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CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout

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Bernier, Nicholas J., and Paul M. Craig. CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 289: R982–R990, 2005. First published June 2, 2005; doi:10.1152/ajpregu.00668.2004.—Hypoxia stress suppresses appetite in a variety of fish species, but the mechanisms mediating this response are not known. Therefore, given their anorexigenic and hypophysiotropic properties, we investigated the contribution of forebrain corticotropin-releasing factor (CRF) and urotensin I (UI) to the regulation of food intake and the hypothalamic-pituitary-interrenal (HPI) stress axis in hypoxic rainbow trout. Exposure to 50 and 35% O_2 saturation for 24 h decreased food intake by 28 and 48%, respectively. The 35% O_2 treatment also increased forebrain CRF and UI mRNA levels, plasma cortisol, and lactate. Exposure for 72 h to the same conditions resulted in similar reductions in food intake, increases in plasma cortisol proportional to the hypoxia severity, and increases in forebrain CRF and UI mRNA levels in the 50% O_2 treatment. Relative to saline-infused fish, chronic intracranial infusion of the CRF receptor antagonist α-helical $CRF_{(9-41)}$ reduced the appetite-suppressing effects of 24-h exposure to 35% O_2 and blocked the hypoxia-induced increase in plasma cortisol. Finally, forebrain microdissection revealed that 50 and 35% O_2 exposure for 24 h specifically increases preoptic area CRF and UI mRNA levels in proportion to the severity of the hypoxic challenge and either has no effect or elicits small decreases in other forebrain regions. These results show that CRF-related peptides play a physiological role in regulating the HPI axis and in mediating at least a portion of the reduction in food intake under hypoxic conditions in rainbow trout and demonstrate that the response of forebrain CRF and UI neurons to this stressor is region specific.

feeding behavior; anorexia; hypothalamic-pituitary-interrenal axis; cortisol; *Oncorhynchus mykiss*

HYPOXIA, caused either by eutrophication or as a result of the natural daily and seasonal fluctuations in O_2 availability, is a common occurrence in a variety of aquatic habitats (2, 19, 49). Although fish have evolved multiple strategies to survive hypoxic conditions and hypoxia tolerance varies considerably among species, chronic exposure to low dissolved O_2 characteristically impairs growth (23, 53). Among the factors that contribute to the growth reduction observed with hypoxia, a reduction in food intake is the main cause (10, 13, 40, 46, 56). Whereas the appetite-suppressing effects of hypoxia in fish are well recognized, the neuroendocrine mechanisms mediating this response are not known. In part, this is a result of our limited understanding of how fish brains perceive stressors such as hypoxia and how the response associated with this perceived threat affects the mechanisms responsible for the control of food intake (6).

Although poorly understood in fish, it is generally recognized that animals perceive stressors via a distinct and complex stress-sensitive brain neurocircuitry that converges on the corticotropin-releasing factor (CRF) neurons located within the hypothalamic paraventricular nucleus (PVN) (24). In turn, the CRF neurons of the PVN play a critical role in the control of the hypothalamic-pituitary-adrenal (HPA) axis and in the coordination of the autonomic and behavioral responses to stress (48). As such, various stressors in mammals, including hypoxia, have been shown to result in the depletion of the CRF-containing neurosecretory neurons of the HPA axis and to elicit increases in the expression of CRF mRNA in the PVN (14, 34). Similarly, in fish, the CRF neurons located within the nucleus preopticus (NPO), the fish equivalent to the PVN, are thought to play an important role in the regulation of the hypothalamic-pituitary-interrenal (HPI) stress axis (30, 33, 52). There is now some evidence in fish that stressors can elicit an increase in CRF expression in the NPO (1, 20). Whether the brain's CRF system is recruited in response to a hypoxic stress in fish is not known, but exposure to severe hypoxia is associated with a marked increase in the final product of HPI axis activation, i.e., plasma cortisol (39, 50).

In addition to their key role in the regulation of the HPI axis, CRF-related peptides are potent anorexigenic factors in fish (5), as in other vertebrates (3, 15). In goldfish (*Carassius auratus*), intracerebroventricular injections of either CRF or the related neuropeptide urotensin I (UI) inhibit food intake in a dose-related manner, and these anorexigenic effects can be reversed by pretreatment with the CRF receptor antagonist α-helical $CRF_{(9-41)}$ [α-$hCRF_{(9-41)}$] (5, 17, 18). Similarly, the appetite-suppressing effects of pharmacological treatments that increase forebrain CRF and UI mRNA levels are reversed by pretreatment with α-$hCRF_{(9-41)}$ (5). Whether endogenous CRF-related peptides also contribute to the regulation of food intake under hypoxic conditions remains to be elucidated.

Thus, to determine whether CRF and UI are involved in mediating the hypoxia-induced stimulation of the HPI axis and reduction in food intake, the objectives of this study were twofold: *1*) determine whether hypoxia exposure in rainbow trout is associated with changes in forebrain CRF and UI mRNA levels, and *2*) assess whether CRF receptor blockade can counteract the increase in plasma cortisol and the reduction in food intake associated with hypoxia exposure.

MATERIALS AND METHODS

Animals

Mixed sex rainbow trout (*Oncorhynchus mykiss*) were obtained from the Alma Aquaculture Research Station (Alma, ON, Canada)

and held in the Hagen Aqualab at the University of Guelph in 90-liter tanks for at least 1 mo before experimentation. Fish were tagged with a 125-kHz passive integrated transponder (PIT tags; Biomark, Boise, ID) and maintained in flow-through well water (temperature, $14 \pm 0.5°C$; dissolved O_2, 8.3 ± 0.2 mg/l; photoperiod, 12:12-h light-dark cycle). All procedures used were approved by the local Animal Care Committee and conform to the principles of the Canadian Council for Animal Care.

Experimental Design

Experiment 1: Effects of hypoxia on food intake and forebrain CRF and UI gene expression. Six groups of 12 fish each with an average weight of 95.4 ± 3.5 g were exposed to one of three O_2 regimes for 24 or 72 h. Two control groups were maintained at a Po_2 of 80% (%dissolved O_2 saturation, 8.3 mg/l), and two groups each were exposed to a Po_2 of 50% (5.2 mg/l) and 35% (3.6 mg/l). To achieve the desired hypoxic conditions, we bubbled N_2 gas into a stripping column that supplied the holding tanks. Levels of dissolved O_2 in the fish tanks were continuously monitored using O_2 sensors (Point-Four Systems, Port Moody, BC, Canada) and a data acquisition system (Argus Control Systems, White Rock, BC, Canada). The desired hypoxic levels were gradually achieved within 2 h and were maintained at $\pm 2\%$ of the target level throughout the experiment by regulating the flow of water and N_2 gas through the stripping column. Using X-ray radiography, food intake was assessed twice on each individual fish: 1 wk before the beginning of the normoxic or hypoxic treatments and at the end of the 24- or 72-h exposure period. Once the treatments were completed, fish were terminally anesthetized in a solution of 2-phenoxyethanol (2 ml/l; Sigma, St. Louis, MO) before decapitation and excision of the forebrain: telencephalon, preoptic region, and hypothalamus. Tissue samples from a given fish were combined in a microcentrifuge tube kept on dry ice, frozen in liquid nitrogen, and stored at $-80°C$ until analysis of CRF and UI mRNA levels. Blood was collected by caudal puncture and centrifuged at 10,000 *g* for 5 min, and the separated plasma was stored at $-20°C$ for later analysis of plasma cortisol and lactate concentrations. The plasma aliquot for determination of lactate was deproteinized with ice-cold perchloric acid (0.6 N) and spun down, and the supernatant was stored as described above.

Experiment 2: Effects of α-$hCRF_{(9-41)}$ infusion on hypoxia-induced changes in food intake. Four groups of 10 fish with an average weight of 189.4 ± 2.4 g were fitted with an intracranial cannula for chronic infusion of either saline or the CRF receptor antagonist α-$hCRF_{(9-41)}$ (American Peptide, Sunnyvale, CA). After surgery, the fish were returned to their respective 90-liter tanks and left undisturbed for 6 days. On the 7th day postsurgery, fish were exposed to one of two O_2 regimes for 24 h. Two groups were maintained at a Po_2 of 80%, and the two other groups were exposed to a Po_2 of 35%, as described for *experiment 1*. Among both the normoxic and hypoxic fish, one group was infused with saline and the other with α-$hCRF_{(9-41)}$. Food intake was assessed twice on each individual fish using X-ray radiography: 1 day before surgery and at the end of the 24-h exposure period. At the end of the trial, fish were terminally anesthetized and sampled as in *experiment 1*. The forebrain was excised to measure CRF and UI mRNA levels, and a blood sample was collected for analysis of plasma cortisol and lactate concentrations.

Experiment 3: Effects of hypoxia on forebrain regional CRF and UI gene expression. Three groups of eight fish with an average weight of 112.7 ± 5.0 g were exposed to one of three O_2 regimes for 24 h. One control group was maintained at a Po_2 of 80%, and two hypoxic groups were exposed to a Po_2 of either 50 or 35%, as in *experiment 1*. At the end of the trial, fish were terminally anesthetized, as described in *Experiment 1: Effects of hypoxia on food intake and forebrain CRF and UI gene expression*, the brains were removed, and the telencephalon, preoptic area, and hypothalamus were regionally

dissected according to the method of Doyon et al. (20). Brain tissues were collected separately in microcentrifuge tubes kept on dry ice, frozen in liquid nitrogen, and stored at $-80°C$ until analysis of CRF and UI mRNA levels.

Experimental Procedures: Quantification of Food Intake

Throughout the study, fish were fed to satiation once daily at 10:00 AM. On experimental days, food intake was quantified from individual fish using X-ray radiography (27). In brief, a commercial trout diet (4PT Classic Sinking fish feed; Martin Mills, Elmira, ON, Canada) was grounded and repelleted with 450-μm hardened cast carbon steel spheres (Draiswerke, Mahwah, NJ) at a ratio of 5% by mass of dry powdered food. Approximately 90 min after a meal of labeled feed, all the fish within a group were anesthetized at once in a buffered (NaHCO$_3$, 1 g/l) solution of tricaine methanesulfonate (0.5 g/l; MS-222; Syndel, Vancouver, BC, Canada), placed on 14×17-in. Konica KF fine-screen cassettes, and X-rayed using an ACU-RAY HFJ portable X-ray unit (50 kVp, 1.05 mAs at 90 cm) and Konica MGL latitude X-ray film (Sterne, Brampton, ON, Canada). Once the radiographs developed, the number of carbon steel spheres present in the gastrointestinal tract of the fish were counted, and the amount of food eaten by each individual was determined from a calibration curve. Preliminary experiments revealed that the labeled feed does not affect palatability and that the X-ray procedure described can be repeated at a 72-h interval without significantly affecting food intake. Between the first (control) and second (postexposure) assessment of food intake by X-ray radiography and during a 2-wk acclimation period before the experiment, the fish were fed the same repelleted diet without the label.

Development of Molecular Probes for Slot-Blot Analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) based on the guanidine isothiocyanate-phenol-chloroform extraction method. Total RNA concentrations were determined using ultraviolet spectrophotometry at 260 nm, and samples were stored at $-80°C$ until used. Reverse transcription-polymerase chain reaction (RT-PCR) was used to prepare rainbow trout CRF, UI, and β-actin DNA probes. In brief, total RNA extracted was reverse transcribed to cDNA by using SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). For PCR amplification, the forward primer (CRF, 5'-ATG AAG CTC AAT TTC CTC GTC-3'; UI, 5'-ATG AAG CCT GTT CCC CTG AT-3'; β-actin, 5'-GTA TCG TCA TGG ACT CCG-3') and reverse primer sequences (CRF, 5'-ATT TCC CGA AGA TCT CCA TCA-3'; UI, 5'-CAC TTT CCA ACT TCA TCC AG-3'; β-actin, 5'-CCA GAC GGA GTA TTT ACG C-3') were based on *O. mykiss* CRF (GenBank accession no. AF296672), UI (GenBank accession no. AJ005264), and β-actin cDNAs (GenBank accession no. AF157514). The PCR products were separated using agarose gel, and the bands of desired size were excised and purified using a Geneclean II kit (Bio 101, La Jolla, CA). The purified DNA fragments were ligated into the plasmid pGEM-T Easy (Promega, Madison, WI) and transformed into the *Escherichia coli* strain XL1 blue (Stratagene, La Jolla, CA) for cloning. Recombinant plasmid DNA containing the cDNA inserts was purified using an alkaline lysis method (8), and both strands of cloned DNA were sequenced in opposite directions to confirm the identity of the PCR fragments. The cloned CRF (502 bp), UI (497 bp), and β-actin (572 bp) DNA fragments were then used as hybridization probes.

Quantification of mRNA Using Slot-Blot Analysis

In *experiments 1* and *2*, forebrain CRF and UI mRNA levels were quantified by slot-blot analysis according to the method of Bernier and Peter (5). Two micrograms of total RNA (initially diluted with sterile water to 10 μl) to which was added 30 μl of denaturing solution [19.7 μl of formamide, 6.4 μl of formaldehyde (37%), and 3.9 μl of 10$\times$

MOPS] was incubated at 65°C for 15 min. The samples were immediately placed on ice, diluted further with 60 µl of ice-cold 20× SSC, and slotted directly onto Hybond-N membranes (Amersham, Amersham, UK) using a Bio-Dot SF manifold apparatus (Bio-Rad, Richmond, CA). The RNA was fixed by baking the membranes at 80°C for 2 h and cross-linked by UV irradiation for 30 s. Rainbow trout CRF and UI hybridization probes were prepared as described above, labeled using a random priming kit (T7 QuickPrime kit; Pharmacia Biotech, Baie d'Urfé, QC, Canada) with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham), and purified using a QIAquick nucleotide removal kit (Qiagen, Mississauga, ON, Canada). The membranes were prehybridized in hybridization solution (0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA) for 3 h at 65°C. The hybridization solution was then changed, and the labeled CRF or UI probe was added. After overnight hybridization at 65°C, the membranes were washed four times (twice for 30 s and twice for 5 min) with washing solution (40 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, and 1% SDS). Signal detection was achieved by exposing the CRF and UI membranes to a PhosphorImager screen and was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). To serve as an internal control, the membranes were stripped and reprobed with the [α-^{32}P]dCTP-labeled rainbow trout β-actin hybridization probe. The CRF and UI mRNA levels were expressed as a ratio to the hybridization signal for β-actin mRNA and then normalized as a percentage of the 80% O_2 saturation control value for each individual treatment. The linearity of the mRNA signals obtained by slot-blot analysis was assessed by determining the CRF and UI signals from blotting dilutions of forebrain total RNA.

Quantification of mRNA Using Real-Time PCR

In *experiment 3*, CRF, UI and β-actin mRNA levels in the isolated telencephalon, preoptic, and hypothalamic brain regions were quantified using real-time quantitative PCR. Real-time PCR was used instead of slot-blot analysis because less total RNA is required for the quantification of mRNA with this technique. Once extracted and quantified as described above, 1 µg of total RNA was treated with DNase I according to the manufacturer's protocol (DNase I amplification grade; Invitrogen) and reverse transcribed to cDNA with SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). The cDNA products were amplified using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Each reaction contained 10 µl of SYBR green PCR master mix (Applied Biosystems), 5 µl of cDNA template, and 2.5 µl each of forward and reverse primers (0.4 µM). 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. This protocol was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between the three different cDNAs, we constructed standard curves for each target by using serial dilutions of cDNA samples known to have high expression levels of the target gene (22). Using the threshold cycle of each unknown, we extrapolated the relative dilution of a given sample by using the linear regression of the target-specific standard curve. To correct for differences in RNA loading and reverse transcriptase efficiencies, we normalized each sample to the expression level of the housekeeping gene β-actin. All samples were assayed in triplicate, and only one target was assayed per well. Finally, non-reverse-transcribed RNA and water only controls were run to ensure that no genomic DNA was being amplified and that reagents were not contaminated. The primers were designed using the software program Primer Express (Applied Biosystems), and to prevent potential coamplification of genomic DNA, the sequence of each forward primer was located on the position of a known exon-exon junction (CRF, GenBank accession no. AY651777; UI, GenBank accession no. AY651778) or a junction deduced on the basis of sequence identity (β-actin). To maximize amplification efficiency, we also designed the CRF (forward, 5′-ACA ACG ACT CAA CTG AAG ATC TCG-3′; reverse, 5′-AGG AAA TTG AGC TTC ATG TCA GG-3′), UI (forward, 5′-AGG AGA CAA AAT ACC GGG CA-3′; reverse, 5′-CTT CAT AGT GCT GGA CAG ACG G-3′), and β-actin primers (forward, 5′-GCC CCC CTC AAC CCC-3′; reverse, 5′-GAA GGT CTC AAA CAT GAT CTG GGT C-3′) to amplify the shortest product possible (CRF, 54 bp; UI, 51 bp; β-actin, 60 bp).

Plasma Analysis

Plasma cortisol was measured using an enzyme immunoassay as described by Carey and McCormick (12) with the following specifications: *1*) the microtiter plates were coated with a rabbit anti-cortisol antibody (product code no. F3-314; Esoterix Endocrinology, Calabasas Hills, CA) at a final dilution of 1:20,000, and *2*) the cortisol-horseradish peroxidase conjugate (Clinical Endocrinology Laboratory, Univ. of California, Davis, CA) was used at a final dilution of 1:60,000. A serial dilution of rainbow trout plasma gave a displacement curve that was parallel to the standard curve, and the lower detection of this assay was 0.60 ng/ml. With the use of a pooled plasma sample, the intra- and interassay variations were 5.7% ($n = 10$) and 12.6% ($n = 5$), respectively. Plasma lactate concentration was measured spectrophotometrically using a NAD^+-linked enzymatic procedure (no. 826-UV; Sigma).

Intracranial Infusion

Intracranial infusions were performed by following procedures described by Levy and Baker (31). Briefly, fish were initially anesthetized by immersion in a 0.1 g/l solution of buffered MS-222, transferred to a moistened foam-lined cradle, and kept sedated throughout the surgical procedure by continuously perfusing the gills with a 0.06 g/l solution of the same anesthetic. A 19-gauge needle was then inserted midline at a 45° angle 8 mm through the brain case to permit subsequent positioning of a treated PE-50 cannula (Clay Adams; Becton Dickinson, Sparks, MD) a few millimeters into the cranial cavity directly above the pineal gland. From its insertion site into the cranium, the cannula was tunneled through the back muscles of the fish to emerge ~3 cm in front of the dorsal fin. An Alzet microosmotic pump (model 1003D; Durect, Cupertino, CA) encased in a layer of dialysis tubing was tied to the fish at the anterior and posterior limits of the dorsal fin, and the pump outflow was connected to the cannula by flexible silicone tubing. On average, each pump was filled with 246.7 ± 4.4 µg of α-hCRH$_{(9-41)}$, and the calculated dosage rate at 14°C (Durect) was 21.2 µg/day. α-hCRH$_{(9-41)}$ was dissolved in teleost physiological saline (11), solubilized with 1 N NaOH (2%), and subsequently diluted. The saline vehicle for controls was treated in a similar manner. In a preliminary experiment, the intracranial cannulation procedure appeared to have no effect on swimming behavior and all cannulated fish ($n = 6$) fed to satiation 72 h postsurgery. Furthermore, postmortem examination confirmed that the cannulas were projecting through the cranial roof and revealed no evidence of hemorrhage or tissue damage at the surface of the brain.

Statistical Analysis

All data are presented as means ± SE. Differences among treatments were assessed using one-way ANOVA followed by a pairwise Tukey's multiple comparison test. Differences between the pre- and postexposure food intake values were assessed using a pairwise Student's *t*-test. The significance level for all statistical tests was $P < 0.05$.

RESULTS

Experiment 1: Effects of Hypoxia on Food Intake and Forebrain CRF and UI Gene Expression

Whereas the 80% O_2 saturation exposure had no effect on food intake, 24-h exposure to 50 and 35% O_2 saturation
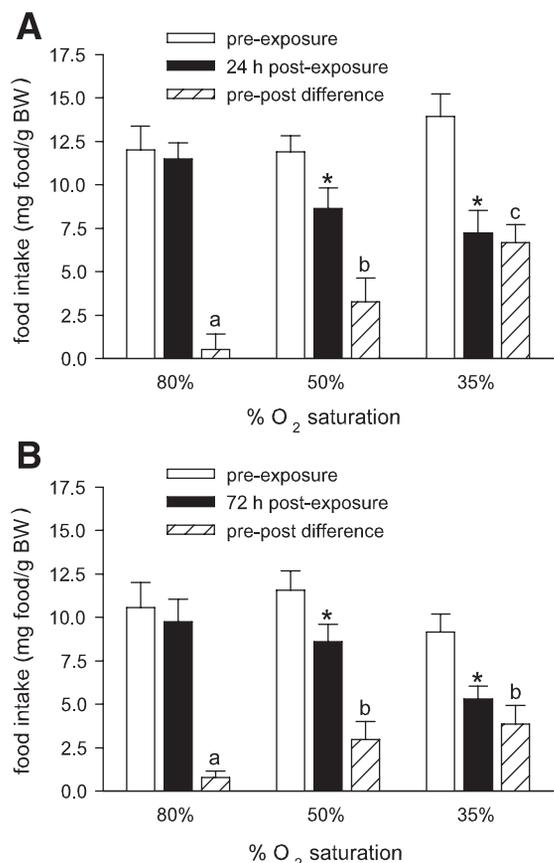
Fig. 1. Effects of a 24- (*A*) or 72-h (*B*) exposure period to 80% (8.3 mg/l), 50% (5.2 mg/l), or 35% (3.6 mg/l) O_2 saturation on food intake in rainbow trout ($n = 8$). BW, body weight. [a,b,c]Pre-post differences that do not share a common letter are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test. Paired Student's *t*-tests were carried out between the pre- and postexposure values for each treatment group, with significant differences indicated (*$P < 0.05$).

significantly decreased food intake by 28 and 48%, respectively (Fig. 1*A*). Similarly, exposure to hypoxia for 72 h suppressed food intake by 26 and 42% in the 50 and 35% O_2 saturation treatments, respectively (Fig. 1*B*). Therefore, although the magnitude of the reduction in food intake paralleled the percent decrease in O_2 saturation, an increase in the duration of the hypoxic treatment did not result in a further reduction in appetite. Hypoxia also elicited changes in forebrain UI and CRF mRNA levels (Fig. 2). Relative to the control 80% O_2 treatment, 24-h exposure to 35% O_2 saturation resulted in nearly a twofold increase in both UI and CRF mRNA levels, and the 50% O_2 treatment had no significant effect. In contrast, after 72-h hypoxic exposure, forebrain UI and CRF mRNA levels were elevated in the 50% O_2 saturation treatment but not in the 35% O_2 treatment. Hence, the magnitude of the changes in forebrain UI and CRF mRNA levels appeared to depend on both the severity of hypoxia and its duration. Similarly, whereas only the 35% O_2 treatment was associated with an increase in plasma cortisol after 24 h, plasma cortisol was elevated in both hypoxic treatments after 72-h exposure, and the increase was inversely related to the O_2 levels (Fig. 3*A*). Finally, independently of exposure duration, hypoxia only

elicited an increase in plasma lactate in the 35% O_2 saturation treatment (Fig. 3*B*).

Experiment 2: Effects of α-hCRF$_{(9-41)}$ Infusion on Hypoxia-Induced Changes in Food Intake

Chronic intracranial infusion of saline or α-hCRF$_{(9-41)}$ had no significant effect on food intake in fish kept in 80% O_2 saturated water (Fig. 4). In contrast, food intake was reduced by 54% in trout exposed to 35% O_2 saturation for 24 h and infused with saline. Although food intake was also significantly reduced by 30% in fish exposed to 35% O_2 saturation and infused with α-hCRF$_{(9-41)}$, the appetite-suppressing effects of the hypoxic treatment were significantly less in the α-hCRF$_{(9-41)}$-infused fish than in the saline-infused fish. Independently of the intracranial infusate, fish exposed to the 35% O_2 treatment for 24 h were characterized by similar increases in both UI and CRF mRNA levels relative to the fish kept in normoxic conditions (Fig. 5). Relative to the normoxic saline- and α-hCRF$_{(9-41)}$-infused fish, exposure to the 35% O_2 treatment for 24 h resulted in a significant increase in plasma cortisol (Fig. 6*A*). However, intracranial infusion with α-hCRF$_{(9-41)}$ before the hypoxic exposure prevented the increase in plasma cortisol. Last, the 24-h 35% O_2 treatment elicited an increase in plasma lactate in both the saline- and α-hCRF$_{(9-41)}$-infused fish (Fig. 6*B*).
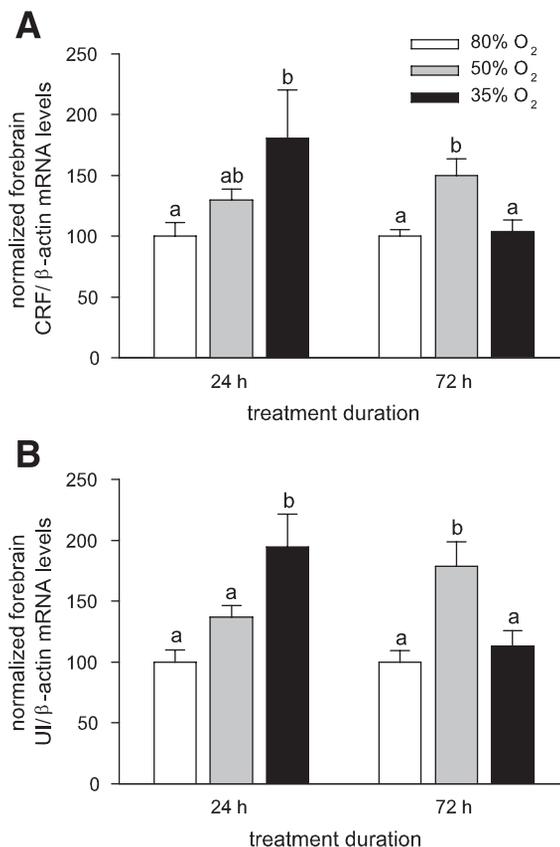

Fig. 2. Effects of a 24- or 72-h exposure period to 80, 50, or 35% O_2 saturation on forebrain corticotropin-releasing factor (CRF; *A*) and urotensin I (UI; *B*) mRNA levels in rainbow trout ($n = 8$). [a,b]Treatment values that do not share a common letter for a given time are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test ($P < 0.05$).

Fig. 3. Effects of a 24- or 72-h exposure period to 80, 50, or 35% O_2 saturation on plasma cortisol (*A*) and lactate (*B*) in rainbow trout ($n = 8$). [a,b,c]Treatment values that do not share a common letter for a given time are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test ($P < 0.05$).
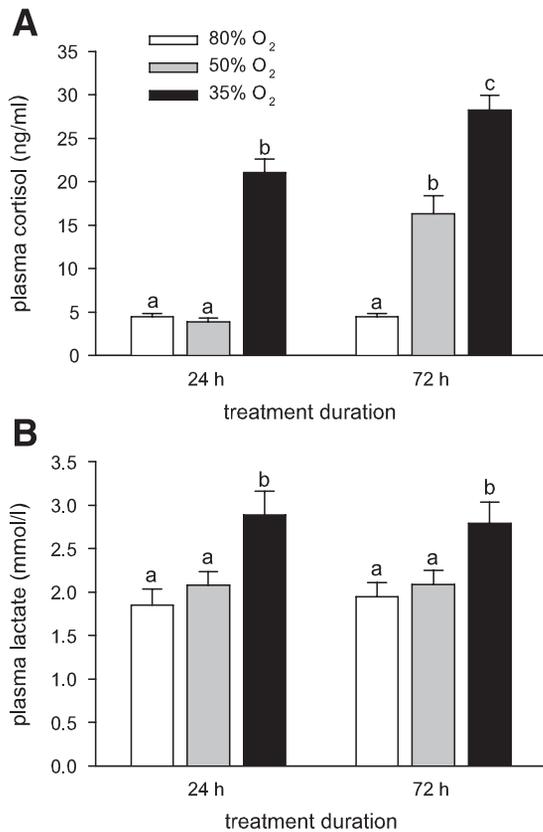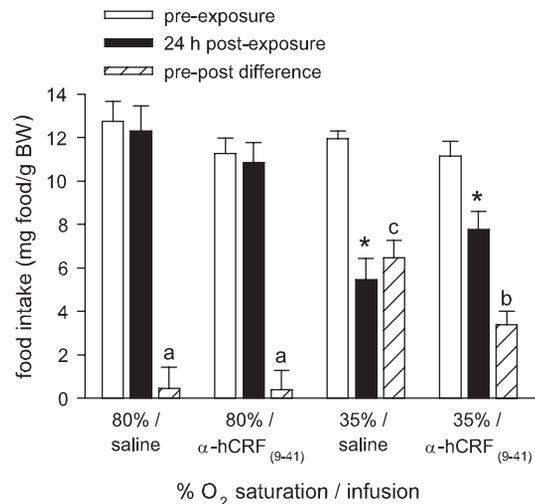

Fig. 4. Effects of a 24-h exposure period to 80 or 35% O_2 saturation on food intake in rainbow trout that received a chronic intracranial infusion of saline or α-helical CRF$_{(9-41)}$ [α-hCRF$_{(9-41)}$] ($n = 10$). [a,b,c]Pre-post differences that do not share a common letter are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test. Paired Student's *t*-tests were carried out between the pre- and postexposure values for each treatment group, with significant differences indicated (*$P < 0.05$).
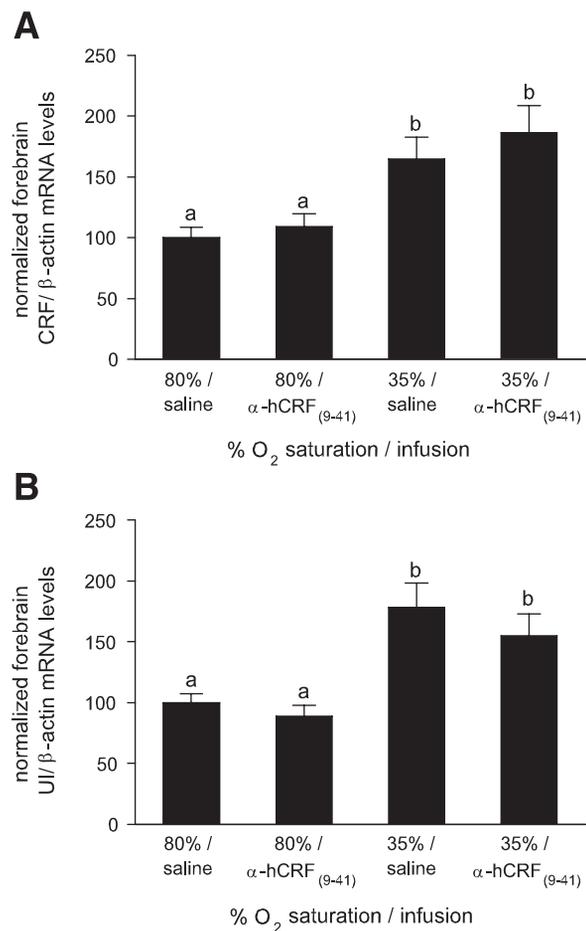
Experiment 3: Effects of Hypoxia on Forebrain Regional CRF and UI Gene Expression

Among the brain regions examined in the 80% O_2 saturation group, the preoptic area had the highest level of CRF and UI gene expression with 83 and 53% of total expression for all three regions, respectively (Fig. 7). Relative to the normoxic conditions, hypoxia elicited significant increases in preoptic area CRF and UI mRNA levels that were proportional to the severity of the hypoxic challenge. In contrast, hypoxia elicited decreases in the mRNA levels of UI and CRF in the telencephalon and of CRF in the hypothalamus, and it had no effect on hypothalamic UI expression.

DISCUSSION

We have shown that hypoxia specifically stimulates CRF and UI gene expression in the preoptic area of rainbow trout forebrain, genes whose products have known hypophysiotropic and anorexigenic effects. After 24 h of hypoxia, preoptic area CRF and UI gene expression are both positively correlated with the severity of the hypoxia-elicited appetite suppression, and in the fish exposed to 35% O_2 saturation, the significant increase in the mRNA levels of these genes is associated with an increase in plasma cortisol. Moreover, CRF receptor blockade partially reverses the reduction in food intake and prevents the increase in plasma cortisol associated with exposure to 24-h 35% O_2 saturation. However, the relationship among the ano-

Fig. 5. Effects of a 24-h exposure period to 80 or 35% O_2 saturation on forebrain CRF (*A*) and UI (*B*) mRNA levels in rainbow trout that received a chronic intracranial infusion of saline or α-hCRF$_{(9-41)}$ ($n = 10$). [a,b]Treatment values that do not share a common letter are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test ($P < 0.05$).

A

B

Fig. 6. Effects of a 24-h exposure period to 80 or 35% (3.6 mg/l) O_2 saturation on plasma cortisol (*A*) and lactate (*B*) in rainbow trout that received a chronic intracranial infusion of saline or α-hCRF$_{(9-41)}$ ($n = 10$). [a,b]Treatment values that do not share a common letter are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test ($P < 0.05$).
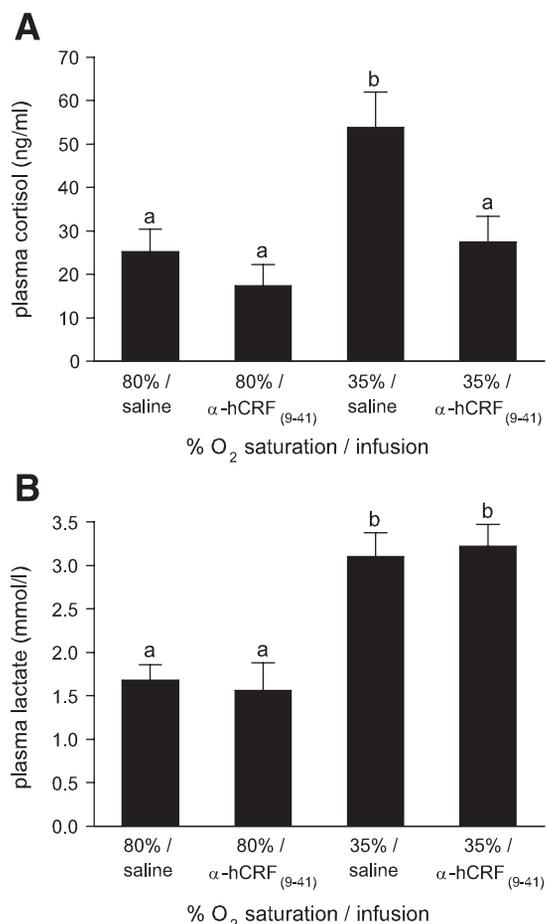
metabolic and respiratory responses to hypoxia are recruited (9). As previously observed in other species (13, 40), the reduction in food intake elicited by the 50% O_2 treatment in this study was not associated with any change in plasma lactate and so, presumably, took place in the absence of any significant recruitment of anaerobic metabolic pathways. In contrast, the more severe anorexia elicited by the 35% O_2 treatment was associated with a sustained increase in plasma lactate over the 72-h exposure. Therefore, among the hierarchy of physiological adjustments associated with hypoxia exposure in fish (2, 9), a reduction in food intake may be a behavioral strategy that is recruited relatively early in the overall response to decreasing O_2 levels.

Although the extent to which hypoxia decreases food intake depends on the severity of the hypoxic exposure, available data suggest that it may be relatively independent of its duration. Compared with the appetite-suppressing effects of the 24-h hypoxic exposures, increasing the exposure duration to 72 h had little effect on the depth of the anorexigenic response. Although there is no other short-term hypoxic exposure study for comparison, in both turbot (*Scophthalmus maximus*) and sea bass (*Dicentrarchus labrax*), the appetite-suppressing effects of hypoxia either remain completely unchanged or decrease modestly over a 6-wk period (40, 41). The sustained appetite suppression associated with chronic hypoxia suggests
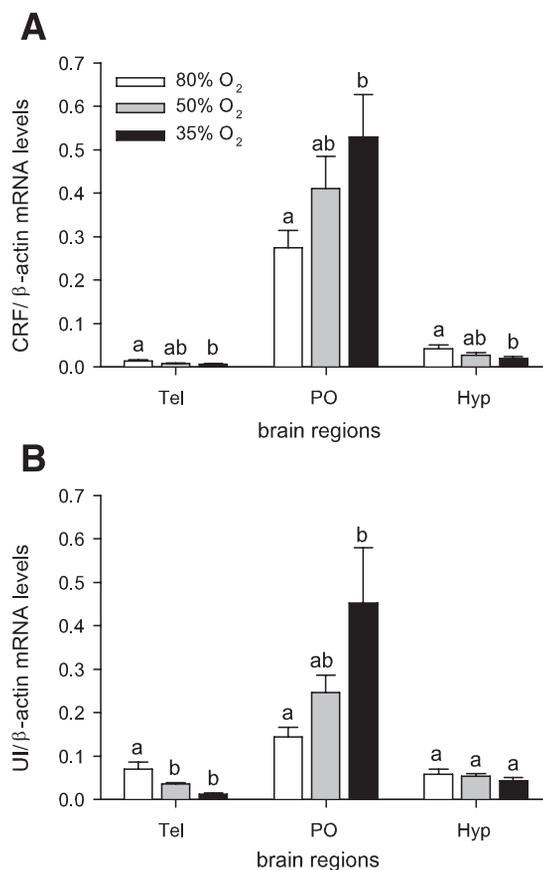
rectic effects of hypoxia, the circulating levels of cortisol, and the forebrain mRNA levels of CRF and UI breaks down after 72 h of hypoxic exposure. Whereas the anorexia is maintained almost unchanged through 72 h of hypoxia and the plasma cortisol values are proportional to the severity of the hypoxic bout, CRF and UI mRNA levels returned to basal conditions in the 35% O_2 treatment. Therefore, although our results implicate CRF-related peptides as potential mediators of the acute stress response and appetite-suppressing effects of hypoxia in rainbow trout, they also suggest that additional mechanisms may be involved in mediating the sustained anorexia that characterizes chronic hypoxia exposure in fish.

Exposure of rainbow trout to 50 and 35% O_2 saturation elicited decreases in food intake that paralleled the reduction in O_2 saturation. These results are consistent with previous observations in several fish species in which food intake is independent of O_2 availability above a species-specific threshold and directly related to ambient O_2 concentration below this value (10, 13, 38, 40, 56). In rainbow trout, the critical O_2 level below which food consumption is affected appears to be $\sim$55–60% O_2 saturation (38). Although exposure to this moderate hypoxic level is associated with a hyperventilatory response (28), it is also above the O_2 level at which most of the

A

B

Fig. 7. Effects of a 24-h exposure period to 80, 50, or 35% O_2 saturation on telencephalon (Tel), preoptic region (PO), and hypothalamus (Hyp) CRF (*A*) and UI (*B*) mRNA levels in rainbow trout ($n = 8$). [a,b]Treatment values that do not share a common letter are significantly different from each other as determined using one-way ANOVA and pairwise Tukey's test ($P < 0.05$).

that this response may be an essential energy-saving strategy for coping with reduced O_2 availability. Routine O_2 consumption measurements in turbot and sea bass support this hypothesis (40, 41). Relative to satiated normoxic fish, both satiated hypoxic fish and normoxic fish pair-fed to hypoxic levels have significantly lower O_2 consumption rates. Appetite suppression confers significant energy savings by reducing specific dynamic action, i.e., the postprandial increase in O_2 consumption associated with digestion, absorption, transformation, and storage of nutrients (25, 26).

As shown by the significant increases in plasma cortisol, in addition to their effects on food intake, the hypoxic treatments also stimulated the HPI axis. Overall, the increases in plasma cortisol in this study were proportional to the severity of the hypoxic bout and its duration. These results are consistent with similar observations in different species acutely exposed to hypoxic levels ranging from moderate to severe (39, 45, 47, 50) and suggest that plasma glucocorticoids are sensitive indicators of short-term hypoxia exposure in fish. Through its effects on intermediary metabolism, the hypoxia-associated increase in plasma cortisol may promote the mobilization of stored energy and increase fuel availability (36). In contrast, in both turbot and sea bass, plasma cortisol levels are unaffected by chronic 6-wk exposure to moderate hypoxia levels similar to those used in this study (40). Similarly, in turbot, sea bass, and Atlantic cod (*Gadus morhua*), plasma lactate levels are unchanged after 6 to 8 wk of chronic exposure to hypoxic conditions as low as 40–45% O_2 saturation (13, 40). Therefore, although further experiments are needed to characterize both the short- and long-term effects of moderate hypoxia on the circulating levels of cortisol and metabolic fuel utilization, it appears that the recruitment of the HPI axis may be a relatively short-term response to moderate hypoxia that is turned off once other physiological and behavioral adjustments enable fish to cope with the hypoxic challenge.

This study also provides original evidence that hypoxia exposure in fish can be associated with an increase in preoptic area CRF and UI mRNA levels. These results are consistent with previous observations in mammals in which acute exposure to hypoxemic conditions in developing sheep (34) or to altitude hypoxia in adult rats (14) is specifically associated with increased PVN CRF mRNA abundance. Given that the NPO in teleosts, like the PVN in mammals, is the principle site of hypophysiotropic CRF fibers (30, 57), our results suggest that CRF is an important stimulus of the HPI axis in fish exposed to hypoxic conditions. In contrast, although hypothalamic UI-expressing cells are thought to innervate the pituitary in fish (30), it is not known whether the preoptic area UI-expressing cells identified in this study directly contribute to the control of ACTH release. Interestingly, in the only study to date to use neural tract tracing techniques to characterize the HPI axis in teleosts, only a small percentage of the NPO cells that project to the pituitary were found to be immunoreactive for CRF; the nature of the remaining cells was not identified (57). Similarly, although CRF and UI are potent anorexigenic agents in fish (5, 17, 18) and our results suggest that the CRF- and UI-expressing cells of the preoptic area may be involved in food intake regulation during hypoxia, the origin of the CRF and UI neural substrate involved in ingestive behavior in fish have not been identified. In rats, although a few different brain nuclei may be involved in mediating the anorexigenic effects of CRF-related peptides (42), several studies have identified the PVN as a potential site for the anorectic actions of CRF and urocortin (16, 29, 51).

An involvement of CRF-related peptides in the regulation of the HPI axis and food intake during hypoxia in trout is further supported by our observation that intracranial infusion of α-hCRF$_{(9-41)}$ blocks the increase in plasma cortisol and partially reverses the reduction in food intake that is associated with hypoxia exposure. Similar results have been observed in mammals in which either central infusion or intracerebroventricular injection of CRF receptor antagonists partially reverses the appetite-suppressing effects associated with various conditions known to activate the CRF system (42). Coadministration of α-hCRF$_{(9-41)}$ in goldfish also partially blocks the increase in plasma cortisol and the reduction in food intake elicited by intracerebroventricular injection of CRF or UI (5). Assuming that NPO CRF and UI neurons secrete their products in response to a hypoxic stress, the preoptic area increase in CRF and UI mRNA levels observed in this study may be in response to depletion of cellular stores. In rats, the altitude hypoxia-elicited increase in PVN CRF mRNA is associated with decreases in PVN and median eminence CRF content (14). Although parallel measurements of CRF-related peptide concentration and gene expression are needed to further characterize the activity of the CRF system under hypoxic conditions in fish, our results strongly suggest that endogenous CRF-related peptides are involved in the mechanisms whereby hypoxia stress suppresses food intake and activates the HPI axis.

Although it is not clear why CRF and UI expression returned to basal values after 72 h of exposure to the 35% O_2 saturation treatment, it is possible that the chronically elevated levels of plasma cortisol in this treatment may have attenuated the response of the CRF system. In fetal sheep, cortisol infusion completely attenuates the hypoxemia-induced increase in CRF mRNA levels (34). In addition, 72 h after injection, cortisol intraperitoneal implants elicit a dose-dependent decrease in forebrain CRF mRNA levels in goldfish (4). Whether cortisol also specifically modulates the response of the CRF system to hypoxia in trout remains to be determined.

Available evidence in mammals suggests that CRF release from PVN neurons into the median eminence during hypoxia is primarily mediated by a norepinephrine (NE) source originating in the brain stem (14, 35). Medullary brain stem catecholaminergic neurons with NE terminals in the PVN are coordinately activated with PVN neurons during hypoxia in fetal sheep (35). Moreover, in rats, the hypoxia-induced median eminence CRF release can be reversed by pretreatment with α_1-adrenoceptor antagonists (14). In return, during hypoxia, the brain stem catecholaminergic system may be activated by afferent fibers from the O_2-sensing glomus cells of the carotid body (21, 43). In fish, afferent fibers from the gill chemoreceptors involved in mediating hypoxic reflexes are known to project to anatomically similar regions in the medullary brain stem (44). However, whether medullary NE excitatory ascending pathways project on the CRF and/or UI neurons of the NPO in fish and whether this ascending pathway can activate the CRF system under hypoxic conditions is not known.

Given the complexity of the neural systems involved in controlling food intake and the stress response in vertebrates

(7, 24), the appetite suppression associated with hypoxia is likely to result from activation of multiple systems rather than being the response of a single pathway such as the CRF-related peptide-expressing cells of the preoptic region. In rats, hypoxia also decreases gastric contractions and delays gastric emptying time (54, 55), physiological effects known to play a major role in the satiation process (7). Although in fish it is not known whether hypoxia effects gastric transit time via effects on peristaltic movements or reduction in food digestive processes or food absorption, acute stress is known to alter the lining of the gastrointestinal tract (37). Finally, the existence of multiple pathways mediating the appetite-suppressing effects of hypoxia also is suggested by the fact that chronic hypoxia can be associated with appetite-suppression without any signs of HPI axis activity (40).

In summary, although several hypophysiotropic factors can stimulate the HPI axis in fish (30) and a variety of anorexigenic signals have been identified (32), our findings suggest that in response to moderate hypoxia in rainbow trout, CRF-related peptides are essential for HPI axis recruitment and for mediating at least a portion of the reduction in food intake. Given that the reduction in specific dynamic action that results from appetite suppression confers significant energy savings and that cortisol can promote the mobilization of stored energy and increase fuel availability, the recruitment and involvement of the CRF system in the regulation of food intake and HPI axis activity may be an important strategy that fish utilize to cope with environmental hypoxia. Further studies are needed to determine whether the CRF and UI neurons involved in the control of food intake are the same as those involved in the control of the HPI axis, and whether CRF, UI, or a related CRF-like peptide plays a lead role in stimulating ACTH release and in regulating food intake during hypoxia stress.

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