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The Role of GnIH in Paracrine/Autocrine Control of Ovarian Function in Zebrafish (Danio rerio)

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The Role of GnIH in Paracrine/Autocrine Control of Ovarian Function in Zebrafish (*Danio rerio*)

by

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Abstract

Gonadotropin inhibitory hormone (GnIH) is a hypothalamic neuropeptide, termed for its observed inhibitory effect on the luteinizing hormone release from cultured pituitary of Japanese quail when it was first discovered in 2000. In vertebrates including fish, GnIH has been found in extra-hypothalamic tissues including gonads, however, information regarding the role of this peptide as extra-pituitary regulator of gonadal function is not available and require further research.

The goal of my thesis was to investigate direct action of GnIH, *in vitro,* on ovarian steroidogenesis, final oocyte maturation, and gene expression using sexually mature zebrafish. In the present study, GnIH did not significantly affect transcript levels for genes involved in the control of gonadal function and steroid biosynthesis. However, GnIH significantly altered hCG-induced estradiol (E_2) release and resumption of meiosis. Thus, the findings provide a support for the hypothesis that GnIH plays paracrine/autocrine role in the regulation of ovarian function in zebrafish.

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Symbol	Definition
3β-HSD	3-β-hydroxysteroid dehydrogenase
17β-HSD-3	17β- hydroxysteroid dehydrogenase-3
17β-HSD-1	17β- hydroxysteroid dehydrogenase-1
ANOVA	One-way analysis of variance
CYP19	Aromatase/cytochrome p450
cDNA	complementary deoxyribonucleic acid
cGnRH-II	chicken gonadotropin-releasing hormone/GnRH type 2
cAMP	cyclic adenosine monophosphate
C-terminal	carboxyl terminal end of peptide
dNTP	deoxyribonucleotide
E2	estradiol
fGRP	frog growth hormone-releasing peptide
FSH	follicle-stimulating hormone
FSH-β	follicle-stimulating hormone β subunit
FSH-R	follicle-stimulating hormone receptor
GABA	*gamma*-Aminobutyric acid
GH	growth hormone
GnIH	gonadotropin inhibitory hormone
gfGnIH	goldfish gonadotropin inhibitory hormone

GnIH-R	gonadotropin inhibitory hormone receptor
GTH	gonadotropin
GnRH	gonadotropin-releasing hormone
h	hour
hCG	human chorionic gonadotropin
HPG	hypothalamic-pituitary-gonadal
icv	intracerebroventricular
ir	immunoreactive
ip	intraperitoneal
IU	International Units
iv	intravenous
LH	luteinizing hormone
LH-β	luteinizing hormone β subunit
LH-R	luteinizing hormone receptor
M	moles/liter
ME	median eminence
mRNA	messenger ribonucleic acid
nm	nanometer
NPFF	neuropeptide FF
NPY	Neuropeptide Y
PACAP	pituitary adenylate cyclase-activating peptide
PGCs	primordial germ cells
PVN	paraventricular nucleus

QPCR	quantitative real time polymerase chain reaction
RACE	rapid amplification of cDNA ends
RP	related peptide
RF-amide	peptides with an arginine and phenylalanine at their C-terminus
RNA	ribonucleic acid
sGnRH	salmon gonadotropin-releasing hormone, GnRH type 3
S.E.M.	standard error of the mean
STAR	steroidogenic acute regulatory protein
α	alpha
β	beta
μL	microliter

Chapter One

Introduction

1.1 Introduction

Zebrafish (Danio rerio) are hermaphrodites during the early stages of development (Takahashi, 1977). At the early stage of development, primordial germ cells move to where gonads are developed (Raz, 2003 and Connaughton and Aida, 1999), and yield ovary-like tissue and eventually develop into male or female (Takahashi, 1977). The gonadal tissue further developed into functional ovaries in females, or turns into testes in males after programmed cell death takes place in oocytes (Slanchev et al., 2005). The absence of germ cells, before early gonads are formed in zebrafish, leads to infertile males (Slanchev et al., 2005), indicating a significant contribution of germ cells in folliculogenesis in the female (Slanchev et al., 2005). In fact, sex determination is a complicated process since zebrafish lack heteromorphic chromosomes (Tokarz et al., 2013). In addition, sex determination has also been a controversial issue regarding the actual manner in which gonadal differentiation of zebrafish takes place. It is not yet certain whether sex determination process is influenced by environmental factors (Lawrence et al. 2008; Corley-Smith et al., 1996) or driven genetically (Tong et al., 2010; Liew et al., 2012).

In adult female zebrafish, all developmental stages of follicles, and progression into maturation are fundamentally regulated by hormones of brain-pituitary-gonadal axis. Basically, gonadotropin-releasing hormone (GnRH), “the key regulator of reproduction in vertebrates”, produced by the hypothalamus, stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. These gonadotropin hormones (GTHs) subsequently act on the ovary and control follicle development, maturity, and local production of growth factors and steroids (Patino et al., 2001; Khan and Thomas, 1999; Wang and Ge, 2004; DiMuccio et al., 2005).

Gonadal sex steroids such as estradiol control initial germ cells development toward folliculogenesis, as ablation of estrogen activity favors development into male testes structures (Brion et al., 2004). Furthermore, follicle growth and vitellogenin is not only regulated by estradiol, but is also promoted by the gonadal peptides, including transforming growth factors beta (TGFß) family and activin βA, known as (*inhba)* (Wang and Ge, 2004a and Wang and Ge, 2004b), which in turn regulate gonadotropin production from the pituitary (Ling et al., 1986). Activin function is modulated by follistatin and countered by inhibin, which is a protein structurally associated with activin subunits (Ling et al., 1986 and Vale et al., 1986). In addition, gonadal inhibin negatively regulates the release of the pituitary gonadotropins via the feedback mechanism (Tsutsui et al., 2010). In 2000, a hypothalamic dodecapeptide was first discovered in quail, and at the time was found to directly inhibit the LH secretion in cultured pituitary; accordingly, this neuropeptide has been named gonadotropin inhibitory hormone (GnIH) (Tsutsui et al., 2000).

Since then, investigations have emerged elucidating the action of GnIH, following its expression along with its G-protein couple receptors in a variety of tissues, including gonads. However, GnIH physiological function is not fully understood as it has shown differential regulation of hormones in both the ovary and testes, and variable effects on vertebrates (Tsutsui et al., 2012). Thus, the objective of my thesis is to investigate the role of GnIH in paracrine/autocrine control of ovarian function in adult zebrafish.

1.1.1 Structure of ovarian follicles in zebrafish

Zebrafish ovarian follicles are composed of oocytes indirectly surrounded by a layer called granulosa cells, which is connected to these oocytes by a vitelline envelope known as zona radiate. Another layer of steroidogenic cells goes around the granulosa cells and

contains blood vessels and fibroblasts, called theca cells (Selman et al., 1993). Even though several studies have found a difference in the structure of follicles between mammals and fish, granulosa and theca cells maintain similar biological functions in both vertebrates (Nagahama, 1994; Nagahama et al., 1994).

Adult female zebrafish are capable of generating all developmental stages of follicles at any time during the year (Ge, 2005). In 1993, Selman and colleagues classified the oocytes, based on their morphology and diameter, into five stages: stage I is the primary growth; stage II is the pre-vitellogenic or cortical alveolus; stage III, at which vitellogenesis occurs, is the vitellogenic; stage IV is the oocyte maturation; and the final stage (V) is the mature egg (Selman et al., 1993). The progress time during which follicle synthesis of the previously mentioned stages occurs in adult females is around 10 days (Wang, Y., Ge, W., 2004b). This certainly makes female zebrafish a remarkable model to study follicle growth and function.

1.1.2 Ovarian follicles formation and steroid production in zebrafish

In the peritoneal cavity of the zebrafish body, the dorsolateral lining is present, producing germinal ridges where teleost gonads originate (Connaughton and Aida, 1999). From these ridges, germ cells migrate to the germinal epithelium of the ovaries, which are not fully mature and still being developed (Connaughton and Aida, 1999). Gametes develop from the primordial germ cells (PGCs), which are considered as a precursor of germ cells that never die (Farrell, 2011), and contain germ plasma enclosing genetic molecules of proteins and RNA (Raz, 2003; Yoshizaki et al., 2002). Northern blotting experiments have shown a protein localizing in germ plasma, which is accepted as a well-known molecular marker for germ cells, called vasa protein (Yoon et al., 1997).

In the very early stages of embryonic development and before gastrulation, PGCs are created and later migrate to the putative gonads (Raz, 2003; Yoshizaki et al., 2002; Yoon et al., 1997). One of the factors that substantially disturb gonadal function if it occurs abnormally is PGCs migration, which emphasizes the importance of this step (Knaut et al., 2003). For instance, if the stromal cell-derived factor 1a (Sdf1a) managing the PGCs migration is altered by a mutation in the gene (*cxcl12a*) from which Sdf1a is encoded, PGCs movement and endurance would fail (Knaut et al., 2003).

21 to 24 days post fertilization, gonadal differentiation and the gender is based on the transformation of the juvenile ovary to either follicles or testes (Slanchev et al., 2005; Brion et al., 2004). However, both males and females will reach sexual maturity in about 90 days (von Hofsten and Olsson, 2005).

In females, estrogen production is stimulated when FSH, which is released from the pituitary, binds to its receptors existing in the theca cells plasma membrane. Hence, in those theca cells, cholesterol transmutes into testosterone after a sequence of chemical catalysis of enzymes. First, Steroidogenic acute regulatory protein (Star), "the key regulator of steroidogenesis", is in charge of cholesterol movement into the mitochondria (Ings and Van Der Kraak, 2006), after which cholesterol is catalyzed by Cyp11a1 (P450scc) into pregnenolone (Nagahama et al., 19O95a and Nagahama et al., 1995b). This first step has been described as "the rate-limiting step" (Nagahama et al., 1995a and Nagahama et al., 1995b). Pregnenolone is then catalyzed by 3β-hydroxysteroid-dehydrogenase (3β-HSD), transforming into progesterone. After that, progesterone is converted to 17α-hydroxyprogesterone by the action of 17α-hydroxylase, and consequently, 17α-hydroxyprogesterone is transmuted to androstenedione by the action of 17,20 lyase. The final step in the theca cells is the conversion of androstenedione to testosterone by the

enzymatic activity of 17β-hydroxysteroid-dehydrogenase (17β-HSD). Later, granulosa cells uptake the released testosterone from the theca cells, aromatizing it by aromatase (P450Arom or Cyp19a) to 17β-estradiol (Clelland and Peng, 2009). Research has documented that gonadal enzymes-encoding genes in zebrafish (Ings and Van Der Kraak, 2006) and biosynthesis of steroids in the female zebrafish ovary is in accordance with the pathway in other vertebrates (Clelland and Peng, 2009).

In general, the role of steroids within the context of reproduction in zebrafish and other vertebrates including mammals is similar and with little difference. In both cases, before ovulation, estradiol levels go up (Busby, et al., 2010). Plus, estradiol affects the brain and pituitary through the negative feedback mechanism, and it regulates vitellogenesis and yolk incorporation in the liver (Busby, et al., 2010; Levi et al., 2009). What does not occur in mammals but occurs in fish is the biological event right before the oocyte maturation. In other words, estradiol concentration declines and 17alpha,20beta-dihydroxy-4-pregnen-3-one (DHP) level, the maturation inducing hormone, increases (Clelland et al., 2009; Thomas et al., 2004), stimulating the resumption of meiosis which is characterized by the germinal vesicle breakdown, chromosomal condensation, and the first polar body (Nagahama & Yamashita, 2008).

1.1.3 Gonadotropin releasing hormone

In fish, maintaining both the ovary and testes and the regulation of their hormones is mainly dependent on the gonadotropin releasing hormone (GnRH), which in turn triggers the release of pituitary FSH and LH to control gonadal function via the hypothalamus-pituitary-gonads (HPG) axis (Holland et al., 2001 and Moles et al., 2007). Accordingly, the feedback mechanism from gonads highlights the expression of GnRH and its production,

which consequently influences FSH and LH, as well as steroids (Schreibman and Magliulo-Cepriano, 1999; Peter et al., 1990). Interestingly, teleosts do not possess portal system in their adenohypophysis, but instead exhibit anatomically that the pituitary is innervated with neuron terminals, producing hormones (e.g. GnRH), coming from the hypothalamus and residing close to the intended endocrine cell (Schreibman and Magliulo-Cepriano, 1999; Peter et al., 1990).

Structurally, GnRH is composed of ten amino acids, a decapeptide, showing a conservation of four different residues, 1, 4, 9 and 10, in fish and mammals (Somoza et al., 2002). In a variety of fish, molecular studies have indicated that the hypothalamus and pituitary have nine or more isoforms of GnRH (Somoza et al., 2002), of which two or more forms of GnRH have been found in all vertebrates' brains (Somoza et al., 2002 and Canosa et al., 2007). The first one is chicken GnRH, or cGnRH, which generally exists in all vertebrates, implicated in the sexual behavior function, and expressed by the neurons located in the midbrain (Somoza et al., 2002 and Canosa et al., 2007). The second one is specific to a given species, playing a vital part to regulating the endocrine activity, and is expressed in the preoptic area and pituitary (Somoza et al., 2002).

In zebrafish, in situ hybridization and expression studies have detected the expression of zebrafish GnRH (zfGnRHII) and its localization in the midbrain (Steven et al., 2003 and Kuo et al., 2005). However, salmon GnRH (sGnRH/GnRHIII) has shown to localize in the olfactory bulb nerve terminus, migrating to the ventral telencephalon preoptic area (POA) and finally to the pituitary to elicit its function (Abraham et al., 2008). Being encoded by two different genes, each of the previous peptides is situated in dissimilar regions of the brain (Nancy et al., 2005). Two types of GnRH receptors have been expressed in fish such as the goldfish, medaka, European sea bass, and zebrafish

(Moles et al., 2007, Somoza et al., 2002, Canosa et al., 2007 and Lethimonier et al., 2004). The genes of these receptors are also expressed in the gonads and their levels of expression rely on the gender and reproductive phase (Levavi-Sivan et al., 2006 and Mathews et al., 2002).

1.1.4 Gonadotropin hormones

Several histochemical trials have been conducted to purify and localize GTHs in teleosts until they were chemically and architecturally described in these species (Levavi-Sivan et al., 2010). Thus, GTHs are heterodimeric glycoproteins, belong to the cysteine knot protein family, and consist of two subunits linked together by a non-covalent bond (Hearn and Gomme, 2000). Accordingly, they are formed as a common α subunit linking to a specific β subunit, which in turn gives the gonadotropin its definite biological activity (Hearn and Gomme, 2000). In situ hybridization techniques in zebrafish showed that GTHs are expressed in two different types of pituitary cells (So et al. 2005). The GTHs are then released and act on the gonads to critically and differentially control spermatogenesis or folliculogenesis (Ge W, 2005). This scenario of regulation by the GTHs is not only mastered by GnRH, but is also influenced by neurohormones such as dopamine, kisspeptin, norepinephrine, GABA, leptin, NPY, PACAP, and ghrelin (Levavi-Sivan et al., 2010). Likewise, gonadal steroids and peptides act through the feedback mode, either on the hypothalamus or pituitary, to regulate GTHs production at the hypothalamus-pituitary level (Kobayashi and Stacey, 1990; De Leeuw et al., 1986b; Khan et al., 1999; Senthilkumaran and Joy, 1996; Bommelaer et al., 1981; Larsen and Swanson, 1997).

2.1 Gonadotropin inhibitory hormone

GnIH peptides possess C-terminus with LPXRFamid (X = L or Q), which has now been characterized as an integral part of the HPG axis in many vertebrates (Tsutsui et al., 2013). This neuropeptide was identified as an inhibitor of gonadotropin production when it was first discovered in quail, exhibiting a similar physiological mode of action in mammals, but showing interestingly variable effects in fish (Tsutsui et al., 2012).

2.1.1 History of RF-amide as neuropeptides

In 1970s, the first RFamide peptides, involving Arg-Phe-NH2 motif, were isolated from the venus clam (Macrocallista nimbosa) ganglia (Price et al., 1977). Later on, a number of RFamide peptides were also found in other invertebrates, functioning as neuromodulators and peripheral hormones. They were later found with the aid of histochemical techniques in a network of nerve cells associated with the pituitary in vertebrates (Tsutsui et al., 2012).

2.1.2 Discovery of GnIH orthologs in vertebrates

Following the detection of RFamide peptides in invertebrates, and equally in vertebrates, results from immunohistochemical analysis anticipated the presence of a similar sequence in the hypothalamus that controls the production of anterior pituitary hormones (Raffa et al., 1988; Rastogi et al., 2001). Accordingly, Tsutsui and his group investigated the existence of an RFamide peptide sequence in the brain of Japanese quail and astonishingly discovered a novel sequence, Ser–Ile–Lys–Pro–Ser–Ala– Tyr–Leu–Pro–Leu–Arg–Phe–NH2 (SIKPSAYLPLRFamide), which was found to apparently obstruct the release of gonadotropin from the quail's pituitary (Tsutsui et al., 2000). Based on this

characterized function, it was named as GnIH (Tsutsui et al., 2000). GnIH cell bodies are detected in the paraventricular nucleus (PVN), whereas the terminals of the neuropeptide neurons are located in the median eminence (ME) (Tsutsui et al., 2000). Consequently, GnIH precursor-encoding cDNA was identified in several avian species including chickens, sparrows, starlings, and zebra finches (Tsutsui, 2009; Tsutsui et al., 2010a; Tsutsui et al., 2010b).

Thereafter, RFamide peptides (avian RFamide peptides) in addition to GnIH related peptides (GnIH-RPs), both with a common C-terminal LPXRFamide (X = L or Q) motif, were discovered in mammals including humans, and in primates (reviewed in Tsutsui, 2009; Tsutsui et al., 2015; Tsutsui et al., 2010a; Tsutsui et al., 2010b; Tsutsui et al., 2012; Tsutsui et al., 2013). Hence, GnIH in mammals and primates is also called RFamide related peptide 1 and 3 (RFRP-1 and -3). Several studies have revealed similar inhibitory effects by either avian GnIH or RFRP-1 and RFRP -3 on the gonadotropin release in various mammalian species (Kriegsfeld et al., 2006; Ubuka et al., 2012; Johnson et al., 2007; Murakami et al., 2008; Rizwan et al., 2009).

In amphibians, GnIH peptide has been characterized in the bullfrog and is known as amphibian GnIH or frog GH-releasing peptide (fGRP), based on its biological effect on the growth hormone in these species (Koda et al. 2002). Molecular studies later detected three more fGRP-RPs (Ukena et al., 2003). RFa peptide carrying LPXRFa motif was also predicted in the European green frog (Chartrel et al., 2002), and four RFa sequences were found by the means of High-Performance Liquid Chromatography (HPLC) in the newt brain (Chowdhury et al., 2011).

In fish, the goldfish (Carassius auratus) was the first fish discovered to have LPXRFNH2 motif peptide (SGTGLSATLPQRF-NH2) (Sawada et al., 2002b), and since

then, it has been known as gfGnIH (Moussavi et al., 2012, 2013). Next, GnIH homologous has been found in zebrafish and in many other teleosts, such as medaka, Takifugu, Tetraodon and stickleback (Zhang et al., 2010). In zebrafish, just like goldfish and medaka, three putative LPXRFamide (X = L or Q) peptide sequences have been found in the GnIH precursor polypeptides (Zhang et al., 2010; reviewed in Ogawa & Parhar, 2014). GnIH sequences, their related peptides, and their used nomenclature are listed in Table 1.1 (modified form Tsutsui et al., 2012). Various GnIH actions in different animals are reported in Table 1.2 (modified from Tsutsui et al., 2012).

2.1.3 Distribution of GnIH and its related peptides in the brain of vertebrates

GnIH localization was primarily determined when it was discovered in quail, as discussed in the previous section. In birds, cell bodies of GnIH neurons assemble mostly in the region of PVN, stretching out to the ME (Tsutsui et al., 2000; Osugi et al., 2004; Bentley et al., 2003; Ukena et al., 2003). This hypothalamic GnIH in Rufous-winged sparrows, nevertheless, does not exist in the ME (Small et al., 2008). Besides, GnIH fibers appear to accumulate in the diencephalic and mesencephalic areas of the nervous system (Ogawa1 and Parhar, 2014).

In mammals, the dorsomedial nucleus of the rodent hypothalamus (DMH) has appreciated distribution of GnIH neural peptide, expanding to both sides of the thalamus (limbic system) (Kriegsfeld et al., 2006). Looking at the abundance of GnIH in the ovine species brain, it has been observed considerably within the DMH, PVN, and the mediobasal hypothalamus (Clarke et al., 2008). An interesting study which emerged in 2014 showed that the previous immunohistochemistry analysis was not able to detect GnIH neurons existing in the ventromedial hypothalamus (VMH) in mammals (Soga et al., 2014). In this

study, EGFP-positive neurons were found in the VMH by employing transgenic rats with an enhanced green fluorescent protein (EGFP) tagged to the GnIH promoter (Soga et al., 2014). A suggesting role of GnIH on the pituitary gland was implied after its detection in the ME outer layer (Ubuka et al. 2009b; Clarke et al., 2008; Gibson et al., 2008). In ewes, the active presence of GnRH in the hypophyseal portal blood was accepted as evidence to strengthen the proposed role of GnIH (Smith et al., 2012). Conversely, in some species including Wistar rats (Rizwan et al., 2009) and hamsters (Ubuka et al., 2012), ME did not show any immunoreactive fibers of GnIH in the brain.

In amphibians, GnIH neurons are seen in the anterior preoptic area (POA), the suprachiasmatic nucleus (SCN), and the dorsal and ventral hypothalamic nuclei in the hypothalamus of the European green frog (Chartrel et al., 2002). Additionally, the areas from the olfactory bulb to the brainstem show a high population of GnIH fibers with some distribution in the ME external layer (Chartrel et al., 2002). Furthermore, in the telencephalon and the diencephalon of the bullfrog brain, GnIH-containing neurons are particularly distributed in the medial septum, the nucleus of the diagonal band of Broca, anterior POA and the SCN (Koda et al., 2002; Chowdhury et al., 2008). The distribution of GnIH-immunoreactive fibers in the newt is in accordance with that of the frogs' brain, since they have been correspondingly accumulated in the mesencephalic, rhombencephalic structures, and ME (Chowdhury et al., 2011).

In fish such as goldfish, mRNA expression of GnIH is found using *in situ* hybridization techniques localizing in the nucleus posterior periventricular (NPPv) in the hypothalamus (Sawada et al., 2002). Likewise, similar localization of GnIH-immunoreactive cells was identified in the sockeye salmon as well as the Indian major carp, which indicates the possibility that teleosts have an equivalent distribution of hypothalamic

GnIH in common (Sawada et al., 2002; Amano et al., 2006; Biswas et al., 2015). In 2010, the distribution of *gnih* gene in zebrafish tissues was studied with the aid of RT-PCR techniques. Zhang and his group (2010) designed primers specific to *gnih* gene after cloning cDNA sequences that encode the gnih. The study highlighted the presence of GnIH in a variety of body organs including the brain, but it did not specify which brain region(s) express the Rfamide sequence (Zhang et al., 2010).

GnIH peptide was shown to diversely exist in the peripheral tissues in both mammalian and avian species including gonads, which suggests tissue specific physiological function of the GnIH (Bentley et al., 2008; McGuire and Bentley, 2010; Li et al., 2012; Anjum et al., 2012). Overall, considering the fact that GnIH gene sequences in non-mammalian vertebrates are still not very well known, GnIH distribution within this class has not been extensively characterized as it has been in mammals (Ogawa and Parhar, 2014).

2.1.4 Functions of GnIH

The presence of GnIH in the brain of vertebrates in proximity to the pituitary gland undoubtedly indicates its implication in the endocrine function. In mammals and birds, GnIH plays a physiological role on GTHs, corresponding to its name given when it was first discovered (Tsutsui et al., 2000; Kriegsfeld et al., 2006). In fish species, however, this role has been interestingly diverse (Tsutsui et al., 2012).

An inhibitory effect of GnIH is exerted on the anterior pituitary, and consequently diminishes the production of gonadotropin in both mammalian and avian species (Tsutsui et al., 2000; Kriegsfeld et al., 2006). It has been demonstrated that RFRP-3 is able to constrain GTHs synthesis, as well as production, in numerous mammals. Similar influence

by RFRP-1 was seen in hamsters (Ubuka et al., 2012). *In vivo* and *in vitro* studies carried out using rats as a model showed that GnIH, at a specific dose, decreased the circulating LH and inhibited GTH release from the cultured pituitary, respectively (Pineda et al., 2010). Suppression of GTH production was again observed in cattle (Kadokawa et al., 2009), ewes (Smith et al., 2012), and pigs (Li et al., 2013).

In teleosts, the role of GnIH was first confirmed in the pituitary culture that was derived from sockeye salmon treated with goldfish LPXRFa peptides (goldfish LPXRFa-1, -2, and -3 peptides), stimulating the GTHs and GH production as a result (Amano et al., 2006). Similarly, but *in vivo,* treatment of the goldfish with GnIH substantially elevated the pituitary gene expression of GTHs, which was found to be dependent on the reproductive stage (Moussavi et al., 2012). Also, when the grass puffer was treated with the gfLPXRFa-1, mRNA abundance of the FSHβ and LHβ was considerably increased (Shahjahan et al., 2011). *In vivo* and *in vitro* studies have shown stimulation effects of GnIH on GTHs release in tilapia (Biran et al., 2014). On the other hand, within the goldfish itself, GnIH inhibited the circulatory LH in the early to later stages of recrudescence (Moussavi et al., 2013; Moussavi et al., 2012). In a similar manner, levels of plasma LH dropped when the goldfish was intraperitoneally treated with zebrafish GnIH (LPXRFa-3) (Zhang et al., 2010; reviewed in Ubuka et al., 2016). Another study used the goldfish and looked at the influence of gfGnIH-II and gfGnIH-III *in vivo* and *in vitro* (Qi et al., 2013b), in which intraperitoneal administration of both peptides reduced the transcription abundance of FSHβ in vivo, while it did not change the production of gonadotropin *in vitro* (Qi et al., 2013b). However, when the pituitary of goldfish was cultured and treated with gfGnIH-III, it did inhibit the GnRH and positively stimulated the synthesis of GTHs (Qi et al., 2013a). To sum up, whereas GnIH action can be regulated by the GnRH in mammals and birds

(Osugi et al., 2004; Rizwan et al., 2012), though it has indicated similar functions, its role in fish species tends to be more influenced by the reproductive stage and accordingly modulated.

2.1.5 GnIH receptors

Investigating the GnIH receptor has helped further understand how GnIH elicits its physiological effects in vertebrates. GnIH receptors have been classified as seven transmembrane G protein-coupled receptors, which are GPR147 and GPR74, which are identified in vertebrates. GnIH-Rs are detected in quail (Yin et al., 2005), chicken (Ikemoto et al., 2005), humans and rats (Bonini et al., 2000), sheep (Dardente et al., 2008), grass puffers (Shahjahan et al., 2011), tilapia (Biran et al., 2014), goldfish (Moussavi et al., 2013), and zebrafish (referred to as GPR147) (Zhang et al., 2010). In quail, the expression of GnIH-R transcription was found in several areas of the brain including the cerebrum, diencephalon, mesencephalon, and the spinal cord (Yin et al., 2005). In the brain of zebrafish, RT-PCR has found three GnIH-Rs (GnIH-R1, GnIH-R2, and GnIH-R3), of which two receptors genes (GnIH-R1 and GnIH-R3) are expressed in the pituitary (Zhang et al., 2010). To date, there has been no study that has provided information regarding the precise distribution of either GnIH or GnIH-Rs in the zebrafish brain.

In humans, RT-PCR was able to detect the presence of GnIH-R (GPR147)-expressing gene in the hypothalamus and pituitary (Ubuka et al., 2009b). Besides, *in situ* hybridization methods have localized the expression of GnIH-Rs in the LH-producing cells (Ubuka et al., 2009b).

The expression of GnIH-Rs has not been found only in the brain, but also in multiple organs including the gonads of vertebrates (Ubuka et al., 2013), by applying the

techniques of immunohistochemistry, *in situ* hybridization, and RT-PCR (Bentley et al., 2010; Tsutsui et al., 2010). Thus, GnIH reveals its contribution in the regulation of ovarian and testicular functions (Zhao et al., 2010; Ubuka et al., 2014). In goldfish (*Carassius auratus*), the expression of two of the GnIH-Rs (*gnrh1* and *gnrh2)* has been determined in both male and female gonads (Qi et al., 2013b). Furthermore, Biran and colleagues (2014) were recently able to clone both GnIH and GnIH-R in tilapia. In contrast, neither GnIH nor its receptor was detected in the grass puffer gonads (Shahjahan et al., 2011). In the female goldfish, GnIH-Rs were found to be associated with the ovarian stage that is before the cortical alveolus phase, whereas they are expressed in the interstitial tissue of the male goldfish (Qi et al., 2013b). In the testis of zebrafish, GnIH and three GnIH receptor genes (GnIh-R1, GnIH-R2, and GnIH-R3) have been detected, whereas in the ovary, only one GnIH-R (GnIH-R3) has been identified along with the GnIH (Zhang et al., 2010).

2.2.5 Gonadal effects of GnIH

In goldfish, sexual dimorphism effects of both gfLPXRFa-2 and gfLPXRFa-3 peptides were observed on cultured testis and ovary. As a result, GnIH was capable of remarkably stimulating the expression of steroidogenic enzymes implicated in the pathway of testosterone synthesis, but inhibiting the conversion of testosterone to estradiol by obstructing the aromatization activity (Qi et al., 2013b; reviewed in Ubuka et al., 2016). Up until the present, nothing has been known regarding the physiological effect of gonadal GnIH in the zebrafish testis and ovary, and how gonadal steroids, enzymes, and subsequent development would locally respond to the RFa peptide. As mentioned before (section 2.1.4), the only physiological action reported regarding zebrafish GnIH was in goldfish in

vivo, the literature has not provided any role of this peptide in Danio rerio (reviewed in Ubuka et al., 2016).

1.3 Hypotheses and Experimental Approach/Design

Based on previous analysis of gonadal gene expression, GnIH and GnIH-R were found to be highly expressed in the zebrafish testis and ovary, suggesting a possible paracrine/autocrine role in gonadal steroidogenesis, spermatogenesis, and ovarian function (Zhang et al., 2010). However, it is not yet known what the exact biological function of GnIH in zebrafish gonads is. Furthermore, recently, *in vitro* findings carried out by Moussavi have shown a significant interaction between gfGnIH and hCG, affecting the expression of vital genes including FSH-R, LH-R, cyp19a, STAR, and activin βA in goldfish gonads (2013). Also, having shown its availability, most studies have used the mammalian gonadotropin human chorionic gonadotropin (hCG) in zebrafish to study the substantial effect of gonadotropins on ovarian and testicular tissue development, and its function *in vivo* and *in vitro*. Hence, due to evidence confirming the local presence of GnIH and GnIHR in the reproductive organs of zebrafish, and seeing that my research focuses on ovarian function, my main hypothesis is that GnIH plays a paracrine/autocrine role in the control of ovarian function in adult zebrafish. Emphasis will be placed on the expression of crucial sex genes in the ovarian follicles and steroid production.

To test the above-mentioned hypothesis, I will use adult zebrafish, which has been an excellent and well-characterized model for the study of vertebrate development. Moreover, these aquatic organisms have the potential to produce eggs anytime during the year, and their embryos take three to four months until they reach sexual maturity and become adult individuals. In 2010, Zhang and his colleagues were able to detect the mRNA signals of

GnIH and its receptors in zebrafish gonads. Based on this information, I investigated tissue-specific expression of GTHR mRNA (FSH-R &LH-R) and steroidogenic genes, and explored estrogen production under the control of GnIH. In general, RT-PCR and ELISA techniques were conducted to test the hypothesis and achieve the following objectives:

Objective 1: Study the effects of GnIH alone or with hCG on the ovarian estradiol release, and on basal and hCG-induced resumption of meiosis *in vitro.*

Objective 2: Examine the effects of GnIH on basal and hCG-induced mRNA expression of ovarian genes involved in gonadal development (STAR, CYP19a/aromatase, activin-beta A, inhibin, 17b-HSD3, and 17b-HSD1 mRNA), in addition to FSH-R, LH-R, zfGnIH, and zfGnIH-R3 *in vitro*

Objective 3: Study the effects of GnIH on 17β-estradiol-induced response of mRNA expression of ovarian genes involved in reproduction (FSH-R, LH-R, CYP19a/aromatase, activin-beta A) (feedback loop on the gonad) *in vitro.*

Basically, I designed *in vitro* experiments to investigate the dose-related effects of zfGnIH on hCG-induced changes in the gene expression of the female zebrafish gonads. Thus, I dissected out the gonads, incubated them in the L-15 media, and treated them with zGnIH alone or with hCG. Another set of experiments were done in a similar way but follicles were treated differently (with zfGnIH alone or with 17β-Estradiol). I used the Qualitative Real Time PCR (QPCR) to measure the expression levels of steroidogenic genes, and values were normalized to a suitable housekeeping gene (beta-actin). I also measured the steroid's release under increasing concentrations of GnIH alone, hCG alone, or with a co-treatment of GnIH and hCG, and I kept the media for estradiol quantification using ELISA. Further more, I looked at the zebrafish oogenesis (resumption of meiosis) by

treating the fully grow oocytes with hCG alone, zfGnIH alone, gfGnIH alone, zfGnIH with hCG, or gfGnIH with hCG.

Table 1.1 the amino acid sequences of GnIH in various vertebrates

Vertebrates	Name	Amino acid Sequences	References
Human	RFRP-1	MPHSFANLPLRFa	Ubuka et al., 2009b
	RFRP-3	VPNLPQRFa	
Macaque	RFRP-1	MPHSVTNLPLRFa	Ubuka et al., 2009a
	RFRP-3	SGRNMEVSLVRQVLNLPQRFa	
Bovine	RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLP LRFa	Fukusumi et al., 2001
	RFRP-3	AMAHLPLRLGKNREDSLSRWVPNLPQRFa	Yoshida et al., 2003
Ovine	RFRP-1	SLTFEEVKDWGPKIKMNTPAVNKMPPSAANLP LRFa	Clarke et al., 2008
	RFRP-3	VPNLPQRFa	
Rat	RFRP-1	SVTFQELKDWGAKKDIKMSPAPANKVPHSAA NLPLRFa	Ukena et al., 2002
	RFRP-3	ANMEAGTMSHFPSLPQRFa	
Hamster	RFRP-1	SPAPANKVPHSAANLPLRFa	Ubuka et al., 2011
	RFRP-3	TLSRVPSLPQRFa	
Quail	GnIH	SIKPSAYLPLRFa	Tsutsui et al., 2000
	GnIH-RP-1	SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVA NLPLRFa	Satake et al., 2001
	GnIH-RP-2	SSIQSLLNLPQRFa	
Chicken	GnIH	SIRPSAYLPLRFa	Ikemoto et al., 2005
	GnIH-RP-1	SLNFEEMKDWGSKNFLKVNTPTVNKVPNSVA NLPLRFa	
	GnIH-RP-2	SSIQSLLNLPQRFa	
Sparrow	GnIH	SIKPFSNLPLRFa	Osugi et al., 2004
	GnIH-RP-1	SLNFEEMEDWGSKDIIKMNPFTASKMPNSVAN LPLRFa	
	GnIH-RP-2	SPLVKGSSQSLLNLPQRFa	
Starling	GnIH	SIKPFANLPLRFa	Ubuka et al., 2008
	GnIH-RP-1	SLNFDEMEDWGSKDIIKMNPFTVSKMPNSVAN LPLRFa	

	GnIH-RP-2	GSSQSLLNLPQRFa	
Zebra finch	GnIH	SIKPFSNLPLRFa	Tobari et al., 2010
	GnIH-RP-1	SLNFEEMEDWRSKDIIKMNPFAASKMPNSVANLPLRFa	
	GnIH-RP-2	SPLVKGSSQSLLNLPQRFa	
Frog	fGRP/R-RFa	SLKPAANLPLRFa	Koda et al., 2002; Chartrel et al., 2002
	fGRP-RP-1	SIPNLPQRFa	Ukena et al., 2003b
	fGRP-RP-2	YLSGKTKVQSMANLPQRFa	
	fGRP-RP-3	AQYTNHFVHSLDTLPLRFa	
Newt	nLPXRFa-1	SVPNLPQRFa	Chowdhury et al., 2011
	nLPXRFa-2	MPHASANLPLRFa	
	nLPXRFa-3	SIQPLANLPQRFa	
	nLPXRFa-4	APSAGQFIQTLANLPQRFa	Sawada et al., 2002
Goldfish	gfLPXRFa-1	PTHLHANLPLRFa	
	gfLPXRFa-2	AKSNINLPQRFa	
	gfLPXRFa-3	SGTGLSATLPQRFa	
Zebrafish	LPXRFa-1	SLEIQDFTLNVAPTSGGASSPTILRLHPIIPKPAHLHANLPL	Zhanga et al. (2010)
	LPXRFa-2	APKSTINLPQRFa	
	LPXRFa-3	SGTGPSATLPQRFa	

Table 1.2 The physiological effects of GnIH peptide in several species

Vertebrates	Name	Peptide effect	Mode of administration	References
Bovine	RFRP-3	Inhibition of GnRH-elicited LH release	i*n vitro*	Kadokawa et al., 2009
		Inhibition of LH	iv	
Ovine	RFRP-3	Inhibition of GnRH-elicited FSH & LH release	*in vitro*	Clerke et al., 2008
		Inhibition of LH release - no effect on FSH	iv	
		Inhibition of GnRH- elicited FSH & LH release	*in vitro*	Sari et al., 2009
Rat	RFRP-3	Inhibition of LH – no effect on FSH	i.c.v	Johnson et al., 2007
		Inhibition of LH	iv	Murakami et al., 2008
		Inhibition of GnRH-elicited LH release	*in vitro*	
Hamster	RFRP-1	Inhibition of LH	icv	Ubuka et al., 2011
	RFRP-3	Inhibition of LH	icv	
		Inhibition of LH	ip	Kriegsfeld et al., 2006
Quail	GnIH	Inhibition of LH	*in vitro*	Tsutsui et al., 2000

		Inhibition of LH synthesis and release-no effect on FSH	ip	Ubuka et al., 2006
Chicken	GnIH	Inhibition of FSH & LH synthesis and release	*in vitro*	Ciccone et al., 2004
Sparrow	GnIH	Inhibition of GnRH-elicited LH release	iv	Osugi et al., 2004
		Inhibition of LH	iv	
		Inhibition of LH	icv	Bentley et al., 2006
Goldfish	gfLPXRFa-1	Stimulation of FSH & LH release	in vitro	Amano et al., 2006
	gfLPXRFa-2	Stimulation of FSH & LH	*in vitro*	
	gfLPXRFa-3	Stimulation of FSH & LH	*in vitro*	
Zebrafish	zfLPXRFa-3	Inhibition of LH	ip	Zhang et al., 2010

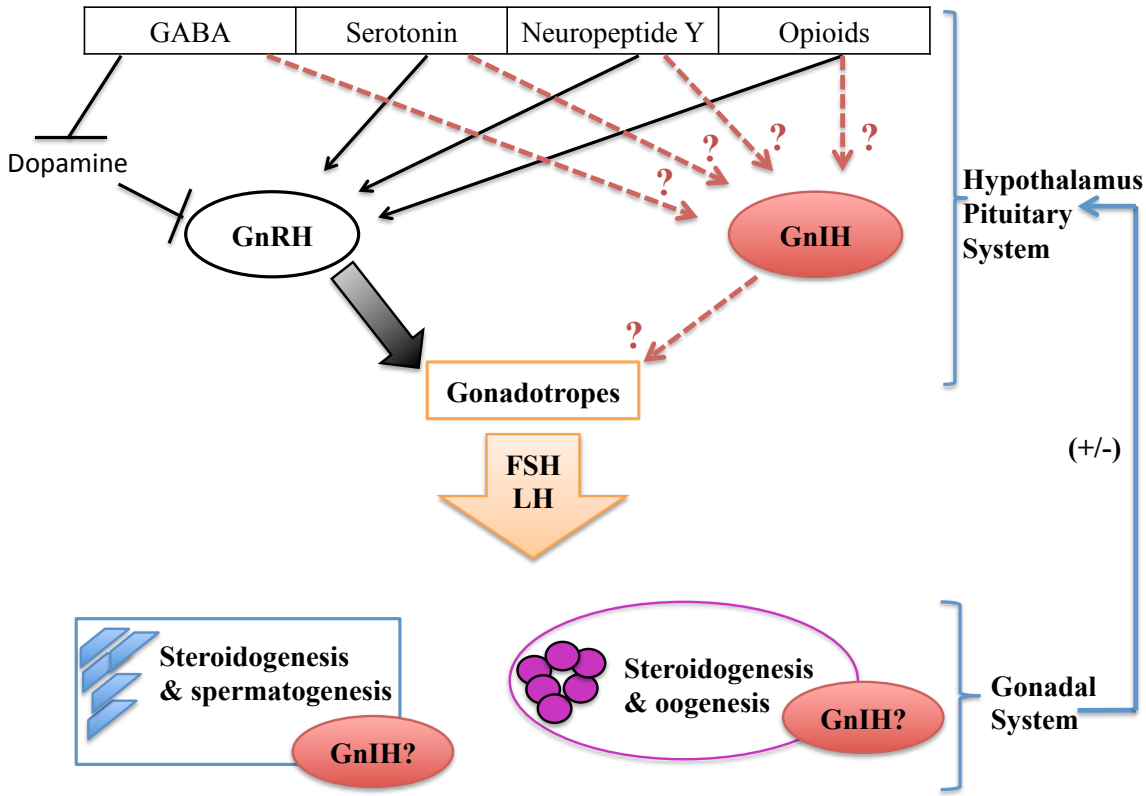


Figure 1.1 Hypothesized model of GnIH function in zebrafish with unknown paracrine/autocrine effect on gonadal steroids, gametogenesis and gene expression.

Chapter 2

Materials and Methods

2.1 Hormones

zfGnIH (SGTGPSATLPQRF-NH_2) was made by SynPeptide Co., Ltd (Beicai pudong new area, Shanghai, China) while gGnIH (SGTGLSATLPQRF-NH_2) was synthesized at the University of Calgary's Peptide Services (Calgary, AB, Canada). hCG was purchased from Sigma Aldrich Canada (Oakville, ON, Canada). A stock solution of zGnIH was constituted in Acetic Acid (0.1M), whereas hCG was dissolved in Ultrapure water purchased from Invitrogen (Burlington, ON, Canada). Then, both solutions were stored at -20°C as aliquots until used. The required concentration of hormones was dissolved prior to each experiment in the culture media.

2.2 Animals

Based on the Canadian Council for Animal Care Guidelines, Tupfel Longfin (TL) zebrafish were maintained and managed. Adult fish were bred in a way that breeding groups involving a ratio of three females to two males were moved to breeding trays in the late afternoon. Then, the next morning and after spawning, fertilized eggs were collected using a mesh strainer. In order to keep embryos' integrity from fungal growth, autoclaved recirculating water system was used to rear embryos in 10 cm culture dishes at 28 °C. Water was replaced on a daily basis with freshly autoclaved water to remove dead embryos and unfertilized eggs. Four days following harvesting, larvae were moved to tanks filled with 3L system water and provided with yellow baby baffle. Larvae were daily fed Ziegler larval diet (0.5g/125ml fish water) for a tank, and brine shrimp was added to their diet when they are 9 days old. Along with this, cleaning tanks was taken place to remove debris via exchanging 1/3 total water. Larvae were moved to the recirculating system, which has a good quality of water, on day14 with maintaining the same routine of feeding and cleaning.

On Day 21, larvae were treated as adults and adult zebrafish food had been given to them. Zebrafish were taken care of until they become adults at three to four months and then dissected for the purpose of the research objectives. The detailed nursery protocol for raising zebrafish was obtained from Dr.Vijayan's lab.

2.3 Dispersed follicles experiments

Zebrafish were anesthetized, euthanized, and their follicles were placed in Leibovitz L-15 media (Gibco, Life Technologies, Burlington, ON, Canada) containing 10,000 U penicillin/ml, 10 mg streptomycin/ml, and 25ug amphotericin B/ml. 5 mM of Sodium bicarbonate (Sigma-Aldrich Canada Co., akville, Ontario Canada) was added to the media for stability and pH was adjusted to 7.6. Follicles were dispersed by aspiration using a Pasteur pipette and separated with fine-tipped forceps.

2.4 Gene expression

2.4.1 RNA extraction and reverse transcription

Total RNA from follicles replicates was extracted using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacture's protocol. Using Nano Drop (Thermo Scientific) spectrophotometric reading at 260/280 nm as well as 260/230 nm, RNA was quantified and its quality verified. Two µg of total RNA was transcribed in a reaction volume of 40 µl to make cDNA using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). cDNA was stored at -20 °C until use for the quantitative real-time PCR.

2.4.2 Quantitative Polymerase Chain Reaction

The relative expressions of mRNA levels were determined by using quantitative real-time PCR (QPCR) on samples derived from follicular tissues. These samples were normalized against the beta-actin gene that was used as an internal control. According to the known and studied sequences in zebrafish, primers were designed and synthesized at the University of Calgary DNA Synthesis Lab. Primer efficiency testing was conducted on serially diluted cDNA to ensure specificity and reproducibility. All forward and reverse primers and their annealing temperature are listed in (Table 2.1).

QPCR amplification was performed in a CFX96 Touch™ Real-Time PCR Detection System using BIO-RAD SsoFast EvaGreen™ Supermix according to the manufacture's protocol (Bio-Rad, Hercules, CA, USA). The conditions for each reaction were as follows: 10 ul Supermix, 75 nM of each primer, 3 ul of cDNA, and 8.65 ul Ultrapure water (Life Technologies, Carlsbad, CA, USA). The protocol on QPCR machine carried out as follows: Initial activation step (95 °C, 2 minutes) for 1 cycle, followed by 40 cycles of denaturing step (95 °C, 10 seconds) and annealing or extension (primer specific temperature, 20 seconds), followed by 1 cycle of pre-melt curve denaturation (95 °C, 10 seconds) with a subsequent series of 0.5 °C incremental increases in temperature from 65 °C to 95 °C for the analysis of melting curve. In respect to the PCR plate, reactions per each sample were performed in triplicate wells. Data among triplicates were accepted with values of standard deviation of no more than 0.3.

2.5 Enzyme-Linked Immunosorbent Assay (Estradiol release)

Supernatants of follicles culture media were collected and stored in -80 °C until use. The assay performed to measure 17β-Estradiol concentrations was conducted according to the instructions provided by the kit manufacture (Enzo Life Sciences Inc., US).

2.6 Statistical analysis

The data for all experiments were subjected to One-Way ANOVA followed by post hoc Tukey's Test to recognize significant comparisons ($P<0.05$) among treatment groups using Prism 5 Statistical Software (Graphpad Software, Inc., USA). Groups were averaged, and the Standard Error of the Mean was calculated.

Table 2.1. QPCR primer sequences and annealing temperature for genes measured in the ovary.

Gene	Primer Sequence (5' – 3')	Annealing Temperature (°C)
β-Actin	F-CGAGCTGTCTTCCCATCCA	75.3
	R-TCACCAACGTAGCTGTCTTTCTG	
Aromatase A	F-TCTGCTTCAGAAGATTCATAAATACTTT	62.6
	R- CCTGCAACTCCTGAGCATCTC	
Activin.βA	F- GGAGGTTAGAGAAGAAGCAAGAG	58.1
	R- GTTCGGAGACAGCCTGATATG	
inhibin	F-AGCCCTTCGAGATCATCACCTTC	53.0
	R-GCCTGCTCCACCACTGACAG	
FSHR	F-TTTTCAACGACCACTGTAAGG	58.5
	R-GCACATTAGAAACCTGGGAAC	
LHR	F-GACGGTTCTGTCAACTCGG	58.5
	R-CAGGTCTGGATAGTGGAAGTC	
17β.HSD.1	F-GCGTGAGAGGACTGCATAAA	57.0
	R-CCGGCATTACACACCAGTAT	
17β.HSD.3	F-AAACATCGAGGGATTGGATATTGGC	62.1
	R-TGGCTTCTGATGTCCTGTCATTGC	
STAR	F-TGTGATAAACTCAGCCGACTAC	55.6
	R-TAGGAGCCAACATACCATTACAC	
zGnIH	F- TGTCCTACTTCGCTCTTCTTTC	59.2
	R- GCAATCTGAGAGCCGTAACT	

zGnIHR3	F- CCCATTTAAGCCCAAACTTACAC	57.6
	R- GCATTAGGACTGACGGAAAC	

Chapter 3

The effects of GnIH on basal and hCG-induced steroidogenesis and resumption of meiosis

1.3 Introduction

The events of oocyte growth and final maturation depend mainly on the GTHs (FSH & LH) released from the pituitary and gonadal steroidogenesis. Ovarian steroids, which are 17β- estradiol (E_2) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP/progesterone), are considered the key regulators of follicle development. In fish, the production of E_2 has been shown to be critical for ovarian growth, beginning at the early stages of development until the vitellogenic stages (Nagahama, 1994; Yoshiura et al., 2003). Yoshiura et al. (2003) demonstrated that mRNA abundance and activity of aromatase A, catalyzing the irreversible production of E_2 from testosterone, is increased after applying hCG treatment to the early vitellogenic phase of the Nile tilapia ovary *in vitro*. Similarly, a rise in E_2 release was found in follicles that had not yet entered the vitellogenic stage, and in follicles that were in the mid-stage of vitellogenesis (Nagahama, 1994, 1997). Eventually, GTHs are central hormones that trigger the initiation of steroidogenesis, by acting on theca cells which are involved in the biosynthesis of testosterone which in turn moves to the granulosa cells for the synthesis of E_2 that is specifically promoted by follicle-stimulating hormone (FSH; Nagahama, 1994, 1997; Yoshiura et al., 2003). On the other hand, the availability of 17α,20β-DP has shown to be important for the resumption of meiosis and oocyte final maturation (Senthilkumaran et al., 2002). Interestingly, it was found that there is steroidogenic shift occurs to increase either the E_2 or 17α,20β-DP depending on the ovarian requirement to undergo successful oogenesis (Senthilkumaran et al., 2002). This process of switching between the E_2 and 17α,20β-DP is influenced by cAMP (the second messenger for steroid synthesis) which later affects the vitellogenin input and oocyte maturation in fish (Senthilkumaran et al., 2004).

Since its discovery, the presence of GnIH and its receptor/s in the gonads suggests a physiological role that this peptide plays to regulate steroidogenesis in vertebrates. In teleosts such as goldfish, administering gGnIH in the female did not change the levels of serum E_2, whereas it had a significant increase on the serum T in males (Qi et al., 2013b). In female mice, GnIH was able to significantly increase the circulating E_2 and decrease the ovarian progesterone release (Singh et al., 2011). To date, there is no study that has been done elucidating the possible action of gonadal GnIH on the gonadal production of E_2 in fish *in vitro*.

In the term of oocyte maturation, the hallmark confirming oocyte final development, or the resumption of meiosis, is the germinal vesicle breakdown (GVBD). Basically, this starts with the migration of germinal vesicle to the micropylar cell, and then GVBD takes place, followed by the conversion of vitellogenin into yolk proteins, ending up with clear oocyte cytoplasm. After that, the oocyte transfers to metaphase II arresting there, it is fertilized when it is termed an egg (Khan & Thomas, 1999; Nagahama et al., 1994; Nagahama et al., 1995a; Nagahama et al., 1995b). In order to achieve the above-mentioned biological events, three processes are important; luteinizing hormone (LH) has to stimulate the maturation inducing hormone (MIH), MIH has to activate the maturation promoting factor (MPF) by its action through the progesterone membrane receptors, and finally, the oocyte needs to be freed from arrest at prophase I by MPF (Nagahama & Yamashita, 2008).

LH/hCG has been found to regulate follicular cells to produce MIH via triggering transcriptional and translational activities (Kagawa et al., 1998, Pang & Ge, 2002a, 2002b). Activin has been shown to mediate the LH effect on oocyte maturation by inhibiting smad2 which blocks the signaling pathway of activin and diminishes the

response of oocyte to hCG to achieve maturity (Tan et al., 2009). Likewise, epidermal growth factor (EGF) demonstrated its paracrine effect by enhancing hCG-induced oocyte maturation, and increasing the activin βA-encoding gene in experiments carried out using female zebrafish oocytes *in vitro* (Pang & Ge, 2002a, 2002b). On the other hand, oocyte maturation can be inhibited by several factors to control folliculogenesis. Accordingly, transforming growth factor β1 (tgfb1) and bone morphogenetic protein 15 (Bmp15), belonging to the TGF β family, were able to prevent oocyte maturation (Kohli et al., 2003) and reduce it (Clelland et al., 2006), respectively.

Maturation inducing hormone or MIH has been known as 17,20βP in species such as salmonids, cyprinids, atherinids, catfishes, or identified as 17,20β-21-trihydroxy-4-pregnen-3-one/20β-S in perciforms and flatfishes (Khan & Thomas, 1999; Nagahama et al., 1994, 1995a, 1995b). Progesterone availability is required for the completion of resumption of meiosis and oocyte final development. Here, the steroidogenesis switches from estradiol production to 17,20βP production. Two enzymes are in charge for this shift, Cyp17a2 (catalyzing progesterone to 17α-hydroxy-progesterone; Nagahama & Yamashita, 2008) and Hsd20b (producing MIH from17α-hydroxy-progesterone; Wang & Ge, 2002). Once MIH is secreted from granulosa cells, it elicits its action via progesterone receptors located in the oocyte cell membrane (oolemma) (Zhu et al., 2003a). MIH receptors have been identified in humans, pigs, mice (Zhu et al., 2003a), rainbow trout (Mourot et al., 2006), goldfish (Tokumoto et al., 2006), and zebrafish (Zhu et al., 2003b). The activity of those receptors is fundamental, as oocyte maturation failed to occur when progesterone receptors were knocked down in zebrafish (Thomas et al., 2004). As in LH-induced oocyte maturation, there has been interesting finding that showed the activation of MIH protein by activin A and inhibition via both tgfb1 and Bmp15 (Tan et al., 2009). Moreover, elevated

concentrations of estradiol have found to promote oocyte growth by sustaining elevated intracellular cAMP levels, which, as a result, inhibits maturation, explaining the shift happening in the biosynthesis of gonadal steroids to drive that oocyte for final maturation (Pang et al., 2008). Thus, cAMP is down regulated within the oocyte immediately after the progesterone receptors are activated, G_i gets stimulated, causing the release of MPF (Nagahama & Yamashita, 2008).

Different from LH- and MIH-stimulated maturation, MPF action has been revealed to influence the entire eukaryotic taxon, and induces mitotic M-phase when initiating oocyte meiosis resumption (Yamashita, 2000). Once MPF is induced, it activates the germinal vesicle dissolution, first polar body is formed, and the oocyte (egg) is ovulated (ready to be fertilized). MPF consists of both cyclin B and cdc2; cyclin B synthesis is stimulated by MIH and consequently binds to cdc2 for the formation of MPF (Nagahama & Yamashita, 2008; Yamashita, 2000).

To summarize, the previous findings demonstrated the importance of steroidogenesis and gametogenesis in mammalian and non-mammalian vertebrates. Detecting GnIH and its receptor in the gonads of zebrafish, indicates a possible autocrine/paracrine effect of this hormone, regulating steroid production and oocyte growth and maturation. In this chapter, I investigated the influence of zfGnIH on steroidogenesis and the influence of both zfGnIH and gfGnIH on the final oocyte maturation. This is the first study has been done *in vitro* looking at the local action of GnIH on E_2 release from cultured ovaries and the oocyte final maturation.

3.2 Materials and Methods

3.2.1 measurement of 17β-Estradiol release (ELISA)

17β-Estradiol was determined according to the product manual provided with the kit. Basically, goat antibody pre-coated 96-well-plate specific to rabbit IgG was used. E2 standards were prepared from the stock 300,000 pg/ml E2 and diluted in the standard diluent as gradual concentrations as follows; (30,000 pg/ml, 7,500 pg/ml, 1,875 pg/ml, 468.8 pg/ml, 117.2pg/ml, and 29.3pg/ml). 100ul of the standards were added into the suitable wells, with 50ul alkaline phosphatase (conjugate), and 50ul rabbit E2 antibody. The well showing maximal binding (Bo) had 100ul of the standard diluent, 50ul conjugate, and 50ul antibody. Non-specific binding (NSB) well used as a negative control and it was received 100ul of the standard diluent, 50ul conjugate, and 50ul antibody. 100ul of media supernatant from the treated ovarian tissues (samples preparation is described in chapter 4 section 4.2.2) were added with 50ul conjugate, 50ul antibody, and the plate was incubated at room temperature for 2h on a plate shaker. After that, by tapping the plate on a paper towel, contents in the wells were removed and all wells were washed three times with washing buffer (200ul). Then, Blank, NSB, Bo, standards, and samples wells were received only p-nitrophenyl phosphate (200ul substrate). The total activity wells (TA) had 5ul conjugate in addition to 200ul substrate. The plate was again incubated for 45 min and Stop Solution (50ul) was subsequently added to all wells to cease the reaction. The plate reader (SpectraMax i3) was used to read the optical density of the contents at 405nm.

3.2.2 Isolation and Incubation of Ovarian Follicles and Germinal Vesicle Breakdown Assay (Maturation assay).

Adult female zebrafish (15 fishes) were anesthetized, immediately and humanely killed by spinal transection. Rapidly, ovaries were dissected out and transferred to sterile 6-well-plate containing 60% of Leibovitz's L-15 media (Incubation medium). Ovaries were washed at least 3 times to remove undesired debris and unwanted tissues. After that, groups of follicles were manually separated through gentle pipetting using Pasteur pipette into smaller groups, followed by careful separation using fine forceps under the dissecting microscope. Then, healthy fully-grown pre-meiotic follicles (diameter: 580–680 mm) were selected and transferred to 24-well-plate (20 oocytes/well) for treatment and incubation. The treatment groups using zfGnIH hormone were as follows; control, hCG (10 IU), zfGnIH (10^{-8} M and 10^{-7} M), and combination treatment of both hCG and zfGnIH, having all hormones diluted in 60% L-15 media. Likewise, experiments carried out using gfGnIH contained treatment groups including hCG (10 IU), gfGnIH (10^{-8} M and 10^{-7} M), and combination treatment of both hCG and zfGnIH, with all hormones diluted in 60% L-15 media. After that, oocytes were incubated at 28° C which is the ideal temperature at which zebrafish normally grows and lives. 4 h incubation time was chosen because spontaneous maturation starts to occur within 2-3 hours of incubation in the control group. Also, 4 h post incubation is the best time to see an obvious difference between the control group and the hCG-treated group which was my positive control. Several studies used incubation time that is between 3 h and 6 h for germinal vesicle breakdown assays (Das *el al*., 2016; Chourasia *et al.,* 2015; Pang and Ge, 2002). After incubation time was terminated, oocytes that became transparent were identified as fully-grown post-meiotic oocytes, and the results were expressed according to the percentage of GVBD for the given number of oocytes.

3.2.3 Statistical analysis

For ELISA, statistical analysis was performed to generate the standard curve, E2 concentrations in the samples of interest were interpolated and values were calculated based on the standards' concentrations. One-Way-ANOVA was carried out to compare the levels of estradiol production among treatment groups followed by Tukey's' test to identify significant differences. In regard to GVBD assay, the statistical analysis was performed using un-paired Student's t-test, and One-Way-ANOVA which followed by Tukey's' test was used to find out whether there were significant differences among treatment groups.

3.3 Results

3.3.1 Effects of zfGnIH on basal and hCG-stimulated steroidogenesis

In order to investigate the paracrine/autocrine role of zfGnIH in the control of ovarian function, I examined the influence of zfGnIH *in vitro* alone and in a combination with hCG on the production of E_2 (Figure 3.1).

Treatment with zfGnIH alone had no effect on the basal levels of E_2 concentration (Figure 3.1.A). However, application of hCG alone increased the E_2 release, resulting in a significant rise at 10 IU (Figure 3.1.B). Co-treatment with 1 IU hCG stimulated the E_2 release in response to 10^{-8} M zfGnIH treatment, which indicated a statistically significant interaction between these two hormones at small doses (Figure 3.1.C). However, combination treatment of zfGnIH (10^{-8} M or 10^{-7} M) and a large dose of hCG (10 IU) did not alter the E_2 production from the ovary (Figure 3.1.D).

3.3.2 Effects of zfGnIH on basal and hCG-stimulated resumption of meiosis

To validate the incubation system of the isolated follicles, I first tested the responsiveness of follicles to hCG (positive control). hCG has a well-documented effect on

enhancing the final oocyte maturation (Li et al. 2015; Nelson &Van Der Kraak 2010a; Selman et al., 1994). Treatment with 10 IU hCG stimulated the zebrafish oocyte maturation within 4h of incubation, compared to the control group (Figure 3.2.A). To examine the involvement of zfGnIH in the oogenesis, I tested its effect on the regulation of oocyte maturation. Treatment with either 10^{-8} zfGnIH or 10^{-7} zfGnIH slightly stimulated the percentage of GVBD when compared to the control group; however, this increase was statistically non-significant (Figure 3.2.B). I also looked at the interactive effect of hCG and zfGnIH on the GVBD. Co-treatment with both hormones did not show significant effects, although it somewhat enhanced the percentage of GVBD when comparing to the groups that were treated with zfGnIH alone (Figure 3.3).

3.3.3 Effects of gfGnIH on basal and hCG-stimulated resumption of meiosis

Since I did not observe a significant effect of zfGnIH on oocyte maturation, I attempted to determine whether gfGnIH might affect ovarian development. Similar to the experiments I have done testing the zfGnIH, after first validating the responsiveness of the oocytes to hCG (section 3.3.2), I also verified it here initially with hCG (Figure 3.4.A). Then, I looked at the effect of gfGnIH. Surprisingly, the results showed that both doses of gfGnIH (10^{-8} M and 10^{-7} M) had a significant stimulatory effect on the GVBD and resumption of meiosis comparing to the control group (Figure 3.4.B). Treatment with gfGnIH alone elevated the percentage of GVBD above that observed by hCG, although this increase was not statistically significant (Figure 3.5). Moreover, I investigated the interactive effect of hCG and gfGnIH, but there was no observable action on the oocyte maturation comparing to the hCG-stimulated GVBD (Figure 3.5). Similarly, the interactive effect of both hormones on the GVBD was not significant compared to gfGnIH-induced oocyte maturation groups (Figure 3.5).

3.4 Discussion

3.4.1 Ovarian GnIH and Estradiol synthesis (steroidogenesis)

This chapter represents the *in vitro* effects of various combinations of GnIH and hCG on the steroidogenesis of adult female zebrafish ovary for the first time. In the current study, I was able to observe the action of zfGnIH on E_2 production in hCG-treated follicles. My results reveal that zfGnIH has a different effect when it is applied in a combination with hCG comparing to when it is applied alone. Interestingly, combinational treatment of lower doses of zfGnIH (10^{-8} M) with hCG (1 IU) stimulated the E_2 production when compared to the basal and hCG-caused steroidogenesis. However, application of zfGnIH treatments (10^{-8} M and 10^{-7} M) alone did not affect the basal E_2 production. Similarly, zfGnIH had no significant effect on hCG-stimulated steroidogenesis at either lower (10^{-8} M) or higher (10^{-7} M) dosages. These results indicate that zfGnIH elicits its action on ovarian steroidogenesis in a dose-dependent manner, depending on the concentration of LH/hCG affecting those ovarian follicles. In other words, these data suggest that low GnIH levels potentiate LH-induced E2 production during early stages of vitellogenesis, as fish needs E_2 to complete the the follicular growth at this stage of ovarian development, when LH levels are low. At higher levels of LH, there is no stimulatory effect of GnIH.

Comparison of the present findings with previous studies that were conducted in avian species showed discrepancies. First, culturing European starlings ovaries with GnIH (0.1 μM, 1 μM and 10 μM for 4h) reduced the basal E_2 release but it did not affect GTH (FSH/LH)-stimulated steroidogenesis (McGuire et al., 2010). Second, similarly to chickens, treating the granulosa cells with chicken GnIH alone (10^{-8} M and 10^{-6} M for 12h) significantly decreased the steroidogenic cells viability, but it had no effect on the GTH (FSH)-stimulated cellular viability (Maddineni et al., 2008). Accordingly, these results taken together suggest that GnIH is able to

diminish the E_2 production by acting on the granulosa cells altering their metabolic activity and decreasing their viability; and that this inhibitory effect is mitigated by the action of GTH. On the other hand, my findings are consistent with the idea supporting the thought of the differential regulatory mechanisms that GnIH has shown in fish species, but not in mammals or birds, which are species and sex dependent.

3.4.2 The effects of zfGnIH and gfGnIH on basal and hCG-stimulated resumption of meiosis

To further understand the role of GnIH within the context of oocyte development and final maturation, I investigated the possible dose response effects of zfGnIH, in addition to the effect of gfGnIH on oogenesis. As a result, I treated pre-meiotic fully-grown follicles with zfGnIH peptide. This stimulated the GVBD but this stimulation was not statistically significant when compared to the control group. Similarly, both doses of zfGnIH had no effect on the hCG-stimulated GVBD. Then, I investigated the possible effect of gfGnIH (10^{-8} M and 10^{-7} M) on the resumption of meiosis in fully-grown follicles. Surprisingly, both doses of gfGnIH were able to release adult female zebrafish oocytes from prophase-I and significantly stimulated the GVBD relative to the oocytes with no treatment. Further more, this increase in GVBD percentage by gfGnIH was even higher than in hCG-induced GVBD, however, it was not statistically significant. Thus, gfGnIH had no effect on the hCG-stimulated oocyte maturation. These data support the idea that the activity of GnIH in fish is not as straightforward as it has been generally inferred in higher vertebrates.

My findings are in accordance with previous results in which GnRH and GnRH analogs were used to investigate its gonadal effects on the oocyte final maturation using goldfish as an *in vitro* study model. The direct action of GnRH variants including sGnRH, cGnRH-II, lGnRH-I,

mGnRH, cGnRH-I and lGnRH-III on the oocyte development was elucidated in fully grown follicles-enclosed oocytes in goldfish (Pati & Habibi, 2000). Pati & Habibi (2000) found that all GnRH forms exerted stimulatory effects on the resumption of meiosis at all tested doses (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M), and the maximum GVBD percentage achieved at the doses 10^{-8} and 10^{-7} (lGnRH-III), and 10^{-6} M (lGnRH-I). Further more, blocking the GnRH by a specific antagonist shown to constrain ovarian extract-stimulated GVBD (Pati & Habibi, 1998).

However, what is in contrast to my findings in the previously mentioned study is that only lGnRH-III and sGnRH, among the tested peptides, prevented the GTH-stimulated oocyte maturation (Pati & Habibi, 2000). Also, in 1988, it was found for the first time that teleost GnRH agonist (10^{-7} M) inhibited the carb GTH-induced GVBD and progesterone-induced maturation in the enclosed fully grown follicles of goldfish *in vitro* (Habibi et al., 1988). To sum up, these reported data supported the hypothesis stating that GnIH plays a significant role in regulating the oocyte maturation and ovarian function.

3.5 Summary

zfGnIH has shown to regulate the steroidogenesis in cultured zebrafish follicles. Even though treatment with zfGnIH peptide had a stimulatory effect on the re-initiation of meiosis, it was statistically insignificant. Conversely, gfGnIH has shown interestingly more potent stimulatory effect than zfGnIH on the GVBD percentage in adult female zebrafish. One possibility of seeing this is that both peptides were synthesized in two different locations. We should have observed similar stimulatory influence by the peptides (zfGnIH and gfGnIH) but where they were manufactured could be a possibility correlated to the difference in their hormonal activity.

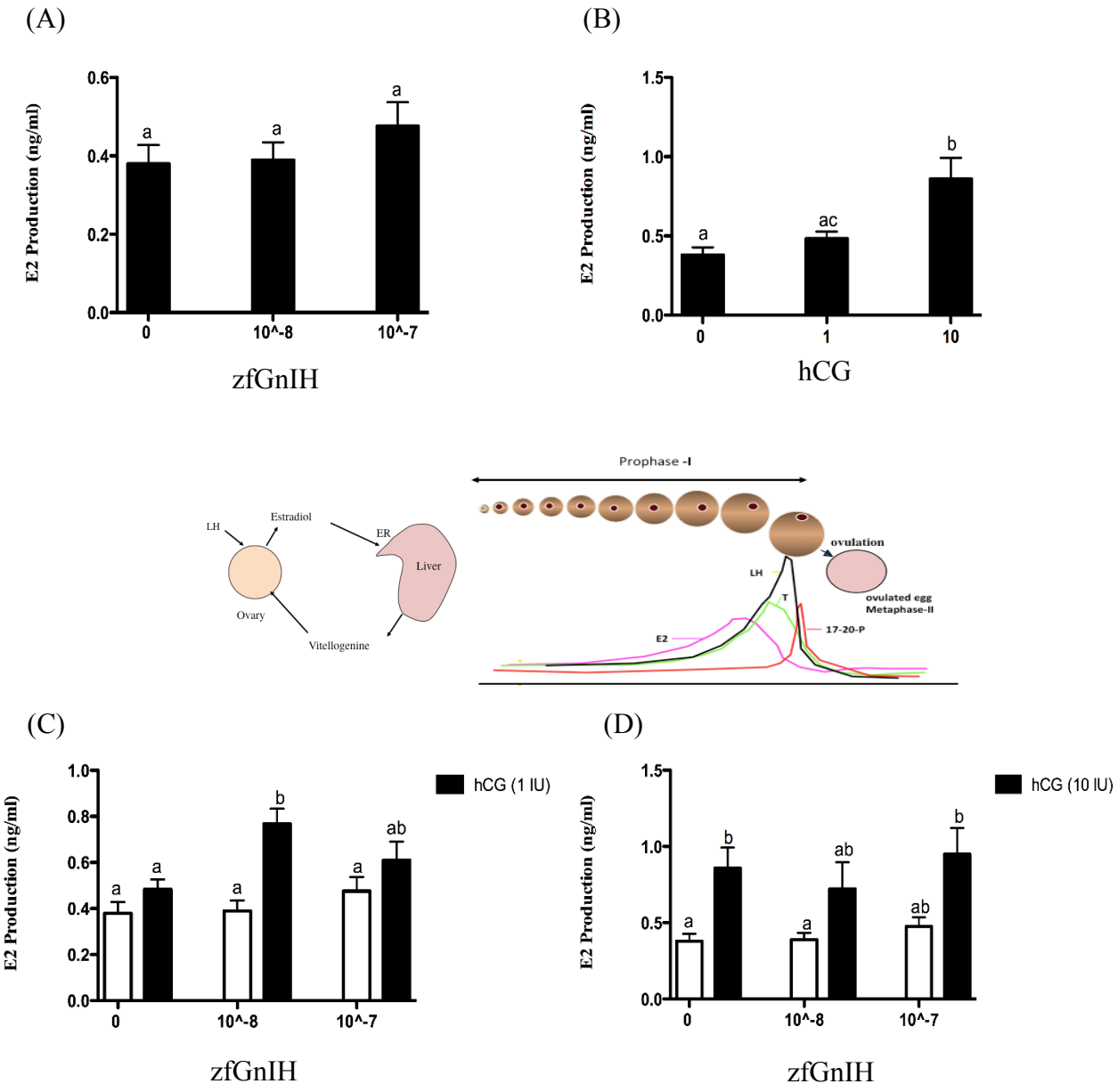


Figure 3.1 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and co-treatment of zfGnIH and hCG on the production of 17β–Estradiol (lower panel). **Values are represented as mean ± SEM (n = 12). Statistically significant differences among treatment groups are denoted with different letters. Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, P < 0.05).**

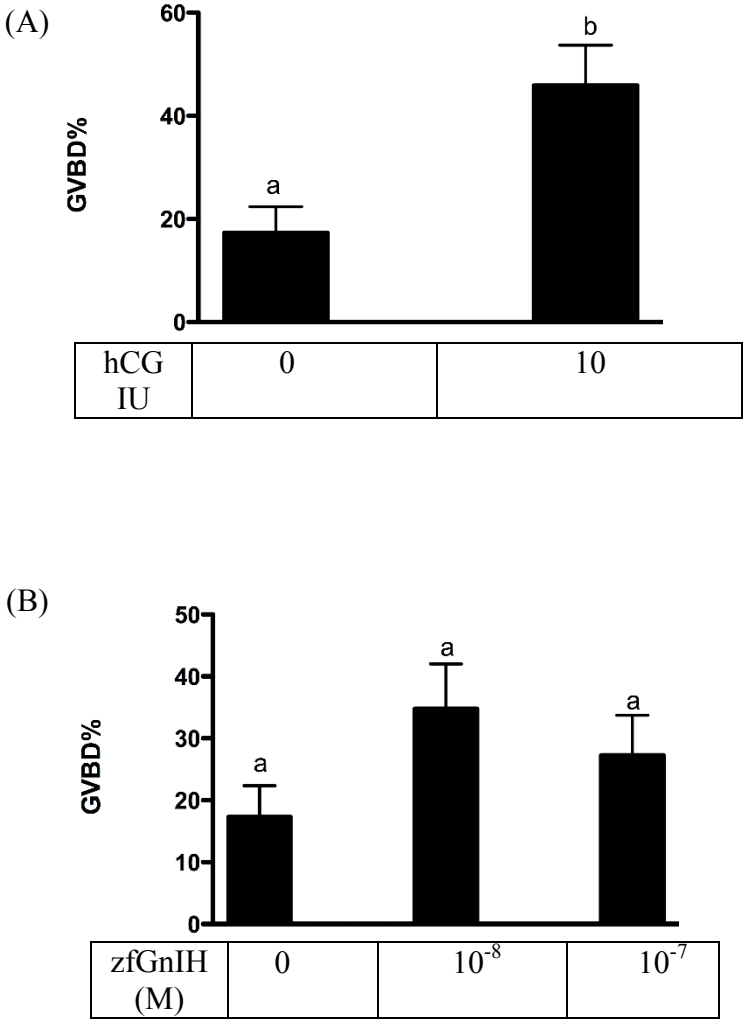
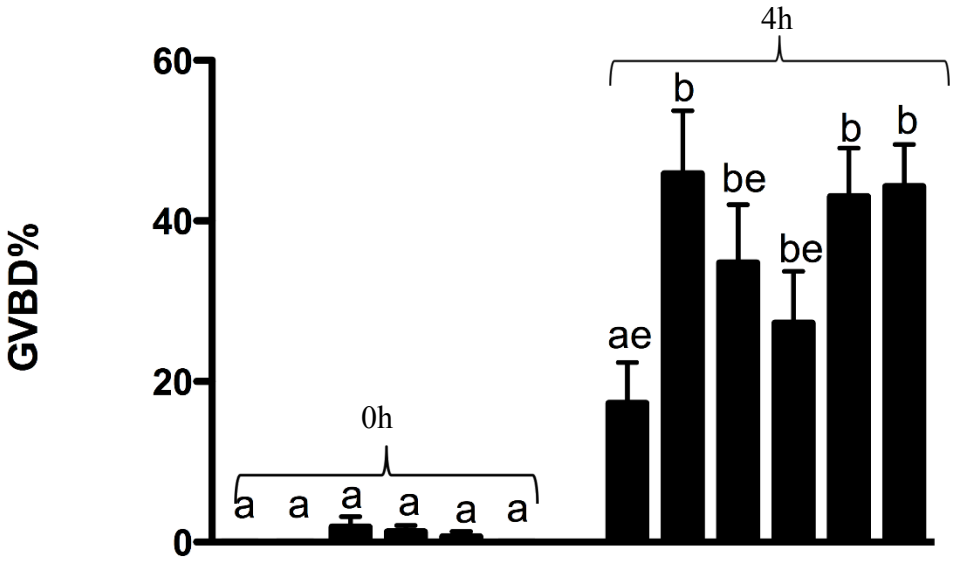


Figure 3.2 The responsiveness of zebarfish pre-meiotic fully-grown oocytes to either hCG (10 IU/ml) (upper panel) or zfGnIH (10^{-8} and 10^{-7} M) (lower panel). Statistically significant differences among treatment groups are represented with different letters. Similar letters indicate insignificant comparisons. (mean ± SEM; n = 8) (A: Student's t-test, B: ANOVA followed by Tukey's test, $P < 0.05$).



hCG (10IU)	-	-	-	-	-	-	-	+	-	-	+	+
zfGnIH (10^-8M)	-	-	-	-	-	-	-	-	+	-	+	-
zfGnIH (10^-7M)	-	-	-	-	-	-	-	-	-	+	-	+

Figure 3.3 The responsiveness of zebrafish pre-meiotic fully-grown oocytes to either hCG (10 IU/ml) or zfGnIH (10^{-8} and 10^{-7} M) alone, and combination of both hCG and zfGnIH. Statistically significant differences among treatment groups are represented with different letters. Similar letters indicate insignificant comparisons (mean ± SEM; n = 8) (ANOVA followed by Tukey's test, $P < 0.05$).

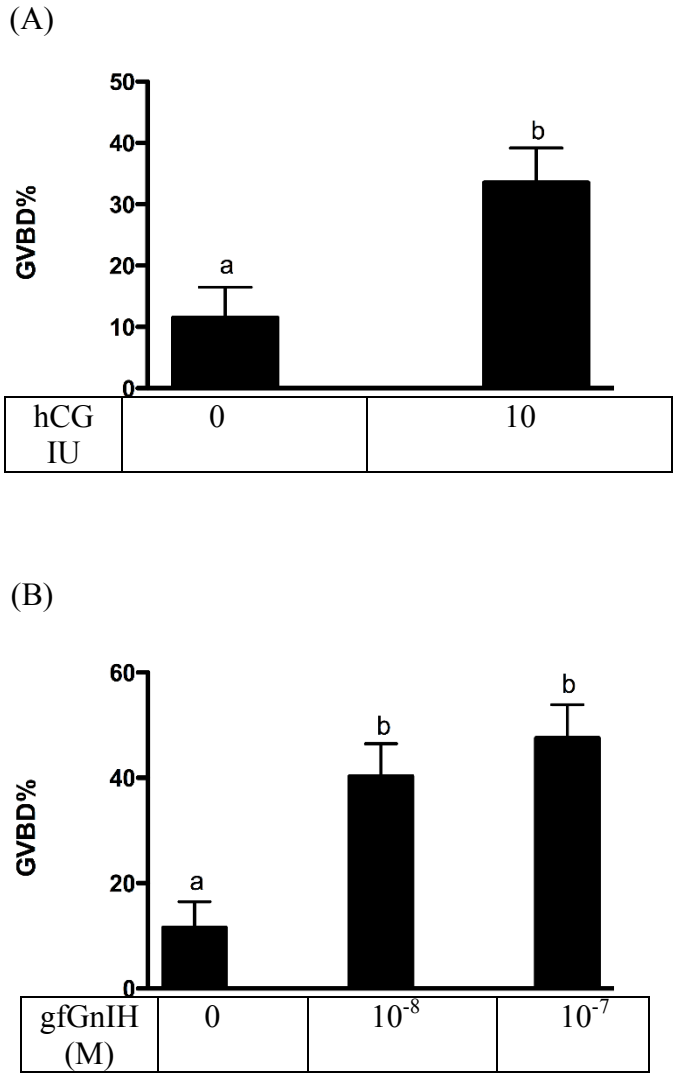
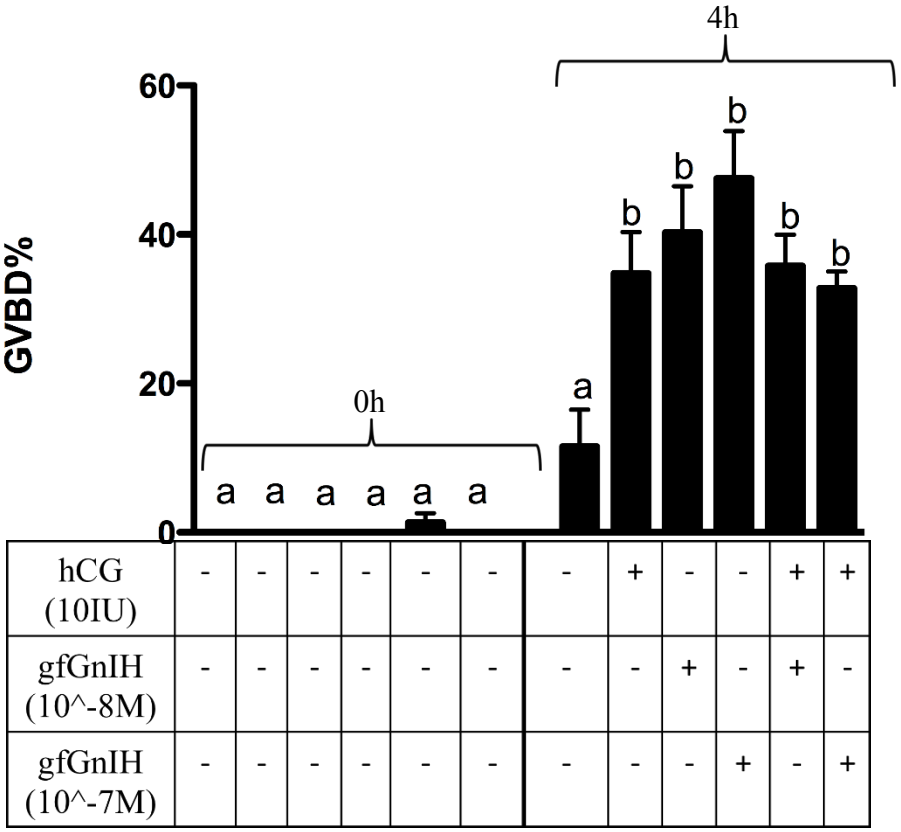


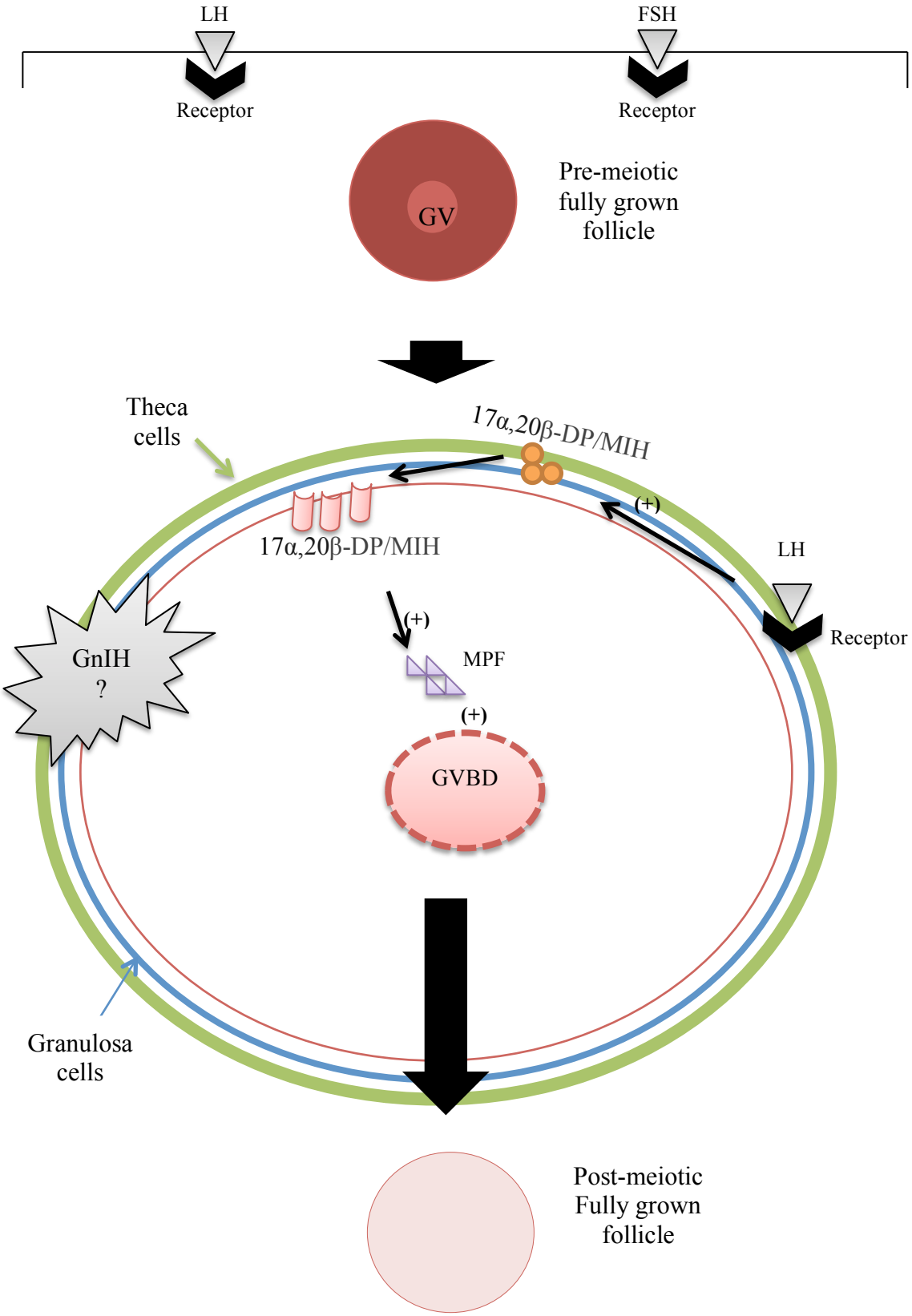
Figure 3.4 The responsiveness of zebrafish pre-meiotic fully-grown oocytes to either hCG (10 IU/ml) (upper panel) or gfGnIH (10^{-8} and 10^{-7} M) (lower panel). Statistically significant differences among treatment groups are represented with different letters. Similar letters indicate insignificant comparisons (mean ± SEM; n = 4) (A: Student's t-test, B: ANOVA followed by Tukey's test, $P < 0.05$).



hCG (10IU)	-	-	-	-	-	-	-	+	-	-	+	+
gfGnIH (10^-8M)	-	-	-	-	-	-	-	-	+	-	+	-
gfGnIH (10^-7M)	-	-	-	-	-	-	-	-	-	+	-	+

Figure 3.5 The responsiveness of zebrafish pre-meiotic fully-grown oocytes to either hCG (10 IU/ml) or gfGnIH (10^{-8} and 10^{-7} M) alone, and combination of both hCG and gfGnIH. Statistically significant differences among treatment groups are represented with different letters. Similar letters indicate insignificant comparisons (mean ± SEM; n = 4) (ANOVA followed by Tukey's test, $P < 0.05$).

Figure 3.6 Hypothesized model of GnIH function in zebrafish ovary with unknown paracrine/autocrine effect on pre-meiotic fully-grown follicle.



Chapter Four

Effects of GnIH on the expression of genes involved in ovarian reproduction

4.1 Introduction

The presence of GnIH in the gonads of vertebrates indicated a possible autocrine/paracrine role for this peptide, controlling gonadal development and growth. In 2008, gonadal GnIH was first discovered in the reproductive system of European starlings and Japanese quail (Bentley et al., 2008). In theses species, ovarian expression of GnIH was verified by the immunocytochemistry analysis in theca and granulosa cell (Bentley et al., 2008), and the relative expression of GnIH-R mRNA was detected using PCR (Maddineni et al., 2008). Furthermore, further evidence of the likely physiological function of GnIH in the context of steroidogenesis is that the immunoreactive GnIH peptides are found in the ovarian theca cells (Bentley et al., 2008). In addition, GnIH existence in the granulosa cells of higher vertebrates such as mice (Singh et al., 2011) and macaques (McGuire & Bentley, 2010) suggests a possible role that the RFa plays to regulate oocytes development (Bentley et al., 2008). As in birds, consequently, mRNA abundance for both GnIH and GnIH-R was found in the gonads of mammalian species (reported by Tsutsui et al., 2013). Moreover, both GnIH and GnIH-R were detected in the testicular and ovarian tissues of teleosts such as goldfish and zebrafish (Zhang et al., 2010; Qi et al., 2013). In general, the literature provides support that GnIH is thought to be implicated in the regulation of gonadal function and reproduction.

The effect of GnIH on the genes participating in steroid biosynthesis in goldfish gonads has been studied *in vivo* and *in vitro* (Qi et al., 2013b). Thus, it was interesting to find out that gfGnIH exerts a dimorphic effect in male and female goldfish (Qi et al., 2013b). Accordingly, testes have shown significant responses to gfGnIH in the gene expression of steroidogenic acute regulatory protein (StAR), which translocates cholesterol into the mitochondria (Stocco, 2000), 3-β-hydroxysteroid dehydrogenase (3-β-HSD), which catalyzes the second step of

steroid biosynthesis (reviewed in Ings & Van Der Kraak, 2006), aromatase A, which catalyzes the production of gonadal estrogen from testosterone (reviewed in Ings & Van Der Kraak, 2006), and LH receptor (LHR) but not FSH receptor (FSHR) (Qi et al., 2013b). Hence, GnIH in goldfish testes affected STAR and 3-β-HSD *in vivo* whereas aromatase A, LH-R, and FSH-R were influenced *in vitro* (Qi et al., 2013b). However, goldfish ovary has revealed another scenario as none of the previous genes exhibited a significant response to the gfGnIH treatment *in vivo* or *in vitro* (Qi et al., 2013b). GTHRs play important roles in the regulation of gonadal physiology as the LHR is activated to stimulate steroid synthesis (reviewed in Pakarainen et al., 2007), while FSHR activity is involved more in the mid vitellogenesis stage of follicular development (Kwok et al., 2005). Hence, these findings support the idea that GnIH is able to target genes/proteins crucially involved in the production of gonadal steroids.

In the fish gonad, gonadotropin hormones (FSH and LH) are believed to bind to their receptors, which causes gametes grow and develop (Kwok et al., 2005; Levavi- Sivan et al., 2010). Therefore, it was surprising not to observe any change in the expression of FSHR in the study carried out in female goldfish by Qi et al. (2013).

In addition to the steroidogenic enzymes and GTHR discussed above, 17 beta dehydrogenases are considered key candidates for stimulating the last steps of steroidogenesis (Mindnich et al., 2004). Among 17 beta dehydrogenases (17β-HSDs), both 17β-HSD type 1, catalysing the production of estrogen from estrone, and 17β-HSD type 3, catalysing the production of testosterone from androstenedione (Mindnich et al., 2004; Reviewed in Ings & Van Der Kraak, 2006), have been implicated in studies examining the gonadal sex differentiation and steroid effect in vertebrates, including teleost species (reviewed in Hunter & Donaldson, 1987; Nakamura et al., 1989). 17β-HSD type 1 is thought to be predominantly

expressed in human female gonadal and other tissues (Luu- The et al., 1990; Martel et al., 1992), similarly, it was found to be expressed in the ovary of adult female zebrafish and in extra-gonadal tissues, but not in testes (Mindnich et al., 2004). On the other hand, 17β-HSD type 3 showed strong and limited expression in mammalian testes, in which it was the main producer of testosterone from androstenedione (Mindnich et al., 2005), but no expression found in the ovary (Geissler et al., 1994; Zhang et al., 1996). In zebrafish, the expression of 17β-HSD type 3 was found in a variety of tissues in the embryo, and in sexually mature males and females (Mindnich et al., 2005). Interestingly, adult zebrafish males showed lower expression of 17β-HSD type 3 in testes comparing to other tissues, whereas the highest level of 17β- HSD type 3 expression in female organs was identified in the ovary (Mindnich et al., 2005). These findings highlighted the significant catalytic activity of 17β-HSDs in the regulation of steroidogenesis and inducing steroid production in the gonads of vertebrates.

Activin and inhibin proteins are known to regulate the secretion of pituitary FSH with no effect on LH, given that inhibin antagonizes activin effects, though they have a similar structure (Ge, 2000). Hence, FSH can be stimulated by activin and inhibited by inhibin, which neutralizes activin action and modulates its receptor activity (Ge, 2000). In zebrafish, activin has indicated a remarkable involvement in final oocyte maturation (Pang & Ge, 1999). In the reproductive organ of fish such as goldfish and zebrafish, activin/inhibin system has been an autocrine/paracrine regulator of ovarian development and function (Ge, 2000).

Due to the limited studies carried out examining the effect of native GnIH on fish gonads, I investigated the possible influence of zfGnIH on basal and on hCG (LH-like hormone) induced gene expression in the ovarian tissue of adult zebrafish females. Zebrafish is an outstanding organism to use as a model for this kind of study because of a large number

of follicles being produced every day. hCG was chosen not only because of its commercial availability, but also because it has a longer half-life, that is a benefit in extended incubation of tissue culture (Ludwig et al., 2003). In addition, follicular tissues were treated with E_2, which is a critical component in ovarian development (Fenske & Segner, 2004), to investigate the possible effects of GnIH on E_2-induced gene expression.

I examined the possible effect of *in vitro* zfGnIH on the genes that are essential for the pathway of steroid biosynthesis, using ovarian tissue culture. QPCR was used to quantify the mRNA abundance of those genes that are FSHR, LHR, STAR, aromataseA, 17β-HSD type 1, 17β-HSD type 3, activinβA, and inhibin, in addition to zfGnIH and zfGnIH-R3. This is the first study examining the direct effect of zfGnIH on the relative mRNA expression of previously stated genes in any vertebrate.

4.2. Materials and methods

4.2.1 Animals

Wild-type Tupfel Longfin (TL) adult female zebrafish were used.

4.2.2 Ovarian tissue culture

Female zebrafish were anesthetized and humanely euthanized by making spinal transection (6-12 fish/experiment). Fish skin in the belly area was lifted up by making a cut just front the pectoral fin along the body cavity where the ovary is suspended. Follicles were collected and placed in sterile 6-well-plate containing 2 ml/well of L-15 media (supplemented with 10,000 U/ml penicillin, 10 mg/ml streptomycin and 25ug/ml amphotericin B, and 5 mM of Sodium bicarbonate), and ovarian tissue from each fish was settled in a separate well. Tissues

were then washed three times to remove unwanted debris. Under the dissecting microscope, follicles that were aggregated in groups were carefully teased apart into smaller groups via gentle pipetting using plastic Pasteur pipette (around 10 times). Most follicles were fully grown (post-vitellogenic stage), transferred one by one using fine forceps and placed in 96-well-plate (as 40 follicles/well) in which there was the incubation media, and then allowed to set for 1 h at 28 °C on the shaker (8 rpm). Each fish contributed an equal number of follicles to all treatment groups to reduce the interanimal variations. Treatments were added to the appropriate wells and tissue incubated for 4 h. The effect of zfGnIH was examined using final concentrations of 10^{-8} and 10^{-7} M, and hCG doses were 1IU and 10IU/ml which is the dosage that has been previously revealed to stimulate E2 production (Ings Van Der Kraak, 2006). After the incubation had been terminated, media was aspired and kept in -80 for ELISA analysis (discussed in chapter 3 section 3.2.1). In addition, another set of ovarian tissue was also dissected out, washed, isolated and treated with the 17β-estradiol hormone which was serially diluted in L-15 media to obtain the desired concentrations (10^{-9} M, 10^{-8} M, and 10^{-7} M). Subsequently, follicular tissues were incubated for 16 h at 28 °C on the shaker (8 rpm). Then, follicles from both experimental sets were harvested and homogenized by Trizol for RNA extraction and transcript levels were measured by QPCR as explained in chapter 2 (section 2.4.1 and 2.4.2).

4.2.3 Statistical analysis

As described in Chapter 2 (section 2.6), statistical analysis was carried out using One-Way ANOVA followed by Tukey's test that was performed to find out significant interactions among multiple comparisons of a single factor.

4.3 Results

4.3.1 Effects of zfGnIH on mRNA expression of basal and hCG-induced changes in gonadal FSH-R and LH-R

Due to the well-documented stimulatory effects of hCG (LH-analogue) in triggering the GTHRs in gonads to regulate the ovarian function, I sought to examine the responsiveness of FSH-R and LH-R expressions to zfGnIH in follicles treated with hCG. I also looked at the possible influence that zfGnIH might have on the expression of zfGnIH-R3. I found no changes in the gene expression of FSH-R (Figure 4.1) and LH-R (Figure 4.2) in response to either hCG or zfGnIH doses. Likewise, treatments with zfGnIH and hCG had no observable effects on the mRNA expression of those receptors (Figures 4.1 and 4.2).

4.3.2 Effects of zfGnIH on mRNA expression of basal and hCG-induced changes in gonadal STAR, 17β-HSD.3, 17β-HSD.1, and aromatase A (CYP19A)

I studied the effects of zfGnIH alone and in a combination with hCG to determine the role of zfGnIH in the control of steroidogenic function of ovarian follicles on the relative mRNA expression of STAR (Figure 4.3), 17β-HSD.3 (Figure 4.4), 17β-HSD.1 (Figure 4.5), and aromatase A (CYP19A) (Figure 4.6). Event though those genes were expressed, none of the treatments induced significant changes in levels of transcript abundance (Figures 4.3, 4.4, 4.5, and 4.6).

4.3.3 Effects of zfGnIH on mRNA expression of basal and hCG-induced changes in gonadal activin βA subunit and inhibin

I examined the influence of zfGnIH alone and in a combination with hCG on the expression of activin βA subunit and inhibin. None of the treatment induced visible changes in the mRNA expression levels. Although treatment with hCG slightly decreased the ability of

zfGnIH to stimulate the expression of activin βA subunit and inhibin, these changes in transcript levels were not significant (Figure 4.7 and 4.8).

4.3.4 Effects of zfGnIH on mRNA expression of basal and hCG-induced changes in gonadal zfGnIH and zfGnIH-R3

To examine the influence of zfGnIH on the ovarian expression of zfGnIH and zfGnIH-R3, I looked at the effect of zfGnIH alone and in a combination with hCG. Both zfGnIH and zfGnIH-R3 were expressed in the ovary of adult female zebrafish, however, the zfGnIH doses did not have a significant effect on the relative mRNA abundance (Figures 4.9 and 4.10).

4.3.5 Effects of zfGnIH on mRNA expression of basal and 17β-estradiol- induced changes in gonadal FSH-R and LH-R

Due to the defined role of 17β-estradiol in maintaining ovarian development, I examined the effect of zfGnIH on the expression of GTH-Rs in follicles treated with various doses of 17β-estradiol (10^{-9} M, 10^{-8} M, and 10^{-7} M). There was no significant change in the levels of mRNA transcription (Figure 4.11).

4.3.6 Effects of zfGnIH on mRNA expression of basal and 17β-estradiol- induced changes in gonadal 17β-HSD.1 and aromatase A (CYP19A)

I studied the influence of zfGnIH on the genes responsible for encoding the enzymes catalyzing the production of estrogen. In the term of 17β-HSD.1 mRNA expression, zfGnIH (10^{-7} M) was able to significantly decrease the stimulatory effect induced by only the smallest dose of 17β-estradiol (10^{-9}) (Figure 4.12). However, none of the treatments had a significant effect on the transcript abundance of CYP19A (Figure 4.12).

4.3.7 Effects of zfGnIH on mRNA expression of basal and 17β-estradiol-induced changes in gonadal activin βA subunit

I found that the expression of activin βA subunit constantly increased as the estradiol doses increased; however, zfGnIH treatment did not cause an effect on the levels of the relative transcript (Figure 4.13).

4.4 Discussion

In this chapter, I investigated the role of zfGnIH on the expression of ovarian genes involved in steroidogenesis and oogenesis. I was able to demonstrate novel information showing the expression of GnIH and GnIH-R3 in the ovary of adult female zebrafish using QPCR. However, treating follicles with zfGnIH had no effect on the mRNA abundance of GnIH or its receptor. Similarly, with the exception of 17β-HSD-1, neither the genes of enzymes crucial for regulating the biosynthesis of steroids, nor the GTH-Rs or gonadal peptides responded to zfGnIH application. Accordingly, the results in this thesis chapter are in accordance with some studies but in disagreement with others. In fact, the dissimilarities could be attributed to the difference in the system of incubating follicles, the specific developmental stage included in a study, or the model (species) of a study.

4.4.1 The effects of zfGnIH on basal and hCG-induced mRNA expression of ovarian genes involved in gonadal development.

GTH-Rs. The ovarian profile of either FSH-R or LH-R expression is not constant. It changes from one developmental stage to another, as FSH-R expressed at the early vitellogenic stage and peaked at the mid-vitellogenic stage. LH-R is expressed mostly at post-vitellogenic stage (Kwok et al., 2005; Rocha et al., 2007). This suggests that FSH-R is implicated in the stages when oocytes are developing, importing the vitellogenin protein. LH-R is more important when the oocyte is fully-grown and about to go through the resumption of meiosis

(Kwok et al., 2005). Since GnIH is expressed by the gonad, it was interesting to see no significant changes in the mRNA abundance of the GTH-Rs (FSH-R and LH-R) in the zebrafish ovary. My results are in agreement with findings from a study carried out exploring the role of GnIH in the goldfish ovary (Qi et al., 2013b). Qi et al. (2013b) have shown that gfLPXRFa played a dimorphic role, as it appeared to significantly regulate the GTH-Rs in males but it had no effect on these receptors in females either *in vivo* or *in vitro*. However, my thesis findings on GTH-Rs disagree with a study performed by Moussavi (2013) using the goldfish ovary. In this study, gfGnIH was shown to markedly control both FSH-R and LH-R. The inconsistency between my results and the data provided by Moussavi (2013) might result from the difference in the developmental stages that were analyzed, as 97 % of the follicles used in my research were at the post- vitellogenic stage, whereas the study by Moussavi (2013) included follicles at early and mid-recrudescence. Another possibility is the incubation time I chose, based on previous study, to stimulate E2 production (between 3-8) under specific dose of hCG (10 IU; Ings & Van Der Kraak, 2006). In my study, E2 was significantly released within the time course (4h) during which also oocytes underwent GVBD and became post-meiotic. In my investigation, oocytes were collected after approximately 60 % of them were at the post- meiotic stage at which the expression of FSH-R and LH-R is correspondingly down-regulated. The expression of GTH-Rs being down-regulated was examined previously in oocytes that exhibited GVBD and went through the resumption of meiosis (Nyuji et al., 2013). Thus, these data suggest that the GnIH function is reproductive stage dependent.

STAR. STAR has been considered an important regulator of the steroidogenesis pathway, as it transfers cholesterol into the mitochondria of steroidogenic cells to initiate steroid biosynthesis (Stocco, 2000). In the current study of gene expression, STAR did not

respond to either zfGnIH treatment alone or to the combinational treatment with hCG. Relating my finding to others shows both similarities and differences. *In vitro* trials done using goldfish as a model demonstrated gfGnIH had no influence on the relative expression of ovarian STAR at the vitellogenic stage (Qi et al., 2013b), whereas gfGnIH increased STAR expression in ovary from early but not mid recrudescence (Moussavi, 2013). The expression pattern of STAR in the zebrafish ovary has been studied throughout the gonadal development (Ings & Van Der Kraak, 2006). Consequently, during post-vitellogenic stage, mRNA expression of STAR was found to be 10-fold less than its expression in the early and mid stages of development, and it was stimulated significantly when treated with hCG (10 IU) *in vitro* at the vitellogenic stage (Ings & Van Der Kraak, 2006). These findings indicated that GnIH might elicit its action based on the ovarian developmental stage.

17β-HSD-3 and 17β-HSD-1. Production of ovarian E_2 is catalyzed by two vital enzymes; indirectly by the catalytic activity of 17β-HSD-3 in producing testosterone substrate (in theca cells), and directly by 17β-HSD-1 in granulosa cells (Mindnich et al., 2004). In the present study, the gene expression of both enzymes did not reflect zfGnIH effects. Since that E_2 synthesis increases at the vitellogenic stage and follicular growth, and the production drops once the oocytes become fully mature, it suggests a following decrease in the catalyzing enzymes/genes. These results indicate that 17β-HSD-3 and 17β-HSD-1 lost the desensitization to hCG (LH-like) treatment in the oocytes when they became post-meiotic. Also, the results suggest that these genes might not be a crucial target could be regulated by the GnIH at this specific stage of development.

Aromatase A (CYP19A). In studies using zebrafish as a model, aromatase A expression has exhibited various expression patterns as the oocyte develops. It reaches its maximum

expression at the pre-vitellogenic, stage but drops sharply at the post- vitellogenic stage, during which the aromatase A is barely detectable (Ings & Van Der Kraak, 2006). A similar expression pattern for Cyp19a after oocytes have completed the vitellogenic stage and became fully-grown was seen in catfish (Kumar et al., 2000), in medaka (Fukada et al., 1997), and tilapia (Chang et al., 1996). Cyp19a was not measurable when oocytes have gone through final maturation in rainbow trout (Nakamura et al., 2005). Moreover, when rat granulosa cells were treated with GTH, aromatase A transcripts were correspondingly stimulated, but expression suddenly reduced as oocytes were about to ovulate (Fitzpatrick et al., 1997). These findings support my results that showed no significant response to incubation with doses of hCG. Also, zfGnIH had no influence on the transcript abundance of aromatase A within this incubation system, or the time course during which the expression might have increased then decreased by the end of incubation time. Similarly, in goldfish, the relative expression of cyp19a did not change when the follicles, from early and mid recrudescence, were incubated with hCG alone or gfGnIH alone (Moussavi, 2013). However, aromatase A expression diminished when both treatments were co-applied (Moussavi, 2013), which disagrees my findings. Qi et al. (2013b) results with goldfish confirmed the findings from Mussavi's (2013) study and my findings that GnIH alone had no effect on ovarian aromatase. These data suggest that GnIH differentially regulates aromatase A within teleost species, which is also emphasized by reproductive stage and species specificity.

ActivinβA and Inhibin. Activin and inhibin system is a downstream of GTH signaling, mediating the regulation of oocyte proliferation and maturation (Knight et al., 2012). Activin activity, modulated by inhibin, is also involved in regulating ovarian function and pituitary FSH secretion through the feedback loop from the gonad (Wang & Ge, 2003). Within the context of

my gene expression study, the expression of activin seems to be higher than inhibin transcripts, and zfGnIH stimulatory effect on both peptides was reversed by hCG, although these effects were statistically insignificant. Looking at the trends but not the significance, my findings regarding activin βA, are in accordance with data generated from goldfish ovaries at the early gonadal recrudescence (Moussavi, 2013). In that study, activin showed a similar response to the interaction of hCG and gfGnIH (Moussavi, 2013). In addition, I found that incubating follicles with hCG did not have a significant effect on the mRNA abundance of activin, even though this treatment increased the level of the transcript in a previous study on zebrafish (Wang & Ge, 2003). However, in their studies, activin expression was stimulated within a short incubation time (2h) after which desensitization to hCG was observed beyond longer treatment time (Wang & Ge, 2003). In addition, Poon et al. (2009) showed that inhibin expression was up regulated in pre-meiotic fully-grown oocytes, but markedly decreased when those oocytes underwent resumption of meiosis. Moreover, in their studies, QPCR detected very low basal expression of inhibin transcripts with incubation time at 3h, 6h, 12h, and 24h compared to 0h, and this expression was time dependent (Poon et al., 2009). Relating these observations to my data suggests that the levels of inhibin transcripts significantly diminished at my incubation time (4h) at which neither hCG nor zfGnIH had an effect on inhibin expression. Taken together, these data support the concept that GnIH action in lower vertebrate gonads varies based on the reproductive stage of the model used in that study.

zfGnIH-R3 and GnIH. This is the first study demonstrating the presence of GnIH and GnIH-R3 messages in the ovary of adult zebrafish using QPCR. The expression of GnIH-R3 and zfGnIH has been verified in the embryo's ovary (Zhang et al., 2010) but not in the gonads of developing or sexually mature zebrafish. In the current study, zfGnIH-R3

showed higher basal expression (~10-fold) compared to the expression of GnIH itself. However, investigating the role of zfGnIH on the zfGnIH-R3 and GnIH in oocytes at advanced stages of development did not reveal any changes in the mRNA abundance of either transcript. Regarding gfGnIH-R, my results are in agreement with trials that used goldfish as a study model (Moussavi, 2013). In this investigation, Moussavi (2013) found that mRNA levels of gfGnIH-R did not respond to treatment with hCG or gfGnIH when follicles were at the mid stage of recrudescence (2013). However, during the early stage of recrudescence, gfGnIH stimulatory effect on the gfGnIH-R was reduced by hCG treatment (Moussavi, 2013). Thus, whether zfGnIH plays a different role during the early and mid stages of zebrafish ovarian development remains unknown.

4.4.2 The effects of zfGnIH on 17β-estradiol-induced response of mRNA expression of ovarian genes involved in reproduction (feedback loop on the gonad)

GTH-R mRNA expression. Herein, I examined the interference of zfGnIH with the 17β-estradiol induced mRNA expression of key steroidogenic enzymes *in vitro*. zfGnIH dosage changed 17β-estradiol induced expression of FSH-R and LH-R, though it was not significant. Also, mRNA abundance of both receptors had similar trends in response to the interactive effect of zfGnIH with gradual doses of 17β-estradiol. These data suggest that zfGnIH is active in the zebrafish gonad, but whether this activity of GnIH would be more apparent at a different stage of ovarian development needs further investigation.

17β-HSD-1 and CYP19A mRNA expression. In the current study, I also examined zfGnIH autocrine/paracrine effect on the 17β-HSD-1 and CYP19A expression, induced by the local feedback exerted by 17β-estradiol in the ovary. Thus, zfGnIH (at a concentration of 10^{-7}

M) significantly decreased the relative expression of 17β-HSD-1 abundance induced by 17β-estradiol (10^{-9} M). However, aromatase A did not show a significant response to the interactive treatment of both hormones. To conclude, these results imply that zfGnIH might preferentially target the genes encoding enzymes regulating steroidogenesis.

Activin βA mRNA expression. Treating follicles with gradual doses of 17β- estradiol continually increased the expression of activin, but zfGnIH had no significant effect on this mRNA abundance induced by estradiol. These data indicate that zfGnIH may not be involved in the control of activin expression at this stage of oocyte development.

4.5 Summary

To summarize, in this chapter, I investigated the likelihood of GnIH effect on the gene expression of transcripts known to regulate pathways of steroids synthesis. Nevertheless, the levels of transcripts quantified were not influenced by the GnIH treatment, which agrees with some studies but disagrees with others (as discussed previously in this chapter). Whether GnIH paracrine/autocrine action was elicited based on the reproductive stage or on sex in zebrafish remains unknown.

FSH-R

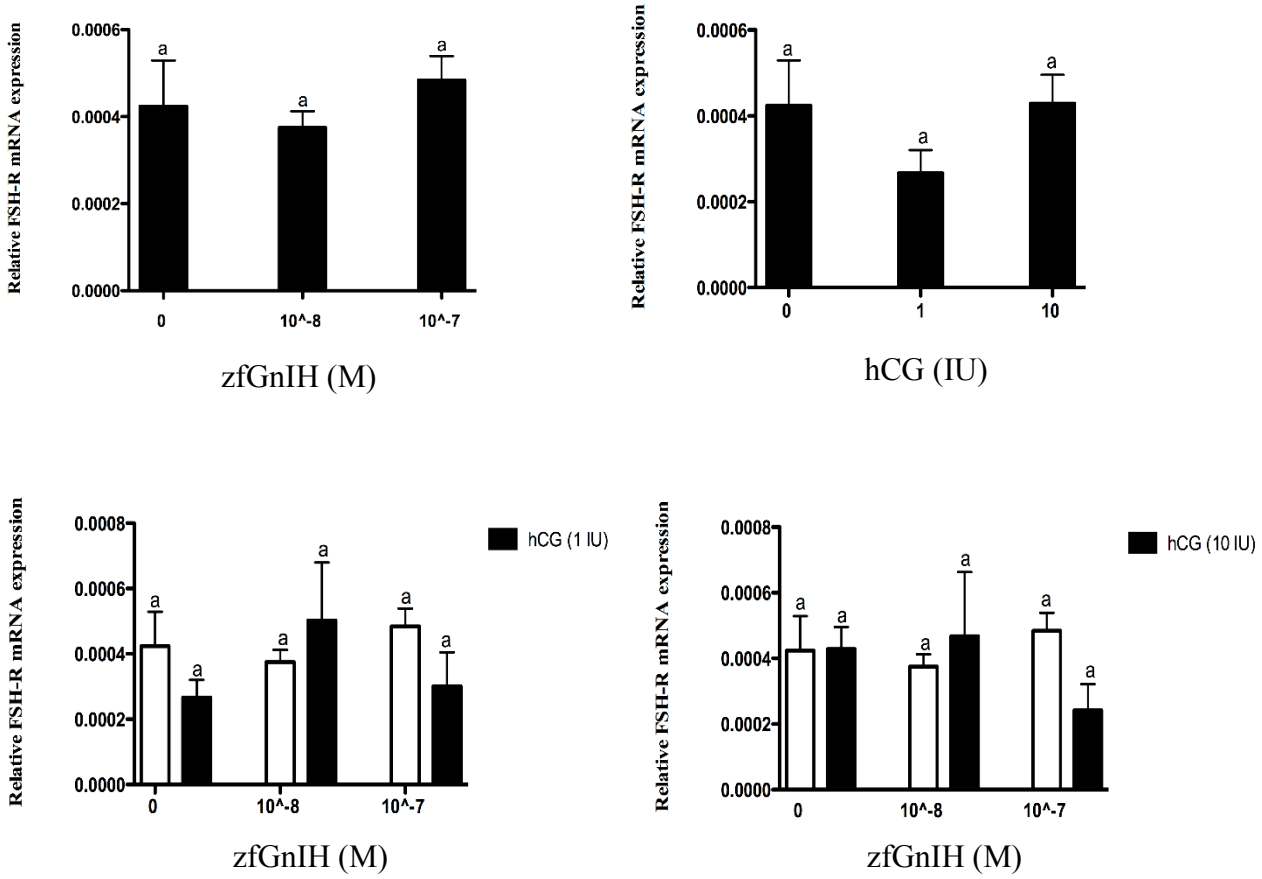


Figure 4.1 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian FSH-R mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

LH-R

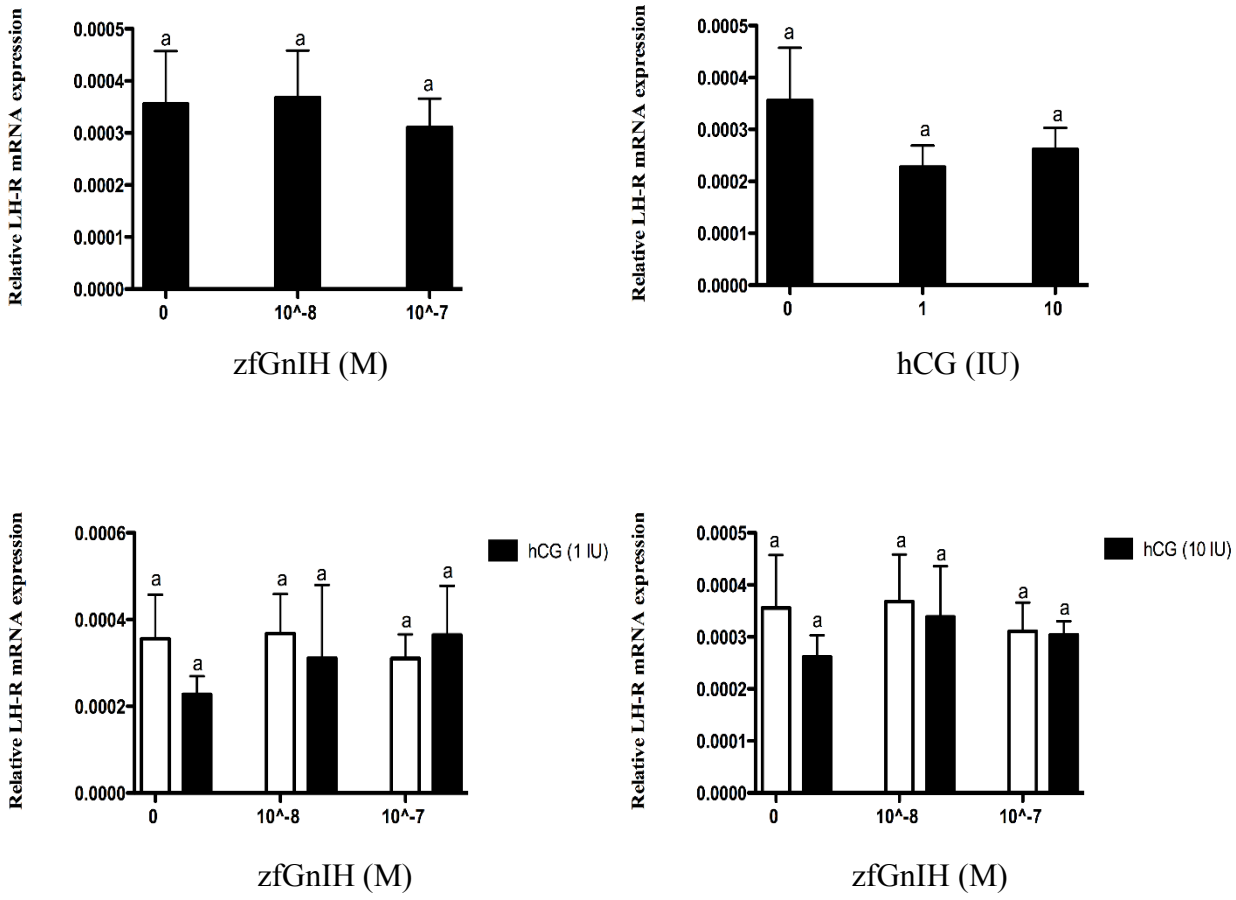


Figure 4.2 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian LH-R mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

STAR

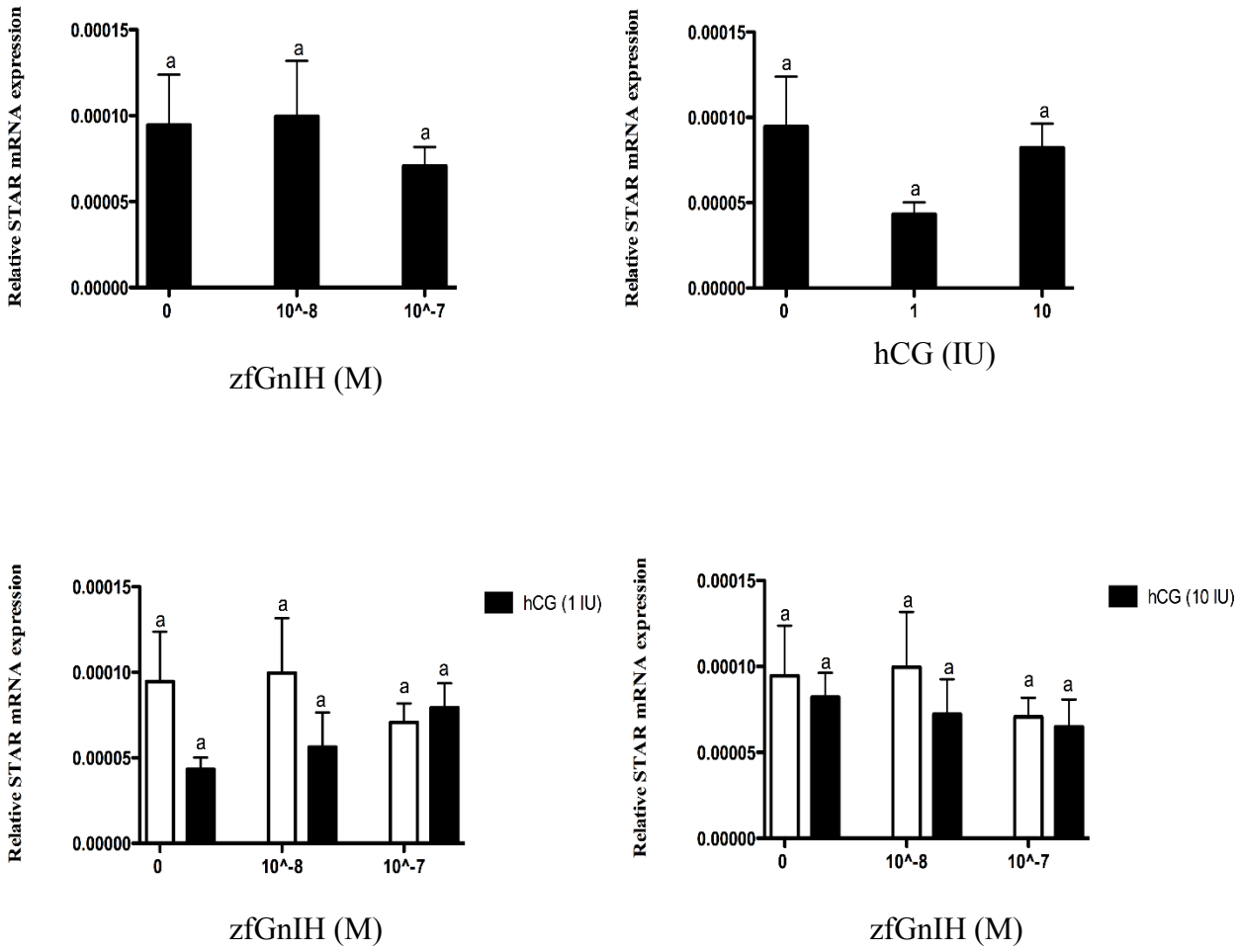


Figure 4.3 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian STAR mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

17β-HSD-3

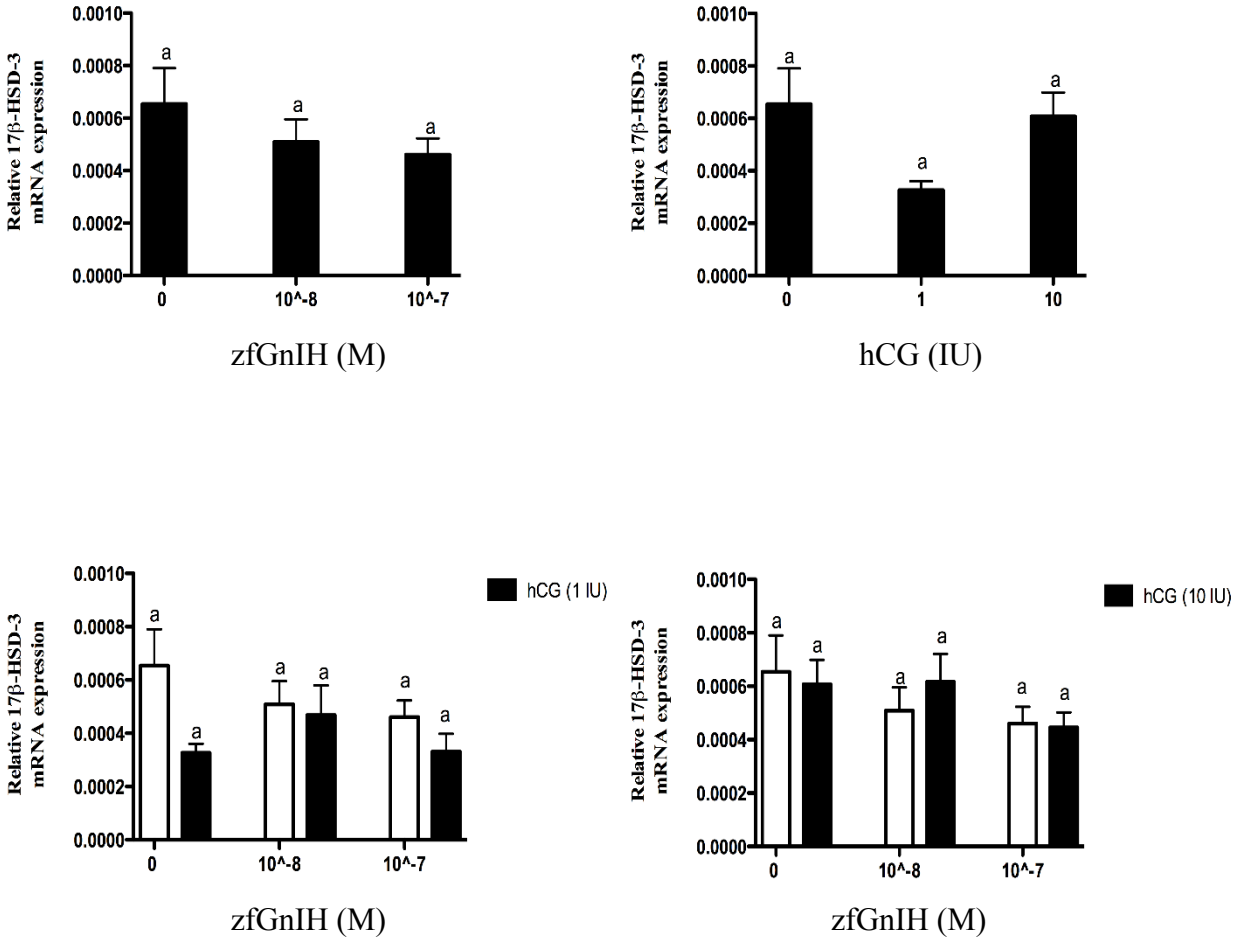


Figure 4.4 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian 17β-HSD-3 mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

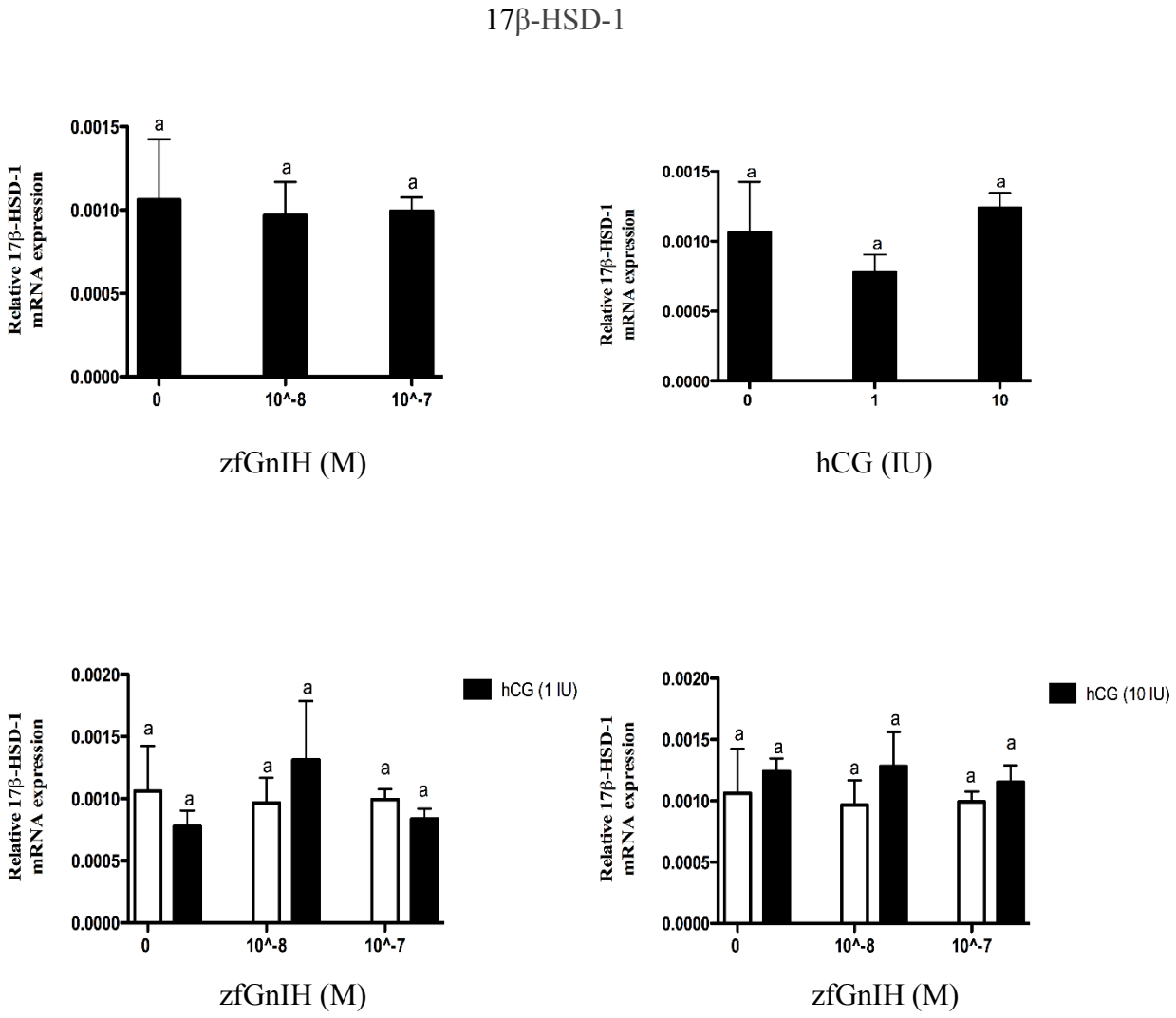


Figure 4.5 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian 17β-HSD-1 mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

CYP19A

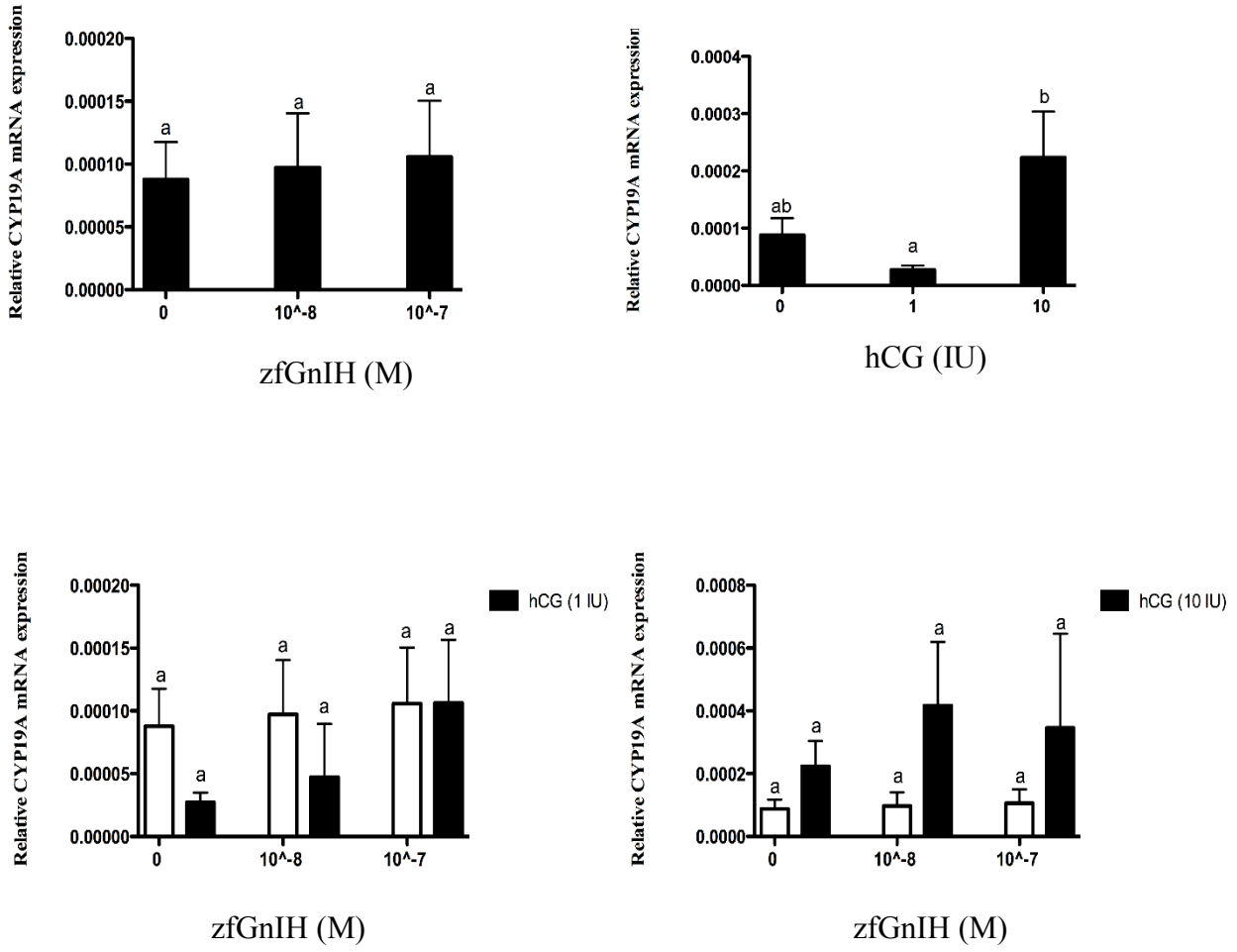


Figure 4.6 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian CYP19a mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

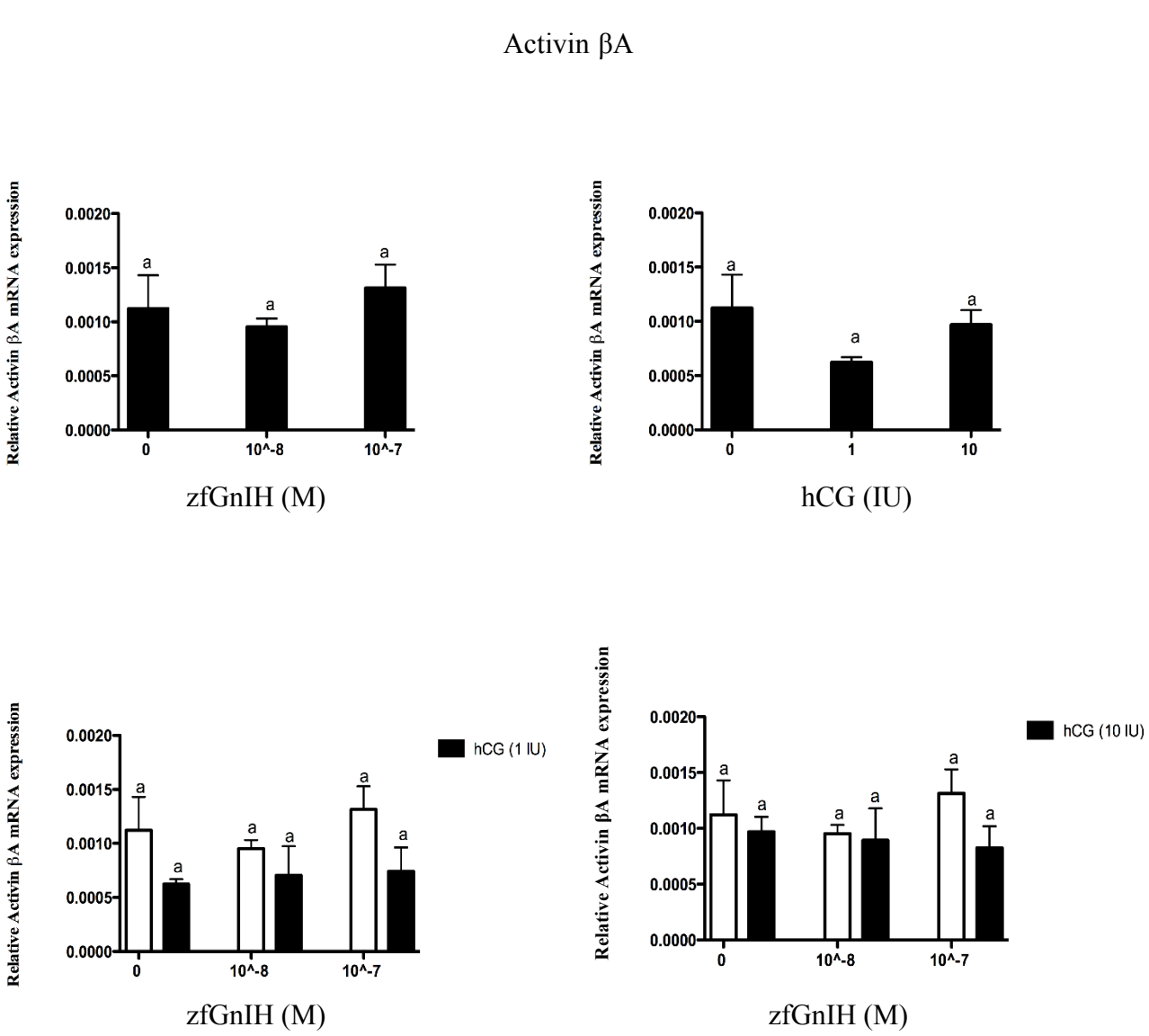


Figure 4.7 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian Activin βA mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

Inhibin

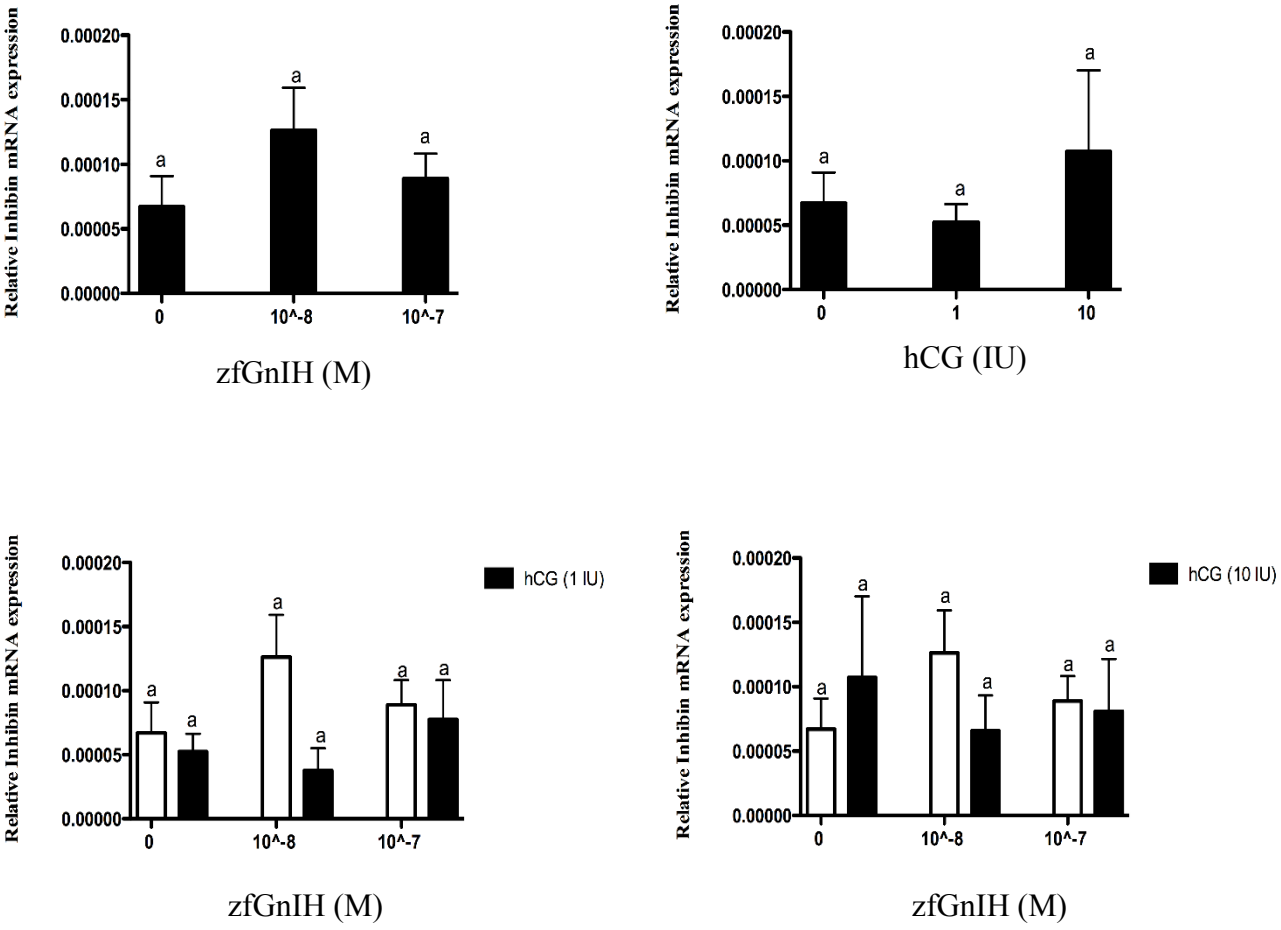


Figure 4.8 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian inhibin mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

zfGnIH-R3

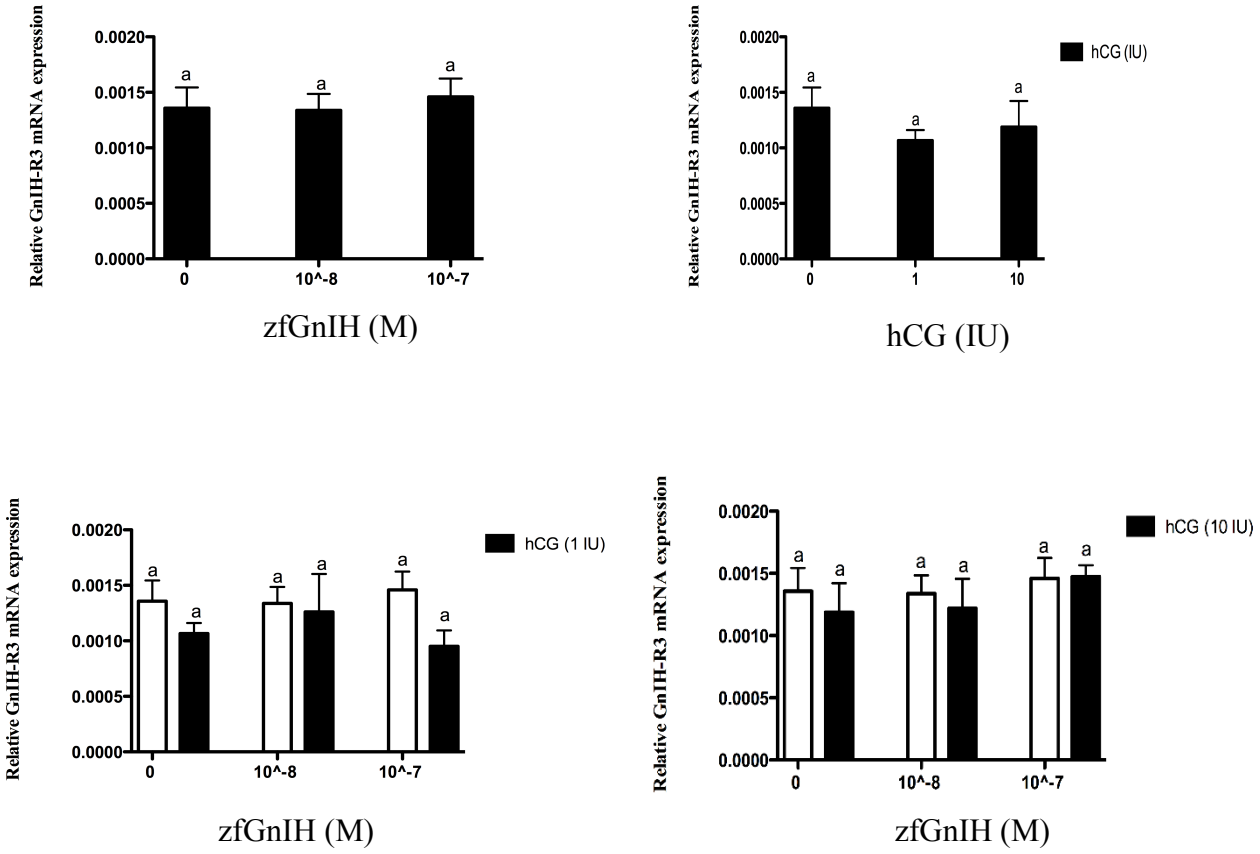


Figure 4.9 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian GNIH-R3 mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

GnIH

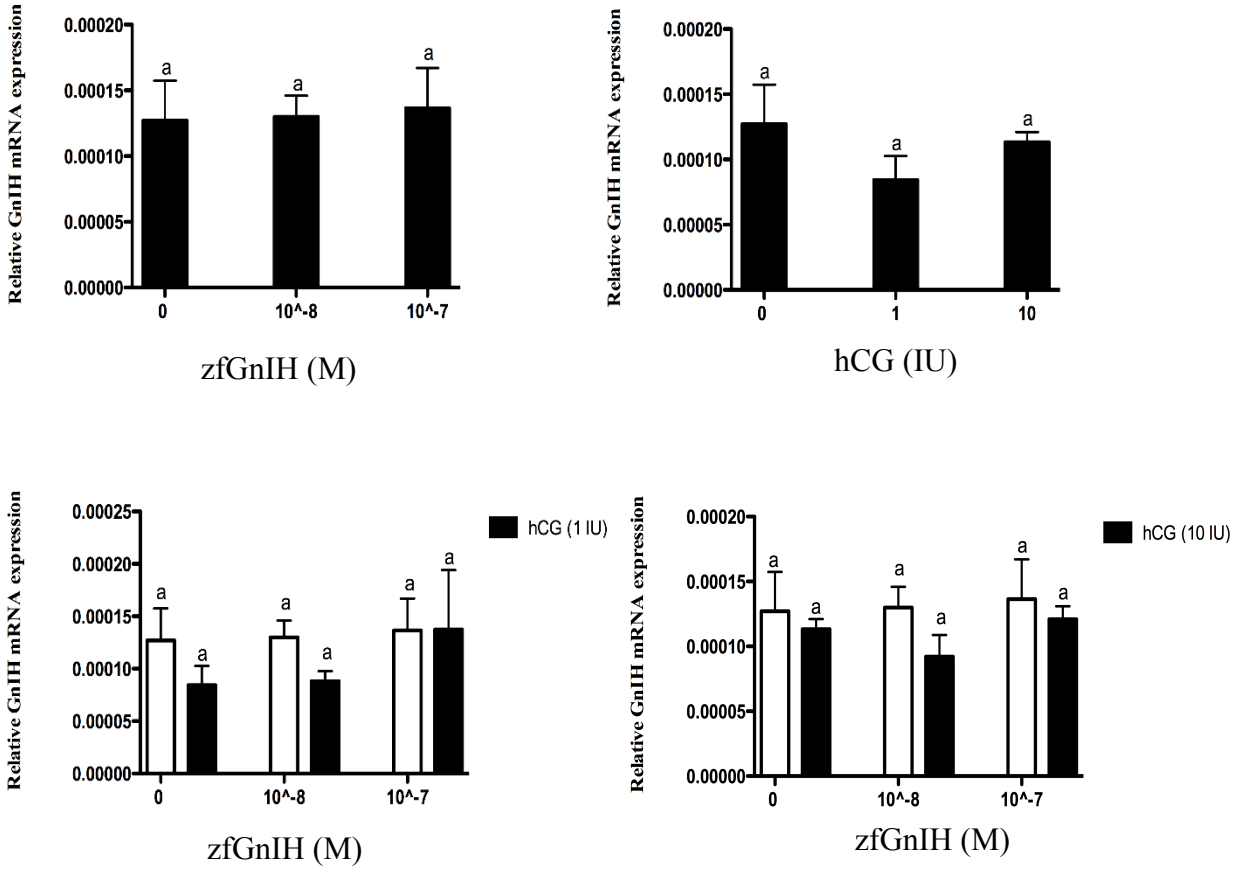


Figure 4.10 *in vitro* effects of either zfGnIH (10^{-8} and 10^{-7} M) or hCG (1 IU/ml and 10 IU/ml) alone (upper panel) and combination of zfGnIH and hCG on ovarian GNIH mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 4). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

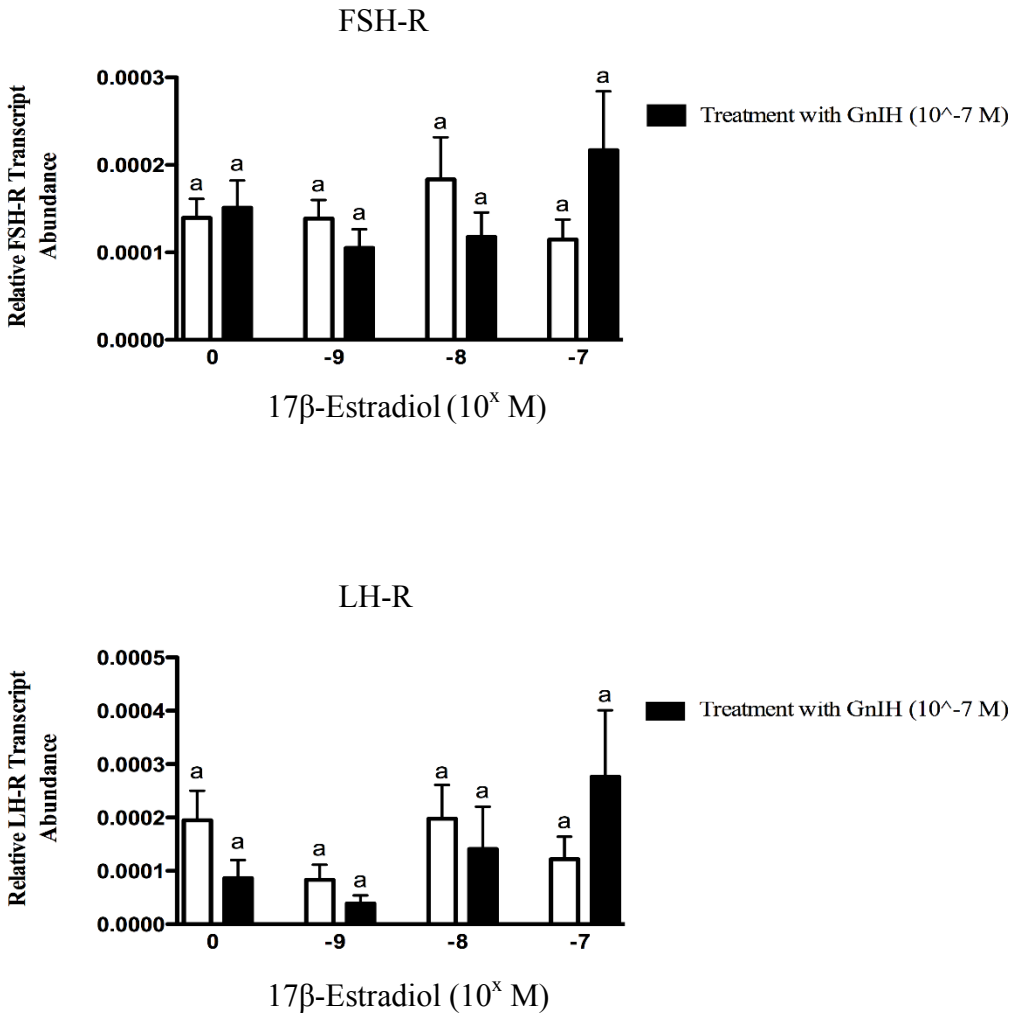


Figure 4.11 *in vitro* effects of either zfGnIH (10^{-7} M) or 17β-Estradiol (10^{-9} M, 10^{-8} M, and 10^{-7} M) alone and co-treatment of zfGnIH and 17β-Estradiol (gradual doses) on FSH-R mRNA abundance (upper panel) and LH-R mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 10). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

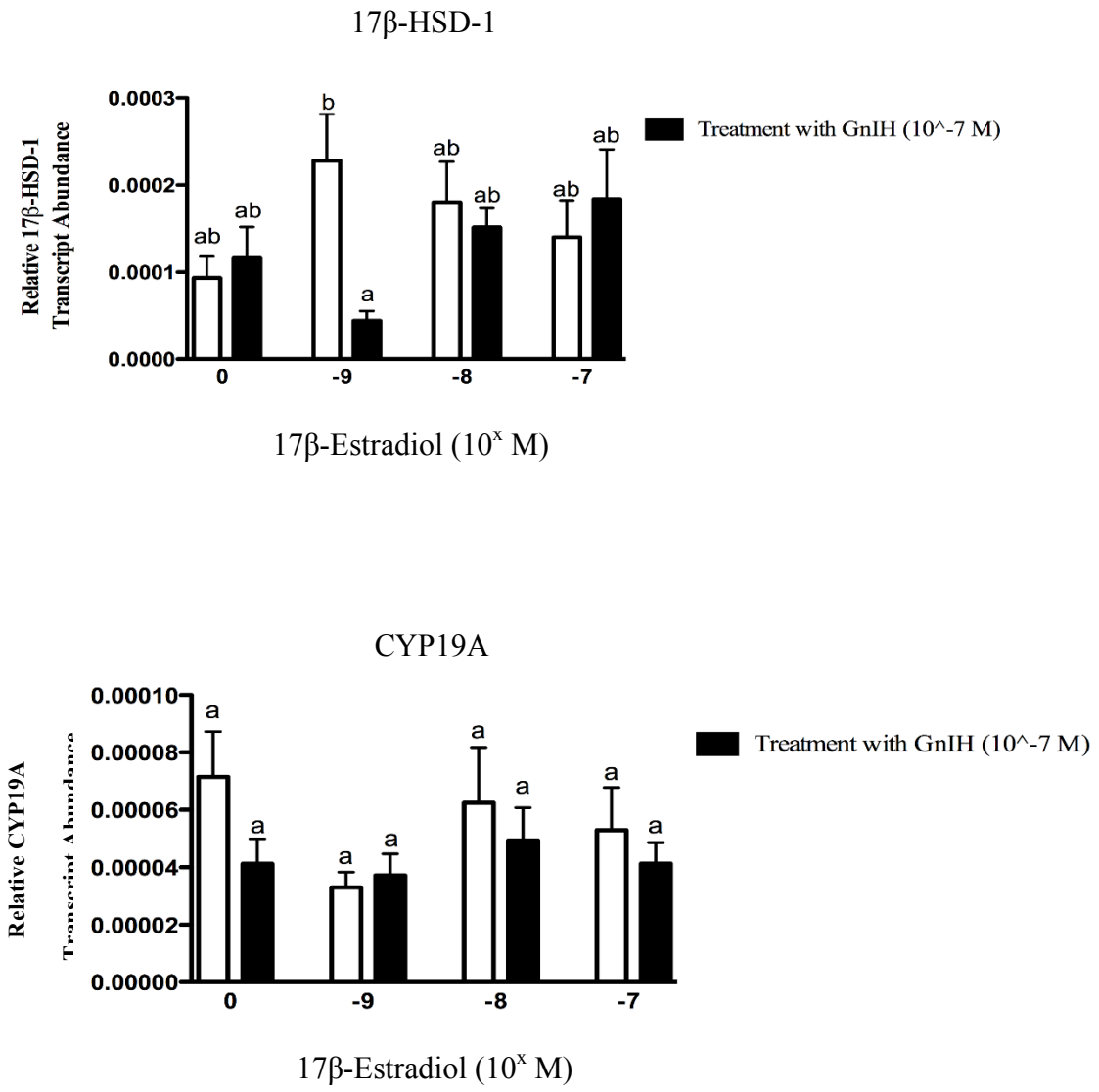


Figure 4.12 *in vitro* effects of either zfGnIH (10^{-7} M) or 17β-Estradiol (10^{-9} M, 10^{-8} M, and 10^{-7} M) alone and co-treatment of zfGnIH and 17β-Estradiol (gradual doses) on 17β-HSD-1 mRNA abundance (upper panel) and CYP19A mRNA abundance (lower panel). Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 14). Statistically significant differences among treatment groups are denoted with different letters. (ANOVA followed by Tukey's test, $P < 0.05$).

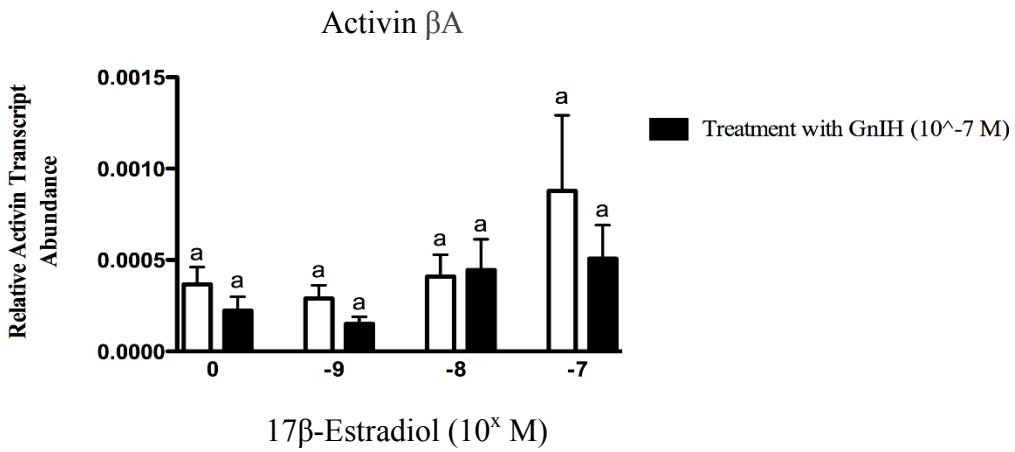


Figure 4.13 *in vitro* effects of either zfGnIH (10^{-7} M) or 17β-Estradiol (10^{-9} M, 10^{-8} M, and 10^{-7} M) alone and co-treatment of zfGnIH and 17β-Estradiol (gradual doses) on Activin βA mRNA abundance. Transcript levels were determined by QPCR and values normalized to β-actin expression (mean ± SEM; n = 14). Similar letters indicate insignificant comparisons (ANOVA followed by Tukey's test, $P < 0.05$).

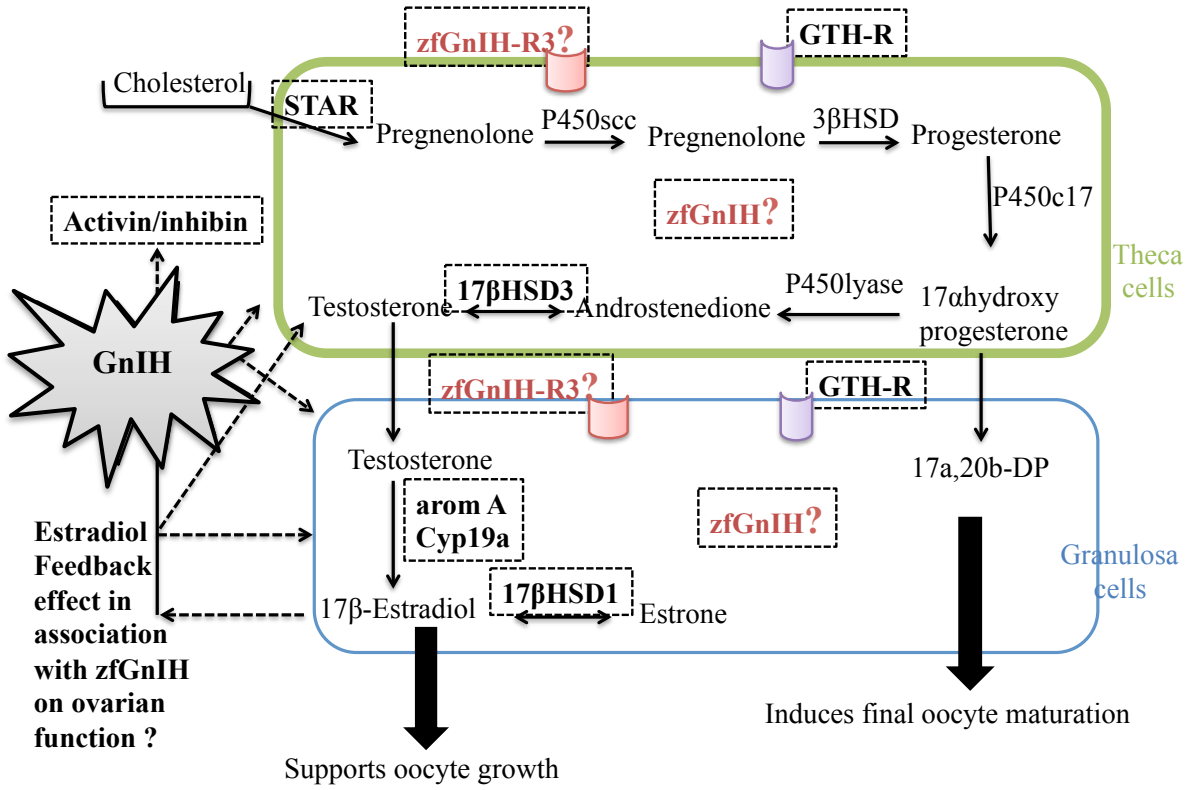


Figure 4.14 Hypothesized model of GnIH role in zebrafish ovary with unknown paracrine/autocrine effect on the gene expression of enzymes involved in steroid biosynthesis (dashed squares represent genes participated in the study).

Chapter Five

General Discussion and Conclusions

5.1 Summary and Conclusions

5.1.1 Overview

The main purpose of my study was to elucidate, *in vitro,* the possible function of GnIH on basal and on hCG-induced steroidogenesis and final oocyte maturation in adult female zebrafish. I also studied the role of GnIH on either hCG- or 17β-estradiol-induced gene expression in the biosynthesis of steroids in the ovary.

Since its discovery, information regarding the significance of GnIH on ovarian E_2 production from cultured ovaries has been examined exclusively in birds (European starling) *in vitro* (McGuire et al., 2010). Several months before I started my study, *in vivo* studies were carried out testing the GnIH influence on E_2 concentration in female goldfish. Administration of GnIH did change E_2 levels (Qi et al., 2013). In terms of oogenesis, I was able to investigate for the first time in any vertebrate the effect of GnIH on the final oocyte maturation, and resumption of meiosis in fully-grown zebrafish oocytes.

In regard to the effect of GnIH on basal or hCG-induced ovarian gene expression, several *in vivo* and *in vitro* trials have explored the responsiveness of various genes encoding enzymes, involved in gonadal steroidogenesis, to GnIH. Thus, Moussavi (2013), from our lab, demonstrated the ability of GnIH, operating on basal or hCG- induced ovarian steroidogenic genes, to significantly influence the abundance of their transcripts, in addition to its apparent effects on the FSH-R and LH-R expressions *in vitro* (discussed in chapter 4 section 4.4.1). However, Qi et al. (2013), using the same animal model, were unable to see any changes in the mRNA abundance of the tested steroidogenic genes when follicles were treated with GnIH alone *in vitro*. The differences that are seen in the two studies might result from the differences in the reproductive stages that were examined, as the first mentioned study was performed on

two different stages (early and mid recrudescence) and the second study was done on one reproductive stage (vitellogenic). I also examined the possible effect of GnIH on E_2-induced ovarian gene expression, which had not been examined in any vertebrate system.

This dissertation investigates for the first time the expression of GnIH and GnIH- R in adult zebrafish reproductive system. To date, there have been no studies performed testing the effect of GnIH in any organ in the zebrafish. Before I initiated my research, the only information that was available regarding GnIH in zebrafish was that this peptide was being expressed in the gonadal and extra-gonadal tissues of developing zebrafish (embryos and larvae; Zhang et al., 2010). Then, the effect of zfGnIH was first tested in goldfish; in which zfGnIH was able to diminish the serum LH just 1h after zfGnIH (1.0 ug/g body weight) was administered (Zhang et al., 2010). My thesis demonstrated not only the expression of GnIH and GnIH-R in the sexually mature zebrafish ovary, but it also indicates the possible physiological function of GnIH on E_2 production, oocyte final maturation, and gene expression. I tested three specific hypotheses and generated novel data on the effect of GnIH within the context of the ovarian function.

The first hypothesis examined whether GnIH may potentially affect the ability of hCG to stimulate gonadal steroid production *in vitro.* The second hypothesis examined whether GnIH may have an influence on the oocyte final maturation *in vitro*. The third hypothesis tested whether or not GnIH has a paracrine/autocrine role on basal and hCG or 17β-estradiol-induced ovarian gene expression *in vitro*.

5.1.2 Paracrine effects of GnIH in zebrafish ovary

Having tested the first hypothesis, I found that GnIH at low, but not high dosages, was able to stimulate the hCG- (1 IU) induced steroid production in the cultured ovary. However, both low and high doses of GnIH did not change either the basal or the hCG- (10 IU) stimulated estradiol release. These results are similar to those from previously performed *in vivo* studies in mice, but dissimilar to *in vitro* findings generated from birds (discussed in chapter 3 section 3.4.1).

In the second part of my thesis, I found that GnIH had a stimulatory effect on oocyte final maturation. Applying a zfGnIH treatment enhanced the resumption of meiosis to a certain extent, compared to an untreated group of oocytes, but this stimulatory effect was not significant. However, and interestingly, when the fully-grown oocytes were treated with gfGnIH, they exhibited significant responsiveness, and GVBD took a place.

In the last part of my work, I was able to detect the expressions of both GnIH and GnIH-R3 in the zebrafish ovary. However, GnIH treatment, excluding 17β.HSD.1, had no significant effects on any of the transcripts examined in this study.

To summarize, GnIH has shown to be an active peptide in the gonad of adult female zebrafish. Although there were no changes at the level of gene expressions, GnIH was capable of influencing the E_2 secretion and regulating oogenesis.

5.1.3 Uncoupling of ovarian transcript expression and hormone release

Uncoupling of gene expression from steroid production has been previously reported in the literature (Ings & Van Der Kraak, 2006) in which zebrafish follicles were treated with hCG (LH-like), and the expression of genes along with the steroids production, were analyzed at different time courses (3h and 8h) *in vitro*. Compared to the untreated group (control), 3h post

incubation, hCG (10 IU) significantly stimulated the expression of STAR, 17β-HSD.3, and P450aromA whereas the E_2 secretion remained unchanged (Ings & Van Der Kraak, 2006). However, compared to the control group, 8h post incubation with hCG has shown a significant increase in the E_2 release but the STAR, 17β-HSD.3, and P450aromA mRNA abundance did not change (Ings & Van Der Kraak, 2006). This might explain the concept that at some time point transcripts levels are up regulated, with no observable hormone release, is needed to synthesize an amount of hormone required by that organ. It is possible that the results from the gene expression study supported this theory, as the amounts of the transcripts I tested were unchanged when follicles were treated with hCG and GnIH at 4h incubation. It is also possible that the expression of those genes already increased a few minutes after the incubation started and were down regulated by the time the incubation was over. There have been findings generated from another study showed the difference between the short incubation and extended incubation time on the Mitogen-activated protein kinases/MAPK, which is an integral component of LH-R down stream signaling controlling gonadal steroidogenesis (Chung & Ge, 2013). In this study, QPCR detected high activin expression at 2h of incubation while MAPK protein was barely seen by western blot during this time point (Chung & Ge, 2013).

5.2 Future direction

This thesis highlighted the engagement of GnIH in affecting zebrafish ovarian function. In zebrafish, as in other fish species, GnIH has been shown not to be a simple inhibitory peptide, as in mammals and birds. Data from this dissertation represent a paracrine stimulatory effect of GnIH at the level of female zebrafish gonads. Since the expression level of GnIH and GnIH-R were found to vary from one reproductive stage to another as the ovary develops in

some vertebrates, it would be interesting to investigate the involvement of GnIH in the early and mid stages of ovarian development. Also, due to the dimorphic function of GnIH that was shown previously in species such as goldfish, it would be beneficial to elucidate the function of RF-amide in the testes of male zebrafish. Furthermore, future studies could test the likely effect of GnIH on the duration of spermatogenesis and spermatogenesis-associated genes using morphological analysis, including cell proliferation assays and immunocytochemistry to determine the spermatogenic phase, and the expression of spermatogenic specific proteins. Androgen synthesis also could be a target of upcoming studies exploring the whether GnIH plays paracrine role in the testicular steroidogenesis in zebrafish. Additionally, it would be useful to characterize the molecular signaling pathway that mediates the action of GnIH, highlighting the importance of adenylate cyclase and cAMP components, in *Danio rerio* gonads. Answers to the previous concerns might provide more information about GnIH in zebrafish gonads, as data on its effect in teleosts are still limited and need further investigation.

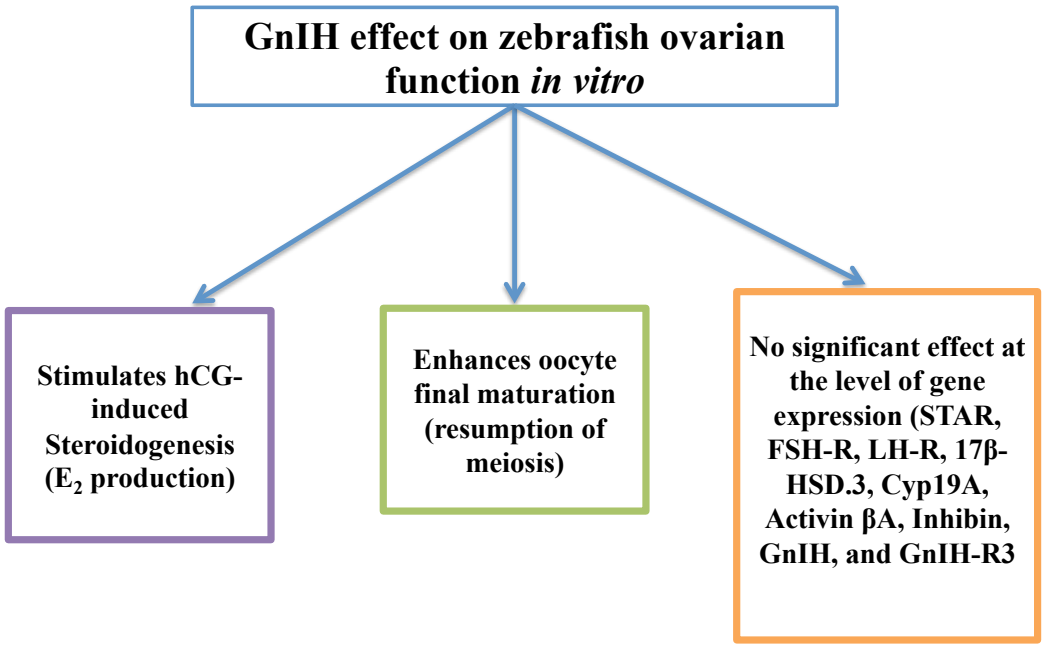


Figure 5.1 Summary of the GnIH effect on the biosynthesis of steroid, gametogenesis, and the gene expression *in vitro* using primary follicles culture.

Chapter Six

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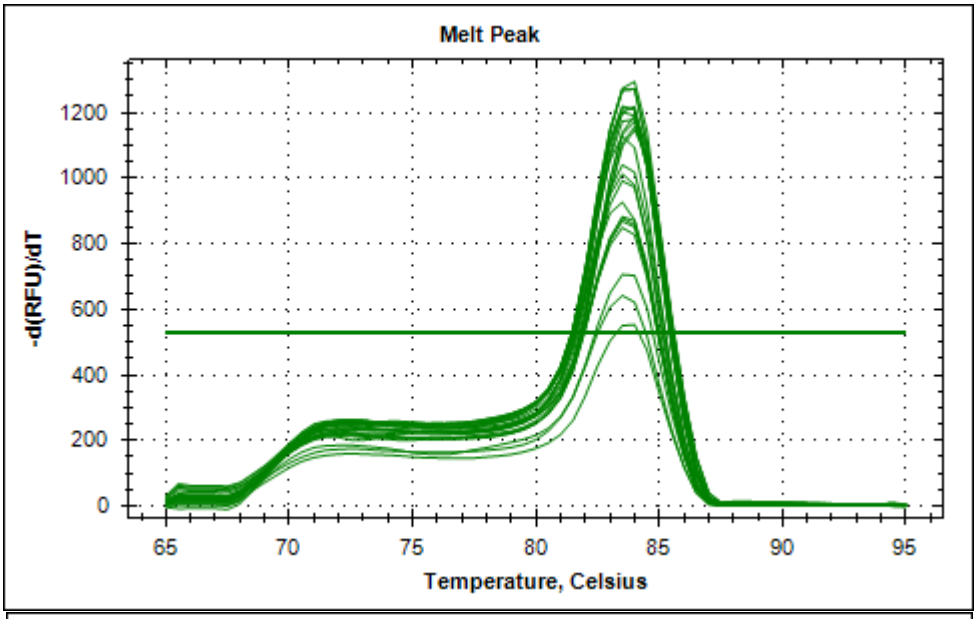
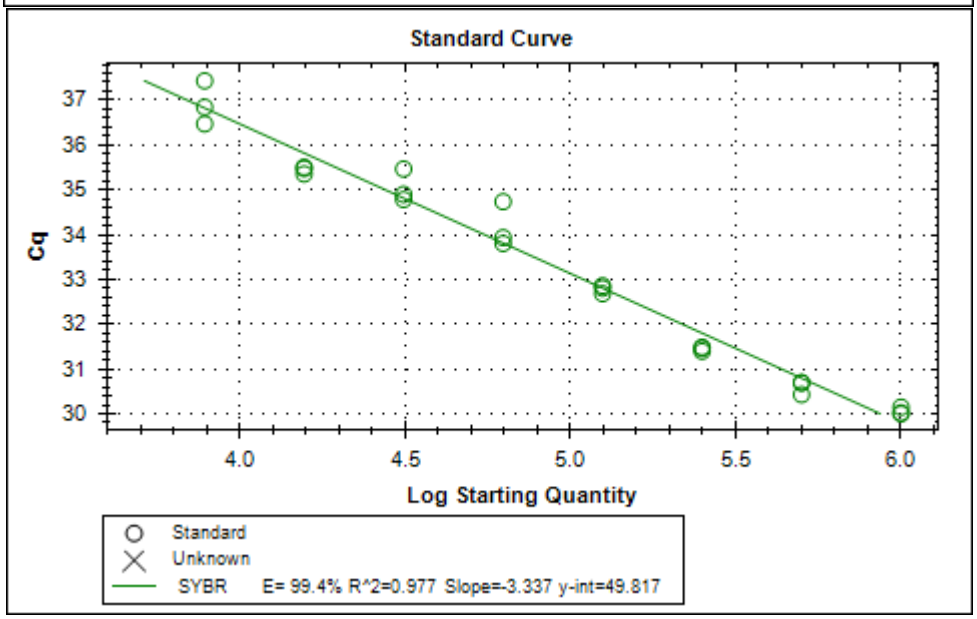
Chapter Seven

Appendix

FSH-R


Melt Peak
-d(RFU)/dT
1200
1000
800
600
400
200
0
65
70
75
80
85
90
95
Temperature, Celsius


Standard Curve
Cq
37
36
35
34
33
32
31
30
4.0
4.5
5.0
5.5
6.0
Log Starting Quantity
Standard
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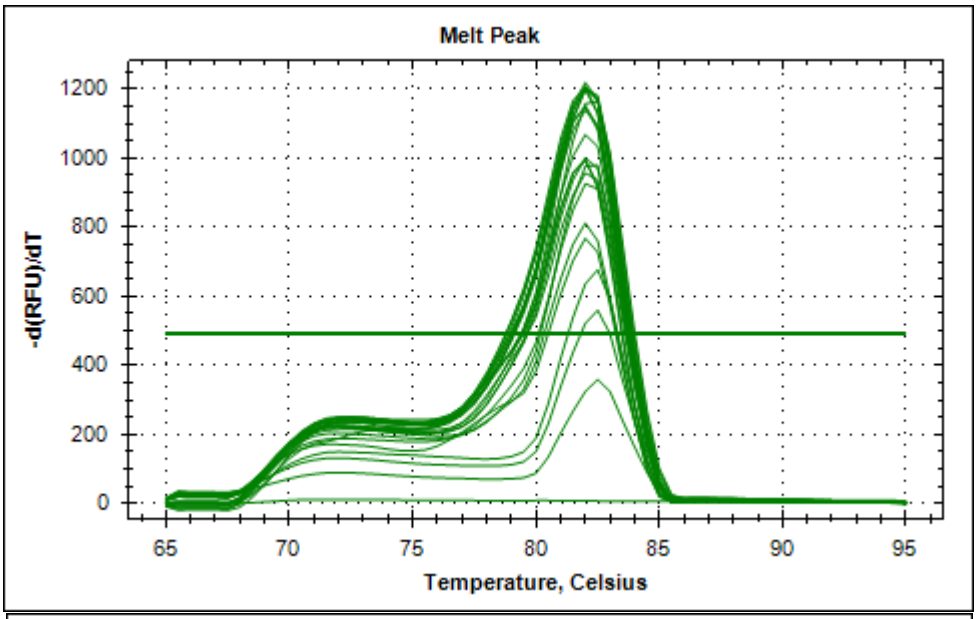
SYBR E= 99.4% R^2=0.977 Slope=-3.337 y-int=49.817

LH-R


Melt Peak
-d(RFU)/dT
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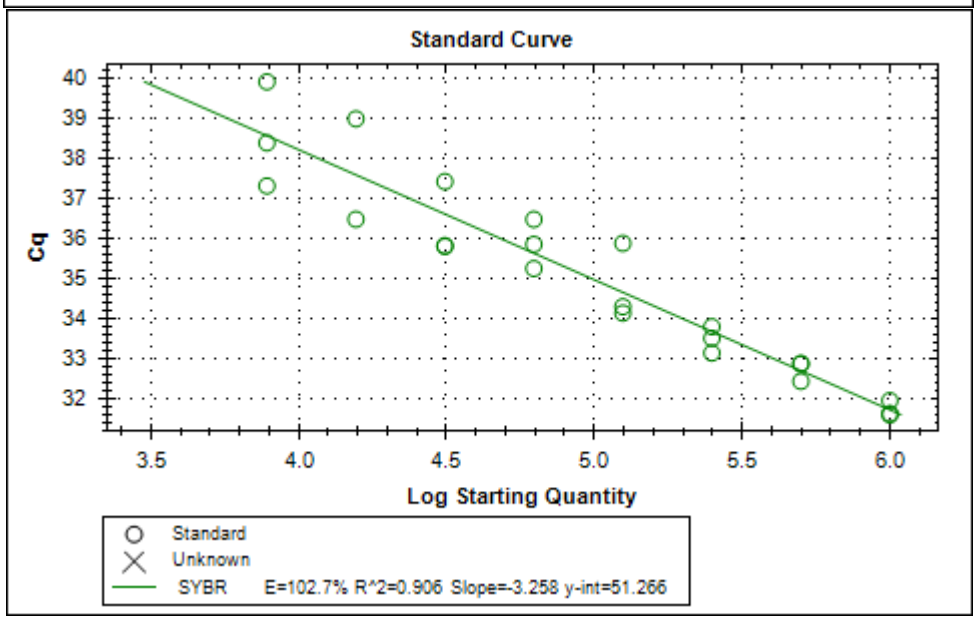
1000
800
600
400
200
0
65
70
75
80
85
90
95
Temperature, Celsius


Standard Curve
Cq
40
39
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36
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34
33
32
3.5
4.0
4.5
5.0
5.5
6.0
Log Starting Quantity
Standard
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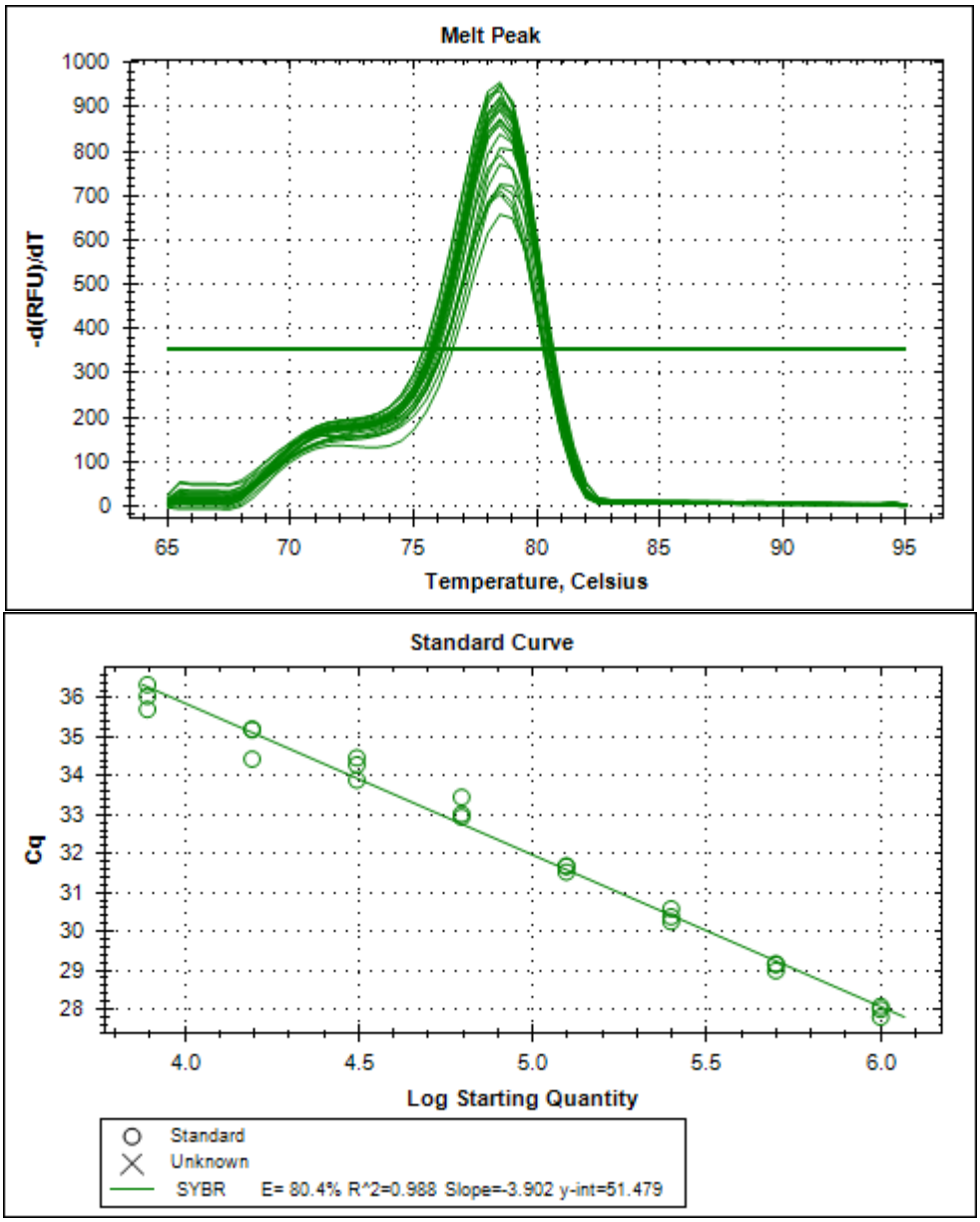
CYP19A


Melt Peak
-d(RFU)/dT
1000
900
800
700
600
500
400
300
200
100
0
65
70
75
80
85
90
95
Temperature, Celsius
Standard Curve
Cq
36
35
34
33
32
31
30
29
28
4.0
4.5
5.0
5.5
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Log Starting Quantity
Standard
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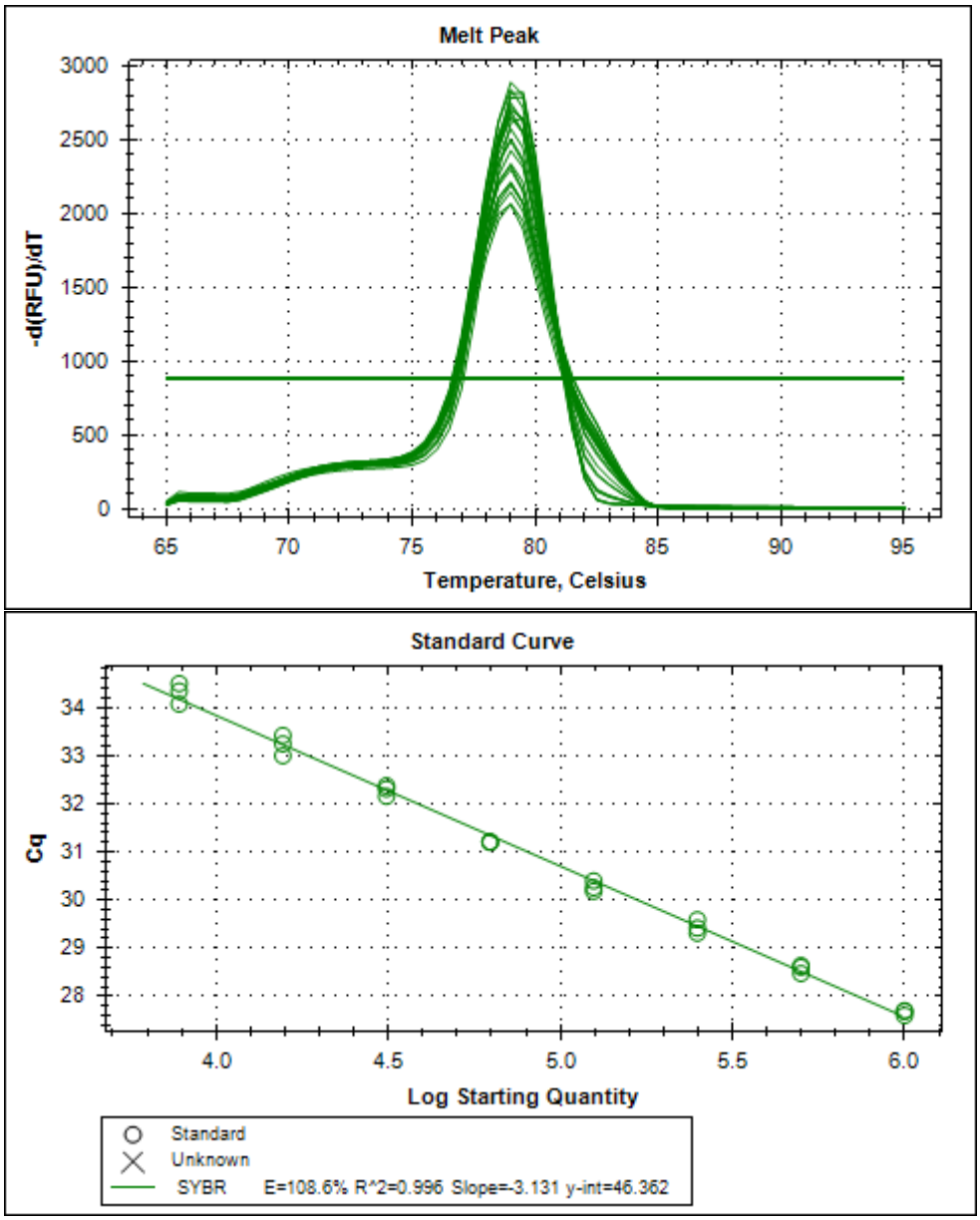
SYBR E= 80.4% R^2=0.988 Slope=-3.902 y-int=51.479

Activin βA


Melt Peak
-d(RFU)/dT
3000
2500
2000
1500
1000
500
0
65
70
75
80
85
90
95
Temperature, Celsius
Standard Curve
Cq
34
33
32
31
30
29
28
4.0
4.5
5.0
5.5
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Log Starting Quantity
Standard
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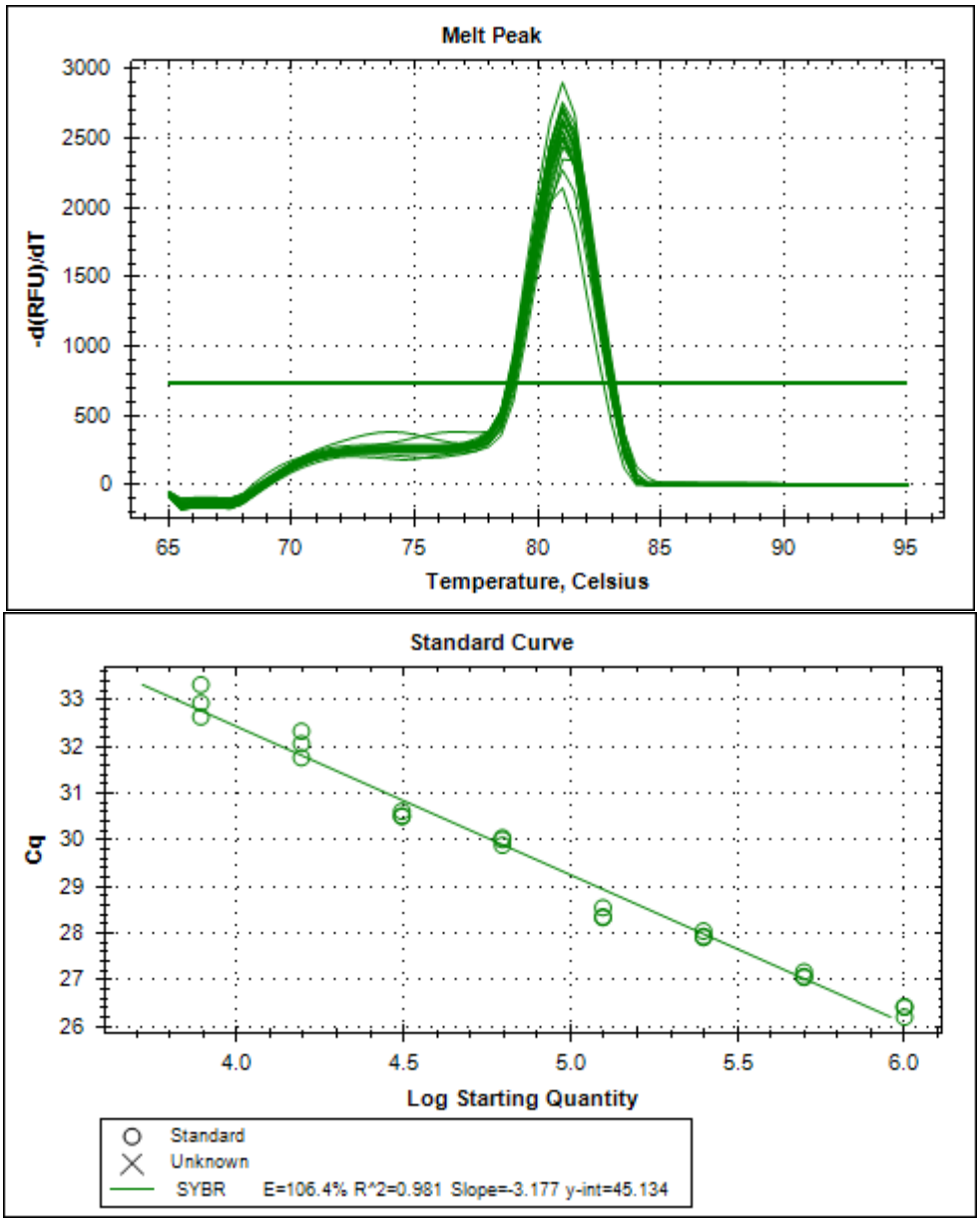
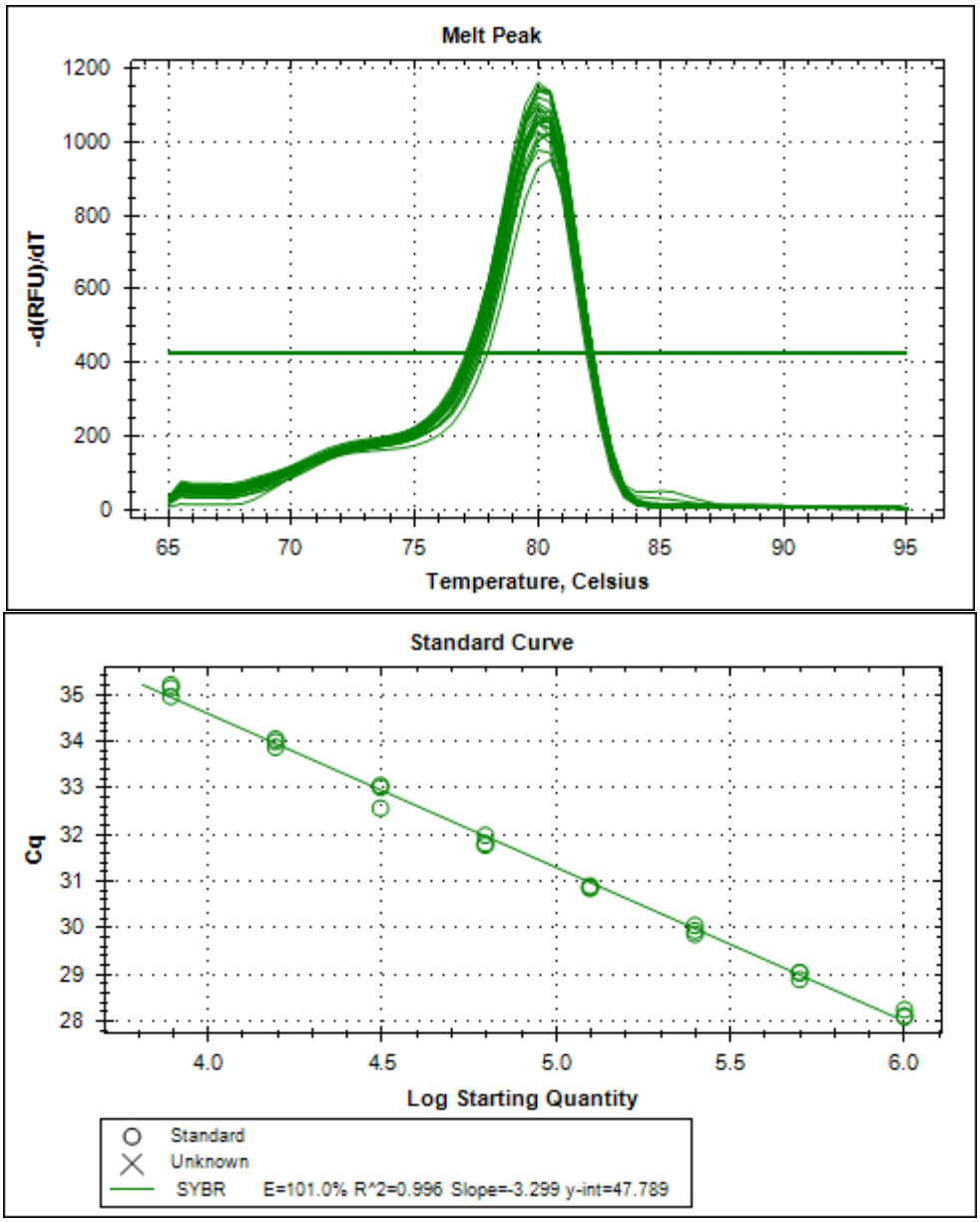
SYBR E=108.6% R^2=0.996 Slope=-3.131 y-int=46.362

17β-HSD-3


Melt Peak
-d(RFU)/dT
3000
2500
2000
1500
1000
500
0
65
70
75
80
85
90
95
Temperature, Celsius
Standard Curve
Cq
33
32
31
30
29
28
27
26
4.0
4.5
5.0
5.5
6.0
Log Starting Quantity
Standard
Unknown
SYBR
E=106.4% R^2=0.981 Slope=-3.177 y-int=45.134

GnRH-R3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-d(RFU)/dT
1200
1000
800
600
400
200
0
65
70
75
80
85
90
95
Temperature, Celsius
Standard Curve
Cq
35
34
33
32
31
30
29
28
4.0
4.5
5.0
5.5
6.0
Log Starting Quantity
Standard
Unknown
SYBR E=101.0% R^2=0.996 Slope=-3.299 y-int=47.789