The Journal of Experimental Biology 211, 3111-3122
Published by The Company of Biologists 2008
doi:10.1242/jeb.019117

AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, *Carassius auratus*

Lindsay A. Jibb and Jeffrey G. Richards*

Department of Zoology, The University of British Columbia, 6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4

*Author for correspondence (e-mail: jrichard@zoology.ubc.ca)

Accepted 23 July 2008

SUMMARY

Cell survival during hypoxia exposure requires a metabolic reorganization to decrease ATP demands to match the reduced capacity for ATP production. We investigated whether AMP-activated protein kinase (AMPK) activity responds to 12 h exposure to severe hypoxia (~0.3 mg O_2 l^{-1}) in the anoxia-tolerant goldfish (*Carassius auratus*). Hypoxia exposure in goldfish was characterized by a strong activation of creatine phosphate hydrolysis and glycolysis in liver and muscle. AMPK activity increased by ~5.5-fold in goldfish liver within 0.5 h hypoxia exposure and this increase in activity was temporally associated with an 11-fold increase in [AMP_{free}]/[ATP]. No changes in total AMPK protein amount were observed, suggesting that the changes in AMPK activity are due to post-translational phosphorylation of the protein. Hypoxia exposure had no effect on the expression of two identified AMPK α-subunit isoforms and caused an ~50% decrease in the mRNA levels of AMPK β-subunit isoform. Changes in AMPK activity in the liver were associated with an increase in percentage phosphorylation of a well-characterized target of AMPK, eukaryotic elongation factor-2 (eEF2), and decreases in protein synthesis rates measured in liver cell-free extracts. No activation of AMPK was observed in muscle, brain, heart or gill during the 12 h hypoxia exposure suggesting a tissue-specific regulation of AMPK possibly related to a lack of change in cellular [AMP_{free}]/[ATP] as observed in muscle.

Key words: energy charge, fish, phosphorylation potential, protein synthesis.

INTRODUCTION

Environmental hypoxia is a common, naturally occurring phenomenon in many aquatic ecosystems, the prevalence of which is increasing due to anthropogenic nutrient loading and eutrophication of both freshwater and marine environments. The major metabolic challenge facing animals living in hypoxic environments is the inhibition of ATP production *via* oxidative phosphorylation, thus hypoxia-intolerant animals quickly succumb to hypoxia due to an inability to maintain cellular energy balance and a loss of cellular [ATP] (Boutilier, 2001). By contrast, hypoxia-tolerant animals are able to maintain cellular energy balance when faced with an O_2 limitation by reducing metabolic demands to match the limited capacity for O_2-independent ATP production. At the cellular level, metabolic rate depression is achieved by reducing rates of protein synthesis and membrane ion movement among other processes (Hochachka et al., 1996). In hepatocytes isolated from the anoxia-tolerant turtle (*Chrysemys picta belli*) ATP-utilization during anoxia exposure falls to ~10% of normoxic rates (Buck et al., 1993) with reductions in protein synthesis making up the largest percentage of this decline (Land et al., 1993). In addition, similar hypoxia-induced reductions in protein synthesis rates have been observed in the anoxia-tolerant crucian carp *Carassius carassius* (Smith et al., 1996). Hypoxia survival necessitates the timely and well-synchronized restructuring of cellular processes involved in both energy provision and utilization; however, little research has focused on how these cellular processes are co-ordinated during hypoxia in order to maintain cellular energy balance.

AMP-activated protein kinase (AMPK) represents an ideal candidate protein to co-ordinate the metabolic responses to hypoxia. AMPK is a heterotrimeric protein kinase composed of a catalytic subunit (α) and two regulatory subunits [β and γ (Carling, 2004)]. Phosphorylation of AMPK at Thr-172 on the α-subunit is essential for its activation (Carling, 2004) and this is brought about *via* the activity of upstream kinases. Two of these upstream kinases have been identified, LKB1 (Sakamoto et al., 2005) and CaMKK (Witters et al., 2006), in mammals. AMPK appears to be continuously phosphorylated; however, the phosphate group is rapidly removed under normal conditions returning AMPK to an inactive form (Hardie, 2007). Binding of AMP to AMPK induces a conformational change and prevents dephosphorylation (Sanders et al., 2007). Upon activation, AMPK inhibits anabolic processes in the cell and activates catabolic processes (Hardie et al., 2006), thereby helping to maintain cellular [ATP]. In mammalian models, AMPK has been shown to inhibit protein synthesis through phosphorylation of eukaryotic elongation factor 2 [eEF2 (Horman et al., 2002)], decrease glycogen synthesis rates through inactivation of glycogen synthase (Nielsen et al., 2002) and decrease fatty acid synthesis rates through phosphorylation of acetyl-CoA carboxylase-1 [ACC-1 (Hardie and Pan, 2002)]. Activation of AMPK has also been shown to result in increased skeletal muscle hexokinase activity, glucose transporter expression [GLUT-4 (Holmes et al., 1999)] and translocation to the membrane (Kurth-Kraczek et al., 1999), and increased phosphofructokinase-2 (PFK-2) activity in rat cardiomyocytes (Marsin et al., 2000), all of which could enhance O_2-independent ATP production. Combined, these actions have led to AMPK being termed the cellular 'energy gauge' because of it critical role in maintaining cellular energy balance. However, the cellular consequences of AMPK activation have been studied mainly in exercise- and ischemia-stressed mammalian models, and no study has assessed the role of AMPK in co-ordinating the cellular responses to environmental hypoxia in a hypoxia-tolerant fish.

At the extreme of hypoxia-tolerance among teleost fishes are the *Carassius sp.*, which are capable of surviving months of anoxia at cold temperature. An important means by which members of this genus accomplish this feat is through a strong hypoxia-dependant depression of metabolic rate and activation of substrate-level phosphorylation. This has been described in the common goldfish, *Carassius auratus*, which depresses metabolic rate by ~70% during anoxic bouts [assessed *via* direct calorimetry (Van Waversveld et al., 1989)]. Metabolic depression during hypoxia/anoxia is key for goldfish survival as it allows for the conservation of endogenous glycogen reserves thereby extending the amount of time that can be spent under O_2-limiting conditions.

Given that AMPK is sensitive to changes in cellular energy status and that its activation leads to a general reduction in anabolic pathways and a stimulation of catabolic pathways, we hypothesize that it may play a role in co-ordinating the processes involved in the metabolic rate depression observed in the goldfish during exposure to severe hypoxia. In the present study, we determined cellular energy status, activation pattern of AMPK and its interactions with a well-characterized target, eEF2, and protein synthesis in liver and skeletal muscle of normoxia- and hypoxia-exposed goldfish. This was carried out in an attempt to determine whether or not AMPK may play a role in co-ordinating metabolic depression during hypoxia exposure in hypoxia-tolerant organisms.

MATERIALS AND METHODS

Animal care

Adult goldfish (*Carassius auratus* L.) weighing 36.0±1.4 g (means ± s.e.m.) were purchased from a local supplier (Delta Aquatics, Richmond, BC, Canada) and held in either flow-through or static renewal dechlorinated City of Vancouver tap water at 16°C. Fish were fed daily with commercial goldfish flakes. All animal procedures adhere to the Canadian Council on Animal Care guidelines as administered by the University of British Columbia Animal Care Committee.

Identification of AMPK subunits

Tissue sampling and gene identification

Goldfish were sampled directly from a holding tank and sacrificed with an overdose of benzocaine (1 g l^{-1}). Samples of brain, eye, heart, gill, intestine, liver, kidney and muscle were rapidly excised, flash-frozen in liquid nitrogen, and stored at –80°C. Total RNA was extracted from these tissue samples following the methods of Chomczynski (Chomczynski, 1993) using Tri Reagent (Sigma-Chemical Co., St Louis, MO, USA). Following isolation, total RNA was quantified spectrophotometrically and the integrity of the two ribosomal bands was assessed by electrophoresis. RNA was stored at –80°C. Reverse transcription reactions and PCR amplification of AMPK sequences were carried out following the methods outlined in Richards et al. (Richards et al., 2003). Briefly, cDNA was synthesized from 4 µg total RNA using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, Burlington, ON, Canada) following the manufacturer's instructions. Partial AMPK subunit sequences were obtained using primers designed from the conserved regions of known AMPK subunit isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) using all available vertebrate sequence information in GenBank, although only primers designed for α1 and β1 yielded amplicons. Primers for AMPKα1a were (forward) 5′-GGG CCA GCG TAA AAC CTT CCT-3′ and (reverse) 5′-GGA GGG GAA CTG TTT GAT TAT AT-3′, and PCR for this gene product consisted of 35 cycles; 1 min at 94°C, 1 min at 51°C and 2 min at 72°C. Primers for AMPKα1b were (forward) 5′-GGA GGG GAG CTA TTT GAT TAT AT-3′ and (reverse) 5′-GGG TTC TTC TTC GTA CAC G-3′, and PCR for this gene product consisted of 35 cycles; 1 min at 94°C, 1 min at 53°C and 2 min at 72°C. Primers for AMPKβ1 were (forward) 5′-GCC GGA AGG AGA GCA TCA GTA CAA GT-3′ and (reverse) 5′-GCG CTA AGA ACC ATC ACG CCA T-3′, and PCR for this gene product consisted of 35 cycles; 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. Primers were designed using GeneTool Lite software (www.biotool.com). PCR products were gel purified and ligated into a plasmid vector (pGEM-T EasyVector System II; Promega, Madison, WI, USA). Ligated plasmids were transformed into heat-shock competent *Escherichia coli* (strain JM109; Promega) and plated onto LB-agar plates. Colonies were grown overnight at 37°C and several colonies containing the ligated insert were selected and grown in liquid culture. Following overnight culture, plasmid DNA was harvested from cultured cells using a GenElute Plasmid Miniprep kit (Sigma Chemical Co.) and sequenced on an Applied Biosystems PRISM 377 sequencer (Foster City, CA, USA).

Tissue distribution of AMPK isoforms

Tissue distribution of goldfish AMPK isoforms was estimated using quantitative real-time PCR (qPCR) and isoform-specific primers designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Primers for AMPKα1a (GenBank accession number EU583380) were (forward) 5′-GCC AAG ATC GCT GAC TTT GG-3′ and (reverse) 5′-CGC AGC TCG TTC TCA GGA A-3′. Primers for AMPKα1b (EU583381) were (forward) 5′-TAA GGA CGA GTT GCG GTT CTC-3′ and (reverse) 5′-GCC CTG CGT ATA ACC TTC CA-3′. Primers for AMPKβ1 (EU580137) were (forward) 5′-GCT GCA GGT GCT CCT CAA C-3′ and (reverse) 5′-GTT GAG CAT CAC ATG GGT TGG T-3′. Total RNA was extracted from brain, eye, heart, gill, intestine, liver, kidney and muscle from fish sampled directly from the holding tank and cDNA was prepared using the same methods as outlined above. Expression was quantified by qPCR using an ABI PRISM 7000 sequence detector (Applied Biosystems). qPCR reactions consisted of 2 µl cDNA (reverse transcribed from 4 µg or total RNA), 4 pmol of each primer and Universal SYBR green master mix (Applied Biosystems) in a total volume of 22 µl. qPCR conditions included initial incubations of 2 min at 50°C and 10 min 95°C, followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. Melt-curve analysis was performed following each reaction to ensure that only a single product was amplified. Additionally, random products were sequenced following the methods outlined above to ensure the amplified product was indeed the product of interest.

Hypoxia exposure

Temperature acclimation

Three weeks before experimentation, a group of ~80 fish were transferred into a 375 l aquarium equipped with a canister filter and a cooling coil. Water temperature was then lowered in the tank at a rate of 2°C per day using a re-circulating water-chiller until it reached 10°C, at which point temperature was maintained for at least two weeks prior to experimentation. Fish were fed commercial goldfish flakes daily throughout the acclimation period.

Hypoxia exposure

Thirty-six hours before experimentation, goldfish were transferred into individual exposure chambers and returned to the aquarium. The exposure chambers consisted of highly perforated plastic beakers that allow for good water exchange between the exposure chamber and the bulk water and were large enough so the fish could

move freely. These chambers were designed so that they slid smoothly into basins that were slightly larger than the exposure chamber and we could remove the fish from the aquarium without air exposure or causing agitation. An overdose of benzocaine (1 g l^{-1}) could then be added to the basin and the fish sampled. To obtain normoxic tissue samples, eight chambers, each containing a fish, were removed and benzocaine added. At complete anaesthesia, which occurred ~3 min following the addition of benzocaine, individual fish were removed, patted dry, and weighted to the nearest 0.1 g. Blood was sampled following caudal severance using haematocrit (Ht) tubes and samples of skeletal muscle, liver, heart, brain and gill were rapidly excised, flash-frozen in liquid nitrogen, and stored at –80°C.

It should be noted that many anaesthetics are known to affect protein phosphorylation [e.g. tetracaine (Nivarthi et al., 1997)] but nothing is known of the impacts of benzocaine on protein phosphorylation; however, since all fish in the present study were sampled in an identical manner, any changes in protein phosphorylation observed are due to hypoxia treatment and not the anaesthetic chosen.

Following the sampling of normoxic fish, the water [O_2] in the experimental tank was lowered over a 1 h period by bubbling nitrogen-gas into the water, until it reached ~0.3 mg l^{-1}. Water [O_2] was monitored throughout the course of hypoxia exposure using an Oakton DO 6 dissolved O_2 meter (Cole Parmer, Montreal, QC, Canada). Eight fish were sacrificed at each of the six time points (0.5, 1, 2, 4, 8 and 12 h hypoxia exposure) in an identical manner to normoxic fish. Water temperature was maintained at 10°C throughout the experiment.

To obtain sufficient tissue for complete biochemical analysis, the acclimation and experimental trials were performed twice. Fish from the first experiment were used for the determination of muscle and liver intracellular pH (pH_i), muscle metabolite concentrations, and muscle and liver AMPK activity, protein content and mRNA expression levels. Fish from the second experiment were used for the determination of haematology, plasma [lactate], liver pH_i, metabolites, eEF2 and phospho-Thr-56 eEF2 protein expression, and analysis of liver protein synthesis rates. Liver pH_i and [lactate] were determined in both experiments and no significant differences were found between the two experiments [data not shown; two-way analysis of variance (ANOVA), $P>0.05$], therefore we consider both experiments to be comparable.

Analytical procedures

Haematology

Blood [haemoglobin] (Hb) was determined spectrophotometrically using Drabkin's reagent (Blaxhall and Daisley, 1973). Haematocrit was determined by centrifugation of whole blood at 5000 ***g*** for 3 min in sealed capillary tubes. Mean cellular haemoglobin content (MCHC) was calculated as [Hb]/Ht.

Tissue processing, pH_i and metabolites

Frozen muscle (~200 mg) was ground to a fine powder under liquid nitrogen and pH_i was determined in an aliquot following the methods of Pörtner et al. (Pörtner et al., 1991) using a thermostatted Radiometer BMS3 Mk2 capillary microelectrode with PHM84 pH meter (Radiometer, Copenhagen, Denmark). The remaining ground muscle tissue was lyophilized for 72 h and stored above desiccant at –80°C. For pH_i determination in liver, ~50 mg of liver was sonicated using a micro-sonicator (Kontes, Vineland, NJ, USA) at medium frequency for ~3 s in 0.2 ml ice-cold metabolic inhibitor (Pörtner et al., 1991). Liver pH_i was measured using an ultra-fine Accumet pH electrode (Cole Parmer).

For metabolite determination, ~20 mg lyophilized skeletal muscle or ~100 mg of frozen liver was homogenized at maximum speed in ice-cold 8% perchloric acid for 30 s using a Polytron homogenizer (Kinematica Inc., Bohemia, NY, USA). Homogenates were then centrifuged at 20,000 ***g*** for 5 min at 4°C and the supernatant adjusted to ~pH 7.6 with 3 mol potassium carbonate. Neutralized extracts were centrifuged at 20,000 ***g*** for 5 min at 4°C and the supernatant was immediately frozen in liquid nitrogen and stored at –80°C until use. These extracts were then used for the enzymatic determination of tissue [lactate], [ATP] and [creatine phosphate] (CrP) (Bergmeyer, 1983). Total [creatine] (Cr) was determined by heating an aliquot of extract in sealed Eppendorf tubes for 20 min at 60°C and assaying for Cr enzymatically (Bergmeyer, 1983). Free [Cr] was calculated for each sample by subtracting [CrP] from total [Cr]. Plasma [lactate] was measured enzymatically on deproteinized plasma [20 µl 8% perchloric acid added to 20 µl of plasma].

Western blotting

Sample preparation, SDS-PAGE and western blotting were carried out according to the methods outlined by Todgham et al. (Todgham et al., 2005). Briefly, liver and muscle samples (~20 mg) were homogenized in a buffer containing: 100 mmol l^{-1} Tris-HCl; 1% sodium dodecyl sulphate (SDS); 5 mmol l^{-1} ethylenediaminetetraacetic acid; 1 µg ml^{-1} aprotinin; 1 µg ml^{-1} pepstatin A; 1 µg ml^{-1} leupeptin; 20 µg ml^{1} phenylmethanesulphonylfluoride; pH 7.5. Homogenates were centrifuged at 5000 ***g*** for 10 min at 4°C, the supernatant was assayed for total protein using the methods of Bradford (Bradford, 1976) and a portion of the supernatant was denatured by boiling it for 3 min in SDS-sample buffer (Laemmli, 1970). Denaturing SDS-polyacrylamide gels were loaded with denatured liver and muscle homogenates at a protein concentration of 20 µg protein per lane and electrophoresed for 15 min at 75 V followed by 75 min at 150 V. An identical control sample was included on each gel to control for gel-to-gel variation. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) using a Trans-Blot semi-dry transfer cell (Bio-Rad). Blots for total AMPKα were blocked using Tween-20 Tris-buffered saline [TTBS: 17.4 mmol l^{-1} Tris-HCl; 2.64 mmol l^{-1} Tris Base; 0.5 M sodium chloride (NaCl); and 0.05% Tween-20 (v/v)] with 2% (w/v) non-fat powdered milk. Blots for eEF2 and phospho-Thr-56 eEF2 were blocked using TTBS with 3% (w/v) bovine serum albumin. All membranes were incubated overnight at 4°C in a 1:1000 dilution of primary antibody [either rabbit IgG anti-AMPKα, rabbit IgG anti-eEF2 or rabbit IgG anti-phospho-Thr-56 eEF2 (Cell-Signalling Technology, Danvers, MA, USA)]. Following washing in TTBS, membranes were incubated in 1:5000 IgG goat anti-rabbit secondary antibody [alkaline phosphatase conjugated (Sigma Chemical Co.)] in TTBS for 1 h. Membranes were developed in alkaline phosphatase buffer containing 5-bromo-4-chloror-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT; Sigma Chemical Co.). Band intensity was quantified using a FluorChem 8800 imager (Alpha Innotech, San Leandro, CA, USA) assisted by AlphaEase FC software (v. 3.1.2; Alpha Innotech), and protein amount was expressed relative to total homogenate protein loaded into each well and normalized to the normoxic control samples.

AMPK activity

AMPK activity was determined following the methods described by Davies et al. (Davies et al., 1989). Briefly, ~150 mg frozen tissue (muscle or liver) was homogenized for 30 s at medium speed in

approximately 3 volumes of ice-cold homogenization buffer [50 mmol l^{-1} Tris-base; 250 mmol l^{-1} Mannitol; 1 mmol l^{-1} EGTA; 1 mmol l^{-1} EDTA; 50 mmol l^{-1} sodium fluoride (NaF); 5 mmol l^{-1} sodium pyrophosphate; 1 mmol l^{-1} phenylmethanesulphonyl fluoride (PMSF); 4 g ml^{-1} trypsin inhibitor; 1 mmol l^{-1} benzamidine; and 1 mmol l^{-1} diothioreitol (DTT)]. Samples were then centrifuged at 4°C for 20 min at 14,000 ***g*** and 360 l supernatant was transferred to a new micro-centrifuge tube, and 40 l of 25% (w/v) polyethylene glycol-6000 (PEG-6000) was added bringing the concentration in the tube to 2.5% PEG-6000. Sample tubes were then vortexed for 10 min at 4°C and subsequently centrifuged at 10,000 ***g*** for 10 min at 4°C. Following centrifugation, 320 l supernatant was transferred to a new micro-centrifuge tube and ~60 l of 25% PEG-6000 was added bringing the concentration in the tube to 6% PEG-6000. Tubes were again vortexed for 10 min at 4°C and centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatant was then removed and discarded and the pellet was washed with 300 l of 6% PEG-6000 (prepared in homogenization buffer) before being centrifuged for a final time at 10,000 ***g*** for 10 min at 4°C. Following centrifugation, the supernatant was removed and discarded and the pellet was resupended in 75 l ice-cold resuspension buffer (50 mmol l^{-1} Tris-base; 250 mmol l^{-1} Mannitol; 1 mmol l^{-1} EGTA; 1 mmol l^{-1} EDTA; 50 mmol l^{-1} NaF; 5 mmol l^{-1} sodium pyrophosphate; 10% w/v glycerol; 0.02% sodium azide; 1 mmol l^{-1} PMSF; 4 mg ml^{-1} trypsin inhibitor; 1 mmol l^{-1} benzamidine; 1 mmol l^{-1} DTT). An aliquot of the purified resuspended protein solution was taken to determine the total protein by the Bradford protein assay [Sigma Chemical Co. (Bradford, 1976)]. Aliquots of 50 l of 1 mg ml^{-1} resuspended protein were prepared for each sample in 0.12% Triton X-100 (Sigma Chemical Co.) made up in resuspension buffer and immediately frozen at –80°C for no longer than two weeks before the activity assays were run. At the time of assay, samples were thawed on ice, and 2.5 l of suspension was assayed for total AMPK activity in a final volume of 25 l, containing: 40 mmol l^{-1} Hepes; 80 mmol l^{-1} NaCl; 8% w/v glycerol; 0.8 mmol l^{-1} EDTA; 0.2 mmol l^{-1} SAMS peptide (GenScript, Piscataway, NJ, USA); 0.2 mmol l^{-1} AMP; 0.8 mmol l^{-1} DTT; 200 mol ATP; 5 mmol l^{-1} magnesium chloride; and [^{32}P]-ATP (~3500 cpm $pmol^{-1}$). Negative controls, where sample was replaced with distilled H_2O, were also run for each sample. After incubation for 5 min at 20°C, 15 l aliquots were spotted onto 2 cm round phosphocellulase paper (Whatman p81, GE Healthcare, Baie d'Urfé, Quebec, Canada) and the phosphorylation reaction immediately stopped by submergence of the spotted papers into 200 ml of 150 mmol l^{-1} phosphoric acid. Spotted papers were washed 10 times for 5 min each in the same volume of fresh 150 mmol l^{-1} phosphoric acid. Ten washes were necessary to reduce non-specific binding to near background levels. Spotted papers were then washed once in 300 ml of acetone for 5 min and air-dried. The amount of bound ^{32}P on the papers was assessed using scintillation counting. AMPK activity was initially expressed as nmol of incorporated ^{32}P min^{-1} mg^{-1} of total protein; however, we did not run T=0 assays to correct for non-specific radioactivity coming down with the protein (although this binding will be consistent across all samples assayed), therefore, we present AMPK activity relative to the normoxia control sample, which was set to T=1.

AMPK gene expression

The expression of AMPKα1a, AMPKα1b and AMPKβ1 mRNA in liver was estimated using qPCR and cDNA synthesized from extracted mRNA using the above protocols. The qPCR primers were identical to those described above and, in this case, the expression of each gene was normalized against the expression of β-actin. qPCR primers were designed for β-actin using available goldfish sequence (Accession No. AB039726) and were (forward) 5′-TGA CCG AGC GTG GCT ACA G-3′ and (reverse) 5′-TCT CCT TGA TGT CAC GGA CAA T-3′. There was no effect of hypoxia exposure on the expression of actin when expressed as a function of total RNA, thus actin appears to be a good control gene for hypoxia studies. To determine the extent of genomic DNA contamination, we developed non-reverse transcribed controls for a random selection of samples. To develop non-reverse transcribed controls, we diluted our RNA samples (containing genomic DNA) to the same extent as our samples used for cDNA synthesis; however, did not reverse transcribe the samples. These samples, along with their paired cDNA sample, were subjected to qPCR and any amplification in the non-reverse transcribed control was due to genomic DNA contamination. Genomic DNA contamination was present in all samples but never constituted more than 1:1024 starting copies for AMPKα1a, 1:32 starting copies for AMPKα1b or 1:524 288 starting copies for AMPKβ1. Genomic DNA, therefore, represents a minor contribution to the total qPCR signal. One randomly selected control sample was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set to assess efficiency of the reaction. All results were expressed relative to these standard curves, and mRNA amounts (in arbitrary units) were normalized to the expression of actin. Expression levels in hypoxia-exposed animals were expressed relative to the mean expression levels in the normoxia control samples. All samples were run in duplicate and the coefficient of variation between duplicate samples was always <10%.

Cell-free protein translation assay

Protein synthesis rates were determined following the methods outlined by Rider et al. (Rider et al., 2006). Briefly, frozen liver was homogenized at 1:5 (w/v) in ice-cold extraction buffer containing: 50 mmol l^{-1} Hepes (pH 7.4); 250 mmol l^{-1} sucrose; 20 mmol l^{-1} NaF; 5 mmol l^{-1} sodium pyrophosphate; 1 mmol l^{-1} EDTA; and 1 mmol l^{-1} EGTA, and then clarified by centrifugation at 14,000 ***g*** for 15 min at 4°C. The resulting supernatant was removed and stored at –80°C until analysis, which was performed within two weeks of extraction. On the day of analysis, Sephadex G-25 columns (GE Healthcare, Piscataway, NJ, USA) were equilibrated with buffer containing: 50 mmol l^{-1} Hepes (pH 7.4); 200 mmol l^{-1} potassium acetate; 5 mmol l^{-1} magnesium acetate; 1 mmol l^{-1} DTT; 5 g ml^{-1} leupeptin; 1 mmol l^{-1} benzamidine; and 1 mmol l^{-1} PMSF as instructed by the column manufacturer. Clarified tissue extracts were thawed on ice and 0.5 ml was gravity filtered through columns to remove endogenous amino acids. Filtrate, containing cellular proteins, was collected and analysed for total protein using the Bradford assay as described above. To determine protein synthesis rates, a 50 l aliquot of the filtrate was added to assay buffer containing, 50 mmol l^{-1} Mops (pH 7.1), 140 mmol l^{-1} potassium acetate, 20 mmol l^{-1} magnesium acetate, 2 mmol l^{-1} DTT, 20 mmol l^{-1} CrP, 20 mmol l^{-1} creatine kinase (CrK), 1 mmol l^{-1} ATP, 0.5 mmol l^{-1} GTP, 0.1 mmol l^{-1} spermidine, 10 U RNaseOUT (Invitrogen, Burlington, ON, Canada), 50 ug ml^{-1} total RNA prepared from goldfish liver using the Tri-Reagent method as outlined above (Sigma Chemical Co.), and 20 mmol l^{-1} of each amino acid (except leucine) to a final volume of 100 µl. The reaction was started with the addition of 0.9 ul of 20 µmol activated leucine stock containing L-[4,5-^{3}H]-leucine (~300 cpm $pmol^{-1}$) and incubated at 25°C for 90 min. Negative controls, where clarified extract was replaced with distilled H_2O, were assayed for each sample.

Following incubation the reaction was immediately stopped with the addition of 1 ml 10% (w/v) trichloroacetic acid and placed on ice for 10 min. Samples were then centrifuged at 10,000 ***g*** for 5 min to collect precipitated proteins and the pellet was resuspended in 0.2 ml of 0.1 mol l^{-1} sodium hydroxide and re-precipitated in 1 ml of 5% (w/v) trichloroacetic acid. After 10 min on ice, proteins were collected by centrifugation and subjected to an additional wash. Following this wash, proteins were solubilized in 1 ml formic acid and 0.9 ml of the solubilized protein solution was taken for counting in 10 ml of Toluene-based scintillant on a LS 1801 liquid scintillation counter (Beckman Coulter, Mississauga, ON, Canada). Protein synthesis rates are expressed as pmol of leucine incorporated per mg of total protein per hour.

Calculations and statistical analysis

Free cytosolic [ADP] and [AMP] were calculated from measured values of [ATP], [CrP], [Cr] and pH_i assuming equilibrium of the creatine kinase and adenylate kinase reactions. Before calculating $[ADP_{free}]$ and $[AMP_{free}]$, metabolite concentrations were converted to molar concentrations using a tissue–water content of 0.8 ml g^{-1} wet mass (Wang et al., 1994). The equilibrium constants for creatine kinase (K'_{CK}) and adenylate kinase (K'_{AK}) were corrected for experimental temperature, ioinic strength, measured pH and free Mg^{2+} [assumed to be 1 mmol l^{-1} (Van Waarde et al., 1990)] according to published protocols (Golding et al., 1995; Teague et al., 1996).

Free cytosolic [ADP] was calculated using the following equation:

$$[ADP_{free}] = \frac{[ATP]\,[Cr]}{[CrP]\,K'_{CK}} \quad (1)$$

and free cytosolic [AMP] was calculated using the following equation:

$$[AMP_{free}] = \frac{[ADP]^2\,K'_{AK}}{[ATP]}. \quad (2)$$

The Gibbs free energy of ATP hydrolysis (Δ_fG'; kJ mol^{-1}) was determined using the following equation:

$$\Delta_fG' = \Delta_fG'^{\circ}_{ATP} + RT\ln\frac{[ADP_{free}]\,[P_i]}{[ATP]}, \quad (3)$$

where R is the universal gas constant (8.3145 J K^{-1} mol^{-1}), T is temperature in K and $\Delta_fG'^{\circ}_{ATP}$ is the standard transformed Gibbs energy of ATP hydrolysis ($\Delta_fG'^{\circ}_{ATP}=-RT\ln K'_{ATP}$) at the measured pH and temperature and estimated free $[Mg^{2+}]$ (Golding et al., 1995). Cytosolic free [Pi] was estimated assuming the inverse of CrP hydrolysis and starting with a resting tissue level of 1 μmol g^{-1} wet mass (Hardewig et al., 1998).

All data are presented as means ± s.e.m. All muscle metabolite concentrations determined on lyophilized tissue were converted back into wet mass using a 4:1 wet:dry ratio. Statistical analysis involved one-way ANOVA followed by Holm–Sidak *post hoc* test to identify where statistical difference occurred. All data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene median test). In cases where data sets did not meet these assumptions, data were log transformed and statistical analyses repeated. For those data sets that still did not meet assumptions following transformation, statistical analysis involved Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's *post hoc* test. Differences were considered statistically significant at $P<0.05$.

RESULTS

AMPK isoforms

We identified two genes coding for AMPKα1 and one gene coding for AMPKβ1 in goldfish. Alignment of the cDNA sequences with the DNA sequences deposited in GenBank revealed homologies of 69–95% for the AMPKα1-catalytic subunit and 78–84% for the AMPKβ1-regulatory subunit. Each of these AMPK genes was expressed in all goldfish tissues examined (Fig. 1). AMPKα1a was expressed most highly in brain, kidney and intestine (Fig. 1A), AMPKα1b was expressed most highly in brain, kidney and gill (Fig. 1B) and AMPKβ1 was expressed at relatively constant levels across tissues with the highest expression in brain (Fig. 1C).

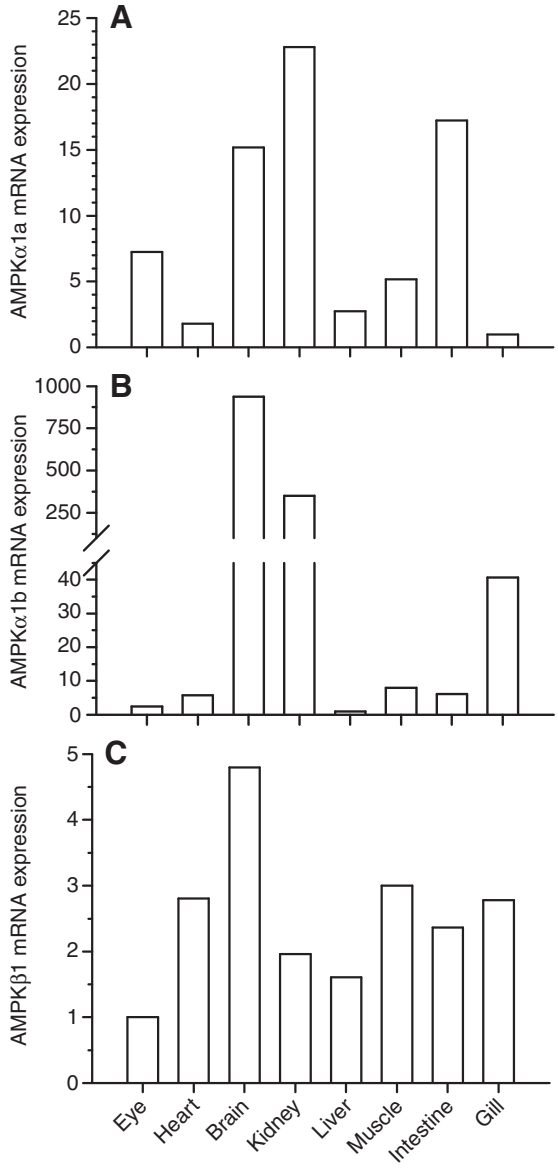

Fig. 1. Distribution of AMPKα1a (A), AMPKα1b (B) and AMPKβ1 (C) mRNA in eye, heart, brain, kidney, liver, muscle, intestine and gill in goldfish. Expression of each gene is relative to the same standard sample and absolute expression is adjusted such that the tissue with the lowest expression for each gene has an expression quantity of 1.

Table 1. Blood haemoglobin (Hb), haematocrit (Ht), mean cellular haemoglobin content (MCHC) and plasma [lactate] in goldfish exposed to normoxia (~9.5 mg O_2 l^{-1}) and 12 h hypoxia (~0.3 mg O_2 l^{-1})

	Time (h)						
Measure	Normoxia	0.5	1	2	4	8	12
Hb	1.18±0.09	1.21±0.12	1.24±0.04	1.23±0.09	1.04±0.13	1.20±0.07	1.06±0.11
Ht	30.2±1.8	35.1±2.9	39.6±3.3	38.2±3.0	35.4±2.3	36.1±1.8	31.2±3.0
MCHC	3.9±0.1	3.4±0.2	3.2±0.2*	3.2±0.1*	3.2±0.1*	3.3±0.1*	3.4±0.1*
Plasma lactate	1.23±0.12	7.71±0.94*	9.03±0.35*	10.40±0.33*	11.73±0.40*	12.05±0.44*	13.44±0.37*

Data are means ± s.e.m. (N=5 to 8). Haemoglobin (Hb) is expressed in mmol l^{-1}; haematocrit (Ht) is expressed in %; mean cellular haemoglobin content (MCHC) is expressed as ([Hb]/Ht); plasma lactate is expressed as μmol g wet tissue^{-1}. *Significant difference from normoxia, P<0.05.

Responses to hypoxia

Whole animal and blood

Goldfish remained quiescent throughout the hypoxia exposure and no fish deaths were observed. Less than 4% of all fish exposed to hypoxia showed signs of distress or lost equilibrium during the hypoxia exposure and these fish were removed from the treatment and placed into a well-aerated tank for recovery and not included in the data analysis. There were no significant effects of hypoxia exposure on blood [Hb] or Ht (Table 1). MCHC decreased significantly compared with normoxia at 1 h hypoxia exposure and remained lower than normoxic values for the duration of the hypoxia exposure (Table 1). Plasma [lactate] increased significantly by ~6-fold over the first 0.5 h of hypoxia exposure and continued to rise, reaching values that were 11-fold higher than during normoxia at 12 h exposure to hypoxia (Table 1).

Liver

Liver [ATP] decreased significantly by ~50% within the first 0.5 h of hypoxia exposure and remained at this lower level for the duration of the hypoxia exposure (Fig. 2A). Over the same time frame, [CrP] decreased significantly to nearly one-quarter of normoxic concentrations and was constant at this level for the remainder of the exposure (Fig. 2B). Total [Cr] was not significantly affected by hypoxia exposure, therefore free [Cr], calculated as the difference between total [Cr] and [CrP], increased significantly at 0.5 h exposure to hypoxia and remained elevated compared with normoxic controls for the 12 h hypoxia exposure (Table 2). Lactate concentrations in liver increased significantly by ~4-fold over the first 2 h hypoxia exposure and continued to rise to values that were 7-fold higher than normoxia by 12 h (Table 2). pH_i decreased rapidly and significantly within the first 0.5 h of hypoxia exposure and remained lower than normoxic samples for the duration of the treatment (Table 2). Calculated [ADP_{free}] was elevated by 1 h exposure to hypoxia and remained elevated for up to 4 h after which point [ADP_{free}] was no longer significantly elevated compared with normoxic controls. Liver [ADP_{free}]/[ATP] and [AMP_{free}] followed similar patterns with values increasing significantly over the first 0.5 h of hypoxia exposure, remaining elevated throughout the 12 h hypoxia exposure (Table 2). Liver [AMP_{free}]/[ATP] increased rapidly following the onset of hypoxia, continued to increase over the first 4 h hypoxia exposure, then stabilized between 8 and 12 h at values that were between 11- and 15-fold higher than observed in normoxic controls (Fig. 2C). The Δ_fG' of ATP hydrolysis in goldfish liver fell significantly over the first 0.5 h of hypoxia exposure and remained lower than normoxic controls for the duration of hypoxia exposure (Table 2).

Liver AMPK activity increased significantly by ~5.5-fold over the first 0.5 h of hypoxia exposure, remained elevated until 8 h exposure to hypoxia to return to levels that were not significantly elevated compared with controls at 12 h exposure to hypoxia (Fig. 3A). Our western blot analysis using antibodies raised against human AMPKα sequence detected two immunoreactive bands of similar size (~62 kDa) that varied in concert with each other. No other immunoreactive bands were detected on the western blots in the present study (full western blots not shown). Quantification of the darker band revealed no significant effect of hypoxia exposure on AMPKα protein expression in liver (Fig. 3B). Hypoxia exposure had no significant effect on AMPKα1a or AMPKα1b mRNA

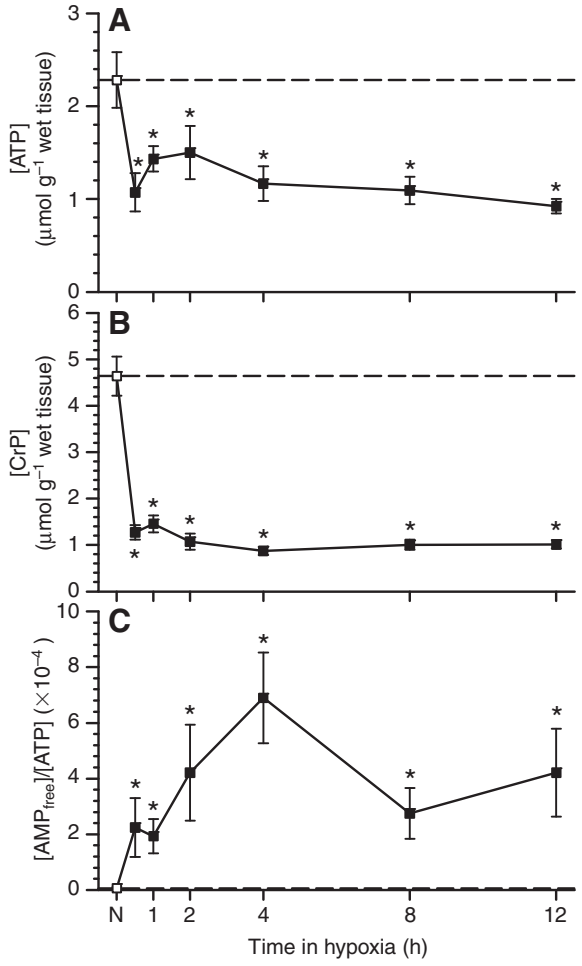

Fig. 2. Liver [ATP] (A), [creatine phosphate] (CrP) (B) and calculated [AMP_{free}]/[ATP] (C) in goldfish exposed to normoxia (N; ~9.5 mg O_2 l^{-1}; open squares) and 12 h of hypoxia (~0.3 mg O_2 l^{-1}; closed squares). Horizontal broken lines through normoxia are shown as a reference. Data are means ± s.e.m. (N=5 to 8). *Significant difference from normoxia, P<0.05.

Table 2. Liver free [Cr], [lactate], pH_i, $[ADP_{free}]$, $[AMP_{free}]$, $[ADP_{free}]/[ADP]$ and Δ_fG' in goldfish exposed to normoxia (~9.5 mg O_2 l^{-1}) and 12 h hypoxia (~0.3 mg O_2 l^{-1})

	Time (h)						
Measure	Normoxia	0.5	1	2	4	8	12
Cr	1.82±0.80	3.71±0.87*	4.02±0.57*	4.81±0.67*	5.39±0.84*	4.13±0.65*	5.01±0.99*
Lactate	1.29±0.20	2.66±0.33	4.27±0.67	5.76±0.64*	5.76±0.21*	6.36±0.61*	9.90±0.82*
pH_i	7.01±0.03	6.89±0.03*	6.90±0.01*	6.88±0.03*	6.87±0.02*	6.86±0.02*	6.85±0.01*
ADP_{free}	6.15±2.65	14.08±4.50	19.22±4.33*	25.69±3.60*	32.19±4.45*	17.93±4.06	17.78±4.47
AMP_{free}	0.02±0.01	0.20±0.08*	0.27±0.09*	0.46±0.15*	0.78±0.19*	0.30±0.11*	0.37±0.15*
ADP_{free}/ATP	0.002±0.001	0.013±0.004*	0.014±0.003*	0.020±0.004*	0.028±0.004*	0.017±0.003*	0.020±0.004*
Δ_fG'	–63.93±1.29	–55.72±1.11*	–55.80±1.01*	–53.75±0.72*	–52.49±0.53*	–54.01±0.78*	–54.11±0.87*

Data are means ± s.e.m. (N=5 to 8). Cr, free creatine; pH_i, intracellular pH; ADP_{free}, free ADP; AMP_{free}, free AMP; Δ_fG', effective Gibbs free energy change of ATP hydrolysis. Cr and lactate are expressed in μmol g wet tissue^{-1}; ADP_{free} and AMP_{free} are expressed in nmol g wet tissue^{-1}; Δ_fG' is expressed in kJ mol.
*Significant difference from normoxia P<0.05.

expression; however, a significant decrease in AMPKβ1 mRNA was noted at 1, 2, 4 and 12 h hypoxia exposure (Table 3).

During hypoxia exposure, there was a rapid (by 0.5 h) and significant 2-fold increase in phospho-eEF2, which remained elevated for the first 2 h of hypoxia exposure and waned following 4 h hypoxia exposure (Fig. 4A,B). There was no significant effect of hypoxia exposure on total eEF2 quantity in goldfish liver (representative western blot shown in Fig. 4B). Protein synthesis rates in liver, as assessed by ^{3}H-leucine incorporated into protein in cell-free extracts, decreased rapidly and significantly over the first 0.5 h of hypoxia exposure and remained depressed compared with the normoxic controls for the full duration of the hypoxia exposure (Fig. 4C).

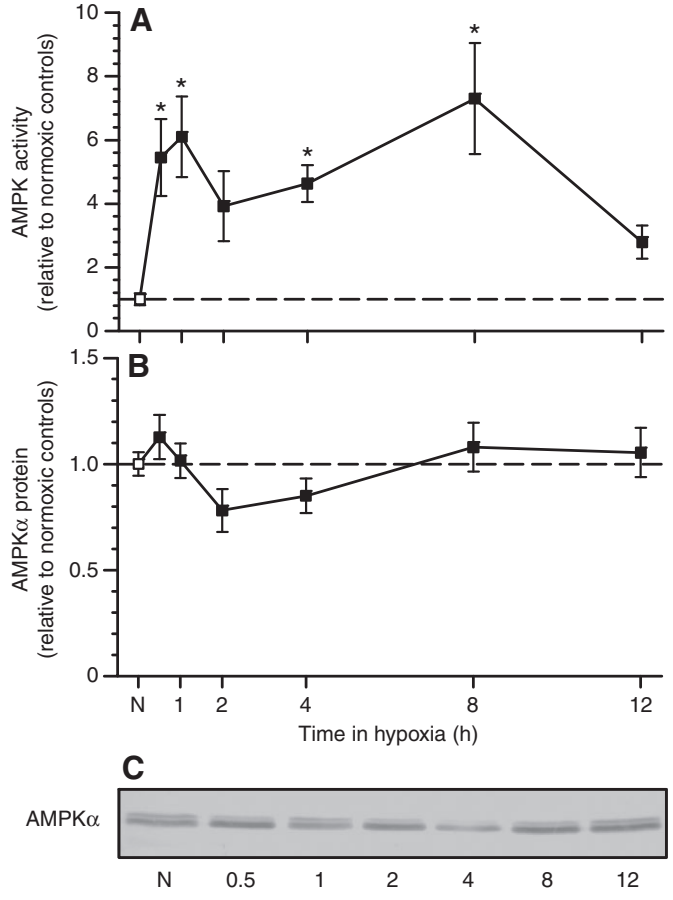

Fig. 3. Liver AMPK activity (A), protein AMPK (B) and representative AMPKα western blot (C) in goldfish exposed to normoxia (N; ~9.5 mg O_2 l^{-1}; open squares) and 12 h hypoxia (~0.3 mg O_2 l^{-1}; closed squares). Horizontal broken lines through normoxia are shown as a reference. AMPK activity during hypoxia exposure is expressed relative to the activity observed in normoxia, which was set to 1. AMPK protein is normalized to total homogenate protein and expressed relative to the values observed in normoxia. C Bands shown on representative western blot are at ~62 kDa. Data are means ± s.e.m. (N=5 to 11). *Significant difference from normoxia, P<0.05.

Muscle

Muscle [ATP] did not change significantly in response to hypoxia exposure (Fig. 5A). Hypoxia exposure caused a significant drop in muscle [CrP] by 2 h hypoxia, which remained lower than normoxic values throughout the hypoxia exposure (Fig. 5B). Total [Cr] levels were not affected by hypoxia exposure (data not shown) thus calculated free [Cr] increased during hypoxia exposure (Table 4). Muscle [lactate] increased significantly by ~4-fold within the first 1 h of hypoxia exposure and continued to increase throughout the hypoxia exposure (Table 4). Muscle pH_i fell significantly by 1 h hypoxia exposure and remained lower than normoxic controls for the duration of treatment (Table 4). There were no significant effects of hypoxia exposure on calculated muscle $[ADP_{free}]$, $[ADP_{free}]/[ATP]$, or $[AMP_{free}]/[ATP]$ (Table 4; Fig. 5C). Muscle $[AMP_{free}]$ was not significantly affected by hypoxia exposure until 12 h, where concentrations increased significantly by nearly ~5.5-fold (Table 4). Furthermore, there was no significant effect of hypoxia exposure on the Δ_fG' of ATP hydrolysis in goldfish muscle until 12 h hypoxia exposure, where Δ_fG' decreased significantly relative to the normoxic controls (Table 4).

Unlike the response observed in liver, AMPK activity in muscle was not affected significantly by 12 h of hypoxia exposure (Fig. 6A). Only a single immunoreactive band at ~62 kDa was observed in muscle (see representative western blot analysis, Fig. 6C) and hypoxia exposure had no significant effect on the amount of AMPK protein (Fig. 6B,C).

Brain, gill and heart

There was no significant effect of 0.5, 8 or 12 h hypoxia exposure on AMPK activity in brain, gills or heart (Table 5).

DISCUSSION

In order to survive hypoxia exposure, hypoxia-tolerant organisms must reorganize cellular metabolism to limit ATP demands to match the limited capacity for O_2-independent ATP production. It has been speculated (Bartrons et al., 2004; Bickler and Buck, 2007; Rider et

Table 3. AMPKα1a, AMPKα1b and AMPKβ1 mRNA expression in the liver of goldfish exposed to normoxia (~9.5 mg O_2 l^{-1}) and 12 h hypoxia (~0.3 mg O_2 l^{-1})

	Time (h)						
Gene	Normoxia	0.5	1	2	4	8	12
AMPKα1a	1.00±0.14	0.81±0.22	1.09±0.29	0.84±0.20	0.91±0.29	1.15±0.32	1.13±0.30
AMPKα1b	1.00±0.17	0.96±0.30	0.65±0.13	0.70±0.17	0.80±0.22	1.19±0.32	0.62±0.15
AMPKβ1	1.00±0.14	1.49±0.12	0.65±0.20*	0.59±0.09*	0.72±0.12*	0.75±0.12	0.58±0.14*

AMPKα1a, AMPKα1b and AMPKβ1 mRNA expression is relative to β-actin expression, and all hypoxia data are relative to normoxic liver samples. *Significant difference from normoxia $P<0.05$.

al., 2006) but never experimentally determined that AMPK may play an important role in co-ordinating the hypoxic cellular response in hypoxia-tolerant animals. The present study is the first to show that AMPK activity increases in response to short-term, severe hypoxia exposure in a hypoxia-tolerant animal, and that this activation is associated with a reduction in protein synthesis rates, potentially mediated through the phosphorylation of eEF2. There was a close temporal relationship between calculated increases in $[AMP_{free}]/[ATP]$ and the activation of AMPK in goldfish liver (Table 2; Fig. 3), supporting the notion that a disruption of cellular

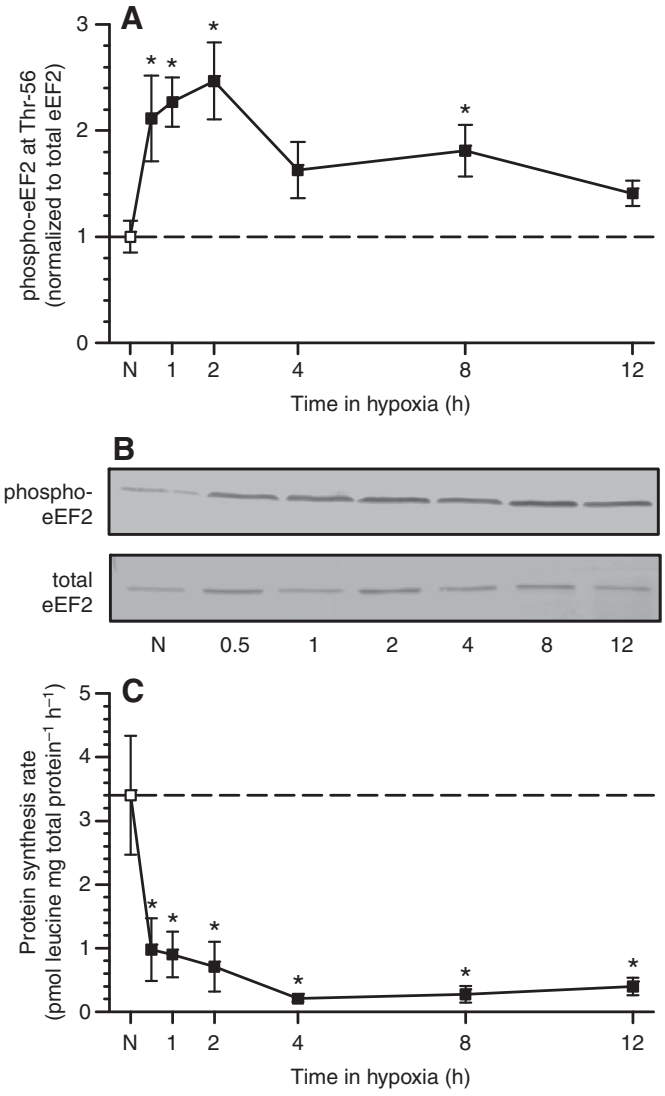

Fig. 4. Liver phospho-eEF2 (A), representative phospho-Thr-56-eEF2 and eEF2 western blots (B) and protein synthesis rate (C) in goldfish exposed to normoxia (N; ~9.5 mg O_2 l^{-1}; open squares) and 12 h of hypoxia (~0.3 mg O_2 l^{-1}; closed squares). Horizontal broken lines through normoxia are shown as a reference. Bands shown on representative western blot are at ~95 kDa. Data are means ± s.e.m. (N=5 to 8). *Significant difference from normoxia, $P<0.05$.

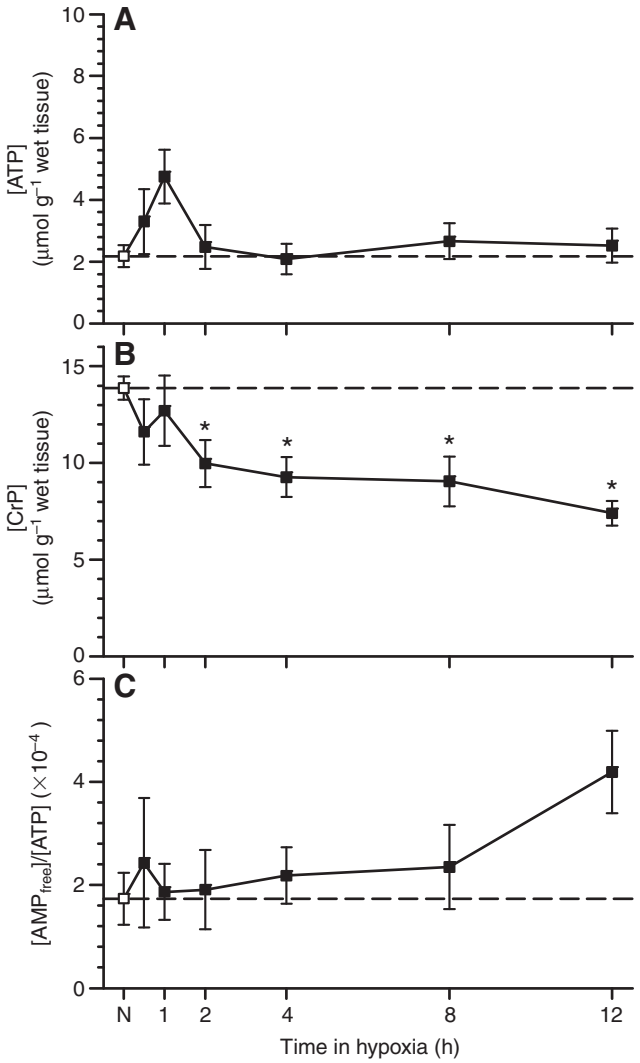

Fig. 5. Muscle [ATP] (A), [creatine phosphate] (CrP) (B) and calculated $[AMP_{free}]/[ATP]$ (C) in goldfish exposed to normoxia (N; ~9.5 mg O_2 l^{-1}; open squares) and 12 h of hypoxia (~0.3 mg O_2 l^{-1}; closed squares). Horizontal broken lines through normoxia are shown as a reference. Data are means ± s.e.m. (N=5 to 15). *Significant difference from normoxia, $P<0.05$.

Table 4. Muscle free [Cr], [lactate], pH_i, $[ADP_{free}]$, $[AMP_{free}]$, $[ADP_{free}]/[ADP]$ and Δ_fG' in goldfish exposed to normoxia (~9.5 mg O_2 l^{-1}) and 12 h hypoxia (~0.3 mg O_2 l^{-1})

	Time (h)						
Measure	Normoxia	0.5	1	2	4	8	12
Cr	13.39±0.92	17.50±0.91*	18.59±1.34*	18.46±1.00*	18.19±1.69*	20.01±1.64*	21.23±1.50*
Lactate	1.02±0.11	3.51±0.68	4.28±0.35*	5.30±0.57*	6.96±0.73*	8.45±0.89*	10.76±0.85*
pH_i	7.36±0.02	7.29±0.02	7.27±0.04*	7.28±0.02*	7.23±0.04*	7.15±0.02*	7.15±0.03*
ADP_{free}	24.67±3.70	47.76±9.21	49.46±6.07	34.97±21.20	37.76±12.36	44.77±18.83	61.22±18.36
AMP_{free}	0.28±0.05	0.56±0.13	0.60±0.11	0.54±0.38	0.58±0.24	0.74±0.37	1.27±0.46*
ADP_{free}/ATP	0.013±0.002	0.015±0.004	0.014±0.002	0.013±0.004	0.015±0.002	0.015±0.004	0.021±0.002
Δ_fG'	–60.54±0.38	–59.65±0.77	–59.91±0.75	–60.71±2.47	–59.01±0.77	–58.82±1.76	–56.96±0.49*

Data are means ± s.e.m (N=5 to 15). Cr, free creatine; pH_i, intracellular pH; ADP_{free}, free ADP; AMP_{free}, free AMP; Δ_fG', effective Gibbs free energy change of ATP hydrolysis. Cr and lactate are expressed in μmol g wet tissue^{-1}; ADP_{free} and AMP_{free} are expressed in nmol g wet tissue^{-1}; Δ_fG' is expressed in kJ mol.
*Significant difference from normoxia P<0.05.

energy status is essential for activation of AMPK. Responses of AMPK to short-term, severe hypoxia exposure were tissue-specific, with responses observed only in the liver and not in other tissues.

It has been suggested that maintenance of a stable cellular [ATP] during hypoxia exposure is the hallmark measure of a hypoxia-tolerant animal (Boutilier, 2001; Hochachka et al., 1996); however, this may be an over simplification. In goldfish, hypoxia exposure caused liver [ATP] to decrease by nearly half during the first 0.5 h but following this initial drop, liver [ATP] stabilized for the duration of hypoxia exposure (Fig. 2). These results are in general agreement with the results of Busk and Boutilier who showed in isolated eel hepatocytes that anoxia caused an initial decrease in [ATP] followed by a stabilization at a new, lower level (Busk and Boutilier, 2005). By contrast, Krumschnabel et al., demonstrated that exposure of isolated goldfish hepatocytes to anoxia did not result in a decrease in [ATP], whereas the same preparation exposed to chemical anoxia (sodium cyanide), showed a decrease in [ATP] (Krumschnabel et al., 1997). This latter decrease in [ATP] was modest when compared with the large decreases in [ATP] observed in anoxia-exposed hepatocytes isolated from the hypoxia-intolerant rainbow trout (*Oncorhynchus mykiss*). The fact that [ATP] is maintained in the liver after an initial disruption, rather than falling to fatally low concentrations highlights the ability of the hypoxia-tolerant goldfish to enter a state of lower ATP turnover. Furthermore, the decrease in [ATP] in fish liver, but not muscle (Fig. 5A), during hypoxia has been described previously in goldfish (van den Thillart et al., 1980) and sole, *Solea solea* (Dalla Via et al., 1994). These authors postulated that the low [CrP] observed in liver results in an inability to adequately buffer [ATP] during the onset of hypoxia. This hypothesis agrees with the present study where liver had ~3-fold lower [CrP] than muscle (cf. Fig. 2B and Fig. 5B), and [CrP] decreased in goldfish liver over the same time period as the decline in [ATP] (Fig. 2). Overall, the initial decline in liver [ATP] results from its hydrolysis in the face of a blunted capacity for ATP production, which leads to the observed accumulation of its breakdown products, $[ADP_{free}]$ and $[AMP_{free}]$ (Table 2). In agreement, $[ADP_{free}]/[ATP]$ and $[AMP_{free}]/[ATP]$ increased significantly during the first 0.5 h and remained elevated for the duration of the hypoxia exposure (Fig. 2; Table 2).

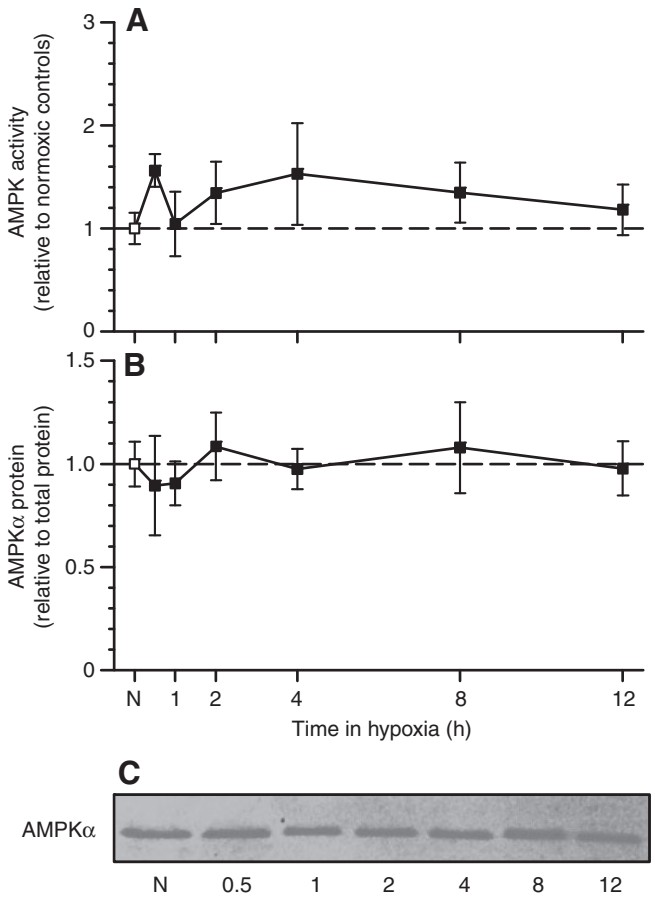

Fig. 6. Muscle AMPK activity (A), AMPK protein (B) and representative AMPK western blot (C) in goldfish exposed to normoxia (N; ~9.5 mg O_2 l^{-1}; open squares) and 12 h of hypoxia (~0.3 mg O_2 l^{-1}; closed squares). Data are means ± s.e.m. (N=6 to 12). See Fig. 3 caption for more detail.
*Significant difference from normoxia, P<0.05.

Table 5. Relative changes in AMPK activity in brain, gill and heart in goldfish exposed to normoxia (~9.5 mg O_2 l^{-1}) and at 0.5, 8 and 12 h hypoxia (~0.3 mg O_2 l^{-1})

	Time (h)			
Tissue	Normoxia	0.5	8	12
Brain	1.0±0.3	1.5±0.2	1.6±0.3	1.6±0.2
Gill	1.0±0.1	0.9±0.3	1.6±0.4	1.4±0.4
Heart	1.0±0.2	1.2±0.2	1.4±0.4	1.3±0.1

AMPK activity during hypoxia exposure is expressed relative to the mean activity observed in normoxia, which was set to 1. Data are means ± s.e.m. (N=5 to 8).

Hypoxia exposure caused a significant disruption of cellular phosphorylation potential in goldfish liver with a rapid (within 0.5 h) and dramatic decrease in Δ_fG' of ATP hydrolysis occurring upon hypoxia exposure (Table 2). Following the initial decrease, there was a stabilization of Δ_fG' of ATP hydrolysis at approximately $-54\,kJ\,mol^{-1}$, which is above the values calculated as being required for the function of ATPases in rat myocardium [–49, –51, –53 and –45 to $-50\,kJ\,mol^{-1}$ for sarcolemmal Na^+/K^+-ATPases, Ca^{2+}-ATPases, sarcoplasmic reticulum Ca^{2+}-ATPases and actomyosin-ATPases, respectively (Kammermeier, 1987; Kammermeier, 1993)]. Species differences in Δ_fG' of ATP hydrolysis requirements exist (Pörtner et al., 1996) and the actual requirements of goldfish ATPases are not known; however, our data suggest that the free energy of ATP hydrolysis in goldfish liver during hypoxia exposure is maintained at a balanced level that continues to allow for the function of integral cellular processes, albeit probably at substantially reduced levels.

Clearly, the liver is capable of readjusting metabolism after a short period of transition and it has been proposed that this occurs through the co-ordinated depression of ATP hydrolysis and increased glycolytic flux to support ATP production (Hochachka et al., 1996). The activation of AMPK, as observed in the present study (Fig. 3A), has been hypothesized to co-ordinate these events (Hardie, 2007). AMPK activation decreases rates of cellular anabolism, in particular the rates of protein synthesis, and upregulates PFK activity in rat cardiomyocytes (Marsin et al., 2000) and increases GLUT-4 transcription and insertion into rat skeletal muscle membranes (Holmes et al., 1999; Kurth-Kraczek et al., 1999). Overall, these effects on PFK and GLUT transporters, if they occur in fish, should enhanced glycolytic ATP production. Furthermore, PFK is allosterically activated by increasing $[AMP_{free}]$ and $[ADP_{free}]$ and inhibited by high [ATP], possibly further enhancing glycolysis and lactate production during hypoxia exposure.

Lactate accumulation in goldfish liver during our hypoxia exposure occurred at a relatively slow rate with a significant 4-fold increase occurring at only 2 h (Table 2). This slow accumulation of lactate tends to argue against a substantial increase in liver glycolytic flux; however, it must be noted that our experimental design does not allow us to assess liver glycolytic flux during hypoxia exposure in goldfish. To calculate glycolytic flux we would need to know the rates of lactate uptake or release from the liver, which is difficult to assess *in vivo*. The observed plasma to liver [lactate] gradient (estimated liver intracellular lactate is $7.2\,mmol\,l^{-1}$ after accounting for an intracellular water content of $0.8\,ml\,g^{-1}$ wet tissue cf. $10.4\,mmol\,l^{-1}$ in plasma) (Tables 1 and 2), opens the possibility that measured liver lactate is of plasma origin and not endogenously produced, although a number of factors play into dictating lactate movement across membranes (Wang et al., 1996). In addition, we exposed the goldfish to severe hypoxia, not anoxia, therefore O_2 is still available for mitochondrial respiration and ATP production, although possibly occurring at reduced levels. Overall, measurements of liver O_2 consumption and liver lactate production rates would be useful to determine the relative roles of metabolic rate depression, enhanced glycolysis and oxidative phosphorylation in longer-term (>1 h) hypoxia survival. However, it remains reasonable to suggest that prolonged hypoxia-survival necessitates a decrease in ATP utilization that can be supplied by either moderately enhanced glycolytic flux or sustained oxidative phosphorylation and that these events appear to be co-ordinated by the activation of AMPK.

The mechanism for the observed AMPK activation in goldfish liver is likely to be post-translational modification through phosphorylation of the α-subunit at Thr-172 (Beauloye et al., 2001; Rider et al., 2006). Decreases in liver [ATP] may be a requisite for the observed AMPK activation as ATP binds to the same allosteric domain as AMP (Hardie, 2007; Scott et al., 2004) competitively inhibiting AMPK (Corton et al., 1995; Hardie et al., 2006). The concurrent increase in $[AMP_{free}]$ and the decrease in [ATP] observed in goldfish liver may thus be required to activate AMPK. To ascertain whether the increase in AMPK activity shown in Fig. 3A was due to phosphorylation, we screened several anti-phospho-Thr-172 AMPK antibodies but discovered that the antibodies were unable to detect the phosphorylated protein in any goldfish tissue tested. Regardless, the rapid and large-scale changes in activation state of liver AMPK are probably only possible by post-translational modification. No changes in total AMPKα protein or mRNA expression and decreases in AMPKβ1 mRNA levels were observed during the 12 h hypoxia exposure (Table 3; Fig. 3B) therefore, upregulation of protein expression cannot explain AMPK activation. The lack of change in AMPK α-subunit expression concurs with previously published findings from human glioblastoma cells, which show no change in AMPKα1 mRNA or protein expression in response to hypoxia exposure and only demonstrate an upregulation of AMPKα2 isoform protein and mRNA after prolonged hypoxia exposure [>24 h (Neurath et al., 2006)].

Regardless of expression pattern, it is known that the activation of AMPK decreases cellular protein synthesis rates, in part through direct phosphoryation of eukaryotic elongation factor-2 kinase [eEF2K (Browne et al., 2004; Horman et al., 2002)], which in turn phosphorylates eEF2 and renders eEF2 unable to bind ribosomes. In the present study, we demonstrate a rapid (within 0.5 h) and significant increase in phosphorylation of eEF2 at Thr-56 in livers of hypoxic goldfish (Fig. 4A). This increase in phosphorylation is temporally associated with a significant decline in the rate of ^{3}H-leucine incorporation into new proteins in cell-free extracts, which fell to ~70% of normoxic values by 0.5 h hypoxia exposure and continued to fall to ~93% of normoxia by 4 h hypoxia exposure (Fig. 4B). Protein synthesis, for its part, accounts for 20–30% of total ATP-coupled O_2 demand (Bickler and Buck, 2007) and has been shown to decrease by ~90% in anoxia-tolerant hepatocyte cultures (Land et al., 1993) and by 56–95% in the liver of crucian carp (Smith et al., 1996) and Amazonian cichlids [*Astronotus ocellatus* (Lewis et al., 2007)] during anoxia/hypoxia exposure. Interestingly, in the present study, both the increase in phosphorylation of eEF2 and the decline in ^{3}H-leucine incorporation into proteins occurs over the same timescale as the increase in AMPK activity (Fig. 3) suggesting that the hypoxia-induced reductions in protein synthesis may be mediated by the activation of AMPK. To demonstrate a causal relationship between AMPK activation and inhibition of protein synthesis in hypoxic goldfish, direct manipulation of AMPK activity using the pharmacological activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) must be performed.

The large decrease in liver pH_i measured in goldfish during hypoxia exposure (Table 2) may also contribute to the regulation of protein synthesis and metabolic rate depression. Tissue acidosis has been shown to cause an increase in eEF2K activity and eEF2 phosporylation, reducing protein synthesis rates (Dorovkov et al., 2002). In addition, beyond the specific effects of acidosis on protein synthesis, decreases in pH_i and extracellular pH are thought to contribute to initiating and sustaining metabolic rate depression in vertebrates and invertebrates through general effects on enzyme function or membrane transport (e.g. Reipschläger and Pörtner, 1996), although not all studies support this notion (e.g. Brooks and Storey, 1989).

Because it appears that AMPK activation can affect metabolically costly processes, like protein synthesis, in hypoxia-tolerant fish and facilitate metabolic rate depression, it is of interest to consider other proteins or pathways, which are important in metabolic rate depression and may be activated by AMPK. For instance, second to protein synthesis, iono-regulation is the largest energy sink in the cell comprising ~20% of ATP demand in hypoxia-tolerant hepatocytes of the western painted turtle, *Chrysemys picta belli* (Hochachka et al., 1996). In goldfish hepatocytes, Na^+ pump activity and K^+ leak pathways are downregulated in a co-ordinated manner during chemical anoxia for energy conservation purposes (Krumschnabel et al., 1996). This ability of hypoxia-tolerant cells to manipulate ion regulatory processes contributes to a large degree to metabolic rate depression and represents an appealing target for regulation by AMPK. Interestingly, epithelial Na^+ channel currents in *Xenopus* oocytes and collecting duct cells in mice are inhibited in an AMPK-dependant manner (Carattino et al., 2005) demonstrating that some iono-regulatory action of AMPK is known.

AMPK α and β subunits are expressed in all tissues examined (Fig. 1) with the highest levels of mRNA being present in the brain, kidney, intestine and gill. However, this tissue-specific expression pattern does not translate into detectable differences in AMPK activation in these tissues during short-term O_2-deprivation. Unlike results demonstrated in the goldfish liver, no activation of AMPK was observed in muscle, brain, heart or gill during 12 h of severe hypoxia exposure (Fig. 6; Table 5). These results are in contrast to those obtained in hypoxia-sensitive mammalian models (Kudo et al., 1995; McCullough et al., 2005; Mu et al., 2001) where AMPK activation in muscle, brain and heart was observed in response to hypoxia exposure. In agreement with our results, the brain and heart of both killifish (*Fundulus grandis*) and trout (*Salmo gairdneri*) showed fewer signs of metabolic stress when exposed to hypoxia than did the skeletal muscle or liver (Dunn and Hochachka, 1986; Martinez et al., 2006) indicating that not all tissues respond in a similar fashion to hypoxia exposure. There are a number of potential explanations for this tissue-specific AMPK activation in goldfish. First, our goldfish were exposed to severe hypoxia rather than complete anoxia thus differential shunting of blood to these organs during hypoxia exposure may explain why the brain, heart and gill displayed no activation of AMPK. Upon hypoxia exposure in fish, essential tissues receive increased blood flow and, therefore, O_2-delivery (Booth, 1979; Gamperl et al., 1995; Soengas and Aldegunde, 2002) and consequently may not experience a metabolic stress to the same degree as liver. Second, the duration of hypoxia exposure may not have been long enough to observe activation of AMPK. Third, AMPK maybe regulated in a tissue-specific fashion with either different upstream regulating kinases expressed in different tissues or the level of cellular disruption required, e.g. degree of increase in $(AMP_{free})/(ATP)$, to observe AMPK activation may differ between tissues.

Within muscle, there was no significant increase in $[AMP_{free}]/[ATP]$ (Fig. 5C) and no apparent activation of AMPK during the 12 h exposure to hypoxia. The maintenance of high muscle [ATP] during hypoxia exposure, as seen in other studies (Fig. 5A) (Richards et al., 2007; van Ginneken et al., 1995; Zhou et al., 2000), may impede AMPK activation since, as mentioned previously, ATP competitively inhibits AMP binding to AMPK. Indeed, in goldfish muscle $[AMP_{free}]/[ATP]$ ratios were unaltered by hypoxia exposure at all sampling times, as were $[ADP_{free}]$ and $[ADP_{free}]/[ATP]$ measurements (Fig. 5C; Table 4). Additionally, there was also no significant change in $[AMP_{free}]$ or Δ_fG' of ATP hydrolysis until 12 h hypoxia (Table 4), suggesting that only at >12 h hypoxia exposure might goldfish muscle experience an energy stress great enough to result in the activation of AMPK and the need to activate biochemical means of reducing ATP demands. Longer-term hypoxia exposures are needed to determine if AMPK is activated in these tissues and plays a role in hypoxic survival.

AMPK has been proposed as an appealing candidate for co-ordinating the metabolic responses of tissues to hypoxia exposure in tolerant organisms (Bartrons et al., 2004; Bickler and Buck, 2007; Rider et al., 2006). Indeed, AMPK activity increased in liver in response to hypoxia exposure and the characteristic interactions between AMPK and the downregulation of protein synthesis were in place and responded to hypoxia exposure. These responses were tissues-specific with no observed activation of AMPK in brain, gill, heart or muscle. AMPK activation was closely associated with increased $[AMP_{free}]$ and decreased [ATP], suggesting that the ratio of these adenylates may have been important for activation. The decreased rates of protein synthesis, a well-known component of metabolic depression, combined with the phosphorylation of eEF2, a downstream target of AMPK, potentially implicate AMPK in the cellular effort to suppress metabolism in tolerant species exposed to hypoxia.

We gratefully acknowledge Milica Mandic and Ben Speers-Roesch for their valuable assistance during sampling. This work was funded by a Natural Science and Engineering Research Council (NSERC) of Canada Discovery Grant to J.G.R.

REFERENCES

Bartrons, M., Ortega, E., Obach, M., Calvo, M. N., Navarro-Sabate, A. and Bartrons, R. (2004). Activation of AMP-dependent protein kinase by hypoxia and hypothermia in the liver of frog *Rana perezi. Cryobiology* **49**, 190-194.

Beauloye, C., Marsin, A. S., Bertrand, L., Krause, U., Hardie, D. G., Vanoverschelde, J. L. and Hue, L. (2001). Insulin antagonizes AMP-activated protein kinase activation by ischemia or anoxia in rat hearts, without affecting total adenine nucleotides. *FEBS Lett.* **505**, 348-352.

Bergmeyer, H. U. (1983). *Methods of Enzymatic Analysis.* New York: Academic Press.

Bickler, P. and Buck, L. T. (2007). Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu. Rev. Physiol.* **69**, 145-170.

Blaxhall, P. C. and Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *J. Fish Biol.* **5**, 771-781.

Booth, J. H. (1979). The effects of oxygen supply, epinephrine, and acetylcholine on the distribution of blood flow in trout gills. *J. Exp. Biol.* **83**, 31-39.

Boutilier, R. G. (2001). Mechanisms of cell survival in hypoxia and hypothermia. *J. Exp. Biol.* **204**, 3171-3181.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brooks, S. P. and Storey, K. B. (1989). Influence of hormones, second messengers and pH on the expresion of metabolic responses to anoxia in a marine whelk. *J. Exp. Biol.* **145**, 31-43.

Browne, G. J., Finn, S. G. and Proud, C. G. (2004). Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. *J. Biol. Chem.* **279**, 12220-12231.

Buck, L. T., Hochachka, P. W., Schon, A. and Gnaiger, E. (1993). Microcalorimetric measurement of reversible metabolic suppression induced by anoxia in isolated hepatocytes. *Am. J. Physiol.* **265**, R1014-R1019.

Busk, M. and Boutilier, R. G. (2005). Metabolic arrest and its regulation in anoxic eel hepatocytes. *Physiol. Biochem. Zool.* **78**, 926-936.

Carattino, M. D., Edinger, R. S., Grieser, H. J., Wise, R., Neumann, D., Schlattner, U., Johnson, J. P., Kleyman, T. R. and Hallows, K. R. (2005). Epithelial sodium channel inhibition by AMP-activated protein kinase in oocytes and polarized renal epithelial cells. *J. Biol. Chem.* **280**, 17608-17616.

Carling, D. (2004). The AMP-activated protein kinase cascade-a unifying system for energy control. *Trends Biochem. Sci.* **29**, 18-24.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* **15**, 532-534; 536-537.

Corton, J. M., Gillespie, J. G., Hawley, S. A. and Hardie, D. G. (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur. J. Biochem.* **229**, 558-565.

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.

Davies, S. P., Carling, D. and Hardie, D. G. (1989). Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.* **186**, 123-128.

Dorovkov, M. V., Pavur, K. S., Petrov, A. N. and Ryazanov, A. G. (2002). Regulation of elongation factor-2 kinase by pH. *Biochemistry* **41**, 13444-13450.
Dunn, J. F. and Hochachka, P. W. (1986). Metabolic responses of trout (*Salmo gairdneri*) to acute environmnetal hypoxia. *J. Exp. Biol.* **123**, 229-242.
Gamperl, A. K., Axelsson, M. and Farrell, A. P. (1995). Effects of swimming and environmental hypoxia on coronary blood flow in rainbow trout. *Am. J. Physiol.* **269**, R1258-R1266.
Golding, E. M., Teague, W. E., Jr and Dobson, G. P. (1995). Adjustment of K' to varying pH and pmol l^{-1}g for the creatine kinase, adenylate kinase and ATP hydrolysis equilibria permitting quantitative bioenergetic assessment. *J. Exp. Biol.* **198**, 1775-1782.
Hardewig, I., Van Dijk, P. L. and Portner, H. O. (1998). High-energy turnover at low temperatures: recovery from exhaustive exercise in Antarctic and temperate eelpouts. *Am. J. Physiol.* **274**, R1789-R1796.
Hardie, D. G. (2007). Balancing cellular energy. *Science* **315**, 1671-1672.
Hardie, D. G. and Pan, D. A. (2002). Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem. Soc. Trans.* **30**, 1064-1070.
Hardie, D. G., Hawley, S. A. and Scott, J. W. (2006). AMP-activated protein kinase-development of the energy sensor concept. *J. Physiol.* **574**, 7-15.
Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.
Holmes, B. F., Kurth-Kraczek, E. J. and Winder, W. W. (1999). Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. *J. Appl. Physiol.* **87**, 1990-1995.
Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C. and Rider, M. (2002). Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr. Biol.* **12**, 1419-1423.
Kammermeier, H. (1987). High energy phosphate of the myocardium: concentration *versus* free energy change. *Basic Res. Cardiol.* **82 Suppl. 2**, 31-36.
Kammermeier, H. (1993). Efficiency of energy conversion from metabolic substrates to ATP and mechanical and chemiosmotic energy. *Basic Res. Cardiol.* **88**, 15-20.
Krumschnabel, G., Biasi, C., Schwarzbaum, P. J. and Wieser, W. (1996). Membrane-metabolic coupling and ion homeostasis in anoxia-tolerant and anoxia-intolerant hepatocytes. *Am. J. Physiol.* **270**, R614-R620.
Krumschnabel, G., Schwarzbaum, P. J., Biasi, C., Dorigatti, M. and Wieser, W. (1997). Effects of energy limitation on Ca^{2+} and K^+ homeostasis in anoxia-tolerant and anoxia-intolerant hepatocytes. *Am. J. Physiol.* **273**, R307-R316.
Kudo, N., Barr, A. J., Barr, R. L., Desai, S. and Lopaschuk, G. D. (1995). High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5′-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J. Biol. Chem.* **270**, 17513-17520.
Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J. and Winder, W. W. (1999). 5′ AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* **48**, 1667-1671.
Laemmli, U. K. (1970). Cleavage of structural protein during assembly of the head bacteriophage T4. *Nature* **227**, 680-685.
Land, S. C., Buck, L. T. and Hochachka, P. W. (1993). Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. *Am. J. Physiol.* **265**, R41-R48.
Lewis, J. M., Costa, I., Val, A. L., Almeida-Val, V. M., Gamperl, A. K. and Driedzic, W. R. (2007). Responses to hypoxia and recovery: repayment of oxygen debt is not associated with compensatory protein synthesis in the Amazonian cichlid, *Astronotus ocellatus*. *J. Exp. Biol.* **210**, 1935-1943.
Marsin, A. S., Bertrand, L., Rider, M. H., Deprez, J., Beauloye, C., Vincent, M. F., Van den Berghe, G., Carling, D. and Hue, L. (2000). Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr. Biol.* **10**, 1247-1255.
Martinez, M. L., Landry, C., Boehm, R., 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*. *J. Exp. Biol.* **209**, 3851-3861.
McCullough, L. D., Zeng, Z., Li, H., Landree, L. E., McFadden, J. and Ronnett, G. V. (2005). Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. *J. Biol. Chem.* **280**, 20493-20502.
Mu, J., Brozinick, J. T., Jr, Valladares, O., Bucan, M. and Birnbaum, M. J. (2001). A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol. Cell* **7**, 1085-1094.
Neurath, K. M., Keough, M. P., Mikkelsen, T. and Claffey, K. P. (2006). AMP-dependent protein kinase alpha 2 isoform promotes hypoxia-induced VEGF expression in human glioblastoma. *Glia* **53**, 733-743.
Nielsen, J. N., Wojtaszewski, J. F., Haller, R. G., Hardie, D. G., Kemp, B. E., Richter, E. A. and Vissing, J. (2002). Role of 5′AMP-activated protein kinase in glycogen synthase activity and glucose utilization: insights from patients with McArdle's disease. *J. Physiol.* **541**, 979-989.
Nivarthi, R. N., Grant, G. J., Turndorf, H. and Bansinath, M. (1997). Effect of intrathecally administered local anesthetics on protein phosphorylation in the spinal cord. *Bichem. Pharmacol.* **53**, 979-986.
Pörtner, H., Boutlier, R., Tang, Y. and Toews, D. (1991). The use of fluoride and nitriloacetic acid in tissue acid-base physiology. II. Intracellular pH. *Respir. Physiol.* **81**, 255-275.
Pörtner, H. O., Finke, E. and Lee, P. G. (1996). Metabolic and energy correlates of intracellular pH in progressive fatigue of squid (*L. brevis*) mantle muscle. *Am. J. Physiol.* **271**, R1403-R1414.
Reipschläger, A. and Pörtner, H. (1996). Metabolic depression during environmental stress: the role of extracellular *versus* intracellular pH in *Sipunculus nudus*. *J. Exp. Biol.* **199**, 1801-1807.
Richards, J. G., Semple, J. W., Bystriansky, J. S. and Schulte, P. M. (2003). Na^+/K^+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J. Exp. Biol.* **206**, 4475-4486.
Richards, J. G., Wang, Y. S., Brauner, C. J., Gonzalez, R. J., Patrick, M. L., Schulte, P. M., Choppari-Gomes, A. R., Almeida-Val, V. M. and Val, A. L. (2007). Metabolic and ionoregulatory responses of the Amazonian cichlid, *Astronotus ocellatus*, to severe hypoxia. *J. Comp. Physiol. B* **177**, 361-374.
Rider, M. H., Hussain, N., Horman, S., Dilworth, S. M. and Storey, K. B. (2006). Stress-induced activation of the AMP-activated protein kinase in the freeze-tolerant frog *Rana sylvatica*. *Cryobiology* **53**, 297-309.
Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame Hardie, D., Ashworth, A. and Alessi, D. R. (2005). Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J.* **24**, 1810-1820.
Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A. and Carling, D. (2007). Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem. J.* **403**, 139-148.
Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G. and Hardie, D. G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* **113**, 274-284.
Smith, R. W., Houlihan, D. F., Nilsson, G. E. and Brechin, J. G. (1996). Tissue-specific changes in protein synthesis rates *in vivo* during anoxia in crucian carp. *Am. J. Physiol.* **271**, R897-R904.
Soengas, J. L. and Aldegunde, M. (2002). Energy metabolism of fish brain. *Comp. Biochem. Physiol., B* **131**, 271-296.
Teague, W. E., Jr, Golding, E. M. and Dobson, G. P. (1996). Adjustment of K' for the creatine kinase, adenylate kinase and ATP hydrolysis equilibria to varying temperature and ionic strength. *J. Exp. Biol.* **199**, 509-512.
Todgham, A. E., Schulte, P. M. and Iwama, G. K. (2005). Cross-tolerance in the tidepool sculpin: the role of heat shock proteins. *Physiol. Biochem. Zool.* **78**, 133-144.
van den Thillart, G., Kesbeke, F. and Waarde, A. V. (1980). Anaerobic energy metabolism of goldfish, *Carassius auratus* (L): influence of hypoxia and anoxia on phosphorylated compounds and glycogen. *J. Comp. Physiol.* **136**, 45-52.
van Ginneken, V., van den Thillart, G., Addink, A. and Erkelens, C. (1995). Fish muscle energy metabolism measured during hypoxia and recovery: an in vivo ^{31}P-NMR study. *Am. J. Physiol.* **268**, R1178-R1187.
Van Waarde, A., Van den Thillart, G., Erkelens, C., Addink, A. and Lugtenburg, J. (1990). Functional coupling of glycolysis and phosphocreatine utilization in anoxic fish muscle. An *in vivo* ^{31}P NMR study. *J. Biol. Chem.* **265**, 914-923.
Van Waversveld, J., Addink, A. D. F. and van den Thillart, G. (1989). Simuultaneous direct and indriect calorimetry on normoxic and anoxic goldfish. *J. Exp. Biol.* **142**, 325-335.
Wang, Y., Heigenhauser, G. J. F. and Wood, C. M. (1994). Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid-base, phosphogen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism. *J. Exp. Biol.* **195**, 227-258.
Wang, Y., Heigenhauser, G. J. and Wood, C. M. (1996). Lactate and metabolic H^+ transport and distribution after exercise in rainbow trout white muscle. *Am. J. Physiol.* **271**, R1239-R1250.
Witters, L. A., Kemp, B. E. and Means, A. R. (2006). Chutes and ladders: the search for protein kinases that act on AMPK. *Trends Biochem. Sci.* **31**, 13-16.
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.