General and Comparative Endocrinology 170 (2011) 365–373

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Differential effects of 17β-estradiol and 11-ketotestosterone on the endocrine stress response in zebrafish (*Danio rerio*)

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ARTICLE INFO

Article history:
Received 22 March 2010
Revised 14 October 2010
Accepted 18 October 2010
Available online 25 October 2010

Keywords:
HPI-axis
Cortisol
Sex steroids
Gene expression
crf
star
cyp11b2
Superfusion

ABSTRACT

Sexually dimorphic stress responses are present in species across all vertebrate taxa and it has been suggested that these effects are mediated by circulating sex steroids. While a few species of fish have been identified as having a sexually dimorphic stress response, there is conflicting evidence as to the effects of sex steroids on the stress axis. In this study, we tested whether zebrafish exhibit a sexually dimorphic cortisol stress response and whether 17β-estradiol (E2) or 11-ketotestosterone (11KT) modulate the activity of the hypothalamic-pituitary-interrenal (HPI) axis. To accomplish this, we quantified the whole body cortisol response to a physical stressor, cortisol release *in vitro*, and the expression of key HPI axis regulating genes of control and E2- or 11KT-exposed zebrafish. Under control conditions no dimorphisms in the HPI axis were apparent at rest or in response to a standardized stressor. In contrast, E2-exposure blunted the cortisol response of male fish *in vivo* and *in vitro* and as well as corticotropin-releasing factor (*crf*) expression in the pre-optic area (POA) of the brain. While the expression of some interrenal genes was suppressed by E2-exposure, these changes occurred in both male and female zebrafish. 11KT-exposure increased whole-body cortisol of males at rest and vortex-exposed females, but had no impact on the rate of cortisol synthesis *in vitro* or on POA *crf* expression. Therefore, while we found no evidence that zebrafish exhibit a sexually dimorphic cortisol stress response, both E2 and 11KT can modulate the activity of the HPI axis in this species and do so via different mechanisms.

1. Introduction

Exposure to stressors is part of daily life for all vertebrates and the stress response is responsible for maintaining homeostasis during these events. While the stress response is highly conserved among vertebrates, the magnitude can vary greatly between species and even between sexes. Sexually dimorphic stress responses have been noted in diverse groups of vertebrates including mammals [8], amphibians [21], and reptiles [10]. In mammals, this sexual dimorphism is responsible for differing susceptibility to many health issues including anxiety, depression, diabetes and heart disease [18]. While few studies have explored this subject in fish, a sexually dimorphic stress response has been noted in some elasmobranchs and teleosts [12,17,24,29,30]. Given our interest in characterizing the stress response in zebrafish (*Danio rerio*) and using this species as a model to understand the regulation of the endocrine stress response in vertebrates [1,2], we investigated in this study whether a sexually dimorphic stress response is present in zebrafish.

The involvement of sex steroids in the regulation of sexually dimorphic stress responses is a common theme among vertebrates. In rodents, 17β-estradiol (E2) and testosterone (T) have been shown to exaggerate and blunt the cortisol stress response, respectively [13,38]. Sex steroids have been shown to exert effects at different levels of the hypothalamic–pituitary–adrenal axis in mammals. Sexual dimorphisms in cortisol production by the adrenal [5] and adrenocorticotrophic hormone (ACTH) production by the pituitary are mediated by sex steroids [18]. Indeed, expression of the corticotropin-releasing factor (*crf*) gene in the paraventricular nucleus is enhanced following binding of estrogen to the estrogen receptor subtype α (ERα) and reduced by T through its actions on androgen receptors (AR) [14,37,42]. As in mammals, E2 exposure or implants exaggerate and T or 11-ketotestosterone (11KT) implants blunt the stress response of salmonids [20,32]. These steroids have been found to modulate cortisol production in the interrenal *in vitro,* suggesting that the interrenal is a target of sex steroids (rainbow trout, *Oncorhynchus mykiss* [45]; rainbow trout and chinook salmon, *Oncorhynchus tshawytscha* [26]).

We previously developed a standardized vortex stressor for zebrafish and found that the endocrine stress response was characterized by dynamic changes in gene expression at all levels of the hypothalamic-pituitary-interrenal (HPI) axis [11]. Using the same stressor, the current study determined whether the stress response

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doi:10.1016/j.ygcen.2010.10.014

of zebrafish is sexually dimorphic and whether sex steroids modulate the stress axis. Accordingly, male and female zebrafish were exposed to the carrier ethanol, E2, or 11KT for 48 h prior to stress exposure and whole-body cortisol levels were examined. 11KT was chosen in lieu of T since it is the primary androgen in male fish and in contrast to T, is not aromatized to E2 in the brain [6]. To determine the sites of action of sex steroids, the interrenals of E2- and 11KT-exposed fish were superfused with ACTH and the rate of cortisol synthesis was measured. In addition, the mRNA levels of pre-optic area (POA) *crf*, interrenal ACTH receptor melanocortin receptor 2 (MC2R), and the interrenal steroidogenic enzymes steroid acute regulatory protein (*star*), 11β-hydroxylase (*cyp11b2*), and 11β-hydroxysteroid dehydrogenase 2 (*hsd11b2*) were assessed in male and female zebrafish after 48 h of exposure to E2.

2. Materials and methods

2.1. Animals

Adult zebrafish (0.3–1.0 g) were obtained from DAP International (Etobicoke, ON) and housed at the Hagen Aqualab (University of Guelph, Guelph, ON) in an Aquatic Habitat for Accelerated Bioresearch (A-HAB) unit in 4 L tanks at a density of 3–5 fish per L at 26 °C with a 12:12 h light/dark (lights on at 7 am) photoperiod cycle. Fish were fed twice daily with a diet of commercial salmon fry fish pellets (Martin Mills, Elmira, ON) which was occasionally supplemented with blood worms. The University of Guelph Animal Care Committee approved care and use of the animals, as per the principles of the Canadian Council for Animal Care.

2.2. Experimental design

2.2.1. In vivo effects of E2 and 11KT on the HPI axis

To test the hypothesis that sex steroids play a role in modulating the stress axis, zebrafish were exposed to E2 or 11KT with the presence or absence of a stressor. Fish were exposed to either 0.01% ethanol (control), 17β-estradiol (100 ng/L; Sigma, St. Louis, MO) or 11KT (100 ng/L; Sigma) for 48 h. The dose of 100 ng/L was chosen from preliminary experiments and from previous findings showing that E2 at a dose of 100 ng/L has physiological effects on the reproductive system of male zebrafish [7,34]. We chose the same dose of 11KT so that the findings could be comparable between the two steroids. Three groups of eight sexually mature zebrafish (approximately 4 males and 4 females) of similar size were housed in 2L glass beakers for each treatment. Groups of six beakers were each on a recirculation circuit and individually supplied with freshly filtered and oxygenated water. Each beaker was covered with mesh and kept in a large (20 L) plastic container (overflow bin) which was outfitted with a tank filter, air stone, tank heater and the recirculation pump. In this setup, fish were fed once daily during a 7 d acclimation period. To expose fish to sex steroids, the water in the setup was replaced with control or steroid treated water at noon of day 0 and day 1 of the 48 h exposure period without disturbing the fish. Charcoal and biological filters were removed from all tanks during the exposure period to prevent removal or biological breakdown of steroids. Water samples were taken from the reservoir tank and from the experimental tank just prior to dosing and 1 h after dosing on day 1 and day 2 as well as at the end of day 2 of each of the experiments. Fish were fasted for 24 h before sampling and were sampled either immediately after the 48 h exposure period, or after the application of a vortex stress. For the vortex stress experiments, magnetic stir bars (Fisherbrand Magnetic Stir Bar, Octagonal, 5 × 1 cm, Fisher Scientific, Toronto,ON) and stir plates (Thermix® Stirrer Model 120S, Fisher Scientific) calibrated to 300 rpm prior to acclimation of fish were used to stress fish for 20 min. Fish were quickly (within 30 s) sacrificed with a lethal dose of 2-phenoxyethanol (1:500, Sigma) and were either flash frozen in liquid nitrogen for cortisol analysis or were placed directly on ice while livers were removed for measurement of vitellogenin mRNA content. All samples were stored at −80 °C until processed. This experiment was conducted twice using E2 in order to confirm observed effects, and once with 11KT.

2.2.2. Tissues within the HPI axis affected by E2 and 11KT

To determine whether sex steroids have direct effects on the HPI axis and/or the synthesis of cortisol, an experiment was performed in which zebrafish were exposed to E2 or 11KT (100 ng/L) for 48 h. After the exposure period, fish were euthanized as previously described and placed on ice. For RNA extraction and measurement of gene expression, POAs and head kidneys of male and female fish (n = 6–8) were removed with the aid of a stereoscopic zoom microscope (Nikon, SMZ1500). The POA was identified as the region rostral to where the optic nerves enter the brain, as depicted by Fuzzen et al. [11]. Additionally, head kidneys were removed for *in vitro* superfusion. Experimental holdings and exposure conditions were the same as described above.

Superfusion methods were modified from those of Metz et al. [27]. Head kidneys from euthanized zebrafish were immediately placed onto cheesecloth discs in a superfusion chamber (two head kidneys from fish of the same sex and treatment per chamber). In zebrafish, head kidney tissue contains the majority of cortisol and is confined to one well-defined organ situated dorsorostrally in the peritoneal wall (Fig. 1). Superfusion chambers were kept at 25 °C with a circulating water bath (RTE 10; Thermo Neslab, Newington NH, USA). Head kidneys were superfused with a 0.015 M HEPES/Tris-buffered medium (pH = 7.4; containing 128 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$ $2H_2O$, 0.25% (wt/vol) glucose, 0.03% (wt/vol) bovine serum albumin and 0.1 mM ascorbic acid) saturated with carbogen (95% O_2-5% CO_2) kept at 25 °C and pumped through the chambers at 50 μL/min using a multichannel peristaltic pump (Miniplus 3; Gilson, Middleton WI, USA). After 60 min, when cortisol release had reached a steady state, 10^{-6} M human ACTH

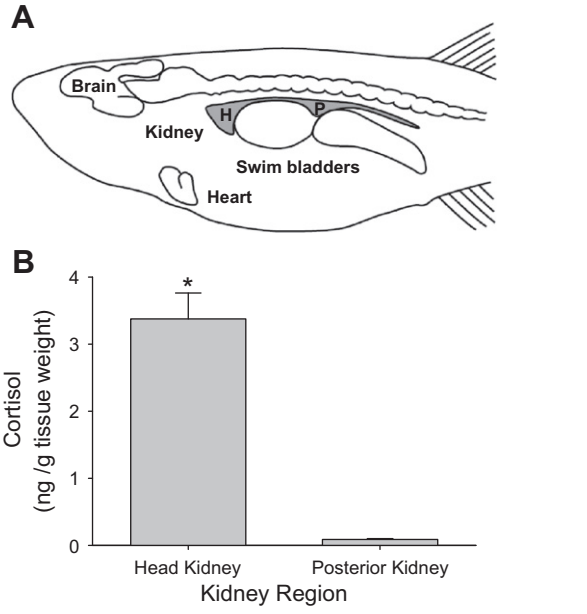

Fig. 1. (A) Diagram of a sagittal section of zebrafish displaying brain, heart, swim bladders, and kidney, subdivided into the head kidney (H) and posterior kidney (P). (B) concentration of cortisol (ng/g tissue) in specific regions of the kidney (n = 4). * Indicates a significant difference (p < 0.005) in cortisol concentration between kidney regions as determined with a Student's t-test.

(American Peptide Company Inc., Sunnyvale CA, USA) was administered for 20 min followed by a 70 min recovery period with the HEPES solution. Fractions of 10 mins were collected, stored on ice, and immediately analyzed for cortisol using a radioimmunoassay (RIA). After superfusion, head kidneys were removed from chambers, sonicated (Vibracell©, Sonics and Materials Inc., Newtown CT, USA) in 500 μL ddH_20 and analyzed for protein content (Bio-Rad Protein Assay with bovine serum albumin standards; Bio-Rad Laboratories, Hercules, CA). A total of 8–10 samples (2 fish per sample) per sex per treatment were superfused from control-, E2- and 11KT-treated fish. Values are expressed as rate of cortisol release (fmol/μg protein/min).

2.3. Experimental procedures

2.3.1. Whole body steroid extraction

Zebrafish were removed from −80 °C storage, weighed, sexed and placed in 5 mL glass tubes with 400 μL homogenizing buffer (80 mM Na_2HPO_4; 20 mM NaH_2PO_4; 100 mM NaCl; 1 mM ethylenediaminetetraacetic acid (EDTA; Fisher Scientific) and 750 counts of ^{3}H-cortisol (Amersham Biosciences, Piscataway, USA). Zebrafish were homogenized using a Euro Turrax T 20b (IKA Labortechnik, Staufen, Germany) mechanical homogenizer for 30 s on ice, and then sonicated (Vibracell©, Sonics and Materials Inc., Danbury CT. USA) for 10 s. Homogenates were extracted three times with 1 mL of methanol. After each addition of methanol, samples were vortexed, incubated for 60 min in the dark at 4 °C, centrifuged at 3000*g*, 4 °C for 5 min, flash frozen at −80 °C for 10 min and decanted into 20 mL scintillation vials. The second and third extractions were performed in the same manner as the first, with the exception of a shortened (30 min) incubation period. The methanol fractions of each sample were combined and dried under N_2 at room temperature.

Samples were purified as described by Lister et al. [22]. Briefly, samples were reconstituted with 300 μL of acetate buffer (2.35 mL glacial acetic acid, 1.23 g sodium acetate trihydrate, in 1 L; pH 4.0) and passed through a C_{18} solid phase extraction column (100 mg octadecyl [C_{18}], 1-mL disposable polypropylene minicolumn, Amersham Biosciences Corp, Piscataway NJ, USA), primed with 1 mL methanol and 1 mL double distilled water. After the addition of samples in the C_{18} columns, 1 mL of ultra-pure water (Fisher Scientific) and 1 mL of hexane (Fisher Scientific) were separately added and the eluates were discarded. Steroids were eluted from the C_{18} columns with 2 mL of ethyl acetate (1% methanol) and collected in 20 mL scintillation vials. Samples were dried under N_2 at room temperature, reconstituted in 3 mL of assay buffer (21.4 mM $Na_2HPO_4.7\ H_2O$; 9.3 mM $NaH_2PO_4.H2O$; pH 7.6; 0.1% gelatin; 0.01% thimerosal) and stored at −20 °C until analysis.

2.3.2. Water steroid extraction

Water samples were analyzed as described by Kramer et al. [16]. Briefly, samples were thawed and 5 mL aliquoted into 10 mL glass test tubes for extraction. Diethyl ether (Fisher Scientific; 3 mL) was added to each sample and the test tube capped prior to vortexing for 30 s. Samples were then flash frozen and the aqueous layer decanted into a 20 mL scintillation vial. Samples were extracted a total of three times with 3 mL of diethyl ether. The extracts for each sample were combined and dried overnight prior to being reconstituted with 650 μL of RIA buffer and analyzed by RIA.

2.3.3. Radioimmunoassay

Cortisol, E2 and 11KT were measured in 200 μL aliquots of samples (in triplicate, values were averaged) by RIA using the methods described by Bernier et al. [4]. Cortisol antibody was obtained from Clinical Endocrinology Laboratory (product code #R4866, University of California; Davis, CA), E2 antibody was obtained from MP Biomedicals (Solon, OH, USA) and 11KT antibody was kindly donated by Dr. T. Owen (Helix Biotech, Vancouver, B.C.). Whole-body extraction efficiency was determined for each sample from the initial 750 counts of ^{3}H steroid added prior to homogenizing and was found to be 74 ± 1% (n = 30). Water steroid extraction efficiency was determined to be 65 ± 3% (n = 7). Interassay and intra-assay variance for cortisol was 5.9% (n = 6), and 1.1% (n = 7) respectively. Intra-assay variance for E2 and 11KT were 2.2% (n = 6) and 1.8% (n = 6) respectively. Whole-body steroid values were presented as ng (cortisol and E2) or pg (11KT) whole-body steroid/g of individual body weight (BW) while water steroid values were presented as ng/L and all values were corrected for individual extraction efficiency.

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

Total RNA from individual (from one fish) tissue samples was extracted using TRIzol Reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One μg total RNA was treated with DNase I (DNase I amplification grade; Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Separate samples were identically treated without the addition of SuperScript II RNase H$^-$ reverse transcriptase or without the presence of RNA to verify the absence of genomic DNA or contaminated reagents. All reactions contained 10 μL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5 μL of 5 times diluted cDNA template, and 2.5 μL each of forward and reverse primers (0.4 μM). The cDNA products were amplified using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The following 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. All samples were assayed in duplicate and values were averaged. A standard curve was generated for each primer pair by serial dilution of RT product in yeast RNA (50 ng/mL; Sigma) to determine the efficiency of the primer amplification. A standard curve was produced for each gene by graphing the negative log of the dilution factor against the relative cycle threshold value. To be considered suitable for analysis, each primer pair was required to have a linear standard curve with an r^2 value above 0.98, have consistency among replicate Ct values, and primer amplification efficiency between 85% and 100%.

To correct for minor variations in template input and transcriptional efficiency, each sample was normalized to the expression level of either elongation factor-1α (*ef1a*; liver samples, 11KT-exposed POAs) or *18s* (E2-exposed POAs and head kidneys). E2-exposed POAs and head kidney samples were normalized against *18s* instead of *ef1a* due to significantly different levels of *ef1a* expression between treatment groups, eliminating it as an acceptable reference gene. Gene specific primer sequences for zebrafish vitellogenin (*vtg1*), *crf*, *star*, *mc2r*, *cyp11b2*, *hsd11b2*, *ef1a* and *18s*, are listed in Table 1, and were designed using Primer Express 3.0 (Applied Biosystems). Expression data were reported as fold change relative to controls.

2.4. Statistical analyses

A two-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was used to determine differences in whole-body cortisol content and mRNA expression using treatment and sex as variables. To determine if ACTH elicited a change in cortisol production over time, a one-way ANOVA followed by Dunnett's Test was performed. To analyze the superfusion experiments, the rate of cortisol production just prior to ACTH dose (at 60 min) and the highest rate of cortisol production after ACTH dose (at 80 or 90 min) were compared using a two-way ANOVA using sex and treatment (dose or no dose) as variables.

Table 1
Sequences of primer pairs used to amplify vitellogenin 1 (vtg1), corticotropin-releasing factor (crf), steroid acute regulatory protein (star), melanocortin 2 receptor (mc2r), 11β-hydroxylase (cyp11b2), 11β-hydroxysteroid dehydrogenase 2 (hsd11b2), *18s*, and elongation factor-1α (ef1a) in real-time reverse transcriptase-polymerase chain reaction assays.

	Sequence (5′ to 3′)	Accession No.
vtg1	F: TGC GTG AAG TTG TCA TGC TTG R: GAT CTC GTG GAT GGG CCT G	NM_001044897
crf	F: CGA GAC ATC CCA GTA TCC AA R: GAT GAC AGT GTT GCG CTT CT	NM_001007379
star	F: ACC TGT TTT CTG GCT GGG ATG R: GGG TCC ATT CTC AGC CCT TAC	NM_131663
mc2r	F: CTC CGT TCT CCC TTC ATC TG R: ATT GCC GGA TCA ATA ACA GC	NM_180971
cyp11b2	F: TGT GCT GAA GGT GAT TCT CG R: GCT CAT GCA CAT TCT GAG GA	NM_001080204
hsd11b2	F: TGC TGC TGG CTG TAC TTC AC R: TGC ATC CAA CTT CTT TGC TG	NM_212720
18s	F: ATT CTT GGA CCG GCG CAA GA R: CAT CTA AGG GCA TCA CAG AC	FJ915075
ef1a	F: GGG CAA GGG CTC CTT CAA R: CGC TCG GCC TTC AGT TTG	NM_131263

F, forward primer; R, reverse primer.

In all experiments, data that did not meet the assumption of normality were log-transformed prior to analysis. Any outliers that were determined to be greater than or less than the 2.0 × interquartile range from the upper quartile or lower quartile, respectively, were removed from the data set (no more than two outliers were found in any one treatment group). The number of samples used in gene expression analysis was variable due to the inability to extract a sufficient amount of total RNA from some samples and due to the elimination of outliers. All statistical analyses were performed using SigmaStat 3.0 (SPSS Inc, Chicago, IL, USA). $p < 0.05$ was considered statistically significant for all tests.

3. Results

3.1. Concentrations of steroid in the exposure water

The concentrations of E2 and 11KT in the reservoir and exposure tanks are given in Tables 2 and 3, respectively. In the E2 exposure experiment, control reservoirs and experimental tanks, receiving vehicle only, had low concentrations of E2 ranging from not detectable to 9.7 ng/L. Each of the reservoir tanks received 99–102% of the intended dose, while the experimental tanks were found to have 87–88% of reservoir concentrations after the water change on each of the dosing days. The concentration of E2 in exposure tanks was quickly depleted and only 18–29% of intended concentrations of E2 were present in the exposure tanks 24 h after application (Table 2). Similar results were found with the 11KT exposure experiment. Control reservoir and experimental tank concentrations of 11KT ranged from not detectable to 6.1 ng/L, reservoir tanks contained 94–100% of intended dose, and experimental tanks contained 87–89% of reservoir concentrations one h after the water change. 11KT levels were also quickly depleted from the experimental tanks with only nominal concentrations present 24 h after the application of sex steroids (Table 3).

Table 2
The concentrations of 17β-estradiol (E2) in water samples collected from reservoir and exposure tanks during a 48 h exposure.

Exposure duration (h)	Water E2 (ng/L)			
	Control treatment		E2 treatment	
	Reservoir tank	Exposure tank	Reservoir tank	Exposure tank
0	9.7	6.3	101.7	10.5
1		7.9		88.0
24	8.5	7.4	98.6	18.2
25		6.7		86.9
48		5.3		29.4

Table 3
The concentrations of 11-ketotestosterone (11KT) in water samples collected from reservoir and exposure tanks during a 48 h exposure.

Exposure duration (h)	Water 11KT (ng/L)			
	Control treatment		11KT treatment	
	Reservoir tank	Exposure tank	Reservoir tank	Exposure tank
0	ND	ND	100.2	ND
1		ND		89.2
24	ND	6.10	93.7	1.7
25		ND		81.4
48		ND		11.3

ND, not detectable.

3.2. In vivo effects of E2 and 11KT on the HPI axis

The vortex stress caused an increase in whole-body cortisol within 20 min. No differences were observed in basal or stressed-induced whole-body cortisol levels between male and female zebrafish under control conditions (Fig. 2A). While exposure to E2 did not affect basal or stress-induced cortisol levels in females, E2-treated males were found to have an attenuated cortisol response to the vortex stressor (Fig. 2A). The same pattern of response for males and females was observed when the E2 exposure and stress experiment was repeated (Fig. 2B). Neither E2 treatment nor the vortex stress had any effect on whole-body E2 levels (Fig. 2C). However, vitellogenin mRNA expression increased 3-fold in E2 exposed male, but not in female zebrafish (Fig. 3A).

11KT exposure increased resting whole-body cortisol levels in male, but not female zebrafish (Fig. 4A). Conversely, 11KT had no effect on the cortisol stress response of male zebrafish, but enhanced the cortisol stress response of females (Fig. 4A). While neither the vortex stress or 11KT exposure had an effect on whole-body 11KT levels in males, 48 h of exposure to 11KT prevented the stressor-induced increase in 11KT observed in females (Fig. 4B). 11KT exposure also caused a 3-fold decrease in male but not female liver *vtg1* mRNA expression (Fig. 3B).

3.3. Tissues within the HPI axis affected by E2 and 11KT

Superfused zebrafish head kidneys responded to ACTH application with a quick increase in cortisol secretion (Fig. 5). Peak secretion rates occurred between 20 and 30 min after the initial application of ACTH and secretion rates returned to pre-ACTH levels quickly after the cessation of ACTH application (Fig. 5). Application of ACTH to zebrafish head kidneys caused an increase in cortisol synthesis that was of comparable magnitude in males and females under control conditions (Fig. 6A). Exposure of zebrafish to E2 for 48 h before superfusion had no effect on basal and ACTH-stimulated secretion rates in female, but it blunted ACTH-stimulated cortisol release from male head kidneys (Fig. 6A). On the other hand, basal and ACTH-stimulated rates of cortisol production were unaltered by 11KT treatment in both sexes (Fig. 6B).

The relative level of expression of *crf* mRNA in the POA region was comparable in males and females under control conditions (Fig. 7A). While E2 exposure had no effect on female *crf* expression, E2-exposed males had 3-fold less *crf* mRNA expression than control males (Fig. 7A). In contrast, 11KT had no effect on *crf* mRNA levels in either sex (Fig. 7B). Male and female zebrafish had similar

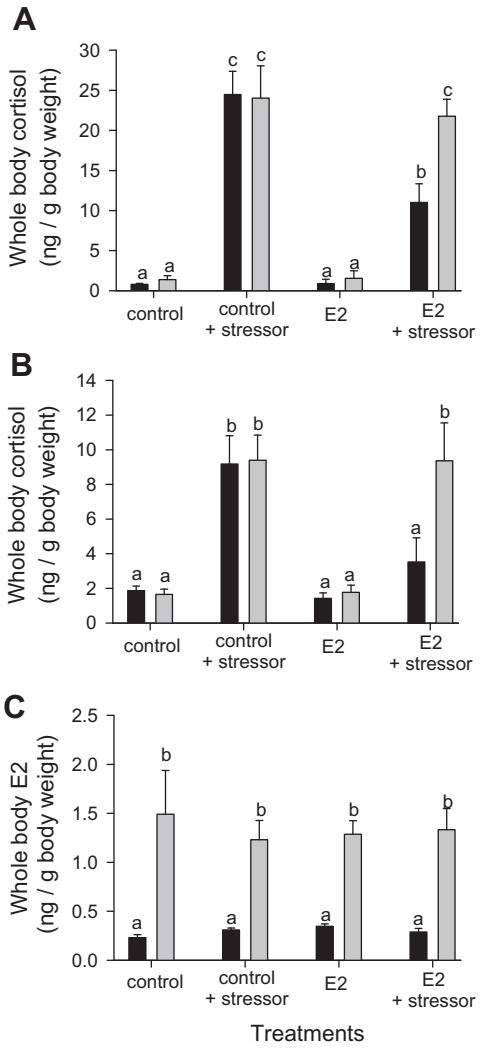

Fig. 2. Whole body cortisol levels (A) from trial one, and whole-body cortisol levels (B) and 17β-estradiol (E2) levels (C) from trial two of male (black bars) and female (grey bars) zebrafish exposed to either 0.01% ethanol (control), or E2 (100 ng/L) for 48 h. Zebrafish were sampled either before or after (+ stressor) a 20 min vortex stress at and intensity of 300 rpm. Values are means ± SEM. (n = 7–12). Bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA ($p < 0.001$) and by pairwise Tukey's test ($p < 0.05$).

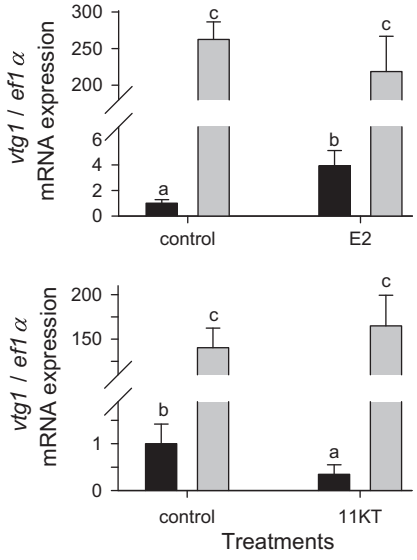

Fig. 3. Changes in liver vitellogenin (*vtg1*) mRNA levels in relation to elongation factor 1α (*ef1a*) mRNA levels of male (black bars) and female (grey bars) zebrafish exposed to 0.01% ethanol (control), 100 ng/L 17β-estradiol (E2; A) or 100 ng/L 11-ketotestosterone (11KT; B) for 48 h. Values are means ± S.E.M. (n = 5–8). For comparative purposes, the expression ratios are presented relative to the average male control value. Bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA ($p < 0.001$) and by a pairwise Tukey's test ($p < 0.05$).

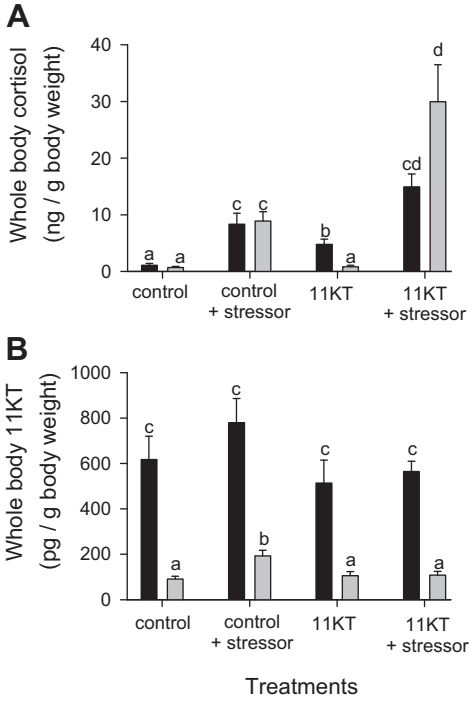

Fig. 4. Whole body cortisol levels (A) and 11-ketotestosterone levels (11KT; B) of male (black) and female (grey) zebrafish exposed to either 0.01% ethanol (control), or 11KT (100 ng/L) for 48 h. Zebrafish were sampled either before or after (+ stressor) a 20 min vortex stress at an intensity of 300 rpm. Values are means ± S.E.M. (n = 7–11). Bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA ($p < 0.001$) and by a pairwise Tukey's test ($p < 0.05$).

levels of head kidney *mc2r*, *cyp11b2* and *hsd11b2* expression under control conditions and with E2 exposure (Fig. 8A, C, and D). Female zebrafish were found to express three times more *star* mRNA in head kidney tissue than males and the expression in both sexes was unaltered by E2 exposure (Fig. 8B). Exposure to E2 caused a 3-fold decrease in the *mc2r* expression and a 2.5-fold decrease in *cyp11b2* expression, but had no effect on the expression of *hsd11b2* mRNA in head kidneys of zebrafish (Fig. 8A–D).

4. Discussion

In this study we compared the cortisol stress response of male and female zebrafish and found no sexual dimorphism in cortisol levels at rest or in response to a vortex stressor. We did, however, observe sexually dimorphic changes in the stress response of E2- and 11KT-exposed zebrafish. The attenuating effects of E2-exposure on male fish were evident in terms of reduced POA *crf* expression and decreased cortisol production by the interrenals. While 11KT-exposed males at rest and stressed females had elevated whole-body cortisol levels, this did not seem to be mediated through the HPI axis at the level of POA *crf* expression nor interrenal cortisol synthesis rate. These results suggest that the sex

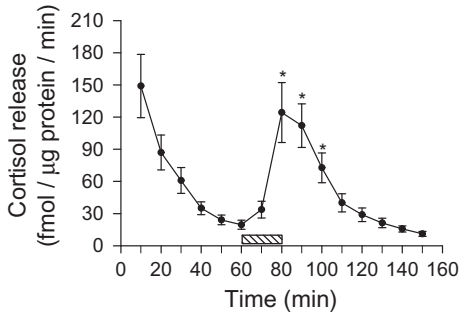

Fig. 5. *In vitro* rate of cortisol release (fmol/µg protein/min) from zebrafish head kidneys ($n = 11$) prior to, during and after superfusion with human ACTH (10^{-6} M; duration marked by dashed bar). Differences from the basal secretion rate (60 min) are indicated by $*$ as determined by a one way analysis of variance test ($p < 0.001$) and a Dunnett's test against the 60 min time point ($p < 0.05$). Note that only the time points between 60 and 150 min were used in the statistical analysis.

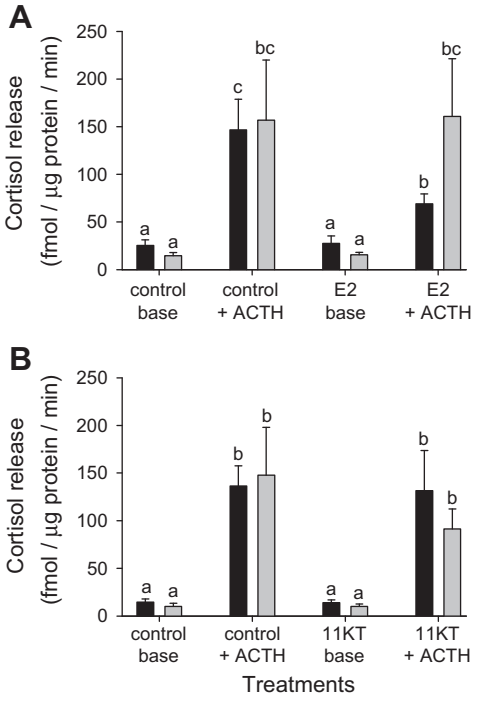

Fig. 6. Effects of 17β-estradiol (E2; A) or 11-ketotestosterone (11KT; B) on the basal (base) and ACTH-elicited cortisol secretion rate from the superfused interrenal tissue of zebrafish ($n = 7–10$). Fish were either kept under control (control) conditions or exposed to steroids for 48 h prior to the removal of the head kidney and quantification of the *in vitro* rate of cortisol release. Measurements were taken after 60 min of superfusion with buffer (base) and after being sitmulated with 10^{-6} M ACTH for 20 min (+ACTH). Bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA ($p < 0.001$) and by a pairwise Tukey's test ($p < 0.05$).

steroids E2 and 11KT modulate the cortisol stress response through different modes of action in zebrafish.

4.1. Testing for sexual dimorphisms in the zebrafish stress response

We observed no sexual dimorphism in either resting whole-body cortisol levels or in the cortisol response to a vortex stressor. There were also no differences in the *crf* mRNA content in the POA, or in resting or ACTH-stimulated cortisol production in the interrenals of male and female zebrafish. While few studies report the effects of stressors on both sexes, there is evidence of sexual

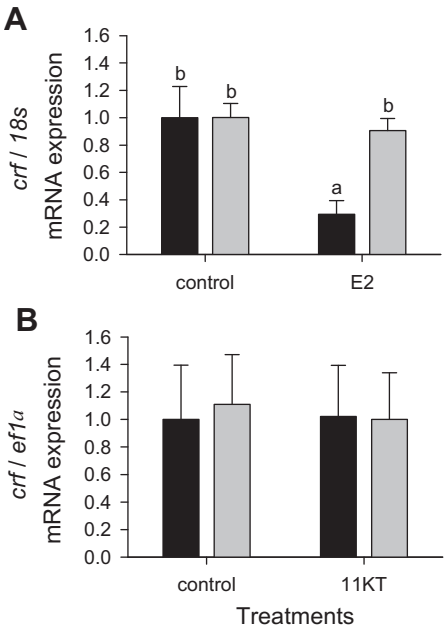

Fig. 7. Effects of 17β-estradiol (E2; A), or 11-ketotestosterone (11KT; B) on pre-optic area corticotropin-releasing factor (*crf*) mRNA levels in relation to *18s* or elongation factor 1α (*ef1a*) mRNA levels depending on which reference gene did not vary its expression levels with steroid treatment. Male (black bars) and female (grey bars) zebrafish were exposed to 0.01% ethanol (control), 100 ng/L E2 or 100 ng/L 11KT for 48 h prior to terminal anesthesia. Values are means ± SEM ($n = 4–11$). For comparative purposes, the expression ratios are presented relative to the average male control value. Bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA ($p < 0.001$) and by pairwise Tukey's test ($p < 0.05$).

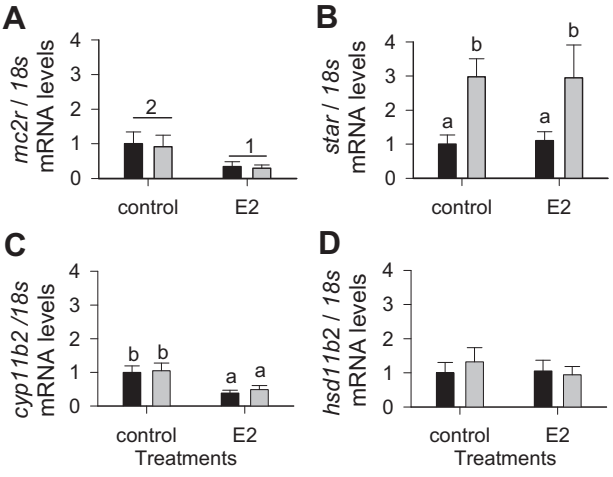

Fig. 8. Effects of 17β-estradiol (E2) on head kidney mRNA levels of melanocortin receptor 2 (*mc2r*; A), steroid acute regulatory protein (*star*; B), *cyp11b2* (C), and *11β*-hydroxysteroid dehydrogenase 2 (*hsd11b2*; D) in relation *18s* mRNA levels. Male (black bars) and female (grey bars) zebrafish were exposed to 0.01% ethanol (control) or 100 ng/L E2 for 48 h prior to terminal anesthesia. Values are means ± SEM ($n = 6–8$). For comparative purposes, the expression ratios are presented relative to the average male control value. Treatment bars that do not share a common letter are significantly different from each other as determined by two-way ANOVA ($p < 0.001$) and by pairwise Tukey's test ($p < 0.05$). Differences in numbers between pairs of bars indicate a significant effect of treatment as determined by two-way ANOVA.

dimorphisms in the stress response of some teleosts (goldfish, temperature stressor [29]; rainbow trout and brown trout, confinement stressor [30]; rainbow trout, confinement stressor [31]; sockeye salmon, confinement stressor [17]. Additionally, Pottinger

et al. [30] found that male rainbow trout had lower plasma ACTH and cortisol levels in response to a stressor compared to juveniles. In goldfish, Peter et al. [29] found that cortisol levels were dependent on sexual conditions. Mature females had the lowest resting levels and also the smallest daily fluctuations, while maturing females, which were undergoing ovarian recrudescence, had the highest levels and largest fluctuations in plasma cortisol in response to a temperature stressor. While our study suggests that the sensitivity and magnitude of the male and female zebrafish cortisol stress response do not differ, future studies are required to confirm this. It is possible that different stressors produce variations in the dimorphism of the HPI axis, as seen in mammals. For example, physical stressors in humans produce similar increases in cortisol in men and women, however, men are known to have larger cortisol responses to short-term psychological stress tasks than women [18]. It is not known whether a similar situation exists in teleosts as the effect of different stressors on this dimorphism has not been tested. Alternatively, although the use of whole-body cortisol levels as an indicator of stress axis activity in small fish has previously been validated [33], it is possible that subtle sex-specific differences in plasma cortisol may not be fully conveyed by whole-body cortisol measurements.

4.2. Effects of estradiol exposure on the stress axis in zebrafish

Analysis of water steroid concentrations revealed that E2 was markedly reduced over the 24 h exposure period. This observation is consistent with studies by Maunder et al. [25] who reported that waterborne E2 and T levels were depleted by stickleback (*Gasterosteus aculeatus*) over a 6 d exposure period. While stickleback were initially exposed to 1000 ng/L E2, E2 levels were reduced to 51–68% of that concentration after 6 h and at the end of the 6 d exposure period were reduced to 19–34%. In contrast, plasma levels of the fish increased quickly and were maintained throughout the exposure period and the authors suggest that this indicates bioaccumulation of steroids in the bodies of the fish [25]. While whole-body E2 levels were not affected by steroid exposure in our study, uptake of the steroid is implied by the decline of steroid in the water over time, as well as an increase in male vtg1 expression. Increases in male *vtg* expression are widely used as a bioindicator of estrogen exposure and our findings are consistent with other studies using zebrafish [34,36,43].

Resting whole-body cortisol levels were also unaffected by 48 h of E2 exposure in males and females. The cortisol stress response of male zebrafish, however, was blunted by E2 exposure while there were no effects on the response in females. This result was repeated in a second trial supporting our original findings despite the fact that the cortisol stress response of fish in the second trial was larger. The few other studies that have examined the effects of E2 on cortisol levels have provided conflicting results. While exposure of juvenile sea bass (*Dicentrarchus labrax L*) to E2 (35 μg/L) for 2 to 24 h had no effects on resting plasma cortisol levels [40], exposure of the same species to E2 (0.2 or 2 μg/L) for 10 d was found to reduce resting plasma cortisol levels [41]. In contrast, immature Atlantic salmon (*Salmo salar*) exposed to E2-containing water (2 μg/L; 21 d) were found to have elevated plasma cortisol levels at rest and after exposure to a confinement stressor [20]. In another study with salmonids, implantation of E2 pellets (20 mg; 24 d) enhanced the stress response of juvenile rainbow trout and juvenile brown trout to confinement [32]. It is interesting to note that the above studies were all conducted on juveniles, while our study was conducted on adult fish. The use of adult fish and the large discrepancy between dosages used may explain some the variation in findings between studies.

Consistent with the *in vivo* results, the tissue superfusion studies demonstrated that E2 decreases the ability of the male zebrafish interrenal to synthesize cortisol. A few other studies have examined the effects of E2 on head kidney cortisol production. One study incubated juvenile Kokanee salmon (*Oncorhynchus nerka*) or juvenile Chinook salmon interrenals with E2 (27–2723 μg/L; 48 h) and found a dose-dependent decrease in pregnenolone-stimulated cortisol production. However, the same study also found that cortisol production of juvenile and mature rainbow trout interrenals was unaffected by E2 incubation (272 μg/L; 48 h; [26]). Similarly, Barry et al. [3], observed no effect of E2 (272 μg/L; 18 h) on basal cortisol secretion from juvenile rainbow trout interrenals. These experiments used concentrations of E2 that were much larger than what was used in our study and also incubated the dissected tissue rather than exposing the animal prior to experimentation. These differences in experimental procedure as well as differences in species and sexual maturity could explain the disparity in the findings. Despite this disparity, our findings in combination with previous studies suggest that E2 can affect the rate of cortisol synthesis in the head kidney and may be responsible for the blunting of the male stress response *in vivo*.

To explore possible mechanisms involved in mediating the effects of E2 on cortisol synthesis, studies were undertaken to examine the expression of the ACTH receptor MC2R and key steroidogenic enzymes in the head kidneys of E2-exposed zebrafish. E2-exposure depressed *mc2r* and *cyp11b2* expression, but did not affect *star* or *hsd11b2* mRNA levels. If the decrease in *mc2r* expression results in a decrease in the MC2R protein, then this would result in a decreased ability of the interrenals to respond to ACTH stimulation. Similarly, if the decrease in *cyp11b2* expression translates into a reduction in enzymatic activity, then the interrenal tissue would have a decreased capacity for cortisol synthesis after E2 exposure. In contrast to our study, head kidneys of juvenile Atlantic salmon exposed to ethinyl estradiol (5 ng/L; 3 d), a potent ER agonist used in human birth control pills, had increased expression of *star*, and *cyp11b2* as well as an increase in StAR protein [23]. Once again, while the direction of change differs from those observed in our study, this may be due to species, experimental, or sexual maturity differences. Despite this, these findings suggest that estrogens are capable of altering steroidogenic genes in the head kidney.

It is not clear from our findings why exposure of zebrafish to E2 blunted cortisol synthesis capabilities in males but not females. While male zebrafish had lower *star* expression than females, this dimorphism was present under control conditions as well as after E2 exposure. Additionally, E2 was found to blunt the expression of *mc2r* and *cyp11b2* at resting levels, but no effect on resting whole-body cortisol was found. It is possible that the expression of these genes are not rate limiting and that other important steroidogenic enzymes, such as cytochrome P450 side chain cleavage (P450*scc*), 17α-hydroxylase or 3β-hydroxysteroid dehydrogenase, play a more prominent role in mediating the effects observed. E2-exposed male zebrafish were found to have less POA *crf* mRNA than control males and E2-exposed females. To our knowledge this is the first account of E2 affecting *crf* gene expression in fish, however there are numerous reports of E2 enhancing *crf* expression in mammals [15,19,35]. It is important to note that while the changes in whole-body cortisol were apparent after exposure to stress, gene expression in the POA was also measured at rest. It is possible that E2 not only affected resting level gene expression, but also played a role in preventing the increase in gene expression known to occur during regular HPI activation [11]. Together these findings suggest that E2 acts on the males stress axis at multiple levels by reducing the quantity of *crf* signaling, the interrenal response to ACTH and the rate of cortisol synthesis. While the main action of E2 seems to be decreasing the rate of cortisol synthesis in males, it is not known whether E2 affects cortisol metabolism. In this study *hsd11b2* expression, which converts cortisol into an inactive from,

was unaffected, however there are many pathways by which cortisol can be reduced or modified to prevent it from acting on GRs [44].

4.3. Effects of 11-ketotestosterone exposure on the stress axis in zebrafish

Analysis of water steroid concentrations revealed that water concentrations of 11KT were rapidly depleted from the exposure water. The rate of depletion was very similar to that of the E2 exposure water, suggesting that the rate of depletion may be dependant on the metabolic rate of the fish. Exposure of zebrafish to 100 ng/L 11KT for 48 h did not alter levels of whole-body 11KT in either sex. This exposure did, however, decrease expression of vtg1 in 11KT-exposed males, an effect that has been also observed with exposure of mummichog (*Fundulus heteroclitus*) to 17α-methyl testosterone [39], as well as exposure of fathead minnow (*Pimephales promelas*) to 17β-trenbolone [9,28]. In combination, the depletion of 11KT in the tank water and the depression of vtg1 expression in males suggest that the treatment regime was effective in altering physiological functions.

11KT exposure increased resting whole-body cortisol levels of males and enhanced the whole-body cortisol response of female zebrafish in response to a stressor. These findings are in contrast to previous studies, where sexually immature rainbow trout and brown trout given 11KT implants were found to have depressed ACTH and cortisol plasma levels after one hour of confinement [32]. While E2 seemed to affect whole-body cortisol levels by acting on components of the HPI axis, our findings suggest that 11KT does not act in a similar manner. 11KT was found to have no effect on POA *crf* mRNA content or on the cortisol synthesis capabilities of the head kidney *in vitro*. While no other studies have examined the effects of 11KT on *crf* mRNA levels in fish, there have been other studies to examine the effects of 11KT on interrenal steroid synthesis. Similar to our findings, McQuillan et al. [26] reported no effect of 11KT (302 μg/L, 48 h) on the rate of cortisol synthesis from the interrenals of Chinook salmon or rainbow trout of either sex. In contrast, Young et al. [45] found that 11KT implants (20 mg; 56 d) decreased head kidney cortisol synthesis abilities of immature, as well as male and female rainbow trout *in vitro*. It is not known why these studies contradict one another, however, methodology and duration of 11KT exposure may play a role. While we did not observe any direct effects of 11KT exposure on *crf* transcription or interrenal cortisol production, it is possible that other effectors of the HPI axis were altered or that 11KT had an impact on the rate of cortisol removal. These are areas for future research.

5. Conclusions

This study provides further evidence of the utility of the zebrafish as a model for investigations of the stress response in teleosts. Our results show that male and female zebrafish respond similarly in terms of cortisol levels at rest or in response to a vortex stressor. This was consistent with comparable production of cortisol by superfused interrenal cells from male and female zebrafish and the expression of genes at various levels within the HPI axis. It is interesting that the application of sex steroids only affected the sex in which it was not the major circulating steroid. It is possible that using an increased dosage of E2 or 11KT would have resulted in an equal modification of the cortisol stress response in males and females, however this theory has yet to be tested. Our finding that exposure to sex steroids effects the magnitude of the cortisol stress response in the sex where these steroids are not normally present at high levels is important to consider for future testing of endocrine disrupting chemicals. Few studies that test for physiological impacts of environmental estrogens and androgens take the stress axis into consideration. The cortisol stress axis is an integral part of overall fish health and a prolonged alteration of the axis, either an enhancement or suppression, would have negative consequences.

Acknowledgments

This research was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to NJB and GVDK.

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