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; Ronnestad 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±0.4°C (mean ± s.d.) and a photoperiod of 16h:8h light:dark with the light phase starting at 06:00h 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 10:00h. 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% O2 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% O2 saturated (0.9 mg l⁻¹). To achieve the desired hypoxic conditions, N2 gas was bubbled into a header tank that supplied the holding tanks. Levels of dissolved O2 in the holding tanks were monitored periodically using a portable OxyGuard dissolved O2 meter (Point-Four Systems, Port Moody, BC, Canada). The desired hypoxic levels were gradually achieved within 2 h and chronically maintained at ±0.5% of the target level for 8 days by regulating the flow of water and N2 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% O2 saturated water and served as a normoxic day 0 control. Three groups were exposed to 10% O2 saturated water as above for 1, 4 or 8 days. The last three groups were maintained in 75% O2 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 Na2EDTA-treated syringe. Aliquots of blood were used immediately to determine haematocrit and haemoglobin concentration. The remaining blood was centrifuged at 14,000 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 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 (Romnestad 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>5′–3′ forward primer</th>
<th>5′–3′ reverse primer</th>
<th>Efficiency (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>agrp</td>
<td>AM498378</td>
<td>GGTGATCACCTGCGCTCTGG</td>
<td>GAAGGGCTTTAAAGAAGACGACTAA</td>
<td>86.8</td>
<td>0.99</td>
</tr>
<tr>
<td>β-actin</td>
<td>M24113</td>
<td>CAACAGGAAAAGATGACACAGTC</td>
<td>GGGACAGCAGCAGCTGATG</td>
<td>102.3</td>
<td>0.99</td>
</tr>
<tr>
<td>cart</td>
<td>AM498379</td>
<td>CCATCTCTACCTGGGAAAG</td>
<td>CTCGCCAACGTGCGCATGTA</td>
<td>94.3</td>
<td>0.99</td>
</tr>
<tr>
<td>cfr</td>
<td>AJ217595</td>
<td>CACCTCGGCTCCTGACGAA</td>
<td>CCAACACGACCTGCGTTAACT</td>
<td>92.3</td>
<td>0.99</td>
</tr>
<tr>
<td>cfr-bp</td>
<td>AJ206881</td>
<td>CAATGATCTAACGCGCTTCCAT</td>
<td>CACCCGAGAAGCTGCAAAA</td>
<td>101.7</td>
<td>0.99</td>
</tr>
<tr>
<td>epo</td>
<td>AJ213394</td>
<td>CACATGAGGCTCCCTAATGGAAG</td>
<td>TCCCATGCTGCTTTAAGTTGACAA</td>
<td>92.2</td>
<td>0.99</td>
</tr>
<tr>
<td>leptin a-I</td>
<td>AJ218537</td>
<td>CATATGATGCTGCCACCCCTTCTG</td>
<td>CCAATTCGACGTTCCTGTGATG</td>
<td>84.4</td>
<td>0.99</td>
</tr>
<tr>
<td>leptin a-II</td>
<td>AJ218516</td>
<td>AGATACGGAAGACAGTTTTGCACA</td>
<td>GGGTTGCTTCCAAGAAAGCA</td>
<td>98.0</td>
<td>0.99</td>
</tr>
<tr>
<td>npr</td>
<td>AF283734</td>
<td>CTCCTAGACGAAAGGATTCCA</td>
<td>GCCGGTATTGAGGTTGATG</td>
<td>94.9</td>
<td>0.95</td>
</tr>
<tr>
<td>lepr</td>
<td>AM498377</td>
<td>AACATGGAAGAGGATTCGA</td>
<td>CTCAATACGACAGTTTTGATG</td>
<td>87.3</td>
<td>0.97</td>
</tr>
<tr>
<td>poma-i</td>
<td>Y13618</td>
<td>TGGCTGATGCTGCTTGTTG</td>
<td>TCACTCTTGCTAGTACACCTGCAT</td>
<td>91.6</td>
<td>0.98</td>
</tr>
<tr>
<td>u1</td>
<td>M11671</td>
<td>GCATTCTGGCTACGACAAGA</td>
<td>GGTTGCTGACGGGATGTCG</td>
<td>101.0</td>
<td>0.89</td>
</tr>
<tr>
<td>40S</td>
<td>AB012067</td>
<td>CGCGTGGGATCACCTGTGCA</td>
<td>TCAGAATGGAACCTGACTGCT</td>
<td>99.9</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Primer efficiency and standard curve R² values are reported for each target gene. The efficiency was calculated from the standard curve using the equation $E=\left(10^{-\frac{1}{\text{slope}−1}}\right)−100$. 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% O₂ 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% O₂ 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 hypoxic 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 npy and agrp (Fig. 3A,B). The pair-fed treatment also had no effect on the expression of npy but was associated with a gradual 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 cort by day 8, the pair-fed treatment did not have a significant effect (Fig. 3C). In general, the expression of hypothalamic pomo-I and lepr decreased in response to the chronic hypoxia and normoxic pair-fed treatments (Fig. 3D,E). However, the reductions in pomo-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 pomo-I mRNA levels by day 4 (Fig. 5A). Although pituitary pomo-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 O₂ saturation. The leptin-a-I mRNA levels increased 1.6-fold between the control and the 10% O₂ saturation treatments, but the 5% O₂ 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% O₂ 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% O₂ saturation treatment and the 5% O₂ 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
hypoxia, like for the stimulation of hepatic anorexigenic signal. Our results also indicate that the proximal cue strategy for coping with reduced O2 availability. Appetite effects of hypoxia in fish highlight the importance of this behavioral persistence and cross-species prevalence of the appetite-suppressing 2001; Bernier and Craig, 2005). In general, the magnitude, (Chabot and Dutil, 1999; Buentello et al., 2000; Pichavant et al., increase in hepatic leptin-a expression, a gene that codes for a potent anorexigeneic 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 anorexigeneic 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 (Ambriosini 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 melastigma (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
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 peptides. Therefore, although our results show that the stimulation of hepatic leptin-a-I and -a-II expression 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., 2010). 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.

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
common carp. In support of a role for CRF-related peptide as appetite regulators in common carp, the first 24 h 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. 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.

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.
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 24h 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 also have stimulated the formation of new red blood cells (Gallaugher and Farrell, 1998; Nikinmaa and Rees, 2005). Indeed, injection of EPO into goldfish stimulates red blood cell production (Taglialatela 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 (Huising 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 (Londraville 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 Huising et al. (Huising 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
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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