

RESEARCH ARTICLE

Activation of oxygen-responsive pathways is associated with altered protein metabolism in Arctic char exposed to hypoxia

Alicia A. Cassidy^{*,‡} and Simon G. Lamarre

ABSTRACT

Fish exposed to fluctuating oxygen concentrations often alter their metabolism and/or behaviour to survive. Hypoxia tolerance is typically associated with the ability to reduce energy demand by suppressing metabolic processes such as protein synthesis. Arctic char is amongst the most sensitive salmonid to hypoxia, and typically engage in avoidance behaviour when faced with lack of oxygen. We hypothesized that a sensitive species will still have the ability (albeit reduced) to regulate molecular mechanisms during hypoxia. We investigated the tissue-specific response of protein metabolism during hypoxia. Little is known about protein degradation pathways during hypoxia in fish and we predict that protein degradation pathways are differentially regulated and play a role in the hypoxia response. We also studied the regulation of oxygen-responsive cellular signalling pathways [hypoxia inducible factor (HIF), unfolded protein response (UPR) and mTOR pathways] since most of what we know comes from studies on cancerous mammalian cell lines. Arctic char were exposed to cumulative graded hypoxia trials for 3 h at four air saturation levels (100%, 50%, 30% and 15%). The rate of protein synthesis was measured using a flooding dose technique, whereas protein degradation and signalling pathways were assessed by measuring transcripts and phosphorylation of target proteins. Protein synthesis decreased in all tissues measured (liver, muscle, gill, digestive system) except for the heart. Salmonid hearts have preferential access to oxygen through a well-developed coronary artery, therefore the heart is likely to be the last tissue to become hypoxic. Autophagy markers were upregulated in the liver, whereas protein degradation markers were downregulated in the heart during hypoxia. Further work is needed to determine the effects of a decrease in protein degradation on a hypoxic salmonid heart. Our study showed that protein metabolism in Arctic char is altered in a tissue-specific fashion during graded hypoxia, which is in accordance with the responses of the three major hypoxia-sensitive pathways (HIF, UPR and mTOR). The activation pattern of these pathways and the cellular processes that are under their control varies greatly among tissues, sometimes even going in the opposite direction. This study provides new insights on the effects of hypoxia on protein metabolism. Adjustment of these cellular processes is likely to contribute to shifting the fish phenotype into a more hypoxia-tolerant one, if more than one hypoxia event were to occur. Our results warrant

studying these adjustments in fish exposed to long-term and diel cycling hypoxia.

KEY WORDS: Salmonid, Protein degradation, HIF, Unfolded protein response, mTOR, *Salvelinus alpinus*

INTRODUCTION

Fish are regularly exposed to fluctuations in oxygen concentrations in the wild, often associated with increased water temperature and eutrophication. The ability to tolerate hypoxia varies between species and individuals, and depends on a fish's capacity to alter its behaviour and/or physiology when oxygen levels decrease (Farrell and Richards, 2009; Richards, 2010). Different species employ a wide range of strategies to increase survival during hypoxia, including increasing gill ventilation and cardiac output to enhance oxygen extraction from the environment (Farrell and Richards, 2009; Richards, 2010). Hypoxia-tolerant species such as crucian carp (*Carassius carassius*), oscars (*Astronotus ocellatus*) and goldfish (*Carassius auratus*) lower their metabolic rate to limit the use of anaerobic metabolism and the accumulation of toxic end-products (reviewed in Boutilier, 2001; Lewis et al., 2007). This is accomplished by suppressing energetically expensive processes such as reproduction, digestion and growth (Boutilier, 2001; Farrell and Richards, 2009).

Metabolic suppression during hypoxia can be partially accomplished by suppressing protein metabolism, which includes both protein synthesis and protein degradation. Hypoxia-tolerant species, such as the Amazonian oscar and crucian carp can suppress protein synthesis by as much as 95% in liver and 55% in muscle (Cassidy et al., 2018; Lewis et al., 2007; Smith et al., 1996) during hypoxia, accounting for a 20–36% decrease in metabolism (Cassidy et al., 2018). The adjustments of protein metabolism, if any, have not yet been characterized in hypoxia-sensitive species such as salmonids. Regulation of protein synthesis during hypoxia is well documented in mammals and is starting to be characterized in fish, yet there is little information on regulation of protein degradation. In mammals, the autophagy pathway is activated during hypoxia (Wouters and Koritzinsky, 2008), with little information on the other protein degradation pathways (ubiquitin proteasome pathway and calpains).

There are three major cellular signalling pathways involved in the hypoxia response: the hypoxia inducible factor (HIF) pathway, the unfolded protein response (UPR) and mTOR pathways (Cassidy et al., 2018; Fang et al., 2015; Wouters and Koritzinsky, 2008). When activated, these pathways eventually inhibit the rate of protein synthesis (Wouters and Koritzinsky, 2008; summarized in Fig. 1). The responses of these pathways are well characterized in mammalian tumours and are starting to be elucidated in fish exposed to hypoxia. During hypoxia, HIF-1 α accumulates in cells and dimerizes with HIF- β which promotes the expression of genes involved in various processes such as angiogenesis and glycolysis (Neufeld et al., 1999; Schofield and Ratcliffe, 2004). HIF may also

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Received 24 March 2019; Accepted 30 October 2019

List of abbreviations and symbols

4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
AKT	protein kinase B
AMPK	AMP-activated protein kinase
ATF4	activating transcription factor 4
ATG4	autophagy-related 4A cysteine peptidase
BNIP3	BCL2/adenovirus E1B 19-kD protein-interacting protein 3
DDIT3	DNA damage-inducible transcript 3
eIF2 α	eukaryotic translation initiation factor 2 α
FbxO25	F-box only protein 25
HIF	hypoxia inducible factor
k_s	fractional rate of protein synthesis
LC3B	microtubule-associated protein 1 light chain 3 beta
mTOR	mammalian target of rapamycin
P70S6K	ribosomal protein S6 kinase beta-1
REDD1	regulated in development and DNA damage responses 1
UBE2H	ubiquitin conjugating enzyme E2 H
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

control the rate of protein synthesis through transcriptional regulation of BNIP3 and REDD1, which inhibit mTOR and protein synthesis (Wouters and Koritzinsky, 2008). In fish, *HIF* transcripts increase during hypoxia and its function during hypoxia appears conserved in fish (Baptista et al., 2016; Heinrichs-Caldas et al., 2019; Rimoldi et al., 2012; Shen et al., 2010). The UPR is activated by an accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) during hypoxia. Its primary functions include degrading misfolded proteins and inhibiting protein synthesis to limit the accumulation of misfolded proteins in the ER (Zhang and Kaufman, 2006). Briefly, PERK (protein kinase RNA-like endoplasmic reticulum kinase) is activated by autophosphorylation and phosphorylates eIF2 α , which directly inhibits translation initiation and, therefore, protein synthesis (Ron

and Walter, 2007). The phosphorylation of eIF2 α during hypoxia was reported in the Amazonian oscar (Cassidy et al., 2018), and transcriptional activation of its downstream targets were reported in the common sole (*Solea solea*) (Mazurais et al., 2014), suggesting that this pathway is also conserved in fish. Finally, the mTOR pathway regulates, among other things, both protein synthesis and degradation in response to various effectors such as hypoxia, food availability or pathogens (Fader et al., 2015; Hay and Sonenberg, 2004; Johnston et al., 2011). mTOR phosphorylates its downstream targets, 4EBP1 and P70S6K, which directly regulate global protein synthesis (Hay and Sonenberg, 2004). During hypoxia in mammals, mTOR is regulated by multiple pathways, including HIF-dependent, and HIF-independent pathways that involve AMPK (Liu et al., 2006; Papandreou et al., 2008). AMPK is an energy sensor that regulates multiple cellular processes including glucose and protein metabolism. AMPK is activated during severe hypoxia, and its activation inhibits mTOR activity, thus inhibiting protein synthesis (Cam and Houghton, 2011; Mungai et al., 2011; Papandreou et al., 2008). There is limited information on the regulation of the mTOR pathway during hypoxia in fish. However, AMPK phosphorylation increased in goldfish and rainbow trout exposed to hypoxia, suggesting that the mTOR pathway should also be inhibited (Jibb and Richards, 2008; Williams et al., 2019). In mammals, there is also significant crosstalk between the three hypoxia-response pathways and each one (HIF, UPR or mTOR) can regulate the activity of one of the other pathways (Wouters and Koritzinsky, 2008).

Protein degradation is also regulated by hypoxia-responsive signalling pathways, although less is known on the regulation of the three major protein degradation pathways (autophagy, ubiquitin proteasome pathway and calpains) during hypoxia. Autophagy and the ubiquitin proteasome pathway are both controlled by cell signalling pathways, including the AKT/mTOR pathway (Johnston et al., 2011). During hypoxia, autophagy is activated by all three hypoxia-response pathways in

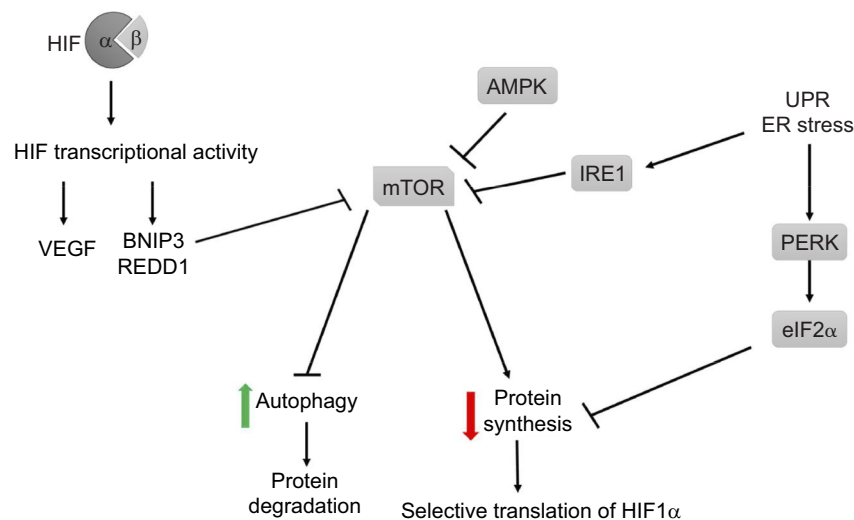


Fig. 1. Summary of the three major hypoxia-response pathways in mammals. During hypoxia, the hypoxia inducible factor (HIF) pathway is activated when HIF α is allowed to accumulate in cells and forms a complex with HIF β . This complex then activates the transcription of several target genes in the hypoxia response, including *VEGF*, *BNIP3* and *REDD1*. mTOR is inhibited during hypoxia by several pathways, including AMPK, which results in a decrease in protein synthesis and increase in autophagy. The unfolded protein response (UPR) is also activated because of increased endoplasmic reticulum stress caused by hypoxia. This activates PERK, which phosphorylates eIF2 α downstream to inhibit global translation initiation. There is also significant cross-talk between the pathways, where mTOR can selectively synthesize HIF proteins and HIF, through BNIP3 and REDD1 can inhibit mTOR. Activation of mTOR and protein synthesis can exacerbate ER stress, while the UPR can inhibit mTOR through the inhibition of IRE1, a positive regulator of mTOR. This information is summarized from Wouters and Koritzinsky (2008) for mammalian tumours.

mammals (Wouters and Koritzinsky, 2008). It is not known, however, whether the ubiquitin proteasome pathway or the calpain system are also regulated by hypoxia-response pathways such as HIF or UPR. Autophagy is responsible for degrading organelles and proteins with long half-lives and includes acidic proteases called cathepsins that degrade proteins and organelles inside vacuoles (Ciechanover, 2012). It is not known whether autophagy is regulated during hypoxia in fish, but a recent study linked autophagy and hypoxia in prawn (*Macrobrachium nipponense*) hepatocytes (Sun et al., 2019). The ubiquitin proteasome pathway is mostly responsible for degrading damaged proteins, and proteins with short half-lives (Glickman and Ciechanover, 2002). This pathway involves three groups of enzymes that tag proteins with a poly-ubiquitin chain that will then be recognized and degraded by the proteasome complex (Ciechanover et al., 1984). In mammals, one study observed a decrease in proteasome activity during hypoxia (Gozal et al., 2003), which is in accordance with a decrease in proteasome subunit transcripts observed in stickleback (*Gasterosteus aculeatus*) (Leveelahti et al., 2011). The third important degradation pathway is the calpain system. Calpains are small, calcium-dependent, proteases that selectively degrade proteins in the cytosol, especially in muscle (Goll et al., 2003). Calpain activity is thought to increase in mammals during hypoxia (Cui et al., 2015; Nanduri et al., 2009); however, this has yet to be evaluated in fish.

Our objective was to determine whether and how protein metabolism is deregulated in tissues of Arctic char submitted to cumulative, graded hypoxia trials. The hypotheses were that: (1) as observed in hypoxia-tolerant species, protein metabolism is regulated during hypoxia in an attempt to maintain energy homeostasis and that this regulation is carried by the aforementioned hypoxia-responsive pathways; and (2) the reorganization of protein metabolism during hypoxia differs among tissues that have drastically different metabolic rates and functions (i.e. liver, white muscle and cardiac muscle). Unlike many cyprinids that tolerate low-oxygen environments, salmonids require higher oxygen availability (Jones et al., 2008). Arctic char, *Salvelinus alpinus*, is the northernmost freshwater fish species, and is considered amongst the most hypoxia-sensitive salmonids (Jones et al., 2008). This low hypoxia tolerance makes Arctic char a good model to study the effects of hypoxia on protein metabolism and to study which hypoxia-responsive pathways are involved in fish tissues.

Arctic char were exposed to a protocol of graded decreases in air saturation (3 h at each of 100%, 50%, 30% and then 15% air saturation) and we measured the fractional rate of protein synthesis and indicators of protein degradation in the liver, white muscle and heart at each oxygen concentration. We found profound alterations of the rate of protein synthesis and the abundance of various markers of protein degradation, but these were highly tissue specific.

MATERIALS AND METHODS

Rearing conditions and experimental design

Juvenile Arctic char [*Salvelinus alpinus* (Linnaeus 1758) Fraser strain; body mass=60.9±1.6 g, mean±s.e.m.] were obtained from the Coastal Zones Research Institute Inc. (Shippagan, NB, Canada). Five hundred fish were held in two 250 liter tanks supplied with flow-through dechlorinated freshwater. Fish were fed a commercial salmon diet (Corey Nutrition Company Inc., Fredericton, NB, Canada) at 2% body weight per day, every other day. Water temperature varied seasonally and was 16°C in the summer, when the experiment occurred.

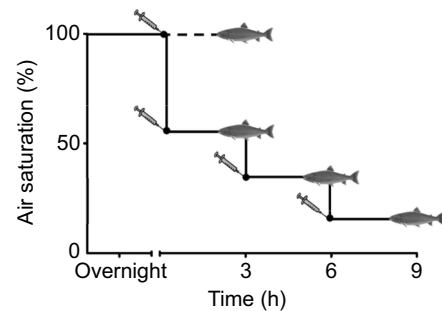


Fig. 2. Experimental protocol to expose Arctic char to a stepwise decrease in air saturation before sampling at 100%, 50%, 30% or 15% air saturation. Using a flooding dose technique, fish were injected with deuterated phenylalanine 3 h prior to sampling, to measure fractional rates of protein synthesis.

The experimental system consisted of three shelves each containing six 9 liter tanks. Each shelf had its own water filtration and biofiltration system. The dissolved oxygen was adjusted by nitrogen injection by means of a programmable proportional–integral–derivative controller (Aquabiotech, Coaticook, QC, Canada). Temperature was maintained at 16±0.1°C. In June 2017, fish were semi-randomly selected (excluding the runts) and transferred to experimental tanks over 5 days. Fish were food deprived for 4 days before the experiment. Twenty fish (5 per group) were exposed to a cumulative, graded hypoxic challenge, with an endpoint of either 100%, 50%, 30% or 15% air saturation (Fig. 2) as in Cassidy et al. (2018). Five fish (1 per tank) were placed in the experimental chambers overnight at 100% air saturation for acclimation and sampled the next day (control group). The next day, 5 additional fish were placed in the chambers overnight at 100% air saturation for acclimation. Air saturation was then dropped to 50%, and maintained at 50% for 3 h before sampling. A third group of 5 fish were then placed in the tanks overnight at 100% air saturation. Air saturation levels were then reduced to 50% for 3 h the following morning, then held at 30% for 3 h before sampling. The final group of fish was placed in the tanks overnight at 100% air saturation. The next morning, air saturation levels dropped to 50% for 3 h, then 30% for 3 h and finally 15% for 3 h before sampling. We did not expose fish to oxygen concentrations lower than 15% since at this level fish were starting to lose equilibrium. Oxygen concentration measured by the system was always confirmed using a handheld oxygen meter (5512-Ft YSI, Yellow Springs, OH, USA). At the end of each hypoxic challenge, fish were killed by severing the spinal cord directly behind the brain. Blood was immediately collected from the caudal vein, centrifuged for 5 min at 2000 g and the plasma frozen. Samples of muscle, liver, heart, gill and blood were collected and immediately frozen in liquid nitrogen. Samples were then stored at –80°C until further analysis. Experiments were conducted with the approval of the animal care committee at the Université de Moncton, New Brunswick, Canada (UdeM17-05).

Lactate

Lactate was measured in plasma, muscle and liver by first deproteinizing tissues with 6% perchloric acid followed by neutralization with 2 mol l⁻¹ KHCO₃. Lactate was measured in glycine buffer (320 mmol l⁻¹ glycine, 320 mmol l⁻¹ hydrazine, 2.4 mmol l⁻¹ NAD⁺) with subsequent treatment of excess lactate dehydrogenase (2 U ml⁻¹) (Brandt et al., 1980). Absorbance was read with a multimode plate reader (Varioskan Flash, Thermo Fisher Scientific, Inc., Waltham, MA USA) at 340 nm wavelength.

Fractional rate of protein synthesis (k_s)

The fractional rate of protein synthesis (k_s) was measured using a flooding dose technique (Garlick et al., 1980) as described and validated for this species (Cassidy et al., 2016; Lamarre et al., 2015). Fish are injected with a solution of 150 mmol l⁻¹ phenylalanine containing 50% [D₅]-L-phenylalanine (ring-[D₅]-PHE, 98%, Cambridge Isotope Laboratories, Inc., Andover, MA, USA), at a dosage of 1 ml per 100 g of fish body mass. Fish were injected 3 h prior to sampling, which is an incorporation time in the optimal range, previously tested by Lamarre et al. (2015). Approximately 60 mg of tissue (but only 20 mg for heart) was homogenized in 1 ml of 0.2 mol l⁻¹ perchloric acid using a sonicating homogenizer (Q55 Sonicator, Qsonica Inc.). Following a 5 min 15,000 g centrifugation at 4°C, the supernatant containing the free amino acid pool was transferred to a new tube and kept at 4°C. The remaining protein pellet was washed three times in 1 ml of 0.2 mol l⁻¹ PCA and once in acetone. Samples were then hydrolyzed in 6 ml of 6 mol l⁻¹ HCl at 110°C for 18 h. Phenylalanine was extracted using solid phase extraction (Bond-Elut C-18, Varian Inc.), and dried. Samples were then derivatized with pentafluorobenzyl bromide and the [D₅]-PHE enrichment of the phenylalanine from the free and protein pools were measured by GC-MS as previously described (Lamarre et al., 2015). The fractional rate of protein synthesis (k_s ; % day⁻¹) is calculated using:

$$k_s = \frac{S_b}{S_a} \times \frac{1440}{t} \times 100, \quad (1)$$

where S_b is the enrichment of the protein pool, S_a is the enrichment of the free amino acid pool, t is the incorporation time (min) and 1440 is the conversion from minutes to days (Lamarre et al., 2015).

Immunoblotting

Western blots were used to analyse specific protein levels in liver, muscle and heart. Tissues were homogenized in 9 volumes of lysis buffer (50 mmol l⁻¹ Tris-HCl, 0.1 mmol l⁻¹ EDTA, 1.0 mmol l⁻¹ β-mercaptoethanol, pH 8) with a sonicating homogenizer and centrifuged at 13,000 g for 15 min at 4°C. Protein concentration was then measured (Bradford protein assay, Bio-Rad, Hercules, CA) (Bradford, 1976). Fifteen mg of protein was loaded in each well and resolved in a 10% SDS-PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), and blocked for 1 h 5% BSA dissolved in TBS. Membranes were probed overnight at 4°C in primary antibodies (Cell Signaling Technologies, Beverly, MA, USA): 4EBP1 (no. 9649), p-4EBP1 (Thr37/46; no. 2855), eIF2α (no. 5324) and p-eIF2α (Ser51; no. 3398), AMPKα (no. 5831), p-AMPKα (Thr172; no. 2535). Protein bands were revealed by probing for 1 h with HRP-conjugated goat anti-rabbit secondary antibody (no. 7074) followed by enhanced chemiluminescence (Clarity Western ECL substrate, Bio-Rad, Hercules, CA). Images were taken with a Chemidoc Touch Imaging System (Bio-Rad). The densitometric analyses were performed using Image Lab v.5.2.1 software (Bio-Rad). Levels of phosphorylation were obtained by calculating the ratio between phosphorylated and total proteins (e.g. phospho-eIF2α/total eIF2α). Protein bands were normalized using the stain-free imaging technology (Gürtler et al., 2013). The relative quantity of polyubiquitinated proteins was determined by dot blots using an antibody detecting polyubiquitinated proteins (mAb FK1, Enzo, BML-PW8805) and an anti-mouse IgM HRP-linked antibody (ab97230, Abcam, Cambridge, MA) as previously described (Cassidy et al., 2016).

RNA extraction and qPCR amplification of mRNAs

Transcripts related to the hypoxia response and protein degradation were amplified via quantitative (q)PCR in liver, muscle and heart. Approximately 50 mg of tissue (15 mg for heart) were homogenized in 1 ml of TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) with a TissueLyser (Qiagen, Venlo, The Netherlands) and 5 mm stainless steel beads for 5 min at 27 Hz. RNA was quantified by UV absorption at 260 nm using a NanoVue spectrophotometer (GE Healthcare, Chicago, IL, USA). RNA was treated with a DNA-free kit (Life Technologies, Carlsbad, CA, USA) and then reverse transcribed with qScript cDNA SuperMix (Quanta Biosciences Inc., Gaithersburg, MD, USA). Primers were designed with Primer3 (v.0.4.0) (Koressaar and Remm, 2007; Untergasser et al., 2012) using Atlantic salmon (*Salmo salar*) gene sequences obtained on NCBI (<http://www.ncbi.nlm.nih.gov/>) (Table S1). Gene sequences were aligned with zebrafish (*Danio rerio*) and mouse (*Mus musculus*) to obtain the most conserved regions of the genes using Mega7 software (Kumar et al., 2016). Primers were validated by sequencing the PCR products (Centre de Recherche du CHUL at Université Laval) and using BLAST (NCBI) searches. qPCR assays were conducted with CFX Connect Real-Time PCR Detection System (Bio-Rad) and respected the minimum information for publication of quantitative PCR experiment guidelines (Bustin et al., 2009). Amplification efficiencies were assessed for each tissue. Triplicates of qPCR reactions contained 5 μl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 4 μl of cDNA, 400 nmol l⁻¹ of forward and reverse primers and RNase-free water to a final volume of 10 μl. Data were normalized by integrating the geometric average of the 3 most stable reference genes into the Pfaffl equation (Pfaffl, 2001; Vandesompele et al., 2002). *E1fα*, *α-tubulin* and *rs11* were the most stable reference genes in liver; *E1fα*, *β-actin* and *rs11* were selected in muscle; and in heart, *E1fα*, *β-actin* and *α-tubulin* were selected. Normalized mRNA values are expressed relative to the average of the control group.

Statistical analyses

Linear models (lm function) with air saturation levels as the fixed factor (one-way ANOVA) and Tukey's *post hoc* were performed in R (<https://www.r-project.org/>) statistical software. Box-cox transformations were applied when needed, to improve the normality of the residuals. P -values smaller than 0.05 were considered statistically significant. The figures were constructed with Prism 7 software (GraphPad Software Inc.).

RESULTS

Graded hypoxia treatment

No mortalities were observed during the cumulative, graded hypoxia treatments although a few fish started losing equilibrium at 15% air saturation. Lactate concentration in plasma and liver showed a gradual increase ($P < 0.001$, $n=5$) as % air saturation levels decreased (Fig. 3). In muscle, a higher concentration of lactate occurred only at 15% oxygen saturation ($P < 0.001$, $N=5$). Lactate concentration was omitted in the heart because of limited availability of tissue.

Protein synthesis

The fractional rate of protein synthesis was measured in gill, liver, digestive system, white and red muscle, and heart of fish at each stage of hypoxia (Fig. 4). In most tissues, protein synthesis decreased with % air saturation, but this trend was not observed in the heart, where protein synthesis remained more consistent

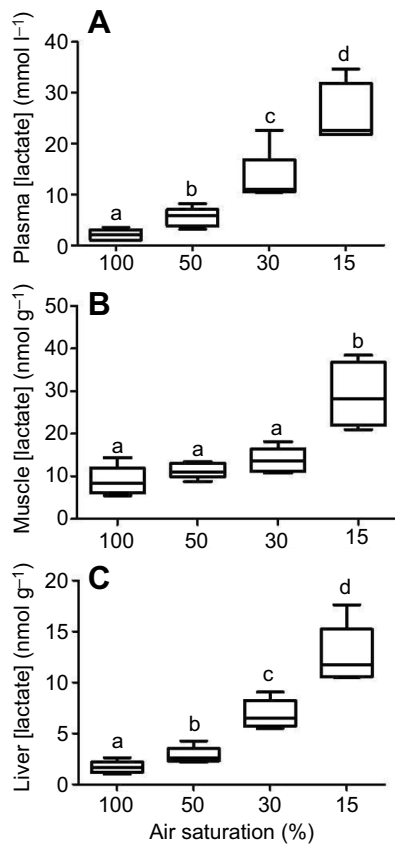


Fig. 3. Lactate concentration in plasma, muscle and liver of Arctic char exposed to varying levels of air saturation. (A) Plasma lactate (mmol l^{-1}). (B) muscle and (C) liver lactate (nmol g^{-1}). Data are expressed as mean \pm s.e.m. Lower case letters indicate significant differences ($P < 0.05$) between air saturation levels. For box plots, whiskers represent the highest and lowest observations, the line in the box represents the median, and the bottom and top of the box are the first and third quartiles, respectively.

(Fig. 4F). The liver and digestive system were the most responsive tissues, with rates of protein synthesis decreasing after only 3 h at 50% air saturation (Fig. 4B,C).

Protein degradation

Markers of the ubiquitin proteasome pathway, calpain and autophagy pathways were measured in liver, muscle and heart (Tables 1, 2 and 3, respectively). Ubiquitin proteasome pathway markers were unaffected in liver and muscle, but in heart, *Fbxo25* and *UBE2H* transcript levels were decreased at 15% air saturation ($P < 0.01$, $N = 5$; Table 3). Polyubiquitinated protein levels were unchanged in all tissues (Fig. S1). In the calpain pathway, *calpain7* transcript levels increased at 15% air saturation in liver and decreased in heart ($P = 0.004$, $N = 5$). *Calpastatin* transcript levels also increased in liver at 50%, 30% and 15% air saturation ($P = 0.003$, $N = 5$), but did not change in muscle and heart. The autophagy-related transcripts *Cathepsin D* and *LC3B* increased in liver as air saturation decreased ($P < 0.001$, $N = 5$; Table 1). In heart, transcripts levels of *Cathepsin L* ($P = 0.04$, $N = 5$), *Atg4* ($P = 0.04$, $N = 5$), and *Beclin1* ($P = 0.001$, $N = 5$) were lowered at 15% air saturation (Table 3).

Activation of oxygen-sensitive signalling pathways

In the mTOR pathway, the phosphorylation levels of AMPK α , 4EBP1 were measured in liver, muscle and heart (Fig. 5). AMPK α phosphorylation increased in the liver and heart at 15% air saturation ($P < 0.001$, $N = 5$; Fig. 5A–C). In all three tissues, phosphorylation levels of 4EBP1 decreased at 15% air saturation compared with controls ($P < 0.001$, $N = 5$; Fig. 5D–F). Activation of the unfolded protein response pathway was assessed by measuring eIF2 α phosphorylation (Fig. 5G–I) and transcript levels of *eIF2 α* , *ATF4* and *DDIT3*. The phosphorylation levels of eIF2 α were higher at 15% air saturation in the liver and muscle ($P < 0.001$, $N = 5$; Fig. 5G,H). In the heart, eIF2 α phosphorylation decreased at 50% and 30%, before returning to control levels at 15% ($P = 0.01$, $N = 5$; Fig. 5I). The hypoxia treatments had no effect on *eIF2 α* transcripts in any of the tissues and levels of *ATF4* transcripts were only affected in the heart ($P = 0.02$, $N = 5$) where transcript levels decreased at 15% air saturation (Table 3). *DDIT3* transcripts showed opposite responses in the liver and muscle. *DDIT3* transcript levels gradually increased in the liver as air saturation decreased ($P = 0.02$, $N = 5$; Table 1), while *DDIT3* transcripts decreased in muscle with decreasing air saturation ($P = 0.01$, $n = 5$; Table 2). No changes occurred for *DDIT3* transcripts in the heart (Table 3). Commercial antibodies for HIF-1 working in salmonids

Table 1. Relative mRNA levels in liver of Arctic char exposed to 100%, 50%, 30% and 15% air saturation

Pathway	mRNA	Relative transcript level (mean \pm s.e.m.)			
		100%	50%	30%	15%
HIF	<i>HIF1a</i>	1.00 \pm 0.08 ^a	1.88 \pm 0.10 ^b	1.67 \pm 0.12 ^b	1.78 \pm 0.10 ^b
	<i>VEGF</i>	1.00 \pm 0.12 ^a	1.80 \pm 0.21 ^b	2.56 \pm 0.86 ^b	1.90 \pm 0.15 ^b
	<i>VHL</i>	1.00 \pm 0.09	1.35 \pm 0.09	1.35 \pm 0.09	1.19 \pm 0.08
UPR	<i>eIF2α</i>	1.00 \pm 0.08	1.30 \pm 0.08	1.03 \pm 0.06	1.13 \pm 0.12
	<i>ATF4</i>	1.00 \pm 0.11	1.10 \pm 0.17	1.12 \pm 0.11	1.05 \pm 0.05
	<i>DDIT3</i>	1.00 \pm 0.09 ^a	1.59 \pm 0.37 ^{a,b}	2.77 \pm 0.58 ^b	2.29 \pm 0.71 ^{a,b}
Proteasome	<i>Fbxo25</i>	1.00 \pm 0.10	1.30 \pm 0.17	1.14 \pm 0.09	1.14 \pm 0.07
Calpain	<i>Calpain7</i>	1.00 \pm 0.04 ^{a,b}	1.05 \pm 0.10 ^{a,b}	0.87 \pm 0.04 ^a	1.33 \pm 0.10 ^b
	<i>Calpain small subunit1</i>	1.00 \pm 0.10	1.18 \pm 0.10	1.24 \pm 0.14	1.16 \pm 0.09
	<i>Calpastatin</i>	1.00 \pm 0.08 ^a	1.43 \pm 0.08 ^b	1.61 \pm 0.26 ^b	1.46 \pm 0.07 ^b
Autophagy	<i>Cathepsin D</i>	1.00 \pm 0.12 ^a	1.45 \pm 0.08 ^b	1.35 \pm 0.09 ^{a,b}	1.36 \pm 0.13 ^{a,b}
	<i>Cathepsin L</i>	1.00 \pm 0.11	1.06 \pm 0.17	1.03 \pm 0.10	1.302 \pm 0.09
	<i>Atg4</i>	1.00 \pm 0.09	0.81 \pm 0.12	0.88 \pm 0.08	0.84 \pm 0.05
	<i>Atg5</i>	1.00 \pm 0.02 ^{a,b}	1.15 \pm 0.04 ^a	0.95 \pm 0.05 ^b	1.09 \pm 0.05 ^{a,b}
	<i>Beclin1</i>	1.00 \pm 0.09	1.26 \pm 0.13	1.11 \pm 0.07	1.22 \pm 0.15
	<i>LC3A</i>	1.00 \pm 0.22	1.29 \pm 0.07	1.29 \pm 0.10	1.48 \pm 0.11
	<i>LC3B</i>	1.00 \pm 0.08 ^a	2.30 \pm 0.26 ^b	2.82 \pm 0.40 ^b	2.72 \pm 0.17 ^b

Transcript levels are normalized with e1f α , α -tubulin and rs11. All data are relative to control levels and expressed as mean \pm s.e.m. Lower case letters indicate significant differences ($P < 0.05$) between air saturation levels.

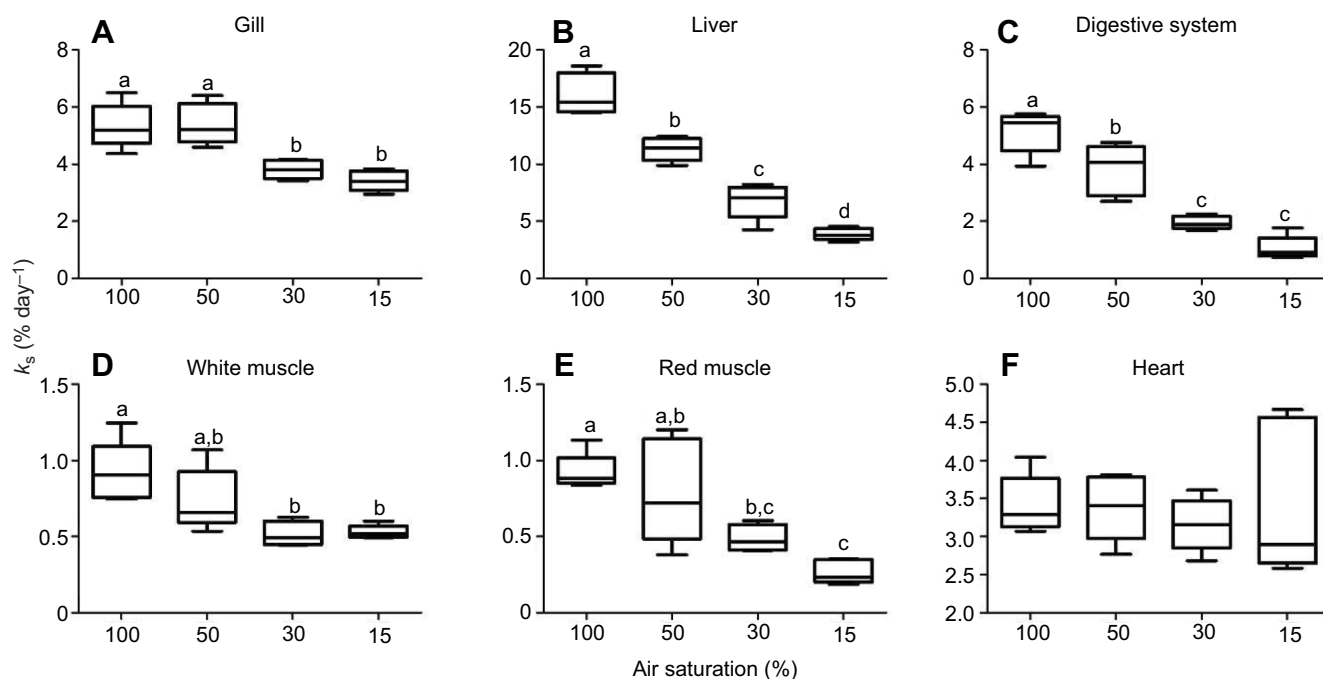


Fig. 4. Fractional rates of protein synthesis in Arctic char tissues after exposure to varying levels of air saturation. Protein synthesis (k_s ; % day⁻¹) in (A) gill, (B) liver, (C) digestive system (D) white muscle, (E) red muscle and (F) heart. Values are expressed as means \pm s.e.m. Lower case letters indicate significant differences ($P<0.05$) between air saturation levels.

are not available, therefore we used the transcript numbers of genes involved in the pathway. In the liver, transcript levels for *HIF1a* ($P<0.001$, $N=5$) and its target *VEGF* ($P=0.007$, $N=5$) were elevated in fish exposed to 50% air saturation, and this elevation was maintained at 30% and 15% (Table 1). *VHL* transcript numbers in the liver were not significantly affected although they showed a similar trend ($P=0.06$, $N=5$) to *VEGF* (Table 1). In muscle, *HIF1a* transcripts remained unchanged, while *VEGF* transcript levels decreased at 30% and 15% air saturation ($P<0.001$, $N=5$), and *VHL* increased at 50% and 30% air saturation ($P=0.004$, $N=5$; Table 2). In the heart, there were no changes for *HIF1a* and *VHL* transcript levels, but *VEGF* transcripts increased at 15% air saturation ($P<0.001$, $n=5$; Table 3).

DISCUSSION

Our objectives were to determine whether protein metabolism was deregulated in different tissues of Arctic char during cumulative, graded hypoxia. The fish were exposed to a graded hypoxia protocol with a first step of hypoxia at 50% air saturation for 3 h. At this concentration, the fish were already in a hypoxic metabolic state, as demonstrated by the elevated plasma and liver lactate concentrations (van Raaij et al., 1996; Via et al., 1997; Wood et al., 2007; Zhou et al., 2005). We should also acknowledge that during each subsequent trial (30% and 15% air saturation) more time was allowed for biochemical responses as well as for gene expression to occur, which could bias the comparisons of the hypoxia response at each level. Nevertheless, our study showed that protein metabolism in Arctic char is altered in a tissue-specific fashion during graded hypoxia, which is in accordance with the responses of the three major hypoxia-sensitive pathways (HIF, UPR and mTOR), as observed in mammalian cells.

Protein synthesis

The rate of protein synthesis was highly responsive to graded hypoxia in Arctic char. The effects of hypoxia on the rate of protein

synthesis is documented in hypoxia-tolerant fish species (Cassidy et al., 2018; Lewis et al., 2007; Smith et al., 1996; Zhou et al., 2005), but to our knowledge, we report for the first time that it is also the case in hypoxia-sensitive species such as Arctic char. At 50% air saturation, some tissues such as the liver and the digestive tract were already responding to hypoxia by decreasing their rate of protein synthesis by $\sim 20\%$ air saturation, while protein synthesis in the heart seemed to be insensitive to graded hypoxia, even at air saturation of 15%. Overall, the liver and digestive systems showed the most dramatic decreases of protein synthesis rates, reflecting the sensitivity of these tissues to changes in oxygen availability and their trivality for short-term survival. Skeletal muscle constitutes over 50% of the fish's body mass; therefore, suppressing muscle protein synthesis by 50% significantly decreases resting metabolic rate. The only tissue that maintained its rate of protein synthesis throughout the graded hypoxia experiment is the heart, contrasting with our previous findings in the hypoxia-tolerant oscar (Cassidy et al., 2018). The heart of most fish, including oscars, receives oxygen from venous blood (Farrell, 2002); therefore, when oxygen is limiting, cardiac functions are impaired (Farrell, 2002). In oscars, since energy demand during hypoxia is so low, a decrease in heart function could have little effect on survival. In the salmonid heart, about 30% of the myocardium is supplemented by a coronary artery (Farrell, 2002), which probably explains how the Arctic char's heart maintained protein synthesis during hypoxia. This anabolic state may also be fuelled by the increase in plasma lactate, one of the preferred metabolic substrates of salmonid hearts (Milligan and Farrell, 1991).

Protein degradation

The effects of hypoxia on protein degradation-related markers have scarcely been studied in fish (Cassidy et al., 2018). Our results provide strong evidence that protein degradation plays an important role in the hypoxia response. Markers of the ubiquitin proteasome

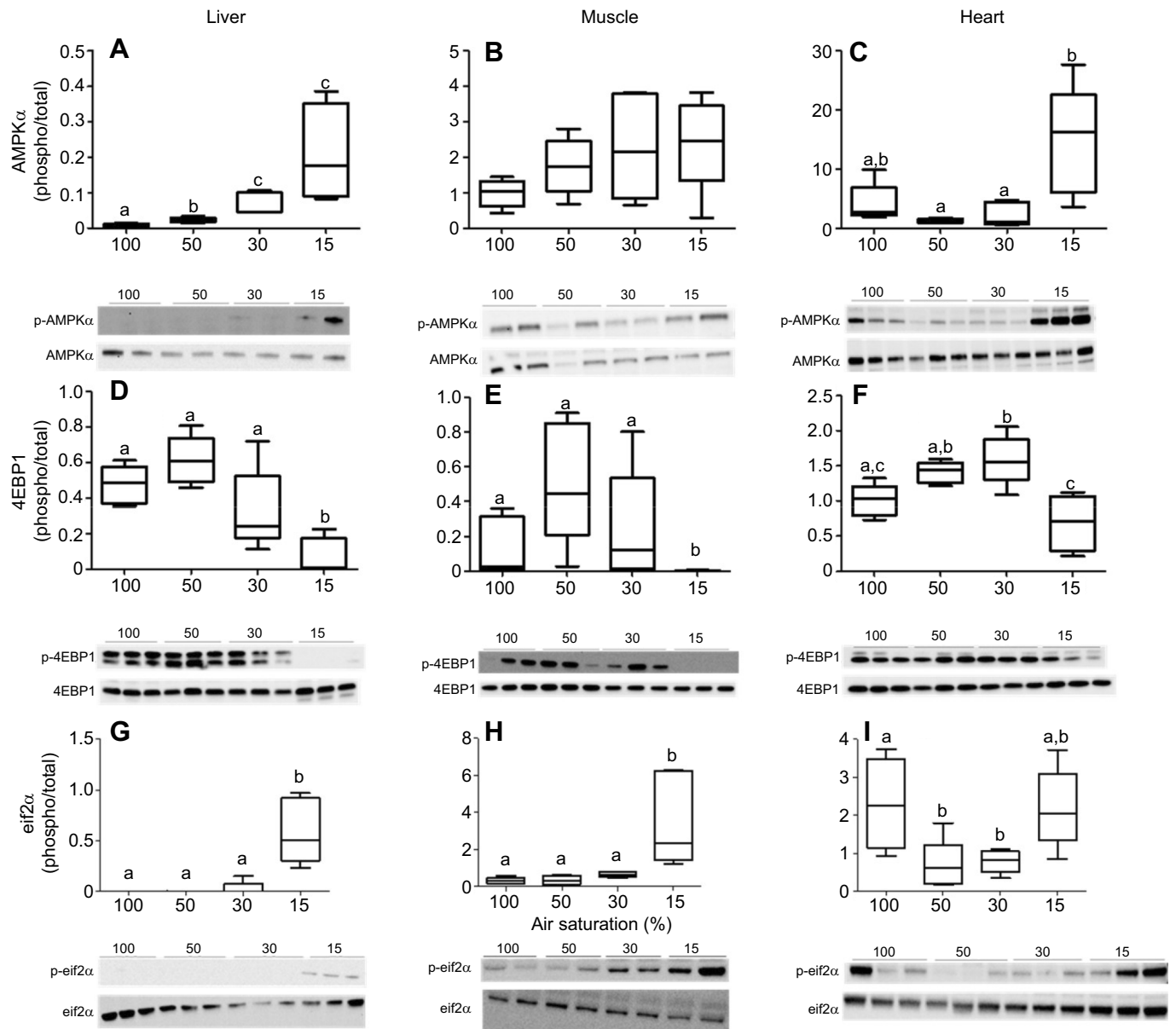


Fig. 5. Phosphorylation levels of proteins involved in the hypoxia-response pathways. Western blots of AMPK α in (A) liver, (B) muscle and (C) heart; 4EBP1 in (D) liver, (E) muscle and (F) heart; eif2 α in (G) liver, (H) muscle and (I) heart. Protein levels were normalized to stain-free gel band volumes and are plotted as ratio of phosphorylated to total protein in box plots. Values are expressed as means \pm s.e.m. Lower case letters indicate significant differences ($P < 0.05$) between air saturation levels.

pathway, autophagy and calpain system showed different trends among tissues. Overall, muscle protein degradation was maintained during hypoxia, supporting the lack of responses measured in the oxygen-response pathways. However, the liver and heart showed large but opposing effects of hypoxia on protein degradation markers. In mammals, hypoxia is a well-documented inducer of autophagy (Fang et al., 2015; Schaaf et al., 2013), but this is not yet verified in fish. In the liver, autophagy was activated during graded hypoxia, as shown by the elevation of *LC3B* transcripts, the hallmark marker of autophagy activation. This increase in autophagy is similar to what is observed in mammals, and likely reflects the liver's sensitivity to hypoxia. Perhaps surprisingly, the ubiquitin proteasome pathway markers were unresponsive to hypoxia in the Arctic char liver, contrasting with a downregulation of proteasome-subunit mRNAs, previously

reported in hypoxic three-spine stickleback (Levelehti et al., 2011). It is likely that this discrepancy results from the different hypoxia protocols the two experiments are using but this issue remains to be clarified by including a higher number of genes and looking at protein abundance.

In the heart, graded hypoxia was accompanied by an unexpected downregulation of protein degradation markers of all three pathways. Ubiquitin proteasome and calpain markers (*Fbxo25*, *UBE2H* and *Calpain7*) decreased, suggesting a downregulation of these two pathways. Transcripts from the autophagy pathway also decreased at 15% air saturation, which is opposite to what we observed in Arctic char liver and what is known in mammals (Mazure and Pouyssegur, 2010; Wouters and Koritzinsky, 2008). We offer two potential explanations for this. First, decreasing protein degradation might protect the heart's main functions by

Table 2. Relative mRNA levels in muscle of Arctic char exposed to 100%, 50%, 30% and 15% air saturation

Pathway	mRNA	Relative transcript level (mean±s.e.m.)			
		100%	50%	30%	15%
HIF	<i>HIF1a</i>	1.00±0.09	0.76±0.06	0.80±0.06	0.96±0.18
	<i>VEGF</i>	1.00±0.12 ^a	0.82±0.08 ^a	0.51±0.03 ^b	0.45±0.02 ^b
	<i>VHL</i>	1.00±0.42 ^a	2.55±0.19 ^b	2.95±0.30 ^b	2.07±0.32 ^{a,b}
UPR	<i>eIF2α</i>	1.00±0.11	0.97±0.08	0.90±0.04	0.81±0.02
	<i>ATF4</i>	1.00±0.1	0.93±0.10	0.92±0.05	0.93±0.15
	<i>DDIT3</i>	1.00±0.12 ^a	0.86±0.10 ^a	0.76±0.08 ^{a,b}	0.53±0.04 ^b
Proteasome	<i>Mafbx</i>	1.00±0.22	0.67±0.12	1.18±0.21	1.43±0.39
	<i>Murf1</i>	1.00±0.26	1.85±0.43	2.34±0.69	1.38±0.31
	<i>Fbxo25</i>	1.00±0.16	0.76±0.03	0.72±0.08	0.67±0.08
	<i>UBE2H</i>	1.00±0.23	0.97±0.05	1.09±0.06	1.08±0.13
Calpain	<i>Calpain7</i>	1.00±0.11	0.72±0.06	0.72±0.10	0.71±0.07
	<i>Calpain small subunit1</i>	1.00±0.22	0.89±0.09	1.06±0.17	0.85±0.06
	<i>Calpastatin</i>	1.00±0.11	1.3±0.35	1.1±0.21	1.21±0.19
Autophagy	<i>Cathepsin D</i>	1.00±0.12	1.13±0.10	1.16±0.16	1.23±0.18
	<i>Cathepsin L</i>	1.00±0.16 ^a	0.82±0.04 ^a	0.44±0.08 ^b	0.46±0.05 ^b
	<i>Atg4</i>	1.00±0.20	1.13±0.14	1.18±0.22	0.80±0.18
	<i>Atg5</i>	1.00±0.14	0.81±0.06	0.88±0.05	0.90±0.07
	<i>Beclin1</i>	1.00±0.23	0.89±0.11	1.13±0.20	0.76±0.16
	<i>LC3A</i>	1.00±0.08	1.08±0.06	1.17±0.06	1.25±0.11
	<i>LC3B</i>	1.00±0.13	0.92±0.08	0.84±0.07	0.79±0.08

Transcript levels are normalized with e1fα, β-actin and rs11. All data are relative to control levels and expressed as mean±s.e.m. Lower case letters indicate significant differences ($P<0.05$) between air saturation levels.

allocating more energy towards other more vital ones, such as contractions and protein synthesis. It is also possible that during hypoxia, the Arctic char heart is in an anabolic state supplied by an increase in plasma lactate. This hypothesis is also supported by the maintained rates of protein synthesis in the heart. A decrease in protein degradation during an anabolic state would maximize protein retention and thereby increase heart growth in the event of long-term hypoxia exposure, or during multiple exposures (diel cycling). An enlarged heart could then increase cardiac output, to increase the Arctic char's ability to deliver oxygen to tissues (Driedzic et al., 1996; Marques et al., 2008). These results warrant further investigation to understand the role of protein degradation inhibition in the heart during hypoxia.

Hypoxia-response pathways

We measured the activation of the three major hypoxia-response cellular signalling pathways (HIF, UPR and mTOR) in liver, muscle and heart by looking at the phosphorylation of key proteins involved in each pathway and/or the transcript numbers of genes under their control. These pathways promote energy conservation and enhance survival under low oxygen conditions (Liu et al., 2006). Our results confirm that these pathways are also regulated in hypoxia-sensitive fish and in a tissue-specific manner.

In liver, the hypoxia-response pathways were regulated as documented in mammals (Wouters and Koritzinsky, 2008). First, HIF was activated, and later UPR was activated during the more severe graded hypoxia exposure, while the mTOR pathway was

Table 3. Relative mRNA levels in heart of Arctic char exposed to 100%, 50%, 30% and 15% air saturation

Pathway	mRNA	Relative transcript level (mean±s.e.m.)			
		100%	50%	30%	15%
HIF	<i>HIF1a</i>	1.00±0.12	1.05±0.14	1.21±0.34	1.09±0.12
	<i>VEGF</i>	1.00±0.13 ^a	0.87±0.11 ^a	0.94±0.11 ^a	1.99±0.20 ^b
	<i>VHL</i>	1.00±0.10	1.07±0.11	1.00±0.10	0.81±0.06
UPR	<i>eIF2α</i>	1.00±0.08	0.89±0.07	0.88±0.04	0.95±0.04
	<i>ATF4</i>	1.00±0.08 ^a	0.80±0.07 ^{a,b}	0.83±0.05 ^{a,b}	0.69±0.03 ^b
	<i>DDIT3</i>	1.00±0.17	1.06±0.16	1.06±0.10	0.87±0.10
Proteasome	<i>Fbxo25</i>	1.00±0.09 ^a	0.93±0.17 ^a	0.73±0.05 ^{a,b}	0.51±0.04 ^b
	<i>UBE2H</i>	1.00±0.14 ^a	0.84±0.13 ^a	0.64±0.05 ^{a,b}	0.54±0.03 ^b
Calpain	<i>Calpain7</i>	1.00±0.06 ^a	0.80±0.10 ^{a,b}	0.65±0.05 ^b	0.61±0.03 ^b
	<i>Calpain small subunit1</i>	1.00±1.6	1.24±0.11	1.24±0.14	0.93±0.08
	<i>Calpastatin</i>	1.00±0.12	1.31±0.35	1.14±0.21	1.21±0.19
Autophagy	<i>Cathepsin D</i>	1.00±0.09	0.94±0.14	0.92±0.04	0.9±0.05
	<i>Cathepsin L</i>	1.00±0.13 ^a	0.78±0.09 ^{a,b}	0.71±0.08 ^{a,b}	0.62±0.04 ^b
	<i>Atg4</i>	1.00±0.07 ^{a,b}	1.11±0.10 ^a	1.05±0.15 ^{a,b}	0.74±0.09 ^b
	<i>Atg5</i>	1.00±0.07	0.92±0.07	0.76±0.09	0.74±0.04
	<i>Beclin1</i>	1.00±0.04 ^a	0.98±0.11 ^a	1.05±0.12 ^a	0.63±0.03 ^b
	<i>LC3A</i>	1.00±0.08	1.19±0.132	1.17±0.12	1.22±0.04
	<i>LC3B</i>	1.00±0.03	0.92±0.08	0.87±0.10	0.79±0.06

Transcript levels are normalized with e1fα, β-actin and α-tubulin. All data are relative to control levels and expressed as mean±s.e.m. Lower case letters indicate significant differences ($P<0.05$) between air saturation levels.

inhibited. The HIF pathway was activated early during hypoxia as shown by the rapid elevation of its target gene, *VEGF* transcripts (Wouters and Koritzinsky, 2008). This might also be supported by the observed increase of *HIF1a* transcripts. Although measuring *HIF1a* transcripts is not directly related to HIF accumulation in tissues, transcript levels increased during hypoxia in several fish species and this has been associated with HIF activation (Heinrichs-Caldas et al., 2019; Rimoldi et al., 2012; Ton et al., 2003). The UPR was activated during severe graded hypoxia, as shown by the accumulation of phosphorylated eIF2 α . This is similar to what is observed in livers in the oscar (Cassidy et al., 2018). The gradual activation of AMPK as the graded hypoxia treatment progresses, eventually translated into an inhibition of the mTOR pathway (shown by the dephosphorylation of 4EBP1). These results are similar to goldfish that increased AMPK activity in the liver during hypoxia (Jibb and Richards, 2008). The dephosphorylation of 4EBP1 results in a depression of cap-dependent translation consistent with the depression of the rate of protein synthesis we observed (Richter and Sonenberg, 2005). Overall, the hypoxia-response pathways were strongly regulated in the liver, and in accordance with the large decrease in protein synthesis and increase of autophagy markers. These results suggest that when oxygen decreases and blood becomes hypoxic, oxygen delivery ceases early in the liver.

In muscle, the hypoxia-response pathways were regulated differently than in the liver. The transcript numbers of *VEGF* decreased as hypoxia progressed which would be consistent with a greater destabilization of HIF in this tissue. Furthermore, the transcript numbers of *VHL*, which is responsible for the selective degradation of HIF (Turcotte et al., 2008), were rapidly elevated during hypoxia. Downregulation of the HIF pathway in muscle during hypoxia is surprising but suggests that muscle vascularization was impeded. To our knowledge, *VEGF* transcripts were never measured in the skeletal muscle of fish during hypoxia. The UPR was activated during the most severe hypoxia treatment in muscle, and mTOR was inhibited (decreased 4EBP1 phosphorylation), which is consistent with the decreased rates of protein synthesis in muscle. The regulation of protein synthesis via signalling pathways is important to reduce energy expenditure and the load of proteins in the ER, even in muscle, which otherwise appeared to have low sensitivity to hypoxia.

Out of the three examined tissues, the heart was the least responsive to hypoxia. There was a late activation of the HIF pathway, as *VEGF* transcripts increased at 15% air saturation and the UPR pathway was not activated. Similarly, AMPK and mTOR pathways responded as if the heart became anabolic during intermediate oxygen concentrations. The pattern of activation of these oxygen-responsive signalling pathways indicates that the heart is one of the last tissues to become hypoxic, probably as a result of its preferential oxygen supply via the coronary artery (Gamperl et al., 1995). The apparent anabolic state of the heart during hypoxia is metabolically fuelled by the circulating muscle-derived lactate as suggested by Lanctin et al. (1980) and Milligan and Farrell (1991).

Conclusion

We show that despite being a hypoxia-intolerant species, Arctic charr demonstrate considerable ability to alter protein metabolism, which could be under the control of hypoxia-responsive cellular signalling pathways (HIF, UPR and mTOR). The activation pattern of these pathways and the cellular processes that are under their control varies greatly among tissues, sometimes even going in opposite direction. This study provides new insights on the effects of hypoxia

on protein metabolism. The adjustments of these cellular processes probably all contribute to shifting the fish phenotype to a more hypoxia-tolerant one. Here, we measured the response of protein metabolism to a single and cumulative, graded hypoxia event in fish that were naïve to hypoxia. Our results warrant studying these adjustments in fish exposed to long-term and diel cycling hypoxia.

Acknowledgements

The authors are grateful to André Dumas and Claude Pelletier at the Coastal Zones Research Institute (Shippagan, NB, Canada) for providing the fish.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.A.C., S.G.L.; Methodology: A.A.C.; Software: A.A.C.; Validation: A.A.C.; Formal analysis: A.A.C.; Investigation: A.A.C.; Resources: S.G.L.; Data curation: A.A.C.; Writing - original draft: A.A.C.; Writing - review & editing: A.A.C., S.G.L.; Visualization: A.A.C., S.G.L.; Supervision: S.G.L.; Project administration: S.G.L.; Funding acquisition: S.G.L.

Funding

A.A.C. was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Postgraduate Scholarship-Doctoral Program (PGSD). S.G.L. was supported by grants from NSERC (435638-2013).

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.203901.supplemental>

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