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Ontogeny of the corticotropin-releasing factor system in zebrafish

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ABSTRACT

The corticotropin-releasing factor (CRF) system in fish functions to maintain homeostasis during stress in part by regulating cortisol production via the hypothalamus–pituitary–interrenal (HPI) axis. Towards understanding the role of the CRF system in vertebrate development, we describe the ontogeny of the CRF system, cortisol, and the stress response in the zebrafish, *Danio rerio*. Early embryonic expression of mRNA encoding CRF, urotensin I (UI), CRF-binding protein (CRF-BP), and two CRF receptors (CRF-R1 and CRF-R2) suggest a function in the early organization of the developing embryo. The expression patterns of CRF, UI, and CRF-BP in the larval brain are consistent with the adult distribution patterns for these genes and support HPI-axis independent functions. The relative amounts of CRF and UI mRNA in the heads and tails of developing and adult zebrafish suggest that CRF functions primarily in the brain while UI also plays an important role in the caudal neurosecretory system. The amount of cortisol in developing zebrafish is low and relatively constant through the first 6 days of development. The commencement of feeding after 4 dpf, however, significantly increases basal cortisol production. Finally, we show that zebrafish larvae are able to respond to an osmotic stressor as early as 3 dpf. Overall, results from this study establish the zebrafish as a model species for research on stress during ontogeny and offer new insights into an HPI-axis independent function for the CRF system during embryogenesis.

1. Introduction

Corticotropin-releasing factor (CRF) is the initial hormone in a signaling cascade that functions to regulate circulating glucocorticoids in vertebrates. In fish, CRF produced in the preoptic area of the brain stimulates the release of corticotropin (adrenocorticotropic hormone; ACTH) from the pituitary, which binds to melanocortin type 2 receptors (MC2R) on kidney interrenal cells to stimulate the production and release of cortisol. This pathway, known as the hypothalamus–pituitary–interrenal (HPI) axis, is activated in response to stress and is homologous to the HP-adrenal (HPA) axis of tetrapods (Flik et al., 2006). CRF and its related peptides, urocortin (tetrapods) and urotensin I (fish), signal via specific G-protein coupled receptors (CRF-R1 and CRF-R2) and are further regulated by a shared binding protein (CRF-BP). Together, these components are referred to as the CRF system and, in addition to their key role in the regulation of the endocrine stress response, they display a variety of functions ranging from food intake inhibition (Bernier, 2006) to behavioral modulation (Koob and Heinrichs, 1999; Lowry and Moore, 2006). Although there are many instances of conserved distribution and function of the CRF system in vertebrates, fish are unique in having another major site of CRF and UI production, the caudal neurosecretory system (CNSS). The CNSS, a neuroendocrine organ located at the caudal end of the spinal cord in fish, expresses CRF and UI at high levels (Lu et al., 2004; Craig et al., 2005; Bernier et al., 2008). Evidence suggests that these peptides are released directly into the circulation in response to salinity changes, allowing direct interaction with interrenal (Arnold-Reed and Balment, 1994; Kelsall and Balment, 1998) and epithelial tissues (Lederis et al., 1985).

Studies on the ontogeny of the HPI axis support a developmental role for the CRF system. CRF mRNA is first detectable in the telencephalon and hypothalamus of zebrafish (*Danio rerio*) embryos at 1 day post fertilization (dpf), and is first localized in the preoptic area at 2 dpf, around the time of hatching (Chandrasekar et al., 2007). Interestingly, this time also marks the beginning of pituitary–interrenal interaction (To et al., 2007) and the onset of steroid hormone biosynthesis (Alsop and Vijayan, 2008). While all components of the HPI axis are in place by 2 dpf, zebrafish do not display a stress-induced increase in cortisol until 4 dpf (Alsop and Vijayan, 2008). Like the zebrafish, many fish species are unresponsive to stress until after hatching (Barry et al., 1995a,b; Feist and Schreck, 2001; Pepels and Balm, 2004; Auperin and Geslin, 2008), however, common carp (*Cyprinus carpio*) embryos exhibit a cortisol response to handling just prior to hatch (Stouthart et al., 1998). Functional roles for the CRF system during ontogeny have been proposed for amphibians and birds. The onset of metamorphosis in several

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amphibian species is linked to central and peripheral expression of CRF and CRF-BP (Miranda et al., 2000; Boorse and Denver, 2002; Boorse et al., 2006), while the timing of hatching in chickens may be influenced by increased preoptic area CRF production and release (Vandenborne et al., 2005).

It is well recognized that exposure to glucocorticoids during development—experimentally, endogenously, or via maternal influence—can pose immediate and permanent consequences to an animal's development and resultant phenotype (Crespi and Denver, 2005; Kapoor et al., 2006; Viltart and Vanbesien-Mailliot, 2007; Wada, 2008; Breuner, 2008). For example, studies in birds show that exposure to elevated glucocorticoids during development alters HPA-axis responsiveness in adulthood (Hayward and Wingfield, 2004), and that the timing of this exposure (pre- vs. post-natal) affects the direction of the effect in fledglings (decrease vs. increase, respectively; Love and Williams, 2008). Given the conservation of the neuroendocrine stress axis among vertebrate taxa, much can be gained by studying the ontogeny of the HPI/A axis in non-mammals in terms of understanding the mechanisms that relate early life experiences to adult fitness. Zebrafish in particular offer several advantages, including a fully sequenced genome, rapid external development, and well-established techniques for genetic manipulation (see e.g., Pogoda and Hammerschmidt, 2007). Therefore, we used the zebrafish as a model organism for studying the early ontogeny of the CRF system, a first step in understanding the role of this important homeostatic regulator in vertebrate development. Using a combination of polymerase chain reaction and *in situ* hybridization, we describe the ontogeny of CRF, UI, CRF-R1, CRF-R2, and CRF-BP, from fertilization to yolk resorption. We further characterize the ontogeny of cortisol and show the influence of exogenous feeding on basal cortisol production following yolk resorption. Finally, we describe the onset of the endocrine stress response using an osmotic stressor (seawater exposure). This study yields a novel perspective on the role of the CRF system during vertebrate ontogeny.

2. Methods

2.1. Animals

Adult zebrafish (*D. rerio*) were held in 10L aquaria in mixed sex groups. Each aquarium was maintained at 26 °C on a 14:10 L:D simulated photoperiod and fed commercial flake food 2–3 times daily. The University of Guelph's Animal Care Committee approved care and use of the animals, as per the principles of the Canadian Council for Animal Care.

2.2. Spawning, rearing, and sampling procedures

Spawning baskets were sunk into each aquarium prior to the end of the photoperiod on the day preceding embryo collection, and removed 15 min following the start of the photoperiod the next day. Embryos were collected and raised at 28.5 °C in egg water (6% w/v Instant Ocean; Spectrum Brands Inc, Atlanta, GA), a portion of which was changed daily. Starting at 4 dpf, fry food (Hatchfry Encapsulon 0; Argent Laboratories, Redmond, WA) was added to the egg cups once daily. Unless otherwise stated, embryos younger than 24 hpf were terminally sampled by direct transfer to dry ice and all other stages were first anaesthetized in tricaine methanesulfonate (~200 mg/L; MS-222; Syndel, Vancouver, BC).

2.3. RT-PCR

To analyze CRF system expression during embryogenesis, embryos (50–150/*n*, $n = 2$) were collected at 6, 12, 24, and 48 h post fertilization (hpf). Unfertilized eggs (100–150/*n*, $n = 2$) were stripped from females to represent 0 hpf. Total RNA was extracted with Trizol according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA) and 5 μg was used for first-strand cDNA synthesis as previously described (Craig et al., 2005). Gene-specific primers were designed for CRF, UI, CRF-BP, and β-actin based on existing GenBank zebrafish sequences (Table 1). A BLAST search of the zebrafish genome using *Carassius auratus* CRF-R1 (AY688836) or *Oncorhynchus keta* CRF-R2 (AJ277158) yielded the respective zebrafish sequences, against which gene-specific primers were designed (Table 1). Each gene was separately amplified by reverse-transcription polymerase chain reaction with *Taq* DNA polymerase (Invitrogen) according to manufacturer's instructions. Thermocycling conditions were adjusted as follows: 5 min initial denaturation at 95 °C; 30–40 amplification cycles of denaturation (95 °C, 30 s), annealing at primer-specific temperatures (30 s), and extension (72 °C, 30 s); and a final extension at 72 °C for 5 min. Product sizes were verified by electrophoresis and product identities were confirmed by sequence analysis.

2.4. In situ hybridization

To describe the expression pattern of the CRF system in early life stages, larvae between 2 and 5 dpf ($n = 3$–6) were anaesthetized with MS-222 as above and fixed overnight in 4% paraformaldehyde at 4 °C. The samples were prepared for cryosectioning as previously described (Alderman and Bernier, 2007). Serial transverse cryosections (12 μm) were thaw-mounted on glass slides, desiccated overnight at 37 °C, and stored at −20 °C until processed for *in situ* hybridization. Antisense and sense riboprobes for CRF, UI, and CRF-BP were synthesized (see Table 1) and hybridized to serial sections as previously described (Alderman and Bernier, 2007). Morphological features (anterior and postoptic commissures, rostral and caudal boundaries of eyes and pharynx, chorda dorsalis) were used to determine the angle of section through each larva, allowing identification of positively stained regions in the larval brain. Nomenclature follows Mueller and Wullimann (2005). Images were taken with a digital camera using OpenLab software (Improvision Incorporated, Lexington, KY). Photomicrographs were matched for brightness and color in Adobe Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA) to increase the quality of the presentation; however, positive staining was not altered or enhanced.

2.5. Ontogeny of CRF and UI in the caudal neurosecretory system by qPCR

Larvae aged 1–4 and 6 dpf (20 larvae/*n*; $n = 3$ independent samples per stage) were anaesthetized and bisected under a dissecting microscope into "head" and "tail" regions at the caudal boundary

Table 1
Primer pair sequences used for RT-PCR* and to generate templates used in the synthesis of gene-specific riboprobes for *in situ* hybridization$^{\gamma}$. Each sequence is listed starting from the 5′-end and its corresponding sequence ID is listed. F, forward primer; R, reverse primer.

Gene	Primer sequence 5′–3′	Sequence ID
CRF*,γ	F: CGA GAC ATC CCA GTA TCC AA R: GAT GAC AGT GTT GCG CTT CT	BC085458
UI*,γ	F: TCC CAT TGG TCC TGC TCA TCA R: CAG GTA TTT GCG GTT CAG T	BX510372
CRF-BP*,γ	F: GCT GTG CTT CCT CCT GTT G R: CCT GAT TGG TGG AGC CTG A	XM678236
CRF-R1*	F: CAG CTC ACC ATG AAT CCA GA R: GAC GAC TGC TTG ATA CTG TG	XM691254
CRF-R2*	F: GAA TCG CTT ACA GAG AGT GT R: ACC ATC CAA TGA AGA GGA AG	XM681362
β-actin*	F: GGT ATC GTG ATG GAC TCT GG R: AGC CTC CGA TCC AGA CAG A	AF057040

of the heart. Additionally, adult whole brains and CNSS (seven terminal vertebrae) were collected ($n = 3$). One μg of Trizol-extracted (Invitrogen) total RNA was used for first-strand cDNA synthesis and CRF or UI were amplified separately using default settings on an ABI Sequence Detection System 7000 (Applied Biosystems Inc, Foster City, CA). Each 20 μl reaction consisted of 1× SYBR Green Master Mix (ABI), 0.1 μM each of gene-specific primers (CRF-fwd: 5′-gccgcgcaaagttcaaaa-3′, CRF-rev: 5′-gcgaggagaatctgtgcgtaa-3′; UI-fwd: 5′-ccgcctgttccctttggt-3′, UI-rev: 5′-tccacctgctcagaaacatg at-3′ based on the reverse complement sequence), and 1.67:20 (v/v) cDNA. Melt curve analysis confirmed the amplification of a single gene product in each reaction. Primer-specific amplification efficiencies were determined by constructing a standard curve of serially-diluted cDNA. For each sample, the relative amount of starting template was determined by fitting the average threshold cycle value to the antilog of the standard curve, then normalizing to the expression of elongation factor-1α (fwd: 5′-gggcaagggctcct tcaa-3′; rev: 5′-cgctcggccttcagtttg-3′).

2.6. Cortisol ontogeny

Embryos and larvae were collected every 24 h from 1 to 6 dpf (10 pooled lavae/n, $n = 3$–12 independent samples per time point). The 4 dpf larvae were collected prior to feeding. Cortisol was methanol-extracted from homogenized samples then purified with C-18 octadecyl-packed minicolumns (Amersham Biosciences, Piscataway, NJ) as previously described (Lister and Van Der Kraak, 2008). The evaporated eluate was reconstituted in 200–250 μl assay buffer and cortisol was quantified with a 96-well colorimetric enzyme immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI). All cortisol data was adjusted to an 87% average extraction efficiency (SE = 1.5%; $n = 15$), and standardized against the protein content of each homogenate (Bio-Rad Protein Assay with bovine serum albumin standards; Bio-Rad Laboritories, Hercules, CA). The extraction protocol was validated with a dilution series of extracted 3 dpf larvae, which ran parallel to the standard curve. The detection limit of the assay is 12 pg/ml.

2.7. Effect of feeding on cortisol production

Embryos were allocated into *6dpf fed* and *6dpf unfed* treatment groups ($n = 5$ independent samples). The *6dpf unfed* group was not offered any food during the course of the experiment, whereas the *6dpf fed* group was supplied fry food once daily from 4 dpf until sampling, for a total of three feedings. One hour after feeding on the day of sampling, larvae were terminally anaesthetized with tricaine. Feeding was confirmed visually by the presence of food in the gut and only larvae that had eaten were collected to dry ice for subsequent cortisol analysis (10 pooled larvae/n). The *6dpf unfed* groups were similarly handled, and time to snap freezing was kept under 1 min for all groups. The remaining larvae in each treatment group were fixed overnight in 4% PFA and individual body lengths (excluding caudal fin) were measured from digital images using OpenLab software. Cortisol was extracted and measured as above.

2.8. Cortisol stress response to an osmotic challenge

Embryos from eight separate spawning events were separated into control and treatment groups and followed through time for up to three time-points (2, 3, or 4 dpf). Only one control and one treatment from each spawning event were used at a given time point, allowing direct comparison of the endocrine stress response in a treatment group to its biological control. Embryos (20 pooled/n; $n = 4$–8) were raised in 100 ml beakers containing mesh-bottomed insert cups to allow quick transfer and exposure of animals to treatment conditions. Two hours after the onset of the photoperiod on day 2, 3, and 4 post fertilization, the insert cups were carefully moved to beakers containing 100 ml of either seawater (SW; 33 ppt) or normal egg water (control). After 15 min of exposure to the osmotic challenge or control conditions, the insert cups were transferred to MS-222 (200 mg/L) and the embryos/larvae were quickly snap-frozen (<1 min). The timing of the stressor used for this study was chosen following a detailed time trial in adult zebrafish exposed to a physical disturbance, where a peak cortisol increase was observed 15 min post-stress (Fuzzen et al., unpublished observation). Preliminary experiments confirmed 100% survival (beating heart) of 4 dpf larvae after a 15 min seawater exposure and 2 h recovery in egg water. Cortisol was extracted and measured as above.

2.9. Statistics

Differences in gene expression in the head and tail of zebrafish were analyzed by a two-way analysis of variance (ANOVA) with developmental age and body region as factors. Group differences were identified by a Holm–Sidak test for multiple comparisons. Changes in cortisol levels through development were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey test for multiple comparisons. Differences in body length and cortisol between fed and unfed fish were analyzed by a Student's t-test. The effect of seawater exposure on cortisol production at 2, 3, and 4 dpf was analyzed by paired t-tests. Any data set that did not meet the assumption of normality was log-transformed prior to analysis. All analyses were performed with SigmaStat 3.0 ($p < 0.05$) and data is expressed as mean ± SE.

3. Results

3.1. Ontogeny of the CRF system

Gene expression of the CRF system during embryogenesis was determined by RT-PCR and is summarized in Fig. 1. Unfertilized eggs (0 hpf) were positive for CRF, CRF-BP, CRF-R1 and CRF-R2 mRNA; however, UI was not detected at this stage. By 6 hpf, endogenous transcription of all genes was evident and, with the exception of the two CRF receptors, continued to be expressed through embryogenesis to 48 hpf. CRF-R1 was not detectable after 6 hpf, and CRF-R2 expression decreased to undetectable levels by 48 hpf.

The mRNA expression patterns in the brain for CRF, UI, and CRF-BP were described using *in situ* hybridization in yolk-sac larvae (2–5 dpf). At all stages examined, CRF was detected in bilateral pairs of cells in the preoptic region, thalamus, and rostral medulla oblongata. Given the consistency of the expression pattern

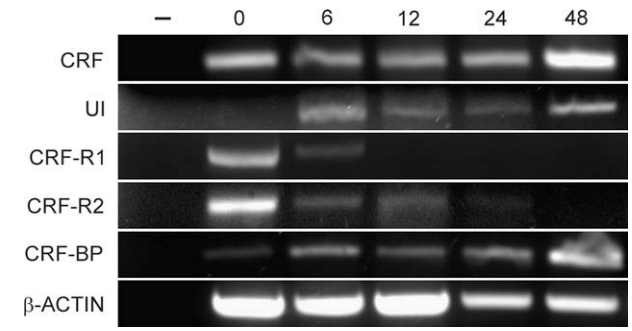

Fig. 1. Developmental expression profile of the CRF System as determined by RT-PCR. Time of development (in hpf) is indicated at the top of each lane. A no-template control reaction (–) was included for all genes, and β-actin was amplified for positive control.

throughout the larval period, we have shown only representative sections from 2 dpf (Fig. 2A and C) and 5 dpf (Fig. 2B and D). In half of the 2 dpf larvae, one bilateral pair of cells in the thalamus was positive for UI expression (Fig. 3A); this pattern was not visible in any of the older larvae. From 2 dpf to 4 dpf, UI mRNA was detected in the medulla oblongata (Fig. 3B–D). No UI expression was detected in the brain of larvae at 5 dpf. CRF-BP was expressed extensively in the larval brain at all stages. Expression in the subpallium and preoptic area was characterized by one or two bilateral pairs of cells (Fig. 4A–B and C–D, respectively). CRF-BP expression in the thalamus was detected as a small cluster of cells just above the dorsal reach of the retina at 2 dpf (Fig. 4E). By 4 dpf, expression in this region was reduced to a single pair of cells (not shown), and by 5 dpf were positioned just below the dorsal edge of the retina, closer to the midline (Fig. 4F). CRF-BP expression in the hypothalamus was first evident as an unorganized cluster of cells at 2 dpf (Fig. 4G). At 3 dpf, these cells formed a horizontal line at the base of the hypothalamus (Fig. 4G inset) and remained as such through to 5 dpf (Fig. 4H). A prominent cluster of CRF-BP-positive cells was found in the medulla oblongata at all stages (Fig. 4I and J). Both CRF and CRF-BP were also detected in the larval retina. CRF was visible in one or two retinal cells starting at 2 dpf (not shown), and the number of positively stained cells increased as development progressed to 5 dpf (Fig. 5A). CRF-BP was first detected in the retina at 4 dpf (not shown) and was more prominent by 5 dpf (Fig. 5B). All sections hybridized with sense probes did not show staining, confirming the specificity of the antisense probes.

The ontogeny of CRF and UI expression was further analyzed by qPCR to describe the separate contribution of brain (head) and

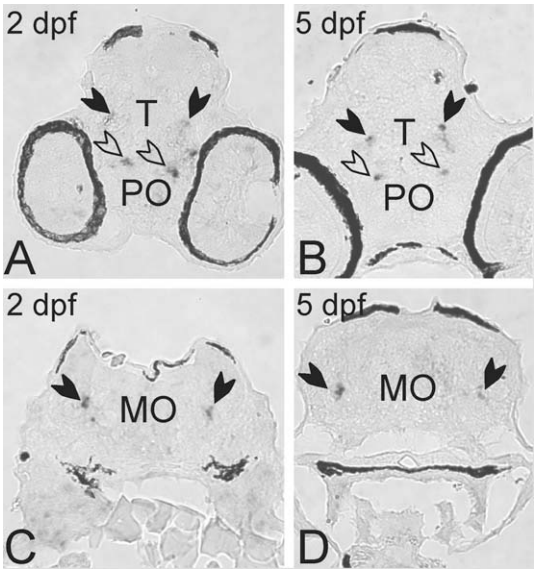

Fig. 2. Representative transverse sections through larva heads showing the distribution of CRF mRNA in the brain, as determined by *in situ* hybridization. (A and B) Paired CRF-expressing cells are stained symmetrically between the heavily pigmented retinas in the thalamus (T, closed arrowheads) and preoptic region (PO, open arrowheads). (C and D) Arrowheads indicate paired cells positive for CRF mRNA in the medulla oblongata (MO). Developmental stages are indicated in days post fertilization (dpf).

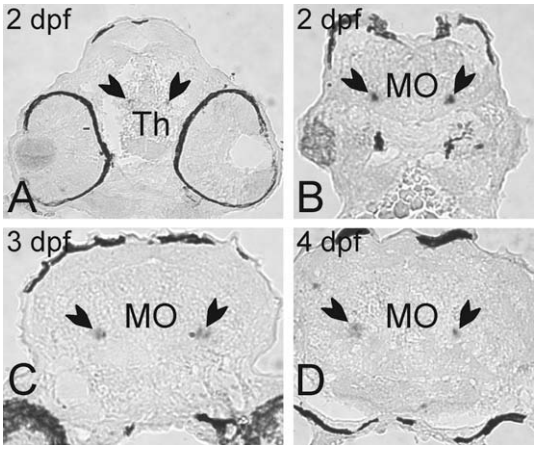

Fig. 3. Representative transverse sections through larva heads showing the distribution of UI mRNA in the brain, as determined by *in situ* hybridization. (A) Paired UI-expressing cells (arrowheads) in the thalamus (Th). (B–D) Arrowheads indicated paired cells positive for UI mRNA in the medulla oblongata (MO). Developmental stages are indicated in dpf.

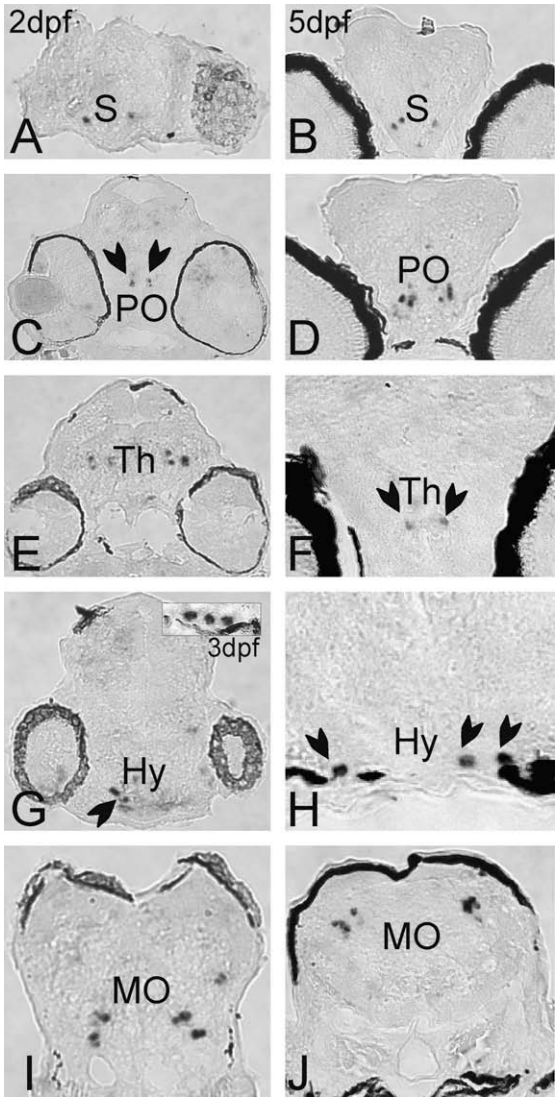

Fig. 4. Representative transverse sections through larva heads showing the distribution of CRF-BP mRNA in the brain, as determined by *in situ* hybridization. All images in the left hand column are of 2 dpf embryos, while all images in the right hand column are 5 dpf embryos, unless otherwise indicated. (A and B) Paired CRF-BP-expressing cells in the subpallium (S). Preoptic area (PO) expression of CRF-BP in pairs (C, arrowheads) or clusters (D) of cells. Expression of CRF-BP in the thalamus (Th) occurred as a small group of cells (E) or a single pair (F, arrowheads). Expression of CRF-BP in the hypothalamus (Hy) was first evident as a small cluster of cells at 2 dpf (G), then appeared as a horizontal row of positively stained cells from 3 dpf (inset G) to 5 dpf (H). (I and J) The medulla oblongata (MO) contained clusters of CRF-BP-expressing cells at all stages.

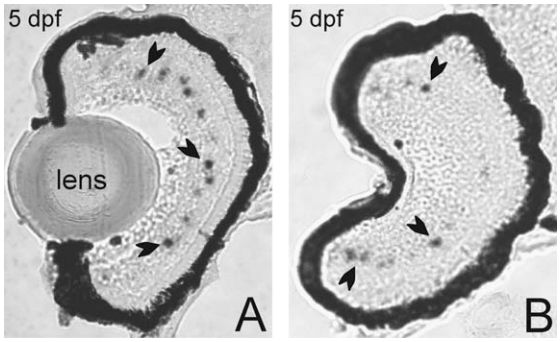

Fig. 5. Representative transverse sections showing CRF (A) and CRF-BP (B) mRNA expression in the retina of 5 dpf larvae, as determined by *in situ* hybridization. Three positively stained cells are labeled with arrowheads in each image. The solid black band seen in both images is the pigment epithelium.

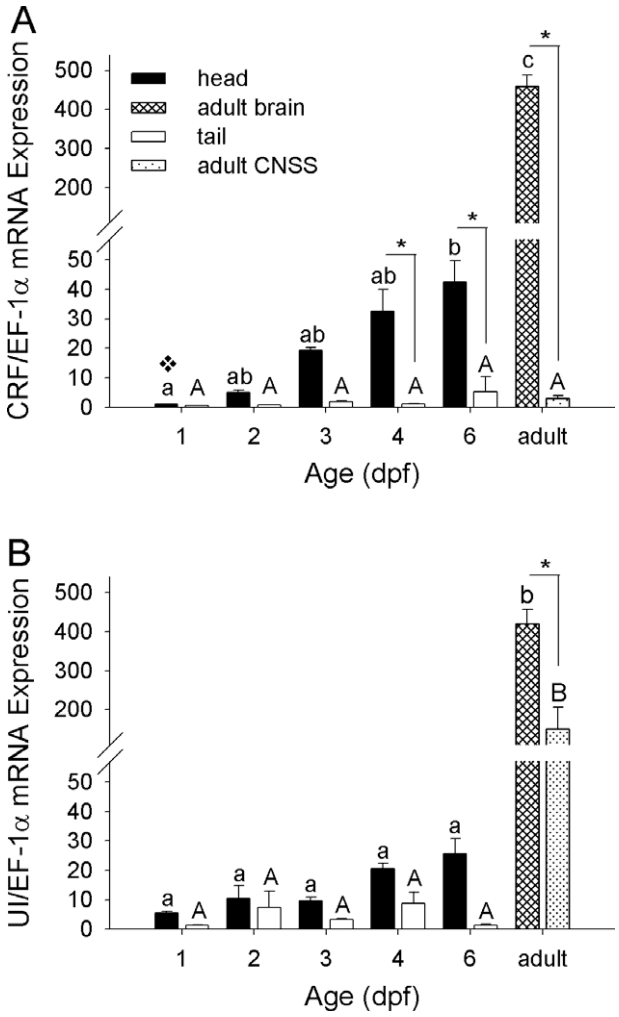

Fig. 6. CRF (A) and UI (B) mRNA expression, relative to elongation factor-1α (EF-1α), in the head and tail of zebrafish aged 1–6 dpf. Gene expression was measured by qPCR from 20 pooled heads or tails ($n = 3$). Amount of CRF and UI in adult whole brain and caudal neurosecretory system (CNSS) are shown for comparison ($n = 3$), and all data is expressed relative to the amount of CRF in embryo heads at 1 dpf (❖). Statistical differences between groups were determined using a two-way ANOVA and Holm–Sidak test for multiple comparisons ($\alpha = 0.05$). Lower case letters show differences in head/brain gene expression, while upper case letters show differences in tail/CNSS gene expression. An ∗ indicates a statistical difference in gene expression within a given age.

CNSS (tail) production. Each gene showed a unique expression profile. CRF expression in the heads of zebrafish increased steadily during development and was 48-fold higher at 6 dpf than at 1 dpf ($p < 0.01$). By 4 dpf, there was significantly more CRF mRNA in the heads of zebrafish larvae than in the tails ($p < 0.05$), and tail expression remained low and constant through to adulthood (Fig. 6A). In contrast, UI expression in zebrafish heads did not significantly increase during the first 6 days of development, nor was there any change in tail UI expression. There was also no difference in the amount of UI mRNA in the heads and tails of zebrafish during development (Fig. 6B). In adult zebrafish, there was significantly more CRF and UI mRNA in the brain than in the CNSS ($p < 0.001$), and the level of expression of the two genes was comparable. UI mRNA in the adult CNSS, however, exceeded CRF expression by approximately 50-fold.

3.2. Ontogeny of basal and stress-induced cortisol production

The amount of cortisol in zebrafish embryos was low and consistent ($0.75 \pm 8.9 \times 10^{-2}$ and 0.84 ± 0.32 pg cortisol/embryo, or $7.4 \times 10^{-2} \pm 4.6 \times 10^{-4}$ and $5.8 \times 10^{-2} \pm 2.3 \times 10^{-2}$ pg cortisol/μg protein at 1 and 2 dpf, respectively). A peak increase in cortisol occurred at 5 dpf (1.2 ± 0.16 pg cortisol/larva or $0.18 \pm 3.5 \times 10^{-2}$ pg cortisol/μg protein), followed by a significant decrease at 6 dpf ($p < 0.05$; Fig. 7A). There was no statistical difference in protein content of fed and unfed larvae, therefore cortisol data for the feeding study is expressed in pg/μg protein. Fed larvae compared to unfed larvae at 6 dpf had 1.8-fold higher whole body cortisol ($0.11 \pm 1.2 \times 10^{-2}$ pg/μg protein vs. $6.0 \times 10^{-2} \pm 8.1 \times 10^{-3}$ pg/μg protein, respectively; $p < 0.01$; Fig. 7B) and were significantly longer (3.53 ± 0.01 mm vs. 3.48 ± 0.01 mm; $n = 119$ and 123, respectively; $p < 0.001$; Fig. 7C).

To determine when zebrafish are first able to increase cortisol production in response to a stressor, we challenged fish at 2, 3, and 4 dpf to an osmotic stressor, seawater exposure. At 2 dpf, no difference in cortisol was observed between control and SW-exposed embryos. At 3 dpf, SW-exposed larvae had, on average, 1.3-fold higher cortisol than their respective controls ($p < 0.05$). By 4 dpf, an average 1.7-fold increase in cortisol was observed in SW-exposed larvae compared to controls ($p < 0.001$). Results of the osmotic challenge are summarized in Fig. 8.

4. Discussion

We present original data on the expression patterns of CRF, UI, their shared receptors (CRF-R1 and CRF-R2) and binding protein (CRF-BP) in the developing zebrafish, representing the first ontogenic description of the entire CRF system in any fish species. We also show for the first time the influence of feeding on basal cortisol production and describe an endocrine stress response as early as 3 dpf. Together, the results of this study establish the zebrafish as a model species for stress ontogeny research and support a developmental function independent of HPI-axis regulation.

4.1. Expression of the CRF system in ontogeny

Although Chandrasekar et al. (2007) were unable to detect CRF by whole-mount *in situ* hybridization prior to 24 hpf, the application of RT-PCR in the current study describes early embryonic expression of not only CRF but the entire CRF system. Prior to fertilization, zebrafish ova contain detectable amounts of all CRF system genes except UI, showing for the first time maternal transcript deposition into the oocytes. Endogenous transcription of the CRF-system also occurs early in development, as all genes are detectable by 6 hpf (mid-blastula transition at 1.5 hpf). In tilapia larval heads (*Oreochromis mossambicus*), CRF content increases exponen-

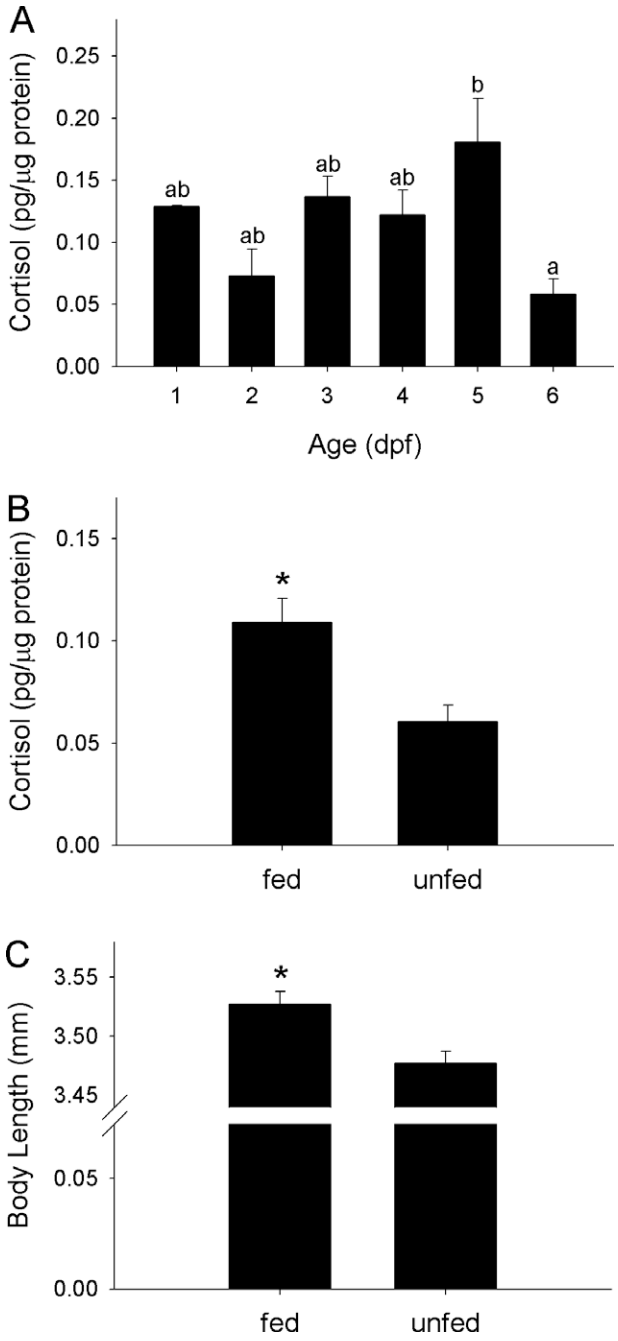

Fig. 7. Whole body cortisol content and influence of feeding in zebrafish aged 1–6 days post fertilization (dpf). (A) Cortisol ontogeny in embryos and larvae from 1 to 6 dpf (n = 3–12 independent samples of 10 pooled individuals). Data was normalized to the protein content of homogenates and is expressed as mean ± SE. Symbols that do not share a common letter are statistically different (one-way ANOVA and Tukey test at α = 0.05). Effect of feeding on basal cortisol levels (B) and body length (C) at 6 dpf (n = 5 independent samples of 10 pooled larvae). Larvae were either not fed or fed three times from 4–6 dpf until sampled. Data is presented as mean ± SE and $*$ indicates a significant difference (t-test; $p < 0.01$ and $p < 0.001$ in B and C, respectively).

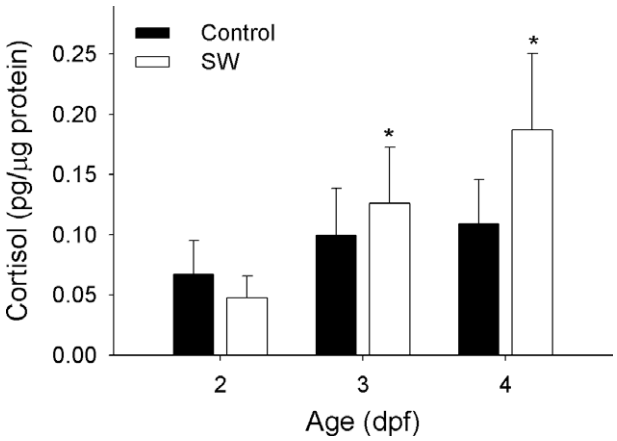

Fig. 8. The ontogeny of the cortisol stress response in zebrafish was determined by exposing larvae to full-strength seawater (SW) for 15 min at 2, 3 and 4 dpf (n = 4–8 independent samples of 20 pooled individuals). Cortisol content was normalized to the protein content of homogenates and is expressed as mean ± SE. Differences between control (black bars) and SW-exposed (white bars) larvae at each time point were determined by paired t-tests (α = 0.05). An $*$ indicates a significant difference within an age group.

tially through development (Pepels and Balm, 2004) which is in line with the age-dependent increase in head expression CRF mRNA observed in this study. The weak CRF receptor expression during mid-embryogenesis and the subsequent undetectable levels may indicate that embryonic production of this gene is minimal. Alternatively, mRNA expression of receptors may be down-regulated as part of a homologous desensitization strategy following ligand binding, as observed with mammalian CRF receptors (Hauger and Dautzenberg, 2000). The expression of CRF-BP throughout embryogenesis and its broad distribution in the larval brain warrant further investigation into the function of this binding-protein. Studies to date suggest that CRF-BP inhibits CRF signaling by sequestering the ligand and preventing receptor binding (Petraglia et al., 1993, 1996; Boorse et al., 2006).

The unique distribution patterns of CRF and UI in the adult zebrafish brain imply differential roles for these peptides in the central nervous system (Alderman and Bernier, 2007). Evidence provided in this study lends further support to this idea. For example, there is a marked increase in head expression of CRF during development through to adulthood yet low and constant expression in the tail/CNSS. Conversely, UI expression remains relatively constant in the head and tail during the first 6 days of development, but shows a pronounced increase in both regions by adulthood. Taken together, these results implicate UI but not CRF in a major functional role in the CNSS. This contrasts results in other fish studies comparing UI and CRF levels in the CNSS. Both genes are expressed at similarly high levels in both rainbow trout (*Oncorhynchus mykiss*; Bernier et al., 2008) and European flounder (*Platichthys flesus*; Lu et al., 2004). More work is needed on a diverse array of Actinopterygians to elucidate the significance of this difference. Similarly, investigations on the affinity of CRF and UI for each receptor subtype and CRF-BP are needed to support differential functions of the CRF-related peptides in fish.

Both CRF and CRF-BP are expressed during zebrafish ontogeny in the retina. There have been several reports of CRF system expression in vertebrate retinas ranging from fish (Chandrasekar et al., 2007; Chen and Fernald, 2008; present study) to humans (Zmijewski et al., 2007). Although the function of the retinal CRF system is not known, there is a marked up-regulation of CRF in the mammalian retina at the time of eye opening, when visual signals begin to be processed (Bagnoli et al., 2003).

4.2. Ontogeny of cortisol and the endocrine stress response

We observed a low and relatively constant level of cortisol in developing zebrafish. Our results coincide well with a previous description of cortisol ontogeny in zebrafish up to 4 dpf, after which we failed to see a sharp increase in whole body cortisol at 6 dpf (Alsop and Vijayan, 2008). Yolk reserves are depleting by 3

dpf (Kimmel et al., 1995), thus an exogenous cholesterol source is needed for cortisol synthesis. In our assessment of cortisol ontogeny, fish were offered food after 4 dpf but individual feeding was not confirmed. Therefore, we tested the hypothesis that food was required to elevate basal cortisol levels after 4 dpf, as observed previously (Alsop and Vijayan, 2008). Despite the clear difference between fed and unfed fish, cortisol in fish confirmed to have fed was not higher than our previous observation (see Fig. 7A). Cortisol ontogeny has been described in a variety of teleosts, including salmonids (*Oncorhynchus* spp.; Barry et al., 1995a; de Jesus and Hirano, 1992; Feist and Schreck, 2001; Auperin and Geslin, 2008), seabream (*Sparus* spp.; Deane and Woo, 2003; Szisch et al., 2005), flounder (*Paralichthys olivaceus*; de Jesus et al., 1991), and common carp (Stouthart et al., 1998). In general, the cortisol level in fertilized eggs is high and gradually decreases through embryogenesis as maternal deposits are depleted. Through endogenous production there is a subsequent rise in cortisol, however, the timing and magnitude of this increase is reportedly variable. Although species differences may exist, there is also evidence in rainbow trout (Barry et al., 1995a; Auperin and Geslin, 2008) and zebrafish (Alsop and Vijayan, 2008; present study) that the ontogeny of cortisol synthesis can vary between studies. Further work is needed to ascertain the source of this variation (genetic, environmental, or both).

Stress experienced early in life can have immediate and lasting phenotypic implications (Crespi and Denver, 2005; Kapoor et al., 2006; Viltart and Vanbesien-Mailliot, 2007; Wada, 2008; Breuner, 2008). In zebrafish, mounting evidence suggests that HPI-axis function begins early in life, around the time of hatching (Fig. 9). At this time, CRF mRNA is being transcribed in the preoptic area (Chandrasekar et al., 2007; present study), which coincides well with pituitary development and proopiomelanocortin (POMC; the mRNA that encodes several peptides including ACTH) expression (Herzog et al., 2003; Liu et al., 2003). Studies on mutant and knockdown zebrafish suggest that functional control of interrenal cells by pituitary hormones also occurs around 2 dpf (To et al., 2007), which corroborates the sudden increase in expression of MC2R (Alsop and Vijayan, 2008). Finally, the mRNA abundance of steroidogenic acute regulatory protein (StAR) and 11β-hydroxylase (11βH), both key enzymes in cortisol synthesis, peak around 2 dpf (Alsop and Vijayan, 2008). Although all necessary components for a functioning HPI axis are in place at hatching, a stress-induced increase in cortisol has not been observed in zebrafish until 3 dpf (osmotic stressor; present study) or 4 dpf (handling stressor; Alsop and Vijayan, 2008). In another study on a cyprinid species (*C. carpio*), a time course experimental approach showed a peak cortisol increase 5 min post-stress in unhatched embryos (Stouthart et al., 1998). A similar approach in zebrafish is needed to confirm whether or not an endocrine stress response can be achieved earlier in development. In other teleosts, an increase in cortisol in response to handling-type stressors is also not observed until after hatching (Barry et al., 1995a,b; Feist and Schreck, 2001; Pepels and Balm, 2004; Auperin and Geslin, 2008). As there are likely timing differences in the development and integration of sensory mechanisms, more studies are needed to determine exactly when an endocrine stress response can occur in a given species, and under what circumstances.

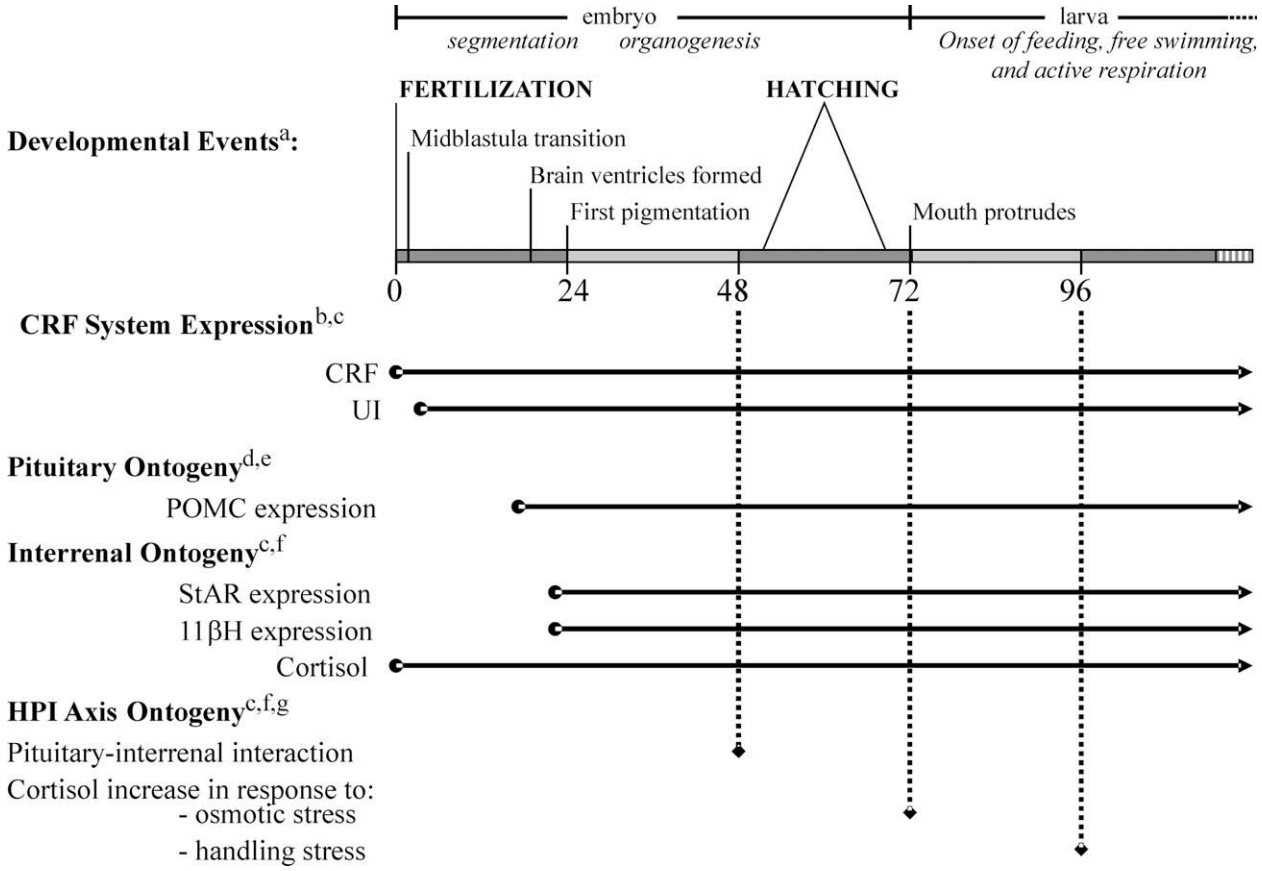

Fig. 9. The early development of the HPI axis in zebrafish is summarized from data presented in this study and literature reports of CRF-related peptide expression, pituitary and interrenal ontogeny, and stress responsiveness. A timeline (in hpf) and select list of developmental events are provided for reference. The expression of mRNA for CRF, UI, POMC, StAR, and 11βH all occur at or before 24 hpf, as indicated by black arrows. Cortisol is present throughout embryogenesis, and endogenous production occurs before hatching. Physiological communication between the pituitary and interrenal cells begins around 48 hpf, as indicated by a dashed vertical line. Zebrafish larvae first exhibit an endocrine stress response (elevated cortisol) at 3 dpf (osmotic stress) or 4 dpf (handling stress; dashed vertical lines). Letters refer to the following citations: a, Kimmel et al. (1995); b, Chandrasekar et al. (2007); c, Alderman and Bernier (present study); d, Hansen et al. (2003); e, Liu et al. (2003); f, Alsop and Vijayan (2008); g, To et al. (2007).

4.3. Developmental implications

The broad distribution of CRF, UI, and CRF-BP in larval brains observed in this study supports an HPI-axis independent function in development. Furthermore, despite the early embryonic expression of the CRF system, HPI-axis function is unlikely to begin prior to 2 dpf in zebrafish (as discussed above). Therefore, the CRF system may function in the growth and organization of the developing embryo. One such function might be to mediate cell survival (Bayatti and Behl, 2005). Several mammalian studies describe localized cytoprotective functions of CRF/UCN in keratinocytes (Mitsuma et al., 2001), cardiac tissues (Okosi et al., 1998; Brar et al., 1999; Ikeda et al., 2002), and neuronal cells (Fox et al., 1993; Pedersen et al., 2001). Although apoptosis signaling pathways are complex and can vary among tissues, two possible mechanisms for CRF-mediated cell survival have been described to date. Firstly, *in vitro* CRF treatment inhibits caspase 3 activity in neurotoxin-exposed GABAergic neurons (Madtes et al., 2004) and explants of metamorphosing tadpole tails (Boorse et al., 2006). This effect is likely achieved by preventing procaspase 3 activation (Radulovic et al., 2003). Secondly, the initiation of cAMP intracellular signaling by CRF may promote neuronal growth and survival by increasing transcription of brain-derived neurotrophic factor (Bayatti et al., 2005).

In addition to being ACTH-releasing factors, CRF-related peptides are also potent secretagogues of pituitary thyrotropin (thyroid-stimulating hormone; TSH) in fish (Larsen et al., 1998) and other non-mammalian vertebrates (Geris et al., 1999; Miranda et al., 2000). This dual function of the CRF system combined with the interactions of thyroid hormones and corticosteroids on peripheral tissues may carry important implications for vertebrate development (see reviews by Kuhn et al., 1998; De Groef et al., 2006). In zebrafish, endogenous thyroid hormone production begins at 3 dpf (Brown, 1997), and CRF is already being transcribed in the preoptic area at this time (Chandrasekar et al., 2007; present study). As in all vertebrates, thyroid hormones have been shown to play a vital role in the normal development of zebrafish (Brown, 1997; Liu and Chan, 2002).

The description of UI immunoreactive (ir) neurons outside the CNSS and its potent ACTH-releasing actions (Lederis et al., 1985) has traditionally supported investigations into non-urophysial actions of UI. Our report of UI mRNA expression at very early stages of embryogenesis encourages further research into the ontogenic-specific function(s) of this peptide. However, the prominent expression level of UI in the adult zebrafish CNSS (present study) and the appearance of UI-ir neurons in the developing CNSS around the time of hatching in tilapia (*Oreochromis niloticus*; Cioni et al., 2000) and chum salmon (*O. keta*; Oka et al., 1993), maintain the notion that UI plays an important role in CNSS function.

5. Conclusions

The ontogeny of the endocrine stress response and the developmental functions of the CRF system are crucial to understanding how early life experience can impact phenotype. Clearly, the tools and advantages of the zebrafish model will prove indispensable in developing this field of research.

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