# REGULATION OF THE ENDOCRINE STRESS RESPONSE AND THE

# MODULATING EFFECTS OF SEX STEROIDS IN ZEBRAFISH (*Danio rerio*)


A Thesis

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# ABSTRACT

# REGULATION OF THE ENDOCRINE STRESS RESPONSE AND THE MODULATING EFFECTS OF SEX STEROIDS IN ZEBRAFISH (*Danio rerio*)

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The purpose of this study was to characterize the endocrine stress response of zebrafish (*Danio rerio*) and to test if this response is modified by sex or the major sex steroids 17β-estradiol (E2) and 11-ketotestosterone (11KT). A standardized stress of having zebrafish swim in a beaker of stirring water resulted in rapid and transient changes in whole-body cortisol. While the cortisol response of males and females were comparable, exposure to E2 (100 ng/L) for 48 h inhibited the cortisol response of males and exposure to 11KT (100ng/L) for 48 h stimulated the cortisol response of females. The inhibitory actions of E2 were mediated in part by decreases in corticotropin releasing factor (crf) expression and decreases in the synthesis of cortisol by interrenal tissue. This research adds to the increasing knowledge of zebrafish physiology and will be useful for future studies concerning the endocrine stress response in fish.

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**CHAPTER 1**

**General Introduction**

The zebrafish (*Danio rerio*) is an important model for many fields of biology. Its rapid development, ease of care, availability and affordability have expanded its original use, the study vertebrate neural development and gene function, to many other disciplines including toxicology, reproductive biology, neurobiology, regenerative medicine and evolutionary theory (Bradbury, 2004). Recently, the zebrafish has been used as a model to study stress endocrinology. While we have some understanding of the development of the stress response (Chandrasekar *et al.*, 2007a; Alsop and Vijayan, 2008; Alderman and Bernier, 2009; Alsop and Vijayan, 2009) and of the stress response in adult fish (Ramsay *et al.*, 2006; Alderman and Bernier, 2007; Barcellos *et al.*, 2007; Ramsay *et al.*, 2009), there is much that we do not know. The purpose of my thesis was to expand our knowledge of zebrafish stress physiology. My objectives were to design a standardized stressor to use in future experiments, outline the timing of the endocrine stress axis, and to determine whether sex or sex steroids alter the magnitude of the endocrine stress response. This chapter provides insight into the regulation of the endocrine stress response in fish and explores what is known about sexual dimorphism and sex steroid-mediated modulation of the stress axis. The thesis objectives are also outlined.

**The Endocrine Stress Response**

All vertebrates experience disruptions of homeostasis. These disruptions, known as stressors, would be detrimental if they were not corrected by the stress response. One major component of the stress response is the endocrine stress response, which results in an increase in glucocorticoids. Cortisol, the major glucocorticoid in most teleosts, allows

the stressed fish to restore homeostasis by increasing the availability of energy

(Wendelaar Bonga, 1997). The endocrine stress response is regulated by the

hypothalamic-pituitary-interrenal (HPI) axis (Fig. 1). When a stressor is perceived

neuropeptides, including corticotropin-releasing factor (CRF) and urotensin (I), are

released from neurons projecting through the hypothalamus. While CRF, the major

secretagogue during a stress event, is located throughout the brain, the production of CRF

for the purpose of stimulating the stress axis occurs in the preoptic area (POA) of the

brain. CRF-binding protein (CRF-BP) is located in the same major regions of the brain as

CRF, and the binding of CRF-BP to CRF is thought to prevent the action of its receptors

(Alderman *et al.,* 2008). When CRF is released from hypothalamic neurons and is

unbound by CRF-BP, it acts on CRF receptors in the anterior pituitary to stimulate the

production and cleavage of pro-opiomelanocortin (POMC), a precursor of

adrenocorticotropic hormone (ACTH). ACTH is released from the corticotrophs of the

pituitary into the bloodstream where it travels to the head kidney and acts on

melanocortin 2 receptors (MC2R) to initiate steroidogenesis and cortisol secretion

(Bernier *et al.*, 2009). The production of cortisol is initiated by the steroid acute

regulatory protein (StAR) which mediates the rate-limiting and acutely regulated

intramitochondrial transfer of cholesterol to the P450 side-chain cleavage enzyme

(P450scc), which then catalyzes the formation of pregnenolone (Fig. 2). Pregnenolone is

then converted through several enzymatic actions to 11-deoxycortsiol. Finally, 11-

deoxycortsiol is converted to cortisol by 11β-hydroxylase. Cortisol is released directly

into the bloodstream and is not stored in the head kidney (Wendelaar Bonga, 1997).

Cortisol itself acts on many aspects of its own production. Negative feedback occurs at all levels of the HPI axis by acting on glucocorticoid receptors (GRs) or mineralocorticoid receptors (MRs) located within diverse target cells. Cortisol bound GRs translocate into the nucleus where they form homodimers with specific glucocorticoid response elements (GRE) present in the promoter region of many hormone-responsive genes. In addition to this, GRs can recruit other transcription factors and promote or inhibit gene transcription (Marino *et al.*, 2006). Steroid hormones can also change the stability of messenger ribonucleic acid (mRNA) and other post-transcriptional modifications, thus affecting the quantity of glucocorticoid-responsive proteins (Tsigos and Chrousos, 2002).

The breakdown and excretion of cortisol from blood plasma is important for the control and maintenance of cortisol levels and can occur by several different mechanisms. At the cellular level, the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) is responsible for converting cortisol to cortisone, which is not an active glucocorticoid in the stress response. Another isoform of this enzyme, 11β-HSD1, is responsible for the reverse reaction and converts cortisone to cortisol. Together, these two enzymes play a role in regulating the action of cortisol on its receptors (Baker, 2004). Since cortisol is a hydrophobic molecule, it must undergo enzymatic modification to make it inactive and to increase its solubility. Several enzymes, including 5α-reductase, 5β-reductase, 3α-HSD, 20α-HSD or 20β–HSD are involved in this process; however 5α-reductase is responsible for the majority of cortisol reduction (Wudy *et al.*, 2007). Conjugation of cortisol or its metabolites with glucuronic acid further increases their solubility by increasing molecular weight and polarity. The conjugated compounds can

then be excreted by the kidney by glomerular filtration or by the liver via the bile into the small intestine by specific transporters (Gomes *et al.*, 2009). Interestingly, the enzyme β-glucuronidase deconjugates steroid hormones in the small intestine, facilitating their movement back into circulation (Croes *et al.*, 2009). Therefore, there is a delicate balance between cortisol synthesis, metabolism, and resorption to maintain steroid hormone levels.

**Sex Steroids and the Stress Response**

Sexually dimorphic stress responses have been noted in diverse groups of vertebrates, including mammals (Critchlow *et al.*, 1963), amphibians (Licht *et al.*, 1983), and reptiles (Elsey *et al.*, 1990). In mammals, this sexual dimorphism is responsible for differing susceptibility to many health issues including anxiety, depression, diabetes and heart disease (Kudielka and Kirschbaum, 2005). While few studies have explored this subject in fish, a sexually dimorphic stress response has been noted in some elasmobranchs and teleosts (Peter *et al.*, 1978; Pottinger *et al.*, 1995; Girard *et al.*, 1998; Kubokawa *et al.*, 2001; Manire *et al.*, 2007).

In rainbow trout, *Oncorhynchus mykiss*, Pottinger *et al.* (1995) found that mature males had lower cortisol and ACTH plasma levels after a stressor than juvenile males. In a subsequent study, Pottinger and Carrick (2000) found that as rainbow trout mature, males have decreasing plasma cortisol levels, but similar responsiveness to confinement; whereas females have increasing basal cortisol levels and a decreased cortisol response to confinement. Similarly, Kubokawa *et al*. (2001) found that female sockeye salmon (*Oncorhynchus nerka*) have higher cortisol levels than males during the spawning season with decreased sensitivity to a confinement stressor. Also, in goldfish (*Carassius*

*auratus*), Peter *et al.* (1978) found that the basal cortisol levels of females depended on their reproductive stage. Vitellogenic females tended to have the lowest levels of cortisol, while females undergoing the final stages of oocyte maturation had the highest levels of plasma cortisol. These studies suggest that sexual dimorphisms do exist in fishes and that sex steroids likely play a role in mediating this phenomenon.

In most teleosts, the main estrogen is 17β-estradiol (E2), the major androgen in males is 11-ketotestosterone (11KT), and the androgen testosterone (T) is present at moderate levels in both sexes (Borg, 1994). A small number of studies have investigated the effects of sex steroids on the stress response. Sexually immature rainbow trout and brown trout (*Salmo trutta*) given T and 11KT implants had depressed stress-induced plasma ACTH and cortisol levels and E2-implanted fish were characterized by an enhanced cortisol stress response to confinement (Pottinger *et al.*, 1996).  Similarly, immature Atlantic salmon (*Salmo salar*) exposed to E2-containing water higher plasma cortisol levels than control fish under both resting conditions and after exposure to a handling stressor (Lerner *et al.*, 2007).  In contrast, studies with juvenile gilthead seabream, *Sparus aurata*, (Teles *et al.*, 2005) and juvenile sea bass, *Dicentrarchus labrax* L, (Teles *et al.*, 2006) have found that E2 injections or exposures depress resting cortisol levels. Finally, Vijayan *et al.* (2001) found no impact of E2 exposure on resting cortisol levels of male Mozambique tilapia (*Oreochromis mossambicus*), and Carrera *et al.* (2007) saw no change in the resting cortisol levels of immature gilthead seabream injected with E2.  Studies conducted with rodents have provided more conclusive findings. It is generally accepted that in rats, E2 enhances while T depresses the stress response (Handa *et al.*, 1994; Torpy *et al.*, 1997; Kudielka and Kirschbaum, 2005). While

the studies conducted in teleosts are equivocal, they suggest that sex steroids have effects

on the stress axis.

Few studies have investigated the mechanism behind sex steroid modulation of

the stress axis. While the aforementioned studies noted effects of 11KT and E2 on plasma

ACTH levels following a confinement stressor (Pottinger *et al.,* 1996), no studies to date

have investigated whether sex steroids act at the level of the brain or pituitary to mediate

changes in plasma cortisol in fish. There have been studies concerning the impact of E2

and 11KT on steroidogenesis by the head kidney.  *In vitro*, the rate of cortisol synthesis

induced by pregnenolone and ACTH was higher in immature rainbow trout than in

mature males, mature males given 11KT implants, or immature females treated with

11KT (Young *et al*., 1996).  However, McQuillan *et al*. (2003) reported no effect of

11KT on the rate of cortisol synthesis from the interrenals of juvenile or mature male or

female rainbow trout and Chinook salmon (*Oncorhynchus tshawytscha*), and noted an

inhibitory effect of E2 on the ability of the head kidney to utilize pregnenolone for

cortisol synthesis. Other studies have shown that immature and mature rainbow trout

interrenals are insensitive to E2 (McQuillan *et al.*, 2003; Barry *et al.,* 1997).  While the

reasons for these differing results are not known, differences in methodological approach

(steroid dose, application and duration), the sex and reproductive status of the fish used in

the various studies, and species-specific differences are likely contributing factors.

**Zebrafish as a Stress Model**

To date, much of our knowledge of stress physiology in teleosts has been obtained

from studies of salmonids. While this research is important due to the impacts of stress in

aquaculture, the fact that the salmonid genome has four copies and that the reproductive

cycle of salmonids is lengthy imposes limitations on our ability to study the impact of stress on reproduction. Zebrafish are an ideal animal model for the study of stress physiology for several reasons. The zebrafish genome contains only one isoform of CRF (Chandrasekar *et al.*, 2007), urotensin I (UI) (Parmentier *et al.*, 2008), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Alsop and Vijayan, 2008) genes, as opposed to two in salmonids. In addition to this, the zebrafish has a short time to maturity (3 months) and reproduces year round; making it an ideal model to study under control conditions. These attributes account for the increasing number of studies concerning the zebrafish, including studies on the stress axis. Over the past two years, the development of the stress response in zebrafish was outlined (Chandrasekar *et al.*, 2007a; To *et al.*, 2007; Alsop and Vijayan, 2008; Alderman and Bernier, 2009; Alsop and Vijayan, 2009), the genes CRF, UI, and CRF-BP were mapped in the adult brain (Alderman and Bernier, 2007), and the timing of the zebrafish stress response was determined (Ramsay *et al.*, 2009). While this surge has increased our knowledge of the zebrafish stress axis, there is much that we do not know.

**Thesis Objectives and Hypotheses**

The purpose of this study was to gain a deeper understanding of the dynamics of the cortisol stress response of zebrafish, and to determine whether sex or sex steroids modulate this response. I had two main hypotheses; the first was that the zebrafish stress axis functions similarly to other fish species. To test this I first needed to design a standardized stressor for zebrafish in order to determine if changes in whole-body cortisol, transcript levels of *crf* in the brain, *pomc* in the pituitary, and *mc2r*, *star*, *11β-hydroxylase* and *11β-hsd2* in the head kidney of zebrafish change in a similar time frame

and magnitude as other fish species. If the HPI axis of zebrafish is similar to that of other teleost species, then whole-body cortisol should increase approximately 10-fold with the application of a moderate to severe stressor and should be accompanied by increases in POA *crf*, pituitary *pomc*, and head kidney *mc2r*, *star*, *11β-hydroxylase* and *11β-hsd2*.

My second hypothesis was that sex steroids act on all levels of the stress axis to alter cortisol production, with E2 enhancing and 11KT depressing stress-induced cortisol levels. This hypothesis was tested first by determining whether the whole-body cortisol response of zebrafish differed between sexes and second by testing whether exposure of zebrafish to environmental E2 or 11KT altered the magnitude of the stress response. Whole-body cortisol of zebrafish from sexually mature males and females were compared at rest and after the application of our standardized stressor in control groups, as well as after exposure to exogenous E2 or 11KT. If E2 acts to enhance and 11KT acts to depress the HPI axis as previously found in mammals, then I would expect female fish to have higher whole-body cortisol levels than males after stress exposure. If this were true I would also expect E2 exposure to increase stress-induced cortisol levels, and 11KT exposure to depress stress induced cortisol levels of zebrafish. To test where in the HPI axis sex steroids act, *in vitro* cortisol synthesis, expression of *crf* in the brain, and expression of *mc2r*, *star*, *11β-hydroxylase* and *11β-hsd2* in the head kidney were compared between control and steroid exposed fish. If sex steroids act at a level higher than the pituitary, then it is expected that E2 exposed fish will have increased, and 11KT exposed fish will have decreased expression of *crf* compared to control fish. If sex steroids mediate cortisol release at the head kidney, cortisol production and gene

expression in the head kidney would be expected to correspond to changes in whole-body cortisol levels.

**Figure 1.**

The hypothalamic-pituitary-interrenal (HPI) axis in teleost fish. Solid arrows indicate release of neuropeptides / hormones and dashed arrows indicate negative feedback by cortisol.
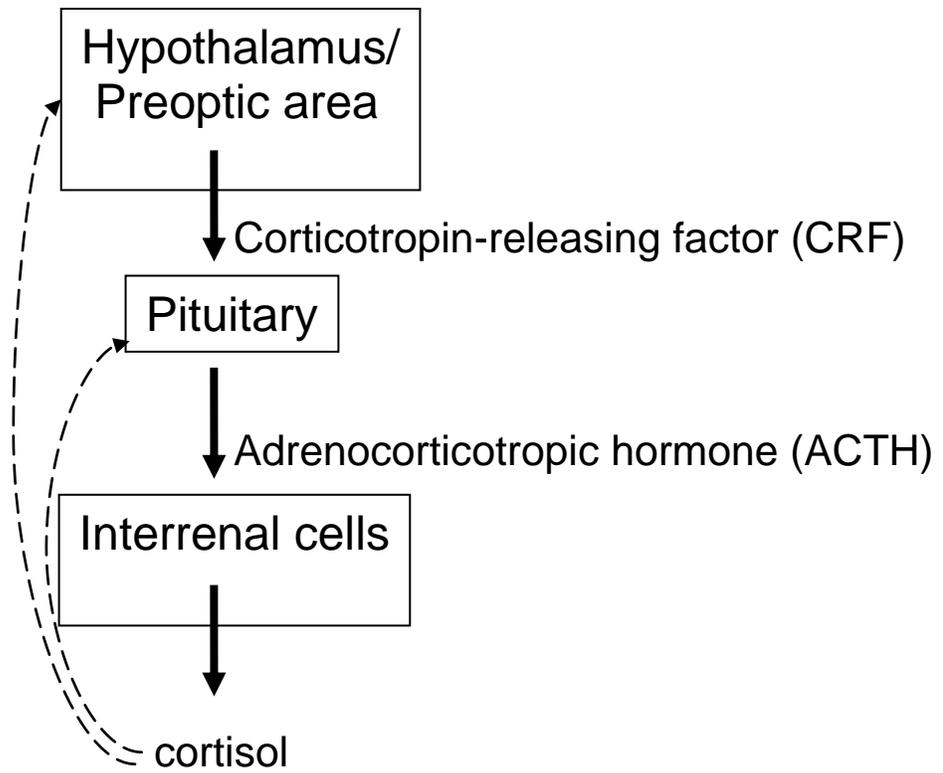
Figure 1

**Figure 2.**

Steroidogenic pathway in teleost fish, depicting the formation of glucocorticoids, estrogens or androgens and the enzymes involved. 1) cholesterol side chain cleavage enzyme (P450scc); 2) 3β-hydroxygsteroid dehydrogenase (3β-HSD); 3)17α-hydroxylase; 4) $C_{17,20}$-lyase; 5) 17β-hydroxysteroid dehydrogenase (17β HSD); 6) P450 aromatase (CYP19A); 7) 11β-hydroxylase (CYP11B); 8) 11β-hydroxysteroid dehydrogenase (11β-HSD); 9) 21-hydroxylase (P450c21). Boxes indicate major circulating hormones.

Figure 2

13
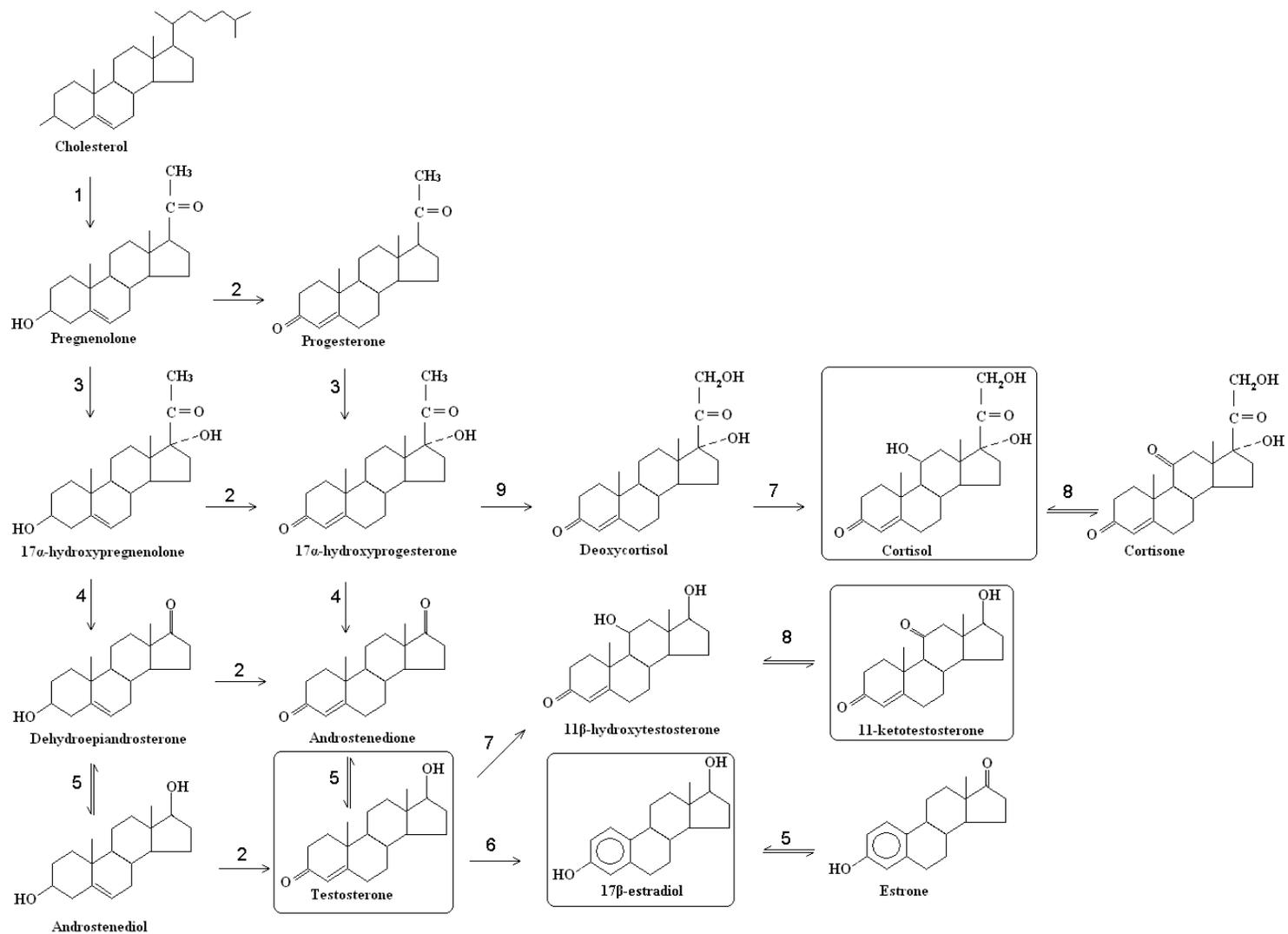
**CHAPTER 2**

**Stirring up new ideas about the regulation of the HPI axis in zebrafish (*Danio rerio*)**

**Abstract**

While the impact of stressors on cortisol levels in fish is well documented, much less is known about the dynamic relationships between changes in cortisol during and after a stressor and expression pattern of the key genes that regulate activity of the hypothalamic-pituitary-interrenal (HPI) stress axis. After establishing a vortex stressor as a standardized stressor for zebrafish (*Danio rerio*), this study characterized its impact at all levels of the HPI axis. Whole body cortisol levels as well as the mRNA levels of corticotropin-releasing factor (*crf*) in the preoptic area, pro-opiomelanocortin α (*pomc*α) in the pituitary, and melanocortin 2 receptor (*mc2r*), steroid acute regulatory protein (*star*), *11β-hydroxylase* and 11β-hydroxysteroid dehydrogenase 2 (*11β-hsd2*) in the interrenal region were quantified to determine the timing and magnitude of the stress response. Whole-body cortisol levels increased linearly with vortex stress speed. Exposure to a moderate vortex speed was associated with rapid and marked increases in whole-body cortisol and *crf* mRNA levels, slower increases in *mc2r*, *star*, *11β-hydroxylase*, and *11β-hsd2*, and a modest delayed increase in *pomc*α. After a short recovery period, whole body cortisol levels and *11β-hsd2* expression were equivalent to resting levels, however, *crf*, *pomc*α, *mc2r*, *star* and *11β-hydroxylase* expression were significantly lower than pre-stress values. These findings suggest that multiple genes at different levels of the HPI axis in zebrafish play an active role in the sequential stimulation and termination of the cortisol response to an acute stressor.

**Introduction**

In order to maintain internal homeostasis in a variable environment, vertebrates have evolved an endocrine coping mechanism known as the stress response. In teleosts, an important component of the stress response is regulated by the hypothalamic-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). When a disruption in homeostasis is perceived, corticotropin-releasing factor (CRF), the principle hypophysiotropic factor in fish, is released from nerve terminals originating in the hypothalamus and acts on the pituitary to release adrenocorticotrophin hormone (ACTH) into the bloodstream (Bernier *et al.*, 2009). In turn, ACTH acts on the interrenal cells of the head kidney by binding to the melanocortin 2 receptor (MC2R) to stimulate cortisol production (Aluru and Vijayan, 2008). Key enzymes involved in the conversion of cholesterol to cortisol include steroid acute regulatory protein (StAR) and 11β-hydroxylase which shuttle cholesterol across the inner mitochondrial membrane and convert 11-deoxycortisol to cortisol, respectively (Mommsen *et al.*, 1999). Limiting the activity of the HPI axis are the enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts cortisol to cortisone, a glucocorticoid that is not involved in the stress response (Mommsen *et al.*, 1999), and the negative feedback effects of cortisol at the hypothalamic, pituitary and interrenal levels of the HPI axis (Bernier *et al.*, 2009).

Though the stress response may be critical for survival, few studies have examined both the activation and recovery of the different levels of the HPI axis in response to a stressor. Using blood oxygen level dependent functional magnetic resonance imaging (BOLD-fMRI) on common carp (*Cyprinus carpio*) of unknown sex, van den Burg *et al.* (2005) showed that a sudden drop in temperature results within 30 s in an activation of

the preoptic area (POA), the brain region where the hypophysiotropic CRF neurons reside. Moreover, the corticotrophs, which are the source of ACTH, were recruited between 90 and 120 s post-stress and plasma cortisol levels were elevated within 5 min of the temperature drop (van den Burg *et al.*, 2005). While the above study provided convincing evidence of the quick activation of the stress axis, it did not identify the specific genes involved in the response or characterize the recovery of the HPI axis following the stressor. To date, studies that have quantified the changes in gene expression of the HPI axis effectors have focused only on one level of the axis. For example, several studies have shown that acute stressors in fish are associated with increases in the gene expression of POA *crf* (Bernier, 2006; Bernier *et al.*, 2009) and pituitary pro-opiomelanocortin (*pomc*), the precursor of ACTH (immature rainbow trout, Gilchriest *et al.*, 2000; male common carp, Metz *et al.*, 2004; juvenile channel catfish, *Ictalurus punctatus*, Karsi *et al.*, 2005). Similarly, there is evidence that acute stressors can stimulate the gene expression of *mc2r* (juvenile rainbow trout, Aluru and Vijayan, 2008), *star*, *11β-hydroxylase* (mature rainbow trout and brook trout, Kusakabe *et al.*, 2002; juvenile rainbow trout, Geslin and Auperin, 2004; juvenile rainbow trout, Aluru and Vijayan, 2006) and *11β-hsd2* (male common carp, Nematollahi *et al.*, 2009) in the head kidney. So while the above studies suggest that multiple genes at different levels of the HPI axis may contribute to the regulation of the stress response in fish, their relative importance to one another during the various phases of the stress response has not been established.

Although zebrafish have been widely used to study many physiological functions, little is known about the regulation of the cortisol stress response of adult fish. While a

few studies have measured whole-body cortisol levels in developing (Alderman and Bernier, 2009; Alsop and Vijayan, 2009) and mature (Pottinger and Calder, 1995; Ramsay *et al.*, 2006; Ramsay *et al.*, 2009) zebrafish in response to a stressor, none of these studies have examined other components of the HPI axis. Despite this, the zebrafish promises to be a good candidate for the study of stress physiology (Alsop and Vijayan, 2009).

The purpose of this study was to determine the timing and magnitude of the zebrafish stress response as well as to determine the relative importance of changes in gene expression in the POA, pituitary and the head kidney during the activation and recovery phases of the cortisol stress response. To accomplish this, we first developed a novel vortex stressor and characterized its impact on the cortisol stress response. Zebrafish were subjected to a moderate vortex current in order to measure changes in whole body cortisol, mRNA levels of POA *crf*, pituitary *pomc*α, as well as interrenal *mc2r*, *star*, *11β-hydroxylase* and *11β-hsd2* during both the activation and recovery from a stress event.

**Materials and Methods**

**Animals**

Adult zebrafish (0.3-1.0g) were obtained from DAP International (Etobicoke, ON, Canada) and housed at the Hagen Aqualab (University of Guelph, Guelph, ON) for at least 1 month in an A-HAB fish containment unit (Aquatic Habitats, Apopka, FL) in 4L tanks at a density of 3-5 fish / L at 26˚C with a 12:12 h light/dark photoperiod cycle (lights on at 7 am). Fish were fed twice daily with commercial salmon fry fish pellets (Martin Mills, Elmira, ON) and their diet was occasionally supplemented with blood worms.

**Experimental Design**

For all experiments, six sexually mature zebrafish (approximately 3 males and 3 females) of similar size were housed in 2L glass beakers. All beakers were on a single recirculation circuit and individually supplied with freshly filtered and oxygenated water. Each beaker was covered with mesh and kept in a large (20L) overflow bin that contained a tank filter, air stone, heater and a recirculation pump. In this setup, fish were fed once daily during a seven day acclimation period.

In all vortex stress experiments, individual magnetic stir bars (Fisherbrand Magnetic Stir Bar, Octagonal, 5cm x 1cm, Fisher) were placed in experimental tanks and stir plates (Thermix® Stirrer Model 120S, Fisher Scientific) were set to desired speed prior to acclimation of fish. Fish were fasted for 24 h before experimentation and vortex stress was always started at noon on the day of sampling. At the start of the stress exposure, control fish were sampled to prevent their exposure to visual stressors and following the 20 min stress exposure, experimental fish were sampled. All fish were quickly (within 30 s of sampling) euthanized with a lethal dose of 2-phenoxyethanol (1:500, Sigma) and either flash frozen in liquid nitrogen and stored at -80˚C for whole body cortisol analysis, or tissues were removed, placed in RNase free tubes, and stored at -80˚C for RNA extraction.

*Experiment 1: Development of a standardized stressor*

The application of a vortex stressor was found to significantly elevate whole-body cortisol in a series of preliminary studies (data not shown). In order to determine the relationship between vortex speed and the magnitude of the cortisol response, one beaker of fish was exposed to 20 min of vortex stress at each speed; 200, 300, or 400 rpm. To

determine the severity of the vortex stressor relative to another type of stressor, a separate

beaker of fish was netted and exposed to air for one min and then allowed to recover in

their beaker for 20 min. The vortex and air exposure treatments were conducted

simultaneously and all fish were sampled as above and later analyzed for whole-body

cortisol.

*Experiment 2: Dynamics of the zebrafish stress response*

In order to determine how the vortex stressor affects whole body cortisol and the gene

expression of several components of the HPI axis through time, fish were exposed to a

vortex speed of 300 rpm (speed chosen based on *Experiment 1*) and sampled after 0, 5,

10, 15, 20, 30, 40, and 60 min of the vortex stress.  Additionally, fish were exposed to the

vortex stress for 60 min and sampled 30 and 90 min after the cessation of the stressor. All

fish were euthanized and immediately flash frozen for whole-body cortisol analysis or

placed on ice for removal of tissues. The pituitary, brain, which was regionally dissected

to isolate the POA, and head kidney were removed from individual fish with the aid of a

stereoscopic zoom microscope (Nikon, SMZ1500) and placed in individual RNase free

tubes on dry ice for analysis of gene expression.  The POA was identified as the region

rostral to where the optic nerves enter the brain, as depicted by Bernier *et al.* (2008).

*Steroid extraction*

Zebrafish were removed from -80˚C storage, thawed on ice, blotted dry, 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-cortsiol (Amersham Biosciences,

Piscataway, USA). Zebrafish were homogenized using a Euro Turrax T 20b mechanical

homogenizer (IKA Labortechnik, Staufen, Germany) 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 and 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 extractions for each sample were combined and dried under $N_2$ at room temperature.

Samples were further purified as described by Lister *et al.* (2008). Briefly, samples were reconstituted with 300 µL of acetate buffer (2.35 ml glacial acetic acid, 1.23 g sodium acetate trihydrate, in 1L;  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 to the $C_{18}$ columns, 1 ml of ultra-pure (Fisher Scientific) water 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.

### *Cortisol Radioimmunoassay*

Cortisol was measured in 200 µl aliquots of samples (in triplicate and values averaged) by radioimmunoassay (RIA) using the methods described by Bernier *et al.*

(2008). The cortisol antibody used for this assay was obtained from the Clinical

Endocrinology Laboratory (product code #R4866, University of California, Davis, CA).

A serial dilution of whole fish extract gave a displacement curve that was parallel to the

standard curve (Fig. 1). Extraction efficiency, interassay and intra-assay variances were

found to be 74 ± 1% (n=30), 5.9% (n=6), and 1.1% (n=7), respectively.  Cortisol values

are presented as ng whole-body cortisol / g of individual body weight (BW) and are

corrected for individual extraction efficiency.

### *Quantification of mRNA by real-time RT-PCR*

Total RNA from each sample (one fish per tissue sample) was extracted using TRIzol

Reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).  One

µg total RNA for head kidney and POA samples, or 0.5 µg of total RNA for pituitary

samples were 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 10µl 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, St. Louis, MO) 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 (Ct) 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 (E) between 85% and 100% based on the equation:

$$E\% = 10 \char94 ((-1/\text{slope})-1)*100$$

Gene expression was quantified with the slope (m) and the y-intercept (y) of the standard curve based on the equation:

$$\text{Value} = 10 \char94 [(\text{Ct-y})/\text{m}]$$

To correct for minor variations in template input and transcriptional efficiency, each sample was normalized to the expression level of either *18s* (POAs and head kidneys) or elongation factor-1α (*ef1α*; pituitaries). In pituitary samples, there were significantly different levels of *18s* expression between treatment groups eliminating it as an acceptable reference gene, but no differences were found in pituitary *ef1α* expression between treatment groups. Gene specific primer sequences for zebrafish *crf*, *pomcα*, *star*, *mc2r*, *11β-hydroxylase*, 11β–HSD2, *18s* and *ef1α* were designed using Primer Express 3.0 (Applied Biosystems) and are listed in Table 1. Expression data are reported as fold change from the time 0 min mRNA levels.

**Statistical Analyses**

A one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons was used to determine differences in cortisol content between treatment

groups in *Experiments 1 and 2* and to determine differences in mRNA expression between treatment groups in *Experiment 2*. Data that did not meet the assumption of normality were log-transformed prior to analysis. A Pearson Product Moment Correlation Test was used to test for potential correlations between vortex speed and whole body cortisol levels. Any outliers that were determined to be greater than or less than the 2.0 x inter-quartile 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.

**Results**

*Experiment 1:  Development of a standardized stressor*

Exposure of zebrafish to the vortex stressor significantly elevated whole-body cortisol levels at all speeds tested. The magnitude of the cortisol response was found to correlate linearly with the vortex speed ($R^2 = 0.652$; $p < 0.001$; Fig. 2). Whole body cortisol levels of fish exposed to the 200, 300 and 400 rpm vortex stressors were 4 ($p = 0.01$), 6 ($p < 0.01$) and 8 fold ($p < 0.001$) higher than control values, respectively. The fastest vortex speed (400 rpm) elicited a cortisol stress response comparable to the response of fish that were exposed to air for one min and allowed to recovery for 20 min ($p = 0.315$; Fig. 2). Cortisol levels were did not vary between sexes ($p = 0.69$) as determined by a students t-test.

*Experiment 2: Dynamics of the zebrafish stress response*

The purpose of this experiment was to determine the time course of the zebrafish stress response, both during and after the 60 min vortex stressor. Whole body cortisol levels increased quickly after the start of the vortex stress and were 6 times higher ($p<0.001$) than controls after 5 min (Fig. 3). The increase in whole body cortisol continued to rise and after 10 min of vortex stress peaked at a value that was 18 fold higher than control levels ($p<0.001$). Cortisol levels then quickly dropped and remained relatively stable between 20 and 40 min at about half of peak values. There was a further decrease in whole body cortisol after 60 min of vortex stress to 3 times control levels ($p<0.01$). Within 30 min of the end of the stressor, cortisol levels had returned to control levels ($p=0.13$).

The expression levels of POA *crf* increased quickly in response to the stirring stress (Fig. 4A). Within 10 min of the initiation of the stress, there was an eight fold increase ($p=0.02$) in *crf* mRNA expression. *crf* expression decreased to levels comparable to control values by 20 min ($p>0.05$) and continued to decrease to half of the time 0 values ($p=0.034$) after the 30 min recovery period. Expression levels of pituitary *pomcα* remained constant through the first 20 min of the vortex stressor, increased modestly to 1.5 fold of control values after 60 min ($p=0.002$) and decreased to half of control values after the 30 min recovery period ($p=0.001$; Fig. 4B). The genes *mc2r* (Fig. 4C), *star* (Fig. 4D), *11β-hydroxylase* (Fig. 4E) and *11β-hsd2* (Fig. 4F) from the interrenal tissue of stress-exposed fish demonstrated similar patterns of expression. The mRNA levels of all four genes were elevated within 10 min (*star*, $p=0.004$; *mc2r*, $p=0.046$; *11β-hydroxylase*, $p<0.001$; *11β-hsd2*, $p<0.001$) of the initiation of the vortex stressor and peaked after 20 min of exposure. The expression of all four interrenal tissue genes decreased after 60

min of vortex exposure either to (*star*, *11β-hydroxylase* and *11β-hsd2*) or well below

control expression levels (*mc2r*; $p<0.001$).  *mc2r* ($p<0.001$), *star* ($p=0.01$), and *11β-hydroxylase* ($p=0.02$) mRNA levels were below that of control values after the 30 min

recovery period.

**Discussion**

In this study, the contribution of the key HPI axis effectors to the activation and

recovery phases of the stress response were characterized simultaneously for the first

time in a fish species. This was accomplished using a novel vortex stress which induced

an intensity- and time-dependent cortisol stress response.  Genes from the POA, pituitary

and interrenal were all found to participate in the rapid onset of cortisol synthesis as well

as the recovery from stress to resting cortisol levels. This data suggests the presence of

negative feedback mechanisms at all levels of the HPI axis to quickly terminate the stress

response.

*Development of a standardized stressor*

Basal whole-body cortisol levels of zebrafish in this study were lower than those

observed in previous studies (Pottinger and Calder, 1995; Ramsay *et al.*, 2006; Barcellos

*et al.*, 2007).  This variation in resting cortisol levels is likely due to differing housing

conditions, sampling techniques and assay detection limits as suggested by Egan *et al.*

(2009).  The range of vortex stressor speeds examined in this study produced increases in

cortisol that were comparable to other mild (visual contact with a predator, Barcellos *et*

*al.* 2007), moderate (tank transfer, Pottinger and Calder 1995; crowding stress, Ramsay *et*

*al.* 2006) and severe (air exposure, Ramsay *et al.* 2009; air exposure in this study)

zebrafish stressors. The linear fold increase in cortisol between 200, 300 and 400 rpm

suggests that the stress response has not reached its maximum at 400 rpm and that a higher speed would elicit a greater response. Informal observations of behavior suggest that while zebrafish were capable of maintaining their position in the water column at 400 rpm, they were swimming vigorously to do so. While we did not observe any differences in the magnitude of the stress response between sexes, in the future it would be beneficial to test this with a larger sample size. The vortex stressor may be useful for future studies of the stress axis in zebrafish and other small aquarium fish. It is easily manipulated, can be used at high or low intensities, and can be used for varying durations. Unlike air exposure, one of the advantages of the vortex stressor is that it can be used to determine how environmental conditions (e.g. temperature, oxygen, ammonia), endocrine disruptors or contaminants can affect the stress response.

### *Dynamics of the zebrafish stress response*

In this study, we observed rapid changes in whole-body cortisol. Cortisol levels were elevated within 5 min of exposure to the vortex stressor and peaked after 10 min before slowly declining over the next 50 min to reach resting levels. These findings are in general agreement with those of the study of Ramsay *et al.* (2009) who reported a peak in adult zebrafish whole-body cortisol after 15 min and a return to resting whole-body cortisol levels after 60 min. The similar timing and magnitude of cortisol increase between this study and that of Ramsay *et al.* (2009) suggest that the zebrafish cortisol response is consistent and resilient.

The timing of the onset and peak of the zebrafish cortisol stress response, although rapid, is comparable to that of other fish species. In male carp acclimated to water at 25˚C, plasma cortisol increased within 5 min of exposure to a drop in water temperature

(van den Burg *et al.*, 2005).  A similar study found that male common carp reached peak

cortisol levels within 30 min of cold shock (Tanck *et al.*, 2000).  In contrast, while cold

water fishes (held at 10-12˚C) such as rainbow trout (*Oncorhynchus mykiss*) can increase

plasma cortisol within 10 min, they take 60 to 90 min to reach peak cortisol levels in

response to an acute handling stressor (Barton and Peter, 1982; Sumpter *et al.*, 1986).

These species differences are most likely due to differences in water temperature.

Assuming $Q_{10}$ effects on the rate of the cortisol stress response in fish range between 2

and 3, one would expect the speed of the cortisol stress response to be approximately 3 to

6 times faster in 26˚C- versus 10˚C-acclimated fish.  Hence, $Q_{10}$ effects alone may

account for the very rapid stress response observed with zebrafish.

The rapid changes in whole-body cortisol observed in this study were mirrored in

timing and magnitude by *crf* expression in the POA. The increase in *crf* mRNA levels in

the zebrafish was transient and followed by a rapid decrease in expression. This is likely

due to negative feedback of glucocorticoids on the POA. Previous studies with mixed sex

goldfish (*Carassius auratus auratus*) have shown that cortisol depresses POA *crf*

expression under resting conditions (Bernier *et al.*, 1999; Bernier *et al.*, 2004). Our

findings of rapid changes in *crf* mRNA expression support previous findings that CRF is

a key regulator of the HPI axis in fish.

The modest changes in *pomcα* mRNA expression observed in this study could be

due to several factors. First, *pomc* expression may not play a large role in the regulation

of the stress response since there are large stores of ACTH available. Second, cortisol

may have a more potent negative-feedback effect on *pomc* expression than on *crf*

expression.  This is supported by studies with immature gilthead sea bream (*Sparus*

*aurata*; Rotlant *et al.*, 2001) and juvenile channel catfish (Karsi *et al.*, 2005), which have demonstrated a decrease in pituitary *pomc* expression with exposure to handling and confinement and low-water stressors, respectively. A third possibility is that little change was observed in whole pituitary *pomc* expression due to contradictory changes in the melanotrophs and the corticotrophs of the pituitary. This theory is supported by reports of *pomc* expression increasing the pars distalis with stress exposure (juvenile rainbow trout, Gilchriest *et al.*, 2000; male common carp, Metz *et al.*, 2004) despite previous reports of a whole pituitary decrease in expression. In addition to this, van den Burg *et al.* (2005) reported differential changes in blood flow in the pars distalis and pars intermedia of male carp exposed to cold water stress. Future studies using quantitative *in situ* hybridization and/or laser dissection technology are needed to determine whether the expression of *pomc* differs between the melanotrophs and corticotrophs during acute stress events in the zebrafish.

Expression of the ACTH receptor *mc2r* changed modestly in this study compared to *crf*. Whether the three fold increase and rapid recovery of *mc2r* mRNA observed in this study reflect changes in the number of MC2R receptors is not known. Only two other studies have measured changes in *mc2r* expression in the head kidney during stress. In male common carp, head kidney *mc2r* expression was down-regulated after a week of restraint stress (Metz *et al.*, 2005). Another study with juvenile rainbow trout noted a 2.5 fold increase in head kidney *mc2r* expression 4 hs after the application of an acute stressor, which fell back to resting levels after a 24 h recovery period (Aluru and Vijayan, 2008). These rapid changes in *mc2r* expression may alter the sensitivity of the head kidney to ACTH, thus increasing or decreasing its ability to stimulate cortisol production.

Previous studies evaluating the effect of acute stress on interrenal *star* mRNA expression in teleosts have yielded variable results. Confinement of male common carp for 3h did not affect *star* expression (Nematollahi *et al.*, 2009), nor did chasing in juvenile gilthead seabream (*Sparus auratua*; Castillo *et al.* 2008). Chronic exposure of immature gilthead seabream to high density living conditions or injection with lipopolysaccharide did however stimulate a three- and four- fold increase in *star* mRNA expression, respectively (Castillo *et al.*, 2008). Equivocal findings were also observed in rainbow trout. A series of capture, anesthesia and confinement stressors caused an increase in mature and juvenile rainbow trout head kidney *star* mRNA (Kusakabe *et al.*, 2002; Geslin and Auperin, 2004). While chasing stress caused no change in one study of immature rainbow trout (Geslin and Auperin, 2004), the same stressor was associated with an increase in head kidney *star* mRNA in another study of immature rainbow trout (Aluru and Vijayan, 2006). These findings are further complicated by *in vitro* studies of interrenal steroidogenesis. In some studies, incubation of interrenals with ACTH had no effect on *star* mRNA levels (juvenile rainbow trout; Geslin and Auperin 2004; juvenile rainbow trout, Hagen *et al.*, 2006), while in other studies an increase was observed (juvenile rainbow trout, Aluru and Vijayan, 2006; juvenile gilthead seabream, Castillo *et al.*, 2008). The changes in *star* mRNA expression observed in this study reflect the pattern of whole-body cortisol changes, supporting the idea that StAR is an important component of the acute stress response in zebrafish. Moreover the transient nature of the response suggests that the timing of sampling in relation to the stressor may be critical to defining the exact nature of the *star* response.

Changes in *11β-hydroxylase* in response to a stressor have been previously noted in studies with juvenile rainbow trout. Aluru and Vijayan (2006) observed an increase in *11β-hydroxylase* expression after exposure to netting and capture stress, as well as with the application of ACTH *in vitro*. Similarly, Hagen *et al.* (2006) noted that *in vitro* incubation of rainbow trout interrenals with ACTH produced a dose-dependant increase in *11β-hydroxylase* mRNA levels. Geslin and Auperin (2004), however, observed no change in *11β-hydroxylase* mRNA levels in response to a capture stress in juvenile rainbow trout. In this study we observed a rapid increase in *11β-hydroxylase* expression and subsequent decrease in concert with whole-body cortisol levels. As in mammals, where ACTH stimulates *11β-hydroxylase* mRNA expression (Wang *et al.*, 2000; Sewer *et al.*, 2007), the present findings suggest that the expression of *11β-hydroxylase* plays an important role in regulating cortisol synthesis during an acute stressor in zebrafish.

Though only one previous study has noted an increase in *11β-hsd* mRNA levels in response to a stressor, a direct correlation between the expression of this enzyme and plasma cortisol levels was observed in male common carp (Nematollahi *et al.*, 2009). In combination with the findings in this study that *11β-hsd2* expression increases with stress exposure, a key role for 11β–HSD in the regulation of cortisol action in teleost head kidneys is suggested. 11β-HSD converts cortisol to cortisone, which is not an active stress hormone. Since 11β-HSD2 works at the cellular level, it is possible that an increased level of this enzyme would prevent the action of cortisol on GR in this tissue. This may reduce the amount of negative feedback on the head kidney and thus prevent de-activation of the cortisol response.

While this study has shown dramatic changes in gene expression of all components of the HPI axis, it has yet to be determined if these transcripts are translated into functional proteins. Studies in mammals have demonstrated that changes in the adrenal expression of *star* results in increases in StAR protein (Fleury *et al.*, 1998; Lehoux *et al.*, 1998). Little research has been conducted, however, to determine whether increases in the expression of other adrenal steroidogenic enzymes results in protein synthesis.

It is interesting to note that all genes involved in the activation of cortisol synthesis were found to have expression comparable to controls after 60 min of stress (except *pomcα*, see above) and significantly lower expression after 30 min of recovery. At the same time whole-body cortisol levels were elevated after 60 min and similar to basal levels after 30 min of recovery. These findings suggest that in zebrafish, cortisol regulates its own production at all levels of the HPI axis during acute stress.

In conclusion, we have determined the whole-body cortisol response of zebrafish to a novel vortex stressor and have examined changes in gene expression at all levels of the HPI axis (POA, pituitary and interrenals) through an acute stress and short recovery period. We hypothesize that while the quick increase in expression of *crf*, *mc2r*, *star*, *11β-hydroxylase* and *11β-hsd2* may all contribute to the rapid increase in whole-body cortisol after the onset of the stress, the rapid decrease and suppression in the expression of all these genes maybe equally important for recovering whole-body cortisol to resting levels.

Table 1. Sequences of primer pairs used to amplify corticotropin-releasing factor (*crf*),

pro-opiomelanocortin α (*pomcα*), steroid acute regulatory protein (*star*), melanocortin 2

receptor (*mc2r*), *11β-hydroxylase*, 11β-hydroxysteroid dehydrogenase 2 (*11β-hsd2*), *18s*,

and elongation factor-1α (*ef1α*) in real-time reverse transcriptase-polymerase chain

reaction assays.

| | Sequence (5' to 3') | GenBank accession no. |
|---|---|---|
| CRF | F: CGA GAC ATC CCA GTA TCC AA | NM_001007379 |
| | R: GAT GAC AGT GTT GCG CTT CT | |
| POMCα | F: CGC AGA CCC ATC AAG GTG TA | NM_181438 |
| | R: CGT TTC GGC GGA TTC CT | |
| StAR | F: ACC TGT TTT CTG GCT GGG ATG | NM_131663 |
| | R: GGG TCC ATT CTC AGC CCT TAC | |
| MC2R | F:  CTC CGT TCT CCC TTC ATC TG | NM_180971 |
| | R: ATT GCC GGA TCA ATA ACA GC | |
| 11β-hydroxylase | F:  TGT GCT GAA GGT GAT TCT CG | NM_001080204 |
| | R: GCT CAT GCA CAT TCT GAG GA | |
| 11β-HSD2 | F: TGC TGC TGG CTG TAC TTC AC | NM_212720 |
| | R: TGC ATC CAA CTT CTT TGC TG | |
| 18s | F: ATT CTT GGA CCG GCG CAA GA | FJ915075 |
| | R: CAT CTA AGG GCA TCA CAG AC | |
| EF1α | F: GGG CAA GGG CTC CTT CAA | NM_131263 |
| | R: CGC TCG GCC TTC AGT TTG | |

F, forward primer; R, reverse primer

**Figure 1.** Radioimmunoassay displacement curve for cortisol standard (black circles) and zebrafish whole-body lipid extraction (clear circles). The zebrafish extract points were phase shifted to the right of the cortisol standard curve for greater visibility. Parallelism of the two curves indicates that no interfering contaminants were present in the zebrafish extract.
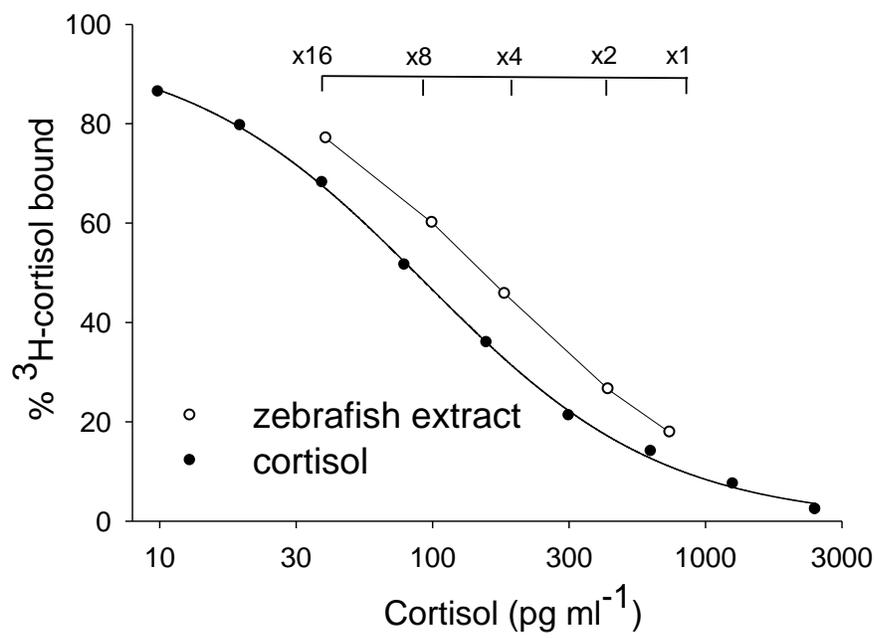
**Figure 1.**

**Figure 2.** Mean (±SE, n= one beaker containing 6-8 fish per treatment group) whole

body cortisol levels (ng/g BW) of adult zebrafish (*Danio rerio*) exposed to a vortex

stress for 20 min at varying intensities (revolutions per minute) or exposed to a 1 min air

exposure and a 20 min recovery period (air). Prior to experimentation, fish were

acclimated in 2L beakers in a flow through setup for one week. Data points with different

letters are significantly different ($p<0.05$) as determined using a one-way analysis of

variance ($p<0.001$). The black line indicates a positive correlation between vortex

intensity and whole body cortisol ($R^2=0.652$; $p<0.001$).
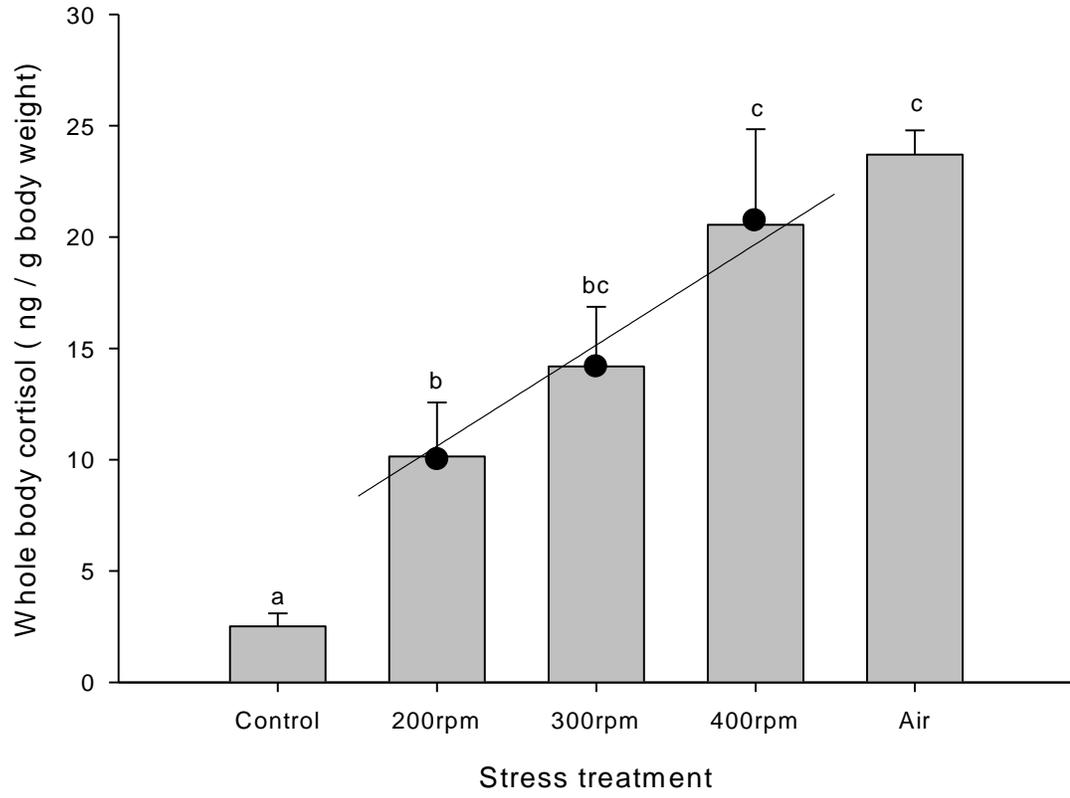
**Figure 2.**

**Figure 3.** Mean (±SE, n = one beaker containing 4-6 fish per time point) whole body cortisol levels (ng/g BW) of adult zebrafish (*Danio rerio*) exposed to a 300 rpm vortex stress (duration indicated by dashed bar) measured over time (min). Data points with different letters are significantly different ($p<0.05$) as determined using a one-way analysis of variance ($p < 0.001$).
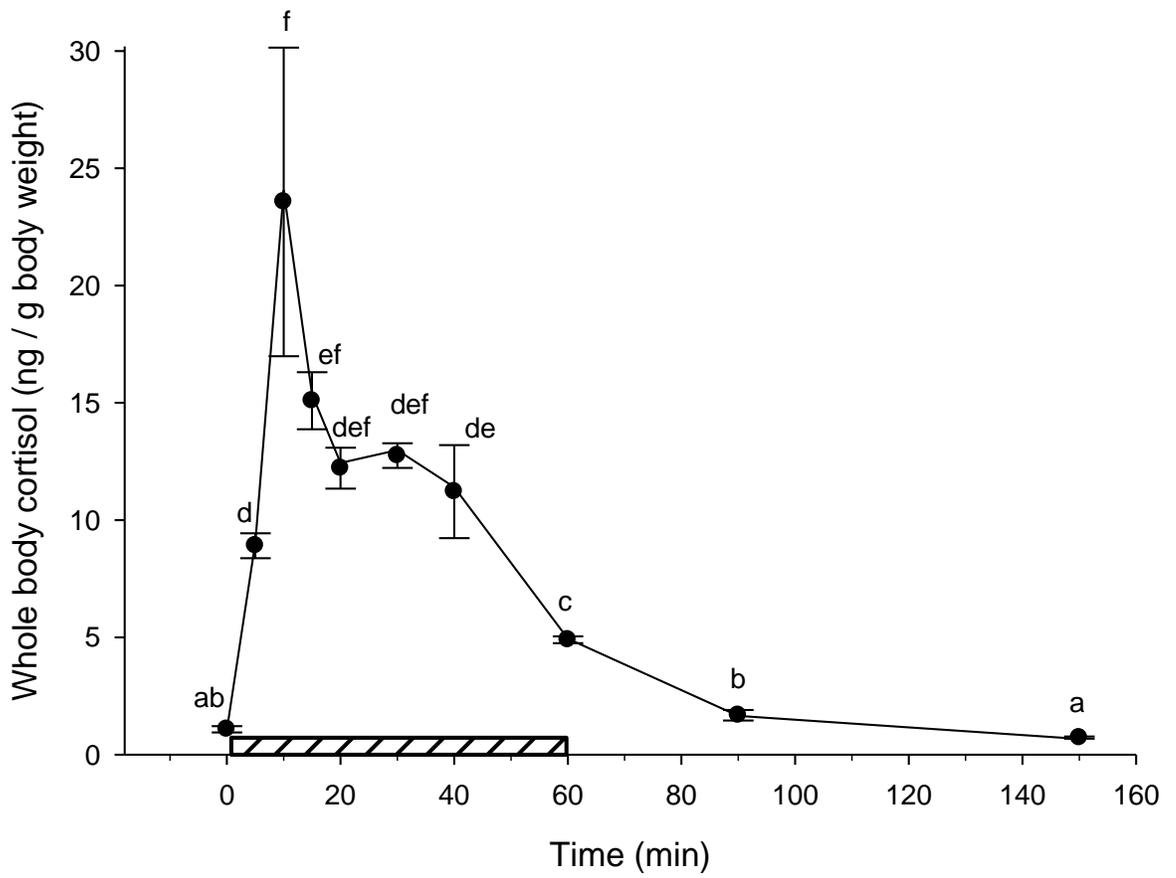
**Figure 3.**

**Figure 4.** Mean (±SE, n= one beaker containing 4-10 fish per time point) expression (gene of interest / reference gene) of adult zebrafish (*Danio rerio*) preoptic areas (A), pituitaries (B) or head kidneys (C-F) exposed to a vortex stress (duration indicated by dashed bar) as measured using quantitative reverse-transcription PCR. Data points with different letters are significantly different ($p < 0.05$), determined using a one-way analysis of variance (A-F, $p < 0.001$).

**Figure 4.**

40
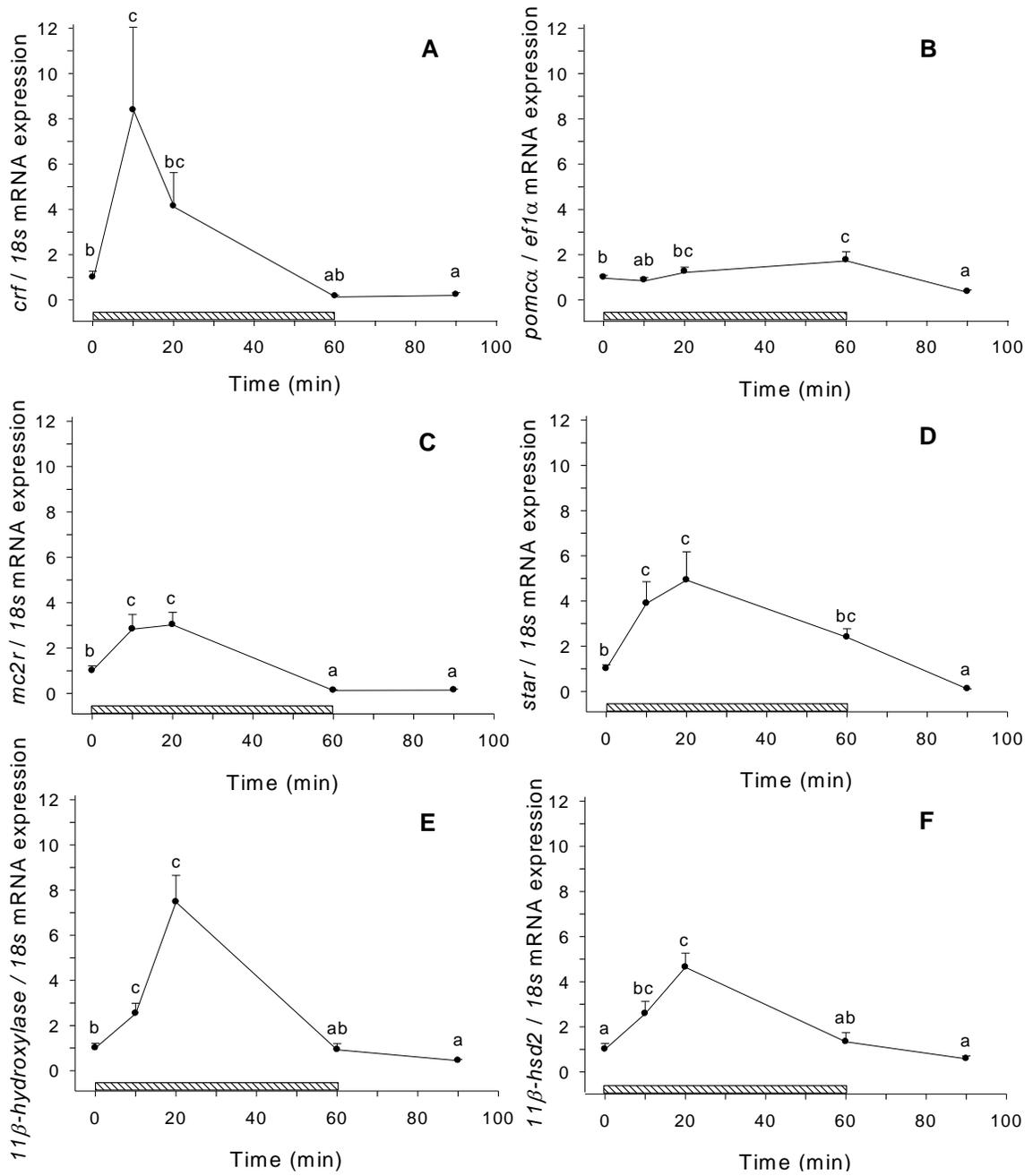
# CHAPTER 3

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

**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 stress response and whether 17β-estradiol (E2) or 11-ketotestosterone (11KT) have modulating effects on the hypothalamic-pituitary-interrenal (HPI) axis. To accomplish this, whole body cortisol levels, cortisol synthesis *in vitro*, as well as gene expression of corticotropin-releasing factor (*crf*) of E2- or 11KT-exposed zebrafish were quantified. Expression of key genes in the head kidney of E2-exposed zebrafish was quantified. No dimorphisms in the HPI axis were apparent at rest or in response to a vortex stressor. Exposure to E2 and 11KT altered the stress response of zebrafish, but only in the sex in which the steroid is not the major circulating sex steroid. E2-exposure blunted the cortisol response of male fish *in vivo* and *in vitro* and as well as *crf* expression. 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 *crf* expression. In summary, zebrafish did not exhibit a sexually dimorphic stress response but E2 and 11KT did modulate the stress response and did so by different mechanisms.

**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 (Critchlow *et al.*, 1963), amphibians (Licht *et al.*, 1983), and reptiles (Elsey *et al.*, 1990). In mammals, this sexual dimorphism is responsible for differing susceptibility to many health issues including anxiety, depression, diabetes and heart disease (Kudielka and Kirschbaum, 2005). While few studies have explored this subject in fish, a sexually dimorphic stress response has been noted in some elasmobranchs and teleosts (goldfish, *Carassius auratus*, temperature stressor, Peter *et al.*, 1978; rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo truta*, confinement stressor Pottinger *et al.*, 1995; yellow perch, *Perca flavenscens*, ACTH challenge, Girard *et al.*, 1998; sockeye salmon, *Oncorhynchus nerka*, confinement stressor, Kubokawa *et al.*, 2001; bonnethead shark, *Sphyrna tiburo* and Atlantic stingray, *Dasyatis sabina*, capture stress, Manire *et al.*, 2007). 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 (Alderman and Bernier, 2007; 2009), 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 (Handa *et al.*, 1994; Seale *et al.*, 2004). 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 (Biernacka-Lukanty *et al.*, 2004) and adrenocorticotrophic hormone (ACTH) production by the pituitary are mediated by sex steroids (Kudielka and Kirschbaum, 2005). 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) (Schouler-Miller *et al.*, 2004; Van De Stolpe *et al.*, 2004; Handa *et al.*, 2009). As in mammals, E2 exposure or implants exaggerate and T or 11-ketotestosterone (11KT) implants blunt the stress response of salmonids (juvenile rainbow trout and juvenile brown trout, confinement stressor, Pottinger *et al.*, 1996; juvenile Atlantic salmon, *Salmo salar*, handling stressor, Lerner *et al.*, 2007). 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, Young *et al.*, 1996; rainbow trout and chinook salmon, *Oncorhynchus tshawytscha*, McQuillan *et al.*, 2003).

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 (Fuzzen *et al*., in preparation). Using the same stressor, the current study determined whether the stress response 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 (Borg, 1994). To determine the sites of action of sex steroids, the interrenals of E2- and 11KT-exposed fish were perfused with ACTH and the rate of cortisol synthesis was measured. In addition, the mRNA levels of preoptic area (POA) *crf*, interrenal ACTH receptor melanocortin receptor 2 (MC2R), and the interrenal steroidogenic enzymes steroid acute regulatory protein (*star*), *11β-hydroxylase*, and *11β-hydroxysteroid dehydrogenase 2* (*11β-hsd2*) were assessed in male and female zebrafish after 48 h of exposure to E2.

**Materials and Methods**

**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.

**Experimental design**

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

To test the hypotheses 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 hrs. 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 (20L) 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 seven day acclimation period. To exposed 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. Fish were fasted for 24 h before sampling and were sampled either immediately after the 48 hour exposure period, or after the application of a vortex stress. For the vortex stress experiments, magnetic stir bars (Fisherbrand Magnetic Stir Bar, Octagonal, 5 cm x 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 had their liver removed for measurement of vitellogenin mRNA content. All samples were stored at -80˚C until processed. This experiment was conducted twice using E2 and once with 11KT.

*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 hrs. At this time the POAs and head kidneys of male and female fish (n=6-8) were removed for RNA extraction and measurement of selected gene expression 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 Bernier *et al.* (2008). 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.* (2005). 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 perfused with a 0.015M HEPES/ Tris-buffered medium (pH=7.4; containing 128 mM NaCl, 2 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 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 (American Peptide Company Inc., Sunnyvale CA, USA) was administered for 20 min followed by a 70 min recovery period with the HEPES solution. Ten minute fractions were collected, stored on ice, and immediately analyzed for cortisol using a radioimmunoassay. After superfusion, head kidneys were removed from chambers, sonicated (Vibracell©, Sonics and Materials Inc., Newtown CT, USA) in 500 μL $ddH_2O$ 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 production (fmol/ μg protein / min).

**Experimental procedures**

*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.* (2008). Briefly, samples were reconstituted with 300 μL of acetate buffer (2.35 ml glacial acetic acid, 1.23 g sodium acetate trihydrate, in 1L; 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 $Na_2HPO_4 \cdot 7\ H_2O$; 9.3 mM $NaH_2PO_4 \cdot H2O$; pH 7.6; 0.1% gelatin; 0.01% thimerosal) and stored at -20˚C until analysis.

### *Radioimmunoassay*

Cortisol, E2 and 11KT were measured in 200 µl aliquots of samples (in triplicate, values were averaged) by radioimmunoassay (RIA) using the methods described by Bernier *et al*. (2008). 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.). Extraction efficiency for all steroids was found to be $74 \pm 1\%$ (n=30). 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. Steroid values were presented as ng (cortisol and E2) or pg (11KT) whole-body steroid / g of individual body weight (BW) and were corrected for individual extraction efficiency.

### *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 10µl 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 (Ct) 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 (E) between 85% and 100% based on the equation:

$$E\% = 10 \wedge ((-1/\text{slope})-1)*100$$

Gene expression was quantified with the slope (m) and the y-intercept (y) of the standard

curve based on the equation:

$$\text{Value} = 10 \wedge [(Ct-y)/m]$$

To correct for minor variations in template input and transcriptional efficiency,

each sample was normalized to the expression level of either elongation factor-1α (*ef1α*;

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 *ef1α* due to significantly different levels of *ef1α* expression between treatment groups, eliminating it as an acceptable references gene. Gene specific primer sequences for zebrafish vitellogenin (v*tg*), *crf*, s*tar*, *mc2r*, *11β-hydroxylase*, *11β-hsd2*, *ef1α* 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.

**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. 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 x inter-quartile 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.

**Results**

*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. 2 A). While exposure to E2 did not affect basal cortisol levels or stress-induced cortisol levels in females, E2-treated males were found to have an attenuated cortisol response to the vortex stressor (Fig. 2 A). 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 estradiol levels (Fig. 2C). However, vitellogenin mRNA expression increased 3-fold in E2 exposed male, but not 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 *vtg* mRNA expression (Fig. 3B).

*Tissues within the HPI axis affected by E2 and 11KT*

Perfused 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 secretion rates or on ACTH stimulated female secretion rates, 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 5-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 levels of head kidney *mc2r*, *11β-hydroxylase* and *11β-hsd2* expression under control conditions and with E2 exposure (Fig. 8 A, C, and D). Female zebrafish were found to express three times more s*tar* 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 *11β-hydroxylase* expression, but had no effect on the expression of *11β-hsd2* mRNA in head kidneys of zebrafish (Fig. 8A-D).

**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 exaggerated whole-body cortisol levels, this did not seem to be mediated through the HPI axis at the level of POA *crf* expression or interrenal cortisol synthesis rate. These results suggest that sex steroids modulate the cortisol stress response through different modes of action in zebrafish.

*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 dimorphisms in the stress response of some teleosts (goldfish, temperature stressor, Peter *et al.,* 1978; rainbow trout and brown trout, confinement stressor, Pottinger *et al.* 1995; rainbow trout, confinement stressor, Pottinger and Carrick, 2000; sockeye salmon, confinement stressor, Kubokawa *et al.,* 2001). Additionally, Pottinger *et al.* (1995) 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*. (1978) 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 in humans, physical stressors 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 (Kudielka and Kirschbaum, 2005). It is not known whether a similar situation exists in teleosts as the effect of different stressors on this dimorphism has not been tested. Therefore, it is necessary to test a variety of stressor types before determining that the HPI axis functions similarly in male and female zebrafish.

*Effects of estradiol exposure on the stress axis in zebrafish*

Resting whole-body cortisol levels were unaffected by 48 h of E2 exposure in males and females. While whole-body E2 levels were not affected by steroid exposure, a physiological effect of the exposure was confirmed by an increase in male *vtg* expression, a response that is consistent with other studies of zebrafish (Rose *et al.,* 2002; Van den Belt *et al*., 2003; Schafers *et al.,* 2007). The cortisol stress response of male zebrafish was blunted by E2 exposure while there were no effects on the whole-body cortisol increase 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 (Teles *et al.,* 2004), exposure of the same species to E2 (0.2 or 2 µg/L) for 10 d was found to reduce resting plasma cortisol levels (Teles *et al.,* 2006). 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 (Lerner *et al.*, 2007). In another study with salmonids, implantation of E2 pellets (20mg; 24 d) enhanced the stress response of juvenile rainbow trout and juvenile brown trout to confinement (Pottinger *et al.*, 1996). 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; 48h; McQuillan *et al.*, 2003). Similarly, Barry *et al*. (1997), 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

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, 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 *11β-hydroxylase* expression, but did not affect *star* or *11β-hsd2* 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 *11β-hydroxylase* expression translates into a reduction in enzymatic activity, then the interrenal would have decreased cortisol synthesis capacity after E2 exposure.  In contrast to our study, head kidneys of juvenile Atlantic salmon exposed to ethinyl estradiol (5 ng/L; 3d),  a potent ER agonist used in human birth control pills, had increased expression of *star*, and *11β-hydroxylase* as well as an increase in StAR protein (Lyssimachou and Arukwe, 2007).  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. It is possible that the expression of 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 (Roy *et al.*, 1999; Jasnow *et al.*, 2006; Lalmansingh and Uht, 2008). It is important to note that while the changes in whole-body cortisol were apparent after exposure to stress, gene expression in both the POA and head kidney were 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 (Fuzzen *et al.* in preparation). 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 *11β-hsd2* 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 and these need to be studied.

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

Exposure of zebrafish to 100 ng/L 11KT for 48 hr did not alter levels of whole-body 11KT in either sex. This exposure did decrease expression of *vtg* in 11KT-exposed males, an effect that has been also observed with exposure of mummichog (*Fundulus*

*heteroclitus*) to 17α-methyl testosterone (Sharpe *et al.,* 2004), suggesting that the treatment regime was effective in altering physiological functions. 11KT exposure also 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 (Pottinger *et al.*, 1996).  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*. (2003) 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.* (1996) found that 11KT implants (20mg; 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.

*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 findings, that exposure to sex steroids at levels that are not normally observed in circulation has an impact on the magnitude of the cortisol stress-response, suggests that these types of responses may only occur when hormone levels are changed acutely. Since zebrafish spawn year round, it is unlikely that there are large fluctuations in sex steroid levels in adult fish under natural conditions. It would be interesting, however, to determine whether juvenile fish undergoing sexual maturation go through periods of altered stress responsiveness. A comparison of the HPI axis of immature and mature zebrafish is an area for future study.

Table 1. Sequences of primer pairs used to amplify vitellogenin (*vtg*), corticotropin-releasing factor (*crf*), steroid acute regulatory protein (*star*), melanocortin 2 receptor (MC2R), *11β-hydroxylase*, 11β-hydroxysteroid dehydrogenase 2 (*11β-hsd2*), *18s*, and elongation factor-1α (*ef1α*) in real-time reverse transcriptase-polymerase chain reaction assays.

|  | Sequence (5' to 3') | GenBank accession no. |
|---|---|---|
| Vtg | F: TGC GTG AAG TTG TCA TGC TTG | NM_001044897 |
|  | R: GAT CTC GTG GAT GGG CCT G |  |
| CRF | F: CGA GAC ATC CCA GTA TCC AA | NM_001007379 |
|  | R: GAT GAC AGT GTT GCG CTT CT |  |
| StAR | F: ACC TGT TTT CTG GCT GGG ATG | NM_131663 |
|  | R: GGG TCC ATT CTC AGC CCT TAC |  |
| MC2R | F:  CTC CGT TCT CCC TTC ATC TG | NM_180971 |
|  | R: ATT GCC GGA TCA ATA ACA GC |  |
| 11β-hydroxylase | F:  TGT GCT GAA GGT GAT TCT CG | NM_001080204 |
|  | R: GCT CAT GCA CAT TCT GAG GA |  |
| 11β-HSD2 | F: TGC TGC TGG CTG TAC TTC AC | NM_212720 |
|  | R: TGC ATC CAA CTT CTT TGC TG |  |
| 18s | F: ATT CTT GGA CCG GCG CAA GA | FJ915075 |
|  | R: CAT CTA AGG GCA TCA CAG AC |  |
| EF1α | F: GGG CAA GGG CTC CTT CAA | NM_131263 |
|  | R: CGC TCG GCC TTC AGT TTG |  |

F, forward primer; R, reverse primer

**Figure 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 in kidney regions as determined with a student's t-test.

**A**



**B**
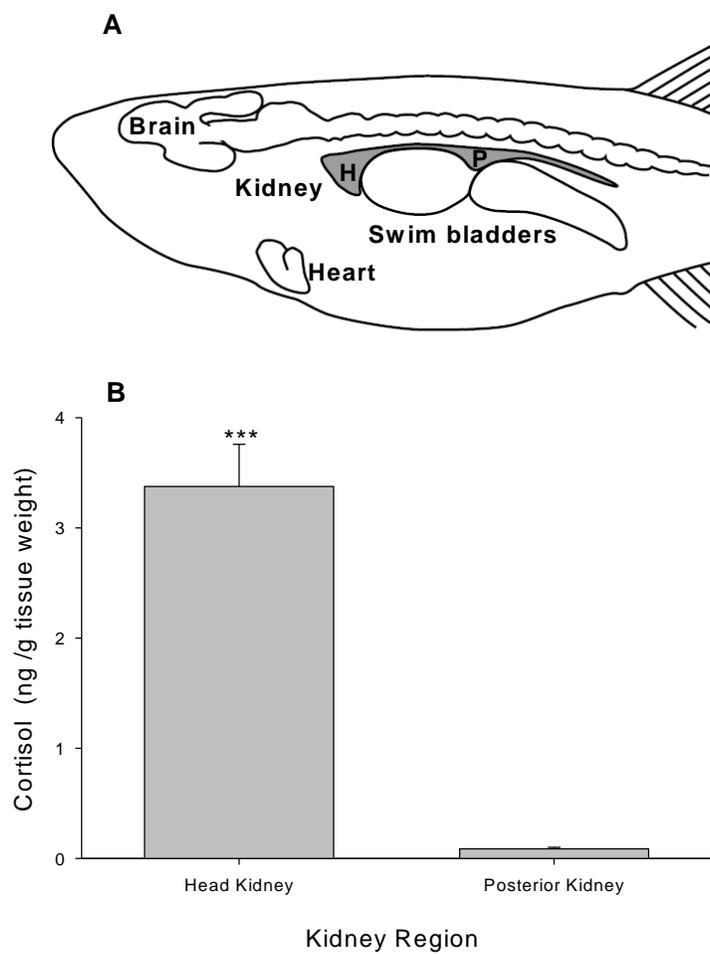


Figure 1

**Figure 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 ± S.E.M. (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 ($p<0.05$).

Figure 2

**Figure 3.** Changes in liver vitellogenin (*vtg*) mRNA levels in relation to elongation factor 1α (*ef1*α) 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 ($p < 0.05$).

Figure 3

**Figure 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 $\pm$ 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 ($p<0.05$).
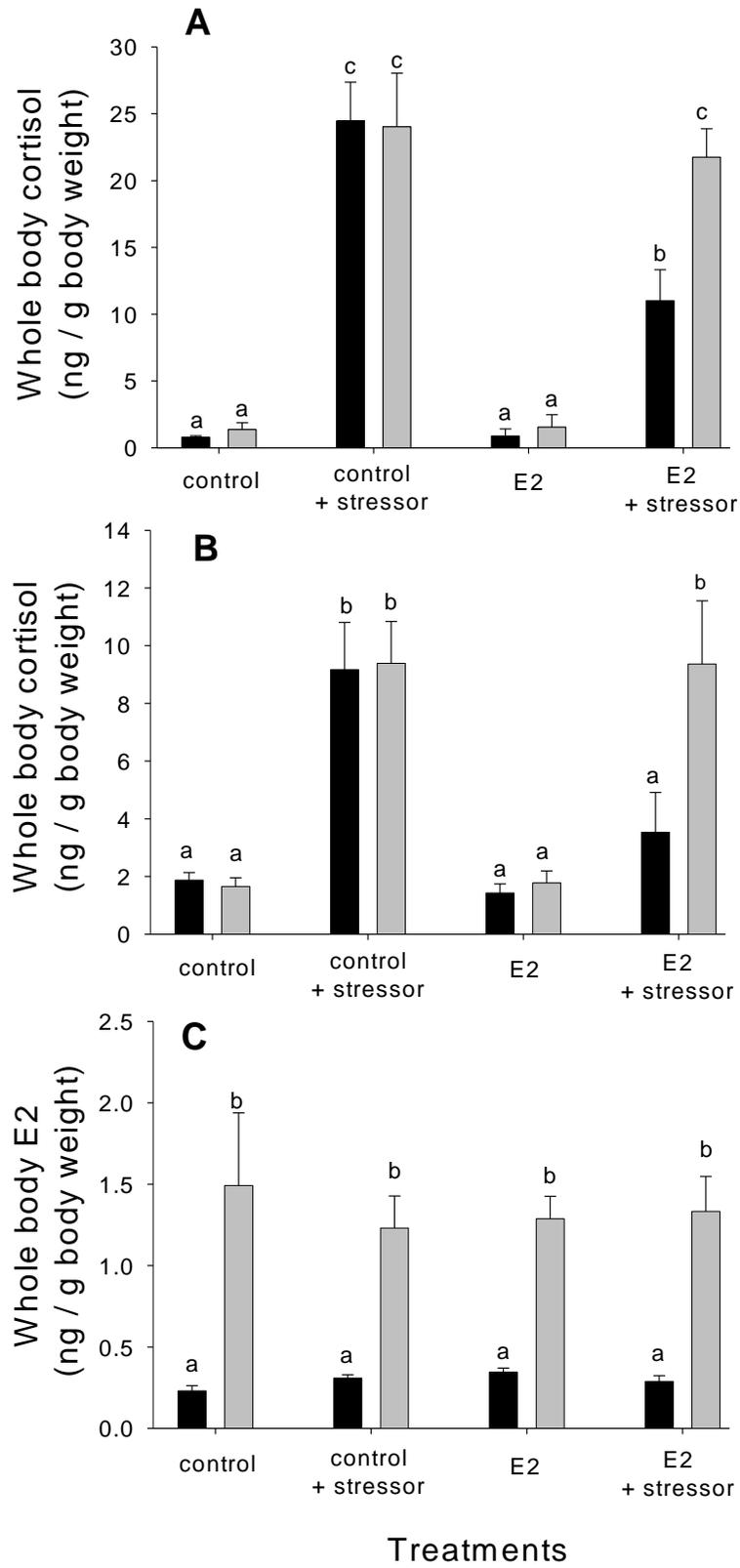
Figure 4

**Figure 5.** Rate of cortisol secretion (fmol/min/μg protein) from zebrafish head kidneys (n=11) perfused with a HEPES buffer followed by $10^{-6}$M human ACTH (duration marked by dashed bar) and subsequent recovery incubation with HEPES. Differences from basal secretion rates (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).
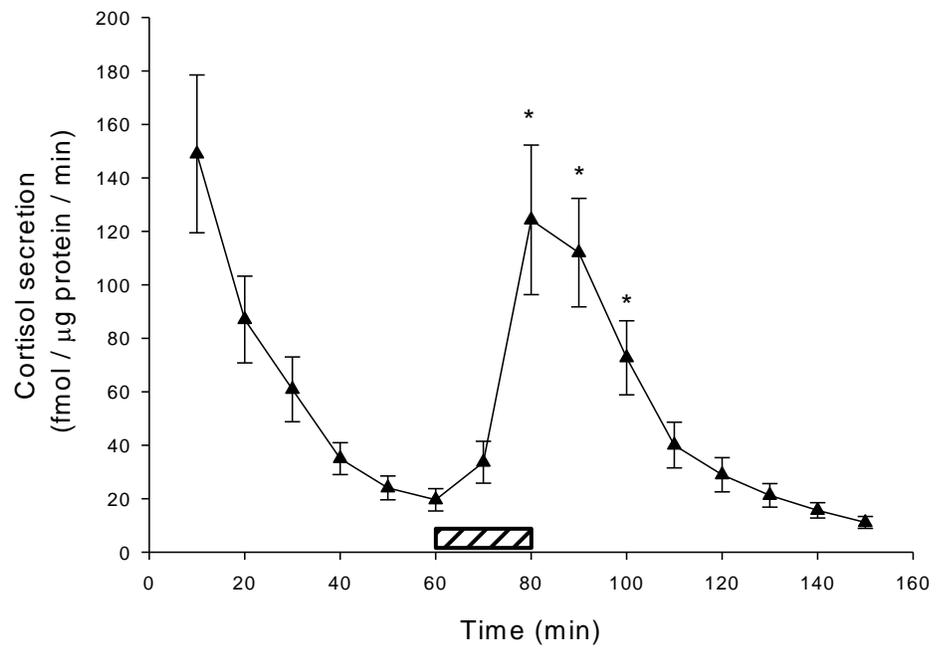
Figure 5

**Figure 6.** Effects of 17β-estradiol (E2; A) or 11-ketotestosterone (11KT; B) on the basal (base) and ACTH- ($10^{-6}$M) elicited cortisol secretion rate from the perfused 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 which was used to quantify cortisol synthesis *in situ*. Measurements were taken after 60 min of perfusion with buffer (base) and after being dosed 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).
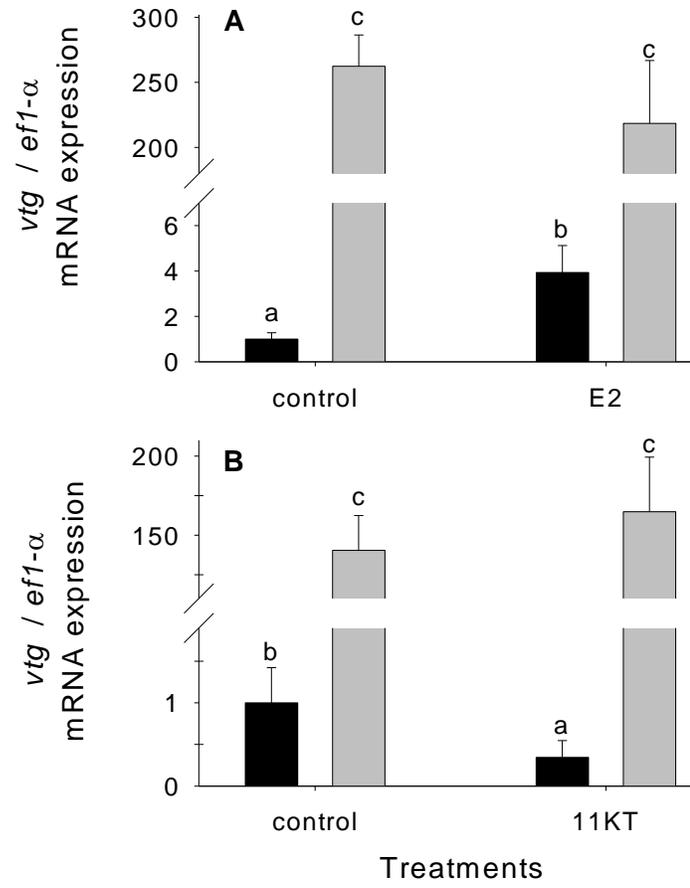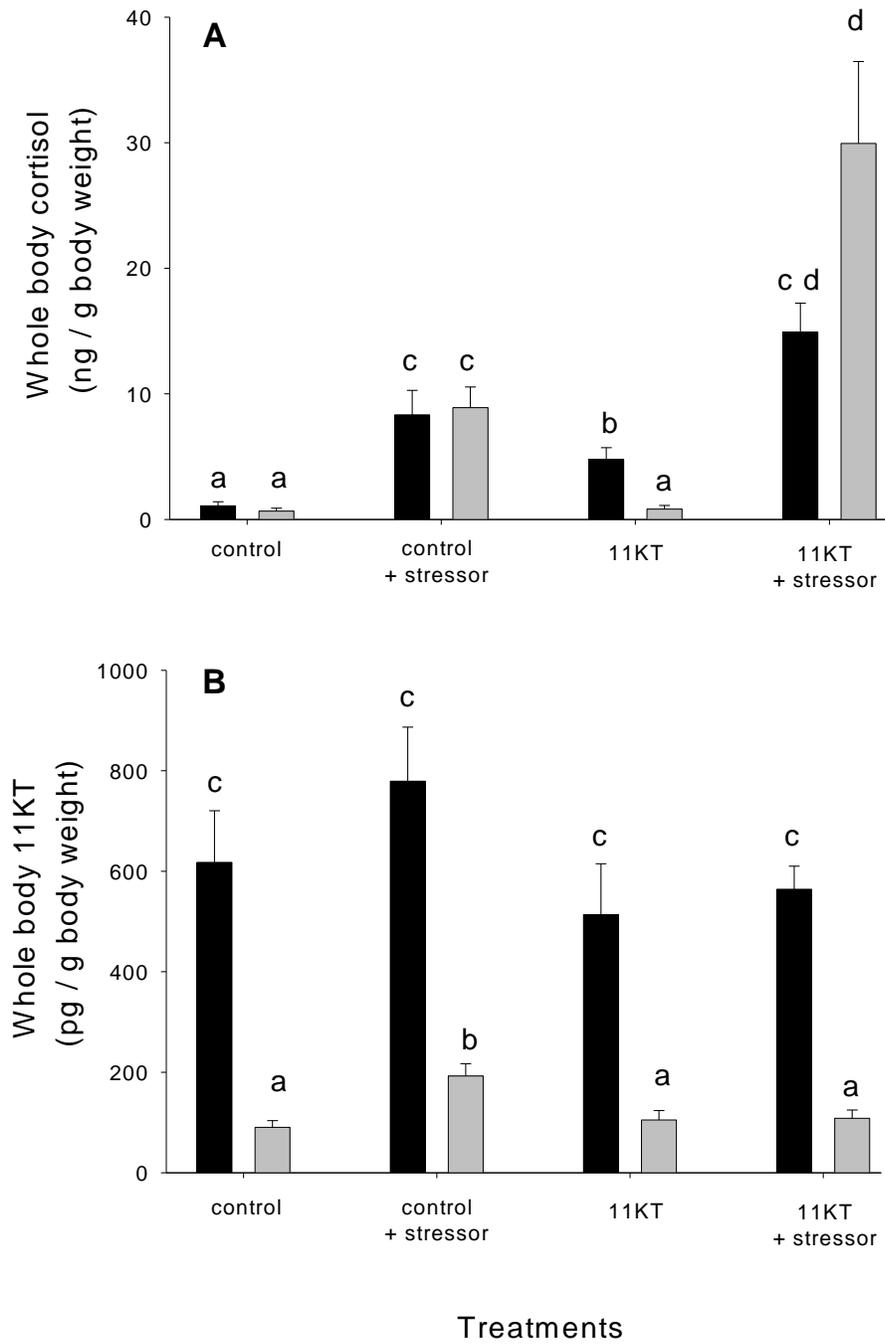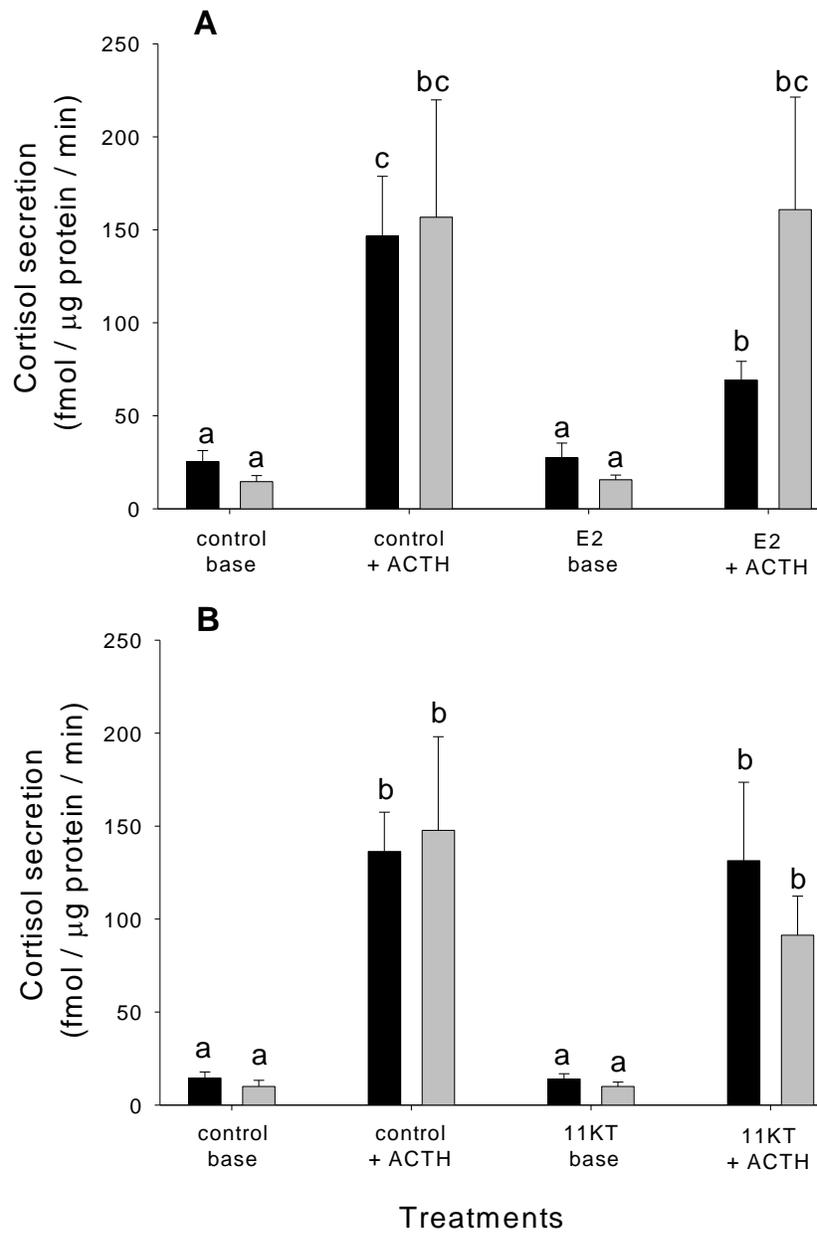
Figure 6

**Figure 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 (*ef1α*) or 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 tissue POA extraction. Values are means ± S.E.M. (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 ($p < 0.05$).

**A**

**B**
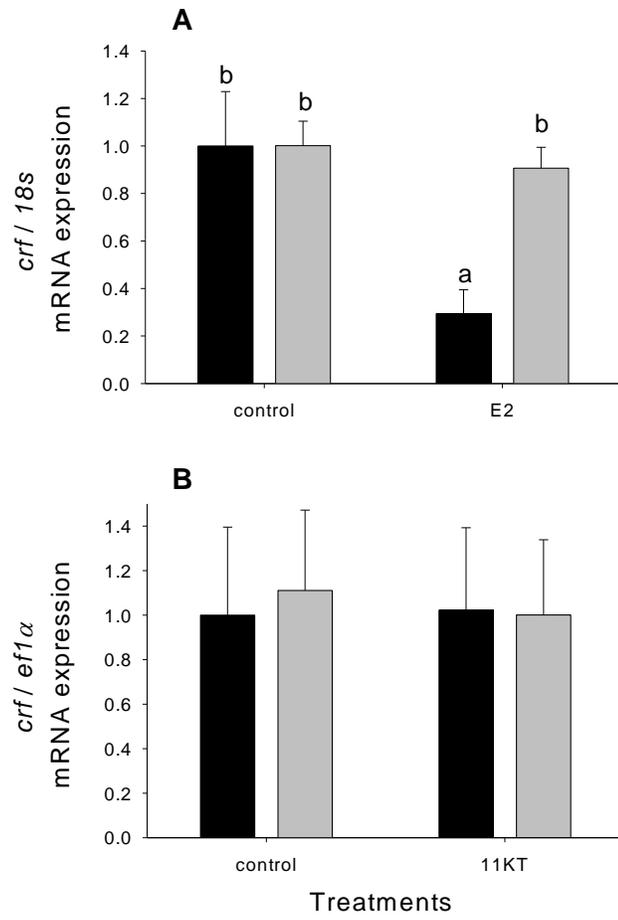
Figure 7

**Figure 8.** Effects of 17β-estradiol (E2) on head kidney mRNA levels of melanocortin

receptor 2 (*mc2R*; A), steroid acute regulatory protein (*star*; B), *11β-hydroxylase* (C), and

*11β-* hydroxysteroid dehydrogenase 2 (*11β-hsd2*; D) in relation *18s*. 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 head kidney removal. Values are means ± S.E.M. (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
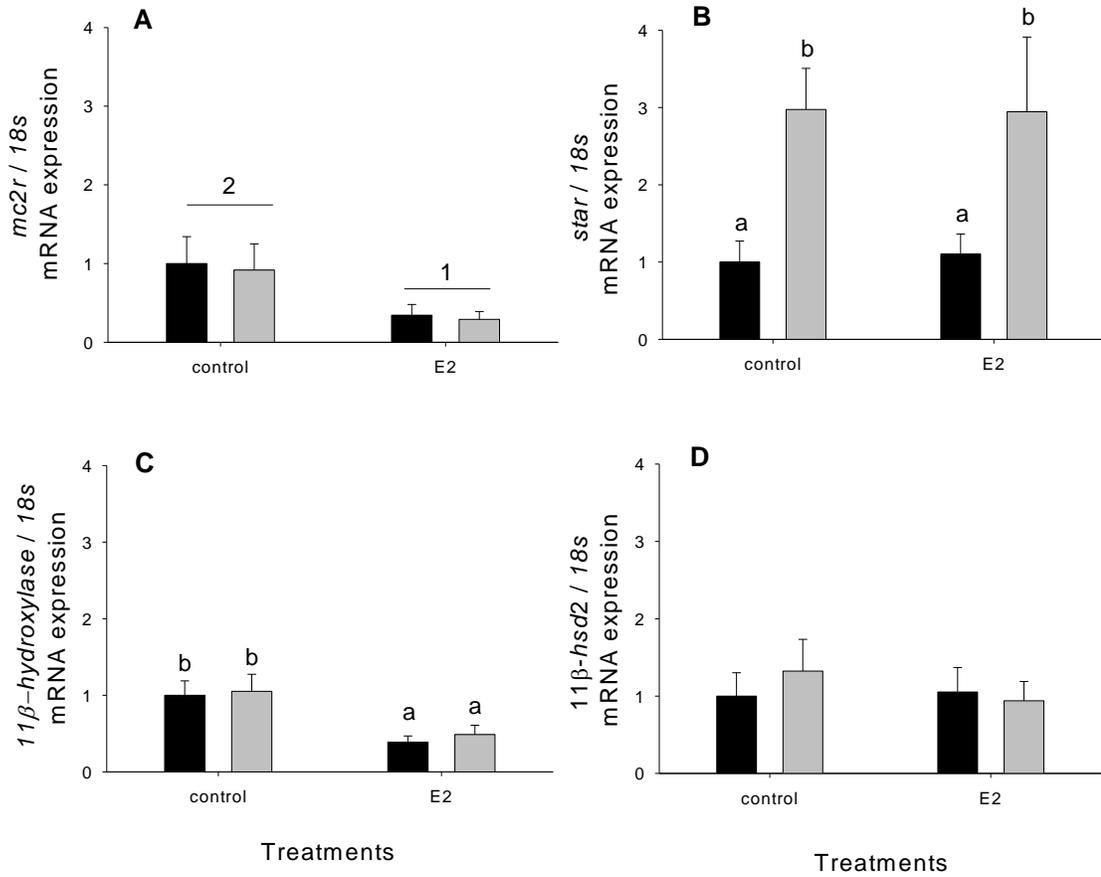
of treatment as determined by two-way ANOVA.

Figure 8

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# CHAPTER 4

## General Discussion

While previous research has been conducted to develop the zebrafish as a stress model, little is known about the dynamics and control of the adult HPI axis under basal and stressed conditions. This study demonstrated that the adult zebrafish stress axis is activated and deactivated quickly and that gene transcription at all levels of the stress axis is involved in this process. It was also found that while sex steroids alter the magnitude of the stress response, there is no intrinsic dimorphism between the male and female stress axis in zebrafish. Collectively, the results of this study support the use of the zebrafish as a model for stress research in future studies.

**Zebrafish as a stress model**

The present studies have demonstrated the utility of using zebrafish to study the cortisol stress response. These studies showed that the cortisol stress response of zebrafish to a standardized stressor was rapid, with an increase in whole-body cortisol occurring within 5 min and a return to resting levels within 1 hr. The magnitude of the stress response was easily manipulated by changing the intensity of our vortex stressor. In addition, the zebrafish stress response between experiments was consistent and changes in whole-body cortisol and *crf* expression were comparable to the stress responses of other teleosts (Bernier *et al.*, 2008). These studies also showed that the zebrafish stress response is not sexually dimorphic, either *in vivo* or *in vitro*. While the physical size of the zebrafish may seem to be a challenge, in this study measurements of whole body cortisol rather than plasma cortisol were taken and the brain was successfully regionally dissected to measure gene expression. In future studies, laser capture technology could be

utilized to isolate specific neuropeptide-producing and steroidogenic cells from tissues. I believe that the study of the adult zebrafish stress response will be important for the future of many areas of research including stress physiology and aquatic toxicology.

In addition to establishing the zebrafish as a good stress model, these studies demonstrated for the first time in a teleost that gene transcription at all levels of the stress axis are activated simultaneously during a stress event. In this study gene expression in the POA and head kidney changed dynamically in response to the vortex stressor. Surprisingly there were only modest changes in *pomc* expression in the pituitary. Given that the pituitary is a heterogeneous tissue and that *pomc* is expressed in different cell types, it is possible that changes in *pomc* expression were blunted due to contradictory changes in different regions of the pituitary. Future studies using quantitative *in situ* hybridization or laser capture technology could be used to determine whether the expression of *pomc* differs between the melanotrophs and corticotrophs during acute stress events in the zebrafish.

While some of the key genes involved in the HPI axis were measured in this study, the stress axis is quite complex. In addition to CRF, there are several other neuropeptides capable of stimulating ACTH release from the corticotrophs including the CRF-related peptide UI, arginine vasotocin, thyrotropin-releasing hormone, angiotensin II and isotocin (Bernier *et al.,* 2009). There is also a CRF-binding protein located in similar regions of the brain as CRF (Alderman and Bernier, 2007), the function of which is not clear, and two CRF receptors in the pituitary which could also alter the activation or magnitude of a stress response (Bernier *et al*., 2009). Therefore future studies would benefit from a comprehensive evaluation of the interaction of these factors during a stress

event. There are many additional questions in the field of stress physiology, such as the role of cortisol in feedback regulation of the HPI axis, which could be addressed using the zebrafish as a model system.

**Effects of sex and sex steroids on the stress axis**

The current studies showed that there were no intrinsic differences between the whole-body cortisol stress response of male and female zebrafish. This finding was confirmed by several studies which suggest that male and female zebrafish sense and respond to a physical stressor in a similar manner. Similar increases in POA *crf* expression and head kidney ACTH stimulation were observed between sexes. Whether other stressor types, such as a social or psychological stressor, would result in the same finding is not known and is an area for future study. If future studies do find that sexes differ in their cortisol response to different stressor types, this would be due to varying stimulation at the level of the brain and pituitary since it was found in this study that there were no differences in the interrenal response to ACTH stimulation between male and female zebrafish.

The application of sex steroids was found to alter the stress response. Interestingly, the application of sex steroids only affected the sex in which the steroid was not the primary circulating sex steroid. E2 blunted the stress response of male zebrafish, and 11KT enhanced the stress response of female zebrafish. These finding suggest that while sex steroids do impact the magnitude of the cortisol stress-response, this may only occur when hormone levels are changed acutely. Since zebrafish are asynchronous spawners capable of spawning daily, it is unlikely that there are large fluctuations in sex steroid levels in adult fish under natural conditions. It would be

interesting, however, to determine whether juvenile fish undergoing sexual maturation go through periods of altered stress responsiveness. A comparison of the HPI axis of immature and mature zebrafish is an area for future study.

The current results also have implications in the field of aquatic toxicology and raise concerns about the effects of waterborne sex steroids and sex steroid mimics on the stress response of aquatic vertebrates. While there is a wealth of literature on the effects of environmental estrogens, anti-estrogens, androgens and anti-androgens on the reproductive biology of fishes, few studies have examined the effects of theses compounds on the stress axis in fish. Despite the lack of focused research on specific sex steroid contaminants, studies have demonstrated altered stress responsiveness of fish living in steroid contaminated waters. Immature marbled sole (*Pleuronectes yokamae*) from highly inhabited parts of Tokyo Bay were found to have a blunted stress response to air exposure. In addition to this, when interrenals were stimulated with ACTH *in vitro*, fish from Tokyo Bay had lower cortisol synthesis rates that fish from reference sites (Kakuta, 2002). Tokyo Bay is known to contain environmental estrogens at high enough levels to elevate vitellogenin levels in male wild founder, *Pleuronectes yokohamae* (Hashimoto *et al.*, 2005; Isobe *et al.*, 2006). These findings are consistent with those of our study in which E2-exposed male zebrafish had a blunted increase in cortisol production after ACTH stimulation. In the future, it will be imperative to examine the role of environmental steroids, including varying doses and duration, in modulating the stress axis of fish.

**Mode and mechanisms of action of sex steroids.**

**17β-estradiol**

The current studies showed that E2 reduced the cortisol stress response in male zebrafish. Several experiments were conducted to determine the possible mechanisms of this effect. At the level of the brain, there was decreased basal *crf* transcription in E2 exposed males suggesting that the blunted stress response of E2 exposed males occurred partly due to a decrease in the stimulation of the HPI axis by CRF. E2 exposure clearly blunted ACTH induced cortisol production *in vitro* in male zebrafish, also supporting the *in vivo* findings. When the expression of key genes in the head kidney of E2 exposed fish was examined it was found that expression of the ACTH receptor, *mc2r*, as well expression of the steroidogenic enzyme *11β-hydroxlyase* were lower than control expression levels in both sexes. While these results do not explain why there was a decrease in the rate of cortisol synthesis in males only, the measurements were taken at rest and there are many steroidogenic enzymes that could also be affected by E2 exposure. Therefore, in the future, it would be beneficial to examine the effects of E2 on stress induced expression levels *crf*, *pomc*, *mc2r*, *star*, *11β-hydroxylase* as well as other steroidogenic enzymes.

While it is not clear how E2 is depressing *crf*, *mc2r* and *11β-hydroxylase* expression in zebrafish, it is likely that steroid receptors are playing a role. Steroid receptors have been shown to play a key role in modulating CRF in rodents, where E2 enhances and T depresses the stress response (Handa *et al.*, 1994; Yao and Denver, 2007). A few key findings in rodents support the role of steroid receptors in modulating sexual dimorphism of the stress response in mammals. Firstly, increases of corticosteroids in E2 exposed rodents are accompanied by increases in CRF levels and ACTH (Handa *et al.*, 1994). Secondly, receptor specific antagonists show that blockade

of ER subtype α (ERα) inhibits E2 enhancing effects, while AR or ERβ blockade prevents the inhibitory effects of androgens on stress axis activity (Lund *et al.*, 2004; Schouler-Miller *et al.*, 2004; Van De Stolpe *et al.*, 2004; Haeger, 2006; Lund *et al.*, 2006). Furthermore, estrogen responsive elements (EREs) have been identified in the promoter region of the human CRF gene (Vamvakopoulos and Chrousos, 1993). Therefore investigating the role of ERs in mediating the effects of E2 on the male zebrafish stress response is an area for future study.

**11-ketotestosterone**

Exposure of zebrafish to 11KT was found to enhance the cortisol stress response of females. While the effects of E2 exposure were found to be mediated at the level of the brain and interrenal, there was no evidence that this was also true for 11KT exposure. Exposure to 11KT for 48 h had no effect on basal *crf* transcription in the POA nor did it have any effect on the cortisol synthesis of either sex. As for E2, it is not known whether 11KT exposure would affect the expression of the various HPI axis genes during a stress response. It is also possible that 11KT does act at the level of the brain, but instead acts on another secretogues of ACTH located in the POA, such as arginine vasotocin (AVT) which is a homologue to mammalian arginine vasopressin (AVP; Bernier *et al.* 2009). Studies with mammals have shown that sex steroids modulate *avp* expression in a sexually dimorphic manner. Castrated rats implanted with E2 or T saw increased *avp* expression and increased labeling of arginine vasopressin-immunoreactive (AVP-ir) neurons, but this effect was exaggerated in males (De Vries *et al.*, 1994). It is not known whether exposure to sex steroids alters *avt* expression in fish and is an area for future investigation.

Another possibility is that instead of acting on the HPI axis, 11KT alters the rate of cortisol removal.  One way this may occur is by altering the expression of one or several cortisol metabolizing enzymes including 11β-HSD2, 5α-reductase, 5β-reductase, 3α-HSD, 20α-HSD or 20β–HSD (Wudy *et al.,* 2007). Several of these enzymes are known to be expressed in a sexually dimorphic manner. In teleosts, males have higher levels of 11β-HSD2 than females due to its role in converting 11-oxygenated androgens to 11KT in the testes (Rebers *et al.*, 2000). In humans, males have a higher rate of cortisol synthesis and clearance. The higher clearance rate of cortisol in males is attributed to an increased 5α-reductase activity compared to females (Wudy *et al.*, 2007). Whether sex steroids effect the production or activity of these enzymes in fish is not known and is an area for future study.

**Conclusions**

Collectively, the results of these studies further the understanding of stress physiology and its effectors. This included the demonstration of an involvement of the POA, pituitary and interrenal in a stress event and a demonstration that sex steroids can alter the magnitude of the stress response in the sex where the steroid is not the major circulating sex steroid. While the effects of sex steroids on the stress response in zebrafish varied from effects observed in salmonid species and the exact mechanism of action remain to be discovered, the fact that sex steroids have any effect on the stress axis warrants future research in this area due to its possible physiological and toxicological implications.

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