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RESEARCH ARTICLE

Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp

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SUMMARY

Appetite suppression is a common response to hypoxia in fish that confers significant energy savings. Yet little is known about the endocrine signals involved in the regulation of food intake during chronic hypoxia. Thus, we assessed the impact of chronic hypoxia on food intake, the expression of the potent anorexigenic signal leptin and its receptor (*lepr*), the mRNA levels of key hypothalamic appetite-regulating genes, and the activity of the hypothalamic–pituitary–interrenal (HPI) axis in common carp, *Cyprinus carpio*. Fish exposed to 10% O₂ saturation for 8 days were chronically anorexic and consumed on average 79% less food than normoxic controls. Hypoxia also elicited gradual and parallel increases in the expression of liver *leptin-a-I*, *leptin-a-II*, *lepr* and *erythropoietin*, a known hypoxia-responsive gene. In contrast, the liver mRNA levels of all four genes remained unchanged in normoxic fish pair-fed to the hypoxia treatment. In the hypothalamus, expression of the appetite-regulating genes were consistent with an inhibition and stimulation of hunger in the hypoxic and pair-fed fish, respectively, and reduced feed intake led to a decrease in *lepr*. Although both treatments elicited similar delayed increases in plasma cortisol, they were characterized by distinct HPI axis effector transcript levels and a marked differential increase in pituitary *lepr* expression. Together, these results show that a reduction in O₂ availability, and not feed intake, stimulates liver *leptin-a* expression in common carp and suggest that this pleiotropic cytokine is involved in the regulation of appetite and the endocrine stress response during chronic hypoxia.

Key words: leptin, hypoxia, appetite, stress, metabolism.

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INTRODUCTION

Large fluctuations in oxygen availability are a common feature of diverse aquatic habitats (Diaz and Brietburg, 2009). As a result, many fish species routinely encounter environmental hypoxia and have evolved a variety of coping strategies (Richards, 2009). One behavioral response to hypoxia observed in both hypoxia-tolerant and -sensitive species is a reduction in food intake (Pedersen, 1987; Chabot and Dutil, 1999; Buentello et al., 2000; Pichavant et al., 2001; Zhou et al., 2001; Bernier and Craig, 2005; Ripley and Foran, 2007). A similar response to hypoxic conditions has also been observed in fruit flies (Harrison and Haddad, 2010), rodents and humans (Quintero et al., 2010). In general, among the hierarchy of responses associated with hypoxia in fish, appetite suppression is a strategy that is recruited relatively early in the overall response to decreasing oxygen levels (Boutilier et al., 1988; Pichavant et al., 2001; Bernier and Craig, 2005). Food intake is typically independent of oxygen availability above a species-specific threshold and directly related to dissolved oxygen concentration below this value (Bernier, 2010). Overall, despite the importance of appetite suppression for the prioritization of oxygen use during a hypoxic challenge and the evolutionary conserved nature of this response, the mechanisms responsible for this strategy are poorly understood.

The regulation of food intake in fish, as in other vertebrates, involves a complex hypothalamic neuronal circuitry that integrates

and processes short- and long-term peripheral signals related to the energetic status of the animal with cognitive information and sensory cues (Volkoff et al., 2009; Bernier, 2010). In return, these hypothalamic neurons produce multiple appetite-stimulating (orexigenic) and -inhibiting (anorexigenic) neuropeptides that participate in the regulation of energy balance and food-seeking behavior (Gorissen et al., 2006; Matsuda, 2009). Among the central factors that promote a decrease in food intake are two related peptides that also play a key role in the regulation of the hypothalamic–pituitary–interrenal (HPI) axis and the coordination of the stress response in fish, namely, corticotropin-releasing factor (CRF) and urotensin I (UI) (Bernier, 2006; Bernier et al., 2009). For example, endogenous CRF-related peptides mediate at least a portion of the reduction in food intake and stimulation of the HPI axis that characterize the short-term response to hypoxia in rainbow trout (Bernier and Craig, 2005). Chronically, however, although the appetite-suppressing effects of hypoxia are sustained (Pichavant et al., 2001; Zhou et al., 2001), CRF-related peptides do not appear to play a role in mediating the anorexia and the mechanisms responsible have yet to be determined (Bernier and Craig, 2005).

A potent anorexigenic signal that may be involved in the regulation of food intake during hypoxic conditions is leptin. Primarily produced by white adipose tissue in mammals, leptin

inhibits food intake by inhibiting the hypothalamic expression of the orexigenic signals neuropeptide Y (NPY) and agouti-related peptide (AgRP), and by stimulating the expression of the anorexigenic signals α -melanocyte-stimulating hormone [a product of the prohormone pro-opiomelanocortin (POMC)] and cocaine- and amphetamine-regulated transcript (CART) (Ahima and Flier, 2000; Myers et al., 2008). Similarly, although fish and mammalian leptins share low sequence identity and the main site of leptin expression in fish is the liver (Kurokawa et al., 2005; Huising et al., 2006), the general consensus is that leptin does inhibit food intake in fish and affects the expression of hypothalamic appetite-regulating genes (Murashita et al., 2008; Li et al., 2010; Copeland et al., 2011; Denver et al., 2011; Murashita et al., 2011). In cyprinids, intracerebroventricular (i.c.v.) injections of murine (Volkoff et al., 2003) or human leptin (De Pedro et al., 2006) in goldfish (*Carassius auratus*) and intraperitoneal (i.p.) injection of recombinant native leptin in grass carp (*Ctenopharyngodon idellus*) caused significant decreases in food intake (Li et al., 2010). Similarly, both i.p. injection of recombinant native leptin (Murashita et al., 2008) and i.c.v. injection of human leptin (Aguilar et al., 2010) reduced food intake in rainbow trout.

In mammals, leptin expression is regulated by adiposity, several hormones and hypoxia (Ahima and Flier, 2000; Ambrosini et al., 2002). For example, fasting leads to a decrease in adipose tissue leptin production (Trayhurn et al., 1995) and chronic hypoxia is associated with an increase in *leptin* expression (Grosfeld et al., 2002). Although there is evidence that *leptin* and leptin receptor (*lepr*) isoforms are also hypoxia-sensitive in fish (Wong et al., 2007; Chu et al., 2010; Cao et al., 2011), in general the effects of fasting on hepatic *leptin* expression are equivocal (Huising et al., 2006; Gorissen et al., 2009; Kling et al., 2009; Rønnestad et al., 2010), and the relationships between food intake, *leptin* expression and the hypothalamic regulation of appetite during hypoxia have yet to be determined.

Thus, in this study, to further our understanding of the mechanisms involved in mediating the appetite-suppressing effects of hypoxia in fish, we assessed the short- (1 day) and longer-term (4 and 8 days) impact of chronic hypoxic conditions on feeding, the expression of hepatic *leptin* and *lepr*, the mRNA levels of key hypothalamic appetite-regulating genes, and the activity of the HPI axis in common carp, *Cyprinus carpio*, a species well known for its hypoxia tolerance (Zhou et al., 2001). Moreover, to differentiate between the proximal effects of a reduction in energy intake from those of hypoxia, we compared the effects of chronic hypoxia with those of restricted feed intake on the above parameters.

MATERIALS AND METHODS

Animals

Common carp (*Cyprinus carpio* Linnaeus 1758) were obtained from Viskweekcentrum Valkenswaard (Valkenswaard, The Netherlands) and kept in 1501 tanks with circulating filtered Nijmegen city tap water at $23.2 \pm 0.4^\circ\text{C}$ (mean \pm s.d.) and a photoperiod of 16h:8h light:dark with the light phase starting at 06:00 h daily. Carp were fed Trouvit sinking dry food pellets (Trouw Nutrition International, Putten, The Netherlands) once daily at a ration of 1.5% of the estimated body mass. Animals were weighed weekly and the amount of food was adjusted accordingly. Before collection of tissue and blood samples, fish were irreversibly anesthetized with 0.1% (v/v) 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA). Animal experiments were performed in accordance with national legislation and approved by the ethical committee of the Radboud University Nijmegen.

Experimental design

Experiment 1: effects of chronic hypoxia on food intake

To assess the effects of chronic hypoxia on food intake, a total of 27 fish (72.4 ± 2.5 g) were used in this experiment. All fish were weighed, randomly assigned to one of three 651 tanks ($N=9$ per tank) and acclimated to these conditions for 2 weeks. During this time and the hypoxic exposure, all fish received a daily excess of pre-weighted feed at 10:00 h. Uneaten food was collected 2 h later. The mass of the uneaten dry food was calculated as the product of the number of uneaten food pellets and the average dry pellet mass. Food intake was determined as the difference between the initial and the calculated uneaten dry food masses. The water was 75% O_2 saturated (6.7 mg l^{-1}) during the acclimation period. Once food intake was assessed on the last day of the acclimation period, the normoxic water source for the tanks was replaced with a hypoxic source that was 10% O_2 saturated (0.9 mg l^{-1}). To achieve the desired hypoxic conditions, N_2 gas was bubbled into a header tank that supplied the holding tanks. Levels of dissolved O_2 in the holding tanks were monitored periodically using a portable OxyGuard dissolved O_2 meter (Point-Four Systems, Port Moody, BC, Canada). The desired hypoxic levels were gradually achieved within 2 h and chronically maintained at $\pm 0.5\%$ of the target level for 8 days by regulating the flow of water and N_2 gas through the header tank.

Experiment 2: effects of chronic hypoxia on the expression of appetite- and stress-responsive genes

To assess potential changes in gene expression of appetite- and stress-responsive genes associated with chronic hypoxia, a total of 63 fish (65.7 ± 1.8 g) were used in this experiment. All fish were weighed, randomly assigned to one of seven 651 tanks ($N=9$ per tank) and acclimated for at least 2 weeks. During this time and over the course of the experiment all fish were fed as in Experiment 1. One group of fish was maintained in 75% O_2 saturated water and served as a normoxic day 0 control. Three groups were exposed to 10% O_2 saturated water as above for 1, 4 or 8 days. The last three groups were maintained in 75% O_2 saturated water and pair-fed for 1, 4 or 8 days to the food intake consumed by the time-matched hypoxic fish the day prior. On the day of sampling, all fish in a given tank were terminally anesthetized at once and a blood sample was collected by caudal puncture using an Na_2EDTA -treated syringe. Aliquots of blood were used immediately to determine haematocrit and haemoglobin concentration. The remaining blood was centrifuged at $14,000 \text{ g}$ for 3 min and the separated plasma was aliquoted and flash frozen in liquid nitrogen prior to storage at -80°C for later analysis of plasma cortisol, lactate, glucose and non-esterified fatty acids (NEFAs). The plasma aliquot for determination of lactate was deproteinized with ice-cold perchloric acid (0.6 mol l^{-1}), spun down and the supernatant was stored as above. The brain, whole pituitary and liver were also collected to quantify the mRNA levels of several genes. The brain was regionally dissected to isolate the hypothalamus and the pre-optic area (POA) as per Bernier et al. (Bernier et al., 2008). All tissues were immediately frozen in liquid nitrogen and stored at -80°C for future analysis.

Experiment 3: effects of graded hypoxia on liver gene expression

To assess the effects of graded hypoxia on the expression of liver appetite- and hypoxia-responsive genes, a total of 32 fish (25.2 ± 0.9 g) were used in this experiment. All fish were weighed, randomly assigned to one of four 651 tanks ($N=8$ per tank) and acclimated for at least 2 weeks. During this time and over the course of the experiment all fish were fed as in Experiment 1. One group

of fish was maintained in 75% O₂ saturated water and served as a normoxic control and one group each was exposed to 20, 10 and 5% O₂ saturated water for 2 days. The desired hypoxic levels were achieved as in Experiment 1. On the day of sampling, all fish in a given tank were terminally anesthetized at once and the collected liver was immediately frozen in liquid nitrogen and stored at -80°C for future analysis.

RNA isolation and cDNA synthesis

Total RNA from all sampled tissues was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the concentrations were quantified using ultraviolet spectrophotometry at 260 nm (Nanodrop, Wilmington, DE, USA). Integrity of the isolated RNA was ensured prior to cDNA synthesis by analyzing a random subset of samples on a 1.5% agarose gel. One microgram of total RNA was treated with DNase I according to the manufacturer's protocol (DNase I amplification grade, Invitrogen) and reverse transcribed to cDNA in a 20 µl reaction mixture containing 300 ng random primers, 0.5 mmol l⁻¹ dNTPs, 10 mmol l⁻¹ dithiothreitol, 10 U RNase inhibitor and 200 U SuperScript II RNase H⁻ reverse transcriptase (Invitrogen).

Real-time quantitative PCR

The cDNA products were amplified using a GeneAmp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each 25 µl reaction mixture contained 12.5 µl Sybr Green Master Mix (Applied Biosystems), 5 µl cDNA template diluted 1:5, 3 µl of both the forward and reverse primers (600 nmol l⁻¹ final concentration) and 1.5 µl DNase-free water. Default cycling conditions were used: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. To account for differences in amplification efficiency between the different cDNAs, standard curves were constructed for each target using serial dilutions of cDNA samples. Using the threshold cycle of each unknown, the relative dilution of a given sample was extrapolated using the linear regression of the target-specific standard curve. To correct for differences in template input and reverse transcriptase efficiencies, each sample was normalized to the expression level of the housekeeping genes *40S ribosomal protein S11* (*40S*) and *β-actin*. Results were confirmed to be very similar following standardization to either housekeeping gene. Therefore, only results standardized to *40S* are shown. All samples were assayed in triplicate, and only

one target was assayed per well. Finally, non-reversed transcribed RNA and water controls were run to ensure that no genomic DNA was being amplified and the reagents were not contaminated. The primer sets used for real-time quantitative PCR were designed using Primer Express software (Applied Biosystems) and are shown in Table 1. Note that the names used to describe carp leptins in this study, leptin-a-I and leptin-a-II, are based on our previous phylogenetic analysis of vertebrate leptins (Gorissen et al., 2009). Also, although leptin receptor isoforms have yet to be identified in common carp, because the primers used to quantify *lepr* amplify a region of *lepr* exon 1 that is conserved among known fish leptin receptor isoforms (Rønnestad et al., 2010; Cao et al., 2011), the *lepr* expression data reported in this paper likely reflect the cumulative expression of different *lepr* isoforms.

Plasma parameters

Plasma cortisol concentrations were measured by radioimmunoassay as described by Metz et al. (Metz et al., 2005) using a commercially available antiserum (Campro Scientific, Veenendaal, The Netherlands) and ¹²⁵I-cortisol (Amersham, Buckinghamshire, UK). Setting the reactivity to cortisol at 100%, the cross-reactivity of the cortisol antibody was 5.9% with 11-deoxycortisol, 2.6% with cortisone, 1.7% with corticosterone, 0.16% with cortisone acetate, 0.4% with 17α-OH-progesterone and 0.02% with progesterone. The detection limit of the assay was 4 pg per tube, and the intra- and inter-assay coefficients of variation were 3 and 5%, respectively. Heparinized capillary tubes containing whole blood were sealed and centrifuged at 12,000 g for 3 min and used to determine haematocrit (Hct). Whole blood samples were also assayed immediately for haemoglobin (Hb) concentration. Briefly, a Hb standard (15.0 g dl⁻¹; Pointe Scientific, Detroit, MI, USA) was sequentially diluted using Drabkin's solution (Sigma-Aldrich) to construct a standard curve. Blood samples were diluted 1:250 using Drabkin's solution and allowed to incubate for 20 min at room temperature; the optical density of samples and standards were then read at 540 nm on a microplate spectrophotometer. Plasma lactate concentrations were determined enzymatically as outlined by Bergmeyer (Bergmeyer, 1985). Plasma glucose was determined using an enzymatic colorimetric test (glucose liquicolor; Human, Wiesbaden, Germany) as per the manufacturer's instructions. Plasma NEFA levels were measured using the NEFA-C kit (Wako Chemicals, Neuss, Germany) according to the manufacturer's instructions.

Table 1. Nucleotide sequences of common carp primers used for quantitative real-time RT-PCR

Gene	Accession no.	5'-3' forward primer	5'-3' reverse primer	Efficiency (%)	R ²
<i>agr</i>	AM498378	GGTCATCACCTGCCCTGTTG	GAAGGCCTTAAAGAAACGACAGTAA	86.8	0.99
<i>β-actin</i>	M24113	CAACAGGGAAAAGATGACACAGATC	GGGACAGCACAGCCTGGAT	102.3	0.99
<i>cart</i>	AM498379	CGCATCTCACTTTGGGAAAAG	CTCCCCAACGTCGCATGTA	94.3	0.99
<i>crf</i>	AJ317955	CATCCGGCTCGGTAACAGAA	CCAACAGACGCTGCGTTAACT	92.3	0.99
<i>crf-bp</i>	AJ490881	ACAATGATCTCAAGCGGTCCAT	CCACCCAGAAGCTCGACAAA	101.7	0.99
<i>epo</i>	AJ831394	CCCATTACGCCCATCTG	TCCCATGCCTCCTTAATGAAA	92.2	0.99
<i>leptin a-i</i>	AJ868357	CATATTGATTGTCCACCCTTCTG	CCATTAGCTGGCTCCTTGGAT	84.4	0.99
<i>leptin a-ii</i>	AJ868356	AGATACGCAACGATTTGTTTACA	GCGTTGTTCTCCAAGAAAGCA	98.0	0.99
<i>npy</i>	AF287347	CTCTAACGGAAGGGTATCCA	GCCGTGTTATGAGGTTGATG	94.9	0.95
<i>lepr</i>	AM498377	AATGTGACGACTGAAGGTGAAG	CTGAAGCAAGGAATTGGATG	87.3	0.97
<i>pomc-i</i>	Y14618	TTGGCTCTGGCTGTTCTGTGT	TCATCTGTGATCAGACCTGCATA	91.6	0.98
<i>ui</i>	M11671	GCACCTGTGTCCAGCATGAA	GGTGCTCAGCGGGATGTG	101.0	0.99
<i>40s</i>	AB012087	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	99.9	0.99

Primer efficiency and standard curve R² values are reported for each target gene. The efficiency was calculated from the standard curve using the equation $E = (10^{-1/\text{slope}} - 1) \times 100$.

AgRP, agouti-related protein; CART, cocaine- and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; CRF-BP, CRF-binding protein; EPO, erythropoietin; NPY, neuropeptide Y; LEPR, leptin receptor; POMC, pro-opiomelanocortin; UI, urotensin I; 40S, 40S ribosomal protein S11.

Statistical analyses

In Experiment 1, a one-way repeated-measures ANOVA followed by a Holm–Sidak test for multiple comparisons was used to determine differences in food intake between fish kept in normoxic and hypoxic conditions. In Experiment 2, differences between the normoxic and hypoxic treatments or between the normoxic and pair-fed treatments were determined by a one-way ANOVA and a pairwise Tukey's test. Differences between the hypoxic and pair-fed treatments at a given time were evaluated with a Student's *t*-test. Differences between treatments in Experiment 3 were assessed by a one-way ANOVA followed by a Tukey's test for all pair-wise comparisons. Data that did not meet the assumption of normality were log-transformed prior to analysis. All statistical analyses were performed using SigmaStat 3.0 (Systat Software, San Jose, CA, USA). A *P*-value of <0.05 was considered statistically significant for all tests.

RESULTS

Experiment 1

Although the mean food intake of common carp maintained in normoxic water was 15.5 mg g^{-1} body mass (BM), fish chronically exposed to 10% O_2 saturation consumed on average 79% less food, i.e. 3.3 mg g^{-1} BM per day (Fig. 1). Peak anorexia was attained after just 24 h of hypoxia exposure and there was no significant recovery over the 8 day treatment.

Experiment 2

Relative to the normoxic treatment, chronic exposure to 10% O_2 saturation resulted in gradual increases in liver *leptin a-I*, *leptin a-II* and *lepr* expression (Fig. 2A–C). By day 8, the mRNA levels of these transcripts in the hypoxic fish were 2.6-, 3.8- and 3.4-fold higher, respectively, than in the normoxic animals. In contrast, the mRNA levels of all three genes remained unchanged in the normoxic fish pair-fed to the hypoxia treatments. Similarly, although hypoxia exposure elicited a time-dependent 2.6-fold increase in the expression of liver *epo*, no change was observed in the normoxic pair-fed fish (Fig. 2D).

In the hypothalamus, chronic hypoxia had no effect on the mRNA levels of *npv* and *agrp* (Fig. 3A,B). The pair-fed treatment also had no effect on the expression of *npv* but was associated with a gradual

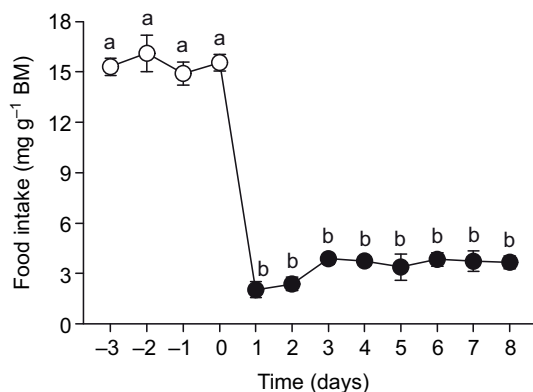


Fig. 1. Food intake in common carp exposed to normoxic (75% O_2 saturation; white circles) or hypoxic (10% O_2 saturation; black circles) water. The normoxic water source was replaced with a hypoxic source immediately after the assessment of food intake on day 0. Values that do not share a common letter are significantly different from each other as determined by repeated-measures one-way ANOVA and a Holm–Sidak multiple comparison test ($P < 0.05$). Values are means \pm s.e.m. ($N = 3$ tanks with nine fish per tank). BM, body mass.

2.1-fold increase in *agrp* mRNA levels by day 8. In contrast, whereas chronic hypoxia elicited a gradual 1.6-fold increase in the expression of *cart* by day 8, the pair-fed treatment did not have a significant effect (Fig. 3C). In general, the expression of hypothalamic *pomc-I* and *lepr* decreased in response to the chronic hypoxia and normoxic pair-fed treatments (Fig. 3D,E). However, the reductions in *pomc-I* and *lepr* mRNA levels were both earlier and more pronounced in the pair-fed treatment than in the chronic hypoxia treatment.

Neither chronic hypoxia nor the normoxic pair-fed treatment had an effect on the expression of pre-optic area *crf* and *ul* relative to the normoxic control fish (Fig. 4A,B). However, *crf* and *ul* mRNA levels were significantly higher in the normoxic pair-fed fish than in the hypoxic fish on days 4 and 8, respectively. Both treatments elicited a transient reduction in the expression of pre-optic area *crf-bp*, but the reduction was more pronounced in the hypoxic fish than in the pair-fed fish (Fig. 4C). The expression of pre-optic area *lepr* was not affected by chronic hypoxia or by pair-feeding (Fig. 4D).

In contrast to the results observed in the hypothalamus, both chronic hypoxia and the pair-fed treatment elicited gradual 3.1-fold increases in pituitary *pomc-I* mRNA levels by day 4 (Fig. 5A). Although pituitary *pomc-I* expression had returned to basal levels by day 8 in both treatments, the hypoxic fish had significantly lower transcript levels than the pair-fed fish. Both treatments also elicited marked increases in the expression of pituitary *lepr*, but in this instance the increase in transcript levels were greater in the hypoxic fish than in the pair-fed fish (Fig. 5B). By day 8, *lepr* mRNA levels in the chronic hypoxia and pair-fed treatments were 74- and 21-fold higher than in the controls, respectively.

The chronic hypoxia and the pair-fed treatments elicited significant increases in plasma cortisol of similar magnitude by day 8 (Fig. 6A). Although Hct values increased significantly in the hypoxic fish, they remained unchanged in the normoxic pair-fed treatment (Fig. 6B). Similarly, the hypoxic treatment was associated with a transient increase in Hb concentration and there was no change in the pair-fed fish (Fig. 6C). Although neither treatment had an effect on plasma lactate levels (Fig. 6D) and chronic hypoxia did not affect the circulating levels of plasma glucose (Fig. 6E) or NEFA concentrations (Fig. 6F), the pair-fed fish were characterized by a sustained decrease in plasma glucose levels and increases in plasma NEFA values. The changes in plasma glucose and NEFA values associated with the pair-fed treatment were most pronounced on day 1 and partially recovered on days 4 and 8.

Experiment 3

To further characterize the relationship between hypoxic conditions and the expression of liver *leptin*, *lepr* and *epo*, we investigated the impact of 48 h exposures to decreasing levels of O_2 saturation. The *leptin-a-I* mRNA levels increased 1.6-fold between the control and the 10% O_2 saturation treatments, but the 5% O_2 saturation treatment did not have a significant effect (Fig. 7A). By contrast, the expression of *leptin-a-II* was proportional to the severity of the hypoxic exposure with an overall 3.0-fold increase in expression between the normoxic and the 5% O_2 saturation treatment (Fig. 7B). The expression of *lepr* followed a pattern similar to that of *leptin-a-I*, with the highest mRNA levels observed in the 10% O_2 saturation treatment and the 5% O_2 treatment having no effect (Fig. 7C). Finally, all three hypoxic treatments elicited similar increases in liver *epo* expression (Fig. 7D).

DISCUSSION

This study provides original evidence that the appetite-suppressing effects of hypoxia in fish are associated with a marked and sustained

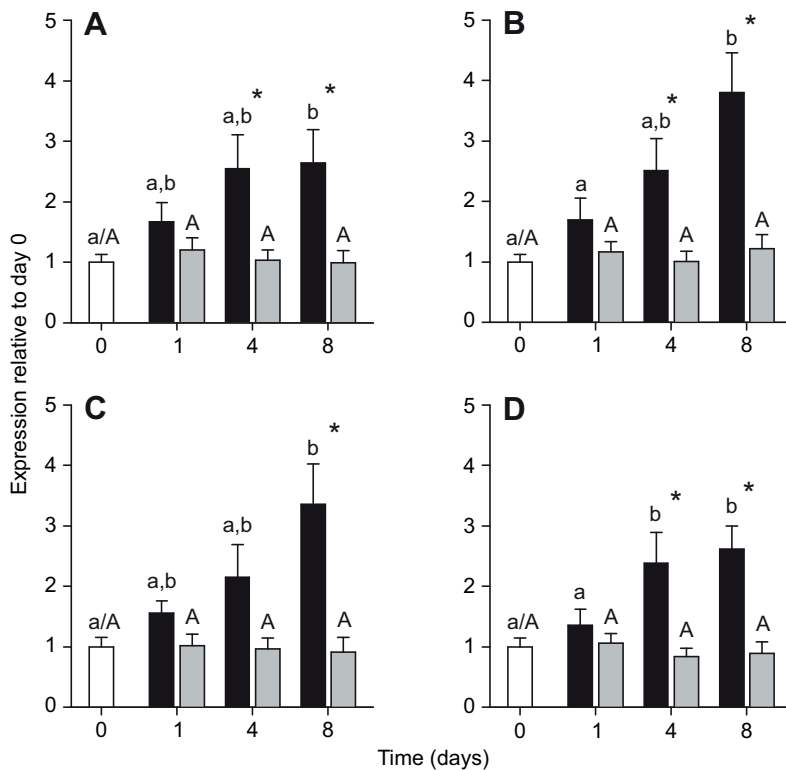


Fig. 2. Effects of normoxic water (75% O₂ saturation; white bars), chronic hypoxia (10% O₂ saturation; black bars) and restricted feeding (pair-fed treatment; gray bars) on the gene expression of liver (A) *leptin-a-I*, (B) *leptin-a-II*, (C) leptin receptor (*lepr*) and (D) erythropoietin (*epo*). Whereas the normoxic and hypoxic treatments were fed to satiation, the pair-fed treatment was fed the food intake consumed by the hypoxic treatment the day prior. Gene expression is normalized to the expression of *40S ribosomal protein S11* and is expressed relative to day 0 values. Normoxic and hypoxic treatments that do not share a common lowercase letter, or normoxic and pair-fed treatments that do not share a common uppercase letter, are significantly different from each other as determined by a one-way ANOVA and a pairwise Tukey's test. An asterisk indicates a significant difference between the hypoxic and pair-fed treatments at a given time as determined by a Student's *t*-test. The significance level for all statistical tests was $P < 0.05$. Values are means + s.e.m. ($N = 9$).

increase in hepatic *leptin-a* expression, a gene that codes for a potent anorexigenic signal. Our results also indicate that the proximal cue for the stimulation of hepatic *leptin-a* and *lepr* expression during hypoxia, like *epo*, is a reduction in O₂ availability, not feed intake. In contrast, comparison of the hypothalamic expression profiles of key appetite-regulating genes in the hypoxic and pair-fed treatments suggest that both feed restriction and leptin influence the regulation of food intake during hypoxia. Beyond its role as an anorexigenic signal, we discuss below how leptin signaling may contribute to the regulation of the HPI axis, O₂ uptake and delivery, and metabolism during chronic hypoxic conditions.

Chronic exposure of common carp to 10% O₂ saturation for 8 days in this study resulted in a marked and sustained reduction in food intake. These results are consistent with previous observations in common carp (Zhou et al., 2001) and in several other fish species (Chabot and Dutil, 1999; Buentello et al., 2000; Pichavant et al., 2001; Bernier and Craig, 2005). In general, the magnitude, persistence and cross-species prevalence of the appetite-suppressing effects of hypoxia in fish highlight the importance of this behavioral strategy for coping with reduced O₂ availability. Appetite suppression confers significant energy savings by reducing the cost of metabolism associated with digestion, i.e. the costs of specific dynamic action (Wang et al., 2009). Combined with a marked reduction in physical activity, the reduction in food intake during hypoxia allows common carp to significantly reduce routine metabolic rate (Zhou et al., 2001).

In addition to appetite suppression, chronic hypoxia in common carp was characterized by increases in hepatic *leptin-a-I* and *leptin-a-II* expression. In general, the relative increases in *leptin* transcript levels were proportional to the duration of the hypoxic exposure and paralleled the changes in *epo* expression, a well-known hypoxia-inducible gene (Stockmann and Fandrey, 2006). These results concur with mammalian studies where hypoxia is known to increase *leptin* expression and circulating leptin levels (Ambrosini et al., 2002;

Quintero et al., 2010), and with recent observations in adult zebrafish where chronic hypoxia increased hepatic *leptin-a* mRNA levels and hypoxia-inducible factor 1 stimulated the transcription of *leptin-a* (Chu et al., 2010). As previously observed in the liver of marine medaka [*Oryzias malastigma* (Wong et al., 2007)] and mice (Baze et al., 2010), hypoxia exposure in common carp also elicited significant increases in hepatic *lepr* expression. Although the physiological significance of this response is not known, an increase in liver leptin receptor abundance could enhance the potential contribution of leptin to the regulation of metabolism during hypoxic conditions. In mammals, however, central leptin signaling plays a dominant role in leptin-mediated regulation of metabolism and the contribution of peripheral leptin signaling appears to be insignificant (Guo et al., 2007).

In the graded hypoxia experiment, the marked stimulation of hepatic *leptin-a-II* expression in the 5% O₂ saturation treatment contrasted sharply with the lack of effect of the same treatment on the expression of *leptin-a-I* and *lepr*. Besides highlighting potential differences in the responsiveness of *leptin-a-I*, *leptin-a-II* and *lepr* to hypoxia, these results also raise the possibility that the widespread repression of total mRNA synthesis seen during severe hypoxic conditions (Johnson et al., 2008) may counter the stimulatory effects of more moderate hypoxic treatments on *leptin* and *lepr* expression. Moreover, because common carp at northern latitudes may be exposed to months of hypoxia under the ice (Ultsch, 1989), it remains to be determined whether the changes observed over a period of 8 days in this study are sustained during long-term hypoxia.

As opposed to the stimulatory effects of hypoxia, restricted feeding in this study had no effect on the expression of liver *leptin-a-I*, *leptin-a-II*, *lepr* or *epo*. Although the effects of restricted feeding on the expression of hepatic *lepr* and *epo* have not been previously documented in fish, our results do concur with those of Huising et al. (Huising et al., 2006), who observed that neither 6 days nor

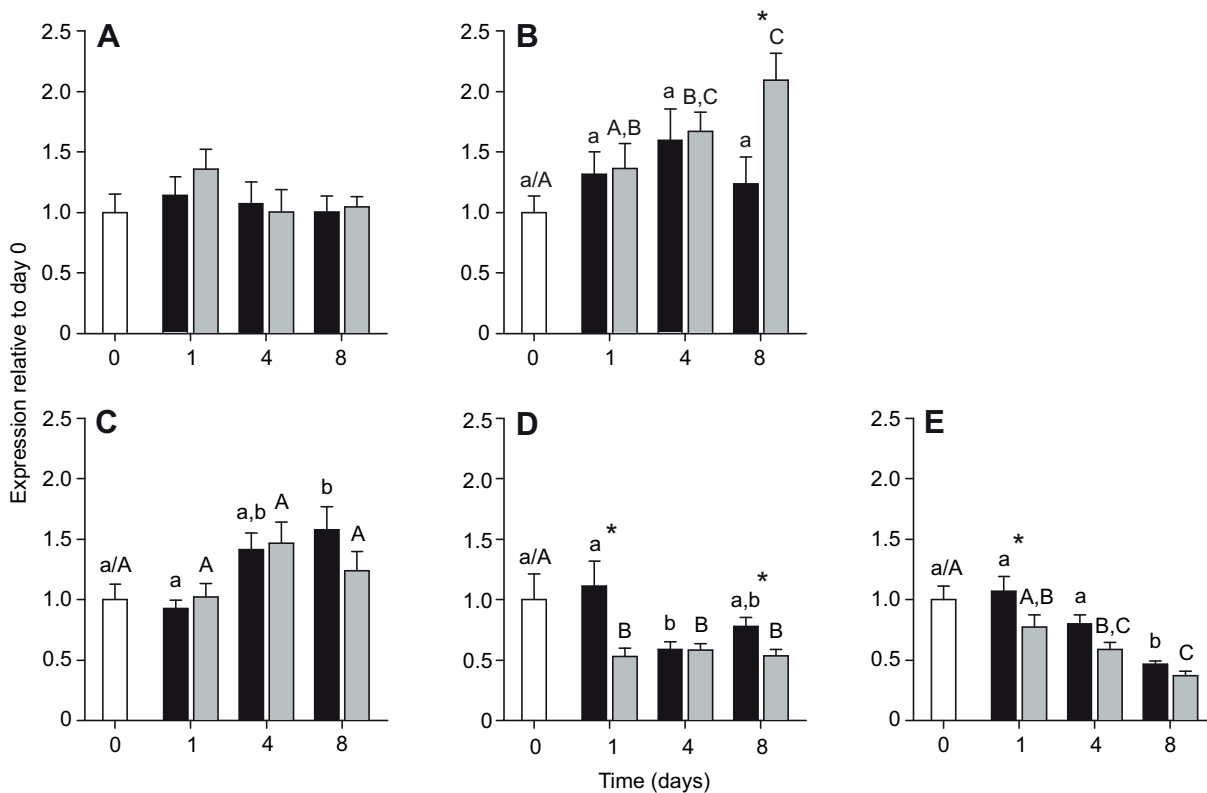


Fig. 3. Effects of normoxic water (75% O₂ saturation; white bars), chronic hypoxia (10% O₂ saturation; black bars) and restricted feeding (pair-fed treatment; gray bars) on the hypothalamic gene expression of (A) neuropeptide Y (*npy*), (B) agouti-related protein (*agrp*), (C) cocaine- and amphetamine-regulated transcript (*cart*), (D) pro-opiomelanocortin-I (*pomc-l*) and (E) leptin receptor (*lepr*). All other details as in Fig. 2.

6 weeks of fasting affected hepatic *leptin-a-I* and *-a-II* expression in common carp. In contrast, fasting consistently reduces the expression and plasma levels of leptin in mammals (Ahima and Flier, 2000) and appears to have varying effects on the expression of *leptin* in other fish species. For example, prolonged rationed feeding of Atlantic salmon (*Salmo salar*) significantly increased the expression of liver *leptin-a-II* and decreased visceral adipose tissue *leptin-a-I*, but had no effect on liver *leptin-a-I*, adipose tissue *leptin-a-II* or circulating plasma leptin levels (Rønnestad et al., 2010). Moreover, although fasting is associated with increased plasma leptin levels in rainbow trout [*Oncorhynchus mykiss* (Kling et al., 2009)] it has no effect on liver *leptin-a* expression in zebrafish (*Danio rerio*) but decreases liver *leptin-b* expression in the same species (Gorissen et al., 2009). In common carp, the expression levels of liver *leptin-b-I* and *-b-II* are too low to measure reliably (Gorissen et al., 2012). Therefore, although our results show that the stimulation of hepatic *leptin-a* genes and *lepr* during chronic hypoxia in common carp is independent of a reduction in nutrient intake, future studies are required to determine whether this observation holds in other fish species.

In general, both the anorexigenic effects attributed to leptin in fish and the changes in the expression pattern of the hypothalamic appetite-regulating genes within the hypoxic treatment support a role for leptin in the regulation of food intake during chronic hypoxia. To date, studies in fish investigating the effects of recombinant native peptides suggest that leptin may suppress food intake by stimulating *pomc* (Murashita et al., 2008; Murashita et al., 2011) and inhibiting *npy* (Murashita et al., 2008; Li et al., 2010). In contrast, the response to prolonged fasting in both mammals and fish is generally

associated with increases in the expression of *npy* and *agrp*, and decreases in *cart* and *pomc* mRNA levels (Ahima and Antwi, 2008; Volkoff et al., 2009). In common carp, however, although prolonged fasting is associated with a reduction in the hypothalamic expression of *pomc*, it has no effect on *npy* mRNA levels (Huising et al., 2006). Therefore, in this study, the lack of change in *npy* expression in the hypoxic and pair-fed treatments is consistent with the known effects of fasting in common carp, but not with those attributed to leptin in fish. In contrast, although the predicted increase in *agrp* expression was observed in the pair-fed treatment, the lack of change in *agrp* transcripts in the hypoxia treatment implies that leptin may have an inhibitory effect on the expression of this gene in common carp. Similarly, the overall changes in *cart* and *pomc-l* mRNA levels in the hypoxia and pair-fed treatments suggest that leptin may stimulate *cart* gene expression during hypoxia and counteract the suppression of *pomc-l* that characterizes feed restriction in this species. Finally, the gradual reduction in hypothalamic *lepr* in the chronic hypoxia and pair-fed treatments suggests that sustained feed restriction, not O₂ availability, may lead to a reduction in leptin signaling within the hypothalamic feeding circuitry. As leptin is unlikely to be the only anorexigenic signal that contributes to the regulation of the hypothalamic feeding circuits during hypoxia, future studies using native leptins are needed to investigate the isolated effects and mechanisms of leptin actions on the appetite-regulating genes of the hypothalamus in common carp.

Although our previous work implicated CRF-related peptides as potential mediators of the acute appetite-suppressing effects of hypoxia in rainbow trout (Bernier and Craig, 2005), the present results provide only limited evidence for such a mechanism in

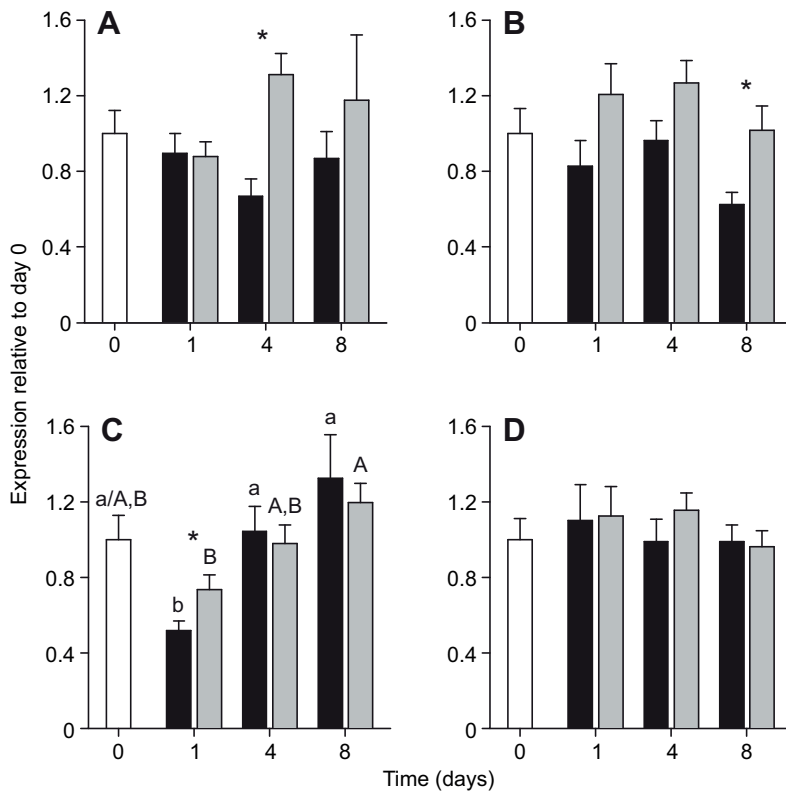


Fig. 4. Effects of normoxic water (75% O₂ saturation; white bars), chronic hypoxia (10% O₂ saturation; black bars) and restricted feeding (pair-fed treatment; gray bars) on the brain pre-optic area gene expression of (A) corticotropin-releasing factor (*crf*), (B) urotensin I (*ul*), (C) CRF-binding protein (*crf-bp*) and (D) leptin receptor (*lepr*). All other details as in Fig. 2.

common carp. In support of a role for CRF-related peptide as appetite regulators in common carp, the first 24h of exposure to 10% O₂ saturation was associated with a ~50% drop in POA *crf-bp* expression, a response that should lead to an increase in the levels of ‘free’ or bioactive CRF-related peptides (Seasholtz et al., 2002). Moreover, both CRF and UI are potent anorexigenic signals in goldfish, a related cyprinid species (Bernier and Peter, 2001). However, and in contrast to our earlier results with hypoxia-sensitive rainbow trout (Bernier and Craig, 2005), chronic hypoxia had no effect on the expression of *crf* and *ul* in the POA of common carp. In fact, the mRNA levels of POA *crf* and *ul* were generally lower in the hypoxic than in the pair-fed fish.

A comparison of the pituitary *pomc-I* mRNA levels and the plasma cortisol data between the hypoxia and pair-fed treatments indicates that a reduction in nutrient availability, not O₂ availability, was responsible for the gradual stimulation of the HPI axis in the hypoxic fish of this study. Similarly, channel catfish [*Ictalurus punctatus* (Peterson and Small, 2004)], gobies [*Gillichthys mirabilis* (Kelley et al., 2001)] and largemouth bass [*Micropterus salmoides*

(Gingerich et al., 2010)] fasted for several days all have elevated resting plasma cortisol levels. Although common carp exposed to deep hypoxia (~1.5% O₂ saturation) are characterized by rapid and marked increases in plasma cortisol levels (Van Raaij et al., 1996), carp have a high tolerance for O₂-deficient waters and our data indicate that the reduction in blood O₂ tension associated with chronic exposure to 10% O₂ saturation does not in itself stimulate the HPI axis. The delayed increase in plasma cortisol levels was also associated on day 8 with a reduction in pituitary *pomc-I* expression and a marked stimulation of pituitary *lepr* transcripts. The negative feedback effects of cortisol on *pomc* are likely responsible for the drop in pituitary *pomc-I* expression between days 4 and 8 and may serve to limit the magnitude of the stress response (Bernier et al., 2009). Similarly, given our recent observation that leptin suppresses basal and CRF-induced ACTH secretion from perfused pituitary glands of common carp (Gorissen et al., 2012), the functional implications of the marked upregulation of pituitary *lepr* expression during chronic hypoxia may include a suppression of the HPI axis.

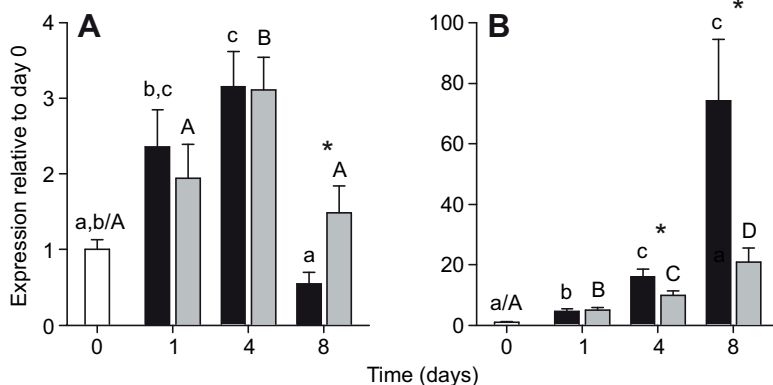


Fig. 5. Effects of normoxic water (75% O₂ saturation; white bars), chronic hypoxia (10% O₂ saturation; black bars) and restricted feeding (pair-fed treatment; gray bar) on the pituitary gene expression of (A) pro-opiomelanocortin-I (*pomc-I*) and (B) leptin receptor (*lepr*). All other details as in Fig. 2.

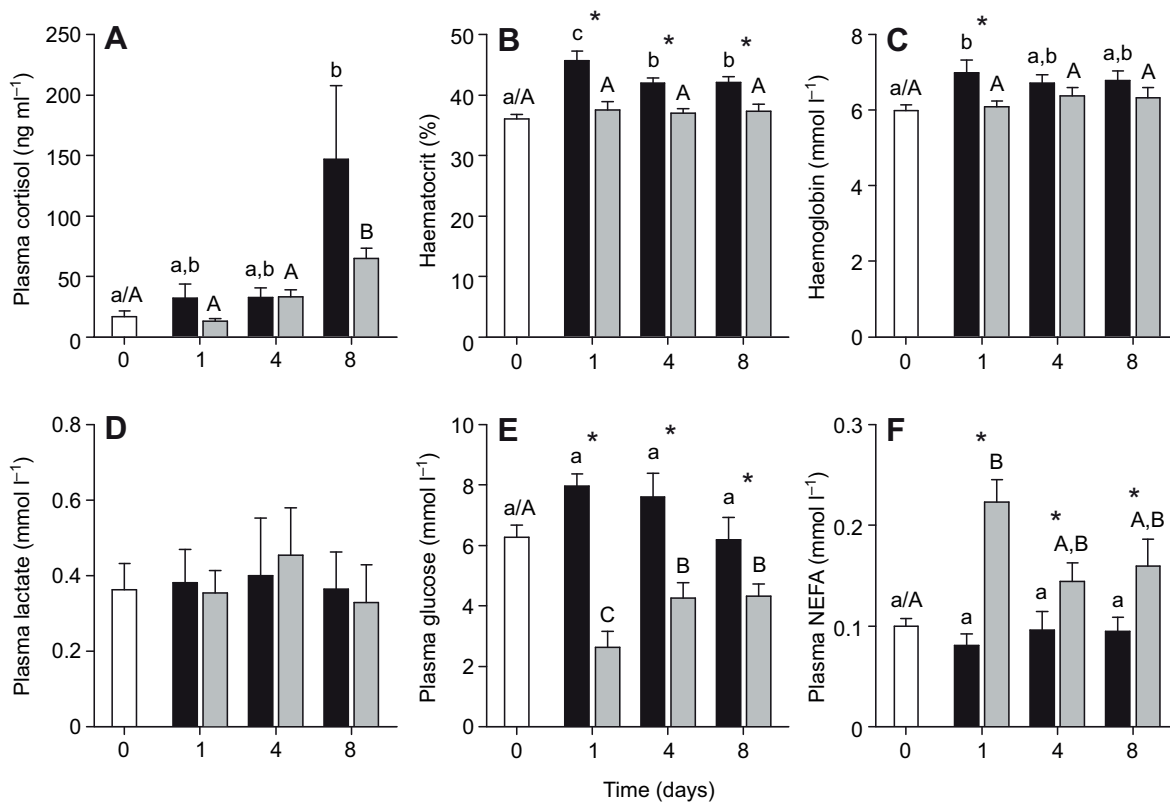


Fig. 6. Effects of normoxic water (75% O₂ saturation; white bars), chronic hypoxia (10% O₂ saturation; black bars) and restricted feeding (pair-fed treatment; gray bars) on (A) plasma cortisol, (B) haematocrit, (C) haemoglobin concentration, (D) plasma lactate, (E) plasma glucose and (F) plasma non-esterified fatty acids (NEFA). All other details as in Fig. 2.

The increased Hct, Hb and liver *epo* expression of the hypoxic fish are consistent with a coordinated response to increase O₂ uptake and delivery. The parallel increases in Hct and Hb after 24 h of hypoxia exposure indicate an increase in erythrocyte number. Although the most likely source of this short-term increase in Hb is a release of erythrocytes from the spleen, erythropoietin (EPO) may have also stimulated the formation of new red blood cells (Gallaughan and Farrell, 1998; Nikinmaa and Rees, 2005). Indeed, injection of EPO into goldfish stimulates red blood cell production (Tagliatela and Della Corte, 1997) and, as observed in this study, hypoxia has now been shown to induce EPO production in several fish species (Lai et al., 2006; Yaqoob et al., 2009; Dangre et al., 2010). Interestingly, mammalian studies have shown that leptin can also enhance the proliferation of hematopoietic stem cells and stimulate the production of erythrocytes (Bennett et al., 1996). Moreover, the angiogenic effects of leptin in humans are equivalent to those of vascular endothelial growth factor (Anagnostoulis et al., 2008). Therefore, an interesting avenue of future research in fish may be to explore the hematopoietic and angiogenic effects of leptin and its overall contribution to O₂ uptake and delivery during chronic hypoxia.

Beyond providing insight into fuel usage during chronic hypoxia and feed restriction, the plasma glucose and NEFA values observed in this study challenge our understanding of the actions of leptin on lipid metabolism and metabolic rate. As previously observed in common carp (Huisling et al., 2006) and in other species (Wang et al., 2006), the marked increase in plasma NEFA levels following feed restriction suggests a shift away from carbohydrate-dominated catabolism towards lipid-dominated catabolism. During chronic

hypoxia, in contrast, the appetite suppression and the sustained increase in liver *leptin-a* and *lepr* expression were not associated with any change in either plasma glucose or NEFA values. In mammals, leptin increases energy expenditure by stimulating lipolysis, fatty acid oxidation and the catabolism of carbohydrates (Reidy and Weber, 2000; Anubhuti and Arora, 2008). Injection of heterologous leptin in goldfish (De Pedro et al., 2006) or green sunfish [*Lepomis cyanellus* (Londrville and Duvall, 2002)] and native leptin in grass carp (Li et al., 2010) suggest that leptin stimulates lipid metabolism in fish. Clearly, the catabolic and lipolytic actions of leptin are at odds with the reduction in lipid oxidation and depression of aerobic metabolism that allow common carp to survive long-term hypoxia (Zhou et al., 2000) and studies are needed to determine the specific contributions of leptin to lipid and carbohydrate metabolism during chronic hypoxia.

Conclusions

Because multiple inhibitory feedback mechanisms promote leptin resistance during obesity, it appears that the primary role of leptin in mammals is to signal energy deficits (Myers et al., 2008). Unlike the situation in mammals, however, leptin in common carp does not appear to be produced in proportion to the nutrient status of the body. As discussed by Huisling et al. (Huisling et al., 2006), without a need to thermoregulate, fish will readily drop their metabolic rate during prolonged periods of fasting and consequently may have a reduced need to signal a deficit in nutrient intake. In contrast, chronic hypoxia survival in fish does require a precise match between energy utilization and production. In this study, we show that oxygen availability is an important physiological parameter for the regulation

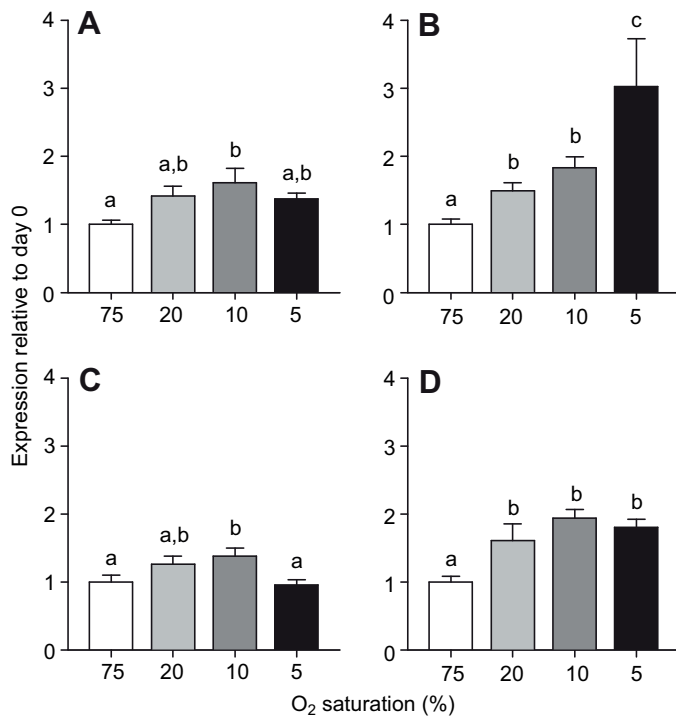


Fig. 7. Effects of a 48 h exposure period to 75% (normoxic control), 20%, 10% and 5% O₂ saturation on the gene expression of liver (A) *leptin-a-I*, (B) *leptin-a-II*, (C) leptin receptor (*lepr*) and (D) erythropoietin (*epo*). Gene expression is normalized to the expression of 40S ribosomal protein S11 and is expressed relative to the normoxic control treatment. Values that do not share a common letter are significantly different from each other as determined by a one-way ANOVA and a pairwise Tukey's test. The significance level for all statistical tests was $P < 0.05$. Values are means \pm s.e.m. ($N=8$).

of *leptin* gene expression in common carp. Although not indicative of the synthesis or release of leptin from the liver, the hypoxia-induced increases in hepatic leptin expression do suggest a functional role for leptin during chronic hypoxia. In addition to a potential role in mediating hypoxia-induced anorexia, our results suggest that leptin may be involved in the regulation of the endocrine stress response during periods of reduced oxygen availability. Therefore, although the overall significance of leptin to hypoxia tolerance remains to be determined, the functions of this pleiotropic cytokine in fish that signal energy deficits linked to oxygen availability may supersede those that communicate deficits in nutrient availability.

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