

**Characterization of the Ovarian Corticotropin-Releasing Factor
System and its anti-steroidogenic effects in Zebrafish**

by

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ABSTRACT

CHARACTERIZATION OF THE OVARIAN CORTICOTROPIN-RELEASING FACTOR SYSTEM AND ITS ANTI-STEROIDOGENIC EFFECTS IN ZEBRAFISH

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The primary objective of this thesis was to characterize the ovarian corticotropin-releasing factor (CRF) system in the zebrafish (*Danio rerio*) and determine its effects on local steroidogenesis. All components of the CRF systems in zebrafish are expressed in the ovary. The mRNA levels of CRFb, the CRF receptor subtype CRF-R1, and the CRF binding protein were found to vary with respect to daily cycles of ovulation. CRF was also shown to inhibit human chorionic gonadotropin-stimulated production of 17β estradiol and testosterone by mid-vitellogenic and full grown follicles, respectively. These actions were mediated by the CRF-R1 receptor and reduced expression of the steroidogenic enzymes StAR and aromatase. Results also suggest that the anti-steroidogenic effects of CRF may be mediated via downstream actions of local ACTH on the melanocortin 2 receptor. Overall, these results provide original evidence of a functional CRF system in the zebrafish ovary with anti-steroidogenic effects.

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INTRODUCTION

The impacts of stress on reproduction and egg development have long been of interest to a wide variety of medical, agricultural and zoological fields. Although most studies show that chronic stress negatively impacts reproductive function (reviewed in Habib et al, 2001; Charmandari et al, 2005; Fuzzen et al. 2011), the details of the hormonal interactions which drive these effects are still under investigation. The interactions between the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis which governs the stress response, and the hypothalamic-pituitary-gonadal (HPG) axis governing reproductive function and steroid production are numerous, and often occur at multiple levels of the two signaling cascades (Murase et al, 2002; Ogawa et al, 2003; Alsop et al, 2009). While best characterized within the central nervous system, components of HPA/HPI signaling have been found in peripheral tissues of numerous vertebrate species in recent years (Mastorakos et al, 1993, 1994; Asakura et al, 1997; Pohl et al, 2001, Boorse and Denver, 2006) where their role remains unclear. The objective of this thesis was to characterize the presence of HPI signaling machinery, namely that of the corticotropin-releasing factor (CRF) family of ligands, receptors and binding protein within the ovary of the zebrafish, *Danio rerio*, with a particular focus on gene expression patterns and interactions of CRF-related peptides with the actions of gonadotropins on gonadal steroidogenesis.

The HPA/HPI Axis

In the physiological sense, stress is a nebulous concept, as the term can refer to an organism's response to almost any negative stimuli, earning it the famous definition by Selye (1973) of "the nonspecific response of the body to any demand made upon it",

or more recently, “A threat, real or implied, to homeostasis” (McEwen and Wingfield, 2002). Generally, sources of stress can be divided into two broad categories; physical and psychological stressors. Physical stressors may include food scarcity (Wendelaar Bonga, 1997), oxidative damage caused by environmental contaminants (Valavanidis et al, 2006), or bodily harm caused by restraint (Paré and Glavin, 1986). Purely psychological stressors, such as anxiety from the presence of a natural predator (deCatanzaro, 1988), stress from overcrowding (Schreck et al, 2001), capture and confinement (Clearwater and Pankhurst, 1997) or social subordination (Øverli et al, 2000) can also indirectly impact the physiology of organisms. In either case, the homeostatic state of the animal is disrupted by stress, placing the survival or general wellbeing of the organism at risk (Wendelaar Bonga, 1997).

The concept of “allostasis” refers to maintaining homeostasis through reactionary bodily or behavioral change, with allostatic load referring to the physiological costs of pursuing homeostasis (reviewed in McEwen and Wingfield, 2002). Allostatic load during stress is the product of signaling from two endocrine cascades, the sympathetic nervous system (SNS), and the HPA/HPI axis, both of which are comprised of central nervous system (CNS) and peripheral components. Activation of the SNS and HPI axis typically occur together to manage stress over different time frames, although the intensity of the stressor will dictate the relative strength of each cascade’s activation (reviewed in Charmandari et al, 2005). The SNS, known as the “fight or flight” response, is most strongly activated in the presence of severe and imminent danger, and in the instance of teleosts, is achieved through the rapid release of catecholamines such as epinephrine or norepinephrine into the plasma from sympathetically innervated

chromaffin cells in the head-kidney of the fish – which is homologous to the adrenals of mammals (Nilsson et al, 1976; Reid et al, 1996; Wendelaar Bonga, 1997). Once in circulation, catecholamines increase heart rate, blood-oxygen carrying capacity, and blood glucose through hepatic glycogenolysis (Sutherland and Cori, 1951; reviewed in Charmandari et al, 2005) in order to promote heightened senses and fast responses from muscles –at the expense of less immediately critical functions such as digestion or sexual response- in order to escape or combat the stressor.

The stress response is also mediated via the HPI axis in teleosts, which serves the same function as the HPA axis of mammals, (reviewed in Barton, 2002; Charmandari et al 2005). Upon the detection of a stressor, the stored neuropeptide CRF is released by the nucleus preopticus (NPO) of the teleost hypothalamus (Alderman and Bernier, 2007; Bernier et al, 2009). CRF acts through CRF type 1 receptors (CRF-R1) in the pars distalis of the pituitary gland to stimulate release of vesicle-stored adrenocorticotrophic hormone (ACTH) into circulation, as well as replenish ACTH supplies via cleavage of its precursor, pro-opiomelanocortin (POMC). ACTH travels to the adrenal-like tissues of the head-kidney where it acts via the melanocortin-2 receptor (MC2R) to promote the synthesis and release of glucocorticoids into circulation (Milano et al, 1997; Flik et al, 2006).

In teleost fish, the primary glucocorticoid is cortisol, which is synthesized from dietary cholesterol in interrenal tissues via a series of enzymatic reactions (Idler and Truscott, 1972). Once in circulation, cortisol fulfills a variety of functions by binding to glucocorticoid receptors in target tissues (reviewed in Bernier et al, 2009). Notably, cortisol signaling increases glucose production through gluconeogenesis in the liver

(De Boeck et al., 2001; Aluru and Vijayan, 2007), suppresses somatic growth via effects on insulin-like growth factors (Peterson and Small, 2005; Madison et al. 2015), and alters electrolyte and solute balance in the blood, such as during the transition between salinities (Redding et al, 1984).

Despite the benefits of short term cortisol signaling, ongoing stressors which elevate cortisol levels on a chronic basis can do more harm than good (Pickering et al, 1987). Allostatic load will accumulate as long as stressors persist, and sustained HPI axis activity through chronic stress (on the order of weeks or months) will eventually lead to a state of energy deficiency called allostatic overload, which is detrimental to the organism through reduced food intake and growth (Barton, 2002; Madison et al, 2015), suppression of the immune system and inflammatory responses (Weyts et al, 1999), and disruption of reproduction in both males and females (Pickering et al, 1987).

The CRF System

CRF is a 41 amino acid peptide, originally isolated from the ovine hypothalamus (Vale et al, 1981), and following a stressful stimulus is the primary mediator of the HPA/HPI stress response. A number of paralogous ligands of CRF exist in vertebrates, creating a larger CRF family of related peptides. In fish, a whole-genome duplication early in the teleost lineage resulted in two CRF genes, *crfa* and *crfb* (Grone and Maruska, 2015). Earlier rounds of whole-genome duplications in vertebrate evolution resulted in additional CRF-related peptides, including the paralogs urotensin 1 (UI) and urocortin 3 (UCN3), among others (Fryer and Lederis, 1983; Lovejoy and Jahan, 2006; Lovejoy 2009). CRFb is the most common CRF peptide in teleosts, and its similarity to other ligands is in keeping with their evolutionary distances from one another; in terms

of amino acid sequence, it is 71% similar to CRFa, 54% similar to UI, and only 18% similar to UCN3 (Bräutigam et al, 2010; Grone and Maruska, 2015). In teleosts, additional CRF-like peptides generated from genome duplications have been lost in evolution or rendered transcriptionally inert, such as CRF2 and UCN2 (Lovejoy, 2009; Grone and Maruska, 2015). The proposed phylogeny of the CRF family in teleosts is provided in Figure 1.

Two CRF receptors have been described, CRF-R1 and CRF-R2, both of which are G protein-coupled receptors (GPCRs). Activation of these receptors causes an increase in intracellular cyclic adenosine monophosphate (cAMP) which through activation of protein kinase A leads to different cellular responses, such as the cleavage of POMC and exocytosis of ACTH in the pituitary (Bale and Vale, 2004; Hollenstein et al, 2013). CRF ligands and receptors interact with varying affinities (summarized in Figure 2; reviewed in Manuel et al, 2014), which are also reflective of their evolutionary separation. CRFa and UI's ability to bind with equal strength to both CRF-R1 and CRF-R2, CRFb's preferential binding to CRF-R1 over CRF-R2 and UCN3's high affinity to CRF-R2 alone (Manuel et al, 2014; Hosono et al, 2015) enables a wide range of signaling responses from a small number of ligands and receptors, creating the framework for a complex signaling system. Finally, CRF binding protein (CRF-BP) is often co-released with CRF ligands and has a high affinity to CRF and UI (Kemp et al, 1998; Seasholtz et al, 2002). As a result, CRF-BP may modulate the impact of CRF-like ligands by sequestering them to end signaling.

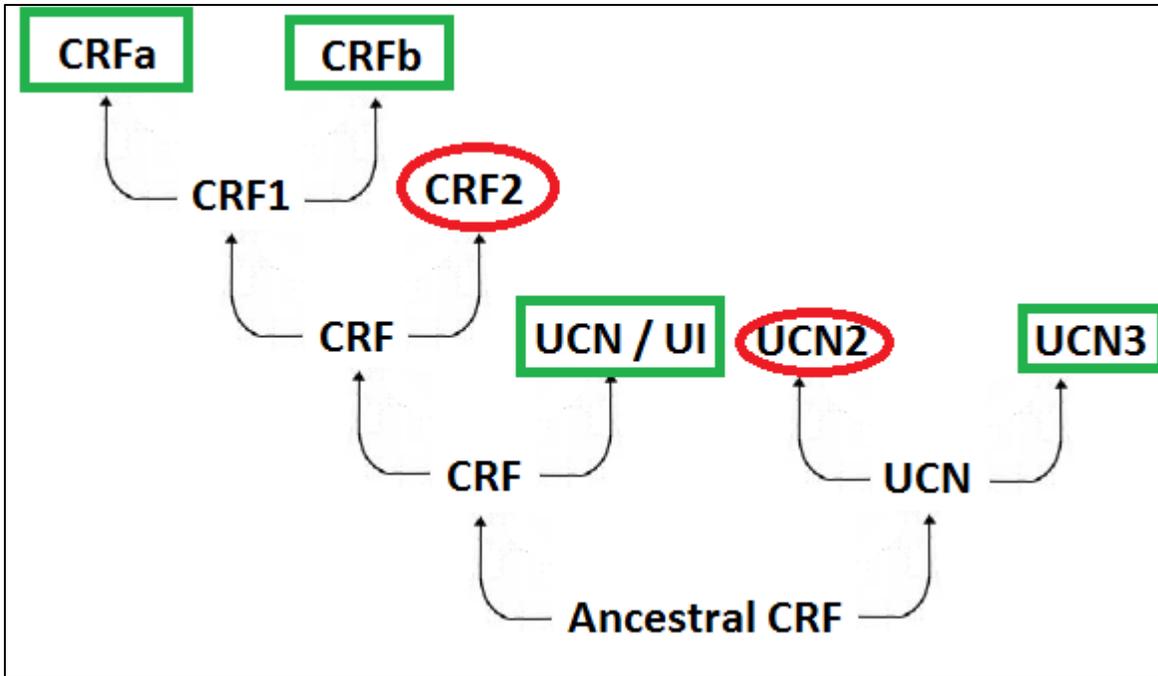


Figure 1: Phylogeny of CRF family ligands in teleosts. Each branching represents the products of an individual whole-genome duplication event in the evolutionary history of teleosts. (Lovejoy, 2009; Grone and Maruska, 2015). Circled ligands are transcriptionally inactive or lost in the teleost genome, while ligands framed in a rectangle are expressed in modern teleosts

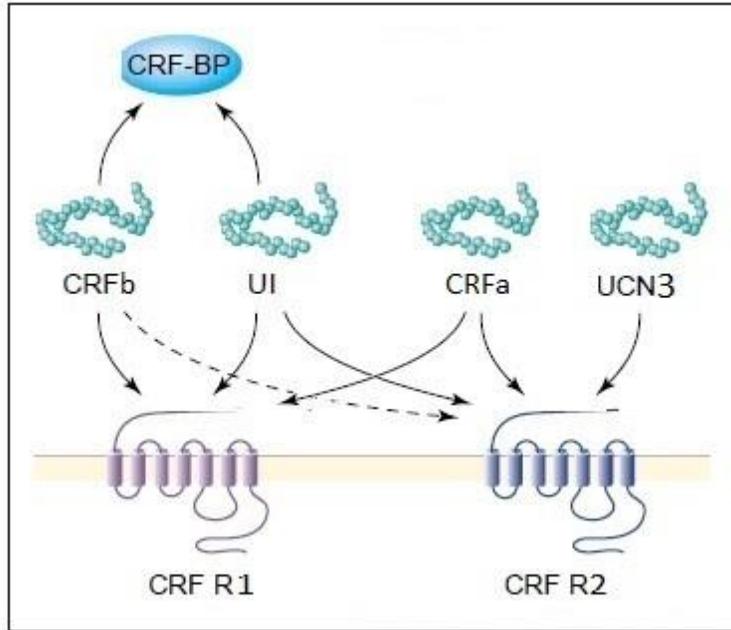


Figure 2: The binding of teleost CRF family members to the receptors CRF-R1 and CRF-R2 and the CRF binding protein. A high binding affinity between CRF ligands and receptors or binding protein is designated by solid arrow whereas a weak binding affinity is designated by a dotted arrow. The data are based on studies by Manuel et al, 2014; Hosono et al, 2015). The diagram is modified from Dautzenberg and Hauger (2002)

HPG Axis and ovarian development in teleosts

Operating in parallel to the HPI/HPA axis is the HPG axis, a cascade promoting gametogenesis, gonadal development, steroidogenesis and cyclic gonadal functions such as ovulation (Nagahama and Yamashita, 2008; Lubzens et al 2010). Following hypothalamic stimulation, the release of gonadotropin-releasing hormone (GnRH) from the preoptic hypothalamus signals to the anterior pituitary gland and promotes the release of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In the theca and granulosa cells surrounding the ovarian follicle, LH and FSH promote production of steroids, such as 17β estradiol (E_2), testosterone (T) and $17,20\beta$ -dihydroxy-4-pregnen-3-one ($17-20\beta P$), which act as major effectors of many gonadal activities (Nagahama et al, 1995; Clelland and Peng, 2009).

Production of steroids from gonadotropic signaling occurs via increased expression of steroidogenic genes, brought about by raised intracellular cAMP, protein kinase A activation, and downstream responses from transcription factors (see Figure 3). A major effector in gonadotropic steroid production is steroidogenic acute regulatory protein (StAR), which rises sharply in expression following LH and FSH signaling (Clark et al 1994; Gyles et al, 2001; Carvalho et al, 2003). StAR is a transport protein which shuttles dietary cholesterol from outer to inner mitochondrial membranes of steroidogenic cells, where it is converted to pregnenolone (P_5) by the enzyme P450 side chain cleavage ($P450_{SCC}$), and subject to further modification to other steroids, such as eventual conversion of T to E_2 via P450 gonadal aromatase ($cyp19a1a$), another enzyme impacted by gonadotropin signaling (Privalle et al, 1983; Miller 1988; reviewed

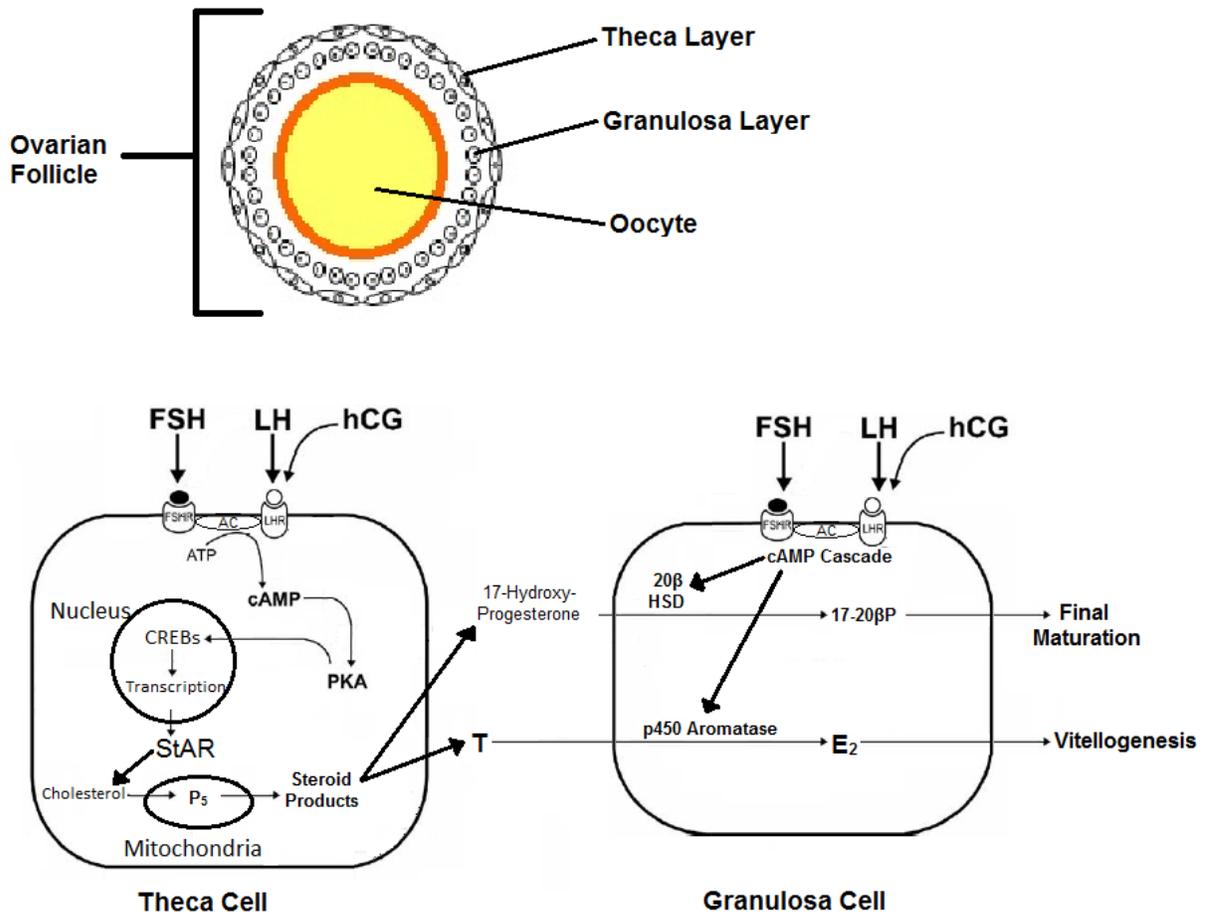


Figure 3: Actions of gonadotropins in the zebrafish ovary. FSH, LH, and hCG signaling in both cell layers produces cyclic-AMP through activation of adenylyl cyclase (AC), leading to phosphorylation of intracellular cAMP response element binding proteins (CREBs), which promote gene expression. Activity of StAR in the theca allows cholesterol to be converted to pregnenolone (P_5), facilitating a series of steroidogenic reactions which produce a variety of downstream effects. Activity of 20β HSD and p450 Aromatase in the granulosa allow conversion of 17-Hydroxyprogesterone and T into 17-20 β P and E_2 , respectively, which are instrumental in various stages of follicular development.

in Lubzens et al, 2010). With this arrangement, StAR and P450_{SCC} act as rate limiting steps for all further steroidogenesis, as no further steroid production can occur in the absence of pregnenolone (Clark et al, 1994; Stocco and Clark, 1996).

Many signals associated with stress, whether in the CNS or periphery, inhibit the actions of the HPG axis in both mammals and teleosts (reviewed in Charmandari et al, 2005; Fuzzen et al, 2011). For instance, the expression and release of GnRH, and with it, gonadotropic activity, has been shown to be inhibited by both CRF (Rivest and Rivier, 1995) and elevated circulating cortisol (Dubey and Plant, 1985). In zebrafish, ACTH suppressed the ability of human chorionic gonadotropin (hCG; an LH analogue) to produce E₂ in a dose-dependent manner (Alsop et al. 2009). Prolonged stress in the red gurnard, *Chelidonichthys cuculus*, leads to reductions of both ovarian E₂ and T, and the developmental processes dependent upon them (Clearwater and Pankhurst, 1997). Surprisingly, several studies have failed to demonstrate a direct inhibitory effect of cortisol on gonadotropin-induced ovarian steroidogenesis in teleosts (Pankhurst et al, 1995; Pankhurst, 1998; reviewed in Fuzzen et al. 2011), which leaves important questions as to how stress affects ovarian function unanswered.

HPA/HPI - HPG interactions and ovarian CRF

Although CRF is classically known for its role in the HPA/HPI axis, there is an abundant body of evidence in mammals that CRF signaling occurs outside of the brain. CRF-R1 has been detected in peripheral sites including the spleen and thymus of rats (Baigent and Lowry, 2000), the gastric tissues of mice (Martinez et al, 2002) and most notably, the theca and granulosa layers of the ovaries of humans and rhesus monkeys (Asakura et al, 1997; Xu et al, 2007), while CRF-R2 has been found in abundance in the

heart, lungs, skeletal muscle, and gastrointestinal tract of rats and humans (Perrin & Vale, 1999; Stenzel et al 1995). Evidence of immunoreactive CRF in ovarian tissues of mice and humans (Mastorakos et al, 1993; 1994) leaves little doubt of active CRF signaling in the mammalian ovary. Given the localization of CRF-R1 receptors in steroidogenic cells, CRF's ties to stress-related signaling, and the ability of chronic stress to suppress gonadal function, including steroidogenesis (reviewed in Rivier and Rivest, 1991), an anti-steroidogenic effect of CRF in the gonads is a distinct possibility.

CRF has anti-reproductive effects when introduced to the ovary. In mice, it has been shown that exogenous CRF application can slow the process of oocyte maturation *in vitro*, through a reduction of the formation of polar bodies, an effect reversed by antalarmin, a CRF-R1 antagonist (Kiapekou et al, 2011). There is also evidence that the addition of CRF to rat ovarian tissues *in vitro* suppresses gonadotropin-mediated E₂ production in a dose-dependent fashion (Ghizzoni et al, 1997; Murase et al, 2002). Similarly, paralogues of CRF have anti-steroidogenic effects. In human granulosa-lutein cells, the addition of UCN3 causes reduction of progesterone (P₄) production (Yata et al, 2009). A study by Calogero et al (1996) of rat granulosa corroborated the ability of CRF to block FSH-mediated increases in steroidogenesis, as well as its ability to significantly decrease the activity of gonadal aromatase. The effects of CRF can be seen at multiple steps in the steroid biosynthetic pathway. For instance, a study of human thecal cells by Erden et al (1998) showed that the ability of LH to promote production of androstenedione and dehydroepiandrosterone, as well as induce expression of p450c17 (intermediate steps to T production) were suppressed by CRF.

These results suggest a generalized inhibition of gonadotropin's activities by CRF, specifically their ability to induce changes to gene expression.

Preliminary analyses of CRF system components expression in peripheral tissues suggest that CRF ligands and receptors may have a functional role in the ovary of teleosts. For example, CRF-R1 expression has been observed in the gonads of the pufferfish, *Fugu rubripes* (Cardoso et al, 2003) and the ovary of chum salmon, *Oncorhynchus keta* (Pohl et al, 2001). Additional discoveries of mRNA for UCN3 in zebrafish testes and UI in ovarian tissue (Bräutigam et al, 2010), as well as high mRNA expression of CRF and CRF-R2 in the ovary of the cichlid, *Astatotilapia burtoni* (Chen and Fernald, 2008) suggest that most or all of the CRF family components are present in the gonads of teleosts. However to date the function of CRF ligands and their receptors in the ovary and testis of teleosts has not been investigated. In addition, whether the mRNA levels of CRF ligands and receptors change in relation to the development and maturation of the ovarian follicle in teleosts is not known.

The possibility of CRF having anti-gonadotropic function in teleosts presents an avenue of study which should be explored. Key steroidogenic genes, such as StAR, gonadal aromatase, and 17 β HSD are governed in their expression by gonadotropins (Goto-Kazeto et al, 2004; Ings and Van Der Kraak, 2006), and the disruption of these enzymes' expression could have severe impacts. The most obvious effect of diminished E₂ production would be the limitation of vitellogenesis, the steroid-dependent recruitment of yolk precursor proteins which the ovulated egg requires for growth following fertilization (Clelland and Peng, 2009). E₂ has other important roles, however, such as its ability to promote the release of LH in goldfish (Peter and Yu, 1997) and its

ability to promote spawning behaviour which typically occurs prior to ovulation (Stacey and Liley, 1974). More generalized inhibition of steroidogenesis could affect a broader range of steroid targets, affecting processes such as the maturation of the oocyte (Nagahama et al, 1995). At present, very little is known about the function of the ovarian CRF system in teleosts. Identifying whether CRF has anti-steroidogenic actions in the ovary of teleosts may provide new insight into the mechanisms responsible for the inhibitory effects of stress on reproduction.

Model Organism:

I characterized the ovarian CRF signaling system and its function in the zebrafish (*Danio rerio*). The zebrafish is a tropical freshwater teleost of the family Cyprinidae, native to India and Pakistan (Zhang et al., 2003). They are practical as a model organism for their small size (2.5-4cm), ease of housing, and rapid onset of sexual maturity from birth (3 months; Mullins and Nusslein-Volhard, 1993). Additionally, their continuous asynchronous ovulatory patterns (Niimi and LaHam, 1974) remove seasonal considerations for reproductive studies.

Ovarian development in zebrafish has been well explored in prior literature, and this information will help structure the characterization of the ovarian CRF system. Five major stages of follicular development have been classified (Selmen et al, 1993; Clelland and Peng, 2009), and the role of steroid production in development is well-understood. E_2 is the primary effector of vitellogenesis, and its production is dependent on gonadal aromatase and StAR, two LH- sensitive steroidogenic enzymes (Ings and Van Der Kraak, 2006; reviewed in Lubzens et al, 2010). A reduction of aromatase expression in later development promotes production of T and 17-20 β P, effectors of

germinal vesicle breakdown and oocyte maturation, respectively (Schuetz, 1967; Nagahama and Yamashita, 2008).

Another appealing property of zebrafish as a model organism comes from established *in vitro* protocols. Current incubation techniques (Ings and Van Der Kraak, 2006; Alsop et al, 2009) allow for measurement of both steroidogenic activity of follicles via enzyme immunoassays, and gene expression measurement via qPCR. This allows a substantial amount of information to be obtained from a relatively small number of sampled animals, as due to the asynchronous ovulatory patterns of the zebrafish (Niimi and LaHam, 1974), multiple follicular stages can be obtained from a single fish.

Thesis Goals, Hypothesis and Predictions:

The goals of this study were to identify which components of the CRF family are expressed in the ovary of zebrafish, to describe the expression pattern of CRF family components across follicular stages and relative to the timing of ovulation, and to determine whether CRF affects ovarian steroidogenesis. I hypothesized that the ovarian CRF system plays a role in the regulation of steroid production during follicular development by inhibiting the gonadotropin-induced expression of steroidogenic enzymes, a signaling system comparable to that observed in mammalian models. From this hypothesis, a number of predictions can be made. First, I predict that components of the CRF family will be expressed in the ovary and that their expression will vary with stage of development, or with respect to ovulatory state. Second, that CRF's signaling effects in the ovary negatively impact gonadotropic steroidogenesis via CRF-R1 signaling. Third, that the ovarian CRF system exerts its effects by modulating the ability of gonadotropins to up-regulate steroidogenic genes.

To test my first prediction, I measured the relative levels of gene expression of various CRF ligands and receptors through qPCR. To determine if the expression of CRF family members was dynamic, I collected ovarian follicles representing multiple stages of development, and also sampled whole ovarian tissue at different time points during a 12 hr period leading to ovulation. To determine CRF's impact on steroidogenesis, I conducted a series of in vitro incubations of ovarian follicles with CRF at multiple stages in development to determine if CRF had an impact on either steroid production or the expression of genes involved in steroidogenesis. Other studies tested the effects of a CRF-R2 specific ligand (UCN3) and antalarmin, a CRF-R1 antagonist, to determine which CRF receptor was mediating the effects of CRF on steroidogenesis

Given the observation of Alsop et al. (2009) that ACTH can inhibit hCG-induced E2 production in the zebrafish, an effect identical to that of CRF, I predicted that CRF's effects are actually mediated through local production of ACTH from POMC, leading to autocrine/paracrine signaling via MC2R. To test this prediction, I conducted a series of experiments to determine if the POMC and MC2R were expressed in the ovary. I then tested if both CRF and ACTH affected steroid production comparably, and finally, if an MC2R receptor antagonist would block the effects of CRF on steroid production.

METHODS

Animals:

Adult female zebrafish were obtained from AQuality Tropical Fish Wholesale (Mississauga, ON, Canada), and housed at the Hagen Aqualab (University of Guelph, Guelph, ON, Canada). Fish were kept in A-HAB fish containment units (Aquatic Habitats, Apopka, FL, USA), in a recirculating water system maintained at 28°C. Fish

were reared at a density of approximately 3/L, and had an artificially controlled 12h light, 12h dark photoperiod. Fish were fed to satiety twice daily with both frozen brine shrimp (Hakari, Hayward, CA, USA) and tropical fish flakes (Tetra Aquarium, Blacksburg, VA, USA). All experiments were conducted in accordance with the animal care protocols approved by the University of Guelph Animal Care Committee on behalf of the Canadian Council on Animal Care.

Chemicals:

Leibovitz medium and penicillin / streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). The peptides rat/human CRF (r/hCRF) and mouse UCN3 (mUCN3) were purchased from American Peptide Company (Sunnyvale, CA, USA). The peptide hACTH₍₁₅₋₂₄₎ (KKRRPVKVYP), a putative MC2R antagonist (Dores and Liang, 2014), was synthesized by GenScript (Piscataway, NJ, USA). Dibutyryl cyclic-AMP (db-cAMP), the selective CRF-R1 receptor antagonist antalarmin, porcine ACTH (pACTH), hCG and all qPCR primers were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design:

Gravid female zebrafish were identified based on morphology including the presence of genital papillae and increased abdominal size, and were anesthetized in an overdose of buffered tricaine methanesulfonate (MS-222, 0.3g/L with 0.6g/L sodium bicarbonate; Syndel Laboratories Inc, Vancouver, BC, Canada) and euthanized by spinal severance. Both whole body weight and ovarian weight were recorded in order to calculate gonadosomatic index (GSI: (ovarian weight/total weight) *100) as a means of assessing the general status of ovarian development.

In vitro incubation protocol:

The ovary was quickly removed from the zebrafish and placed in 60% Leibovitz L15 medium with penicillin/streptomycin to prevent bacterial growth (120 IU/mL and 120 µg/mL, respectively; Invitrogen, Carlsbad, CA, USA). Follicles at different stages of development (as described in Selmen et al, 1993) were separated using fine forceps under a light microscope, based on follicle size and visual cues such as cortical alveoli formation or opacity from yolk accumulation. The stages were as follows: stage I; primary growth, stage II; cortical alveolar / early vitellogenic, stage III-1; mid vitellogenic, stage III-2; late vitellogenic, and stage IV; full grown / post-vitellogenic. Isolated follicles were placed in a 24-well polystyrene plate (Corning Life Sciences, Corning NY, USA), 20 to a well. Follicles were incubated in 500µl of 60% L15 along with other treatments which varied by experiment. In tests of abundant follicular stages (full grown and mid-vitellogenic), 120 follicles obtained from a single fish were placed in one of six wells corresponding to six treatments. This procedure was repeated 4 times per plate and across 3 plates, with each repeat using follicles from a new fish (n=12). Less abundant follicular stages (early vitellogenic) were incubated using a modified protocol (see experiment 8). All incubations were conducted in darkness at 28°C with gentle agitation (60 rpm). Following incubation, follicles and media were separated and stored at -80°C until RNA extraction or EIA, respectively.

Experiment 1: Basal CRF family expression within and across follicular stage

The first experiment examined the levels of CRF system gene expression across the five stages of ovarian follicle development. This was an important first step in determining whether the various components of the CRF system are expressed within

the zebrafish ovary, as well as providing initial insight into how expression varies by follicle stage. Untreated follicles sampled from 12 female zebrafish were sorted into five stages of development based on Selmen et al (1993). All samples were taken at 10AM to control for any circadian changes in gene expression (addressed in *Experiment 2*). Tissue collected for each follicular stage was stored at -80°C for later determination of gene expression by RNA extraction, reverse transcription, and quantitative real-time PCR (qPCR).

Experiment 2: Periovoluntary changes in the expression of CRF system components

A second experiment was conducted to quantify the periovoluntary gene expression pattern of the various CRF system components. To accomplish this, whole ovarian tissue was sampled from five groups of 12 female fish at three h intervals throughout the night, beginning at 10PM and ending at 10AM and stored at -80°C for later quantification of CRF family gene expression via qPCR. By collecting samples at and around the approximate time of ovulation in our zebrafish colony (7AM), this experiment sought to observe the expression of CRF family genes in relation to the ovulatory process.

Experiment 3: Effects of CRF on steroidogenesis

Ovarian follicles were isolated from zebrafish sampled at 10AM. Two separate experiments were performed using mid-vitellogenic follicles to study the production of E₂, and full grown follicles to quantify the effects on T production. In the case of full grown follicles, the expression of gonadal aromatase is reduced, thus leading to greater production of T (Lubzens et al, 2010; Ings and Van Der Kraak 2006). All experiments

were comprised of 12 wells per treatment, with each well originating from a unique fish (n=12).

Follicles were incubated in 500 μ l of 60% L-15 medium with various treatments: 1) a control treatment with L-15 alone, 2) 10^{-7} or 10^{-9} M r/hCRF, in keeping with concentrations shown to elicit a response *in vitro* (Calogero et al, 1996; Erden et al, 1998; Murase et al, 2002) and to assess if the response is dose-dependent, 3) 20 IU/ml hCG to stimulate steroidogenesis, and 4) hCG with either 10^{-7} or 10^{-9} M r/hCRF to assess the effects of CRF on hCG's actions. r/hCRF was chosen for its 93% amino acid similarity to zebrafish CRFb, and its broad availability. Follicles were pre-treated with L15 media or r/hCRF for 1h to allow CRF to exert its effects in advance of hCG application, followed by 6h incubation. Incubation medium was collected and separated from follicles for determination of E_2 or T concentration by enzyme immunoassays (EIA). The EIA procedures are described below.

Experiment 4: Determination of the CRF receptor subtype mediating the actions of CRF on steroidogenesis

Two experiments were conducted to determine which of the two CRF receptors (CRF-R1 and CRF-R2) mediate the effects of CRF on steroidogenesis. Both experiments used an incubation protocol with a similar design to *Experiment 3*. All experiments were comprised of 12 wells per treatment, with each well originating from a unique fish (n=12). The initial experiment tested the effects of r/hCRF or the paralog mUCN3 at 10^{-8} M alone or in combination with 20IU/ml hCG on E_2 and T production in mid vitellogenic and full grown follicles, respectively. As UCN3 binds preferentially to CRF-R2 in fish (Manuel et al, 2014), a comparable anti-steroidogenic effect shared

between CRF and UCN3 would suggest an R2-mediated interaction. Follicles were pre-incubated with r/hCRF or mUCN3 for 1h prior to 6h of hCG treatment.

In a second experiment, the CRF-R1 antagonist antalarmin was used to determine if blocking CRF-R1 would affect the actions of r/hCRF on hCG stimulated E₂ and T production by mid vitellogenic and full grown follicles, respectively. The effects of antalarmin (10⁻⁷ M) on basal steroidogenesis, hCG (20 IU/ml) and hCG plus CRF (10⁻⁸M) - mediated steroidogenesis were tested. Treatment groups were incubated with antalarmin and/or 10⁻⁸ M r/hCRF for 1 h prior to the addition of hCG, followed by 6h incubation.

Experiment 5: Effects of CRF on the expression of steroidogenic genes

A series of experiments using full grown follicle incubations was performed to test for the effects of CRF on the expression of StAR and gonadal aromatase, two genes involved in steroid production. These experiments tested the same treatment groups described in *Experiment 3*, using 12 wells per treatment, each well obtained from a unique fish (n=12). The experiment was repeated twice, incubating follicles for either 3 or 6 h. Following incubation, tissues were stored at -80°C for later determination of *star* and *cyp19a1a* gene expression by qPCR.

Experiment 6: Effects of CRF on db-cAMP-stimulated steroid production

Two experiments used db-cAMP, a cell permeable and degradation-resistant analogue of cAMP which is known to activate cAMP-dependent protein kinases. Both experiments used 12 wells of full grown follicles per treatment, with each well containing follicles obtained from a unique fish (n=12). The effects of r/hCRF on db-cAMP's ability

to promote steroidogenesis were tested to infer where in the gonadotropic cascade CRF may exert its effects.

The first experiment tested the effects of a gradient of db-cAMP concentrations over 6h to determine a dosage that significantly raised production of T. In the second experiment, full grown follicles were incubated for 6h with db-cAMP (2.5 mM) either alone or in the presence of a high (10^{-7} M) dose of r/hCRF. Separate incubations included 20 IU/ml hCG and hCG + 10^{-7} M r/hCRF groups for comparison. The amounts of T released to the media were determined by EIA. Follicles were incubated with db-cAMP or r/hCRF for 1h prior to the addition of hCG.

Experiment 7: Expression of POMC and MC2R in the ovary

Although Alsop et al. (2009) demonstrated that ACTH can inhibit steroidogenesis in zebrafish ovaries, they did not assess whether the ACTH precursor, POMC, or the ACTH receptor, MC2R, are expressed in isolated follicles. Therefore, ovarian tissue was obtained from 12 fish (n=12) and the basal expression of *pomc* and *mc2r* were assessed with qPCR in isolated mid-vitellogenic and full grown follicles. The basal expression of CRFb was also assessed in these samples as a means of comparison.

Experiment 8: Effects of an MC2R antagonist on hCG, ACTH and CRF stimulated steroidogenesis

Two experiments were conducted to investigate the link between ovarian CRF signaling and ACTH production and their effects on ovarian steroid production. The first experiment determined the effects of 1 IU/ml pACTH alone and in combination with 10^{-7} M hACTH₍₁₅₋₂₄₎, a putative MC2R receptor antagonist, on basal and 20 IU/ml hCG-stimulated E2 production by mid-vitellogenic follicles. A second experiment was

conducted to determine if hACTH₍₁₅₋₂₄₎ would block the effects of r/hCRF on hCG-induced E₂ production. This experiment examined the basal effects of 10⁻⁷M hACTH₍₁₅₋₂₄₎, its effects on 20IU/ml hCG signaling in the absence of r/hCRF, and its ability to counter the effects of 10⁻⁸ M r/hCRF on hCG. Both experiments used pooled early and mid-vitellogenic follicles originating from multiple fish, due to the scarcity of these follicular stages. Pooled follicles from approximately 6-8 fish were incubated with each treatment in quadruplicate per plate, repeated over three plates, with each drawing from a new pool of follicles (n=3). Pre-incubations with r/hCRF and/or hACTH₍₁₅₋₂₄₎ were for 30 min prior to introduction of hCG, and incubation for a further 1.5h before the measurement of E₂.

Analytical techniques

RNA extraction and cDNA synthesis

RNA extraction was performed as described in Ings and Van Der Kraak (2006). Briefly, total RNA was extracted from ovarian tissue or separated follicles using Trizol reagent (Life Technologies, Burlington, ON, Canada). RNA was reconstituted in 5-10 µl DNase/RNase-free water followed by quantification using a NanoDrop 8000 (Thermo Scientific, Waltham MA, USA). Sample quality was determined by calculating the ratio of the absorbance of nucleic acids at 260 nm and the absorbance of proteins at 280 nm. A ratio of 1.8–2.2 was considered to be free from contaminating proteins or reagents. DNase treatment and reverse transcription followed the protocols of Nelson and Van Der Kraak (2010). Each reverse transcription reaction used 1 µg of RNA and was treated with DNase 1 (Sigma, St. Louis, MO, USA) as per manufacturer's instructions. To ensure no external RNA contamination, a water control (without RNA) was

processed with the samples. A “no-RT” control (without reverse transcriptase) was performed on selected samples to confirm absence of genomic DNA contamination. cDNA samples were stored at -20°C until used for quantification of gene expression.

Quantification of gene expression

Relative levels of gene expression were measured using qPCR. Primer sequences used and their respective ideal annealing temperatures (as determined via temperature gradient tests) are included in Table 1. cDNAs were diluted 10-fold prior to use. The reaction mixture contained 3.75 µL of diluted cDNA, 1.875 µL of both forward and reverse primers (1.6 µM), and 7.5 µL of Perfecta SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD, USA). All samples were run in duplicate on a CFX96 Real-Time system (Bio-Rad Laboratories, Mississauga, ON, Canada), including an inter-assay control of amalgamated cDNA from random samples to adjust for between-plate differences. The cycling conditions used were: 2 min at 50°C, 5 min at 95°C, followed by 40 cycles of 1 sec at 95°C and 30 sec at the specific annealing temperature of each primer pair.

The qPCR results for *Experiments 2, 5 and 7* were normalized to the expression of the two housekeeping genes, elongation factor 1 α (*ef1 α*) and β -actin (*actb1*), using the reference residual normalization method (Edmunds et al. 2014). Standard curves for quantifying expression of each gene were developed using pooled cDNA from across different follicular stages serially diluted to six separate concentrations.

Experiment 1 tested two reference genes and expressed results relative to *ef1 α* , which showed lower variability within stage (*actb1* results, while not included, were comparable). As *ef1 α* expression was not uniform across stages, differences in

expression were accounted for by normalizing to a control group via the technique used by Essex-Fraser et al (2005): individual input within a group / (mean input within a group / mean value of control group), using inputs derived from standard curves. Expression of *ef1α* within the primary follicle stage was used as the control group in both approaches. Expression of *crfb* was set to 1 when comparing genes within stage, and primary follicle expression was set to 1 when comparing a single gene across stages. This method was employed in place of reference residual normalization due to the lack of inter-plate controls in Experiment 1 and reference gene stability.

Steroid measurement:

Duplicates of 50 µl of medium separated from follicles post-incubation were tested for steroid production using EIA kits for T and E₂ as per the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). The detection limit of the T and E₂ assay were 6 and 15 pg/ml, respectively. Intra-assay and inter-assay variations were compared for both assays and were ~6.3% and 6.4% respectively for T and 10.8% and 7.1% respectively for E₂.

Statistical Methods:

Statistical analysis of data was done using SigmaPlot V12.5 (SigmaStat, San Jose, CA, USA), with significance set to $\alpha=0.05$. For data originating from pooled follicle incubations (both stages of *Experiment 8*), each quadruplicate was condensed into a single mean value. Homogeneity of variance within measurements of gene expression and steroid production were tested using Levene's Test, and in instances where the assumption of normality could not be met, log-10 transformation of the data was performed. Significance between groups was tested using one-way ANOVA tests followed by a Holm-Sidak test for multiple comparisons

Table 1: Nucleotide sequences, annealing temperatures, and efficiencies of primers used for qPCR

Gene (Annealing T°C)	Sequence (5' - 3')	Efficiency	Database ID
<i>actb1</i> (60C)	F: ACAGGGAAAAGATGACACAGATCA R: CAGCCTGGATGGCAACGTA	91.8%	NM_131031
<i>Crfa</i> (59C)	F: ACAGCAGACTCTCACCGACAA R: CGGTAATCTCAGTCGGTGTCC	101.9%	ENSDARG00000093401
<i>Crfb</i> (59C)	F: GCCGCGCAAAGTTCAAAA R: GCGAGGAGAATCTGTGCGTAA	94.7%	ENSDARG00000027657
<i>Crfbp</i> (60C)	F: CGAGAGTTACCAGAGGAGTTTGTGTA R: ACCCTCTACGGCCACCATATC	100.9%	NM_001003459.1
<i>crfr1</i> (59C)	F: AGGCAGGCTGTTACCTCAT R: CTGTTGCCCGGAGATTAGTGTA	90.0%	XM_691254.4
<i>crfr2</i> (60C)	F: TTACCAAGGGCCTGTGATTCTAG R: GCGCACAATGTTGAAAAGAAAC	91.0%	NM_001113644.1
<i>cyp19a1a</i> (62C)	F: AGTTCAACTGGCACACGCAG R: AGCTCTCCATGGCTCTGAGC	96.2%	AF_406757.1
<i>ef1α</i> (60C)	F: GATCACTGGTACTTCTCAGGCTGA R:GGTGAAAGCCAGGAGGGC	98.5%	NM_131263
<i>mc2r</i> (62C)	F: CTCGGTTCTCCCTTCATCTG R: ATTGCCGGATCAATAACAGC	104.0%	NM_180971.1
<i>Pomca</i> (58C)	F: CGCAGACCCATCAAGGTGTA R: CGTTTCGGCGGATTCT	98.2%	AY125332.2
<i>Star</i> (59C)	F: ACCTGTTTTCTGGCTGGGATG R: GGGTCCATTCTCAGCCCTTAC	90.2%	NM_131663
<i>ucn3</i> (60C)	F: AGCCCAACTTTCTCTGCAACA R: CGGCTGCCTCTTATCATTCG	87.9%	NM_001082954.2
<i>uts1</i> (60C)	F: CACGCTTCCTCACCGCTACT R: CAGTCGCGCACTTCTCGAT	85.0%	NM_001030180.1

actb1, β actin; *crfa*, corticotropin-releasing factor a; *crfb*, crf b; *crfbp*, crf binding protein; *crfr1*, crf receptor 1; *crfr2*, crf receptor 2; *cyp19a1a*, p450 aromatase; *ef1 α* , elongation factor 1 α ; F, forward; *mc2r*, melanocortin receptor 2; *pomca*; pro-opiomelanocortin; R, reverse; *star*, steroidogenic acute regulatory protein; *ucn3*, urocortin 3; *uts1*, urotensin I

RESULTS

Experiment 1: Basal CRF family expression within and across follicular stage

Basal expression of CRF family ligands and receptors was assessed across five stages of follicular development (primary growth, cortical alveolar or early vitellogenic, mid vitellogenic, late vitellogenic, and full grown; see Figure 4). Among the ligands, *crfb* was the most highly expressed at all stages, often 10-fold in excess of the ligands *uts1* and *ucn3*, which had approximately equal expression, while *crfa* had the lowest expression levels at all stages. Depending on the stage, the expression of *crfbp* was either equal to the expression of *uts1/ucn3* or slightly higher. The expression of *crfr1* was ~15-20 fold higher than *crfb* across the first four follicular stages, increasing to 54 fold higher in full grown follicles. Finally, the mRNA levels of *crfr2* were ~5-10 times higher than the expression of *crfb*.

Examining the data between follicular stages (Figure 5), expression levels of all ligands (*crfb*, *crfa*, *uts1*, *ucn3*), *crfbp* and *crfr2* remained unchanged. In contrast, the expression of *crfr1* was significantly elevated in full grown follicles when compared to prior stages (One-way ANOVA; $F = 16.562$, $P = <0.001$).

Experiment 2: Perioovulatory changes in the expression of CRF system components

Whole ovarian tissue was sampled at 3 h intervals between 10PM and 10AM to quantify the perioovulatory gene expression pattern of CRF system components. Overall, the perioovulatory expression patterns of *crfb* ($F=4.979$, $P=0.002$), *crfr1* ($F=28.452$, $P<0.001$) and *crfbp* ($F=4.127$, $P=0.006$) varied significantly, whereas those of *uts1*, *ucn3* and *crfbp* did not (Figure 6). However, the near-significant changes in *ucn3* and *uts1* ($P= 0.11$ and 0.07 , respectively) may warrant further examination. The mRNA levels of

crfb rose sharply between 1AM and 7AM ($P < 0.001$), seeing an approximately 2-fold increase in expression. The expression of *crfr1* ($P < 0.001$) and *crfbp* ($P = 0.017$) between 1AM and 7AM follow similar patterns, seeing approximately 6 and 3 fold increases, respectively.

Experiment 3: Effects of CRF on steroidogenesis

In full grown follicles, CRF and hCG treatments produce significant effects on T production ($F = 9.786$, $P < 0.001$; Figure 7A). The addition of a low dose of r/hCRF (10^{-9} M) had no effect on basal level T production, or on the ability of hCG to stimulate steroid production ($P < 0.001$). However, a higher dose of r/hCRF (10^{-7} M), while still not affecting basal production, reduced hCG-stimulated T production to an intermediate level, not statistically significant from either hCG or control treatments.

The production of E_2 in mid-vitellogenic follicles followed a similar pattern across treatment groups ($F = 24.671$, $P < 0.001$; Figure 7B). Addition of 20 IU/ml hCG elicited a ~3-fold increase of E_2 production ($P < 0.001$). Low dose r/hCRF (10^{-9} M) had no impact on either basal levels of E_2 or the production of E_2 induced by hCG. Higher doses of r/hCRF (10^{-7} M), though not affecting basal production, significantly reduced hCG-stimulated E_2 production by ~35% ($P < 0.001$).

Experiment 4: Determination of the CRF receptor subtype mediating the actions of CRF on steroidogenesis

The effects of CRF and hCG were consistent with the results of *Experiment 3* ($F = 42.07$, $P < 0.001$; Figure 8A). Incubating full grown follicles with 20 IU/ml hCG elicited a 4-fold increase in T production relative to basal levels ($P < 0.001$). Neither 10^{-8} M r/hCRF nor mUCN3 had an effect on basal T levels. While 10^{-8} M r/hCRF reduced

hCG-stimulated T production by 44% ($P < 0.001$), 10^{-8} M mUCN3 did not affect the steroidogenic actions of hCG.

Mid-vitellogenic incubations saw similar patterns of significance ($F = 31.075$, $P < 0.001$; Figure 8B). Incubation with 20 IU/ml hCG induced a 6-fold increase in E_2 production above basal ($P < 0.001$). Basal levels of E_2 were not affected by either 10^{-8} M r/hCRF or mUCN3. Although 10^{-8} M r/hCRF reduced the stimulatory effects of hCG on E_2 production by 59% ($P < 0.001$), a similar dose of mUCN3 had no effect.

Antalarmin incubations with full grown follicles yielded noteworthy results ($F = 42.59$, $P < 0.001$; Figure 8C), the inhibitory effects of 10^{-8} M r/hCRF on hCG-stimulated T production ($P < 0.001$) were prevented by the addition of 10^{-7} M antalarmin ($P = 0.010$). Similar results were found in mid-vitellogenic follicles ($F = 13.67$, $P < 0.001$; Figure 8D). The inhibitory effects of 10^{-8} M r/hCRF on hCG-stimulated E_2 production ($P = 0.003$) were blocked by the addition of 10^{-7} M antalarmin ($P = 0.005$). On its own, antalarmin (10^{-7} M) had no effect on basal T or E_2 production, or on hCG-stimulated steroidogenesis (data not shown).

Experiment 5: Effects of CRF on the expression of steroidogenic genes

Significant effects of CRF and hCG on StAR production were found at both 3 and 6 hours ($F = 22.41$, $P < 0.001$ / $F = 38.18$, $P < 0.001$). Incubating full grown follicles with 20 IU/ml hCG for 3 or 6h elicited a ~3-fold increase in *star* expression ($P < 0.001$ for each; Figure 9A and B, respectively). Neither 10^{-9} or 10^{-7} M r/hCRF had an effect on basal *star* mRNA levels, but both r/hCRF dosages significantly inhibited hCG-stimulated *star* expression at both 3 and 6h ($P < 0.001$, in all four instances). Expression of *cyp19a1a* followed a similar pattern at both 3 ($F = 21.45$, $P < 0.001$) and 6h ($F = 5.32$, $P < 0.001$). hCG

treatment elicited significant increases in *cyp19a1a* expression after both 3 and 6h of incubation ($P < 0.001$, $P = 0.002$, respectively; Figure 9C-D). While 10^{-9} M r/hCRF had no effect on hCG-stimulated *cyp19a1a* expression, co-incubation with 10^{-7} M r/hCRF prevented the stimulatory effect of hCG on *cyp19a1a* mRNA levels at both 3 ($P < 0.001$) and 6h ($P = 0.003$). Finally, neither dosage of r/hCRF affected basal *cyp19a1a* expression.

Experiment 6: Effects of CRF on db-cAMP-stimulated steroid production

Concentrations of db-cAMP \geq to 2.5 mM elicited a significant dose-dependent increase (One-way ANOVA; $F = 15.592$, $P = < 0.001$) in T production in full grown follicles (Figure 10A). Both hCG and db-cAMP interacted significantly with r/hCRF ($F = 32.58$, $P < 0.001$; Figure 10B). Relative to basal T levels, incubating full grown follicles with 2.5 mM db-cAMP or 20 IU/ml hCG for 6 h elicited ~2- and 4-fold increases in T production, respectively ($P < 0.001$, for both). Pre-incubation with 10^{-7} M r/hCRF completely blocked db-cAMP-stimulated steroidogenesis ($P < 0.001$). Similarly, as previously observed in *Experiment 3*, 10^{-7} M r/hCRF partially inhibited the stimulatory effect of hCG on T production ($P < 0.001$).

Experiment 7: Expression of POMC and MC2R in the ovary

Both *pomca* and *mc2r* were shown to be expressed in mid-vitellogenic (Figure 11A) and full grown (Figure 11B) zebrafish follicles. The basal expression of *pomc* is ~3- and 6-fold higher than *crfb* in mid-vitellogenic and full grown follicles, respectively.

Experiment 8: Effects of an MC2R antagonist on hCG-, pACTH- and CRF- stimulated steroidogenesis

There were significant interactions between ACTH, hCG, and hACTH₍₁₅₋₂₄₎ ($F=12.46$, $P<0.001$; Figure 11C). Treatment with 1 IU/ml pACTH for 1.5h had no effect on basal E₂ production in mid–vitellogenic follicles, but blocked ($P<0.001$) the observed stimulatory effects of 20 IU/ml hCG on the production of this steroid ($P=0.001$). On its own, the putative MC2R antagonist, hACTH₍₁₅₋₂₄₎ (10^{-7} M), had no effect on basal E₂ production. However, pre-incubation with hACTH₍₁₅₋₂₄₎ reversed the inhibitory effects of ACTH on hCG-stimulated E₂ secretion ($P<0.001$).

There were significant interactions observed between CRF, hCG and hACTH₍₁₅₋₂₄₎ as well ($F=23.62$, $P<0.001$; Figure 11D). While hACTH₍₁₅₋₂₄₎ alone had no effect on hCG-stimulated E₂ secretion, it reversed ($P<0.001$) the inhibitory effects of 10^{-8} M r/hCRF on hCG-stimulated E₂ secretion ($P<0.001$).

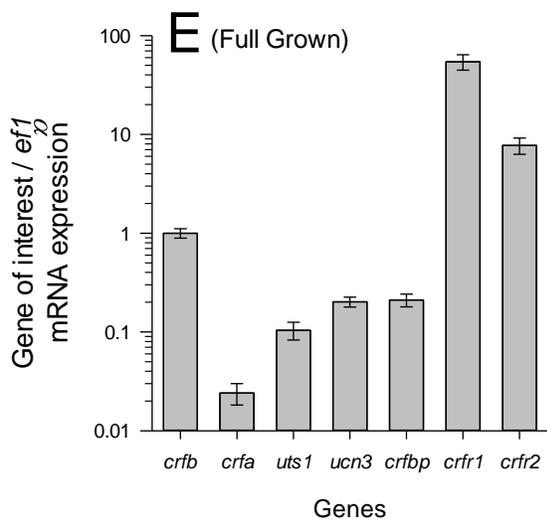
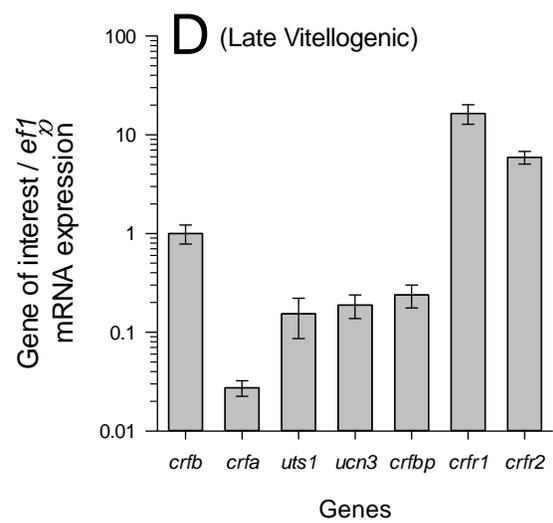
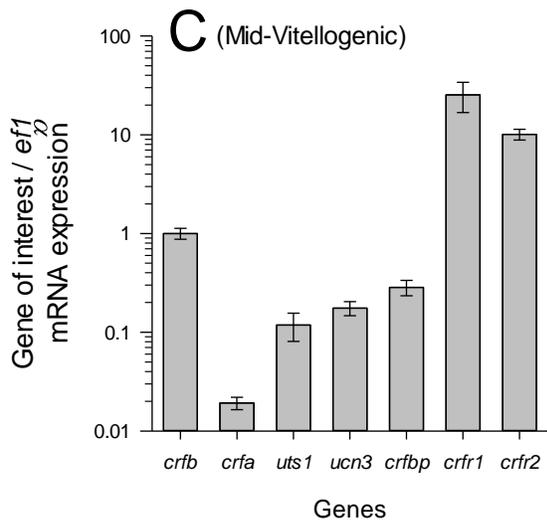
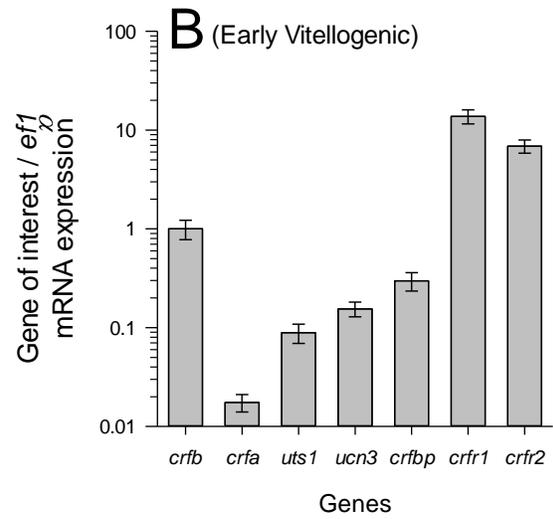
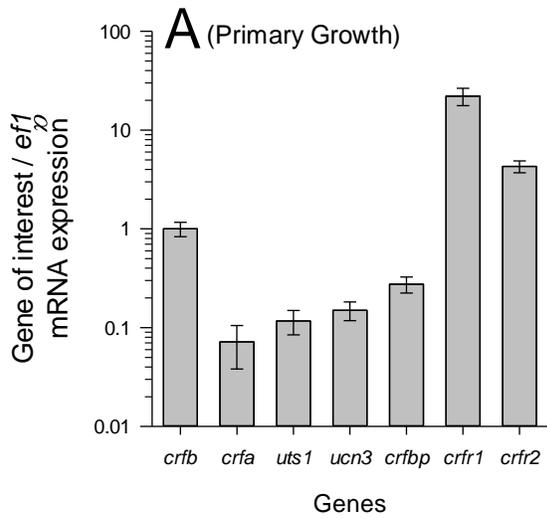


Figure 4: Expression of CRF family members including ligands, receptors and binding protein, in zebrafish ovarian follicles at individual stages of development. Ovarian follicles were separated into Primary, Early Vitellogenic, Mid Vitellogenic, Late Vitellogenic and Full Grown stages based on size and appearance (A-E, respectively). Gene expression was normalized to the housekeeping gene *ef1a*, with the expression of *crfb* within each stage set as 1. Data represent the mean \pm S.E.M of readings from multiple wells of follicles, with each well's contents obtained from a different fish (n=12).

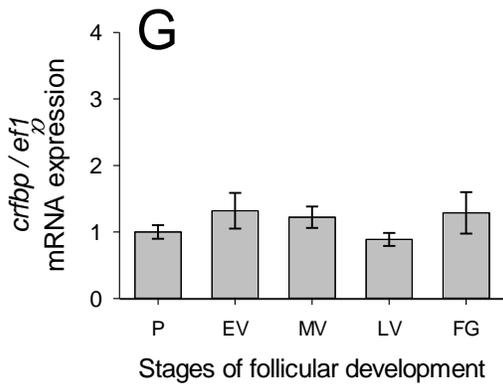
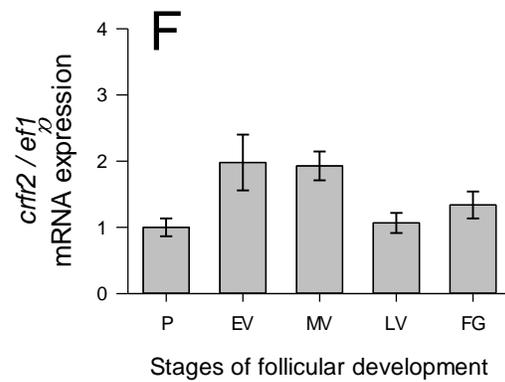
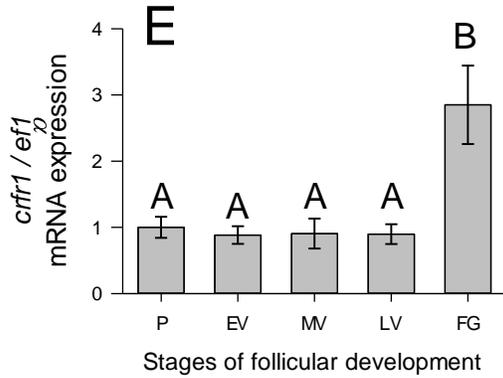
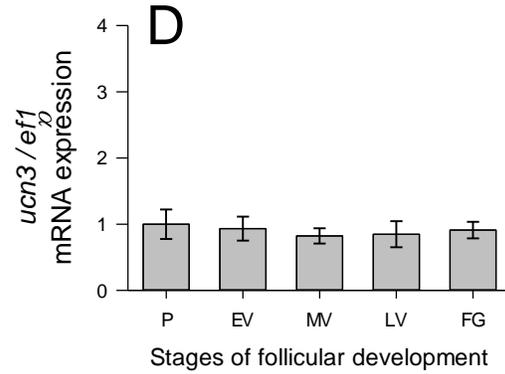
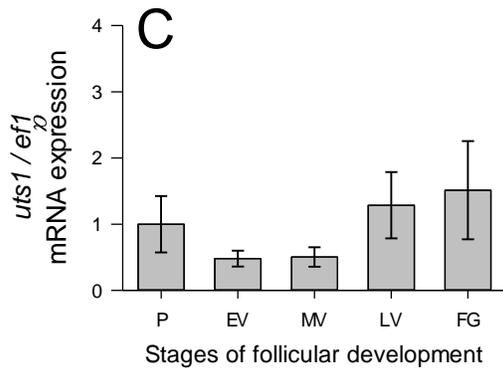
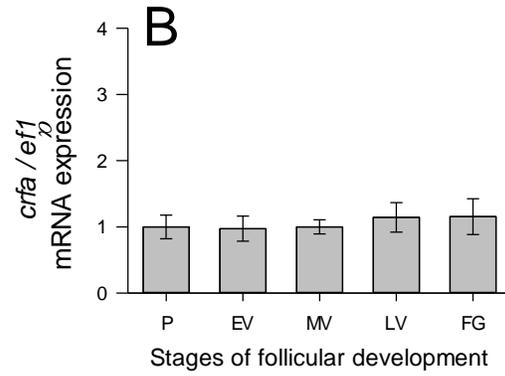
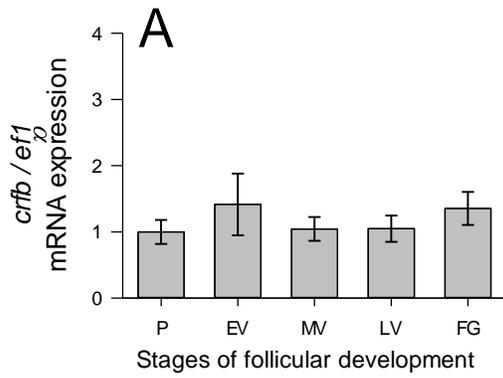


Figure 5: Expression of each CRF family member, including ligands, receptors and binding protein in zebrafish ovarian follicles across multiple stages of development. Ovarian follicles were separated into primary (P), early vitellogenic (EV), mid-vitellogenic (MV), late vitellogenic (LV) and full grown follicles (FG) based on size and appearance. Gene expression was normalized to the housekeeping gene *ef1a* with the expression of primary follicles set as 1. Data represent the mean \pm S.E.M of readings from multiple wells of follicles, with each well's contents obtained from a different fish (n=10-12). Differences in the expression of the individual CRF family members were determined by ANOVA and the Holm-Sidak test with significant differences indicated by different letter designations.

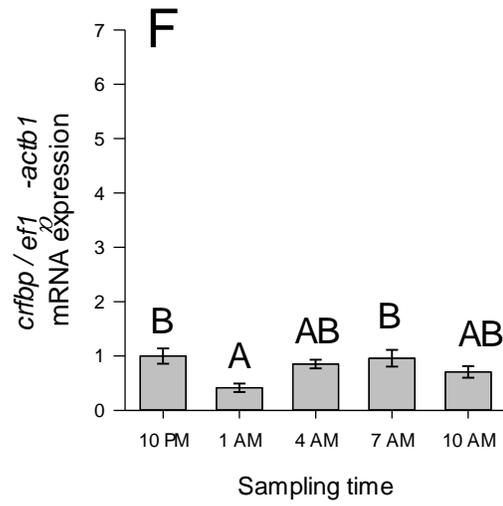
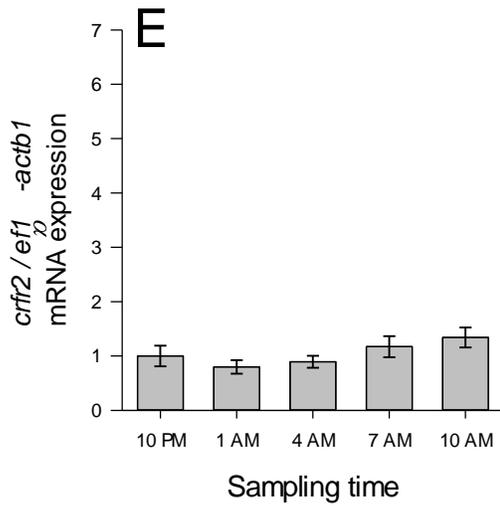
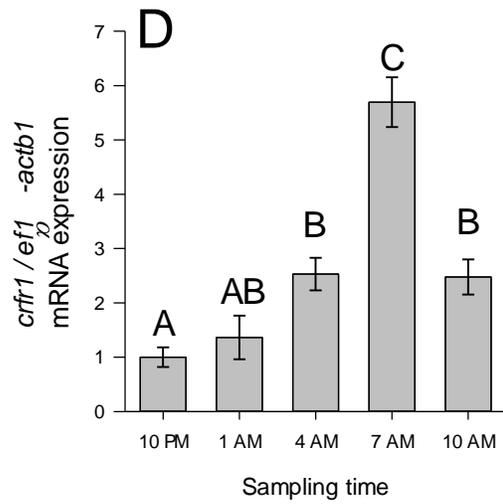
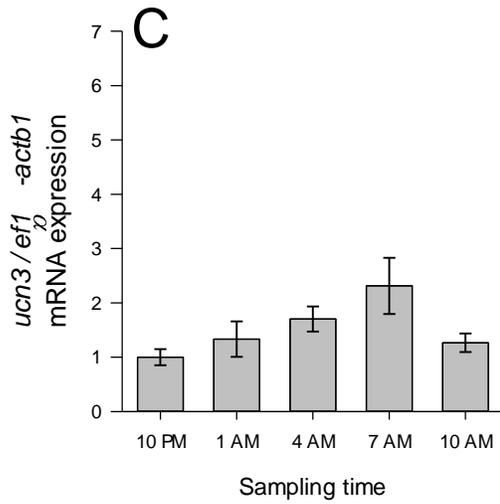
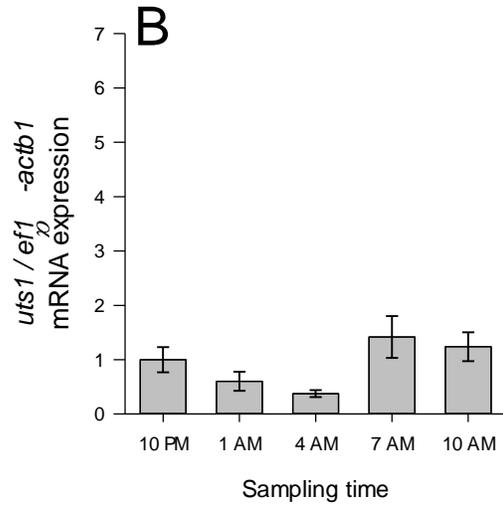
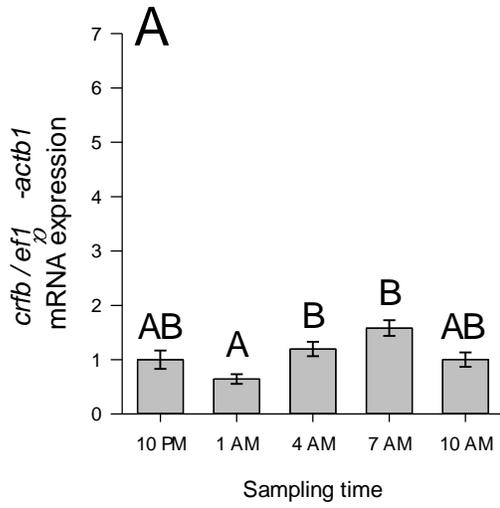


Figure 6: Perioovulatory changes in the expression of CRF family members including ligands, receptors and binding protein in whole ovarian tissue sampled at various times between 10PM and 10AM. Gene expression was normalized to the housekeeping genes *ef1a* and *actb1*, with the expression of follicles sampled at 10PM set as 1. Data represent the mean \pm S.E.M of readings from multiple wells of follicles, with each well's contents obtained from a different fish (n=10-12). Differences in the expression of the individual CRF family members were determined by ANOVA and the Holm-Sidak test with significant differences indicated by different letter designations.

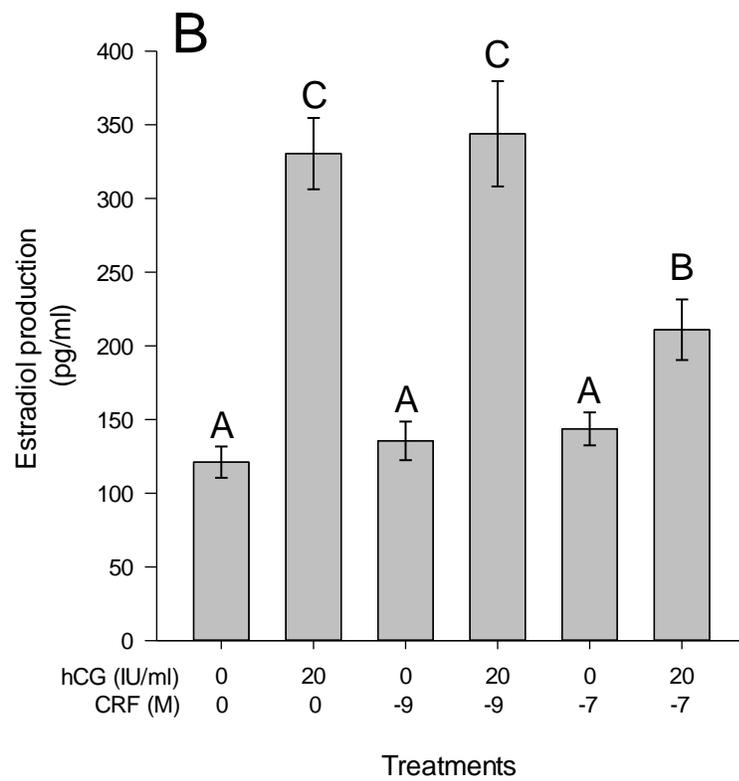
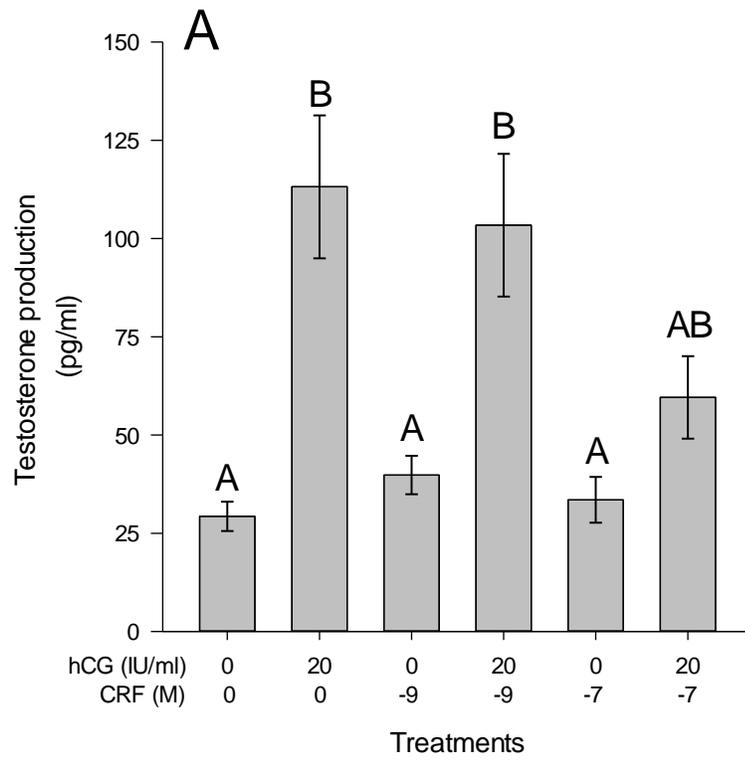


Figure 7: Effects of r/hCRF alone (10^{-9} and 10^{-7} M) and in combination with human chorionic gonadotropin (hCG; 20 IU/ml) on T production by full grown follicles (A) and E_2 production by mid-vitellogenic follicles (B), respectively. Follicles were pre-incubated with buffer or r/hCRF for 1 h prior to the addition of hCG and incubated for 6 h thereafter. Values represent the mean \pm S.E.M of steroids released to the well media, obtained from 12 wells, each containing follicles from a unique fish (n=10-12). ANOVA and the Holm-Sidak test were used to determine significant differences in steroid production and these were indicated by different letter designations.

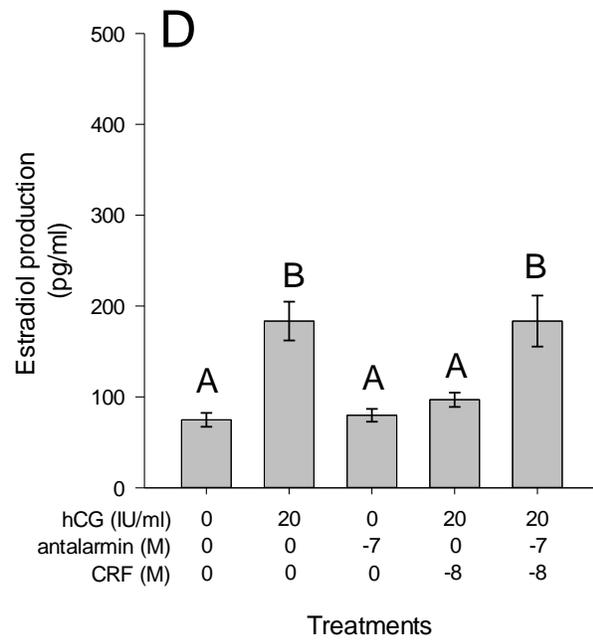
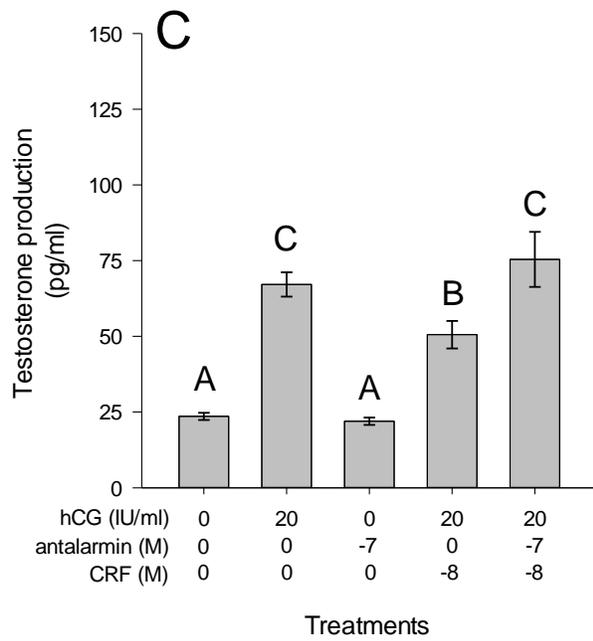
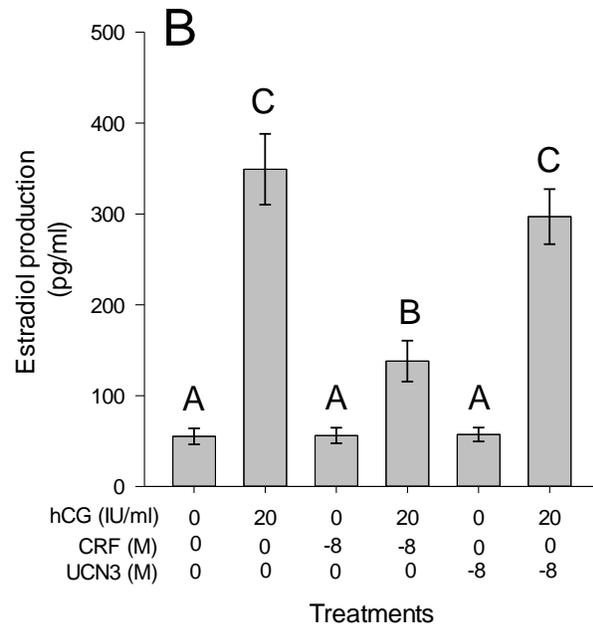
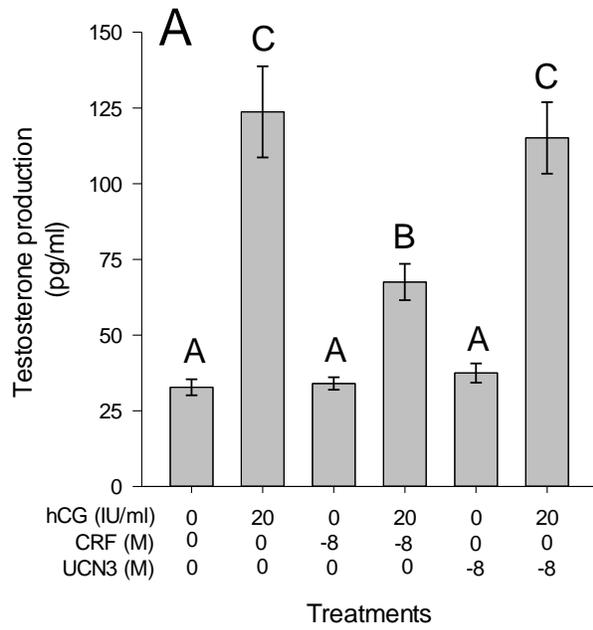


Figure 8: Determination of receptor dominance in the anti-gonadotropic effects of r/hCRF. 10^{-8} M r/hCRF and mUCN3 were applied to both full grown and mid-vitellogenic follicles (A and B, respectively) to determine their effects on the actions of 20IU/ml hCG on the production of either T or E₂. The ability of the R1-specific antagonist antalarmin (10^{-7} M) to block the anti-gonadotropic effects of r/hCRF in both full grown and mid-vitellogenic follicles was tested (C and D, respectively). Follicles were pre-incubated with buffer or r/hCRF / m/UCN3 / antalarmin for 1hr prior to the addition of hCG and incubated for 6hr thereafter. Values represent the mean \pm S.E.M of steroids released to the well media, obtained from multiple wells, each containing follicles from a unique fish (n=10-12). ANOVA and the Holm-Sidak test were used to determine significant differences in steroid production and these were indicated by different letter designations.

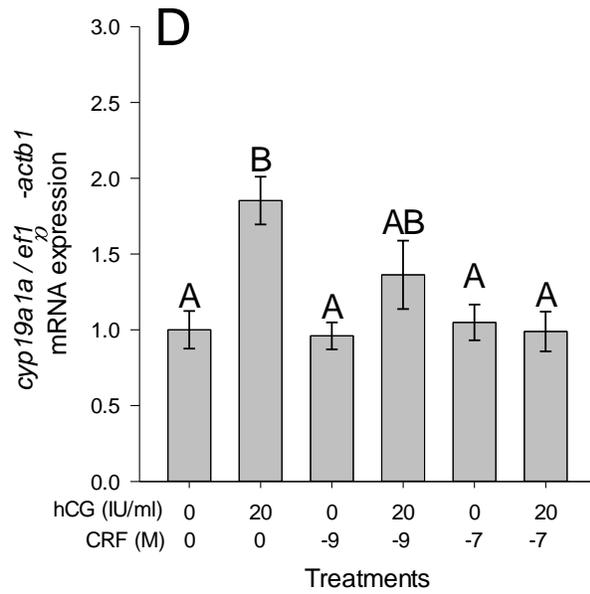
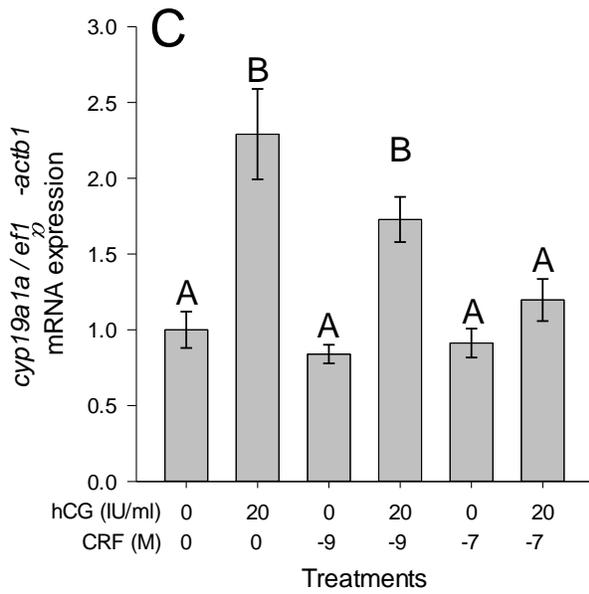
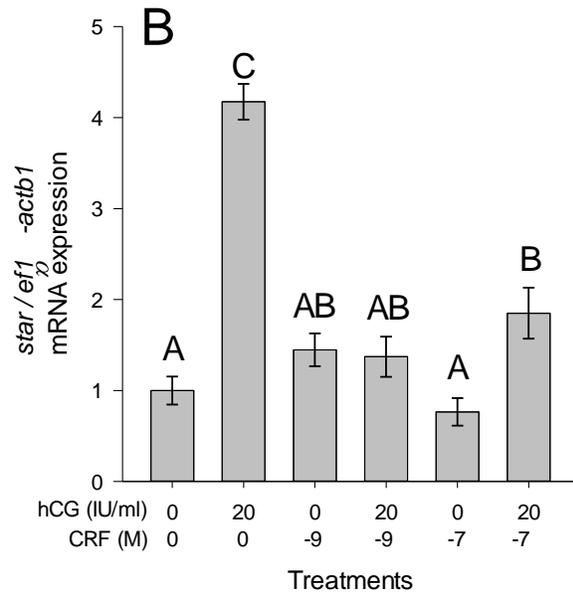
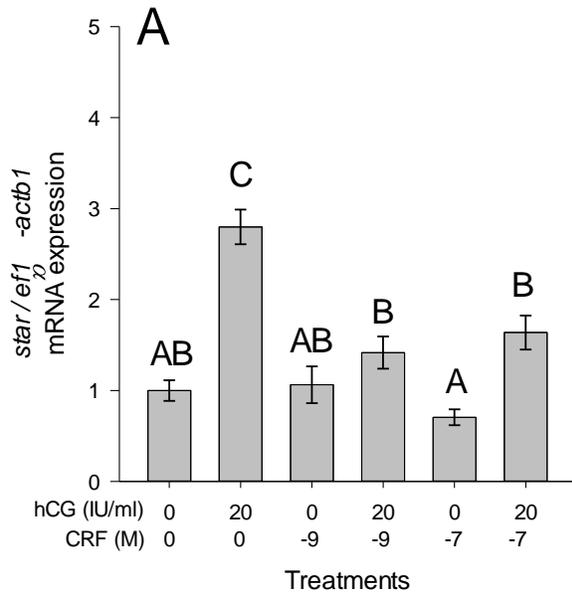


Figure 9: Expression of steroidogenic enzymes in response to r/hCRF / hCG signaling in full grown follicles. The response in expression of the genes for steroidogenic acute regulatory protein (*star*; A and B) and p450 aromatase (*cyp19a1a*; C and D) to hCG (20IU/ml) in the presence or absence of r/hCRF (10^{-7} or 10^{-9} M) were measured after a 1 hour pre-incubation with r/hCRF and incubations of either 3 or 6 hours (A,C and B,D, respectively) thereafter. Gene expression was quantified by qPCR and normalized to the housekeeping genes *ef1a* and *actb1* with the expression of control groups set as 1. Values represent the mean \pm S.E.M of expression obtained from 12 wells, each containing follicles from a unique fish (n=10-12). ANOVA and the Holm-Sidak test were used to determine significant differences in steroid production and these were indicated by different letter designations.

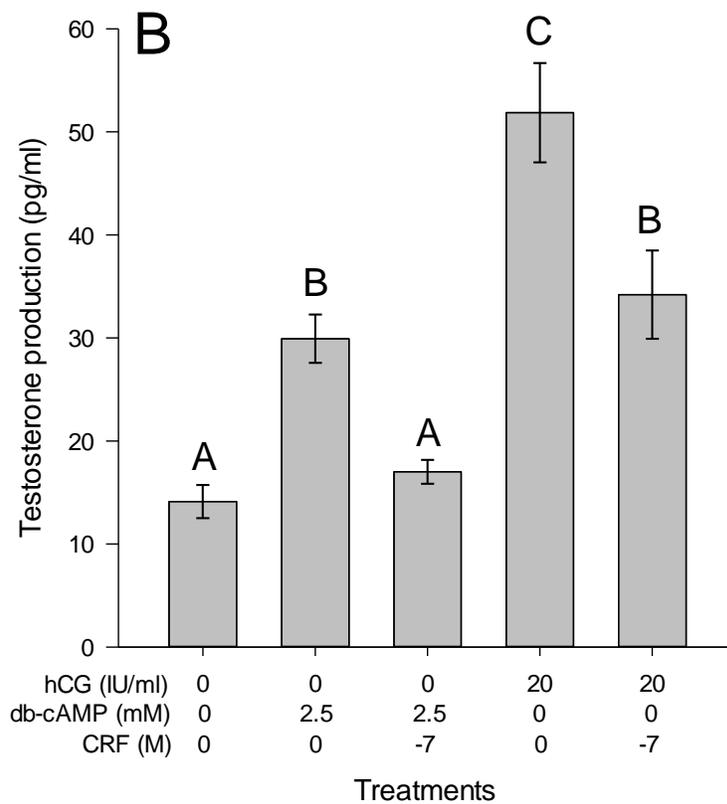
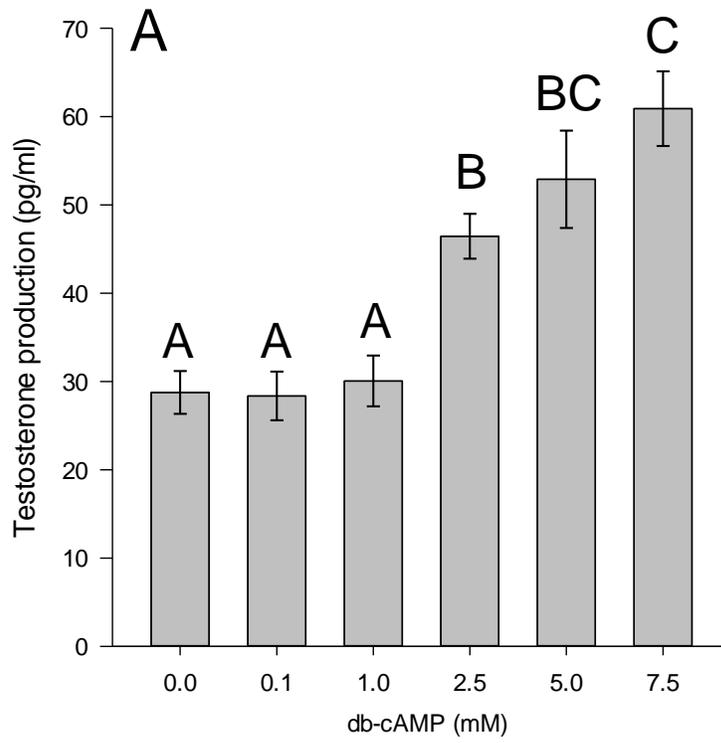


Figure 10: Effects of r/hCRF on the actions of dibutyryl cyclic-AMP. The ability of dibutyryl cyclic-AMP to enhance T production in stage IV full grown follicles was tested following a 6 hour incubation (A). The steroidogenic effects of db-cAMP (2.5mM) were compared to those of hCG (20IU/ml), and the ability of r/hCRF (10^{-7} M) to disrupt either was tested. Follicles were pre-incubated with buffer or r/hCRF for 1hr prior to the addition of hCG or db-cAMP and incubated for 6hr thereafter. Values represent the mean \pm S.E.M of steroids released to the well media, gathered from multiple wells, each containing follicles from a unique fish (n=11-12). ANOVA and the Holm-Sidak test were used to determine significant differences in steroid production and these were indicated by different letter designations.

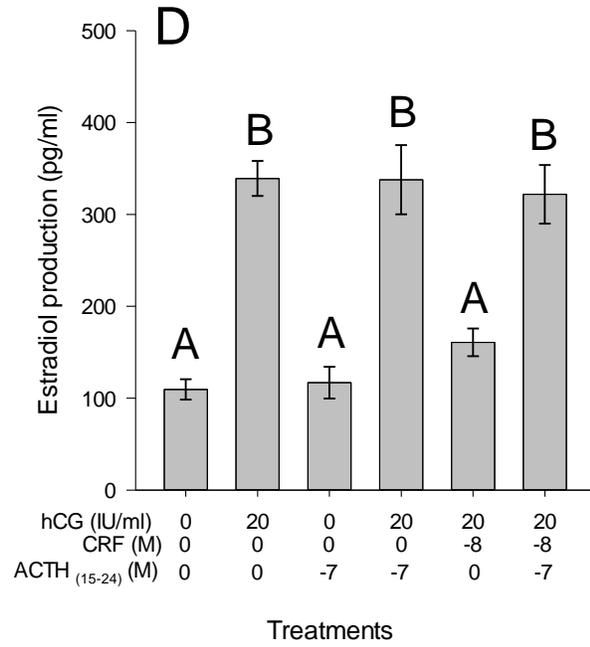
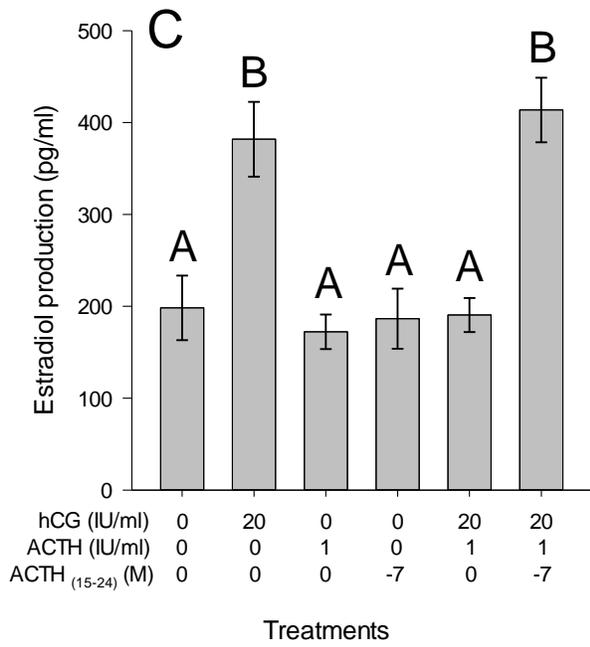
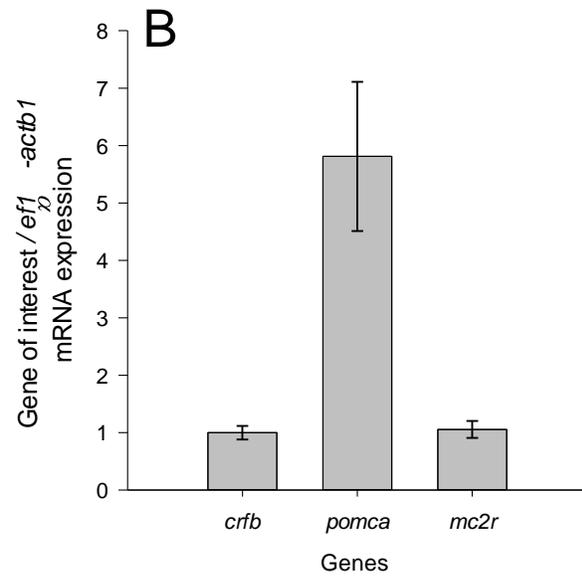
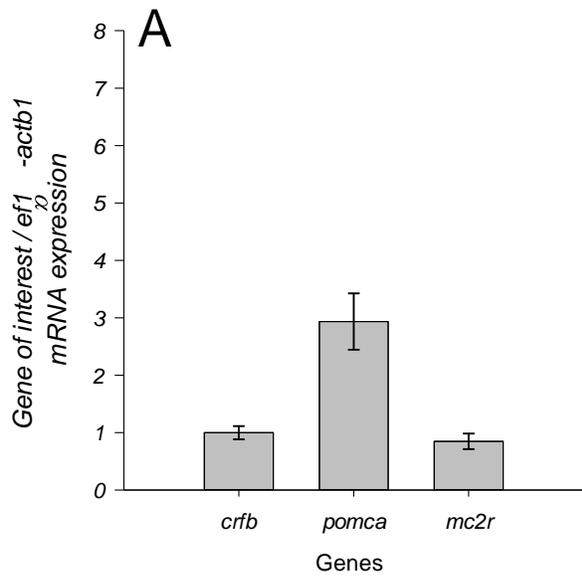


Figure 11: Characterization of ovarian ACTH components and the effects of ACTH in the OV-CRF system. The expression of genes for pro-opiomelanocortin (POMC) and the MC2R receptor were assessed in mid-vitellogenic and full grown follicles (A and B, respectively). Gene expression was quantified by qPCR and normalized to the housekeeping genes *ef1a* and *actb1* with the expression of *crfb* set as 1. The effects of porcine ACTH (1IU/ml) in inhibiting the actions of hCG (20IU/ml) were tested, as was the efficacy of hACTH₍₁₅₋₂₄₎ (10^{-7} M) as an MC2R antagonist (C). Interference of hACTH₍₁₅₋₂₄₎ (10^{-7} M) with the actions of hCG (20IU/ml) as well as its ability to impede the anti-gonadotropic effects of r/hCRF (10^{-8} M) were tested (D). Values represent the mean \pm S.E.M of steroids released to the well media, gathered from 3 separate experiments examining 3 groups of pooled follicles (n=3), with 20 follicles incubated per well. ANOVA and the Holm-Sidak test were used to determine significant differences in steroid production and these were indicated by different letter designations.

DISCUSSION

This thesis sought to investigate the presence and function of the CRF system in the ovary of zebrafish. My results demonstrate that all members of the teleost CRF family are expressed in zebrafish ovarian follicles throughout all stages of development, with both *crfr1* and *crfr2* having a high level of expression relative to ligands. The increase in *crfr1* mRNA levels in both full grown follicles and whole ovarian tissue near the time of ovulation suggest that dynamic and time-sensitive mechanisms are involved in regulating the expression of this CRF receptor. Similarly, the comparable though less pronounced periovulatory changes in whole ovary *crfb* and *crfbp* mRNA levels suggest a potential role for the ovarian CRF system in the regulation of ovulation. *In vitro*, CRF inhibited hCG-induced T and E₂ production in full grown and mid-vitellogenic follicles, respectively, via the CRF-R1 receptor. These anti-steroidogenic effects of CRF are at least partially mediated by an inhibition of hCG-stimulated *star* and *cyp19a1a* gene expression. Moreover, the inhibitory effects of CRF on the steroidogenic actions of gonadotropins likely occur after cAMP generation, as db-cAMP-stimulated steroid production was blocked by CRF. Both *pomca* and *mc2r* are expressed at appreciable levels in multiple ovarian follicular stages, raising the possibility of ACTH production within this tissue. Finally, the fact that the inhibitory effects of CRF on hCG-stimulated E₂ secretion can be reversed by an MC2R inhibitor, suggests that the anti-steroidogenic effects of CRF in the ovary involves local ACTH production. The proposed mechanism by which CRF inhibit steroidogenesis in the zebrafish ovary is summarized in Figure 12.

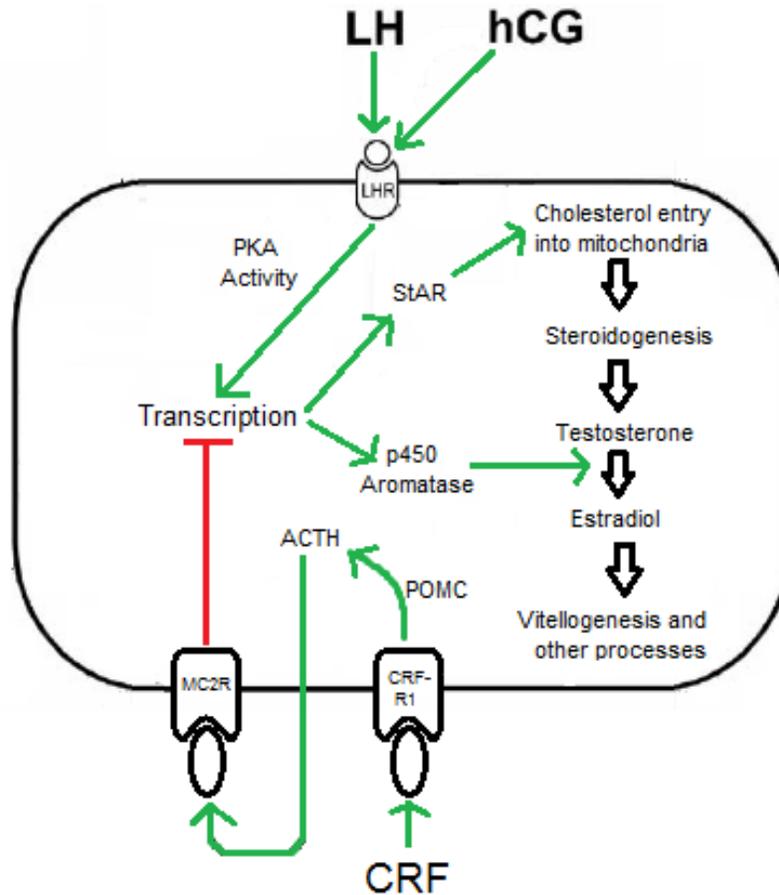


Figure 12: Proposed mechanism of CRF in the zebrafish ovary. Interactions are shown, with green and red lines indicating activation and inhibition, respectively. Activation of CRF-R1 triggers the conversion of locally produced POMC to ACTH, which engages in autocrine/paracrine signaling within the ovary. Activation of MC2R by ACTH inhibits gonadotropin-mediated increases in the transcription of steroidogenic genes such as StAR and p450 aromatase, thereby inhibiting testosterone and 17 β -estradiol production.

Spatial gene expression:

This study demonstrates the presence and relative expression levels of all teleost CRF family ligands within the zebrafish ovary at each stage of follicular development. The presence of ovarian mRNA for CRF ligands has been shown in several teleost species, including *crfb* in the cichlid *Astatotilapia burtoni* (Chen and Fernald, 2008) and *crfb*, *crfbp*, and *uts1* mRNA in unfertilized oocytes (Alderman and Bernier, 2009) and follicles (Bräutigam et al, 2010) of zebrafish. The findings of ovarian CRF family members in zebrafish are consistent with what is known in other vertebrates. Boorse and Denver (2006) detected in *Xenopus laevis* the ovarian expression of *crf*, *ucn1* (the amphibian equivalent of *uts1*), *ucn3* and *crfbp*. Other studies have also detected CRF mRNA in the ovaries of macaques (Xu et al, 2006) and humans (Muramatsu et al, 2001).

This study has additionally characterized expression patterns of both CRF receptors in the zebrafish ovary. The detection of mRNA coding CRF receptors in ovarian tissues has been documented in ovulated oocytes of zebrafish (Alderman and Bernier, 2009) and whole ovaries of the chum salmon, *Oncorhynchus keta* (Pohl et al, 2001) and the Japanese pufferfish, *Fugu rubripes* (Cardoso et al, 2003). These receptors are also found in broader range of vertebrates (Muramatsu et al, 2001; Boorse and Denver, 2006), showing a level of consistency across species.

This study distinguishes itself from prior works, as it is the first to provide a quantitative assessment and an analysis of the expression of the various components of the ovarian CRF system across follicular stages. Prior studies of the ovarian CRF family have been primarily conducted with whole ovarian tissue, rather than separated follicles.

Additionally, much of the characterization of the ovarian CRF system has been done for phylogenetic purposes and is qualitative in nature (Alderman and Bernier, 2009; Bräutigam et al, 2010). The relative levels of CRF related mRNA had until now been uncharacterized in the stages of follicular development. The additional level of detail with respect to developmental stage given by this study provides a foundation on which to understand ovarian CRF family signaling. The high expression of *crfr1*, *crfr2* and *crfb*, and the dynamic elevation of *crfr1* in the final stages of follicular maturation, suggest that some or all of these CRF family members play a role in regulating ovarian functions.

Temporal gene expression:

This study has demonstrated a time-dependent change to whole-ovary expression of *crfr1*, *crfbp*, and *crfb*, with the mRNA levels of all three genes increasing around 7AM, the approximate time of ovulation in our zebrafish stocks. Temporal characterization of ovarian CRF family gene expression has not yet been studied in other teleosts. There are, however, some parallels with what has been reported in the mammalian literature. Asakura et al (1997), using *in vitro* immunohistochemistry of human ovaries, showed a qualitative increase of both *crf* and *crfr1* in maturing ovarian follicles relative to preantral follicles. Xu et al (2006, 2007) found declining levels of both *crf* and *crfr1* in the ovaries of rhesus monkeys during the luteal phase following ovulation. While not comprehensive, the timing of these studies, when considered alongside the results of the present study, is consistent and suggests an increase of CRF family expression around the time of ovulation. These findings present the

possibility of ovarian CRF family signaling being related to the ovulatory process, if not another process occurring within a similar time frame.

Steroidogenic effects of CRF and gonadotropins

The present study demonstrated *in vitro* anti-gonadotropic effects of CRF in the ovaries of zebrafish, and is the first such study in a teleost. Although not documented in the teleost literature, there is an abundant body of mammalian research on the impacts of CRF on ovarian function and steroidogenesis, which bears remarkable consistency with the results of this study. CRF application to granulosa-lutein cells in culture suppresses, in a dose-dependent fashion, the ability of gonadotropins such as hCG and pregnant mare serum gonadotropin to elevate production of E₂, an effect seen in rats, mice and humans (Calogero et al, 1996; Ghizzoni et al, 1997; Murase et al, 2002; Dinopoulou et al, 2013). The dose range over which CRF inhibits the actions of gonadotropins is consistent across models, including zebrafish tested in the present study (10⁻⁹ to 10⁻⁷M). The actions of CRF appear to be mediated via CRF receptors as alpha-helical CRF, a non-specific CRF antagonist, eliminates the anti-steroidogenic effects of CRF (Ghizzoni et al, 1997; Murase et al, 2002).

Though the mammalian literature focuses primarily on the effects of ovarian CRF on E₂ production, the peptide has a broader range of anti-steroidogenic action, as well as the ability to disrupt steroid-dependent reproductive functions. The results of the present study corroborate the notion of multiple types of steroidogenic inhibition by CRF, including T and E₂. A study by Kiapekou et al (2011) in mice demonstrated that oocyte maturation, as measured by first polar body extrusion, was slowed significantly in the presence of a sufficient dose (10⁻⁹ M or greater) of CRF, suggesting that CRF

may inhibit a broader range of steroids. In human ovarian theca cells, CRF also inhibits the LH-stimulated production of DHEA and androstenedione, early precursors for the biosynthesis of testosterone and estradiol (Erden et al, 1998). This effect occurs within a narrow range of CRF dosages (10^{-8} to 10^{-7} M). The fact that CRF inhibits the synthesis of a variety of steroids suggests that CRF either inhibits multiple stages of steroid production or an early stage of the steroidogenic process. This raises the possibility of a much wider range of ovarian CRF effects *in vivo* than the literature might suggest. Although the ultimate function of this system is not yet clear, the consistency of CRF's presence and effect in the ovaries of so many organisms implies an important reproductive function.

Another finding of the current study is that CRF-R1 is the dominant receptor mediating the anti-gonadotropic effects displayed by CRF. In both mice and rats, the effects of ovarian CRF can be blocked by antalarmin, a CRF-R1 receptor specific antagonist (Calogero et al, 1996; Kiapekou et al, 2011). In vertebrates as a whole, the role of CRF-R2 in ovarian CRF signaling remains comparatively unexplored, despite its expression in both whole ovarian tissue (Boorse and Denver, 2006) and isolated ova (Alderman and Bernier, 2009) of various species.

Effects of CRF and gonadotropins on gene expression

This study demonstrates the ability of CRF to inhibit gonadotropin-elicited increases in steroidogenic gene expression, a novel finding in teleosts. The influence of gonadotropins on steroidogenic genes is well characterized in teleosts and mammals alike, as one of their major functions is mediation of steroidogenic gene expression (Ge, 2005; reviewed in Nagahama et al, 2008). Ings and Van Der Kraak (2008) reported

strong increases to both *star* and *cyp19a1a* expression in the presence of hCG over both 3 and 8 h incubations, using a protocol comparable to the present study. Moreover, mammalian models demonstrate strong *star* and *cyp19a1a* responses to gonadotropins, demonstrating conservation of the HPG response across multiple vertebrate classes (Clark et al, 1994; Fitzpatrick and Richards, 1991).

The discovery of CRF-based inhibition of gonadotropic gene expression is a novel finding among teleosts, but is not without precedent in a wider range of vertebrates. Calogero et al (1996) have shown that FSH-induced increases of aromatase activity are suppressed by CRF in rat granulosa cell cultures, potentially indicating changes in gene expression. Yu et al (2016) further demonstrated that intravenous introduction of CRF leads to a marked decrease in both *cyp19a1a* expression in porcine ovarian tissues and E₂ production.

While the stimulatory effects of gonadotropins on *star* gene expression have been documented (Clark et al, 1994; Ings and Van Der Kraak, 2008), the inhibition of this response by CRF is unprecedented in teleost or mammalian literature. Notably, the inhibitory effects of CRF on *star* expression are greater than its effects on *cyp19a1a* expression. At both 3 and 6 h, *star* underwent a greater fold change from hCG signaling compared to *cyp19a1a*. Additionally, *star* was more sensitive to r/hCRF inhibition of hCG than *cyp19a1a*, with a significant reduction to gonadotropin-mediated expression at both doses. The inhibitory effects of CRF on either gonadotropin-induced *star* or *cyp19a1a* gene expression could be responsible for the diminished E₂ production previously observed in mammalian experiments (Calogero et al; 1996, Murase et al, 2002). Since StAR regulates a key rate-limiting step in steroid production, the inhibitory

effects of CRF on gonadotropin-induced *star* expression will have broader effects on gonadal steroidogenesis by limiting the availability of pregnenolone, reducing both T and E₂ (Privalle et al, 1983; Miller 1988).

The mechanism of CRF's anti-gonadotropic effects

The role of CRF in the inhibition of cAMP signaling is another novel teleost finding of the present study. In both mammals and teleosts, activation of the luteinizing hormone receptor increases cAMP production which in return activates protein kinases and CREBs resulting in gene expression changes (Dufau, 1998; Ryu et al, 1998). Though CRF signaling does not alter cAMP production in the presence of gonadotropins in *in vitro* rat studies (Calogero et al, 1996), it does still counter its downstream effects on aromatase activity and steroidogenesis. These mammalian findings are similar to the results of the present study, wherein the steroidogenic impacts of dibutyl cAMP are blocked by CRF. Murase et al (2002) showed that CRF application can, in a dose-dependent fashion, block the steroidogenic increase brought about by forskolin (an adenylyl cyclase activator) in both rat and human granulosa cells. Along with CRF's inability to alter cAMP levels in the presence of gonadotropins (Calogero et al, 1996) and its inhibition of cAMP-dependent steroidogenesis, the present study corroborates with extant literature by demonstrating that CRF exerts anti-gonadotropic effects after cAMP generation. Additional inquiry is needed to determine where along the cAMP-dependent cascade inhibition occurs, be it on protein kinases, the actions of CREBs, or elsewhere. As the present study implicates ACTH as the final effector of anti-gonadotropic signaling, its role in effecting the cAMP cascade should be explored as well.

Implication of ACTH in the ovarian CRF system

The final findings of this study demonstrate the expression of *pomca* and *mc2r* in the zebrafish ovary, as well as a link between ovarian CRF signaling and the local activation of MC2R to facilitate anti-gonadotropic effects. The second of these findings is unprecedented in vertebrate research, although prior teleost literature does contain findings which are consistent with such a system. RNA transcripts for *mc2r* have been detected both in the whole ovary of rainbow trout, *Oncorhynchus mykiss* (Aluru and Vijayan, 2008), and the isolated ovarian follicle of zebrafish (Alsop et al, 2009). These studies have found *mc2r* expression levels comparable in ovarian tissue to those of interrenal tissue, where the receptor contributes to the HPA/HPI axis, suggesting the possibility of ACTH based signaling in the ovary. The presence of *mc2r* transcripts in zebrafish follicles was confirmed in the present study, along with an elevated expression of *pomca*. This raised the possibility that local ACTH production in ovarian follicles may be occurring. Although *pomca* itself has not been reported in studies with teleost ovaries, it has been found extensively in the rat (reviewed in Autelitano et al, 1989). However, certain findings in teleost literature are potentially explained by the presence of local POMC generation. Significant immunohistochemical detection of α -MSH, a POMC and ACTH derivative (reviewed in Leatherland et al, 2010) in ovarian follicular layers of two teleost species (Mosconi et al 1994) suggest that local ACTH signaling occurs within the ovarian follicle.

The association between ovarian CRF and ACTH/MC2R signaling is another novel finding for teleosts. Alsop et al (2009) demonstrated that ACTH, when incubated with ovarian follicles in a protocol comparable to that of the present study, produced

similar anti-gonadotropic effects to CRF (that is, a dose-dependent suppression of gonadotropic steroidogenesis). An *in vivo* study of cows (Biran et al, 2015) found anti-steroidogenic effects of ACTH, with chronic administration of ACTH inducing a reduction of both E₂ and androstenedione, again suggesting a broad inhibition of steroidogenesis. Additionally, this study found a strong reduction of *cyp19a1a* mRNA in preovulatory follicles following ACTH administration. The concurrent effects of cortisol on LH receptor prevalence leaves the contributions of ACTH to direct gene change in the ovary unclear in this study, although the findings of Alsop et al (2009) demonstrate clearly that a cortisol-independent inhibition of gonadotropins occurs via ACTH.

In this study, the anti-steroidogenic effects of ACTH and CRF in zebrafish follicles were both reversed by pre-incubation with the MC2R antagonist, hACTH₍₁₅₋₂₄₎. By inhibiting the effects of CRF through blockage of MC2R, this study has demonstrated that CRF's anti-steroidogenic actions occur through local ACTH production and signaling, as ACTH could not have arrived from an external source to activate MC2R in an *in vitro* study. Although the contribution of circulating ACTH originating from the pituitary can't be ruled out as a contributor to gonadal steroid inhibition *in vivo*, the present study demonstrates that autocrine/paracrine ACTH signaling can mediate the inhibitory effects of CRF on gonadotropin-induced steroidogenesis.

Future Directions

An important detail as of yet not broached in the teleost ovarian CRF system is the distribution of CRF receptors with respect to follicular layers and the oocyte itself. In mammalian species, CRF-R1 is present in both theca (Mastorakos et al 1993; Erden et al 1998; Kalantaridou et al 2004) and granulosa cells (Ghizzoni et al 1997; Murase et al,

2002). This pattern of expression could reasonably be anticipated in zebrafish as well, as enzymes present in both theca (*star*) and granulosa (*cyp19a1a*) cells were inhibited by CRF via CRF-R1 in this study. However, maternally derived mRNA is often deposited to the growing oocyte by somatic cells (reviewed in Lubzens et al, 2010), and the contribution of this mRNA to qPCR readings of whole follicles should be addressed. Alderman and Bernier (2009) identified the presence *crfb*, *crfbp*, *crfr1* and *crfr2* mRNAs in the ovulated oocyte of zebrafish, with a profile of expression levels which, qualitatively, resembled that of the present study. These maternal mRNAs, involved in the ontogeny of the embryo rather than gonadal steroidogenesis, obscure the follicle-specific patterns of expression of the CRF family with regards to its role in steroidogenesis. The fact that CRF treatment affected the expression of both theca- and granulosa-derived enzymes, and had steroidogenic effects attributable to these changes, indicate that *crfr1* is expressed in both follicular layers, but the relative prevalence in each layer remains an important factor in understanding the ovarian CRF system's ultimate function.

The scope of CRF's effects and its purpose *in vivo* will depend on which follicle layer it interacts with most prominently under physiological conditions. The disruption of gonadal aromatase by CRF in the granulosa in this study and in mammalian species (Calogero et al, 1996) has the potential to induce cessation of vitellogenesis in teleosts via disruption of E₂ production (Clelland and Peng, 2009). Keeping in mind the current study's demonstration of elevated *crfr1* in full grown follicles, those nearing the end of vitellogenesis, CRF signaling may be implicated in the termination of that process. Meanwhile, StAR disruption would induce numerous downstream effects, including

reduction of E_2 and the associated effects, the prevention of oocyte maturation via reduction of 17-20 β P (Nagahama et al, 1995), or disruption of germinal vesicle breakdown through reduction of T (reviewed in Lubzens et al, 2010). This more general inhibition of steroidogenesis would result in a near complete cessation of ovarian function and could be involved in regulated inhibition of reproduction such as that seen during chronic stress (Clearwater and Pankhurst, 1997; Chrousos et al, 1998; Charmandari et al, 2005; Fuzzen et al. 2011).

Although other CRF family members such as *uts1*, *ucn3* and *crfr2* exhibited reduced expression throughout development compared to *crfb* and *crfr1*, their potential roles in ovarian CRF signaling should not be ignored. The differential binding affinities of paralogs to the two CRF receptors (Manuel et al, 2014) present the possibility of greater complexity in the ovarian CRF system than reported here, which warrants further examination. Although *crfr2* was appreciably expressed relative to *crfr1* in all stages of follicular development, its role in steroidogenesis and ovarian function is not clear. Interestingly, there is some evidence of CRF-R2-mediated effects on steroidogenesis in mammals. Used at concentrations that are too low to activate CRF-R1, UCN3 inhibits P_4 production from cultured human granulosa-lutein cells (Yata et al, 2009). Although the present study demonstrated that UCN3 and CRF-R2 have no role in steroidogenic changes of T and E_2 , there may be additional components of steroidogenesis impacted by CRF-R2 as of yet undiscovered. Additionally, the role of CRF-BP, which had comparable whole-ovary dynamics to both *crfb* and *crfr1* over time has not yet been explored. Given the co-release and modulation of CRF signaling by CRF-BP in other

systems (Kemp et al, 1998; Seasholtz et al, 2002), its actions may contribute to the complexity of this system as well.

A greater level of understanding of the ovarian CRF system's ultimate function is needed. Whole ovary results demonstrate elevated *crr1* expression around 7AM, and comparison of gene expression across follicular stages sampled at 10AM implies that CRF-R1 is elevated in full-grown follicles. These findings are consistent with expression patterns in both human and xenopus models (Asakura et al, 1997; Xu et al 2006, 2007). Given appreciable expression of *pomca* transcripts at both stages of follicular growth, the present study appears consistent with prior studies which show POMC derivatives to be elevated in the ovaries of reproductive and post-reproductive teleosts (Mosconi et al, 1994). Taken together, these results suggest that CRF's ovarian signaling has the greatest potential to disrupt gonadotropic steroidogenesis at or nearing the time of ovulation. The notion of CRF having a hand in the control of ovulation has been proposed before, as in mammals it has been implicated in polycystic ovary syndrome, menopause, and anovulation (reviewed in Mastorakos et al, 1994; Chrousos et al, 1998). However, at present the ultimate function of teleost ovarian CRF inhibition of steroidogenesis is only speculative. Additional work, such as additional sampling times of isolated follicles, or the aforementioned examination of CRF receptors within different follicular layers via immunohistochemistry or layer-specific qPCR, is needed. These studies would provide a more complete picture both of CRF's potential signaling activity throughout development, and the likely impacts and function of CRF signaling *in vivo*.

The characterization of the ovarian CRF system remains to be explored *in vivo*. Although the effects of CRF on steroidogenesis are well characterized *in vitro* (Ghizzoni

et al, 1997; Murase et al, 2002; present results), and there is some evidence in porcine models to suggest that *in vivo* CRF application induces similar effects (Yu et al, 2016), the natural function of this system remains unclear. The factors which induce endogenous CRF signaling in the ovary remain unknown, which makes its function in the organism difficult to predict. At present, several potential (and not mutually exclusive) functions of CRF in regulating ovulation, egg maturation, or stress-suppression of reproduction stand as feasible possibilities.

Given the present study's findings that CRF's effects occur via local ACTH production and signaling, there is some evidence that HPA/HPI activation may exert control over ovarian CRF activity. Although cortisol does not directly inhibit ovarian steroidogenesis (reviewed in Fuzzen et al. 2011), *in vitro* administration of cortisol to stage I and II zebrafish follicles does increase *mc2r* gene expression (Sousa et al 2015). In this way, elevated cortisol could potentiate the CRF/ACTH ovarian response without directly activating it. This elevated receptivity to CRF/ACTH signaling may partially explain the reduced sex steroid levels typically associated with elevated cortisol levels (Pickering et al, 1987; Clearwater and Pankhurst, 1997). Moreover, as cortisol exposure *in vivo* impacts T production on a more rapid time scale than E₂ (Pankhurst and Van Der Kraak, 2000), it is likely that these effects occur early in the steroidogenic cascade, an effect which could be explained through CRF/ACTH disruption of StAR. However, as cortisol also is known to impact the HPG axis through GnRH disruption, which can produce similar anti-reproductive effects (Dubey and Plant, 1985), the relative contribution of ovarian ACTH signaling *in vivo* remains unclear. Further study of ACTH's effects on steroidogenesis, and in particular, determining if its local production

occurs separately from or concurrently with cortisol elevation will be an important first step in understanding this emerging endocrine system.

Conclusion

This study is the first to characterize the ovarian CRF family in teleosts, not only in terms of gene expression through time, but also in terms of anti-steroidogenic effects. The presence of *crfb*, related peptides *uts1*, *ucn3* and *crfa*, *crfbp*, and receptors *crfr1* and *crfr2* across all stages of ovarian development suggest a complex system of CRF signaling within the ovary. Increases to *crfr1* with respect to both follicular maturity and daily cycling suggest a role in the regulation of ovulation or a closely timed event. CRF-R1 and MC2R-dependent inhibition of both T and E₂ production by CRF via reduction of gonadotropic promotion of *star* and *cyp19a1a* demonstrate broad anti-steroidogenic function of CRF. Additional work is needed to localize the expression of CRF receptor subtypes within the zebrafish ovary and determining their ultimate function *in vivo*, but the present study provides a strong initial foundation on which such further works may be based.

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