton University Press.

Jensen, F. B., Nikinmaa, M. and Weber, R. E. (1993). Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. In *Fish Ecophysiology* (ed. J. C. Rankin and F. B. Jensen), pp. 161-179. London: Chapman & Hall.

Johnston, I. A. and Bernard, L. M. (1982). Ultrastructure and metabolism of skeletal muscle fibres in the tench: effects of long-term acclimation to hypoxia. *Cell Tissue Res.* **227**, 179-199.

Kraemer, L. D. and Schulte, P. M. (2004). Prior PCB exposure suppresses hypoxia-induced up-regulation of glycolytic enzymes in *Fundulus heteroclitus*. *Comp. Biochem. Physiol.* **139C**, 23-29.

Kramer, D. L. (1987). Dissolved oxygen and fish behavior. *Environ. Biol. Fishes* **18**, 81-92.

Loughna, P. T. and Goldspink, G. (1984). The effects of starvation upon protein turnover in red and white myotomal muscle of rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **25**, 223-230.

Lushchak, V. I., Bahnjukova, T. V. and Storey, K. B. (1998). Effect of hypoxia on the activity and binding of glycolytic and associated enzymes in sea scorpion tissues. *Braz. J. Medical Biol. Res.* **31**, 1059-1067.

Martínez, M., Dutil, J.-D. and Guderley, H. (2000). Longitudinal and allometric variation in indicators of muscle metabolic capacities in Atlantic cod (*Gadus morhua*). *J. Exp. Zool.* **287**, 38-45.

Martínez, M., Guderley, H., Dutil, J.-D., Winger, P. D., He, P. and Walsh, S. J. (2003). Condition, prolonged swimming performance and muscle metabolic capacities of cod *Gadus morhua*. *J. Exp. Biol.* **206**, 506-511.

Milligan, C. L. (2003). A regulatory role for cortisol in muscle glycogen metabolism in rainbow trout *Oncorhynchus mykiss* Walbaum. *J. Exp. Biol.* **206**, 3167-3173.

Mommsen, T. P., French, C. J. and Hochachka, P. W. (1980). Sites and patterns of protein and amino acid utilization during the spawning migration of salmon. *Can. J. Zool.* **58**, 1785-1799.

Mommsen, T. P., Danulat, E., Gavioli, M. E., Foster, G. D. and Moon, T. W. (1991). Separation of enzymatically distinct populations of trout hepatocytes. *Can. J. Zool.* **69**, 420-426.

Nikinmaa, M. and Rees, B. B. (2005). Oxygen-dependent gene expression in fishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R1079-R1090.

Opie, L. H. and Newsholme, E. A. (1967). The activities of fructose 1,6-diphosphatase, phosphofructokinase and phosphoenolpyruvate carboxykinase in white muscle and red muscle. *Biochem. J.* **103**, 391-399.

Pelletier, D., Guderley, H. and Dutil, J.-D. (1993). Effects of growth rate, temperature, season, and body size on glycolytic enzymes activities in the white muscle of Atlantic cod (*Gadus morhua*). *J. Exp. Zool.* **265**, 477-487.

Pelletier, D., Blier, P. U., Dutil, J.-D. and Guderley, H. (1995). How should enzyme activities be used in fish growth studies? *J. Exp. Biol.* **198**, 1493-1497.

Pierce, V. A. and Crawford, D. L. (1994). Rapid enzyme assays investigating the variation in the glycolytic pathway in field-caught populations of *Fundulus heteroclitus*. *Biochem. Genet.* **32**, 315-330.

Pierce, V. A. and Crawford, D. L. (1997). Phylogenetic analysis of thermal acclimation of the glycolytic enzymes in the genus *Fundulus*. *Physiol. Zool.* **70**, 597-609.

Shaklee, J. B., Christiansen, J. A., Sidell, B. D., Prosser, C. L. and Whitt, G. S. (1977). Molecular aspects of temperature acclimation in fish: contributions of changes in enzyme activities and isozyme patterns to metabolic reorganization in the green sunfish. *J. Exp. Zool.* **201**, 1-20.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

Soengas, J. L. and Aldegunde, M. (2002). Energy metabolism of fish brain. *Comp. Biochem. Physiol.* **131B**, 271-296.

Sokal, R. R. and Rohlf, F. J. (1981). *Biometry: The Principles and Practice of Statistics in Biological Research*. San Francisco, CA: W. H. Freeman.

Sollid, J., de Angelis, P., Gundersen, K. and Nilsson, G. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, 3667-3673.

Somero, G. N. and Childress, J. J. (1980). A violation of the metabolism-size scaling paradigm: activities of glycolytic enzymes in muscle increase in larger-size fish. *Physiol. Zool.* **53**, 322-337.

Stierhoff, K. L., Targett, T. E. and Grecay, P. A. (2003). Hypoxia tolerance of the mummichog: the role of access to the water surface. *J. Fish Biol.* **63**, 580-592.

Storey, K. B. (1987). Tissue-specific controls on carbohydrate catabolism during anoxia in goldfish. *Physiol. Zool.* **60**, 601-607.

Sullivan, K. M. and Somero, G. N. (1983). Size- and diet-related variation in enzymic activity and tissue composition in the sablefish, *Anaplopoma fimbria*. *Biol. Bull.* **164**, 315-326.

Timmerman, C. M. and Chapman, L. J. (2004). Behavioral and physiological compensation for chronic hypoxia in the sailfin molly (*Poecilia latipinna*). *Physiol. Biochem. Zool*. **77**, 601-610.

Van den Thillart, G. and Smit, H. (1984). Carbohydrate metabolism of goldfish (*Carassius auratus* L.): Effects of long-term hypoxia-acclimation on enzyme patterns of red muscle, white muscle and liver. *J. Comp. Physiol. B* **154**, 477-486.

Van den Thillart, G. and Van Waarde, A. (1985). Teleosts in hypoxia: aspects of anaerobic metabolism. *Mol. Physiol.* **8**, 393-409.

Van den Thillart, G., Dalla Via, J., Vitali, G. and Cortesi, P. (1994). Influence of long-term hypoxia exposure on the energy metabolism of *Solea solea*. I. Critical O_2 levels for aerobic and anaerobic metabolism. *Mar. Ecol. Prog. Ser.* **104**, 109-117.

Virani, N. A. and Rees, B. B. (2000). Oxygen consumption, blood lactate and inter-individual variation in the gulf killifish, *Fundulus grandis*, during hypoxia and recovery. *Comp. Biochem. Physiol.* **126A**, 397-405.

Wannamaker, C. M. and Rice, J. A. (2000). Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States. *J. Exp. Mar. Biol. Ecol.* **249**, 145-163.

Webster, K. A. (2003). Evolution of the coordinate regulation of glycolytic enzyme genes by hypoxia. *J. Exp. Biol.* **206**, 2911-2922.

Zhou, B. S., Wu, R. S. S., Randall, D. J., Lam, P. K. S., Ip, Y. K. and Chew, S. F. (2000). Metabolic adjustments in the common carp during prolonged hypoxia. *J. Fish Biol.* **57**, 1160-1171