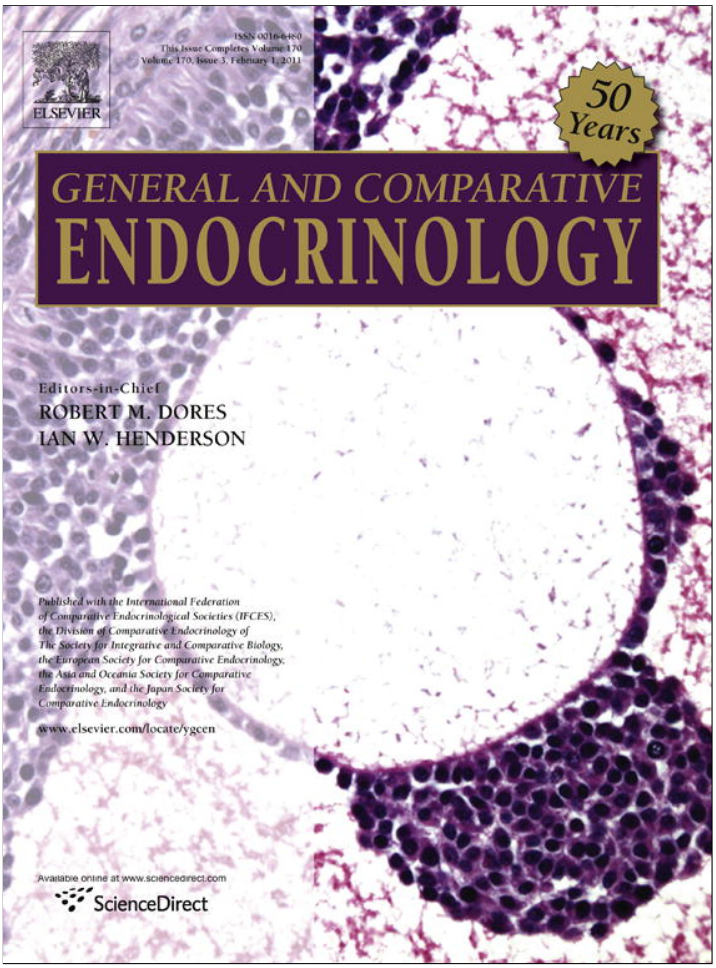

General and Comparative Endocrinology 170 (2011) 604–612

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Ontogeny of the corticotropin-releasing factor system in rainbow trout and differential effects of hypoxia on the endocrine and cellular stress responses during development

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ARTICLE INFO

Article history:
Received 23 July 2010
Revised 23 November 2010
Accepted 24 November 2010
Available online 2 December 2010

Keywords:
Stress
Development
Ontogeny
Embryo
HPI-axis
CRF
CRF-BP
Cortisol
Hypoxia
Hsp70

ABSTRACT

To further our understanding of the development of the stress axis and the responsiveness of embryonic and larval fish to environmental stressors, this study examined the ontogeny of whole-body cortisol levels and of the corticotropin-releasing factor (CRF) system in rainbow trout, as well as the endocrine and cellular stress responses to hypoxia. After depletion of a maternal deposit, *de novo* synthesis of cortisol increased exponentially between the 'eyed' stage and first feeding. Whole body CRF mRNA levels dominated over those of the related peptide urotensin I (UI) from hatch through complete yolk sac absorption. The mRNA levels of CRF-binding protein (CRF-BP) closely paralleled those of CRF and UI throughout ontogeny except at first feeding when an increase in CRF gene expression was not matched by change in CRF-BP transcript abundance. In the hypoxia challenge, fish were exposed to 15% O_2 saturation for either 90 min or 24 h at three key developmental stages: hatch, swim up and first feeding. While the embryos were unaffected, chronic hypoxia elicited a transient 2-fold increase in whole-body cortisol levels in the larval stages. The hypoxia challenge also generally suppressed the mRNA levels of CRF and CRF-BP, had no effect on the expression of UI, but had a marked stimulatory effect on heat shock protein 70 (Hsp70) gene expression. Taken together, these results suggest a role for the CRF system in the ontogenic regulation of corticosteroidogenesis and show that hypoxia has developmental stage-specific effects on the endocrine and cellular stress responses in rainbow trout.

1. Introduction

Exposure to stressors during development can have a profound impact on the physiology and health of an organism later in life [43,77]. Mammalian studies have shown that glucocorticoids, the end product of hypothalamic–pituitary–adrenal (HPA) axis activation, play a key role in the programming of brain structures that can alter stress responsiveness [47,66,71]. In fact, permanent changes in stress phenotype due to stressors experienced during development have now been demonstrated in mammals [47,53], birds [51], amphibians [38], and fish [8]. Overall, however, we know very little about the ontogeny of the neuroendocrine stress axis in teleosts and even less about the responsiveness of embryonic and larval fish to environmental stressors.

The neuroendocrine stress response in fish is regulated by the hypothalamic–pituitary–interrenal (HPI) axis. At the hypothalamic level, among the multiple factors with hypophysiotropic actions, the peptide corticotropin-releasing factor (CRF) is considered to be the major regulator of adrenocorticotropic hormone (ACTH) secretion from the pituitary [19]. Urotensin I (UI), a piscine member of the CRF peptide family, is also a potent *in vitro* stimulator of ACTH secretion [34,78]. ACTH, in turn, is recognized as the principle stimulator of cortisol release from the head kidney interrenal cells [33]. The binding protein (BP) for CRF-related peptides, CRF-BP, may also be an important player in the regulation of HPI axis activity [19]. CRF-BP is localized with CRF and UI in the nucleus preopticus (NPO) of the preoptic area [2,4], a major site of hypophysiotropic peptide production, and various stressors affect the expression of CRF-BP in this region of the brain [4,39]. Similarly, various environmental, social and physical stressors in adult fish are associated with increases in the expression of preoptic area CRF and/or UI [18,16,39,52].

During ontogeny, all components of the CRF system are expressed as early as 6 h post-fertilization (hpf) in zebrafish (*Danio rerio*) embryos [3] and CRF mRNA is first localized in the NPO by 48 hpf, i.e. just prior to hatch [23]. Synthesis of the ACTH precursor molecule, pro-opiomelanocortin (POMC), occurs prior to hatch in rainbow trout (*Oncorhynchus mykiss*) [65] and zebrafish [73], and POMC is sensitive to feedback regulation by cortisol around the time of hatching in zebrafish [73]. Key enzymes involved in corticosteroidogenesis are expressed during organogenesis in the inter-

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doi:10.1016/j.ygcen.2010.11.022

renal cells of zebrafish [5] and *de novo* cortisol synthesis prior to hatch has been demonstrated in several fish species [5,8,61]. In contrast, most studies have shown that a stressor-mediated increase in cortisol synthesis does not take place until sometime after hatch [5,11,12,32,40,44,67]. The cause of this lag is unknown, although it has been suggested that there may be a disconnect between the perception of a stressor and the release of CRF peptides from the hypothalamus [6]. To date, the impact of stressors on the expression of CRF system components in embryonic or larval fish has not been assessed. In contrast, several studies have shown that the expression of heat shock proteins (HSPs) is affected by diverse stressors during embryogenesis [27,48,72]. HSPs, a component of the cellular stress response, may therefore serve as useful indicators of stress during early embryonic development [28].

Unable to escape and without the protection of an intrauterine environment, fish embryos may be particularly susceptible to the developmental impact of environmental insults. Hypoxia, resulting either from natural events or anthropomorphic activities, is a common feature of various aquatic habitats [30]. In salmonid redds, for example, O_2 levels within egg pockets can vary from ~20% to 90% saturation [25,60]. Despite clear evidence that hypoxia retards embryonic growth and delays development [24,37,42,80], our understanding of the impact of hypoxia on the activity of the HPI axis during development in fish remains cursory [32,59].

In this study, to further our understanding of the development of the HPI axis and the responsiveness of embryonic and larval fish to environmental stressors, we investigated the ontogeny of the CRF system and whole-body cortisol levels in rainbow trout, as well as the endocrine and cellular stress responses to hypoxia at key ontogenetic transitions.

2. Materials and methods

2.1. Animals

Fertilized rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada) and raised at the Hagen Aqualab (University of Guelph, ON, Canada). Eggs were incubated in mesh bottom Heath trays (length 62.9 cm × width 52.1 cm × depth 6 cm) in darkness with continuous flow-through local well water (10 °C, 10.4 mg/L O_2, pH 7.9; water hardness 411 mg/L as $CaCO_3$; ion concentrations in mmol/L: 2.6 Ca^{2+}, 1.5 Cl^-, 1.5 Mg^{2+}, 0.06 K^+, 1.1 Na^+). Hatching occurred between 30 and 34 days post-fertilization (dpf). At 56 dpf, larvae were transferred to a floating basket with a simulated 12:12 light:-dark photoperiod and fed ground trout pellets *ad libitum* with an automatic belt feeder. While the eggs used to determine the ontogeny of whole-body cortisol and the CRF system (see Section 2.2) and those used to determine the effects of chronic hypoxia exposure on the stress response (see Section 2.3) originated from the same brood stock, they were collected at different times of year. The University of Guelph's Animal Care Committee approved of the care and usage of all animals in this study, as per the principles of the Canadian Council for Animal Care.

2.2. Ontogeny of whole-body cortisol and the CRF system

To determine the ontogeny of whole-body cortisol and the CRF system in rainbow trout, eggs, embryos and larvae were collected at 2 week intervals between 0 and 84 dpf. These intervals represented key stages in the development of rainbow trout embryos and larvae [76]. The 0 dpf samples were unfertilized eggs, 14 dpf embryos (stage 21) represented the 'eyed' stage, 28 dpf embryos (stage 30) were just prior to hatching, 42 dpf larvae (stage 32) were transitioning to the 'swim up' stage, 56 dpf larvae (stage 34) were beginning the transition to exogenous feeding, 70 dpf fish (stage 36) represented the near completion of yolk sac absorption and the transition to complete exogenous feeding, and 84 dpf fish (stage 37) represented the completion of yolk sac absorption and fully exogenous feeding fish. Unfertilized eggs (n = 50) were collected at the trout farm immediately after they were recovered from brood stock. Between 14 dpf and 84 dpf, embryos or larvae were quickly netted and euthanized in a lethal dose of 2-phenoxyethanol (0.2%; Sigma–Aldrich, St. Louis, MO, USA) and individually flash frozen in liquid nitrogen. Samples were stored at −80 °C for future quantification of cortisol (n = 5) and CRF, UI, or CRF-BP mRNA levels (n = 4–8).

2.3. Effects of chronic hypoxia exposure on the stress response

To characterize the impact of environmental hypoxia on the stress response during ontogeny, embryos (28 dpf) and larvae (42 and 56 dpf) were either sampled directly from normoxic conditions (92% O_2 saturation; 10.4 mg/L O_2) or exposed to hypoxia (15% O_2 saturation; 1.7 mg/L O_2) for 90 min or 24 h. Hypoxic conditions were created by bubbling N_2 gas into a header tank of water which continually fed the Heath tray. The oxygen level in the Heath tray was monitored intermittently throughout the experiment using an oxygen meter (YSI 55; YSI, Yellow Springs, OH, USA). Oxygen levels remained very stable throughout the duration of the exposures and did not vary by more than 2% of the target value. At the time of sampling, embryos/larvae were euthanized as above and stored at −80 °C for future cortisol measurement (n = 6–8) or quantification of the mRNA levels for CRF, UI, CRF-BP, and Hsp70 (n = 4–8).

2.4. Cortisol determination

Frozen embryos/larvae were individually weighed and homogenized in ice-cold buffer (80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl; 1 mM ethylenediaminetetraacetic acid (EDTA; pH 7.4)) using a Euro Turrax T 20b (IKA Labortechnik, Staufen, Germany) homogenizer. Homogenates were extracted three times with methanol, and purified with C_{18} solid phase extraction columns (100 mg octadecyl [C_{18}], 1-ml disposable polypropylene minicolumn, Amersham Biosciences Corp, Piscataway NJ, USA) as described by Lister and Van Der Kraak [49]. Samples were reconstituted in 650 μL assay buffer (21.4 mM $Na_2HPO_4 \cdot 7\ H_2O$; 9.3 mM $NaH_2PO_4 \cdot H_2O$; pH 7.6; 0.1% gelatin; 0.01% thimerosal) and cortisol content was determined in triplicate by radioimmunoassay (RIA) as described by Bernier et al. [18]. A serial dilution of whole embryo extract gave a displacement curve that was parallel to the standard curve (Fig. 1), indicating that there were no contaminants interfering with the assay. Inter- and intra-assay variance were found to be 5.9% (n = 6) and 1.7% (n = 7), respectively. Cortisol values are presented as ng whole-body cortisol / g of individual body weight (BW) and are corrected for average extraction efficiency (59.1 ± 1.7%; n = 10).

2.5. Quantification of mRNA by real-time RT-PCR

Total RNA from individual embryos and larvae was extracted using TRIzol Reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Five μg of total RNA was treated with DNase I (Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen) according to the manufacturer's protocols. The cDNA products were amplified in duplicate with gene-specific primers (Table 1) using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the quantification of CRF, CRF-BP, and UI, reactions contained 12.5 μL 2X TaqMan Universal Master Mix (Applied Biosystems), 5 μL of 5× diluted cDNA tem-

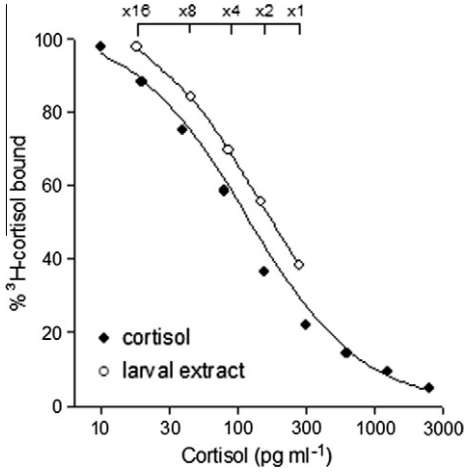

Fig. 1. Serial dilution of a cortisol standard (dark circles) and extract from whole bodies of 42 days post-fertilization rainbow trout larvae (white circles) measured using a radioimmunoassay. Larval extract values were phase shifted to the right from the serial dilution of the standard for better visibility.

plate, and 2.5 μL each of forward and reverse primers (9 μM) and 2.5 μL probe (2.5 μM). For Hsp70 quantification, reactions contained 10 μL SYBR Green PCR Master Mix (Applied Biosystems), 5 μL of 5× diluted 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. To account for differences in amplification efficiency, a standard curve was constructed for each target using serial dilutions of cDNA. Input values were obtained by fitting the average threshold cycle value to the antilog of the standard curve. To correct for variations in template input and transcriptional efficiency, each sample was normalized to the expression level of two reference genes, β-actin and elongation factor-1α (EF1α). Finally, non-reverse transcribed RNA and water controls were run to ensure that no genomic DNA was being amplified and the reagents were not contaminated.

2.6. Statistical analysis

A one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was used to determine differences in cortisol content between developmental stages. Two-way ANOVAs followed by Tukey's test for multiple comparisons were used to determine differences in mRNA levels between the various transcripts and developmental stages during ontogeny. Two-way ANOVAs followed by Tukey's test for multiple comparisons were also used to determine differences in cortisol content or mRNA levels between the various treatments and developmental stages in response to the hypoxic challenge. Data that did not meet the assumption of normality was log-transformed prior to analysis. If the transformed data still did not conform to the assumptions of ANOVA, a Kruskal–Wallis one-way ANOVA on ranks followed by a Dunn's test for multiple comparisons were performed. A Pearson product-moment correlation test was used to test for potential correlations between CRF and CRF-BP, CRF and UI, or UI and CRF-BP mRNA levels of individual fish. All analyses were performed using SigmaStat 3.0 (SPSS, Inc., Chicago, IL, USA) and data expressed as mean ± SE was considered statistically significant when $p < 0.05$.

3. Results

3.1. Ontogeny of whole-body cortisol and the CRF system

Unfertilized embryos contained, on average, 3.36 ± 0.23 ng/g BW cortisol (Fig. 2A). The lowest cortisol content during ontogeny was observed 2 weeks later at 14 dpf, the eyed-stage (0.03 ± 0.01 ng/g BW). Cortisol increased exponentially between the 14 and 56 dpf developmental stages, peaking at 7.10 ± 0.64 ng/g BW. Cortisol levels fell once again by the first sampling stage after the onset of exogenous feeding, 70 dpf (0.88 ± 0.16 ng/g BW), but rebounded by the end of the feeding transition at 84 dpf (4.17 ± 0.75 ng/g BW).

Independent of whether the mRNA levels of CRF, UI or CRF-BP were normalized to the expression levels of β-actin or EF1α, the overall changes in the expression pattern of each gene throughout ontogeny and in response to hypoxia were conserved. However, since the standard deviation in expression values for β-actin both within and between sampling times were slightly lower than those for EF1α, β-actin was identified as a more suitable normalization gene in this study. Individual unfertilized eggs contained insufficient quantities of total RNA to quantify gene expression at 0 dpf (Fig. 2B). Similarly, the expression of all three transcripts could not be reliably detected in 14 dpf embryos. Low levels CRF and CRF-BP mRNA were detected in 25% and 50% of embryos tested,

Table 1
Sequences of gene-specific primer pairs and probes used to amplify corticotropin-releasing factor (CRF), CRF-binding protein (CRF-BP), urotensin I (UI), heat shock protein 70 (Hsp70), β-actin and elongation factor-1α (EF1α) in real-time reverse transcriptase-polymerase chain reaction assays. The GenBank accession number for each gene is indicated. + indicates a gene that was amplified with TaqMan chemistry and ∗ indicates a gene that was amplified with SYBR green chemistry.

Gene	Sequences (5′–3′)	GenBank accession no.
CRF^{+}	F: AAA TAC CTA AAA TCC AGG GAC ACA A R: GAG GTA CCA GTG ATC ATG TTC TTG A P: [DFAM] CAA CTG AAG ATC TCG TTG AAC CCC TGA CA [DTAM]	AF296672
CRF-BP^{+}	F: CAT CAC CCA GCC ATC AAA CAC R: GAG TAT GAC AGC GTT GAC ATC GA P: [DFAM] TTG ATG AAG TCT CCT CCC CT [DTAM]	AY363677
UI^{+}	F: GTG CAA CTT TTT CAG CAT TAA GGA R: GTG CTG GAC AGA CGG ACA AAA P: [DFAM] ACA AAA TAC CGG GCA CCG [DTAM]	AJ005264
Hsp70*	F: AGG CCC AAC CAT TGA AGA GA R: GCA ATG TCC AGC AAT GCA ATA	AB176855
β-actin^{*+}	F: GAC CCA GST CAT GTT TGA GAC CTT R: CGT AGC CCT CGT AGA TGG GTA P: [DFAM] ACT CCG GTG ACG GCG TGA CCC[DTAM]	AJ438158
EF1α^{*+}	F: CCA TTG ACA TTT CTC TGT GGA AGT R: GAG GTA CCA GTG ATC ATG TTC TTG A P: [DFAM] ACC GGC AGG TAC TAC GTC ACA ATC ATT GA [DTAM]	AF498320

F, forward primer; R, reverse primer; P, probe.

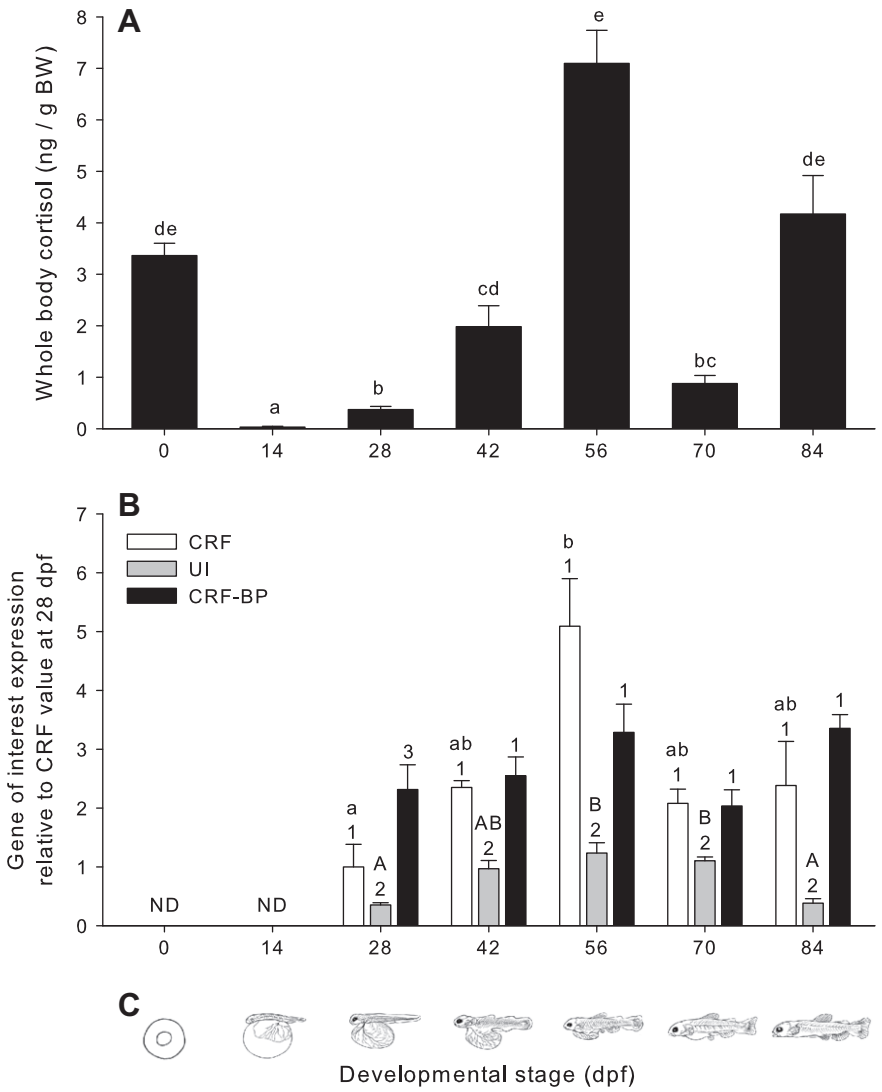

Fig. 2. (A) Whole-body cortisol ($n = 5$) and (B) expression of corticotropin-releasing factor (CRF), urotensin I (UI), and CRF-binding protein (CRF-BP) ($n = 4–8$) in (C) rainbow trout unfertilized eggs (0 days post-fertilization; dpf), embryos (14 and 28 dpf), and larvae (42–84 dpf). All gene expression data is standardized to the expression of β-actin and shown as fold-change from the CRF mRNA levels at 28 dpf. For the cortisol data, bars that do not share a common letter are significantly different from each other as determined by one-way ANOVA and pairwise Tukey test ($p < 0.05$). Differences in gene expression were assessed by two-way ANOVA and pairwise Tukey test ($p < 0.05$). For a given gene, bars that do not share a common letter are significantly different (there were no significant differences in CRF-BP expression). At a given developmental stage, bars that do not share a common number differ from one another. BW, body weight; ND, not detected (see text for further details).

respectively, but UI could not be amplified. Between 28 and 84 dpf, the overall changes in basal CRF mRNA levels were similar to the changes in basal cortisol content. CRF mRNA levels increased 5-fold between the 28 dpf embryos and the 56 dpf larvae and then decreased at 70 dpf and 84 dpf. A similar profile was observed in the ontogenic gene expression of UI, with a steady increase in expression between 28 and 56 dpf and a subsequent decrease in 84 dpf larvae. In contrast, CRF-BP mRNA levels did not change between the 28 and 84 dpf developmental stages. Throughout ontogeny, the whole body gene expression levels of CRF and CRF-BP were consistently greater than those of UI. Whereas the mRNA levels of CRF-BP where greater than those of CRF at 28 dpf, the mRNA levels of these two genes were comparable at all later stages of development.

At the 28, 42, 70, and 84 dpf stages, a strong positive correlation was found between CRF and CRF-BP mRNA levels ($R^2 = 0.650$; $n = 43$; $p < 0.001$; Fig. 3A). Significant but weaker correlations were also observed between CRF and UI mRNA levels ($R^2 = 0.320$; $p < 0.01$; not shown) as well as between CRF-BP and UI mRNA levels ($R^2 = 0.279$; $p < 0.01$; not shown) at these stages. To facilitate visual interpretation, data from all four stages were combined in Fig. 3A, however positive correlations were found to be significant in each separate developmental stage. In contrast, no correlations were found for any of the comparisons in the 56 dpf larvae ($R^2 < 0.05$; $n = 20$; $p > 0.05$; Fig. 3B, only CRF vs CRF-BP shown).

3.2. Effects of chronic hypoxia exposure on the stress response

Compared to their respective normoxic control group, exposure to 15% O_2 saturation for 90 min increased cortisol levels 2-fold in the 42 and 56 dpf larvae, but had no effect on the 28 dpf embryos (Fig. 4A). After 24 h of continuous hypoxia exposure, whole-body cortisol levels did not differ from the control values at any of the developmental stages tested.

The 15% hypoxia treatment was also characterized by a 2-fold reduction in CRF mRNA levels after 90 min of exposure in all developmental stages. After 24 h of hypoxia exposure, CRF mRNA levels remained depressed in the 28 dpf embryos but recovered to

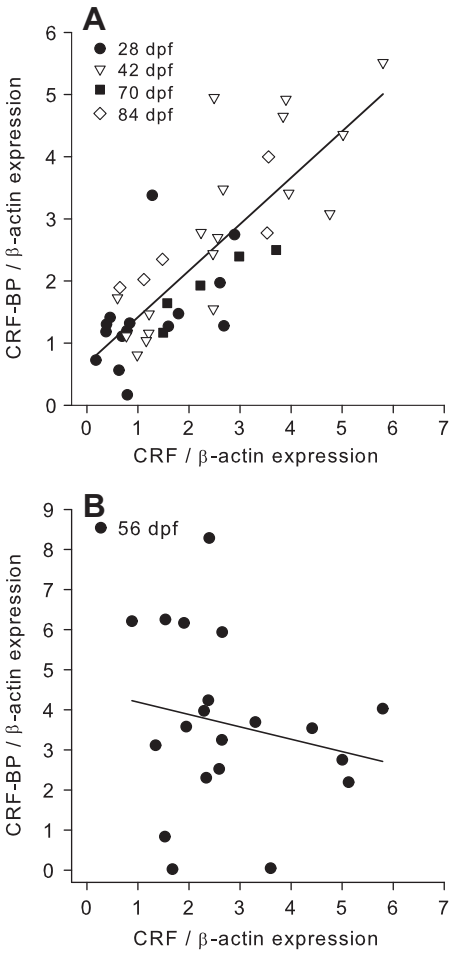

Fig. 3. Relationship between corticotropin-releasing factor (CRF) and CRF-binding protein (CRF-BP) gene expression in rainbow trout embryos and larvae at (A) 28, 42, 70, and 84 days post-fertilization (dpf; $R^2 = 0.65$, $p < 0.001$), and at (B) 56 dpf ($R^2 = 0.04$, $p > 0.05$). All gene expression data is standardized to the expression of β-actin. The relationships were analyzed with the Pearson product-moment correlation test.

control values in the 42 and 56 dpf larvae (Fig. 4B). The hypoxia exposure had no effect on UI gene expression at any of the developmental stages or sampling times (Fig. 4C). In the 28 dpf embryos and 42 dpf larvae, hypoxia exposure was associated with decreases in CRF-BP mRNA levels that paralleled the changes in CRF gene expression (Fig. 4D). In contrast, hypoxia had no effect on CRF-BP mRNA levels in the 56 dpf larvae.

Basal expression of Hsp70 mRNA levels increased throughout ontogeny, with a 13-fold increase between the 28 and 56 dpf developmental stages (Fig. 4E). Hypoxia had a marked stimulatory effect on Hsp70 gene expression in the 28 dpf embryos, increasing mRNA levels 15-fold after 90 min and 172-fold after 24 h. At 42 dpf, hypoxia increased Hsp70 mRNA levels 7-fold and 12-fold after 90 min and 24 h, respectively. Finally, at 56 dpf, the hypoxia treatment was associated with a 3-fold increase in Hsp70 mRNA levels after 90 min and a 2-fold increase in gene expression after 24 h.

Strong positive correlations were observed between CRF and CRF-BP mRNA levels in the control and hypoxia-exposed 28 dpf embryos ($R^2 = 0.561$, $n = 12$, $p < 0.01$; Fig. 5A) and 42 dpf larvae ($R^2 = 0.773$, $n = 18$, $p < 0.001$; Fig. 5B). In the same fish, there were also positive but weaker correlations between CRF and UI mRNA levels ($R^2_{28\ dpf} = 0.545$, $n = 12$; $p = 0.058$; $R^2_{42\ dpf} = 0.274$, $n = 18$; $p < 0.05$; not shown), and between CRF-BP and UI mRNA levels ($R^2_{28\ dpf} = 0.608$, $n = 18$, $p < 0.05$; $R^2_{42\ dpf} = 0.394$, $n = 18$, $p < 0.01$; not shown). In contrast, no correlations were found between any of the above transcripts in the 56 dpf larvae ($R^2 = 0.01$, $n = 20$; $p > 0.05$; Fig. 5C only CRF vs CRF-BP shown).

4. Discussion

4.1. Ontogeny of whole-body cortisol and the CRF system

The cortisol ontogeny described in the present study corroborates similar reports in developing embryos and larvae of the genus *Oncorhynchus* [8,11,12,26,32]. A maternal deposit of cortisol was detected prior to fertilization and was depleted by the start of organogenesis (14 dpf). The subsequent rise in cortisol prior to hatching (28 dpf) results from *de novo* synthesis [61]. As previously observed in rainbow trout [11] and chum salmon (*O. keta*) [26], post-hatch cortisol levels increased exponentially as larvae approached the onset of exogenous feeding (56 dpf) and declined sharply right after (70 dpf). By 84 dpf, when the energy requirements of larvae were derived completely from exogenous sources, basal cortisol levels rose once again.

The transition from yolk-derived nourishment to exogenous feeding in teleosts is associated with high mortality rates and is a major factor in the life history and survival of fish [10,74]. After exogenous feeding is initiated, larvae use a combination of yolk and captured food to meet their energy requirements until the yolk sac is completely depleted [46]. The high cortisol levels observed at the onset of exogenous feeding in this study and others [8,11,26,59], and the coinciding peak in glucocorticoid and mineralocorticoid receptor mRNA levels in zebrafish larvae [5], suggest an involvement of cortisol in the initiation of feeding and/or the regulation of intermediary metabolism during this key ontogenetic transition. In tadpoles [38], seabird chicks [45] and rodent pups [22] treatment with corticosteroids stimulate feeding and nutritional deficits are characterized by elevated corticosteroid levels and hyperphagia. In contrast, zebrafish larvae that are fasted throughout the yolk sac resorption phase have lower cortisol levels when compared to the fed larvae [3]. Manipulation of caloric intake studies during the ontogenetic transition from endogenous to exogenous feeding are needed to help elucidate the causal relationships between exogenous feeding, HPI axis activity and cortisol levels in larval fish.

The pattern of CRF expression through ontogeny was similar to that of cortisol with a peak in CRF mRNA levels occurring at 56 dpf followed by a decrease at 70 dpf. The parallel ontogenic changes in CRF expression and whole-body cortisol observed in this study suggest hypophysiotropic regulation of basal cortisol levels as early as hatching in rainbow trout. Similarly, CRF mRNA expression in zebrafish larvae increases between hatching and exogenous feeding [3] and discrete CRF-expressing cell clusters are localized to the preoptic region during embryonic and post-embryonic development [23]. In general, the pattern of basal UI expression in developing rainbow trout was more variable than the pattern of CRF, peaking at 56 and 42 dpf in the ontogeny and hypoxia experiments, respectively. As previously observed in zebrafish [3], UI was also consistently expressed at a lower level than CRF throughout the larval period in rainbow trout. In zebrafish, whereas the preoptic area is a principal site of CRF synthesis in the developing brain, UI is primarily expressed in distinct neurons of the embryonic midbrain and hindbrain, and only sparsely expressed in the preoptic nucleus [3,21,23].

The quantification of CRF-BP mRNA expression during ontogeny in a teleost is described for the first time in this study. Whereas the basal expression level of CRF-BP remained constant between hatch and complete yolk sac absorption in the ontogeny experiment, a small but significant increase was observed post-hatch in the hypoxia experiment. Interestingly, a strong positive correlation between the expression levels of CRF-BP and either CRF or UI

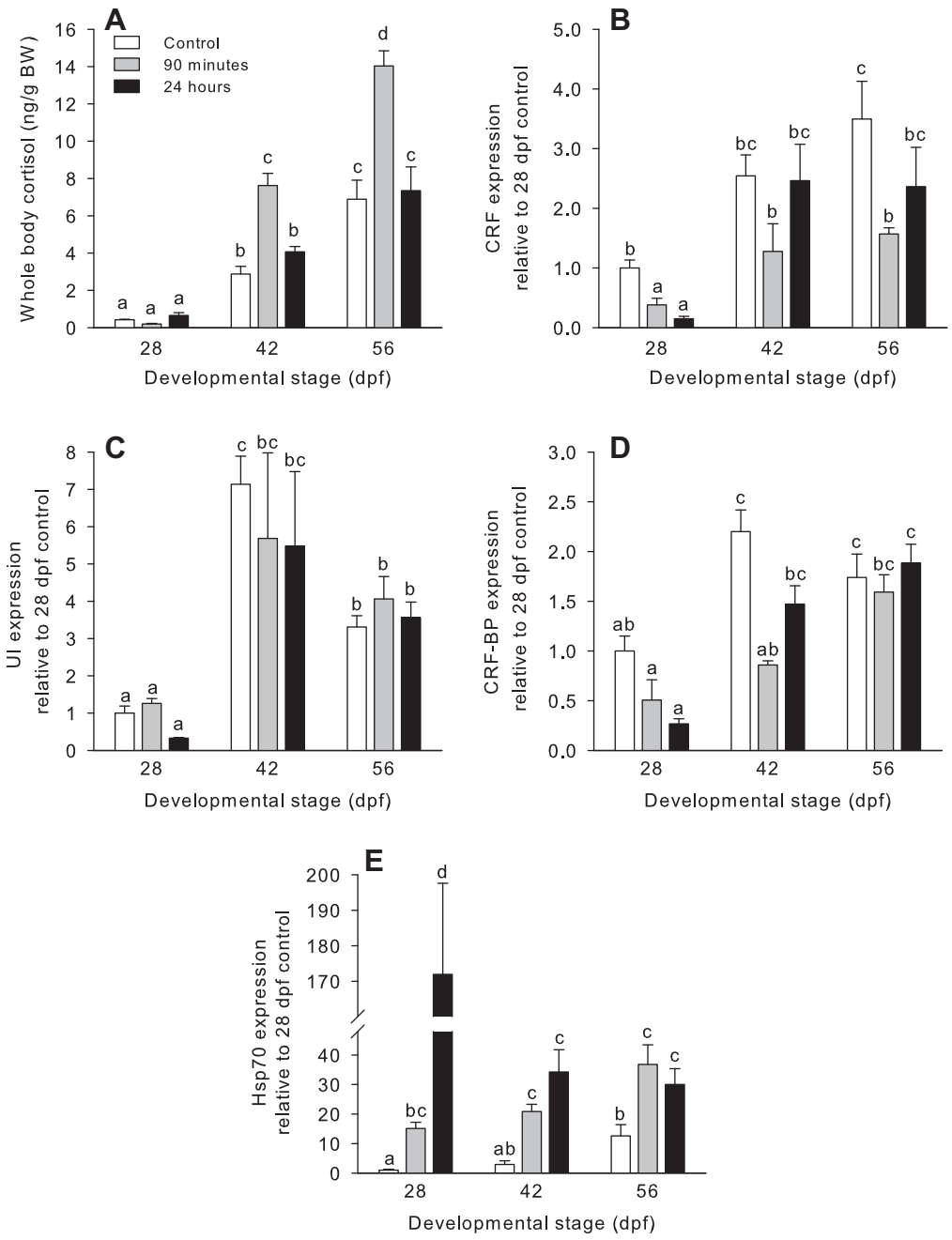

Fig. 4. Effects of hypoxia exposure (15% O_2 saturation; 1.7 mg/L) on (A) whole-body cortisol and on the expression of (B) corticotropin-releasing factor (CRF), (C) urotensin I (UI), (D) CRF-binding protein (CRF-BP), and (E) heat shock protein 70 (Hsp70) in rainbow trout embryos (28 days post-fertilization; dpf) and larvae (42 and 56 dpf). All gene expression data is standardized to the expression of β-actin. For a given gene, the expression data is reported as fold-change from the respective 28 dpf control normoxic value. Bars for a given gene that do not share a common letter are significantly different from each other, as determined by a two-way ANOVA followed by Tukey's test for multiple comparisons ($n = 4–8$; $p < 0.05$). BW, body weight.

within individual embryos/larvae was observed at all developmental stages except at 56 dpf. These positive correlations, or lack thereof at 56 dpf, existed despite differences in the average expression levels of the three genes and were also observed in the chronic hypoxia experiment. Although the ability of CRF-BP to bind CRF and UI has not been determined in any teleost, the strong conservation of CRF-BPs across vertebrates [39,79] and the ability of mammalian CRF-BPs to bind CRF and UI with high affinity [15,69] suggest that CRF-BP may also be involved in mediating the signaling activity of CRF-related peptides in fish. If so, strong positive correlations between the expression of CRF-BP and both CRF and UI may be indicative of a large potential to sequester ligands and block the activation of CRF receptors by either CRF or UI. In contrast, since CRF-BP can attenuate CRF-stimulated ACTH release in mammals [62,69], the lack of correlation between CRF-BP expression and CRF/UI expression at 56 dpf in rainbow trout larvae may be responsible, at least in part, for the peak cortisol levels observed during the transition to exogenous feeding. Clearly, future studies aimed at determining the affinity of teleost CRF-related peptides for CRF-BP are needed to determine the physiological significance of the correlations reported in this study.

4.2. Effects of chronic hypoxia exposure on the stress response

We observed ontogenic differences in the stress response of rainbow trout to chronic hypoxia. Consistent with previous studies

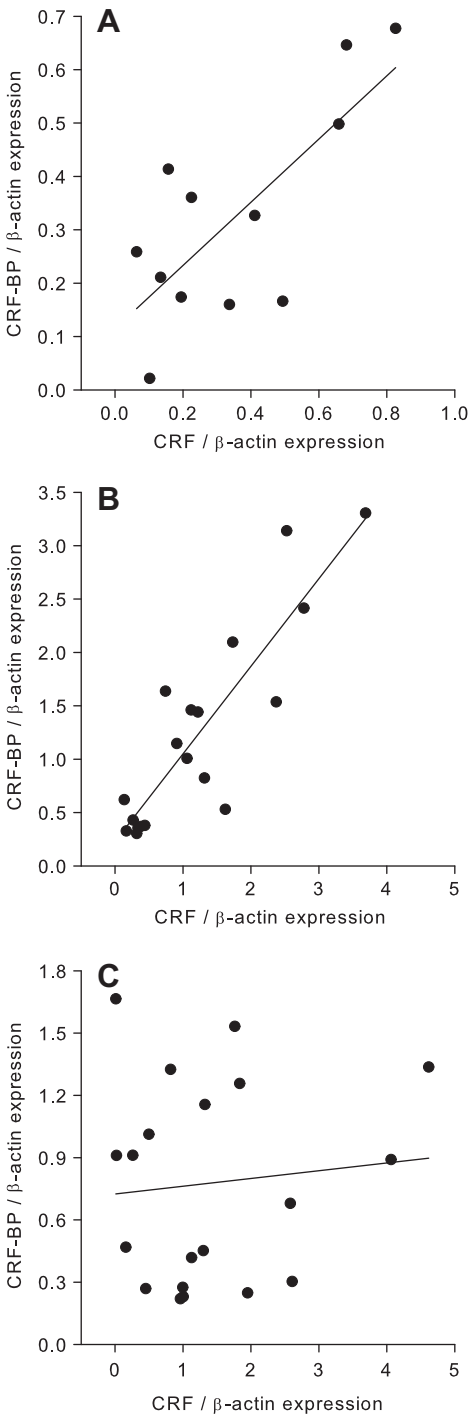

Fig. 5. Relationship between corticotropin-releasing factor (CRF) and CRF-binding protein (CRF-BP) gene expression of normoxic control and hypoxia-exposed (15% O_2 saturation; 1.7 mg/L) rainbow trout embryos and larvae at (A) 28 days post-fertilization (dpf; $R^2 = 0.56$, $p < 0.01$), (B) 42 dpf ($R^2 = 0.77$, $p < 0.001$), and (C) 56 dpf ($R^2 = 0.01$, $p > 0.05$). All gene expression data is standardized to the expression of β-actin. The relationships were analyzed with the Pearson product-moment correlation test.

investigating the impact of various stressors in rainbow trout [8,11,12,63], Chinook salmon (*O. tchawyschtcha*) [32], red drum (*Sciaenops ocellatus*) [7,59] and zebrafish [5], the ability of rainbow trout to secrete cortisol in response to chronic hypoxia was not observed until after hatch. The inability of embryos to activate the HPI axis during hypoxia exposure may be due in part to immaturities in the afferent sensory pathway involved in relaying this stressor to the brain and/or in immaturities of HPI axis components. For example, differentiation and innervation of O_2-sensing gill neuroepithelial cells in zebrafish occurs after hatch (see review by Jonz and Nurse) [41]. While the development of rainbow trout embryos is clearly affected by hypoxic conditions [24,55], the ontogeny of central hypoxia sensory integration is not known. Similarly, although ACTH can stimulate *in vitro* cortisol production in rainbow trout at hatch [11] and POMC is present in pituitary corticotropes at this time, the synthetic activity of these cells remains very low until ~4 weeks post-hatch [65,70]. Moreover, the expression level of the enzyme responsible for the proteolytic conversion of POMC into ACTH, prohormone convertase 1, remains low in zebrafish until after hatch [56]. Our results also suggest ontogenic differences in the impact of chronic hypoxia on the expression of CRF and CRF-BP. While the hypoxic treatment resulted in a sustained and marked reduction in the transcript abundance of CRF and CRF-BP in 28 dpf embryos, the effects were transient and less pronounced in 42 and 56 dpf larvae. Thus, the developmental differences in the impact of hypoxia on the HPI axis of rainbow trout appear to be the product of multiple factors.

To our knowledge, this study provides the first evidence of a hypoxia-induced increase in whole-body cortisol in larval fish. In contrast, a previous experiment on larval red drum found that moderate hypoxia had no impact on whole-body cortisol [59]. Although the chronic 24 h 15% O_2 saturation treatment used in this experiment would be quite severe for adult hypoxia-intolerant rainbow trout [17,20,31], it elicited a mere 2-fold increase in cortisol of larvae after 90 min and a return to basal conditions after 24 h. In preliminary experiments, exposure of 42 and 56 dpf rainbow trout larvae to 30% O_2 saturation for 24 h had no effect on whole-body cortisol. In contrast, adult rainbow trout chronically exposed to 35% O_2 saturation for 24 h were characterized by a 4-fold increase in plasma cortisol levels [18,16]. Overall, our results suggest that the HPI axis of larval rainbow trout is less responsive to hypoxic stressors than the HPI axis of adults.

The activation of the HPI axis in adult rainbow trout exposed to 30–35% O_2 saturation for 24 h is associated with increases in the mRNA levels of preoptic area CRF, UI, and CRF-BP [4,18,16]. In contrast, the increase in HPI axis activity observed in hypoxic larvae was generally accompanied by a transient decrease in the transcript levels of CRF and CRF-BP, and UI gene expression was unaffected. Since there is evidence that cortisol can exert negative feedback effects on the HPI axis in larval fish [58,75], it is tempting to speculate that the difference in the transcriptional response of CRF and CRF-BP to hypoxia between adult and larval fish is the product of feedback regulation. However, chronic hypoxia elicited a more pronounced and sustained reduction in CRF and CRF-BP gene expression in embryos, a developmental stage that lacks a stress response, than in larvae. Our results, therefore, suggest developmental differences in the effects of hypoxia on the CRF system in rainbow trout whereby an initial suppression of the CRF system during ontogeny may be gradually replaced by a stimulation of the same HPI axis regulators later in life.

Beyond ontogenic differences in the impact of chronic hypoxia on the expression of the CRF system, methodological differences may also have contributed to the above differences. While our studies on adult rainbow trout used isolated brain regions for gene expression measurements, we report whole body mRNA levels in this study. The CRF system has many functions beyond the endocrine stress response and is widely expressed in the brain and body of fish [2,4,21,23,57]; thus by reporting whole-body changes in transcript levels, the specific changes in preoptic area expression of the CRF system may been masked or diluted by expression in other tissues.

Hypoxia-exposed rainbow trout embryos and larvae also displayed a cellular stress response. Although all three developmental stages where characterized by increases in Hsp70 mRNA levels

with hypoxia exposure, the response was significantly more pronounced in the embryos than in the larvae. Whether this difference indicates an enhanced cellular stress response in embryos or a suppressed cellular stress response in larvae is not clear. In adult rainbow trout, both exogenous cortisol and stress-induced increases in circulating cortisol can depress the Hsp70 response to heat shock [1,14,13]. Assuming cortisol also modulates the cellular stress response during ontogeny, the development of a functional stress response in larvae may be associated with a reduced Hsp70 response to stressors. Interestingly, exposure of rainbow trout to 33% O_2 saturation for 6 h had no effect on myocardial Hsp70 protein expression [35]. Moreover, exposure of Altantic cod (*Gadus morhua*) to 35% O_2 saturation for 4 h [54] or Nile tilapia (*Oreochromis niloticus*) to 5% O_2 saturation for ~24 h [29] induced the expression of Hsp70 proteins in some tissues but not in others. While anoxia was associated with a large increase in Hsp70 gene expression in the brain and heart of crucian carp (*Carassius carassius*) held at 13° C, the response was muted at 8° C [68]. Taken together these results suggest that the effects of O_2 deprivation on the expression of Hsp70 in fish are species-specific and dependent on environmental factors and developmental stage.

Numerous studies have shown that HSPs play an important role in protein folding and stabilization in fish as in other organisms [28]. Increasingly, there is also evidence that Hsp70 may be particularly important for protection against the physiological strain of chronic hypoxia. Induction of Hsp70 promotes cardiac and brain protection from hypoxic injury in mammals [36,50], freshwater turtles [64] and fruit flies [9], and increases survival of adult *Drosophila* exposed to chronic hypoxia [9]. Functionally, Hsp70 may protect against hypoxia by inhibiting apoptosis, reducing protein aggregation [36] and by stabilizing newly synthesized hypoxia-inducible factor-1α [81], the master transcription factor responsible for the regulation of key biological functions during hypoxia. Whether the hypoxia-induced increase in Hsp70 gene expression in rainbow trout embryos and larvae can be attributed similar physiological roles is not known and certainly warrants further investigation.

5. Conclusion

This study presents novel data on the ontogeny of the CRF system and the impact of hypoxia on the endocrine and cellular stress responses in developing rainbow trout. Overall, the ontogenic profiles of CRF and CRF-BP transcript levels relative to the developmental changes in whole-body cortisol suggest an active involvement of the CRF system in the regulation of the HPI axis in larval rainbow trout. As previously observed with other stressors in developing fish, the responsiveness of the HPI axis to environmental hypoxia was only observed post-hatch. However, the transient increase in cortisol and general suppression of CRF gene expression with chronic hypoxia at the swim up and first feeding stages suggest that the HPI axis of larval rainbow trout may be less responsive to O_2 deprivation than in adults. In contrast, hypoxia elicited a marked increase in Hsp70 mRNA levels in embryos and a more modest cellular stress response after the formation of a functional HPI axis in larvae. Future studies are needed to determine the short- and long-term physiological significance of these developmental stage-specific effects of hypoxia exposure on the endocrine and cellular stress responses.

Acknowledgments

We would like to thank Ian Smith for help with figure formatting and presentation, and Bob Frank for technical assistance in the Hagen Aqualab. This research was supported through a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to N.J.B.

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