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RESEARCH ARTICLE

Diel cycling hypoxia enhances hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*): evidence of physiological and metabolic plasticity

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ABSTRACT

Many fish naturally encounter a daily cycle of hypoxia, but it is unclear whether this exposure hardens hypoxia-intolerant fish to future hypoxia or leads to accumulated stress and death. The rainbow trout (*Oncorhynchus mykiss*) is a putatively hypoxia-sensitive species found in rivers and estuaries that may routinely experience hypoxic events. Trout were exposed to one of four 135 h treatments in a swim-tunnel respirometer: (1) air-saturated control (20.7 kPa P_{O_2}); (2) diel cycling O_2 (20.7–4.2 kPa P_{O_2} over 24 h); (3) acute hypoxia (130 h at 20.7 kPa P_{O_2} followed by 5 h at 4.2 kPa P_{O_2}); and (4) the mean oxygen tension (12.4 kPa P_{O_2}) experienced by the diel cycled fish. Some responses were similar in diel O_2 cycled and mean P_{O_2}-treated fish, but overall, exposure to ecologically representative diel hypoxia cycles improved hypoxia tolerance. Diel hypoxia-induced protective responses included increased inducible HSP70 concentration and mean corpuscular hemoglobin concentration, as well as reduced plasma cortisol. Acclimation to diel hypoxia allowed metabolic rates to decline during hypoxia, reduced oxygen debt following subsequent exposures, and allowed fish to return to an anabolic phenotype. The data demonstrate that acute diel cycling hypoxia improves hypoxia tolerance in previously intolerant fish through the activation of cellular protective mechanisms and a reduction in metabolic O_2 requirements.

KEY WORDS: Intermittent hypoxia, HSP70, Anabolism, Preconditioning, AMP-activated protein kinase

INTRODUCTION

Hypoxia is a common, naturally occurring phenomenon in aquatic ecosystems (Dan et al., 2014; Richards, 2011; Yang et al., 2013). The principal causes are the effect of temperature on oxygen solubility (Culberson and Piedrahita, 1996), water column density stratification (Justic et al., 2017) and biological O_2 demand, which is closely linked to anthropogenic eutrophication (Duncan et al., 2012; Richardson and Jørgensen, 1996). Diel oxygen cycling, or diel cycling hypoxia (DCH), is a common phenomenon in aquatic systems where O_2 partial pressure (P_{O_2}) increases during daylight hours with photosynthetic activity and decreases overnight, sometimes leading to hypoxic conditions (<30% O_2 saturation), as a consequence of biological O_2 demand (Dan et al., 2014; Yang et al., 2013). Organisms that are able to alter physiological and biochemical processes to maintain energy balance when exposed to hypoxia are more likely to survive than those that are not (Boutilier, 2001; Nilsson and Renshaw, 2004). Hypoxia-tolerant organisms respond to oxygen deprivation by modifying O_2 uptake, O_2 delivery and metabolism to guard against arterial blood O_2 deficiency (hypoxemia) and cellular energy exhaustion (Boutilier, 2001). In contrast, the physiological responses mounted by hypoxia-intolerant fish are insufficient to effectively avoid hypoxemia and balance energy supply and demand, so they tend to behaviorally avoid hypoxic waters.

Physiological plasticity in response to hypoxia has been demonstrated in fish (Borowiec et al., 2015); a prior exposure to hypoxia can increase tolerance to a subsequent event in a process known as preconditioning (Gamperl et al., 2001, 2004). Gamperl et al. (2001, 2004) showed that preconditioned hearts and isolated cardiomyocytes from rainbow trout (*Oncorhyncus mykiss*) were better able to maintain function under subsequent hypoxic stresses. Cardioprotective processes were also activated and included an increased reliance on anaerobic glycolysis, fueled by exogenous glucose (Gamperl et al., 2001). Numerous signal transduction pathways have been identified as vital for hypoxic preconditioning, including those associated with oxygen sensing (Sheldon et al., 2014), which activate downstream pathways to improve oxygen transport/delivery (Xiao, 2015), erythropoiesis (Chen et al., 2010), angiogenesis (Bolli, 2001) and energy metabolism (Manchenkov et al., 2015).

The majority of work on DCH or similar repeated hypoxia exposures in fish has been carried out on hypoxia-tolerant species (Dan et al., 2014; Lykkeboe and Weber, 1978; Yang et al., 2013), and it is not clear whether more-sensitive species respond similarly. The epaulette shark (*Hemiscyllium ocellatum*) naturally experiences intermittent hypoxia in tidepool environments, and repeated exposure to hypoxia triggers aerobic metabolic depression and a decrease in critical oxygen tension (P_{crit}) (Nilsson and Renshaw, 2004). In juvenile qingbo (*Spinibarbus sinensis*), DCH increased feeding and growth rates, while decreasing P_{crit}, loss of equilibrium threshold and aquatic surface respiration threshold (Dan et al., 2014). Similar results were found in southern catfish (*Silurus meridionalis*) in terms of P_{crit}, loss of equilibrium threshold and aquatic surface respiration threshold, but DCH-acclimated catfish exhibited reduced feeding and growth rates, combined with a lower maximum aerobic metabolic rate and improved critical swimming speed (Yang et al., 2013). Numerous physiological and morphological changes directed at improving O_2 uptake, delivery and metabolism were also noted in killifish (*Fundulus heteroclitus*) exposed to DCH (Borowiec et al., 2015). Summer flounder (*Paralichthys dentatus*) growth rate did not acclimate to DCH and

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mortality increased significantly following 2–3 weeks of extreme DCH (Davidson, 2015), which was representative of highly eutrophic estuaries. In cases where DCH ostensibly improved hypoxia tolerance, it is not clear whether changes were triggered by fluctuations in P_{O_2} or by the average decrease in P_{O_2} experienced by fish exposed to DCH conditions. If subcritical DCH is capable of preconditioning hypoxia-sensitive fish in a similar manner as for hypoxia-tolerant fish, it may allow these fish to persist in the increasingly unfavorable conditions predicted to occur with the progression of climate change (Henson et al., 2017; Stocker et al., 2013).

Relative to DCH, there are numerous studies examining the protective effect of temperature cycling in stress-sensitive species such as rainbow trout (Callaghan et al., 2016) and Atlantic salmon (Tunnah et al., 2016b). Diel thermal fluctuations were shown to mediate metabolic remodeling by inducing an increasingly less catabolic phenotype as well as sequestering and regenerating metabolic substrates in the liver with successive high temperature exposures (Callaghan et al., 2016). The mean temperature from the fish's original habitat appeared to affect responses to diel thermal cycling in Atlantic salmon (Tunnah et al., 2016b), but it is unclear whether such an effect is triggered by DCH. Because both high temperature and low P_{O_2} impose energetic stresses on fish, it is possible that the metabolic response to DCH may parallel the catabolic-to-anabolic phenotype transition exhibited in response to diel thermal cycling.

The goals of this study were to (1) determine whether exposure to DCH improves hypoxia tolerance in a putatively sensitive fish, the rainbow trout, and (2) characterize changes in key metabolic regulators involved in the maintenance of energy balance under stress. As climate change progresses, temperature and P_{O_2} are likely to become more variable in aquatic environments (Caissie et al., 2013; Stocker et al., 2013). There is also increasing recognition that the responses of animals to variable environments differs from their responses to the mean of that variation (Morash et al., 2018). It is therefore essential to understand not only how animals will cope with static, chronic stressors, but also to variable and stochastic environments. Rainbow trout were used as a model as they are hypoxia sensitive (Gamperl et al., 2001), have a well-characterized capacity for stress preconditioning (Gamperl et al., 2001, 2004), and are an economically and ecologically important species (Behnke et al., 2002; Ward and Post, 2014). We specifically focused on responses exhibited during the initial onset of diel P_{O_2} cycles, when animals are transitioning from a stable normoxic environment to an environment with chronically fluctuating P_{O_2} levels. Rainbow trout were thus exposed to ecologically relevant diel P_{O_2} cycles over 5 days and examined for a suite of responses associated with the maintenance of O_2 uptake, energy homeostasis, and physiological and cellular stress indicators. We then characterized key signal transduction pathways to understand whether DCH triggers a protective remodeling of energy metabolism.

MATERIALS AND METHODS

Animal collection and care

Female hatchery-reared rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792); n=26; body mass 632.0±25.2 g, length 34.1 ±0.6 cm] were held in a 750 liter tank of filtered, partially recirculated freshwater at 16±0.5°C on a 12 h:12 h day:night cycle for a minimum of 2 weeks prior to study. Fish were fed to satiation daily with sinking pellets. Food was withheld for 24 h prior to, and for the entire duration of, all experiments. Fish mass and length were not significantly different (P=0.824) between treatment groups. All procedures were performed with the approval of the Mount Allison University Animal Care Committee (protocol no. 16-08).

Intermittent flow swim tunnel respirometry

A Loligo swim tunnel respirometer (Tjele, Vilborg, Denmark) was used to measure O_2 consumption rates ($\dot{M}_{O_2}$) in each animal as a proxy of aerobic metabolic rate. The system consisted of a darkened 30 liter holding chamber within a 120 liter reservoir. The water temperature was held at 16±1°C. P_{O_2} and temperature readings were collected via a DAQ-M data acquisition unit and AutoResp software (Loligo Systems). Each $\dot{M}_{O_2}$ measurement cycle consisted of a 900 s flush, 200 s wait and a 100 s measurement, culminating in one $\dot{M}_{O_2}$ reading every 20 min. Water flow speed was set at 0.5 body lengths per second for all fish.

Hypoxia was achieved by attenuating aeration and bubbling N_2 gas through air stones submerged in the swim tunnel reservoir and connected to a valve controlled by the AutoResp software. A Clark-type O_2 probe interfaced with the system measured P_{O_2} levels in the reservoir and AutoResp utilized these data to control N_2 flow to reach desired P_{O_2} levels. Re-oxygenation was achieved by suspending N_2 gas influx and resuming aeration of the external reservoir.

Treatment regimens

All fish were held under normoxia (20.7 kPa P_{O_2}) for 24 h following insertion into the respirometer. Rainbow trout were exposed to one of four protocols for 135 h (Fig. 1, Table 1). (1) Acute hypoxia (AH, n=7): fish were held for 130 h at normoxia prior to an acute hypoxic exposure (5 h, 4.2 kPa P_{O_2}). In preliminary studies, 4.2 kPa was the lowest P_{O_2} at which naive rainbow trout could consistently survive for 5 h (data not shown). (2) Diel hypoxia fluctuation (DC, n=7): fish were exposed to 130 h of environmentally relevant diel cycling hypoxia (Tyler et al., 2009). Deoxygenation occurred at ∼2.0 kPa h^{-1} until P_{O_2} reached 4.2 kPa, where the fish were held for 4 h before re-oxygenation. Re-oxygenation occurred at ∼5.0 kPa h^{-1} until P_{O_2} reached a maximum of 20.7 kPa where the fish were held until the next cycle of deoxygenation. Following the final deoxygenation, DC fish were held for 5 h at 4.2 kPa P_{O_2}. (3) Air-saturated control (SC, n=6): for the entirety of the protocol. (4) Mean P_{O_2} control (MC, n=6): fish were exposed to 12.4 kPa P_{O_2} for the entirety of the protocol, which was the mean P_{O_2} experienced by DC-treated fish. Treatment regimens were limited to 135 h to avoid prolonged fasting and to facilitate comparisons with previous studies on responses to diel thermal cycling in salmonids (Callaghan et al., 2016; Tunnah et al., 2016b). Deoxygenation rates were identical for hypoxia exposures in the AH and DC treatments.

Tissue sampling

Prior to insertion into the respirometer, each fish was anesthetized with buffered tricaine methanesulfonate (MS-222; 0.19 g l^{-1}) and pre-treatment blood samples (∼1.0 ml) were taken from the caudal vein. Mass and length were measured and used for $\dot{M}_{O_2}$ and swimming velocity calculations.

Following the experiment, a second blood sample was collected. At the time of sampling, SC fish were at 20.7 kPa, MC fish were at 12.4 kPa, and AH and DC fish were at 4.2 kPa P_{O_2}. Fish were then placed in an anesthetic bath (300 mg l^{-1} tricaine methanesulfonate buffered with 600 mg l^{-1} $NaHCO_3$) until ventilation ceased, and killed by spinal transection. Heart ventricle and liver were quickly collected, frozen in liquid N_2 and stored at −80°C. Hemoglobin and hematocrit levels were measured on fresh blood immediately following sampling. Blood samples were then centrifuged (5000 ***g***

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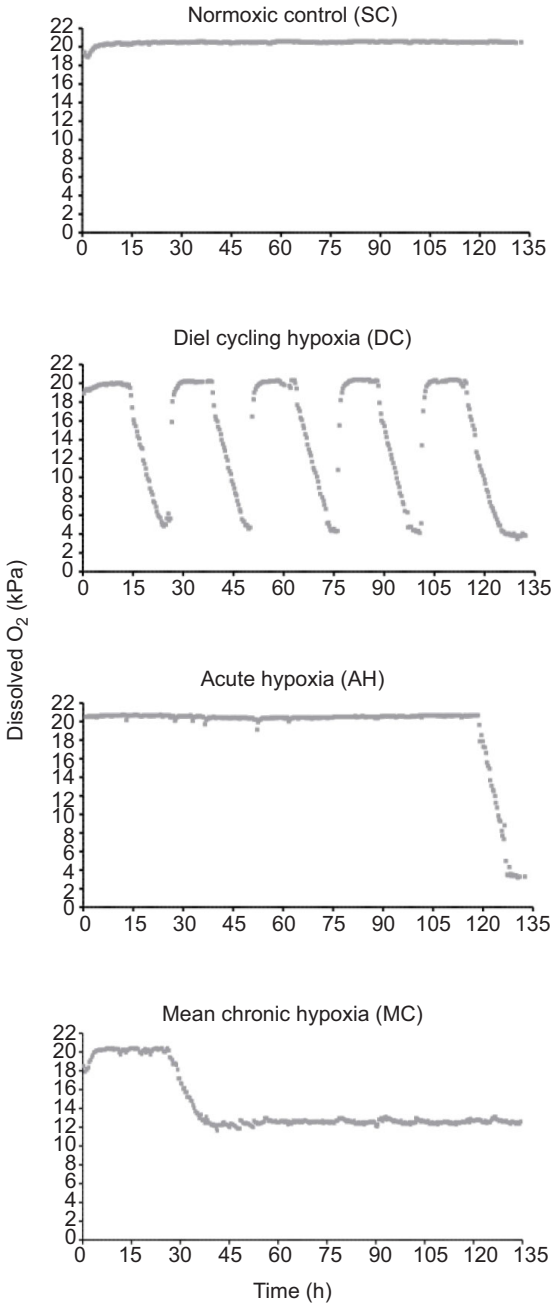

Fig. 1. Swim-tunnel respirometer parameters for each experimental treatment group. Rainbow trout (*Oncorhynchus mykiss*) were held at 16°C throughout the treatment regime and were exposed to either normoxic (SC, n=6), diel cycling hypoxia (DC, n=7), acute hypoxia (AH, n=7) or mean oxygen tension (MC, n=6). Dissolved oxygen saturation (%) was modified at specific time points and rates (kPa O_2 h^{-1}). Blood and tissue samples were collected at the 135 h mark at the oxygen tension that the fish was held during the last phase of their treatment regime.

for 10 min, at 4°C), and plasma was collected, frozen in liquid N_2 and stored at −80°C.

Hematological analyses

Hemoglobin levels were measured using a HemoCue HB 201+ (HemoCue America, CA, USA). Hematocrit levels were determined by measuring the height of packed erythrocyte versus that of total blood [(height of packed red blood cells/total blood height)×100%] following centrifugation in micro-hematocrit capillary tubes. Mean corpuscular hemoglobin concentration (MCHC) was calculated as described in Tunnah et al. (2016a). Plasma cortisol levels were measured by ELISA according to the manufacturer's instructions (Neogen Corp., KY, USA).

Plasma and tissue metabolites

Plasma and tissue samples were homogenized in cold 6% perchloric acid and centrifuged at 10,000 ***g*** for 3 min, and supernatants were collected for glucose and lactate determination. Glucose was quantified spectrophotometrically at 340 nm by following the production of NADPH using a hexokinase and glucose-6-phosphate dehydrogenase coupled assay (MacCormack et al., 2003). Lactate was also quantified spectrophotometrically at 340 nm by following the oxidation of NADH by lactate dehydrogenase in a glycine–hydrazine buffer (MacCormack et al., 2003). Glycogen in liver and heart was extracted in 2% Na_2SO_4 and absolute ethanol, hydrolyzed to glucose by boiling in 1.2 mol l^{-1} HCl, and enzymatically quantified as glucose using the above assay (Callaghan et al., 2016). Triacylglycerol (TAG) content was measured as glycerol equivalents using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Protein extraction and immunoblotting

Tissue samples were initially homogenized in TRIS buffer [50 mmol l^{-1} TRIS (pH 8), 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} β-mercaptoethanol, pH 8, containing 1 mmol l^{-1} PMSF and 1 mmol l^{-1} sodium orthovanadate (as inhibitors of proteases and phosphatases, respectively)] using a sonicating homogenizer (Q55 Sonicator, Qsonica) and then centrifuged (10,000 ***g*** for 3 min). Soluble protein was further prepared for SDS-PAGE as previously detailed (Capaz et al., 2017; Lamarre et al., 2016). Protein concentration was determined using a Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

Protein (15 µg per well) was loaded into 4–15% TGX Gels (Bio-Rad) with an anti-rabbit compatible MagicMark™ XP Western Standard protein ladder (Thermo-Fisher Scientific). Proteins were electrophoresed in TGX gels and then transferred onto PVDC membranes using semi-dry Trans-Blot® Turbo Modules (Bio-Rad). A stain-free image of the membrane was taken for protein normalization using a Bio-Rad Chemidoc Touch Imaging System. Membranes were blocked in 5% BSA in 1× tris-buffered saline with Tween 20 (TBST). All primary and secondary antibodies were validated for use in fish tissue (Callaghan et al., 2016; Lamarre et al., 2012; Tunnah et al., 2016b) and are detailed in Table 2. Relative phosphorylation of a specific protein was calculated as the ratio of phosphorylated protein versus total protein expression.

Data analysis and statistics

Statistical analyses were carried out using either Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) or R (https://www.r-project.org/). Data were tested for normality via the Shapiro–Wilk test by analyzing all cases together, and normally distributed data were then tested for homogeneity of variance via a Levene's test. In all instances, significance was assigned at $P \leq 0.05$, and all values are presented as means±s.e.m.

All $\dot{M}_{O_2}$ measurements based on P_{O_2} slopes with an R^2 less than 0.75 were excluded (63 of 2912 measurements; 2.1%). Between-treatment comparisons of $\dot{M}_{O_2}$ are based on the timeline of the DC group, where the stages were: post-insertion recovery, hypoxia 1, recovery 1, etc., until the final extended hypoxia. The mean $\dot{M}_{O_2}$ for each treatment at each stage was calculated once P_{O_2} plateaued at a maximum or minimum state (20.7 and 4.2±0.3 kPa) until the next

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Table 1. Swim-tunnel respirometer parameters for each experimental treatment group (see Fig. 1)

Oxygenation profile	Time (h)	Range (% O_2)	Δ % O_2	Ramp rate (kPa O_2 h^{-1})	Min./max. duration (h)
Normoxic control (SC)	24–135	95–100	5	–	–
	0–24 (recovery)	100	0		
Diel cycling hypoxia (DC)	0–24 (recovery)	95–100	5	–	–
	24–32	100–20	60	2	3–4
	36–42	20–100	60	5	6–8
	48–56	100–20	60	2	3–4
	60–66	20–100	60	5	6–8
	72–80	100–20	60	2	3–4
	84–90	20–100	60	5	6–8
	96–104	100–20	60	2	3–4
	106–108	20–100	60	5	6–8
	112–130	100–20	60	2	5
Acute hypoxia (AH)	0–24 (recovery)	95–100	5	–	–
	24–118	100	0	–	–
	118–130	100–20	60	2	5
Mean chronic hypoxia (MC)	0–24 (recovery)	95–100	5	–	–
	24–38	100–60	40	2	–
	38–135	60	0	–	97

All experiments were performed at 16°C.

deoxygenation or re-oxygenation. The mean $\dot{M}_{O_2}$ across the different stages was analyzed by a two-way repeated-measures ANOVA and Bonferroni *post hoc* tests. Critical O_2 tension (P_{crit}) was determined for each hypoxic stage of the DC treatment and the single hypoxic exposure in the AH treatment using a segmented regression analysis according to established methods (Yeager and Ultsch, 1989). P_{crit} values across the DC treatment were compared using a repeated-measures ANOVA or Tukey's HSD. P_{crit} values for the final extended hypoxia in AH and DC treatments were compared using a two-tailed *t*-test.

Excess post-hypoxia O_2 consumption (EPHOC) in DC fish was determined using R. First, the standard metabolic rate (SMR) of each fish was estimated based on quantile analyses of $\dot{M}_{O_2}$ data collected >300 min after return to normoxia. Previous researchers have used the 0.2 quantile to estimate SMR (Chabot et al., 2016); here, the 0.25 quantile was chosen to ensure a minimum of 10 data points were included in this estimate. Quantiles were calculated using the calcSMR function of the fishMO$_2$ package (https://www.researchgate.net/project/fishMO2-a-R-package-to-calculate-and-plot-SMR-O2crit-and-SDA). To estimate EPHOC, $\dot{M}_{O_2}$ at hypoxia exposure recovery times was modeled as a general additive model using the 'gam' function of the mgcv package (Wood, 2011). Fitted $\dot{M}_{O_2}$ curves were produced for each fish, except for one in the DC treatment regime that was identified as an outlier using a Grubbs outlier test. For all gam models, arguments included a *k* value of −1, 'Gamma' as family, an fx of 'F', and a 'tp' bases. EPHOC duration ($EPHOC_{duration}$) was estimated as the time elapsed since returning to normoxia until the point where fitted recovery $\dot{M}_{O_2}$ and SMR no longer differed; specifically, where fitted recovery $\dot{M}_{O_2}$ minus the associated s.e.m. was first ≤SMR plus a 5% tolerance limit. Total EPHOC was determined by subtracting the area under the SMR curve over the $EPHOC_{duration}$ from the area under the fitted recovery $\dot{M}_{O_2}$ curve.

Hematological parameters were analyzed using two-way ANOVAs with Tukey *post hoc* tests. Metabolite and protein expression measurements were analyzed using one-way ANOVAs with Tukey *post hoc* tests. Protein expression was normalized to total protein and then SC protein levels.

RESULTS

Physiological and cellular stress

Whole-organism stress was assessed using plasma cortisol and lactate, and protein poly-ubiquitylation and HSP70 relative concentration. Pre-treatment cortisol and lactate levels were 9.5±0.5 ng ml^{-1} and 1.9±0.1 mmol l^{-1}, respectively, and were not different between groups (P=0.67 and P=0.79, respectively; data not shown). AH fish had post-treatment cortisol concentrations 2.5- to

Table 2. Western blot antibodies used to assess carbohydrate, lipid and protein metabolism, as well as physiological stress responses

Signal transduction category	Protein	Target	Dilution	Provider	Catalog no.
Carbohydrate metabolism	AMPKα	$Arg^{3/7}$	1:1000	Cell Signaling Technology	5831
	pAMPKα	$pThr^{172}$	1:1000	Cell Signaling Technology	2535
	AMPKβ	His^{233}	1:1000	Cell Signaling Technology	4150
	pAMPKβ	$pSer^{108}$	1:1000	Cell Signaling Technology	4181
Lipid metabolism	ACC	Ser^{523}	1:1000	Cell Signaling Technology	3676
	pACC	$pSer^{79}$	1:1000	Cell Signaling Technology	11818
	Akt	Cys^{67}/Glu^{7}	1:1000	Cell Signaling Technology	4691
	pAkt	Ser^{473}	1:1000	Cell Signaling Technology	9271
Protein metabolism	4E-BP1	$His^{11/53}$	1:1000	Cell Signaling Technology	9644
	p4E-BP1	$Thr^{37/46}$	1:1000	Cell Signaling Technology	2855
	eIF-2α	$Asp^{3/7}$	1:1000	Cell Signaling Technology	5329
	peIF-2α	$Ser^{51}/Arg^{8/9}$	1:1000	Cell Signaling Technology	3398
Physiological stress response	PolyUb	Lys-48 linked poly-ubiquitin	1:1000	Abcam Canada	ab190061
	Inducible HSP70	B5X4Z3	1:10000	Agrisera Antibodies	AS05 061A
Secondary antibody	N/A	Anti-rabbit immunoglobin	1:5000	Cell Signaling Technology	7074
	N/A	Anti-rabbit HSP70 immunoglobin	1:5000	Agrisera Antibodies	AS09 602

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10-fold higher than those of other groups (P=0.001). Cortisol concentrations did not significantly differ between the SC, MC and DC fish (Fig. 2A). Post-treatment plasma lactate levels were 1.5-3 fold higher in AH fish relative to other treatments (P<0.001; Fig. 2B). Fish in the DC group exhibited plasma lactate concentrations ∼2-fold higher than those of SC fish (P<0.001), but not different from those of MC fish. Plasma lactate levels in MC fish were not significantly different from those in the SC or DC groups.

Liver protein K48 poly-ubiquitylation was increased by 2- to 6-fold in AH fish (P<0.001; Fig. 2C), whereas no changes were noted in other treatments. In heart, poly-ubiquitylation was elevated in both AH and DC fish relative to SC and MC fish (P=0.041).

HSP70 concentration was significantly higher (P<0.001; Fig. 2D) following the DC treatment in both liver (∼2000-fold increase) and heart (∼18-fold increase) relative to all other treatments (Fig. 2D). In AH fish, liver HSP70 levels were significantly higher than in SC and MC fish, but this was not the case for the heart.

Aerobic metabolism and O_2 uptake capacity

The $\dot{M}_{O_2}$ of each fish was continuously measured in each of the four treatment regimens (Fig. 3). Approximately 10 h following insertion into the respirometer, fish reached a baseline $\dot{M}_{O_2}$ of 124±5.7 mg O_2 kg^{-1} h^{-1} (Fig. 4A). The $\dot{M}_{O_2}$ of the AH fish was not significantly different from that of the SC fish at any point, even during the acute hypoxia exposure. The mean $\dot{M}_{O_2}$ of the AH fish during hypoxia was similar to that of the DC fish during their first hypoxia exposure. DC fish exhibited depressed $\dot{M}_{O_2}$ during each subsequent hypoxia exposure that was significantly lower than those of all other groups at the same time points. This corresponded

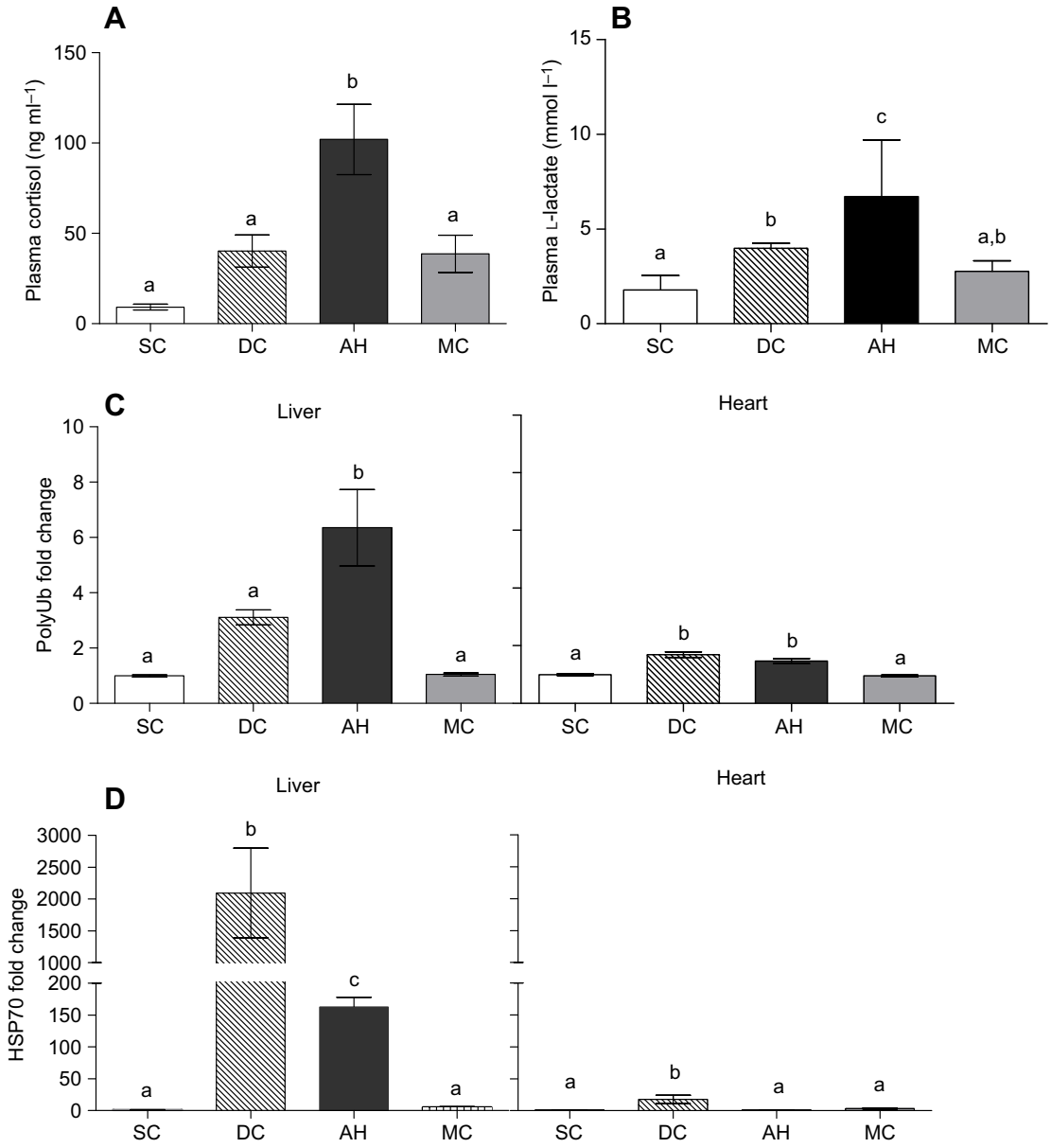

Fig. 2. Physiological stress response markers in rainbow trout (*O. mykiss*) exposed to normoxic (SC, *n*=6), diel cycling hypoxia (DC, *n*=7), acute hypoxia (AH, *n*=7) or mean oxygen tension (MC, *n*=6) treatments. (A) Post-treatment plasma cortisol, (B) pre- and post-treatment plasma lactate, and (C,D) fold change of liver and heart poly-ubiquitin (PolyUb) and heat shock protein 70 (HSP70) relative to SC treatment. Data are expressed as means±s.e.m. and statistically significant differences (P<0.05, two-way ANOVA with Tukey *post hoc* tests) are indicated by dissimilar letters.

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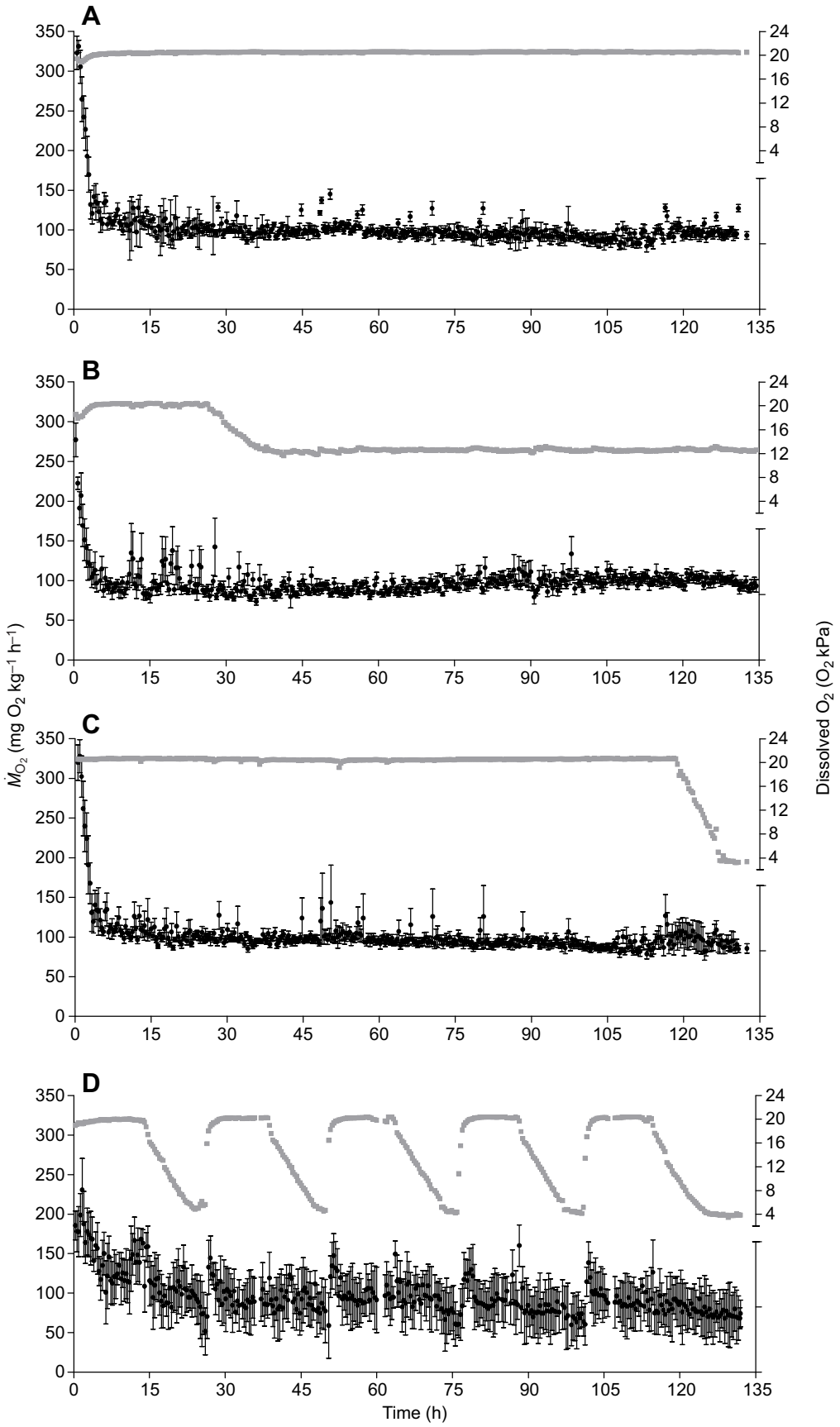

Fig. 3. Aerobic metabolic rate ($\dot{M}_{O_2}$; black) and measured ambient partial pressures of O_2 (O_2 kPa; gray) in each of the four treatment groups studied. (A) Normoxic (SC; *n*=6), (B) diel cycling hypoxia (DC; *n*=7), (C) acute hypoxia (AH; *n*=7) and (D) mean O_2 tension (MC; *n*=6). $\dot{M}_{O_2}$ data represent the means±s.e.m. of the $\dot{M}_{O_2}$ at each time point for all fish in a treatment group.

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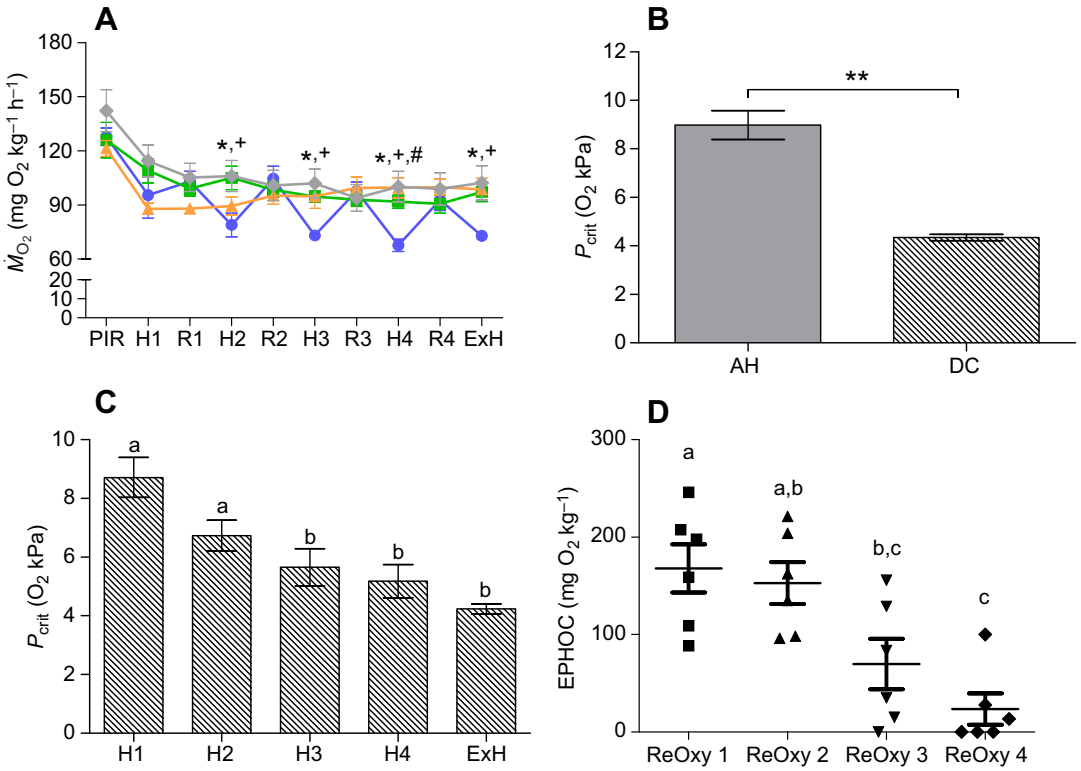

Fig. 4. Average aerobic metabolic rates ($\dot{M}_{O_2}$), critical oxygen tension (P_{crit}) and excess post-hypoxia oxygen consumption (EPHOC) in rainbow trout (*O. mykiss*) exposed to normoxic (SC, *n*=6), diel cycling hypoxia (DC, *n*=7), acute hypoxia (AH, *n*=7) or mean oxygen tension (MC, *n*=6) treatments. (A) Mean $\dot{M}_{O_2}$ for each treatment group relative to the DC treatment group: post-insertion recovery (PIR), hypoxic 1 (H1), recovery 1 (R1), until the extended hypoxia (ExH). Statistically significant differences (P<0.05, two-way repeated-measures ANOVA and Bonferroni *post hoc* tests) are represented by * (DC versus AH), # (DC versus MC) or + (DC versus SC). (B) P_{crit} during the final extended hypoxia for DC and AH fish (**P<0.001, repeated-measures ANOVA and Tukey's HSD *post hoc* tests). (C) P_{crit} values obtained for each successive hypoxia exposure for DC-treated fish. (D) EPHOC following each re-oxygenation in the DC treatment group. For C and D, significant differences between either stages or treatment groups are indicated by dissimilar letters (P<0.05 repeated-measures ANOVA and Tukey's HSD *post hoc* tests).

with a reduction in EPHOC so that by the third re-oxygenation, it was reduced by >6-fold (Fig. 4D). By the final extended hypoxia exposure, DC fish exhibited an $\dot{M}_{O_2}$ of 72±1.8 mg O_2 kg^{-1} h^{-1} compared with 97±4.6 mg O_2 kg^{-1} h^{-1} in SC and MC fish (P<0.001; Fig. 4A). Evidence of improved hypoxia tolerance in DC fish was also demonstrated by a progressive decrease in P_{crit}: the P_{crit} of DC fish was half that of AH fish by day 5 (P=0.003; Fig. 4B). DCH did not affect SMR under normoxia, as in DC fish it was not different from the other treatment groups by the end of each normoxic recovery (Fig. 4A).

Pre-treatment hematocrit and MCHC were not different among the treatment groups (P=0.3 and P=0.6, respectively; Fig. 5). Post-treatment hematocrit was significantly elevated in DC fish relative to the SC treatment group. Hemoglobin and MCHC were significantly elevated in DC fish relative to all other treatments. The apparent increase in O_2 uptake and transport capacity, along with the depression of hypoxic $\dot{M}_{O_2}$, also likely explains why plasma lactate levels in DC fish were low relative to those of AH fish following the extended hypoxia (P=0.001; Fig. 2B).

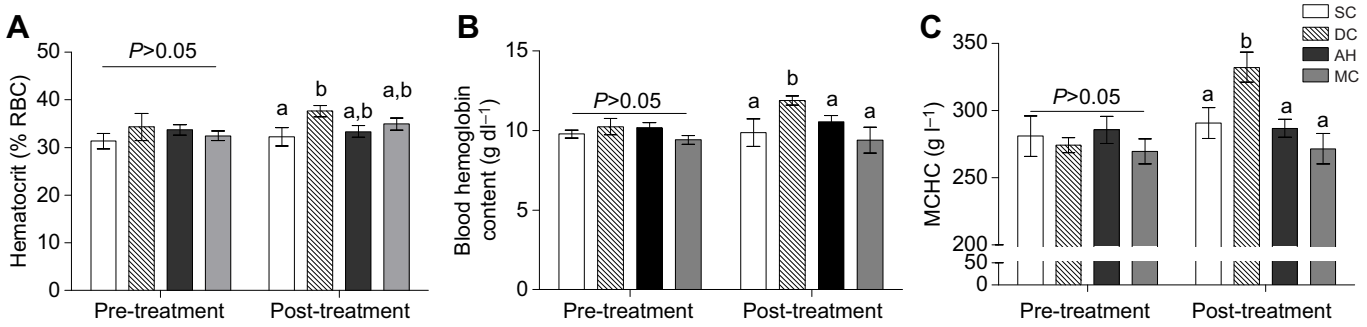

Fig. 5. Pre- and post-treatment hematocrit, hemoglobin and mean corpuscular hemoglobin concentration (MCHC) in rainbow trout (*O. mykiss*) exposed to normoxic (SC, *n*=6), diel cycling hypoxia (DC, *n*=7), acute hypoxia (AH, *n*=7) or mean oxygen tension (MC, *n*=6) treatments. (A) Hematocrit, (B) blood hemoglobin concentration and (C) MCHC. Significant differences (P<0.05, two-way repeated-measures ANOVA and Bonferroni *post hoc* tests) between treatment groups are indicated by dissimilar letters.

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Tissue energy metabolism

Data on the phosphorylation state of AMPKα and β, and tissue glycogen content provided insight into the effects of DCH on the regulation of carbohydrate metabolism (Fig. 6). The phosphorylation of liver AMPKα increased in AH fish relative to other groups (Fig. 6A), but no changes were noted in either DC or MC fish. Heart AMPKα phosphorylation did not differ among treatments, and there were no changes in AMPKβ phosphorylation in either liver or heart (Fig. 6B). Therefore, AMPK was active in all fish as the compulsory phosphorylation of AMPKβ was similar

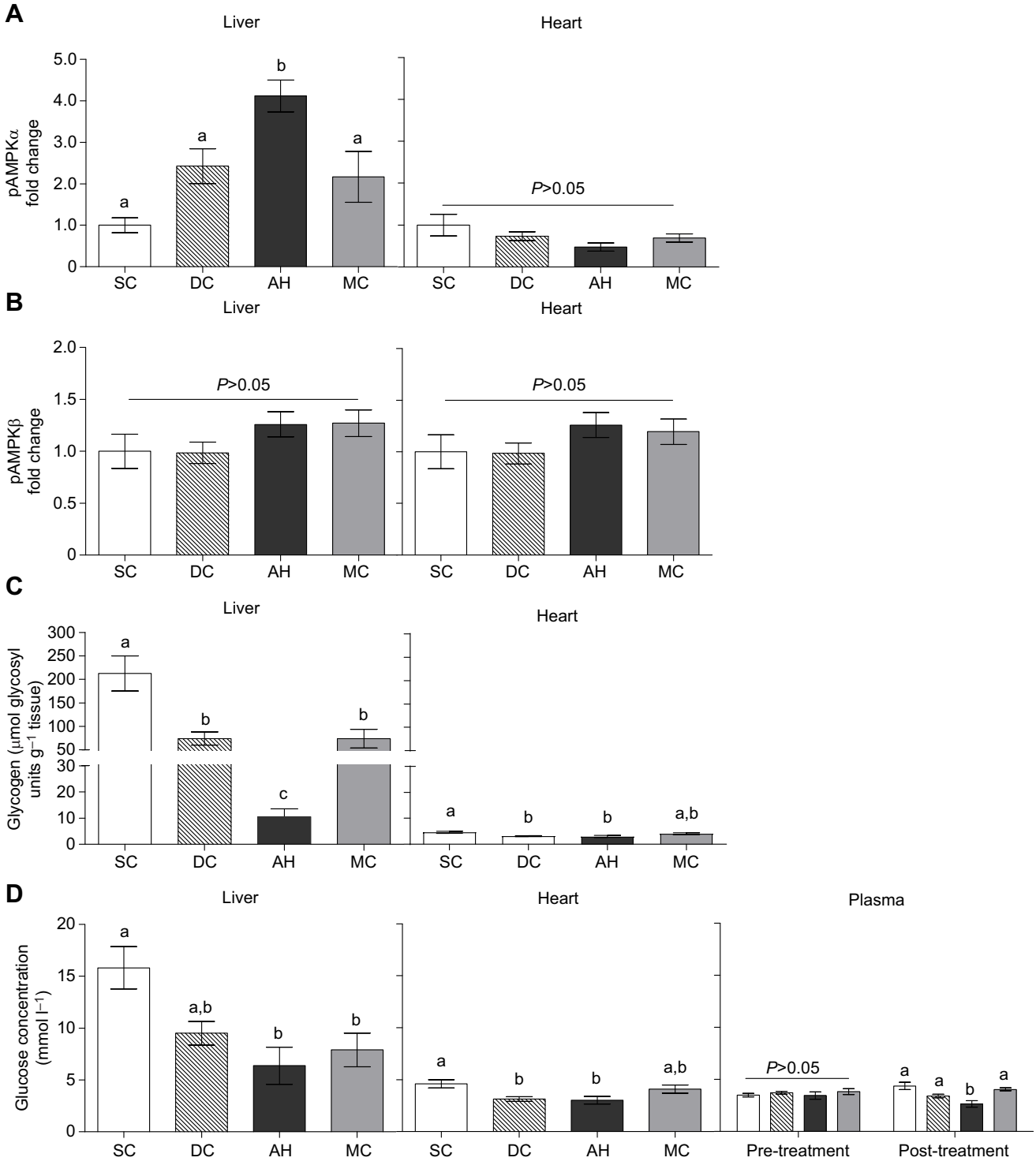

Fig. 6. Markers of carbohydrate metabolism, 5′ AMP-activated protein kinase (AMPK) α/β, glycogen and glucose in rainbow trout (*O. mykiss*) exposed to normoxic (SC, *n*=6), diel cycling hypoxia (DC, *n*=7), acute hypoxia (AH, *n*=7) or mean oxygen tension (MC, *n*=6) treatments. The control of cellular carbohydrate metabolism was assessed by the phosphorylation of AMPKα/β (A,B) in liver and heart, normalized by the SC group. Glucose utilization and storage was assessed through measurements of liver and heart glycogen (C), as well as liver, heart and plasma free glucose (D). Significant differences (P<0.05, one-way ANOVA and Tukey *post hoc* tests) between treatment groups are indicated by dissimilar letters.

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throughout, but AH fish exhibited the highest level of activation owing to elevated AMPKα. The activation of AMPKα in the liver of AH fish likely led to the significant depletion of glycogen in these animals (Fig. 6C). Heart glycogen was lower in both DC and AH fish relative to SC fish. Liver free glucose content was lower in AH and MC fish relative to SC fish, but the difference between DC and SC fish was not significant (Fig. 6D). Heart glucose was lower in DC and AH fish relative to SC fish. Furthermore, there was a significant post-treatment reduction in plasma glucose in AH fish relative to other treatments.

Liver lipid metabolism was not affected by any treatment but a number of changes were observed in the heart (Fig. 7). Heart acetyl-CoA carboxylase (ACC) phosphorylation was higher in DC than MC fish, but it did not differ from other treatments. Heart TAG stores were significantly depleted in both DC and AH fish relative to the SC group.

Protein synthesis regulator activation in liver was depressed in AH fish relative to controls (Fig. 8), as suggested by the relative phosphorylation of Akt's downstream protein synthesis effectors, i.e. the significant reduction of 4E-BP1 phosphorylation and the increase in eIF-2α phosphorylation. The inverse phosphorylation of 4E-BP1 and eIF-2α would initiate the deactivation of energetically costly survival and proliferation pathways such as glycogen, lipid and protein synthesis under acute stress conditions (Callaghan et al., 2016). DC fish exhibited elevated levels of liver Akt phosphorylation relative to controls, but 4E-BP1 and eIF-2α phosphorylation did not differ, potentially indicating initial activation of survival pathways. eIF-2α could not be detected in the heart of any fish, and other markers of cardiac protein synthesis were unaffected by the treatments (Fig. 8C).

DISCUSSION

Many fish are naturally exposed to chronic cycles in environmental O_2 availability, but we know little about the mechanisms used to survive such conditions (Borowiec et al., 2015; Dan et al., 2014; Remen et al., 2012; Yang et al., 2013). Here, we show that a putatively hypoxia-sensitive species responds differently to diel cycling than to acute hypoxia. Trout progressively adopted a more hypoxia-tolerant phenotype as cycling progressed, likely increasing O_2 uptake and transport through increases in hematocrit and MCHC, reducing hypoxic $\dot{M}_{O_2}$, re-activating anabolic metabolism, and massively upregulating chaperone protein levels. Plasma cortisol in DC fish was also lower than in AH fish, suggesting that physiological stress also diminished as animals acclimated to DCH. Metabolic phenotype switching was also exhibited by rainbow trout exposed to diel thermal cycling (Callaghan et al., 2016), suggesting the response is not unique to a particular stressor and may be a general approach for maintaining homeostasis during cyclic and/or chronic energetic challenges. These findings have important implications for understanding how aquatic animals will cope with increases in environmental variability associated with the progression of climate change (Henson et al., 2017).

DCH tempers the stress response and promotes HSP70

Fish in the DC group presumably responded to their first hypoxia exposure in a manner similar to that of animals in the AH group, with a pronounced increase in plasma cortisol (Figs 2–4) aimed at mobilizing energy stores to fuel an escape response (Das et al., 2018). Under chronic stress, however, heightened activity and sustained catabolism can rapidly deplete energy stores and endanger survival, or at minimum impair growth and reproduction (Mommsen et al., 1999). After the fifth hypoxia exposure, cortisol was lower in DC than in AH fish and was similar to that of normoxic animals, suggesting a reduction in stress. Cortisol levels can normalize with continued stress (Vijayan and Leatherland, 1990), but this interpretation is supported by

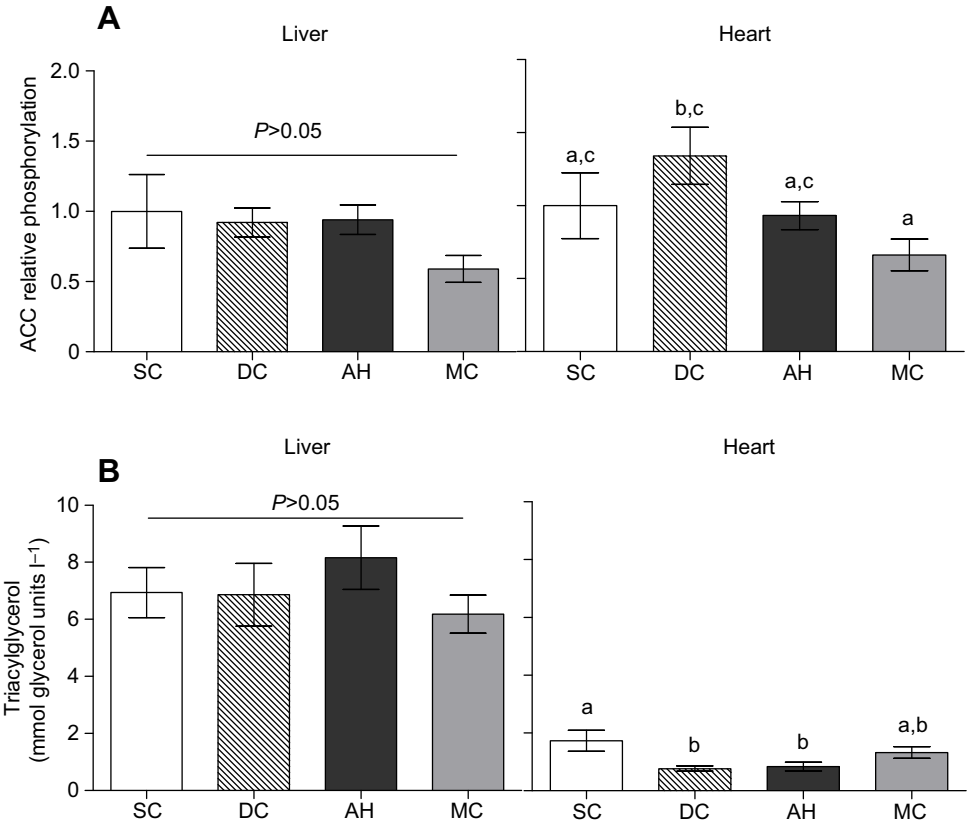

Fig. 7. Markers of lipid metabolism, acetyl-CoA carboxylase (ACC) and triacylglycerol in rainbow trout (*O. mykiss*) exposed to normoxic (SC, *n*=6), diel cycling hypoxia (DC, *n*=7), acute hypoxia (AH, *n*=7) or mean oxygen tension (MC, *n*=6) treatments. The control of liver and heart lipid metabolism was assessed by the relative phosphorylation of ACC (A) normalized by the SC group. Lipid utilization and storage was assessed through measurements of tissue triacylglycerol (B). Significant differences (P<0.05, one-way ANOVA and Tukey *post hoc* tests) between treatment groups are indicated by dissimilar letters.

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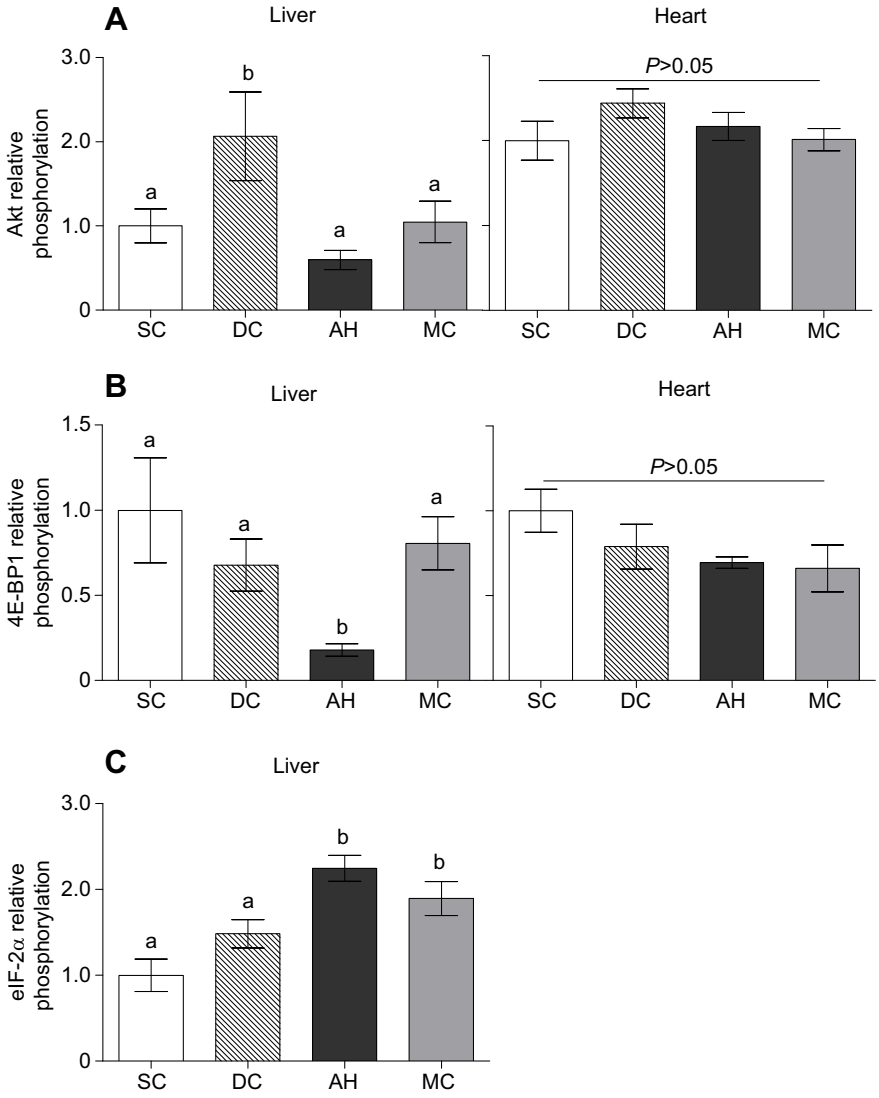

Fig. 8. Markers of protein metabolism, protein kinase B (Akt), Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and Eukaryotic translation initiation factor 2A (eIF2A) in rainbow trout (*O. mykiss*) exposed to normoxic (SC, *n*=6), diel cycling hypoxia (DC, *n*=7), acute hypoxia (AH, *n*=7) or mean oxygen tension (MC, *n*=6) treatments. The control of cellular protein metabolism was assessed by the relative phosphorylation of Akt (A), 4E-BP1 (B) and eIF-2α (C) in both the liver and heart normalized by the SC group. Note that eIF-2α could not be detected in heart tissue. Significant differences (P<0.05, one-way ANOVA and Tukey *post hoc* tests) between treatment groups are indicated by dissimilar letters.

attendant declines in plasma lactate, liver protein poly-ubiquitylation and evidence of metabolic remodeling (see below).

Heat shock proteins are vital to maintain protein functionality under stress and cellular dysfunction (Basu et al., 2002), as the accumulation of misfolded proteins is energetically expensive and cytotoxic. The relative concentration of liver HSP70 was 14- to 2000-fold higher in DC fish than in others (Fig. 2D), with smaller increases observed in the heart. The absolute concentration of HSP70 was not quantified, so comparisons with other species are restricted to the relative magnitude of the heat shock response and do not account for differences in basal HSP70 expression. Acute hypoxia increases hepatic HSP70 levels in hypoxia-sensitive Atlantic cod (Methling et al., 2010), but not in the more tolerant Nile tilapia (*Oreochromis niloticus*) (Delaney and Klesius, 2004), and only relatively minor changes are noted in anoxia-tolerant carp (*Cyprinus carpio*) under chronic hypoxia (Poon et al., 2007). To our knowledge, this is the first examination of chaperone levels in fish exposed to DCH and the magnitude of the liver heat shock response implies that it plays a vital role in the response to this stressor. In an analogous study on thermal treatments, liver HSP70 levels were lower in Atlantic salmon exposed to diel thermal cycles than to an acute thermal stress (Tunnah et al., 2016b). Species- and stress-specific differences make it difficult to infer whether the observed increase in HSP70 leads to improvements in hypoxia tolerance or whether it reflects a response to accumulating damage in DC fish. Reoxygenation following severe hypoxia causes oxidative stress in liver (Welker et al., 2012); the upregulation of HSP70 levels in DC fish may occur in response to the accumulation of oxidized proteins and any resulting cytotoxicity or expression may be upregulated to prevent such damage. Chronic studies incorporating longer DCH treatments and additional biochemical endpoints are required to clarify this ambiguity and assign causation.

DCH improves whole-animal oxygen regulation during hypoxia

The majority of truly hypoxia-tolerant species exploit some form of metabolic depression to conserve ATP stores (Boutilier, 2001). Although the reduction in $\dot{M}_{O_2}$ exhibited by DC fish during successive hypoxic exposures (Fig. 3A) may not qualify as metabolic depression, it does suggest the fish are adopting a more tolerant phenotype. Animals exposed to stable, mean P_{O_2} conditions (12.4 kPa) showed some initial signs of a reduction in $\dot{M}_{O_2}$, but it never reached significance and did not persist for the duration of the exposure. It should also be noted that the fish were only treated for 5 days and were not fed during this time, so the observed phenotypes may not represent that of fish exposed to chronic DC

in the wild. The mean P_{O_2} exposure was not sufficiently stressful to induce changes in cortisol or HSP70 levels, implying that fluctuations in P_{O_2} triggered the phenotype change in DC fish, not the average decrease in P_{O_2}. However, this mean P_{O_2} exposure was limited because the fish within this treatment group were never exposed to sub-critical hypoxia (4.15 kPa). This would have allowed for the assessment of whether mean P_{O_2} exposure could promote protective metabolic regulation when fish encountered sub-critical hypoxia like the DC-treated fish. Therefore, if future studies include a mean P_{O_2} treatment, they should also include a treatment group in which fish would be exposed to mean P_{O_2} for the majority of the treatment regimen and then exposed to an acute hypoxia stress.

The progressive decrease in hypoxic $\dot{M}_{O_2}$ exhibited by DC fish was also associated with a marked reduction in P_{crit} and EPHOC, further demonstrating increased hypoxia tolerance. The reduction in P_{crit} indicates that DC fish became better oxy-regulators, a response exhibited by hypoxia-tolerant fish under both chronic (Richards, 2009) and cycling hypoxia (Dan et al., 2014; Yang et al., 2013). This was in part likely facilitated by increases in hematocrit, hemoglobin (Hb) and MCHC (Fig. 5) to improve oxygen uptake and delivery, avoid hypoxemia, and minimize the need for anaerobic metabolism and the concomitant EPHOC. It is likely that Hb–O_2 affinity also increased, but this was not measured in the current study. Allosteric regulation of Hb through changes in red blood cell organic phosphate levels can markedly alter Hb–O_2 affinity over time scales relevant to this experiment (Val, 2000). Several studies have illustrated that P_{crit} correlates with the P_{O_2} at which blood is 50% saturated with O_2 (i.e. P_{50}; e.g. Mandic et al., 2009; Speers-Roesch et al., 2012), so an increase in Hb–O_2 affinity may underlie the observed reductions in P_{crit}. Similar hematological responses have been observed in the hypoxia-tolerant killifish (*Fundulus heteroclitus*) following long-term (28 days), but not short-term (7 days), intermittent hypoxia (Borowiec et al., 2015). Atlantic cod (*Gadus morhua*), a relatively hypoxia-sensitive species, exhibited a variety of hematological and physiological responses to chronic hypoxia acclimation (>6 weeks at 8–9 kPa), but acute hypoxia tolerance was not improved relative to normoxic control animals (Petersen and Gamperl, 2011). Routine $\dot{M}_{O_2}$ was also significantly elevated and maximum $\dot{M}_{O_2}$ decreased in cod exposed to these conditions (Petersen and Gamperl, 2010). Without a DC treatment of comparable duration in rainbow trout, it is impossible to determine whether the response differences noted above are species specific or whether they are associated with treatment duration. Fish in the MC treatment were not exposed to acute hypoxia at the end of the experiment, so we are unable to determine whether the mean decrease in P_{O_2} experienced by the DC-treated fish was sufficient to trigger the observed reduction in P_{crit} after 5 days. The data set is similarly unable to provide information on the potential effects of fasting on P_{crit} and EPHOC, as animals were not fed during the protocol. Regardless, our data strongly suggest that hypoxia-sensitive species such as rainbow trout have considerable capacity for plasticity in their sensitivity to environmental stress, at least over the short term, a trait that may facilitate their persistence in habitats progressively impacted by climate change.

DCH promotes metabolic phenotype switching in the liver

The phosphorylation and activation of the major regulatory AMPK subunit AMPKβ was similar across all treatments, indicating a baseline level of activation in all fish (Fig. 6A) (Carling, 2004). As a result, liver fatty acid biosynthesis was uniformly inhibited across the treatments, as AMPK phosphorylates and inhibits ACC (Carling, 2004). Liver TAG concentrations were similar between treatment groups. As expected, AH fish increased their reliance on carbohydrate catabolism, largely exhausting their glycogen reserves and accumulating plasma lactate. This was likely initiated by the 2-fold increase in AMPKα activation and reduction in inhibitory Akt phosphorylation relative to DC and MC fish, responses that should promote catabolic processes such as glycogenolysis and glycolysis (Carling, 2004). The response to DCH was quite different: DC fish reduced catabolic AMPK activation, partially recovered their glycogen stores, increased plasma glucose and decreased lactate concentrations relative to AH fish. A remarkably similar response was observed in rainbow trout exposed to diel thermal cycling, where AMPK was deactivated and fuel stores recovered following multiple thermal cycles (Callaghan et al., 2016). In DC fish, the reduction in hypoxic $\dot{M}_{O_2}$, along with the demonstrated and presumed improvements in the O_2 transport cascade, decreased reliance on anaerobic metabolism and glycogen stores during hypoxia, limited EPHOC, and thus facilitated recovery during normoxia. Given the severity of the DC exposure, it is unlikely they could recover their normal anabolic state, but these responses clearly shift the balance from catabolism to anabolism.

Interestingly, Chen et al. (2015) and Xia et al. (2017) found in mice and cancer cells that AMPK activation through the AMPK–HDAC5 pathway is critical for HIF-1α activation and accumulation of HSP70. Inhibiting the AMPK–HDAC5 pathway led to reduced HIF-1α/HSP70 expression/activation and survivorship, whereas prior exposure to hypoxia induced increased AMPK/HIF-1α/HSP70 with reduced ischemia–reperfusion injury. Analogous data are also available in fish; in the epaulette shark (*Hemiscyllium ocellatum*), repeated hypoxia exposure, but not a single acute exposure, activates transcription of HIF-1α pathway components and downstream protective pathways in a tissue-specific manner (Rytkönen et al., 2012). Therefore, the observed activation of AMPK in AH-treated fish may be required to initiate the protective upregulation of HSP70 and the suite of physiological responses that contributed to the apparent increase in hypoxia tolerance in the DC fish.

Previous work on diel cycling hypoxia in Atlantic salmon suggests that the responses observed here on isolated animals in the laboratory may translate to the population level. Remen et al. (2012) found that salmon exposed to DCH for extended periods exhibited similar patterns of change in plasma cortisol, lactate, hematocrit and MCHC, suggesting an initial stress followed by recovery. They also showed that feed intake, which was initially reduced in proportion to the severity of exposure, largely recovered after 2–3 weeks and that animals continued to grow under diel cycling conditions (Remen et al., 2012). Together, these findings reinforce the contention that hypoxia-sensitive fish such as salmonids possess considerable metabolic plasticity, which may allow them to thrive (or at least survive) in increasingly inhospitable habitats.

Protein synthesis can account for 23–42% of a fish's metabolic rate (Cassidy et al., 2018), and it is frequently downregulated under hypoxia to maintain energy homeostasis (Richards, 2009). The decrease in phosphorylation of 4E-BP1 and increase in eIF-2α phosphorylation suggests that protein synthesis is shut down in the liver of AH fish (Fig. 8B,C). This was expected, as acute hypoxia exposure inhibits protein synthesis in trout hepatocytes *in vitro* (Krumschnabel et al., 2000). Poly-ubiquitylation also increased in AH fish, suggesting that either ubiquitin ligases are more active or that the proteasome is less active. Protein degradation via the proteasome is energetically expensive and downregulating it may

limit ATP demand under hypoxia (Lamarre et al., 2012). This was not the case in DC animals, where the relative phosphorylation of protein synthesis effectors and poly-ubiquitylation recovered to levels similar to those of SC fish. In the short term and under stressful conditions, situation-specific reactivation of protein synthesis would allow for the production of key proteins such as HSP70 and hemoglobin to promote survival while the overall rate of protein synthesis is suppressed through Akt-mTORC$_1$ pathway (Callaghan et al., 2016).

DCH does not alter the metabolic phenotype of the heart

In the heart, metabolic signaling markers were largely unchanged across the treatment groups, implying that metabolic organization remains relatively constant (Figs 5, 6 and 7). Rainbow trout have a well-developed coronary circulation to supply oxygenated blood to the compact myocardium and coronary flow increases with hypoxia (Cox et al., 2016). It is probable that coronary O_2 delivery was sufficient to prevent the compact myocardium from becoming severely hypoxic under these conditions, negating any requirement for major metabolic remodeling. Regardless, cardiac glycogen and TAG stores were reduced in AH fish and there was no evidence of recovery in DC fish. The importance of exogenous glucose and lactate as metabolic fuels for the heart is enhanced under such conditions (Driedzic and Gesser, 1994), with liver glycogen acting as a substrate.

Conclusions

Our results clearly demonstrate that an ostensibly hypoxia-sensitive species maintains the capacity for considerable physiological and metabolic plasticity, which can facilitate the transition to a far more hypoxia-tolerant phenotype. Our previous work has illustrated analogous plasticity in Salmonidae challenged with diel thermal cycling (Callaghan et al., 2016; Tunnah et al., 2016b), including a similar transition from an initial catabolic phenotype followed by a return to an anabolic phenotype (Callaghan et al., 2016), suggesting there may be some common strategies for coping with repetitive energetic stressors. Although this study focused on relatively short-term responses in fasting fish, the findings have important implications for understanding and predicting how species will cope with increasingly variable and challenging environments. Additional work is necessary to characterize how chronic DCH combined with food availability may impact hypoxia tolerance and metabolic organization. The impacts of DCH on feeding behavior and how specific dynamic action is fueled and prioritized will likely vary between species and will be a challenging question to address. The intensifying eutrophication of freshwater systems, particularly around large urban centers (Smith, 2003), will undoubtedly increase the frequency and severity of DCH (Smith, 2003) and climate change models predict similar effects on temperatures (Caissie et al., 2014). There is also growing recognition that the response of animals to variable conditions cannot be accurately predicted based only on their response to the stable average of those conditions (Morash et al., 2018). Our current findings further underscore the necessity of incorporating environmentally relevant variability into studies examining the potential impacts of climate change on animals.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.J.W., T.M.; Methodology: K.J.W., A.A.C., S.L., T.M.; Software: C.E.V.; Validation: K.J.W.; Formal analysis: K.J.W., C.E.V.; Investigation: K.J.W., A.A.C.; Resources: K.J.W., A.A.C., S.L., T.M.; Data curation: K.J.W.; Writing - original draft: K.J.W.; Writing - review & editing: K.J.W., A.A.C., C.E.V., S.L., T.M.; Visualization: K.J.W., T.M.; Supervision: S.L., T.M.; Project administration: S.L., T.M.; Funding acquisition: S.L., T.M.

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