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**Molecular Characterization of the Mummichog
(*Fundulus heteroclitus*) Ovarian Steroidogenic
Pathway and Implications for Exogenous
Estrogen Effects during Follicular Development**

By

Thiviya Kanagasabesan

HBSc, University of Waterloo, 2013

Thesis

Submitted to the Department of Biology

Faculty of Science

In partial fulfilment of the requirements for the

Master of Science in Integrative Biology

Wilfrid Laurier University

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Abstract

Unlike many teleosts, the estuarine killifish, *Fundulus heteroclitus* (mummichog), does not demonstrate a shift from estrogens (such as 17 β -estradiol; E₂) to progestogens (such as maturation inducing steroid; MIS) during ovarian development. While these hormonal changes associated with ovarian development have been previously described in mummichog, the primary focus of the current study was to extend those studies by investigating whether these hormonal patterns are associated with changes in gene expression within the steroidogenic pathway. Blood (plasma), ovary (tissue and ovarian follicles), liver, and brain were collected from female mummichog at various stages of maturation, classified as cortical alveolus, vitellogenic, early mature, late mature, and ovulating. Testosterone (T), E₂ and MIS, measured from plasma and *in vitro* ovarian follicle incubations, confirmed that T and E₂ production correlate with vitellogenesis and final maturation in mummichog, whereas MIS correlates with only final maturation, further establishing the lack of an E₂ drop prior to maturation. To determine patterns in mummichog steroidogenic and hormone signalling pathways, gene expression of ovarian StAR (steroidogenic acute regulatory protein), cytochrome P450 enzymes, estrogen receptors (ERs), and gonadotropin receptors, along with hepatic vitellogenin (VTG1) and ERs, and brain P450 aromatase (P450arom; CYP19b), were determined by qPCR across ovarian development. The expression of P450arom (CYP19a1; converts T to E₂), ER α , StAR (initiates cholesterol transport), P450scc (P450 side chain cleavage; converts cholesterol into pregnenolone), and hepatic VTG1 are associated with patterns of E₂ levels in maturing mummichog as expression remained continuously high or increased into late maturation; however, these genes did not follow trends exhibited in other

teleosts. CYP17 expression (converts pregnenolone to 17α -hydroxypregnenolone and then dehydroepiandrosterone, or converts progesterone to 17α -hydroxyprogesterone and then androstenedione) was not associated with E_2 patterns in mummichog, or other teleosts; expression decreased across maturation in mummichog. The secondary focus of this thesis was to determine the effects of an exogenous estrogen (EE_2 ; 17α -ethinyl estradiol) on maturing follicles. Although many teleost species exposed to environmentally-relevant concentrations (e.g., <10 ng/L EE_2) *in vivo* respond with a decrease in egg production and reproductive steroid levels, mummichog do not exhibit similar effects at much higher concentrations (e.g., >100 ng/L EE_2). Ovarian follicles (cortical alveolus, vitellogenic, early mature, and late mature) were exposed *in vitro* to 50 – 250 nM of EE_2 *in vitro* for 24 hours. While other studies have suggested that ovarian LHR and P450arom expression are estrogen-responsive in fish, addition of EE_2 *in vitro* had no effect; T and MIS production were also unaffected. Overall, these studies confirm high levels of follicular E_2 across maturation, accompanied by increasing or continuously high expression of ovarian $ER\alpha$, StAR, P450scc, and P450arom, hepatic VTG1 and brain P450arom in maturing fish. The tolerance exhibited by mummichog to exogenous estrogens could be partially attributable to continuously high levels of E_2 present in the maturing ovary, as EE_2 during follicular incubations has no effect on presumed E_2 -responsive genes.

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List of Acronyms

11-KT	11-ketotestosterone
17 α -HP	17 α - hydroxyprogesterone
17,20 β -P	17 α , 20 β -dihydroxy-4-pregnen-3-one
ANOVA	Analysis of variance
cDNA	Complementary DNA
E ₂	17 β -estradiol
EE ₂	17 α -ethinyl estradiol
EM	Early mature fish
ESR1	Estrogen receptor α
ESR2a (ER β 1)	Estrogen receptor β 1
ESR2b (ER β 2)	Estrogen receptor β 2
EDC	Endocrine disrupting compound
FM	Fully mature fish
FSH	Follicle stimulating hormone
FSHr	Follicle stimulating hormone receptor
GnRH	Gonadotropin releasing hormone
Gth	Gonadotropin hormone
GSI	Gonadosomatic index
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotropin
HPG axis	Hypothalamic pituitary gonadal axis

HSD	Hydroxysteroid dehydrogenase
L-15	Leibovitz-15 medium
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor
LSI	Liversomatic index
MIS	Maturation inducing steroid
MPF	Maturation promoting factor
OV	Ovulated fish
P450 _{scc} (CYP11)	Cytochrome P450 side-chain cleavage
P450 _{c17} (CYP17)	Cytochrome P450 c17
P450 _{arom} (CYP19)	Cytochrome P450 aromatase
R	Regressed fish
RNA	Ribonucleic acid
StAR	Steroidogenic acute regulatory protein
T	Testosterone
V	Vitellogenic fish
VTG	Vitellogenin
qPCR	Quantitative polymerase chain reaction

Chapter One: General Introduction

1.0 Introduction

This thesis is focused on characterization of aspects within the reproductive endocrine system during ovarian development in the estuarine killifish, *Fundulus heteroclitus* (mummichog). In previous studies, ovarian E₂ (17 β -estradiol) during maturation was observed to be regulated differently than in other teleosts. This difference is explored in the context of mummichog's relative insensitivity to exogenous estrogen exposure in comparison to other fish species. Chapter one provides an overview of the fish reproductive endocrine system in general, and the mummichog system in particular. Chapter two describes the experimental work undertaken for the thesis in manuscript format. Chapter three is a summary including a description of the integrative nature of the work.

1.1 Female Fish Reproductive Physiology

In the classical definition, endocrine glands secrete chemical messengers (hormones) directly into the circulatory system, to be transported to a target organ; the hormones control responses such as growth, reproduction, and metabolism (Rushton, 2009). Reproductive processes and strategies have evolved extensively among fish (Miller and Kendall, 2009), and are controlled and regulated by a balance and interconnectivity among the hormones of the hypothalamus, pituitary and gonads, referred to as the hypothalamus-pituitary-gonadal (HPG) axis (Ramezani-Fard *et al.*, 2013). Gonadal hormones control reproductive processes such as spawning, spermatogenesis (production of sperm), and oogenesis (production of eggs) (Miller and Kendall, 2009).

1.1.1 The Hypothalamus-Pituitary-Gonadal (HPG) Axis (Overview)

The HPG axis controls reproduction in fish, through the development of reproductive tissues and coordination of complex processes that occur during the annual reproductive cycle (**Figure 1.1**) (Thomas, 2008). The brain facilitates the release of gonadotropin-releasing hormones (GnRHs) (Yu *et al.*, 1997). The GnRHs are tropic peptide neurohormones produced in the neurons of the hypothalamus, which are released from their neural terminals to stimulate the release of gonadotropin hormones (GtH), from gonadotropes of the pituitary (Parhar *et al.*, 2002; Zohar *et al.*, 1995). Binding of GnRH to specific receptors on the plasma membrane of gonadotrope cells in the pituitary causes the activation of intracellular signaling pathways, which regulate the release of gonadotropins from storage (Thomas, 2008). The pituitary gland is able to facilitate reproduction in fish by using a dual GtH system: FSH (follicle stimulating hormone) and LH (luteinizing hormone), which are produced in two distinct gonadotropes of the pituitary (Ohkubo *et al.*, 2013).

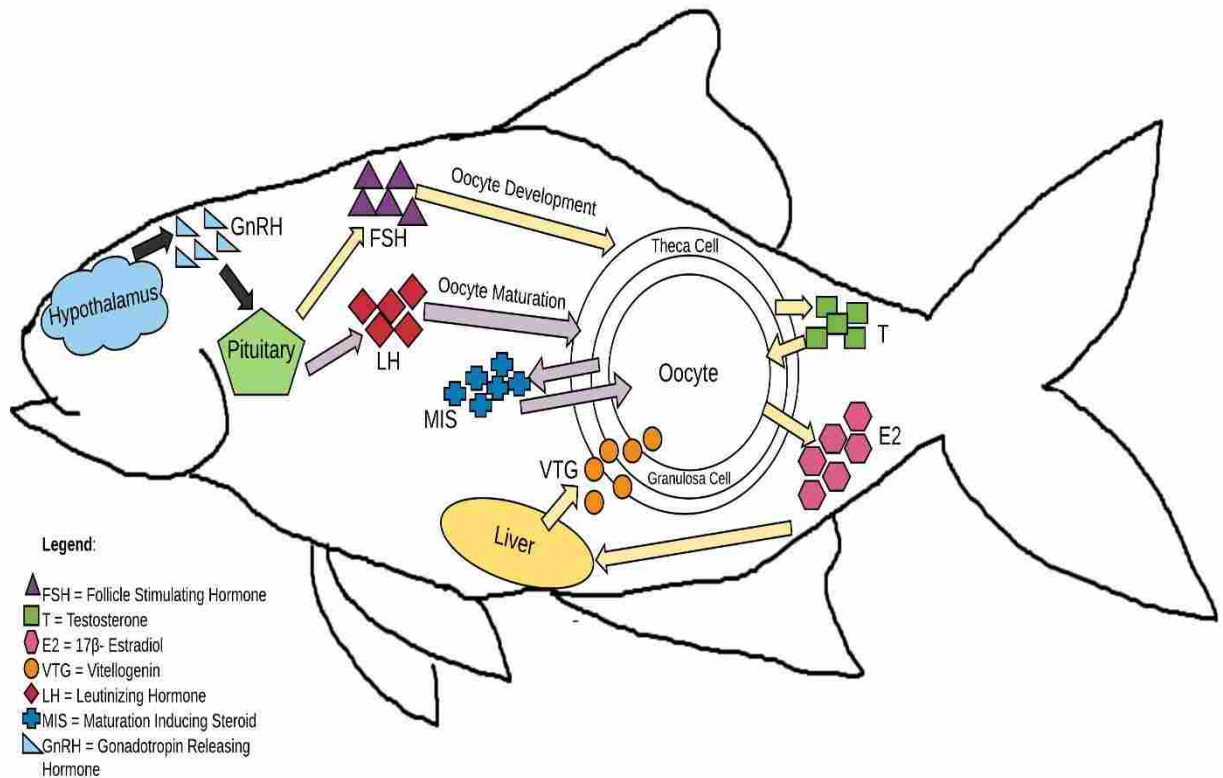


Figure 1.1. Stimulatory processes in the hypothalamus-pituitary-gonadal (HPG) axis.

Descriptions of the processes can be found in sections 1.1.1 and 1.1.2. A primary role of FSH is to stimulate E₂ production, and a primary role of LH is to stimulate MIS production. (Modified from Clelland and Peng, 2009.)

1.1.2 Gonadotropin, Steroid and Vitellogenin Regulation

FSH and LH are glycoprotein hormones consisting of both an alpha subunit and a beta subunit; the beta subunit is unique for each gonadotropin (Ohkubo *et al.*, 2013). In female fish, FSH and LH are released into the bloodstream and then bind to their respective cell surface ovarian receptors (FSHr and LHR) (Ohkubo *et al.*, 2013). FSHr and LHR activation stimulates gonadal functions, including the synthesis and release of certain steroid hormones involved in reproduction (Evans, 1999; Peter and Yu, 1997; Wang and Ge, 2004). It was first determined in coho salmon, *Oncorhynchus kisutch*, that FSH is

primarily involved in regulating gonadal steroidogenesis in the early ovarian developmental stages, especially during vitellogenesis (Swanson *et al.*, 1991; Ohkubo *et al.*, 2013). During vitellogenesis, vitellogenin (a female-specific lipoprotein) is synthesized in the liver under the stimulation of ovarian 17β -estradiol (E_2), and is secreted into the blood and transported to the ovary where it is taken up into the oocytes to stimulate egg yolk protein production during development of the oocyte (Hoar *et al.*, 1983). FSH's dominant role in vitellogenesis is to stimulate E_2 production and release from ovarian follicles into the bloodstream (Nagahama and Yamashita, 2008). In teleosts, E_2 plays a crucial role in sexual differentiation, gonadal development and steroidogenesis (the synthesis of steroids) during the reproductive cycle (Menuet *et al.*, 2005). E_2 also influences pituitary gonadotrope activity, which will in turn control the gonads through the synthesis of gonadotropins (Menuet *et al.*, 2005).

LH's primary function, on the other hand, is to regulate the final stages of ovarian development and spawning (Breton *et al.*, 1998; Tyler *et al.*, 1997), partially due to its ability to stimulate $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha, 20\beta$ -P), or maturation inducing steroid (MIS), production from the granulosa cell layer of the ovarian follicle (**Figure 1.1**; Clelland and Peng, 2009; Nagahama and Yamashita, 2008). In most teleosts, $17\alpha, 20\beta$ -P is the most effective steroid in the induction of GVBD (germinal vesicle breakdown) which occurs once a follicle is mature (Nagahama, 1983; Nagahama, 1994). MIS is responsible for inducing resumption of meiosis in teleost oocytes (Nagahama and Yamashita, 2008). The mechanism of MIS activity involved in oocyte maturation involves a complex interaction between oocyte MIS receptors and signal transduction pathways via inhibitory G-proteins (Nagahama and Yamashita, 2008). The MIS signal

induces the formation of maturation-promoting factor (MPF) and further induces GVBD and oocyte maturation (Nagahama and Yamashita, 2008).

1.1.3 Estrogen Receptor Activity

The principal mediator of E₂ activity is the nuclear estrogen receptor (Marino *et al.*, 2006). Analyzing estrogen receptor activity furthers our understanding of how E₂ is regulated and how it is involved in other signaling pathways. The activation of steroids, such as estradiol, can occur by both nuclear and membrane receptors. With membrane receptors, the steroid binds to receptors on the cell surface, resulting in activation of ion channels or intercellular secondary messengers (Thomas, 2008). With nuclear receptors, steroids bind to the hormone response elements on a gene and alter their transcription rates (Thomas, 2008). The gene expression of nuclear receptors is regulated by steroid hormones, so expression rates will fluctuate in response to changes in steroidogenesis (Thomas, 2008). The genomic effects of E₂ are mediated by specific nuclear receptors (ERs), which are able to modulate specific gene activity by acting as ligand-dependent transcription factors; when receptors are bound to the dependent ligand, the target genes will be activated (Menuet *et al.*, 2002). In teleosts, two ER subtypes have been identified: ER α and ER β ; there are two distinct forms of ER β : ER β 1 and ER β 2 (Menuet *et al.*, 2002).

1.2 Steroidogenesis

Hormones are derived from cholesterol in the process known as steroidogenesis (**Figure 1.2**), which involves a number of intermediates and enzymatic conversions prior to their release from the gonads (Arukwe, 2008; Leusch and MacLatchy, 2003; Young *et al.*, 2005). The synthesis of the different classes of gonadal steroids depends on the

delivery of the substrate cholesterol, and its conversion by three enzymes belonging to the cytochrome P450 superfamily (Young *et al.*, 2005). Steroidogenic acute regulatory protein (StAR) is a sterol transport protein which initiates cholesterol transport into the mitochondria (Rone *et al.*, 2009). Cytochrome P450 side-chain cleavage (P450 scc) is located at the inner mitochondrial membrane and converts cholesterol to pregnenolone (Young *et al.*, 2005). Pregnenolone is a basal steroid that can serve as a substrate for cytochrome P450 C17; P450 C17 can catalyze the hydroxylation of pregnenolone to yield 17 α -hydroxypregnenolone and then dehydroepiandrostone, or the hydroxylation of progesterone to yield 17 α -hydroxyprogesterone (17 α -HP) and then androstenedione (Young *et al.*, 2005). Testosterone (T) is created from the conversion of androstenedione with the enzyme 17 β -HSD (HSD: hydroxysteroid dehydrogenase), which is later converted into 11-ketotestosterone (11-KT) in males. The synthesis of estrogens depends on cytochrome P450 aromatase (P450arom), which uses T as a substrate for conversion into E₂; changes to P450arom are responsible for the drop in estrogen levels that can occur in many teleosts prior to maturation (Young *et al.*, 2005). During the maturation of oocytes, post-vitellogenic follicles have to synthesize a large amount of MIS (Arukwe, 2008). MIS is produced from the conversion of 17 α -hydroxyprogesterone to 17 α , 20 β -P, with the enzyme 20 β -HSD, which is believed to be initiated with a surge in LH production (Barry *et al.*, 1990; Young *et al.*, 2005).

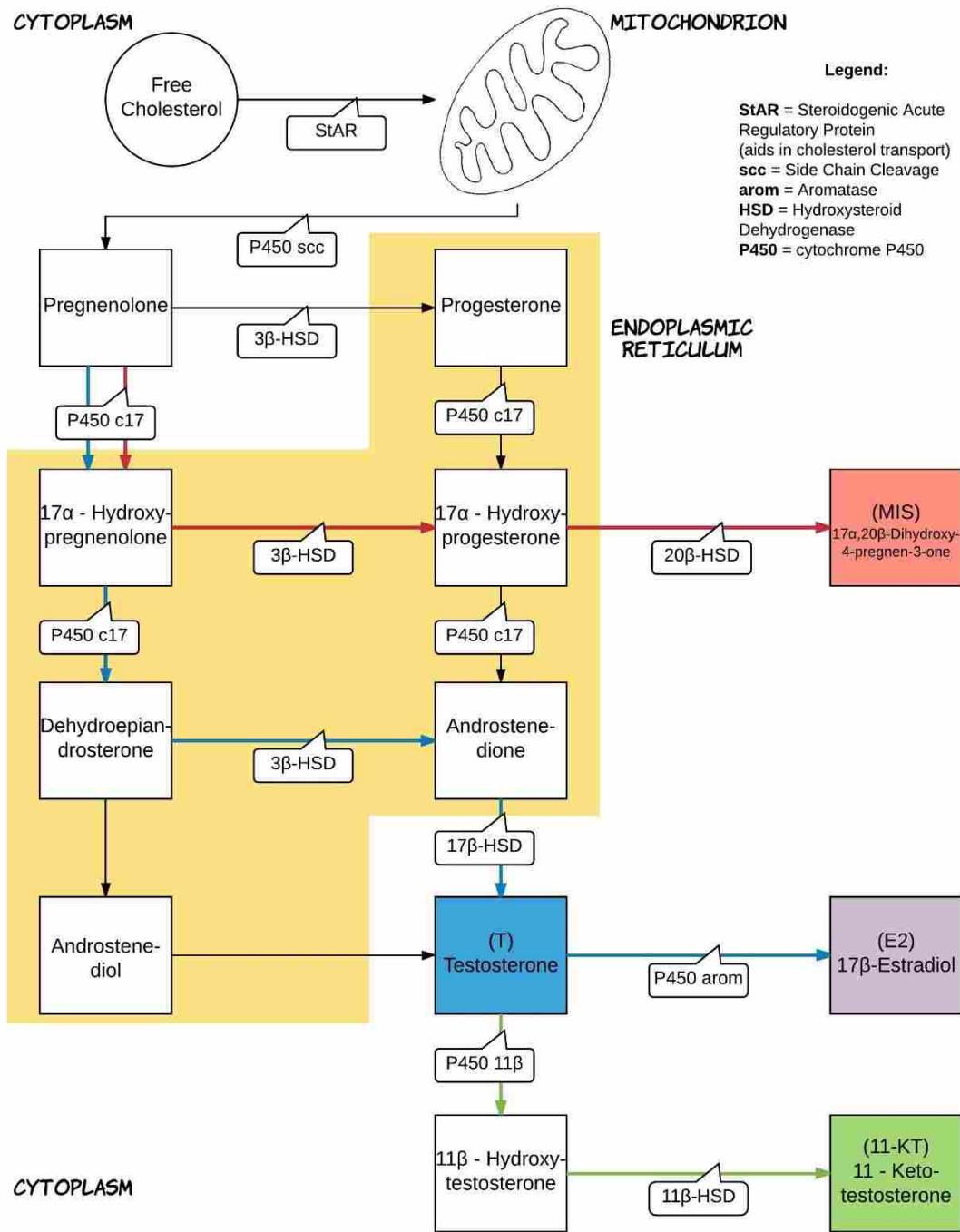


Figure 1.2. The steroidogenic pathway, responsible for the production of sex steroids such as MIS, T and E₂ from cholesterol through multiple enzymatic reactions as explained in section 1.2. (Modified from Young *et al.*, 2005).

1.3 The Ovarian Development Cycle

Ovarian development in teleosts varies from species to species, in both the duration of the cycle as well as the mechanisms involved in regulating the cycle; the duration of the cycle in fish can vary from a few days to a few years (Khan and Thomas, 1999). Oocyte maturation is the process whereby ovarian follicles develop (with an enormous growth of oocytes due to accumulation of yolk proteins in cytoplasm) and become mature (including germinal vesicle breakdown, chromosome condensation, assembly of the meiotic spindle and formation of the first polar body) prior to ovulation, which is required for successful fertilization (Nagahama and Yamashita, 2008).

Oogenesis (oocyte maturation) can be described in six major steps: formation of PGCs (germline segregation), transformation of PGCs into oogonia (sex differentiation), transformation of oogonia into oocytes (onset of meiosis), growth of oocytes while under meiotic arrest, resumption of meiosis (maturation), and expulsion of the ovum from its follicle (ovulation) (Patino and Sullivan, 2002).

The ovarian follicle, which surrounds each oocyte, consists of two major cell layers: an outer thecal cell layer and an inner granulosa cell layer (**Figure 1.3**; Clelland and Peng, 2009). As oocytes grow, follicle cells multiply and form a continuous follicular layer (granulosa cell layer); the follicular layer changes in order to support, nourish and regulate oocyte development in a continuous manner (Nagahama, 1994). In teleosts, growth and development of ovarian follicles are dependent on the actions of gonadotropin FSH and the steroid E₂; whereas maturation of ovarian follicles is regulated by gonadotropin LH and the steroid MIS (Nagahama and Yamashita, 2008). In the two-cell type model, the thecal cell layer will secrete androgen substrates, such as T and 17 α -

HP, while under the influence of gonadotropins. In the granulosa cells, 17α -HP and testosterone will be converted into MIS and E_2 (Nagahama and Yamashita, 2008; Yamashita *et al.*, 1995). In this model, prior to ovulation, E_2 levels decrease and allow for significantly increased levels of MIS, which is required to complete maturation of the oocyte (Nagahama and Yamashita, 2008). Human chorionic gonadotropin (hCG) is used in experimental studies of ovarian development, as it is effective in inducing *in vitro*, and sometimes *in vivo*, oocyte maturation and ovulation in some teleosts; hCG mimics LH activity (Harvey and Hoar, 1979; Zuberi *et al.*, 2011).

The stages of oogenesis in commonly-studied teleosts, e.g., zebrafish (*Danio rerio*), are generally divided into growth/developmental stages and maturational stages (**Figure 1.4**). The developmental stages of oogenesis encompass the primary growth, cortical alveolus, and vitellogenic stage, and are predominately regulated by increasing levels of E_2 and FSH. During primary growth (prior to stage 1), oocytes begin to grow in size while the germinal vesicle (GV; oocyte nucleus) appears in the center of the oocyte, and a single layer of follicle cells surrounds the oocyte, eventually forming a vitelline envelope (Lyman-Gingerich and Pelegri, 2007). Stage 1 (cortical alveolus) is when the cortical alveoli (bound vesicles) appear in the ooplasm and the vitelline membrane contains three separate layers which surrounds the oocyte (Lyman-Gingerich and Pelegri, 2007). Stage 2 (vitellogenesis) is when oocytes undergo vitellogenesis and acquire large amounts of vitellogenin, displacing cortical alveoli to the cortex of the oocyte (Clelland and Peng, 2009; Lyman-Gingerich and Pelegri, 2007). Stages 3-5 are maturational stages, which are commonly initiated with the drop in E_2 levels and rise in MIS and LH levels. The maturational stages of oogenesis allow for drastic morphological changes in

accordance with the progression of meiosis, and allow for GVBD as well; the GV, which is usually located in the center of the oocyte, migrates to the animal pole with hormonal stimulation, where GVBD can occur (Suwa and Yamashita, 2007). Stage 3 (early maturation) is when oocytes are able to respond to LH, secreted from the pituitary, and MIS, synthesized and secreted from the follicle following MPF (maturation promoting factor) stimulation (Yamashita, 2000). Stage 4 (late maturation) is when the germinal vesicle migrates from the center of the oocyte to the periphery and the nuclear membrane breaks down (Selman *et al.*, 1993). Stage 5 (ovulated) is when mature eggs are ovulated (oocyte is released from follicle complex and are ready for spawning; Lyman-Gingerich and Pelegri, 2007).

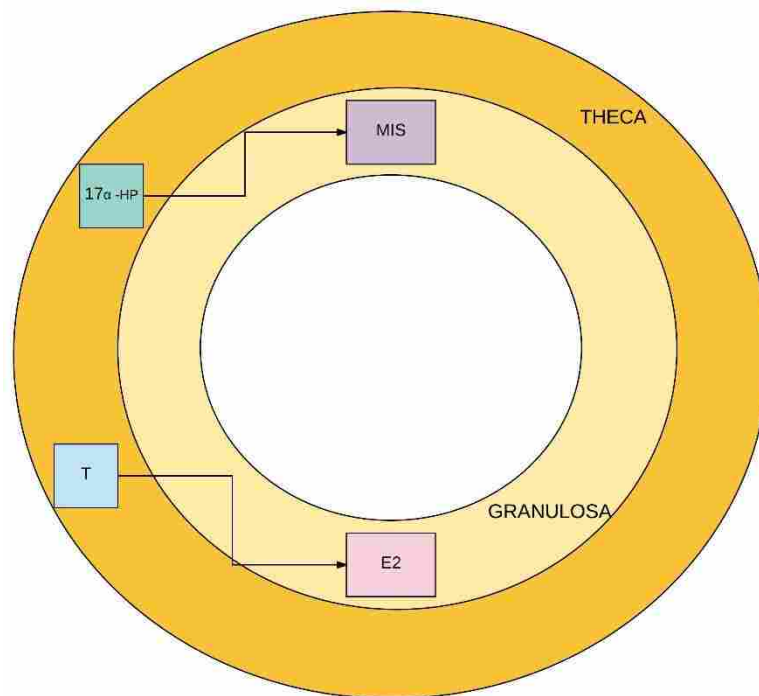


Figure 1.3. The two-cell type model in teleost oocytes. Major hormones, E₂ (17β-estradiol), MIS (maturation inducing steroid), T (testosterone) and 17α-HP (17α-hydroxyprogesterone), involved in oocyte growth and maturation outlined in the two layers (theca and granulosa) of the oocyte are shown. Descriptions of these processes can be found in section 1.3. (Modified from Clelland and Peng, 2009).

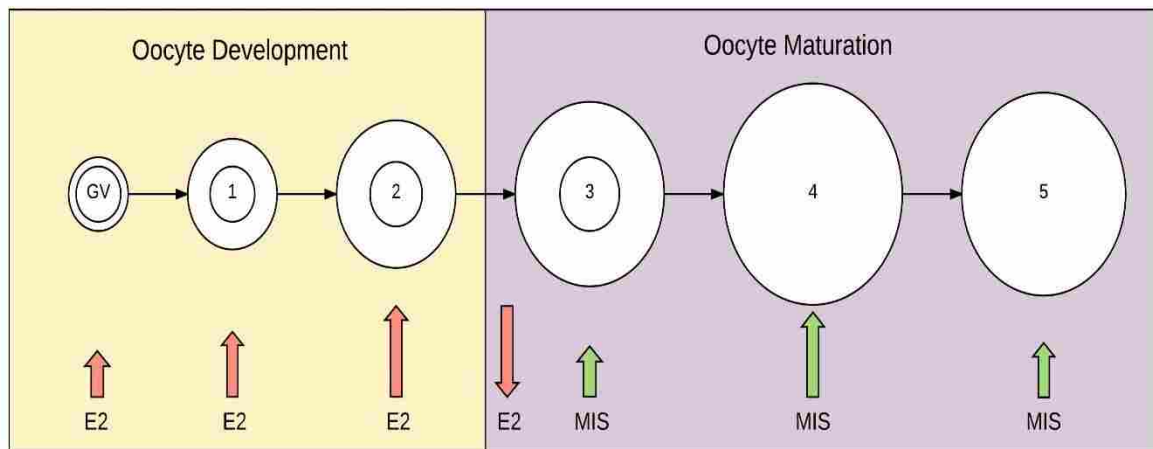


Figure 1.4. Stages of ovarian development in commonly-studied teleosts with a two-cell type model, which are regulated by processes as explained in section 1.3 and Figure 1.3, separated by oocyte development and oocyte maturation stages. Primary growth stage = prior to stage 1; stage 1 = cortical alveolus stage; stage 2 = vitellogenic stage; stage 3 = early maturation stage; stage 4 = late maturation stage; stage 5 = ovulated stage. Germinal vesicle (GV) breakdown occurs when oocytes become fully mature in stage 4. Regulation of hormones E₂ (17β-estradiol) and MIS (maturation inducing steroid) are depicted with relative increasing and decreasing arrows. (Modified from Selman *et al.*, 1993).

In mummichog, however, thecal cells (endocrine cells which play an essential role in producing androgen substrate required for ovarian estrogen biosynthesis (Magoffin, 2005)) are not evident in the thecal layer (Nagahama, 1994). In mummichog, granulosa cells are the major site of steroid synthesis, and respond to mummichog pituitary extract *in vitro* (Petrino *et al.*, 1989). The production of E₂ and 17,20β-P does not require the involvement of two cell types (Petrino *et al.*, 1989). Petrino *et al.* (1989) also concluded that 17, 20β-P plays a major role as a maturation inducing steroid in mummichog, even though it is not the only active steroid produced by maturing follicles (as E₂ production remains high).

1.3.1 Shift in Steroidogenesis Prior to Oocyte Maturation

In most teleost ovarian development processes studied to date, there is a shift that occurs between the developmental stages and maturational stages in the ovarian development cycle; a shift from estrogenic to progestational steroid production when follicles progress from vitellogenesis to a prematuration stage (Nagahama and Yamashita, 2008). As mentioned earlier, E₂ (regulated by FSH) is produced in correlation with vitellogenesis; whereas MIS (regulated by LH) is produced in correlation with the onset of maturation of oocytes. It has been demonstrated in several teleost fish that the steroid 17α, 20β-P (MIS) is the most effective steroid in stimulating oocyte maturation (Nagahama, 1983; Nagahama, 1987). In goldfish, *Carassius auratus*, E₂ production reduces greatly in the prematuration stage when compared to vitellogenic follicles (Kagawa *et al.*, 1984). Maximal production levels of T were reported in the tertiary yolk stage follicles with hCG (LH analogue) stimulation (Kagawa *et al.*, 1984). In coho salmon,

the concentration of E₂ was reduced while the production of T and MIS increased with advancing oocyte development (Van Der Kraak and Donaldson, 1986).

In mummichog, however, there is no shift detected as the levels of E₂ are consistently high in all stages of follicles (Lin *et al.*, 1987). Ultimately, high levels of E₂ coexist with high progestagen levels in mummichog (Lin *et al.*, 1987). Lin *et al.* (1987) also demonstrated that mummichog gonadotropins show a noticeable species specificity, as mummichog follicles exhibit both a seasonal and size dependent responsiveness to gonadotropins; the steroidogenic responses of mummichog ovarian follicles were found to be very dependent on the stage of follicular development.

1.4 Endocrine Disrupting Compounds

There are numerous natural and anthropogenic chemicals discharged into freshwater and estuarine systems that are capable of disrupting the endocrine systems of aquatic organisms (Sumpter, 2005). Endocrine-disrupting chemicals (EDCs) have been defined by the World Health Organization's International Programme on Chemical Safety (2002), as: "an exogenous substance which alters function(s) of the endocrine system and ultimately causes adverse health effects in an organism, its offspring or (sub) populations." EDCs have the potential to disturb sensitive hormone pathways that regulate growth and reproductive functions in aquatic organisms (Arcand-Hoy and Benson, 1998).

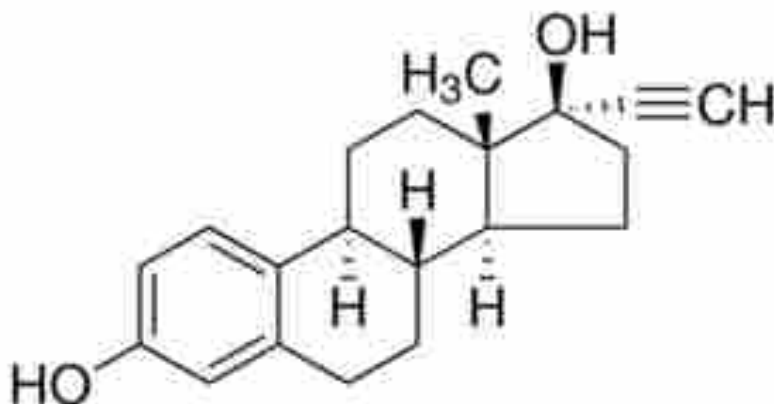
Fish are primary targets for waterborne endocrine disruptors, many of which are xenostrogens (synthetic or natural estrogen-mimicking or -blocking compounds) (Menuet *et al.*, 2005), making the impact of xenostrogens on aquatic ecosystems and fish a subject of great interest. Reports of reproductive problems in North American and

European freshwater and marine fish populations have sparked a wave of research, and raised awareness on the potential risks of EDCs to human and environmental health (Diamanti-Kandarakis *et al.*, 2009; UNEP/WHO, 2013). The feminization of male fish due to exogenous estrogen exposure has been demonstrated in several studies such as in Kidd *et al.*'s (2007) whole-lake study with fathead minnow (*Pimephales promelas*), which showed feminization in males following exposure to 5-6 ng/L of 17 α -ethinyl estradiol (EE₂), and ultimately a near extinction of the species studied from the lake. Schwindt *et al.* (2014) suggested that fish populations exposed to environmentally-relevant EE₂ concentrations may not recover from exposure, and recommended improved wastewater processing technology to improve removal of bioactive chemicals such as environmental estrogens. Understanding the mechanisms of action for xenostrogens is an important step in evaluating the impact of these substances on fish reproductive physiology, and of assessing risk to fish populations in vulnerable ecosystems (UNEP/WHO, 2013).

1.4.1 EE₂: 17 α -Ethinyl Estradiol

The synthetic steroid EE₂ (Figure 1.5A), is one of the most commonly-used active ingredients for oral contraception and related medicines (Lange *et al.*, 2001). Estrogenic EDCs (including estrone, E₂, estriol, and EE₂) have been shown to enter the aquatic environment via effluent discharges from sewage treatment works (Sun *et al.*, 2014). The hormones present in pharmaceuticals (including synthetic estrogens) are not completely broken down through municipal wastewater sewage treatment plants (Ternes *et al.*, 1999). The chemical structure of EE₂ differs slightly from the chemical structure of the natural estrogen, E₂ (Figure 1.5B), by an additional ethinyl group in EE₂; this difference

leads to changes in biodegradability, making EE₂ more resistant to degradation of microorganisms in wastewater treatment systems (Ternes *et al.*, 1999). As a result, wastewater effluent from municipal treatment plants discharge EE₂ into receiving waters in both treated and untreated waste waters, and can be found at 0 to 34 ng/L in different bodies of water (rivers, estuaries, bays, lagoons, surface water, etc) internationally, as well as in sediments within those bodies of water (Aris *et al.*, 2014; Monteiro and Boxall, 2010). Compounding their reproductive effects, EE₂ levels are a concern in aquatic environments because of EE₂'s high resistance to the process of degradation and its tendency to absorb organic matter, accumulate in sediment and concentrate in biota (Aris *et al.*, 2014; De Wit *et al.*, 2010). Effluents from municipal wastewater contain mixtures of various estrogens and their mimics, varying in toxicity (Desbrow *et al.*, 1998). Within this group of substances, EE₂ is one of the more potent synthetic estrogens present, and has been linked to serious effects on fish development and reproductive status (Desbrow *et al.*, 1998).



A

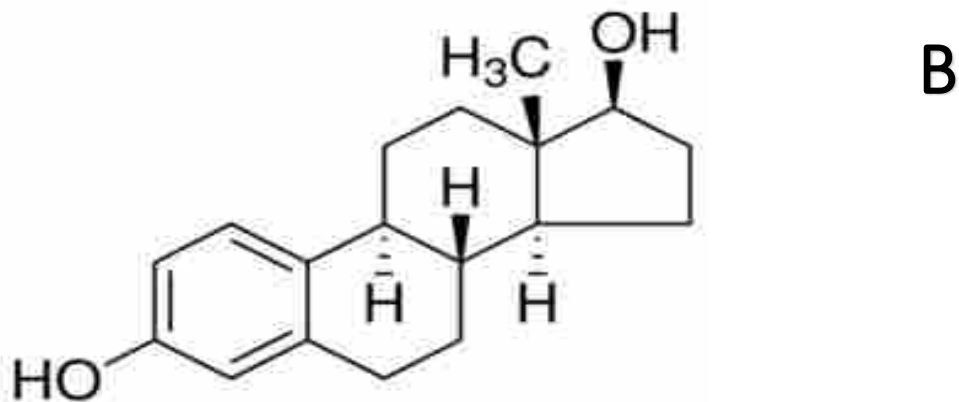


Figure 1.5: The chemical structure of the synthetic estrogen, 17 α -ethinylestradiol (EE₂; A), and the natural estrogen, 17 β -estradiol (E₂; B) (Sigma-Aldrich, 2015).

1.4.2 Effects of EE₂ on Teleosts

It has been demonstrated that EE₂ can negatively impact the reproductive system and related processes in several teleosts. These effects on the reproductive status and development of fish can have detrimental long-term effects on fish populations, which can also impact the ecosystem. Mummichog, however, typically respond at higher concentrations of EE₂ (generally 100 ng/L or higher) compared to the levels (<10 ng/L) to which other fish respond. For instance, this can be seen when comparing population and reproductive health effects between commonly-studied freshwater teleosts and mummichog. EE₂ exposure caused intersex (feminization) and induced vitellogenin (female egg yolk precursor protein) production in fathead minnow at 5-6 ng/L of EE₂ during a 7-year whole-lake experiment (Kidd *et al.*, 2007), and in zebrafish when exposed to 5 ng/L EE₂ in a full lifecycle exposure (Nash *et al.*, 2004). Parrott and Blunt (2005) demonstrated significant effects on fathead minnow fertilization success, sex ratio, male secondary sex characteristics, GSI (gonadal somatic index), LSI (liver somatic index) and growth at 0.32-23 ng/L EE₂ (nominal), or 0.0-1.5 ng/L of EE₂ after RIA

(radioimmunoassay) detection, 150 days post hatch. In mummichog, reproductive endpoints do not appear to be affected at similar concentrations of EE₂, when compared to other teleosts. In mummichog, induced male plasma vitellogenin and vitellogenin mRNA has been demonstrated at higher concentrations of 100 ng/L EE₂, after 21-day and 14-day (respectively) exposures (Hogan *et al.*, 2010; Peters *et al.*, 2007). However, EE₂-treated mummichog larvae showed complete feminization (all treated larvae exhibited female phenotype) at 10 weeks post hatch, including a low exposure concentration of 10 ng/L (Chehade, 2012), similar to effects in other species.

Reproduction is impacted by EE₂ exposure in teleosts, as egg production was significantly reduced in fathead minnow at concentrations of 0.47-3.92 ng/L after a 21-day exposure (Armstrong *et al.*, 2016). Other freshwater species such as Chinese rare minnow (*Gobiocypris rarus*) exposed to 0.2 ng/L EE₂ (Zha *et al.*, 2008) and zebrafish exposed to 1 ng/L EE₂ (Lin and Janz, 2006) or 25 ng/L for seven days (Schilling, 2015) exhibited significantly reduced egg production. EE₂ does not have such a consistent low-concentration effect on reproduction in mummichog, as egg production decreased in females and fertilization decreased in males at only 100 ng/L, as assessed at 28 days (Peters *et al.*, 2007). A study by Bosker *et al.* (2016), has further shown that the cumulative egg production per female was unaffected by high concentrations of EE₂ at 100 ng/L (nominal), or 84.1 ng/l ± 6.0 actual, after 28 days of exposure.

EE₂'s strong impacts on fish reproduction may be mediated by effect on sex steroids that regulate reproductive processes. In fish, estrogen mimics compete with naturally-occurring estrogen for binding to the estrogen receptors and disrupt the steroidogenic pathway, which can consequently depress plasma hormone levels (Hogan

et al., 2010). Hormones T, 11-KT and E₂ were notably lower in male fathead minnow after exposure to 10 ng/L EE₂ for 21 days (Salierno and Kane, 2009). In mummichog, some hormones were affected by EE₂ at high (e.g., 100 ng/L) concentrations. Female plasma T and E₂ significantly decreased after exposure to EE₂ at 100 ng/L for 21 days; plasma E₂ also decreased at 10 ng/L EE₂ after 28 days (Peters *et al.*, 2007). Hogan *et al.* (2010) demonstrated different effects, as male and female plasma T levels did not differ significantly among treatments of 100 and 500 ng/L (nominal) of EE₂ for 14 days, or 67.9 ± 7.4 and 247.9 ± 12 (average) ng/L. Gillio Meina *et al.* (2013) demonstrated decreases in female E₂ and male 11-KT at 250 ng/L EE₂. These data substantiate the general trend of EE₂ effects on mummichog at higher than environmental levels, unlike what has been found in most other fish species studied.

EDCs have been shown to exert direct effects on gonadal steroidogenesis in teleosts through various mechanisms (Thomas, 2008). EDCs, such as exogenous estrogens, may disrupt the steroidogenic pathway by interfering with the gene expression of specific enzymes at key steps (Hogan *et al.*, 2010). For instance, steroidogenic enzymes (P450_{scc}, P450_{c17}, and 3 β -HSD) expression decreased with EE₂ exposure (8 days of oral treatment at 20 mg/kg) in rainbow trout (Baron *et al.*, 2005). In fathead minnow, steroidogenic enzymes StAR, P450_{scc} and P450_{c17} were downregulated with 2-50 ng/L EE₂; P450_{arom} was not significantly altered, but showed a downward tendency (Garcia-Reyero *et al.*, 2009). On the other hand, there was no effect on steroidogenic enzymes StAR, P450_{scc} and P450_{arom} in mummichog ovaries after a 14-day exposure to 250 ng/L EE₂ (Doyle *et al.*, 2013).

1.4.3 Potential Mechanisms for High Sensitivity to EE₂ in Mummichog

The mechanistic reasons in mummichog for the differences in effects following EE₂ exposure compared to other freshwater teleosts is not known. Previous studies have tried to determine the factors which cause lower sensitivity in mummichog by focusing on environmental and physiological variables. Environmental variables that have been investigated to some extent include salinity, temperature and hypoxia. Salinity was analyzed as mummichog live in estuarine environments with fluctuating salinity, and most fish commonly used in EE₂ exposure studies are freshwater fish. Salinity increases EE₂ uptake (Blewett *et al.*, 2013), but has no significant effect on plasma steroids or gonadal steroidogenesis (Gillio Meina *et al.*, 2013). Increased temperature results in greater EE₂ uptake (Blewett *et al.*, 2013). Increased temperature at 26°C, compared to 10°C and 18°C, resulted in increased gonad weight in male and female mummichog and decreased LSI, yet there was no interaction between temperature and EE₂ on either plasma E₂ or T in females, or plasma T and 11-KT in males (Gillio Meina *et al.*, 2013). In hypoxia, there is little change on the effect on EE₂ uptake in mummichog (Blewett *et al.*, 2013).

Tissue distribution of EE₂ accumulation after exposure in mummichog has also been examined through a comparative study to other species. In mummichog, gallbladder and liver accumulated the largest amount of radiolabeled EE₂ (50%); the carcass and the gut were the next highest accumulators (Blewett *et al.*, 2014). Alternatively, EE₂ was quickly associated with the carcass (50%) in fathead minnow, goldfish, zebrafish and rainbow trout (Blewett *et al.*, 2014). This could indicate that EE₂ is potentially being metabolized and cleared more quickly in mummichog compared to other teleosts, thus

protecting the reproductive system from deleterious effects of EE₂. This hypothesis has not been further investigated and, to date, is the most evidence-based explanation for the differences in EE₂ sensitivity between mummichog and other fish species.

1.5 Objectives

The hypothesis for this thesis is that the differences in E₂ regulation during ovarian development between mummichog and other teleosts may play a part in the species-specific sensitivity of mummichog to EE₂. In most teleosts, the shift between the developmental stages and maturational stages in oogenesis is accompanied by a shift from estrogenic (E₂) to progestational (MIS) steroid production when follicles progress from vitellogenesis to a prematurational stage (Nagahama and Yamashita, 2008). These key steroids, such as E₂, T and MIS regulate the ovarian development cycle. E₂ is stimulated by FSH and is produced in correlation with vitellogenesis (involved in developmental stages); whereas MIS is stimulated by LH and is produced in correlation with the onset of maturation of oocytes (involved in maturational stages). In mummichog, however, there is no shift detected as the levels of E₂ are consistently high in all stages of follicles and coexist with high progestagen levels in mummichog (Lin *et al.*, 1987). Because mummichog have continuously high levels of E₂ across maturation, this may cause a decrease in ovarian sensitivity to EE₂, possibly through molecular pathways in the steroidogenic pathway. EDCs, such as EE₂, have been shown to exert direct effects on gonadal steroidogenesis in teleosts through various mechanisms, and also potentially alters gonadal steroid production at specific sites within the steroidogenic pathway (MacLatchy *et al.*, 2003; Hogan *et al.*, 2010; Peters *et al.*, 2007; Thomas, 2008). Therefore, the present study focused on the molecular characterization of the ovarian

steroidogenic pathway across the maturational stages in mummichog to firstly, better understand the molecular changes that occur during steroidogenesis and secondly, the effects of EE₂ *in vitro* on maturing ovarian follicles.

The approach used was to initially replicate and confirm the current understanding of mummichog maturation as it relates to ovarian steroidogenesis (e.g., changes in plasma and follicular steroids during maturation), and to then add to the body of knowledge by characterizing for the first time the gene expression changes across the ovarian development cycle, using *in vitro* ovarian follicle incubations. With this deeper mechanistic understanding of mummichog ovarian steroidogenesis, it was then possible to undertake a preliminary *in vitro* ovarian follicle exposure across maturational stages to EE₂, to determine the effect on gene expression endpoints known to be estrogen sensitive in other teleost species. Together, these studies have progressed our understanding of mummichog ovarian development and provided a potential addition to the mechanistic understanding of the general resistance of mummichog to environmentally-relevant levels of EE₂.

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Chapter Two

2.1 Abstract

This study investigated ovarian development in *Fundulus heteroclitus* (mummichog), through molecular characterization of key mediators in the ovarian steroidogenic pathway; these included ovarian StAR (steroidogenic acute regulatory protein), cytochrome P450 enzymes, estrogen receptors (ER α , ER β 1 and ER β 2), and gonadotropin receptors (FSHr and LHr); additionally, hepatic vitellogenin (VTG1) and ERs, and brain P450 aromatase (P450arom; CYP19b) were studied. Fish were staged into cortical alveolus, vitellogenic, early mature, late mature, and ovulating stages of maturation; blood (plasma), ovary (tissue and isolated ovarian follicles per stage), liver, and brains were collected. Testosterone (T), 17 β -estradiol (E₂) and maturation inducing steroid (MIS), measured from plasma and *in vitro* ovarian follicle incubations, confirmed that T and E₂ production correlate with vitellogenesis and final maturation in mummichog, whereas MIS correlates with only final maturation in mummichog; no E₂, as is present in other teleosts, drop prior to maturation was observed. The expression of P450arom (CYP19a1), ER α , StAR, P450 side-chain cleavage, and hepatic VTG1, determined by qPCR methods, were associated with patterns of E₂ in maturing mummichog, but did not follow patterns exhibited in other teleosts. CYP17 expression was not associated with E₂ patterns in mummichog, or other teleosts. To determine EE₂ (17 α -ethinyl estradiol) effects during maturation, follicles grouped per stage were exposed to 50 - 250 nM of EE₂ *in vitro* for 24 hours; there was no effect on P450arom or LHr expression, or on T or MIS production. The tolerance exhibited by mummichog to exogenous estrogens *in vitro* could be partially attributable to continuously high levels of

E₂ present in the maturing ovary, as EE₂ during follicular incubations had no effect on presumed E₂-responsive genes.

2.2 Introduction

In most teleost ovarian development processes studied to date, there is a shift that occurs from estrogenic (17β -estradiol or E_2) to progestational (maturation inducing steroid or MIS; 17α , 20β -dihydroxy-4-pregnen-3-one) steroid production when follicles progress from vitellogenesis to a prematurational stage (Nagahama and Yamashita, 2008). E_2 is produced in correlation with early developmental stages and rises during vitellogenesis (the process of vitellogenin synthesis in the liver, under the stimulation of E_2 , and uptake into the oocytes; Hoar et al., 1983), whereas MIS is produced in correlation with the onset of maturation of oocytes (Nagahama and Yamashita, 2008). MIS role in maturation is to induce resumption of meiosis and germinal vesicle breakdown (GVBD) in teleost oocytes (Clelland and Peng, 2009; Nagahama and Yamashita, 2008). In mummichog there is no progestagen shift; the levels of E_2 are consistently higher in all stages of follicles tested and coexist with increasing progestagen during mummichog (Lin *et al.*, 1987). Similar to other teleosts, MIS continues has a major role in maturation of mummichog (Petrino *et al.*, 1989).

Ovarian development in teleosts varies from species to species, in both the duration of the cycle as well as the mechanisms involved in regulating the cycle (Khan and Thomas, 1999). Selman and Wallace (1986) characterized five stages of oogenesis; the first stage is the primary growth stage (definitive follicle is formed), the second stage is the cortical alveolus stage (contains randomly dispersed cortical alveoli, or “yolk vesicles”), the third stage is vitellogenesis (immense growth of the follicle occurs due to uptake of vitellogenin), the fourth stage is oocyte maturation, further separated into early maturation (follicular size increases due to hydration and protein uptake) and late

maturation (germinal vesicle breakdown occurs), and the fifth stage is ovulation (mature egg which is ready for ovulation). These stages resemble those as described for other fish species, including zebrafish (Clelland and Peng, 2009; Lyman-Gingerich and Pelegri, 2007) and rainbow trout (Van Der Hurk and Peute, 1979).

Ovarian development in teleost fish is controlled and regulated by a balance and interconnectivity among steroids and gonadotropins, through signalling pathways (Ramezani-Fard *et al.*, 2013). In teleosts like mummichog, gonadotropins FSH (follicle stimulating hormone) and LH (luteinizing hormone) are released into the bloodstream from the pituitary and bind to their respective receptors (FSHr and LHr) on the ovary (Ohkubo *et al.*, 2013; Shimizu *et al.*, 2003). FSH is primarily involved in regulating gonadal steroidogenesis in the early developmental stages (primary growth – completion of vitellogenesis), as it correlates with E₂ production, whereas LH is primarily involved in regulating oocyte maturation (early maturation – ovulation), as it correlates with MIS production (Ohkubo *et al.*, 2013; Swanson *et al.*, 1991). E₂ action is mediated by estrogen receptors (ERs) which have the subtypes ER α (ESR1) and ER β (including both ER β 1, or ESR2a, and ER β 2, or ESR2b; Menuet *et al.*, 2002). It is presumed that the gene expression of ERs is regulated by steroid hormones, so expression rates will fluctuate in response to changes in steroidogenesis (Thomas, 2008); however, ESR1 is the only subtype that has been linked to biological function in previous studies, such as stimulation of vitellogenin synthesis in liver and E₂ regulation (Chakraborty *et al.*, 2011).

All sex steroids are derived from cholesterol via a number of enzymatic conversions in a process known as steroidogenesis (Arukwe, 2008; Leusch and MacLatchy, 2003). Little is known about the expression of the genes involved in

regulation of the steroidogenic pathway across ovarian development in mummichog. These steroidogenic enzymes include: steroidogenic acute regulatory protein (StAR), which is a sterol transport protein that initiates cholesterol transport into the mitochondria (Rone *et al.*, 2009); cytochrome P450 side-chain cleavage (P450_{scc}, or CYP11), which produces pregnenolone (basal steroid) from cholesterol (Young *et al.*, 2005); cytochrome P450 C17 (P450C17, or CYP17), which can catalyze the hydroxylation of pregnenolone to yield 17 α -hydroxypregnenolone and then dehydroepiandrostone, or the hydroxylation of progesterone to yield 17 α -hydroxyprogesterone and then androstenedione by 3 β -HSD (Young *et al.*, 2005); and cytochrome P450 aromatase (P450_{arom}; CYP19a1 for ovarian, or CYP19b for brain), which converts T into E₂ and is potentially responsible for the drop in estrogen levels that occur prior to maturation (Young *et al.*, 2005).

EDCs (endocrine disrupting compounds) have been shown to exert direct effects on gonadal steroidogenesis in teleosts through various mechanisms; EE₂ (17 α -ethinyl estradiol; a synthetic estrogen commonly found in municipal waste waters via birth control and hormone replacement therapy pharmaceuticals) can alter gonadal steroid production at specific sites within the steroidogenic pathway (Hogan *et al.*, 2010; Thomas, 2008). EE₂ is commonly used as a model compound to study the impact of estrogenic compounds on aquatic organisms, due to its environmental relevance and inability to be broken down in municipal wastewater treatment (Bosker *et al.*, 2016). Mummichog exhibit a greater tolerance to EE₂ when compared to other small-bodied species. For instance, a seven-year whole lake EE₂ exposure demonstrated induced vitellogenin and the feminization of male fathead minnow (*Pimephales promelas*) at 5-6 ng/L of EE₂, and an eventual population collapse. Egg production was significantly

reduced in fathead minnow at concentrations of 0.47-3.92 ng/L of EE₂ after a 21-day exposure (Armstrong *et al.*, 2016), in Chinese rare minnow (*Gobiocypris rarus*) and zebrafish (*Danio rerio*) at concentrations as low as 0.2 ng/L EE₂ and 1 ng/L EE₂, respectively (Lin and Janz, 2006; Zha *et al.*, 2008). Alternatively, mummichog respond to higher levels of EE₂ at concentrations of 100 ng/L (Doyle *et al.*, 2013; Gillio Meina *et al.*, 2013; Hogan *et al.*, 2010; Peters *et al.*, 2007). Confirming previous studies, Bosker *et al.* (2016) demonstrated that cumulative egg production per female was unaffected by high concentrations of EE₂ at 100 ng/L (nominal), or 84.1 ng/l \pm 6.0% of nominal, after 28 days of exposure.

Previous studies have tried to determine factors which cause lower sensitivity to EE₂ in mummichog, by investigating environmental and physiological variables. It was determined that there was no interaction between temperature and EE₂, or salinity and EE₂ on plasma steroid levels in mummichog (Gillio Meina *et al.*, 2013). A tissue distribution study, however, showed that EE₂ may be metabolized and cleared more quickly in mummichog, as mummichog accumulate EE₂ to a greater degree in the gallbladder and liver, compared to the carcass in zebrafish and fathead minnow (Blewett *et al.*, 2014). Because ovarian physiology differs in mummichog, primarily the lack of an E₂ drop during maturation, it may be at least partially attributable to the decreased sensitivity of mummichog to EE₂.

The objectives of this study were to confirm the current understanding of mummichog maturation as it relates to ovarian steroidogenesis and to characterize the gene expression changes across the ovarian development cycle for the first time. With this deeper mechanistic understanding of mummichog ovarian steroidogenesis, it was

then possible to undertake a preliminary *in vitro* ovarian follicle exposure across maturational stages to EE₂, to determine the effect on gene expression endpoints known to be estrogen sensitive in other teleost species. Together, these studies have progressed our understanding of mummichog ovarian development and provided a potential addition to the mechanistic understanding of the general resistance of mummichog to environmentally-relevant levels of EE₂.

2.3 Materials and Methods

2.3.1 Fish collection and holding

Adult mummichog (6.07 – 21.1 g) were collected in June 2015 from Shemogue Harbour, NB, Canada (46° 10' 35" N, 64° 8' 55" W) by seine net (size of mesh: ¼"). The fish were transported to Wilfrid Laurier University in aerated plastic totes, and quarantined in 200 L plastic reservoirs for 14 days at room temperature with frequent water changes before being transferred and maintained in recirculating fish holding units. Fish were housed in 380 L single stock tanks (Aquabiotech Inc, Coaticook, QC, Canada) or held in a G-HAB unit consisting of 40 L and 60 L glass aquaria (Pentair, Apopka, FL, USA). Routine water quality tests (ammonia, nitrite and nitrate levels) and water changes (25% water change every two weeks and 50% water changes every month) were conducted to maintain conditions within desired levels. For all holding conditions, the water was maintained at 16-18 ppt salinity by the addition of artificial sea salt [Crystal Sea Marine Mix sea salt (Marine Enterprises International, Baltimore, MD, USA)] to City of Waterloo dechlorinated and reverse osmosis water.

2.3.2 Characterization of the estrogen biosynthetic pathway during ovarian development

In order to obtain ovaries at different stages during the reproductive cycle, photoperiod and temperature were adjusted in the stock tanks to mimic seasonal changes. The temperature and photoperiod began at 4°C and 8 h light: 16 h dark (held at these conditions for two weeks) to simulate a colder season and obtain regressed stages of ovarian follicles. The females were also separated from the males during this period and were fed commercial trout pellets (2mm; EWOS Pacific, Vancouver, BC, Canada) once a

day. Once sampling was complete (December 2015), the temperature and photoperiod was slowly raised to 12°C and 12 h light: 12 h dark (temperature was increased 1°C each day) to simulate warmer seasons and stimulate ovarian recrudescence and maturation. Males were added to the stock tanks and fish were fed commercial trout pellets twice a day; the temperature was also further increased slowly to 16°C. Once sampling was complete for the vitellogenic stage (April 2016), the temperature and photoperiod was slowly raised to 22°C and 16 h light: 8 h dark (spawning conditions) and fish were fed commercial trout pellets twice a day, and supplemented with freeze-dried blood worms (*Glycera dibranchiata*; BrineShrimpDirect, Ogden, UT, USA) once every other day. Maturing stages were easily collected at these conditions (April-May 2016).

i) Sampling

When female mummichog reached desired stages of ovarian development (as estimated from the increased size and softness of the female abdomen as it becomes larger with increasing ovarian development), fish were anaesthetized with ~0.05% ethyl 3-aminobenzoate methanesulfonate salt (Sigma-Aldrich, Oakville, ON, Canada), bled from the caudal vasculature using heparinized 25 G 5/8 needles on 1 mL syringes, and killed by spinal severance after being weighed, as simplified in Figure S2.4. Fish were sampled on these dates: December 1st, 2015; April 12th, 15th and 20th, 2016; May 3rd, 2016. The stage of ovarian development was classified with gross morphological assessment of the ovary following criteria listed in Table 2.1; the dominantly present stage (dominant stage ~ >75% of follicles in ovary) in samples were verified with histological assessment. Collected blood was kept on ice then centrifuged at 8000 x g for 10 minutes at 4°C to isolate plasma. Plasma was pipetted and stored at -20°C in 1.5 mL

centrifuge tubes (Fisher Scientific, Ottawa, ON). E₂, T and MIS levels were measured from plasma samples obtained from 15, 12, 16, 14, and 4 fish respectively classified as having ovaries with stage 1-5 follicles dominantly present.

The ovary, liver, and brain (pituitaries were not isolated because of difficulty due to size) were dissected and removed, and ovary and liver were weighed. The ovary and liver weights, along with the whole-body weight, were used to calculate the GSI (gonadal somatic index) and LSI (liver somatic index) of each fish.

$$\text{GSI} = \frac{\text{ovary weight}}{\text{body weight}} \times 100 \qquad \text{LSI} = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

The liver and brain were snap-frozen on dry ice, then stored at -80°C, to be used later for RNA extractions and molecular analysis. The ovary was divided into two sections: one section was snap-frozen on dry ice and stored at -80°C (utilized later for RNA extractions and molecular analysis) and another section was placed in 75% Leibovitz-15 (L-15) medium [L-15 powder with L-glutamine (Sigma-Aldrich) diluted with ultrapure water as per manufacturer's instructions; medium was balanced to a pH of 7.5 and contained 10 mL of penicillin streptomycin (Thermo Fisher Scientific, Burlington, ON, Canada)]. Gene expression was measured from ovary (tissue), liver (tissue) and brain (whole) samples obtained from 9-12, 10-11, 14-15, 14, and 4 fish classified as having ovaries with stage 1-5, respectively, follicles dominantly present.

ii) *In vitro methodology*

Whole ovary tissue was placed into glass petri dishes containing 75% L-15 medium and gently teased apart using forceps to separate different stages of follicles. In order to classify follicles into stages, an EVOS XL digital inverted microscope imaging system (Life Technologies, Thermo Fisher Scientific) was used to identify morphological

characteristics and measure follicle diameter, which corresponded to criteria in Table 2.1. Example images of staged follicles are shown in Figure 2.1. The incubation protocol was modified from McMaster *et al.* (1995) and MacLatchy *et al.* (2003). Follicles grouped by stage were incubated in 1 mL (stages 1-3) or 2 mL (stages 4-5) of culture medium (75% L-15) for 24 hours at 18°C, as simplified in Figure S2.6A. Each well contained 24 mg of tissue (3 pieces of 7-8 mg) for stage 1 (cortical alveoli) ovaries or 15 follicles for stage 2-5 follicles, from individual fish. Samples from each fish were distributed into 2-6 wells, depending on the available tissue or follicles. The wells were divided into two treatments: control [basal (medium only)] or hCG (human chorionic gonadotropin; Lee Biosolutions, Maryland Heights, MO, USA) treated at 20 IU/mL. Preliminary results showed that this concentration of hCG stimulated steroid production by follicles (data not shown) and has been used in other studies (MacLatchy *et al.*, 2003). hCG is an analogue to LH (luteinizing hormone), an endogenous gonadotropin responsible for oocyte maturation and stimulation of steroidogenesis. After incubation, medium was removed and stored at -20°C, while tissue was removed and stored at -80°C. The medium was later used for steroid hormone measurement of E₂, T and MIS through enzyme immunoassay (EIA) analysis; medium was obtained from 10, 15, 12, 16, and 10 fish for stages 1-5, respectively.

Table 2.1. Size and morphology classification of ovarian stages (Modified from Selman and Wallace, 1986), verified through examination under an EVOS XL digital inverted microscope.

Stage of Follicle	Stage of Fish	Follicle Diameter	Description
(1) Cortical Alveolus	(R) Regressed	175-550 μm	Appearance of cortical alveoli (“yolk vesicles”)
(2) Vitellogenesis	(V) Vitellogenic	550-1350 μm	Ooplasm is opaque, membrane-bound yolk spheres occur & germinal vesicle visible under microscope
(3) Early Maturation	(EM) Early Mature	1350-1700 μm	Ooplasm becomes more translucent as oocyte matures & several oil droplets appear (dispersed)
(4) Late Maturation	(FM) Fully Mature	1700-1900 μm	Ooplasm becomes more transparent, Germinal vesicle breakdown, lipid droplets continue to coalesce
(5) Ovulated	(OV) Ovulated	1900 μm +	Transparent follicles with no apparent membrane, covered by coat material “jelly” & oil droplets migrate to one pole

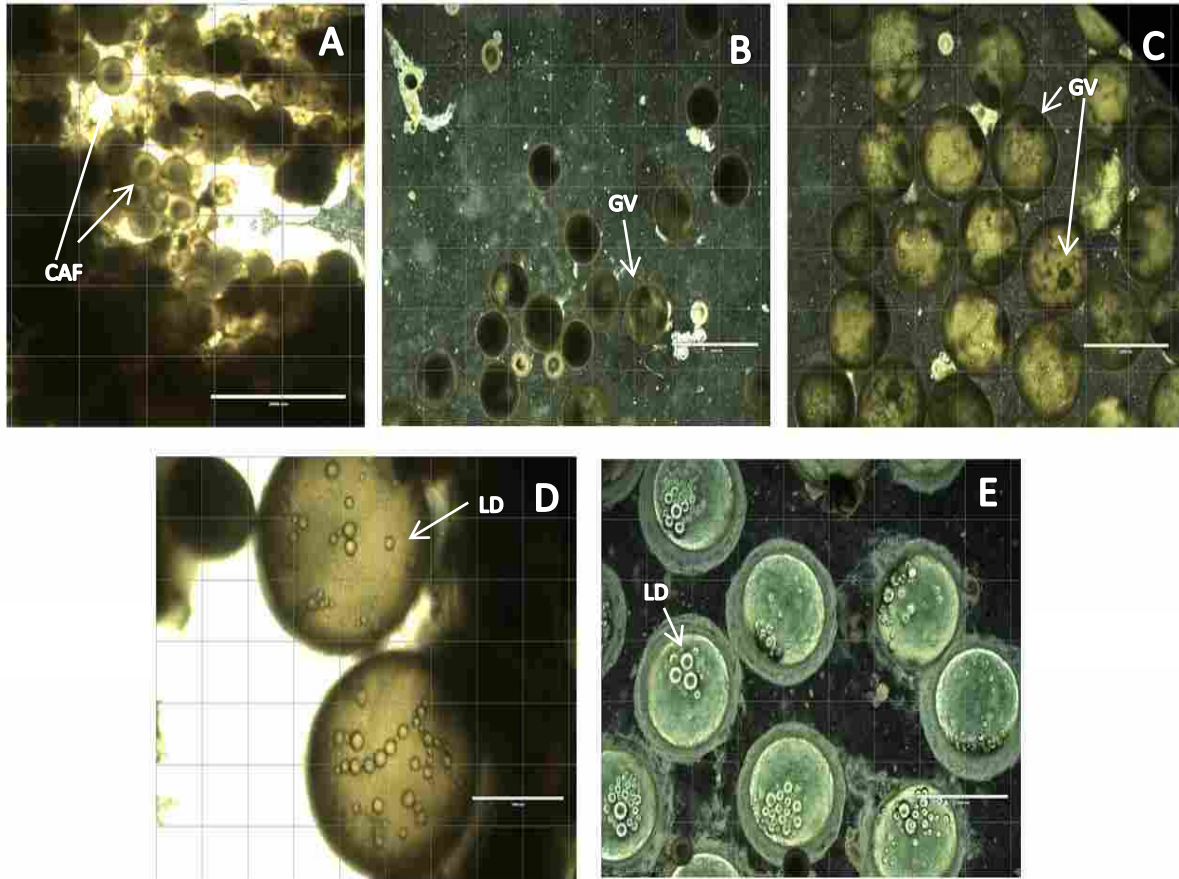


Figure 2.1. Images of staged follicles across the ovarian development cycle in mummichog: (A) cortical alveolus, (B) vitellogenesis, (C) early-mid mature, (D) late mature, (E) ovulated. Scale bar is 2000 μm for images A-C and E; scale bar is 1000 μm for image D. CAF = cortical alveolus follicle; GV = germinal vesicle; LD = lipid droplets. Images obtained from the EVOS XL digital inverted microscope imaging system.

iii) Measurement of steroid hormone levels by EIA

Steroids were extracted from plasma samples using ethyl-ether (according to Gillio-Meina *et al.*, 2013). The extracted samples were dried overnight at room temperature and then reconstituted in 500 μL of EIA buffer (Cayman Chemical, Ann

Arbor, MI, USA) and stored at -80°C. Media samples from the *in vitro* incubations were assayed without extraction. EIAs for E₂, T, and MIS were conducted as per the manufacturer's (Cayman Chemical) instructions. The appropriate dilution for each steroid stage and basal or stimulated treatment, was determined by analyzing serial dilutions of pooled samples; dilutions used on plasma or media samples were either 2x or 4x. To determine if there was interference in the EIA caused by the L-15 media samples, standard curves were generated with both L-15 medium and EIA buffer; no differences were found. Therefore, media samples were assayed directly in the EIA. Interassays were created for each hormone measured; following EIA, the coefficient of variation was calculated to be 7% for E₂, 13% for T, and 11% for MIS [standard deviation of samples/mean of samples x 100], which indicates that the method is reproducible and reliable. The samples were read at a wavelength of 420 nm using a Molecular Device SpectramaxPlus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Mean fold increase (shown to compare hCG-stimulated production to basal production, per treatment and stage) was calculated as follows:

$$\frac{(hCG\ value/basal\ value)\ of\ sample\ 1\ +\ (hCG\ value/basal\ value)\ sample\ of\ 2\ \dots\ +\ (hCG\ value/basal\ value)\ of\ sample\ N}{N}$$

iv) RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted from frozen ovary (whole ovary or isolated follicles), liver and brain tissue, using TRIzol reagent (Invitrogen, Burlington, ON, Canada). The protocol was carried out as per the manufacturer's instructions with minor modifications. Ovary and liver samples (20-50 mg), whole brains, or separated follicles (15-40, depending on experiment) were added to centrifuge tubes with 800 µL of TRIzol reagent, and homogenized via mechanical disruption using a hand-held homogenizer (Knotes

Pellet Pestle Cordless Motor, Fisher Scientific), or a 21G (0.8 mm x 25 mm) needle on a 3 mL syringe. Chloroform (160 μ L) was added for the extraction of RNA into the top, clear supernatant. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C, after which the top RNA layer was decanted into 400 μ L of iso-propanol of a correspondingly labelled tube, and then centrifuged again at the same parameters. The supernatant was discarded, and 800 μ L of 75% ethanol was added to the tubes containing the RNA pellet, then the tubes were centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol layer was poured off and the pelleted RNA was dissolved in 10-60 μ L (depending on size of pellet) of RNase-free water. The samples were then vortexed briefly, heated at 55-58°C for 5-10 minutes to fully dissolve the RNA pellet, and stored at -80°C.

RNA yield was verified using a Nanodrop 8000 (Thermo Fisher Scientific, Burlington, ON) with 2 μ L of samples at two dilutions of 10 and 50x to allow for an accurate calculation of the undiluted RNA. RNA samples were then diluted to 1000 ng/ μ L with molecular grade water, for use in the cDNA reactions. RNA sample quality was checked at a 260/280 nm wavelength, and samples were read between 1.6 and 2.0. A subset of 10x diluted RNA samples were randomly selected from each experiment to verify RNA integrity using RNA StdSens Chips on the Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Mississauga, ON, Canada) as per the manufacturer's instructions.

RNA samples were treated for potential genomic DNA contamination with the AMP-D1 kit (Sigma Aldrich) according to the instructions prior to cDNA synthesis. 2 μ L of 1000 ng/ μ L RNA was added to a 0.2 mL PCR tube containing 6 μ L of RNase-free water, 1 μ L of 10 x Reaction Buffer and 1 μ L of DNase enzyme. The samples were

heated at 70°C for 10 minutes [using a C1000 PCR thermocycler (Bio-Rad Laboratories)] and then chilled on ice. Reverse transcription of total RNA was performed with 4 µL of iScript Reverse Transcription Supermix (Bio-Rad Laboratories) and followed the manufacturer's instructions, with parameters of 5 min at 25°C for priming, 30 min at 42°C for RT (reverse transcription), 5 min at 85°C for inactivation, and cooling to 4°C. The resulting cDNA was stored at -20°C until real-time quantitative PCR (qPCR) analysis.

qPCR was performed in duplicate for each sample using SsoFast EvaGreen Supermix and the CFX96 Real-Time System (Bio-Rad Laboratories) in a reaction volume of 10 µL containing 2.5 µL of cDNA, 5 µL EvaGreen, 1.25 µL of each forward and reverse primers. The qPCR parameters were 30 seconds at 95°C, followed by 40 cycles of 95°C for 1 second and 55°C for 5 seconds, which was the annealing temperature determined to be best suited for all of the primers through a series of temperature gradient tests. To demonstrate primer specificity and the amplification of a single amplicon, dissociation curves were conducted on all reactions. Genomic DNA and RT-water controls were conducted on approximately 10% of the samples and were found to be free of contamination.

Oligonucleotide primers for most genes of interest were designed using IDT PrimerQuest Tool, and obtained from either Sigma-Aldrich or IDT [Integrated DNA Technologies (Coralville, IA, USA)]. Table 2.2 presents the primer sequences and their NCBI GenBank accession numbers. The relative standard curve qPCR method was used and six-point standard curves were generated for each gene with primer efficiency values shown in Supplementary Table S2.1.

Genes of interest were normalized to a reference gene, which was shown to not change expression levels in female mummichog undergoing ovarian development (data shown in Figure S2.1, S2.2, and S2.3). The reference genes used were estrogen receptor β 1 (ESR2a) in the ovary, 18sRNA in the liver, and EF1 α in the brain.

Table 2.2. Forward (FWD) and reverse (REV) primer sequences used for qPCR analysis with accession numbers and commercial source.

Gene		Sequence 5'-3'	Accession #	Company
B-Actin	FWD	GCA CGG TAT TGT GAC CAA	AF397164	Sigma
	REV	GGG TGT TGA AGG TCT CAA		
EF1α	FWD	TCA CCA TCA GCT TTT ATA AAG	AY735180	Sigma
	REV	GAC GCT CCA TTG CCT ACT GTC		
18s-RNA	FWD	GTC GTA GTT CCG ACC ATA AAC	M91180	IDT
	REV	CAC CCA CAG AAT CGA GAA AG		
ESR1	FWD	ACT CTA CCA CTG GCT ACT ACT C	AY571785	IDT
	REV	GCT GGT GAC TGG ATC TGT AAA		
ESR2a	FWD	GAG GAG CAT CCA AGG ACA TAA C	AY570922	IDT
	REV	TTG GTC ATG CCG ACT TCA TAG		
ESR2b	FWD	CTC AGC CGA ACC ACA GTA ATC	AY570923	IDT
	REV	CTC CAG CCA GCA ACA CTT TA		
LHr	FWD	TGG AAA CCA TCG AGG CAT TAG	AB295491	IDT
	REV	GAC ATC AGG GAA GAG CGT TAT C		
FSHr	FWD	CCA TCT CCT TCT TTG CCA TCT	AB295490	IDT
	REV	CCA TCC AGA CTC AGC GTA TTC		
StAR	FWD	CCT TCA AAA GAT CGG ACA GG	Doyle <i>et al.</i> , 2013	IDT
	REV	TTT CTG AGG CAT TTG TG		
CYP11a1	FWD	TTC AAG GCA GAG GGT CAA TAT C	AB471800	IDT
	REV	AGG TCC GTG GTC CAT TTA TTC		
CYP17a1	FWD	CTG CTT CAA CTC CTC CTA TTC	XM_012852 073 (Predicted Sequence)	IDT
	REV	CCT TGT GCT CGT TGT ACT T		
CYP19a1	FWD	GTC CAC TCT TGT CTT ATT TG	AY428665	Sigma
	REV	GTC TCC TCT CCA TTG ATC		
CYP19b	FWD	CTG TGA GGA CTC AGA AA	AY428666	IDT
	REV	TCA GTG CGA GGT TAT AC		
Vtg1	FWD	GTT GGC ATA CAC TGA GAA	AAA93123	Sigma
	REV	AGA CGT AAC TTA ATG TTG TG		

v) ***Histological assessment***

At the time of dissection and based on gross morphology, ovaries were classified according to Table 2.1 if approximately $\geq 75\%$ of the follicles present within the ovary appeared to be in the desired stage (1 – 5). Histological assessments of approximately 25% (N= 3-4 per stage) of the ovaries were conducted to ensure the ovaries contained a majority of the desired stage of follicle (Figure 2.2). Follicle diameter was measured with the EVOS XL digital inverted microscope imaging system (Figure 2.1). Histological assessments of subsamples of the ovaries verified these classifications (Figure 2.2).

Pieces of whole ovary were placed into cassettes [7x12 mm Tissue Path MACROSETTE Processing/Embedding Cassettes (Fisher Scientific)], immersed in 10% buffered formalin (Fisher Chemical, Ottawa, ON, Canada) and processed by the University of Guelph Animal Health Laboratory. Samples were paraffin embedded, cross-sectioned at 4 μm at 3 different levels, stained with standard haematoxylin and eosin, and mounted on slides using Tissue Tek permanent mounting medium (Sakura Finetek, Torrance, CA, USA). Slides were examined under a Zeiss Axio Upright Observer Microscope (Carl Zeiss, Toronto, ON) under 5-10x magnification, using ZEN Pro Software (Carl Zeiss, Toronto, ON) to determine the stage of ovarian development and to allow for measurements of the diameters of the follicles as shown in Table 2.1.

2.3.3 Molecular characterization of the biosynthetic pathway of ovarian follicles

In order to characterize the expression of genes across follicular stages of development and provide a comparison of the expression levels determined in whole ovarian tissue, individual follicles were separated and classified by stage. The females used for this work were held at conditions known to be favourable for the continuous

production of mature eggs (spawning conditions: 22°C with 16h L: 18h D, as per demonstrated in Peterson *et al.*, 2010). The fish were from the same stock used in the work described in Section 2.3.2, but were sampled on February 10th and 17th 2017, to collect sufficient numbers of follicles at each stage from multiple fish.

i) Sampling

Sampling was followed as outlined in section 2.3.2.i and ii with few exceptions. The whole ovary was dissected and placed directly into petri dishes containing 75% L-15 medium, and underwent the sorting process (similar to section 2.3.2.ii). When collecting ovulated follicles (stage 5), the abdomen of the fish was slightly squeezed to release the ovulated follicles from the abdominal cavity, through the ovipositor. After sorting, the isolated follicles were removed from the medium and placed in sterilized 1.5 mL Flat Top Microtubes (Diamed, Mississauga, ON) in groups of ~30-40 follicles that were snap-frozen and stored at -80°C. The total time for this process was under 10 minutes, as each fish was processed individually. Gene expression was measured from grouped ovarian follicle samples obtained from 7-8 fish for each stage.

ii) RNA extraction and qPCR

The RNA extraction and qPCR protocol was followed as outlined in section 2.3.2.iv with minor modifications. Total RNA was extracted from frozen ovarian follicles (30-40 per sample), using TRIzol reagent. Following the extraction procedure total RNA was pelleted, rinsed with 75% ethanol, reconstituted in 10-40 µL RNase-free water and incubated at 56-59°C for 5-10 minutes to fully dissolve the RNA pellet. Gene expression values were normalized to adjusted values of ESR2a, based on the approach described by Ings and Van Der Kraak, 2006 (Table S2.1), due to the inability to find a reference gene

which was steadily expressed across all stages. Stage 5 (ovulated) follicles produced non-detectable amounts of gene expression, so they were excluded from gene expression analysis data.

iii) Histology assessment

The histology protocol was followed as outlined in section 2.3.4.v, with the exception of storing the isolated follicles in 15mL disposable, polypropylene centrifuge tubes (Fisher Scientific) containing ~5mL of 10% buffered formalin. These centrifuge tubes were sent to the University of Guelph Animal Health Laboratory to be processed for histological analysis.

2.3.4 Effect of EE₂ on basal and hCG-stimulated ovarian follicles: steroid production and gene expression

To assess the effect of EE₂ on gene expression and hormone production at the gonadal level in mummichog, staged ovarian follicles were exposed to 50-250 nM of EE₂ over a 24 h incubation period, as simplified in Figure S2.5. The fish used in this experiment were from the same stock used in the work described in Section 2.3.2 and 2.3.3., but were sampled from October 21st 2016 to December 21st 2016, over 11 days in order to collect sufficient numbers of follicles at each stage for each treatment, from multiple fish. Stock tanks were held at spawning conditions (22°C with 16h L: 18h D) in order to obtain ovaries with follicles at various maturation stages.

i) Sampling

Sampling was followed as outlined in section 2.3.3.i with minor modifications. After the sorting process, the separated follicles were removed from the medium and

placed in 1-2 mL of fresh medium in *in vitro* plate wells. Wells each contained 25 follicles of a specific stage; the mass of tissue for each well was recorded prior to incubation. Regressed ovaries (stage 1) were cut into pieces of 7-8 mg, while in medium, with a total of 3 pieces per well (total ~24 mg).

ii) *In vitro methodology*

The *in vitro* protocol was followed as outlined in 2.3.2ii with a few exceptions, as simplified in Figure S2.6B. The follicles were obtained from multiple fish for every stage (3-4 individual fish per pool). Each pool was distributed into 11 wells. Each well contained 24 mg of tissue (three pieces of 7-8 mg) for regressed (cortical alveoli) ovaries or 25 follicles for vitellogenic to ovulated ovaries. There were 11 treatments per stage, with a total of 7-8 reps per stage ($n = 7-8$). The first treatment was time zero; after follicles were sorted, samples from each pool were removed from the medium and directly snap-frozen, then stored at -80°C . There were five treatments: no solvent control (only L-15 medium), solvent (ethanol) control, 50 nM EE₂, 100 nM EE₂, and 250 nM EE₂; these treatments were either basal or hCG-stimulated, resulting in a total of 10 treatments plus one time zero sample per pool/stage. Concentrated stocks of EE₂ (Sigma-Aldrich) were prepared in ethanol at concentrations of 10, 20, and 50 μM , and stored in glass vials at -20°C . EE₂ and ethanol were added to wells at 5 μL per 1 mL of total solution (as per McMaster *et al.*, 2005), resulting in final concentrations of 50, 100 and 250 nM per well (concentrations are equivalent to 14.82 $\mu\text{g}/\text{mL}$, 29.64 $\mu\text{g}/\text{mL}$, and 74.1 $\mu\text{g}/\text{mL}$ respectively). After incubation, the media were removed, stored at -20°C , and used later for steroid hormone measurement of T and MIS through EIA analysis, on a

subset of samples (all stage 2, 3 and 4 samples). The tissue was also removed, stored at -80°C and later used for gene expression analysis.

iii) RNA extraction and qPCR

The RNA extraction and qPCR protocol were followed as outlined in section 2.3.3.ii with the exception that total RNA was extracted from frozen ovarian follicles (25 per sample), using TRIzol reagent. Gene expression values were normalized to β -actin.

2.3.5 Statistics

Statistical analysis was performed using SPSS 23 (IBM Canada Ltd, Markham, ON, Canada). One-way analysis of variance (ANOVA) was performed to assess plasma and *in vitro* steroid hormone levels (E_2 , T & MIS), or gene expression (ERs, P450 enzymes, StAR, LHr, FSHr), across the different stages of maturation. Two-way ANOVA was performed to assess basal T and MIS production across different stages of follicle maturation with different treatments of EE_2 (from *in vitro* exposure). Assumptions of normality and homogeneity of variance were tested using Shapiro-Wilk's W test and Levene's test, respectively. If the data were not normally distributed, or did not test positive for homogeneity of variance, the data were log transformed or square-root transformed (rarely) through SPSS, and the parameters were tested again. If the normality and variance tests still failed after data transformation, then the non-parametric Games-Howell test was used; Tukey's test was used for all parametric post hoc analyses. In all cases, a p-value of 0.05 was chosen to indicate significance.

2.4 Results

2.4.1 Characterization of the estrogen biosynthetic pathway during ovarian development

i) Morphometric Data

Morphometric data, such as body weight, gonad weight and liver weight, were recorded from sampled fish and analyzed to determine the mean GSI and LSI for each stage of fish. Mean GSI in fish increased with increasing stage of ovarian development [minimum at R, 1.72 ± 0.17 %; maximum at OV, 12.36 ± 3.2 %; $p = 0.00$ (Table 2.3)], as did body weight [minimum at R, 10.1 ± 0.55 g; maximum at OV, 17.4 ± 1.0 g; $p = 0.00$ (Table 2.3)], and gonad weight [minimum at R, 0.17 ± 0.01 g; maximum at OV, 2.09 ± 0.45 g; $p = 0.00$ (Table 2.3)]. Mean LSI in fish remained relatively the same across stages, except for an increase in regressed fish [minimum at FM, 3.87 ± 0.32 %; maximum at R, 6.53 ± 0.24 %; $p = 0.00$ (Table 2.3)], and there were no significant changes in liver weight across stages [minimum at V, 0.55 ± 0.05 g; maximum at OV, 0.80 ± 0.11 g; $p = 0.20$ (Table 2.3)].

ii) Plasma hormones in females across the reproductive cycle

Plasma E₂ production was highest compared to T or MIS levels across all stages of fish examined (Figure 2.3). R (regressed) refers to fish containing primarily cortical alveoli follicles; V (vitellogenic) refers to fish containing primarily vitellogenic follicles; EM (early mature) refers to fish containing primarily early mature follicles; FM (fully mature) refers to fish containing primarily late mature follicles; OV (ovulated) refers to fish containing primarily ovulated follicles. Plasma T levels increased from regressed to

fully mature fish and were highest in fully mature fish; fully mature fish were significantly higher than regressed or vitellogenic fish [minimum at V, 88.5 ± 21.3 pg/mL; maximum at FM, 250.0 ± 32.0 pg/mL; $p = 0.00$ (Figure 2.3 A)]. Mean plasma E₂ significantly increased from regressed to fully mature fish, and dropped in ovulating fish [minimum at R, 26.5 ± 3.09 pg/mL; maximum at FM, 2550 ± 339 pg/mL; $p = 0.00$ (Figure 2.3 B)]. Mean plasma MIS had no significant changes from regressed to early mature fish, but significantly increased from early mature to ovulating fish [minimum at V, 0.3 ± 0.1 pg/mL; maximum at OV, 5.0 ± 1.2 pg/mL; $p = 0.00$ (Figure 2.3 C)].

iii) *In vitro* steroid production across follicular stages of ovarian development

Basal E₂ production was highest compared to T or MIS levels across all stages of follicles examined (Figure 2.4). Mean basal and hCG-stimulated levels of E₂, T and MIS (Figure 2.4) dropped between stages 1-2. The decline in basal *in vitro* hormone production seen in stage 2 follicles is most likely due to the difference in tissue weight per well (a methodological issue), and the results are focussed on stage 2 to stage 5 follicles. Basal T significantly increased during stage 4, while having no significant differences across stages 2, 3 or 5 [minimum at stage 3, 10.2 ± 1.5 pg/mL; maximum at stage 4, 26.5 ± 5.0 pg/mL; $p = 0.00$ (Figure 2.4 A)]. Basal E₂ increased from stage 2 to stage 4 [minimum at stage 5, 84.7 ± 25.7 pg/mL; maximum at stage 4, 2096 ± 261 pg/mL; $p = 0.00$ (Figure 2.4 C)]. Basal MIS levels significantly increased from stage 2 to stage 4, and remained at high levels during stage 5 [minimum at stage 2, 0.96 ± 0.2 pg/mL; maximum at stage 5, 18.45 ± 7.8 pg/mL; $p = 0.00$ (Figure 2.4 E)]. hCG-stimulated T, E₂ and MIS production was highest at stage 3, had no significant differences between stages 3-4, and had low production during stage 2 and 5 ($p = 0.00$ for

each hormone; Figure 2.4 B, D, F). hCG-stimulated hormone production was highest for E₂, then MIS, and lowest for T.

Fold increase of hCG-stimulated to basal production of steroids by separated and staged (stages 1 to 5; refer to Table 2.1) follicles were examined to compare hormone levels across different stages of maturation, without being influenced by follicle size or weight. Early mature (stage 3) follicles showed the greatest capacity to respond to hCG treatment and the MIS response seemed most pronounced at this stage given that hCG had little stimulatory effect on MIS at all other stages (Figure 2.5). The fold increase (hCG-treated to basal hormone levels) of T, E₂ and MIS at stage 3 were 25.8 ± 6.9 [$p = 0.00$ (Figure 2.5 A)], 5.0 ± 0.8 [$p = 0.00$ (Figure 2.5 B)], and 243.2 ± 66.7 [$p = 0.00$ (Figure 2.5 C)], respectively.

iv) Gene expression in females across the reproductive cycle

Gene expression was determined in whole ovary pieces from fish that were sampled over the reproductive cycle having group-synchronous ovaries, characterized as containing >75% of a desired stage (based under gross examination under a microscope). Follicles were later verified through histological assessment (Figure 2.2) and stage of fish was supported with morphometric data (Table 2.3). Ovarian expression data were normalized to estrogen receptor ER β 1 (ESR2a) and displayed as the fold change relative to the regressed stage. The constant expression of ESR2a across all ovarian developmental stages is shown in Supplemental Figure S2.1.

Ovarian expression of ESR1 significantly increased in vitellogenic fish and had no significant changes in expression throughout the rest of maturation [minimum at R, 1.0 ± 0.2 ; maximum at FM, 4.8 ± 0.5 ; $p = 0.00$ (Figure 2.6 A)]. Ovarian ESR2b

expression peaked in regressed fish, dropped in vitellogenic fish and had no significant differences in expression throughout the rest of maturation [minimum at OV, 0.2 ± 0.1 ; maximum at R, 1.0 ± 0.3 ; $p = 0.00$ (Figure 2.6 B)]. Expression of FSHr in ovary peaked in early mature fish and had no significant differences between early maturation to ovulation [minimum at R, 1.0 ± 0.2 ; maximum at EM, 8.9 ± 2.5 ; $p = 0.00$ (Figure 2.6 C)]. Ovarian expression of LHr increased from regressed to fully mature fish, and decreased in ovulating fish [minimum at R, 1.0 ± 0.3 ; maximum at FM, 56.6 ± 15.7 ; $p = 0.00$ (Figure 2.6 D)].

All steroidogenic enzymes increased in expression throughout the ovarian development cycle. StAR expression was highest in ovulating fish [minimum at R, 1.0 ± 0.1 ; maximum at OV, 184.1 ± 92.9 ; $p = 0.00$ (Figure 2.7 A)]. CYP11 expression was highest in fully mature fish [minimum at R, 1.0 ± 0.1 ; maximum at FM, 21.4 ± 6.8 ; $p = 0.00$ (Figure 2.7 B)]. CYP17 expression was highest in fully mature and ovulating fish [minimum at V, 0.7 ± 0.1 ; maximum at OV, 4.3 ± 1.3 ; $p = 0.002$ (Figure 2.7 C)]. CYP19a1 peaked in fully mature fish [minimum at V, 0.7 ± 0.2 ; maximum at FM, 6.2 ± 3.0 ; $p = 0.00$ (Figure 2.7 D)].

Liver gene expression was analyzed from whole liver tissue dissected from fish that were sampled over the reproductive cycle having ovaries characterized as containing >75% of a desired stage. Liver expression data were normalized to 18sRNA and displayed as the fold change relative to the regressed stage. The constant expression of 18sRNA across all ovarian developmental stages is shown in Supplemental Figure S2.2.

Expression of ESR1 in the liver increased throughout the cycle, and was highest in ovulating fish [minimum at R, 1.0 ± 0.1 ; maximum at OV, 2.5 ± 0.4 ; $p = 0.00$ (Figure

2.8 A)]. VTG1 expression is associated with ESR1 expression trends [minimum at R, 1.0 ± 0.2 ; maximum at OV, 26.8 ± 1.8 ; $p = 0.00$ (Figure 2.8 D)]. Expression of ESR2a decreased throughout the cycle, with no significant changes between early maturation to ovulation [minimum at FM, 0.2 ± 0.0 ; maximum at R, 1 ± 0.1 ; $p = 0.00$ (Figure 2.8 B)]. Expression of ESR2b peaked at in vitellogenic fish, with low expression and no significant changes in all other stages [minimum at R, 1.0 ± 0.13 ; maximum at V, 11.1 ± 1.8 ; $p = 0.00$ (Figure 2.8 B)].

Brain gene expression was analyzed from whole brain tissue dissected from fish that were sampled over the reproductive cycle having ovaries characterized as containing >75% of a desired stage. Brain expression data were normalized to EF1 α and displayed as the fold change relative to the regressed stage. The constant expression of EF1 α across all ovarian developmental stages is shown in Supplemental Figure S2.3. There were no significant changes in expression of CYP19b across the ovarian development cycle [minimum at OV, 0.8 ± 0.3 ; maximum at V, 1.2 ± 0.1 ; $p = 0.61$ (Supplemental Figure S2.4)].

2.4.2 Molecular Characterization across Follicular Stages in the Maturation Cycle

Using additional fish, gene expression in follicles isolated by 100% stage were assessed. Gene expression in stages 1-5 (refer to Table 2.1) were measured and verified through histological analysis. Stage 5 (ovulating) was excluded as qPCR results showed non-detectable expression (with the exception of ESR2b). Follicular expression data were normalized to adjusted values of ESR2a and displayed as the fold change relative to stage 1. The formula used for adjusting ESR2a values is found in Supplementary Table S2.1.

Follicular expression of ESR1 had no significant differences amongst stages 1-3; however, stage 4 (late mature) was significantly lower than stage 2 (vitellogenic) [minimum at stage 4, 0.7 ± 0.1 ; maximum at stage 2, 1.6 ± 0.1 ; $p = 0.00$ (Figure 2.9 A)]. Expression of ESR2b decreased across maturation [minimum at stage 5 (ovulated), 0.0 ± 0.0 ; maximum at stage 1 (cortical alveoli), 1 ± 0.3 ; $p = 0.00$ (Figure 2.9 B)]. FSHr expression peaked during stage 2, and had low expression with no significant differences across all other stages [minimum at stage 4, 0.7 ± 0.3 ; maximum at stage 2, 6.4 ± 0.7 ; $p = 0.00$ (Figure 2.9 C)]. LHr expression increased across maturation until stage 3 (early mature) [minimum at stage 1, 1 ± 0.3 ; maximum at stage 4, 19.0 ± 2.1 ; $p = 0.00$ (Figure 2.9 D)].

Follicular expression of StAR and CYP11 increased throughout the cycle. StAR had highest expression during stage 4 (late mature) [minimum at stage 1, 1 ± 0.09 ; maximum at stage 4, 171.5 ± 76.0 ; $p = 0.00$ (Figure 2.10 A)], and CYP11 peaked during stage 3 (early mature) [minimum at stage 1, 0.9 ± 0.2 ; maximum at stage 3, 6.1 ± 1.4 ; $p = 0.00$ (Figure 2.10 B)]. Expression of CYP17 peaked during stage 1 (cortical alveoli) and had minimal expression across stages 2-4 (vitellogenic – late mature) [minimum at stage 3, 0.0 ± 0.0 ; maximum at stage 1, 1 ± 0.2 ; $p = 0.00$ (Figure 2.10 C)]. There were no significant changes in expression of CYP19a1 between stages 1-3 (cortical alveoli – early mature); however, there was a significant decrease in expression at stage 4 (late mature) [minimum at stage 4, 0.2 ± 0.1 ; maximum at stage 2, 1.3 ± 0.2 ; $p = 0.00$ (Figure 2.10 D)].

2.4.3 Effect of EE₂ on Basal and hCG-stimulated Follicular Steroid Production and Gene Expression

Gene expression in follicles (stages 1-4; cortical alveoli, vitellogenic, early mature, and late mature) isolated by stage (e.g., stage 2 contained 100% follicles at stage 2) were assessed after *in vitro* incubation with varying EE₂ treatments. The medium was removed and utilized for hormone analysis and the tissue was utilized for gene expression analysis. The basal and hCG-stimulated steroid results of the *in vitro* incubations from ovarian follicles (stages 2-4), treated with 50, 100 and 250 nM EE₂ are shown in Table 2.4. As expected, basal T and MIS levels increased significantly with increased maturational stage of the ovarian follicles [for T, $p = 0.00$ from Two-way ANOVA; for MIS $p = 0.00$ from Two-way ANOVA (Table 2.4)]; however, the steroid levels were unaffected by EE₂ treatments (50, 100 and 250 nM) at all stages examined. There was no significant effect of EE₂ on basal T levels (for stages 2-4, $p = 0.11, 0.09, \text{ and } 0.67$, respectively (Table 2.4)) or hCG-stimulated T levels (for stages 2-4, $p = 0.51, 0.36, \text{ and } 0.57$, respectively (Table 2.4)) for any stage of maturation analyzed. There was no significant effect of EE₂ on basal MIS levels (for stages 2-4, $p = 0.88, 0.26, \text{ and } 0.97$ (Table 2.4)) or hCG-stimulated MIS levels (for stages 2-4, $p = 0.44, 0.69, 0.98$, respectively (Table 2.4)) for any stage of maturation analyzed. hCG-stimulated incubations produced significantly higher levels of hormone for both T and MIS during stages 2-4 ($p < 0.05$; data not shown).

Follicular expression data were normalized to β -actin, and displayed as the fold change relative to the time 0-hour control. There were no significant effects of EE₂ treatment on CYP19a1 expression for any stage of follicular development (Table 2.5).

There was a significant increase in CYP19a1 expression with hCG stimulation for stages 2, 3 and 4 (for stages 1-4, $p = 0.00, 0.03, 0.00, 0.00$). Time 0 expression was greater than basal 24 h expression for all stages, but varied per stage for hCG-stimulated expression. Stage 1: time 0 > 24h expression, stage 2: time 0 = hCG-stimulated 24 h expression, stage 3 and 4: time 0 < hCG-stimulated 24 h expression. There was no significant effect of EE₂ treatment on LHr (luteinizing hormone) expression for any stage of follicular development as well (Table 2.5). hCG stimulation had no effect on LHr expression for either stage (for stages 1-4, $p = 0.21, 0.71, 0.09, 0.43$). Time 0 expression was equal to 24 h expression levels for stages 1-4, however the basal control was significantly lower than time 0 expression at stage 3.

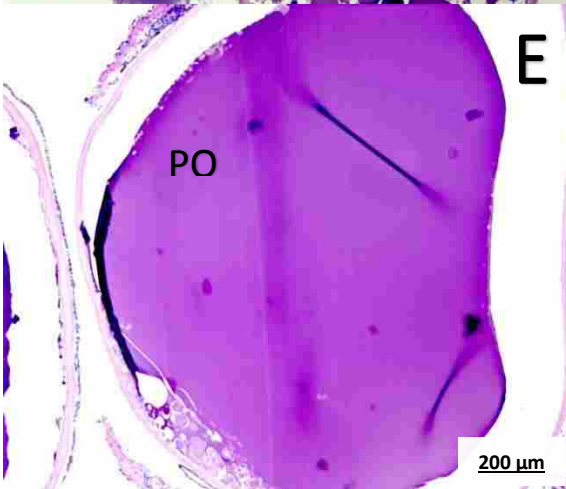
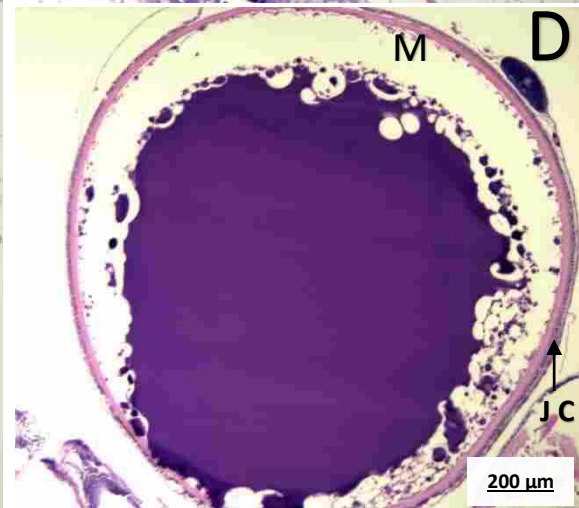
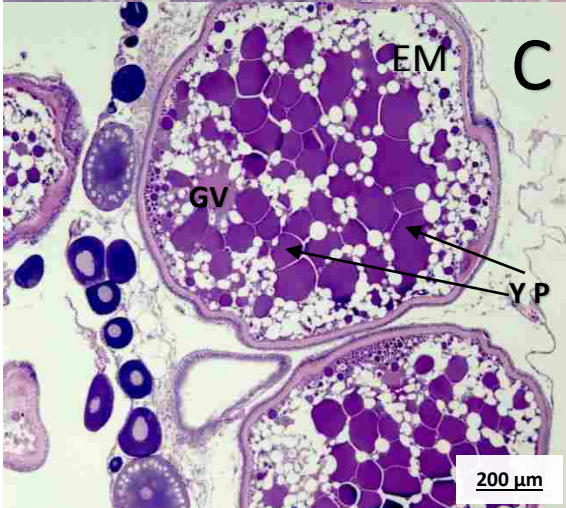
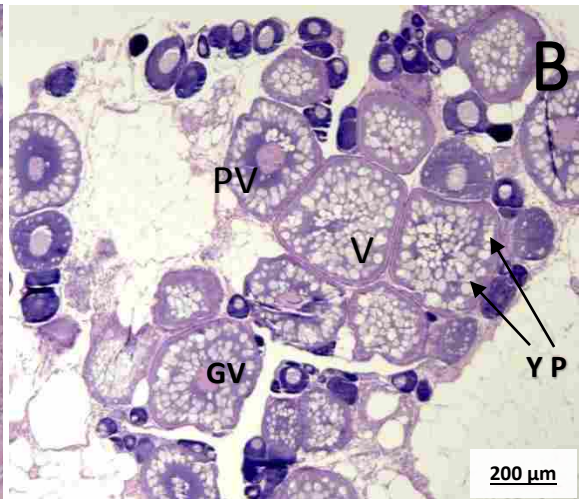
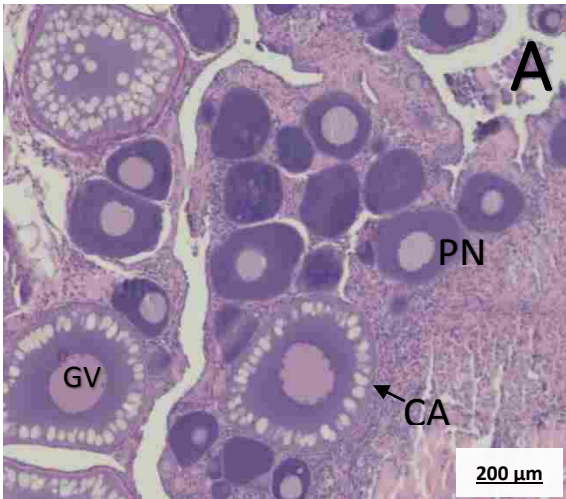


Figure 2.2 Images of ovarian tissue sampled from mummichog over the developmental cycle to provide examples of the ovaries staged as A) regressed (primarily stage 1), B) vitellogenic (primarily stage 2), C) early mature (primarily stage 3), D) fully mature (primarily stage 4), E) ovulated (primarily stage 5). Scale bar is 200 μm . PN = perinucleolar; CA = cortical alveolar; GV = germinal vesicle; PV = previtellogenic; V = vitellogenic; YP = yolk proteins; EM = early mature; M = mature; PO = post ovulated; JC = jelly coat. H&E stained sections were prepared by the University of Guelph Histology Laboratory.

Table 2.3. Mean (\pm SEM) body weight, gonad weight, liver weight, gonadosomatic and liversomatic index of female mummichog sampled with ovaries containing a majority of cortical alveoli (regressed), vitellogenic, early mature, late mature (fully mature) or ovulated follicles. Different letters indicate significant differences for the GSI and LSI. $P = 0.00$ for body weight, gonad weight, GSI and LSI. $P = 0.198$ for liver weight.

Sample Date	Stage	Body Weight (g)	Gonad Weight (g)	Liver Weight (g)	GSI (%)	LSI (%)	N
December 2015	Regressed	10.12 \pm 0.55 ^A	0.17 \pm 0.01 ^A	0.65 \pm 0.04 ^A	1.72 \pm 0.17 ^A	6.53 \pm 0.24 ^A	20
April 2016	Vitellogenic	13.39 \pm 0.47 ^{AB}	0.44 \pm 0.07 ^B	0.55 \pm 0.05 ^A	3.30 \pm 0.51 ^B	4.12 \pm 0.39 ^B	10
April 2016	Early Mature	15.58 \pm 0.85 ^{BC}	1.07 \pm 0.10 ^C	0.66 \pm 0.07 ^A	6.78 \pm 0.34 ^C	4.21 \pm 0.43 ^B	14
April 2016	Fully Mature	14.54 \pm 0.77 ^{BC}	1.23 \pm 0.08 ^C	0.56 \pm 0.05 ^A	8.58 \pm 0.57 ^{CD}	3.87 \pm 0.32 ^B	14
May 2016	Ovulating	17.40 \pm 1.02 ^C	2.09 \pm 0.45 ^D	0.80 \pm 0.11 ^A	12.36 \pm 3.2 ^D	4.54 \pm 0.36 ^B	4

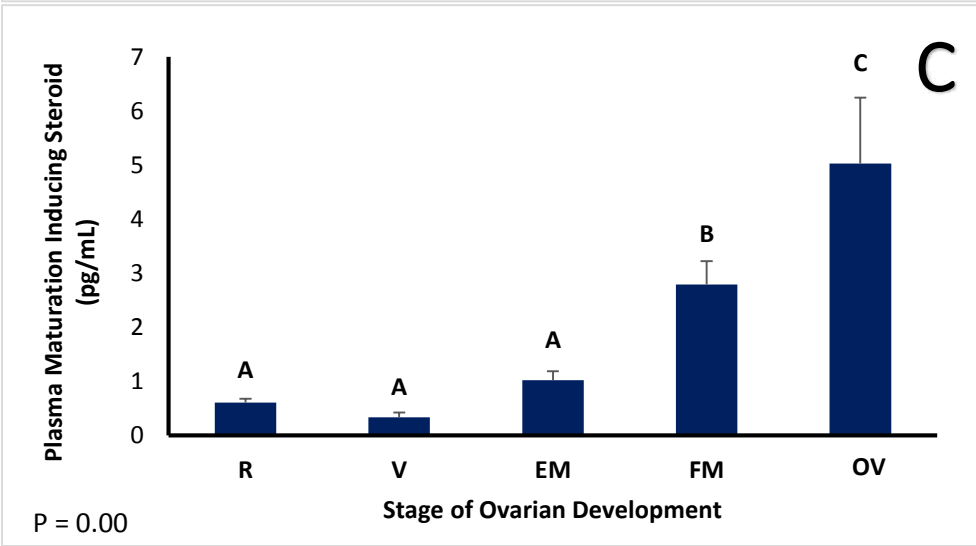
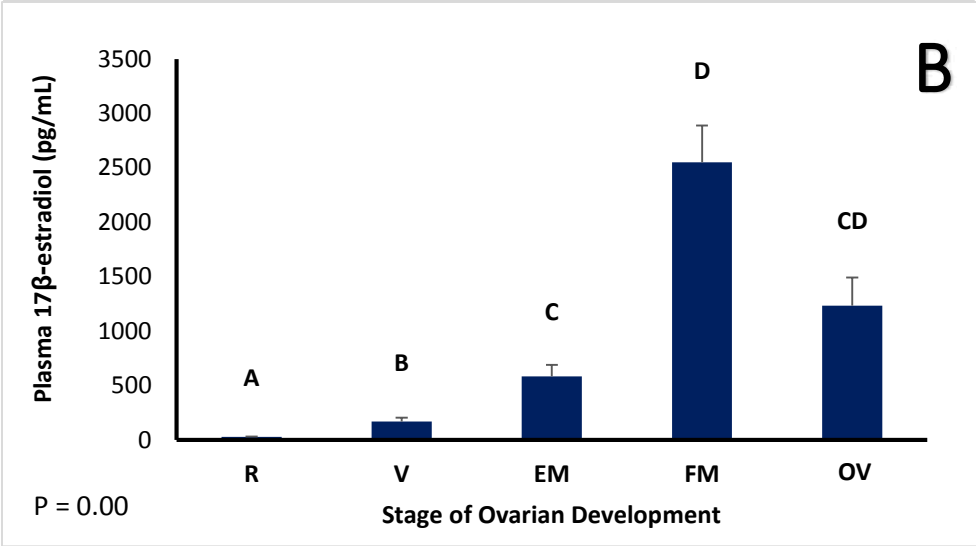
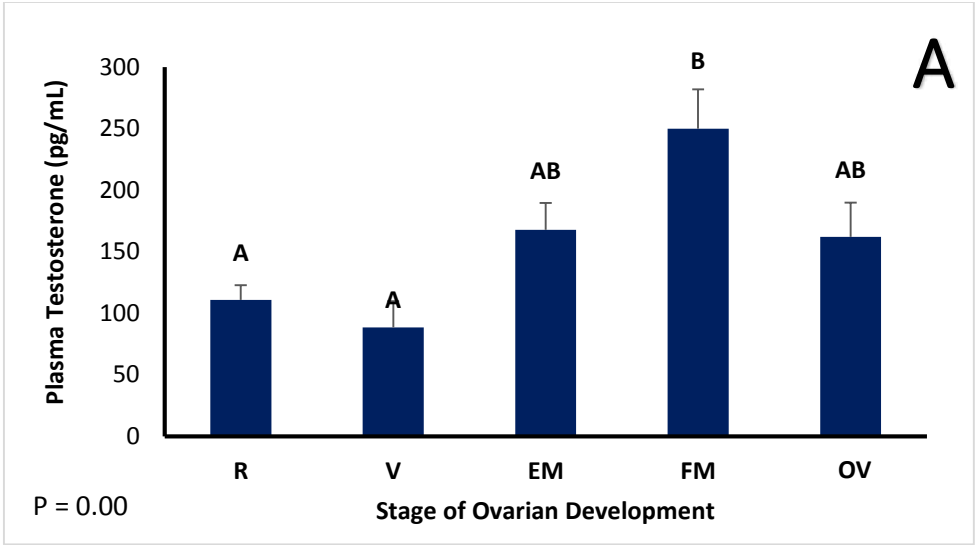


Figure 2.3 Mean (\pm SEM) plasma testosterone (A), 17β -estradiol (B), and maturation inducing steroid (C) levels (pg/mL) characterized over the developmental cycle in female mummichog with ovaries containing a majority of cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Different letters indicate significant differences. The number of females sampled at each point were 15, 12, 16, 14, and 4, respectively, for each hormone.

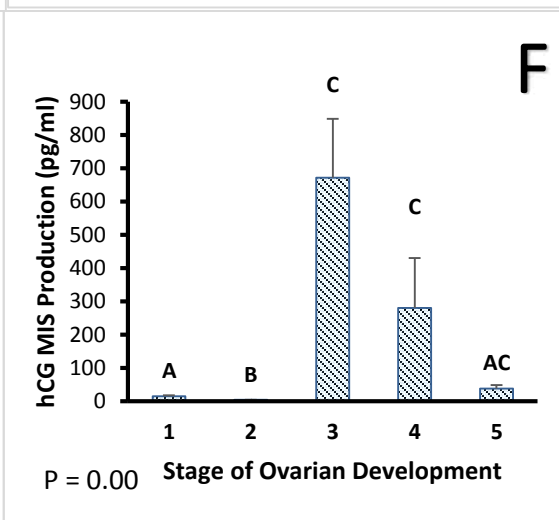
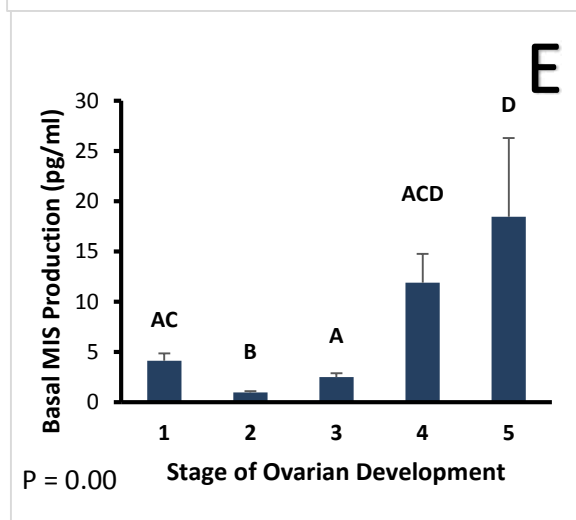
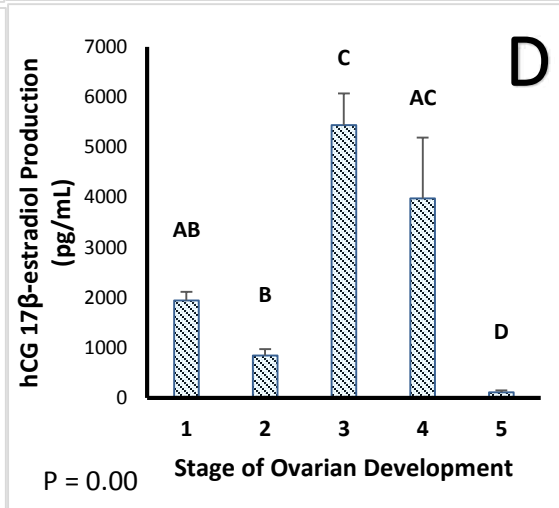
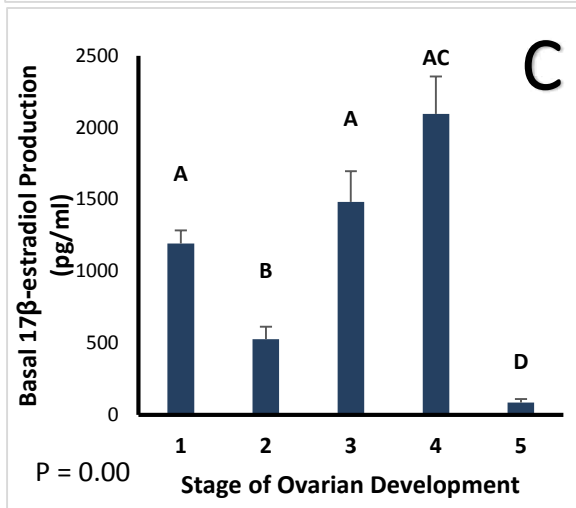
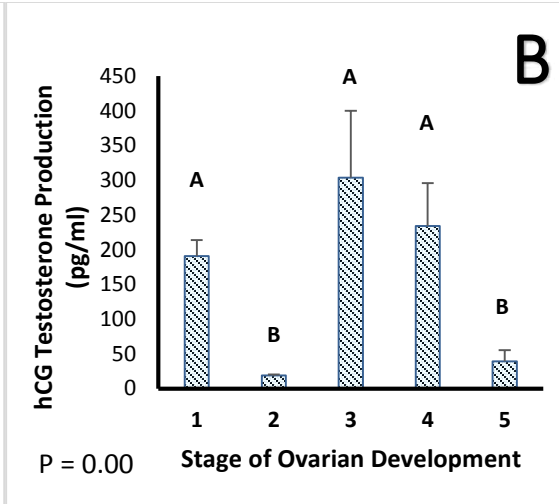
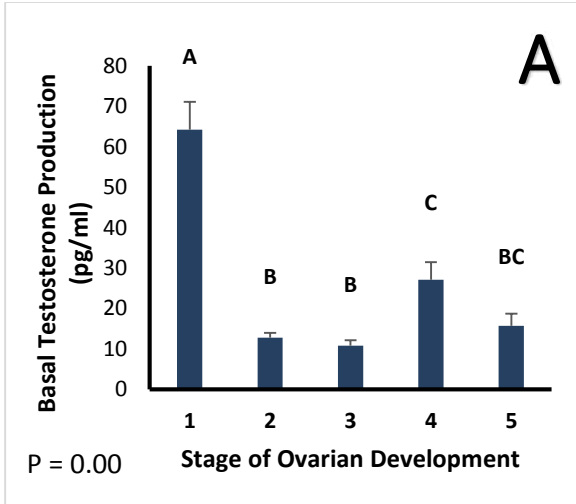


Figure 2.4. Mean (\pm SEM) follicular *in vitro* production of basal testosterone (A), hCG-stimulated testosterone (B), basal 17 β -estradiol (E₂; C), hCG-stimulated E₂ (D), basal maturation inducing steroid (MIS; E), and hCG-stimulated MIS (F) levels (pg/mL), characterized over the ovarian development cycle in cortical alveoli (stage 1), vitellogenic (stage 2), early mature (stage 3), late mature (stage 4) and ovulated (stage 5) follicles. Different letters indicate significant differences. N = 10, 14-15, 11-13, 13-15, and 8-9 for stages 1-5, respectively.

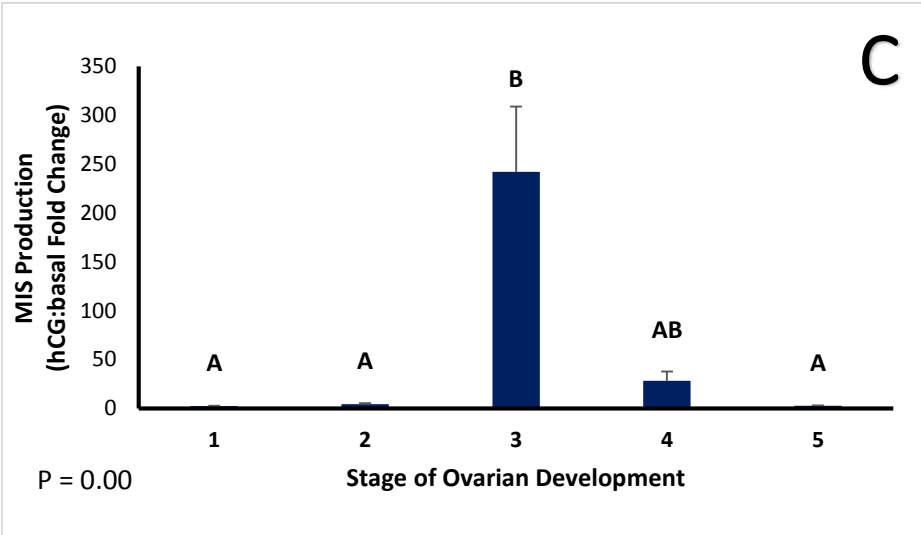
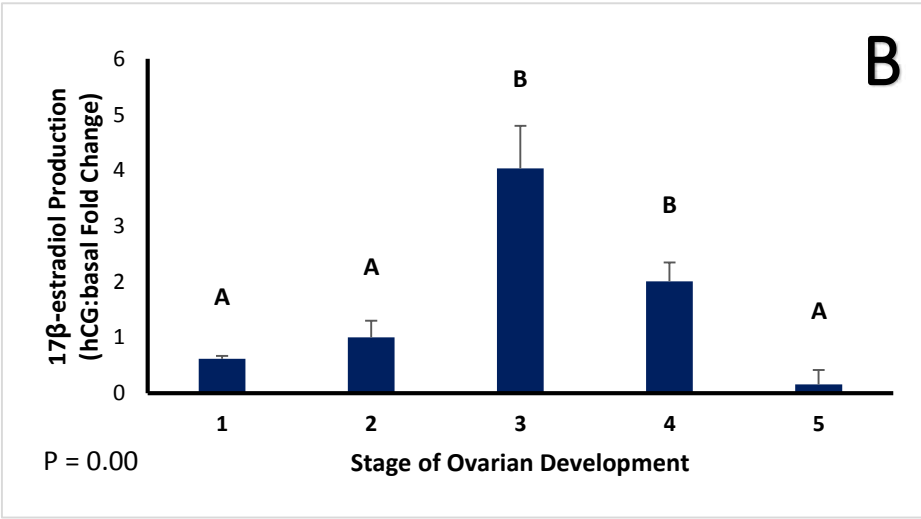
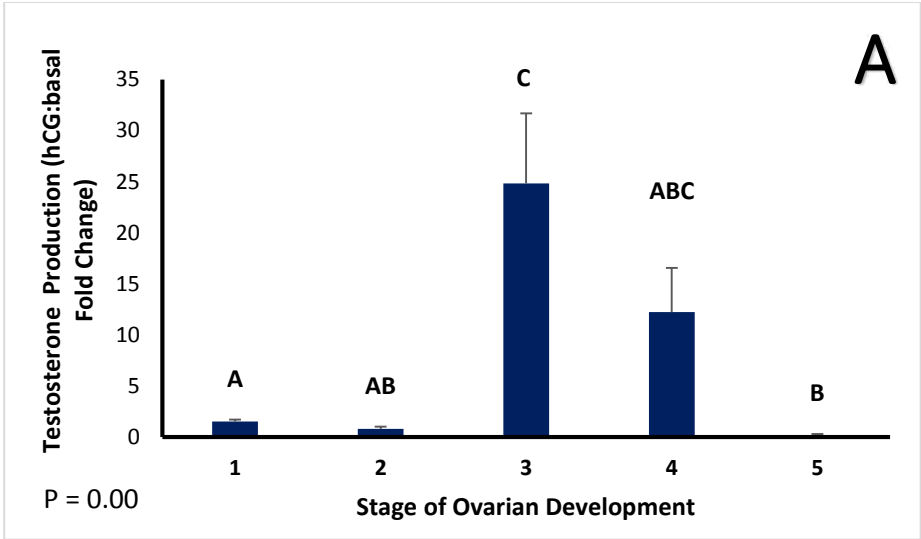


Figure 2.5. Fold increase of hCG-stimulated to basal hormone production of testosterone (A), 17β -estradiol (B) and maturation inducing steroid (C) characterized over the ovarian development cycle in cortical alveolus (stage 1), vitellogenic (stage 2), early mature (stage 3), late mature (stage 4) and ovulated (stage 5) follicles. Different letters indicate significant differences. N = 10, 14-15, 11-12, 13-15, and 8-9 for stages 1-5, respectively.

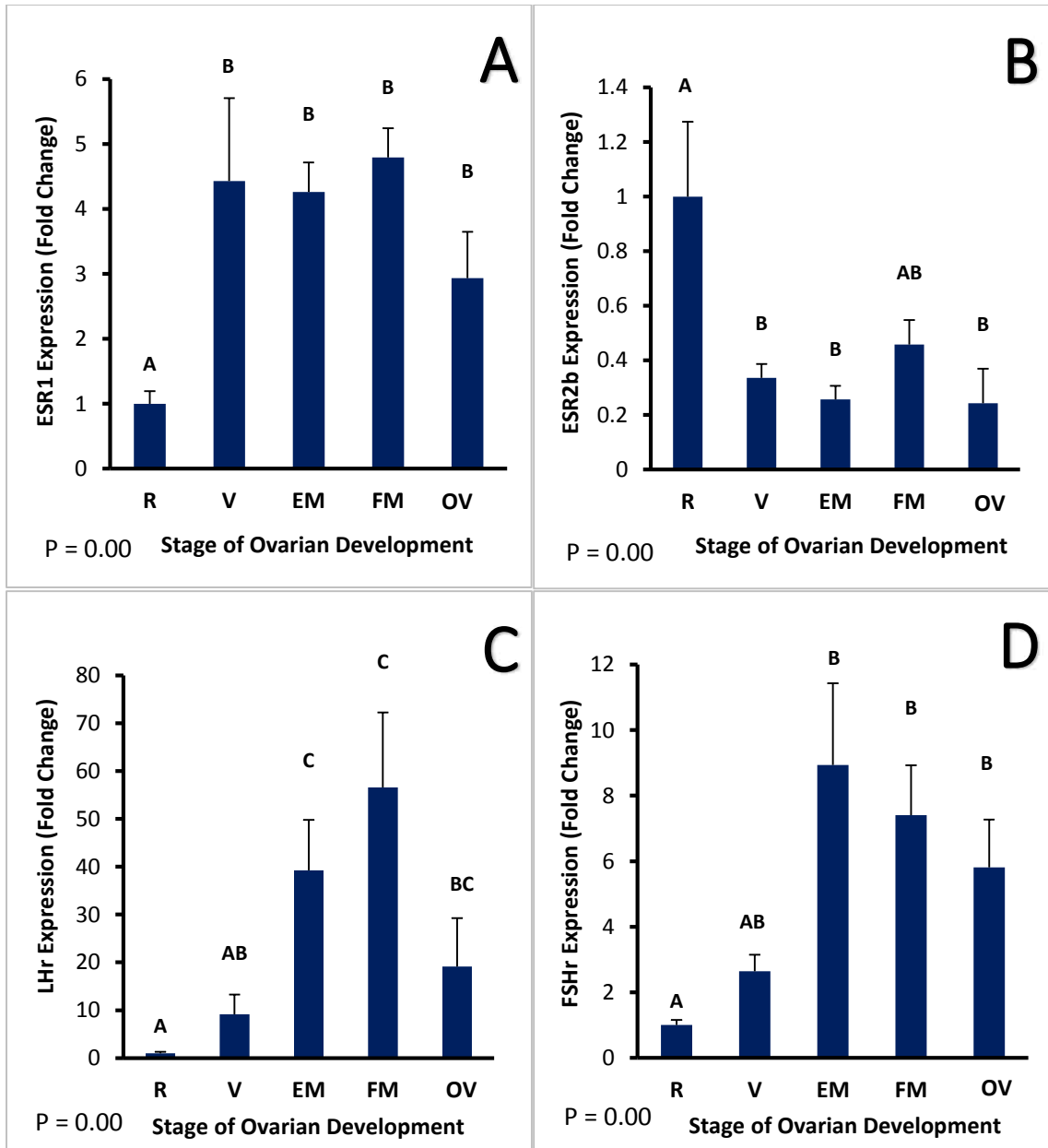


Figure 2.6. Expression of ESR1 (A), ESR2b (B), FSHr (C), and LHr (D) in mummichog whole ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Data were normalized to ESR2a and displayed as the fold change relative to the R stage. Values represent the mean \pm S.E.M. N= 8-9, 10, 14, 14, and 4, for stages 1-5, respectively, as determined by qPCR. Different letters indicate significant differences.

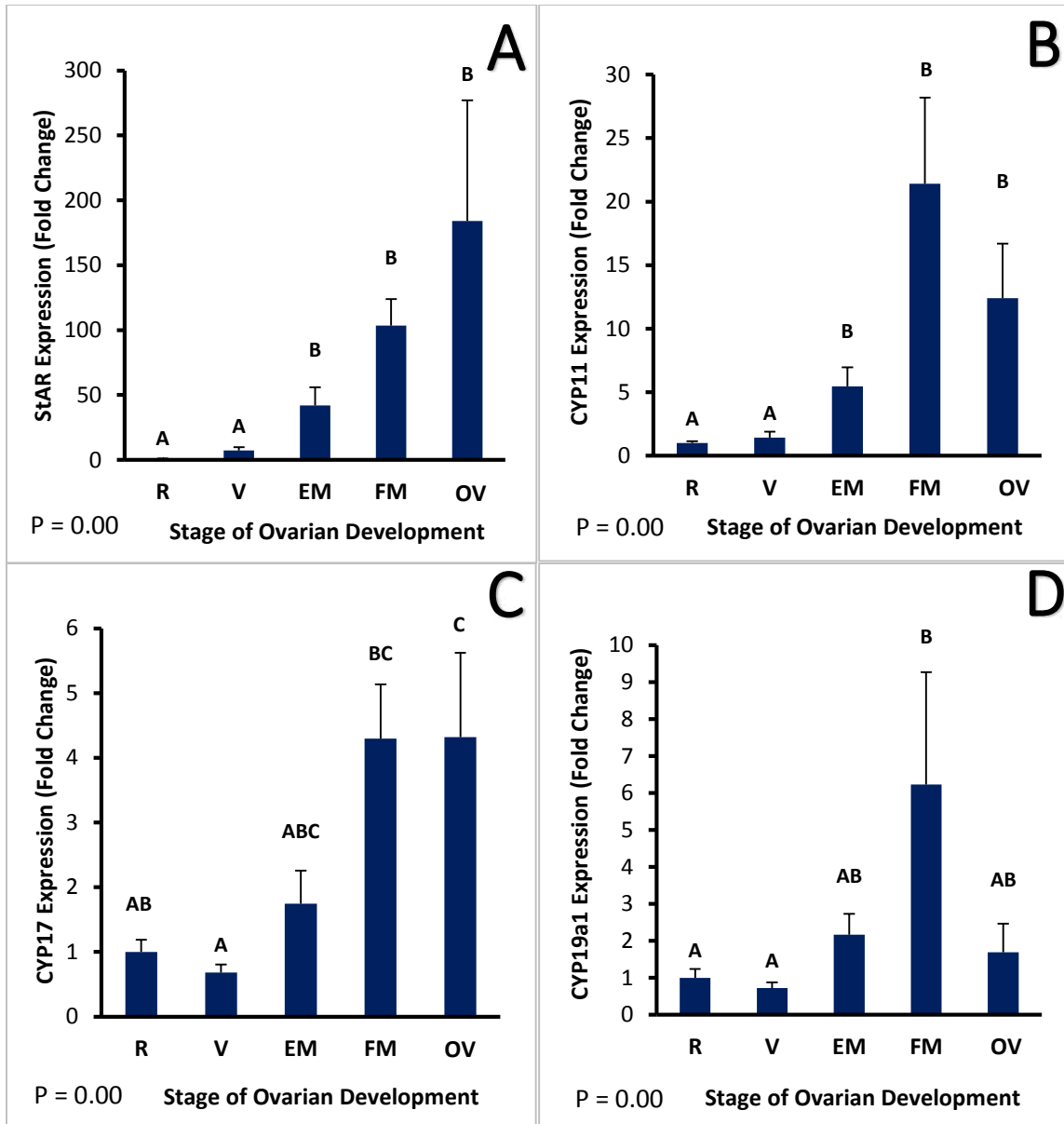


Figure 2.7. Expression of StAR (A), CYP11 (B), CYP17 (C), and CYP19a1 (D) in mummichog whole ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Data were normalized to ESR2a and displayed as the fold change relative to the R stage. Values represent the mean \pm S.E.M. N= 9, 10, 14, 14, and 4, for stages 1-5, respectively, as determined by qPCR. Different letters indicate significant differences.

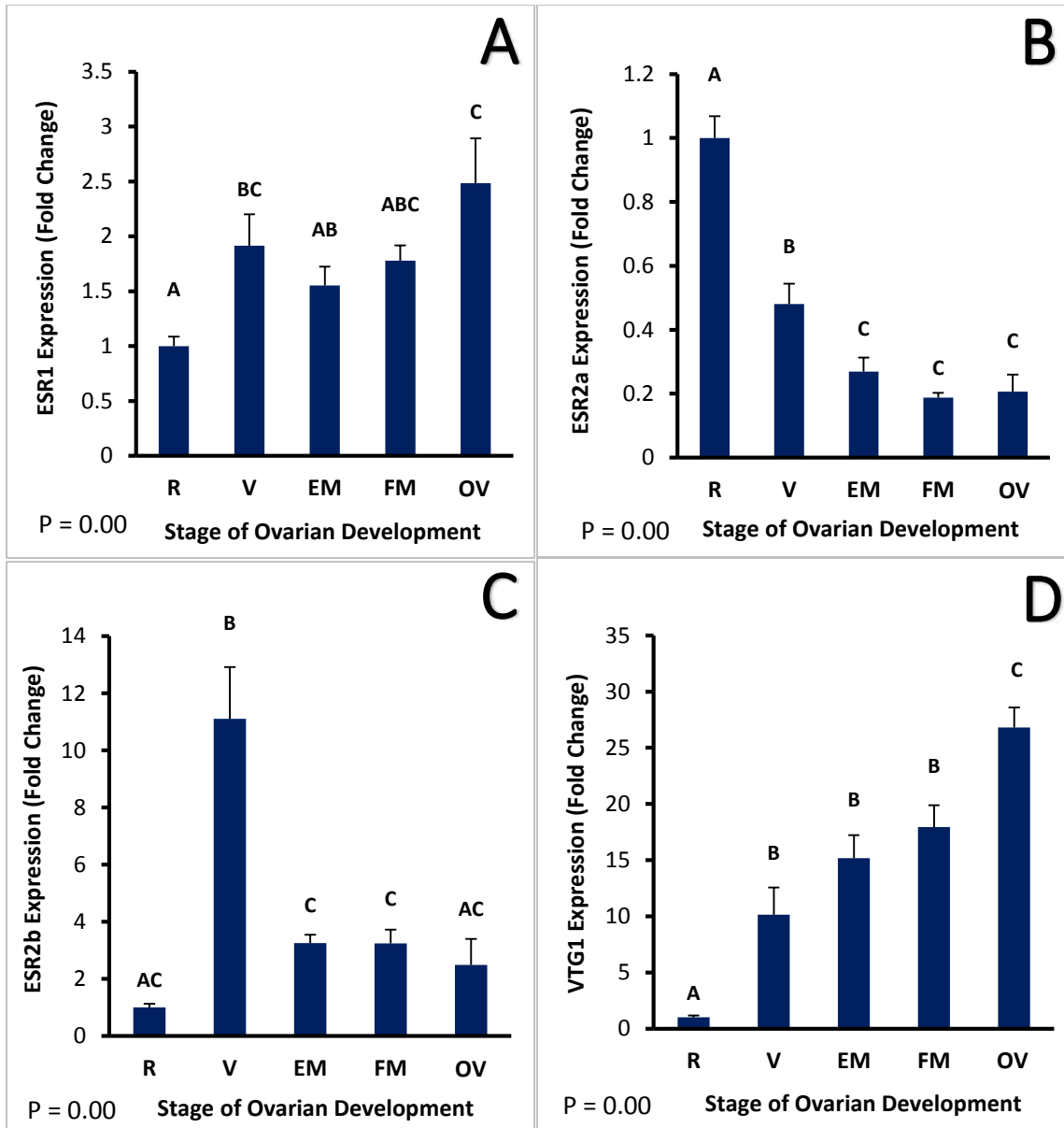


Figure 2.8. Liver expression of ESR1 (A), ESR2a (B), ESR2b (C), and VTG1 (D) in mummichog with ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Data were normalized to 18sRNA and displayed as the fold change relative to the R stage. Values represent the mean \pm S.E.M. N= 12, 10, 13-14, 12-14, and 4, for stages 1-5, respectively, as determined by qPCR. Different letters indicate significant differences.

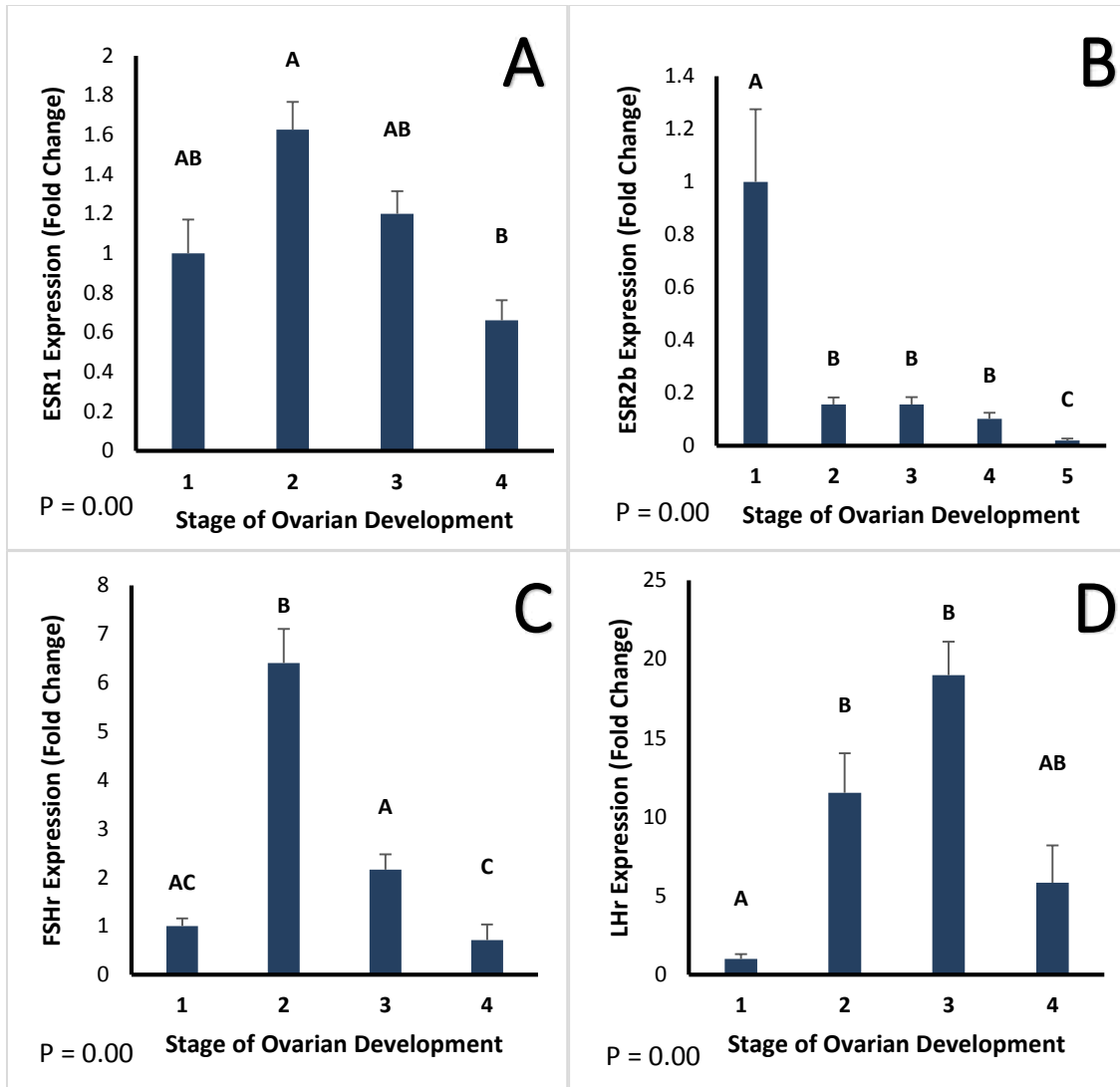


Figure 2.9. Stage specific expression of ESR1 (A), ESR2b (B), FSHr (C), and LHr (D) in mummichog with cortical alveoli tissue (stage 1), vitellogenic (stage 2), early mature (stage 3), and late mature (stage 4) follicles. Data were normalized to adjusted values of ESR2a and displayed as the fold change relative to stage 1. Values represent the mean \pm S.E.M. N= 8-9, 8, 9, and 7-8, for stages 1-4, respectively; N= 7 for stage 5 (ESR2b only) as determined by qPCR. Different letters indicate significant differences.

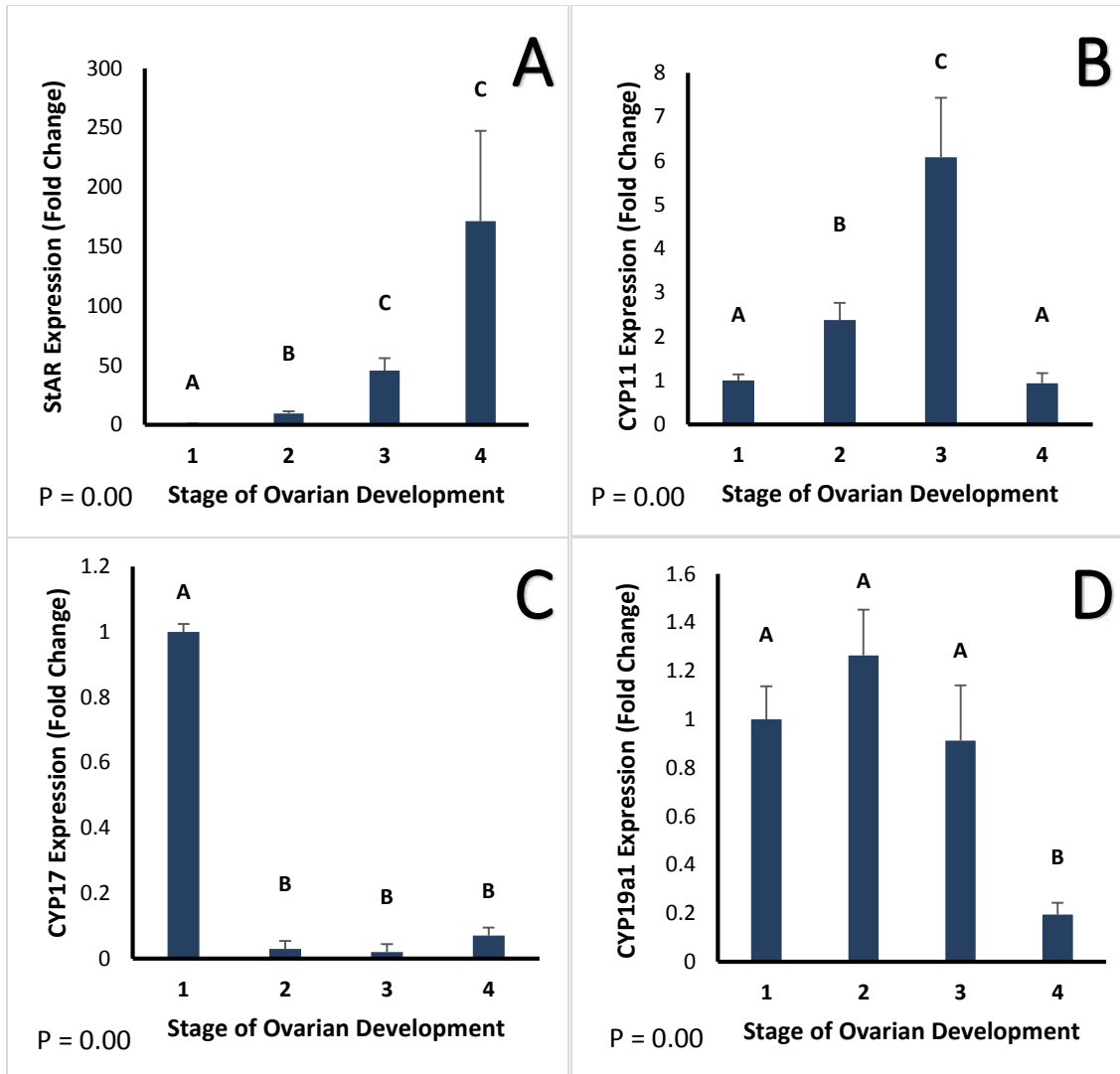


Figure 2.10 Stage specific expression of StAR (A), CYP11 (B), CYP17 (C), CYP19a1 (D) in mummichog with cortical alveoli tissue (stage 1), vitellogenic (stage 2), early mature (stage 3), and late mature (stage 4) follicles. Data were normalized to adjusted values of ESR2a and displayed as the fold change relative to stage 1. Values represent the mean \pm S.E.M. N= 8-9, 8, 8-9, and 7-8, for stages 1-4, respectively, as determined by qPCR. Different letters indicate significant differences.

Table 2.4 Unstimulated (basal) and human chorionic gonadotropin (hCG)-stimulated testosterone (T) and maturation inducing steroid (MIS) levels from ovarian follicles at different stages of maturation [stage 2 (vitellogenic), stage 3 (early mature), stage 4 (late mature)], exposed to solvent control (ethanol; s ctrl) and increasing concentrations of 17 α -ethinyl estradiol (EE₂). The hormone levels are presented as mean \pm S.E.M. values (pg/mL). Mean fold increase is shown to compare hCG-stimulated production to basal production, per treatment and stage. There are no significant differences in T or MIS production among treatments (solvent control, 50 nM EE₂, 100 nM EE₂, 250 nM EE₂) per stage, within groups (basal or hCG-stimulated). There are no significant differences in fold increase among treatments, per stage. hCG-stimulated hormone production is significantly higher than basal hormone production, per stage, for each hormone. Average basal hormone production significantly increases with stage, for each hormone. N = 7-8 for each treatment per stage. P-values from statistical tests performed on T and MIS data are found in Supplementary Table S2.2.

Hormone	EE ₂ Treatment	Stage 2			Stage 3			Stage 4		
		Basal	hCG+	Fold Increase	Basal	hCG+	Fold Increase	Basal	hCG+	Fold Increase
T	<i>S Ctrl</i>	8.3 ± 1.2	30.3 ± 4.9	4.0 ± 0.6	26.2 ± 4.1	235.5 ± 52.0	14.1 ± 4.1	66.2 ± 16.5	237.2 ± 59.9	5.63 ± 2.4
	<i>50 nM</i>	9.6 ± 1.1	24.3 ± 3.8	2.0 ± 0.5	25.5 ± 4.2	143.8 ± 15.7	9.7 ± 3.9	74.6 ± 13.2	193.3 ± 68.4	5.05 ± 2.2
	<i>100 nM</i>	11.8 ± 0.9	21.5 ± 3.1	1.9 ± 0.3	28.7 ± 4.3	217.4 ± 35.1	9.5 ± 2.7	64.4 ± 11.4	267.0 ± 58.9	4.9 ± 1.5
	<i>250 nM</i>	13.5 ± 1.0	32.3 ± 6.7	2.4 ± 0.6	32.8 ± 4.1	249.4 ± 37.9	7.6 ± 1.4	61.2 ± 12.5	247.6 ± 47.9	5.8 ± 1.5
MIS	<i>S Ctrl</i>	1.8 ± 0.4	30.8 ± 17.2	48.8 ± 20.0	12.2 ± 2.1	1456.2 ± 508.8	216.5 ± 90.7	35.0 ± 8.4	247.4 ± 45.7	14.9 ± 5.8
	<i>50 nM</i>	1.9 ± 0.5	43.1 ± 16.9	46.3 ± 13.4	13.0 ± 3.3	1220.3 ± 199.6	187.0 ± 64.5	36.4 ± 9.4	396.3 ± 126.6	14.3 ± 4.0
	<i>100 nM</i>	1.7 ± 0.4	24.3 ± 8.0	23.1 ± 12.1	12.7 ± 3.1	1486.1 ± 436.2	188.6 ± 53.4	35.7 ± 8.9	301.1 ± 68.3	19.0 ± 6.9
	<i>250 nM</i>	2.1 ± 0.3	56.7 ± 18.4	32.3 ± 9.38	12.9 ± 2.7	1518.4 ± 286.9	135.1 ± 31.7	34.1 ± 8.8	485.5 ± 176.0	28.3 ± 10.9

Table 2.5 Unstimulated (basal) and human chorionic gonadotropin (hCG)-stimulated P450 aromatase (CYP19a1) and luteinizing hormone receptor (LHr) gene expression of ovarian follicles at different stages [stage 1 (cortical alveolus), stage 2 (vitellogenic), stage 3 (early mature), stage 4 (late mature)] of maturation exposed to solvent control (ethanol; s ctrl) and increasing concentrations of 17 α -ethinyl estradiol (EE₂). Gene expression is presented as fold increase to the 0-hour control, per stage, normalized to β -actin (mean \pm S.E.M). values (pg/mL). One-way ANOVA was completed to assess gene expression of CYP19a1 or LHr among treatments (basal or hCG-stimulated 0-hour control, solvent control, 50 nM EE₂, 100 nM EE₂, 250 nM EE₂) per stage. Different letters, or astericks, indicate significant differences among treatments, within stages of an individual gene. There were no significant differences among treatments, within stages of LHr expression. N = 7-8 for each treatment per stage. ($p > 0.05$, ANOVA followed by Tukey post-hoc test). P-values from statistical tests performed on CYP19a1 and LHr data are found in Supplementary Table S2.3.

Gene	EE ₂ Treatment	Stage 1		Stage 2		Stage 3		Stage 4	
		Basal	hCG+	Basal	hCG+	Basal	hCG+	Basal	hCG+
CYP19a1	<i>O h</i>	1 ± 0.24 *		1 ± 0.15 ^B		1 ± 0.21 ^C		1 ± 0.32 ^A	
	<i>S Ctrl</i>	0.23 ± 0.04	0.23 ± 0.06	0.06 ± 0.01 ^A	1.22 ± 0.40 ^B	0.07 ± 0.01 ^A	6.11 ± 1.65 ^B	0.19 ± 0.04 ^A	5.67 ± 2.12 ^B
	<i>50 nM</i>	0.18 ± 0.02	0.20 ± 0.04	0.07 ± 0.02 ^A	1.82 ± 0.40 ^B	0.09 ± 0.02 ^A	7.37 ± 1.82 ^B	0.27 ± 0.10 ^A	5.90 ± 1.71 ^B
	<i>100 nM</i>	0.21 ± 0.05	0.18 ± 0.02	0.08 ± 0.02 ^A	1.14 ± 0.27 ^B	0.09 ± 0.02 ^A	6.22 ± 1.26 ^B	0.28 ± 0.07 ^A	7.01 ± 2.38 ^B
	<i>250 nM</i>	0.18 ± 0.01	0.25 ± 0.04	0.10 ± 0.03 ^A	1.94 ± 0.53 ^B	0.09 ± 0.01 ^A	5.10 ± 0.90 ^B	0.30 ± 0.06 ^A	7.06 ± 1.88 ^B
LHr	<i>O h</i>	1 ± 0.14		1 ± 0.27		1 ± 0.13		1 ± 0.13	
	<i>S Ctrl</i>	1.00 ± 0.14	1.19 ± 0.14	1.18 ± 0.30	0.91 ± 0.20	0.65 ± 0.09	0.62 ± 0.09	0.54 ± 0.13	0.71 ± 0.12
	<i>50 nM</i>	1.62 ± 0.12	1.10 ± 0.21	1.44 ± 0.51	0.92 ± 0.18	0.86 ± 0.12	0.67 ± 0.11	0.65 ± 0.25	0.59 ± 0.05
	<i>100 nM</i>	1.30 ± 0.17	1.37 ± 0.09	1.18 ± 0.24	0.69 ± 0.17	0.79 ± 0.11	0.62 ± 0.05	0.63 ± 0.11	0.76 ± 0.16
	<i>250 nM</i>	1.07 ± 0.13	1.47 ± 0.35	1.18 ± 0.35	0.74 ± 0.14	0.76 ± 0.14	0.71 ± 0.08	0.64 ± 0.12	0.79 ± 0.12

2.5 Discussion

This study further develops the estuarine mummichog as a model species for understanding the reproductive physiology of teleosts. The study confirmed that, similar to other work in mummichog (Lin *et al.*, 1987), E₂ is produced by the ovarian follicles from the cortical alveolus stage to late maturation, distinguishing this species from many other teleosts, in which E₂ levels rise from cortical alveolus to completion of vitellogenesis and drop prior to maturation (Clelland and Peng, 2009). It is the first study to demonstrate changes in gonadal steroidogenic enzymes and ERs, hepatic VTG1 and ERs, and brain aromatase gene expression across the stages of female mummichog maturation. The expression of ERs, VTG1 and CYP19a1 are associated with patterns of plasma and *in vitro* E₂ levels throughout ovarian development, while CYP17 expression is not associated with the patterns of E₂. While other studies have suggested that ovarian LHr and aromatase gene expression are estrogen-responsive in fish (Liu *et al.*, 2013; Lyssimachou *et al.*, 2006), addition of EE₂ to *in vitro* ovarian follicle incubations had no effect. Overall, these studies support the hypothesis that the tolerance exhibited by mummichog to exogenous estrogens could be partly attributable to continuously high levels of E₂ present in the maturing ovary.

Plasma T and E₂ were measured in female mummichog throughout recrudescence, in reproductively regressed to ovulating fish; plasma hormone patterns of T and E₂ were generally similar to those previously demonstrated in mummichog (Lin *et al.*, 1987; Shimizu 2003). Plasma T levels were highest in fully mature fish, with no significant differences between regressed and early mature stages. This is similar to Shimizu (2002), in which plasma levels of T dramatically increased in pre-spawning

periods; T levels dropped dramatically during the late spawning-post spawning period (Shimizu 2002). Given T's role as the precursor to E₂ in the maturing ovary (Nagahama and Yamashita, 2008; Sabet *et al.*, 2009), it is not surprising in the present study that higher levels of plasma T generally associated with higher levels of plasma E₂. Plasma E₂ levels increased dramatically from regressed to fully mature stages with significant differences between the progressive stages of maturation. These findings are similar to Shimizu (2002), in which a substantial increase in plasma E₂ was demonstrated during pre-spawning periods, and eventually dropped significantly during the late-spawning to post-spawning period. T and E₂ production trends in this study differ from other fish species, as plasma T and E₂ levels peak during the pre-spawning period in rainbow trout (*Oncorhynchus mykiss*; Whitehead *et al.*, 1978), goldfish (*Carassius auratus*; Schreck & Hopwood, 1974), and caspian kutum (*Rutilus kutum*; Sabet *et al.*, 2009). Plasma T rose in association with E₂ and reaching reproductive maturity in common carp (*Cyprinus carpio*; Taghizadeh *et al.*, 2013), and ribbed gunnel (*Dictyosoma burger*; Hwang *et al.*, 2012). Plasma T and E₂ levels rose in association with vitellogenesis in catfish (*Heteropneustes fossilis*; Lamba *et al.*, 1983), rainbow trout (Zohar and Billard, 1984), and Persian sturgeon (*Acipenser persicus*; Hosseinzade *et al.*, 2012). Plasma E₂ levels from previous studies ranged from 1500 pg/mL in spring to 11000 pg/mL in the summer, 750 pg/mL in autumn, and back to 1500 pg/mL in late autumn/early winter (Shimizu *et al.*, 2003). Plasma E₂ levels from the current study are dependent on stage of ovarian development and appear to be expressed at a lower range when compared to Shimizu *et al.* (2003) work; however, this could be caused due to the differences in sub-species of mummichog utilized in the studies, as well as differences in methodology. Although

difficult to compare between species and studies, plasma E₂ levels in mummichog within the current study were lower than plasma E₂ levels measured in rainbow trout and Persian sturgeon during peak production; however, plasma E₂ in mummichog were much higher than plasma E₂ levels in common carp (Hosseinzade *et al.*, 2012; Taghizadeh *et al.*, 2013; Whitehead *et al.*, 1978). Reproductive processes in teleost fish are controlled and regulated by a balance and interconnectivity among various hormones; these hormones also regulate other processes such as growth and development and osmoregulation (Menuet *et al.*, 2005; McCormick, 2011; Ramezani-Fard *et al.*, 2013). The association of changes in gonadal steroid plasma levels with gonad condition has been proven to be valuable in understanding the endocrine control of reproduction in teleosts, as hormone levels detected in plasma can be produced from tissues other than the gonads, such as the interrenal glands (Cornish, 1998). T and E₂ plasma results from the current study suggest plasma T and E₂ are dependent on the stage of ovarian development, verifies previous mummichog work, and confirms T and E₂ are regulated differently in mummichog as compared to other teleosts.

T and E₂ production were measured from basal (control) and stimulated (with hCG: analogue to LH) ovarian *in vitro* incubations from reproductively maturing fish (regressed to ovulating). The *in vitro* steroid production in regressed ovaries is most likely artificially high because of a higher tissue weight per well for regressed ovarian tissue compared to vitellogenic follicles. Fold changes of hCG-stimulated to basal steroid production, between regressed and vitellogenic ovaries, did not differ; therefore, the decline in hormone production seen in vitellogenic follicular *in vitro* incubations is most likely due to the difference in tissue weight per well, and therefore, the interpretation of

results is focussed on vitellogenic to ovulated stages. Focussing on vitellogenic to ovulated stages, higher levels of unstimulated *in vitro* T generally associated with high levels of unstimulated *in vitro* E₂. Unstimulated *in vitro* T levels were highest during late maturation, and had no significant differences in expression among the vitellogenic, early maturation and ovulated stages, whereas E₂ levels significantly increased throughout the cycle and peaked during early to late maturation. These results are similar to previous mummichog work, in which Lin *et al.* (1987) determined that T production from basal follicles peaked during maturational stages, and that E₂ production from basal follicles consistently increased throughout the cycle and peaked during maturational stages. This differs from other teleosts, as a shift is usually present when E₂ levels rise throughout the earlier stages of the ovarian development cycle, and drop prior to early maturation to allow for significantly increased levels of MIS, which are required to complete maturation of the oocyte (Nagahama and Yamashita, 2008). This shift was not demonstrated in the current study, nor in previous mummichog work (Lin *et al.*, 1987). In zebrafish, unstimulated *in vitro* T and E₂ levels increase during growth stages of the cycle and drop prior to early maturation (Clelland and Peng, 2009). *In vitro* T and E₂ progressively rise through the ovarian development cycle and drop during the late stages of vitellogenesis in rainbow trout (Breton *et al.*, 1975), amago salmon (*Oncorhynchus rhodurus*; Kagawa *et al.*, 1983), and goldfish (Kagawa *et al.*, 1984). *In vitro* E₂ production from ovarian follicles across development in mummichog from the current study are similar to previous mummichog studies as Lin *et al.* (1987) demonstrated a range of ~900-1500 pg/mL of E₂, varying by stage of ovarian development. However, E₂ levels from the current study is much higher than E₂ production in amago salmon ovarian

follicles (Kagawa *et al.*, 1983). In the current study, unstimulated *in vitro* T and E₂ trends associated with their respective plasma trends; this suggests that the majority of T and E₂ produced is from the gonads, similar to most female vertebrates (Thomas, 2008). This study confirms previous mummichog studies and suggests that T and E₂ production correlate with vitellogenesis, as well as with final maturation in mummichog without an E₂ drop prior to maturation; as noted in the following, this warrants further investigation into the regulation of the steroidogenic pathway during maturation in mummichog.

This is the first study to measure plasma MIS levels in recrudescing mummichog. MIS levels increased significantly from early maturation to ovulating stages, and were dissimilar to T and E₂ as MIS did not drop off after maturation. These findings from the current study are similar to other species such as rainbow trout, in which serum MIS levels were highest in female rainbow trout, rockfish (*Sebastes taczanowskii*) and amago salmon undergoing final oocyte maturation and ovulation (Campbell *et al.*, 1980; Nagahama *et al.*, 1991; Young *et al.* 1983), or in ribbed gunnel during spawning and post-spawning (Hwang *et al.*, 2012). In the current study, unstimulated *in vitro* MIS trends followed similar trends as MIS plasma, with the exception that *in vitro* MIS increased significantly between vitellogenic and late maturation ovarian follicles, and plasma significantly increased between late maturation and ovulation. This suggests a time lag between gonadal production and manifestation in the plasma of MIS, perhaps due to the low levels of MIS produced relative to T and E₂. These findings are similar to Lin *et al.* (1987) which demonstrated that MIS production from stimulated follicles (basal MIS levels were non-detectable) peaked during late vitellogenesis – early maturation stages. These findings from the current study are also similar to *in vitro* MIS production

trends in other teleosts, even though no dramatic shift in the steroidogenic pathway from E₂ to MIS was detected prior to oocyte maturation. Follicular MIS levels peak during early maturation in zebrafish (Clelland and Peng, 2009), and in maturing and ovulated follicles in amago salmon (Young *et al.*, 1983). MIS was confirmed to be the primary inducer of GVBD (germinal vesicle breakdown) in amago salmon, rainbow trout and goldfish post-vitellogenic oocytes (Nagahama, 1983). This study suggests the rise in MIS is the primary event which triggers final oocyte maturation, as opposed to the preceding decline in E₂ levels usually found in other teleosts. The action of MIS on final oocyte maturation is not direct as it is mediated by a complex interaction of several factors, such as cytoplasmic factor and MPF (maturation promoting factor) (Nagahama and Yamashita, 2008; Senthilkumaran *et al.*, 2004); therefore, the cause of increasing plasma and *in vitro* MIS levels in ovulating mummichog can be influenced by multiple other factors. This study has only considered the presence of T, E₂ and MIS across maturation in mummichog. Other steroid hormones and gonadotropins, such as progesterone, FSH and LH, are involved in ovarian development and can possibly influence the production of plasma and *in vitro* T, E₂ and MIS, directly or indirectly (Hosseinzade *et al.*, 2012), which warrants further studies.

hCG-stimulated *in vitro* T, E₂ and MIS hormone production was significantly higher than unstimulated *in vitro* hormone production. hCG is able to stimulate oocyte maturation and ovulation in many teleosts, in the same fashion as LH (Harvey and Hoar, 1979; Zuberi *et al.*, 2011). Oocyte maturation in teleosts is typically first initiated by an LH surge in maturing oocytes, which triggers the production of 17 α -hydroxyprogesterone from the thecal cells, which is then converted into MIS (Nagahama and Yamashita, 2008;

Patino and Sullivan, 2002). The current study demonstrated that the steroidogenic response of ovarian follicles to hCG is highly dependent on the stage of maturation; early mature, followed by late mature ovarian follicles, were most responsive to hCG, as they exhibited the highest increase in hormone production from unstimulated *in vitro* production. This differs from previous studies, as Lin *et al.* (1987) showed a maximal increase in hormones T, E₂ and MIS in late vitellogenic follicles with FPE (*Fundulus* pituitary extract; acts similar to LH in terms of maturation induction) stimulation; however, the differences in stage response could be due to differences between FPE and hCG. In other teleosts, such as catfish, hCG production causes maximal production of T in maturing follicles, and a maximal production of E₂ in vitellogenic follicles (Kagawa *et al.*, 1984). Similarly, in amago salmon, SG-G100 (purified chinook salmon gonadotropin) could stimulate E₂ in vitellogenic follicles, whereas T could be stimulated in maturing follicles (Kagawa *et al.*, 1983). Since hCG displays LH bioactivity as it binds to gonadal LH receptors and stimulates ovarian development and maturation (Mananos *et al.*, 2009), the present study suggests that maturing follicles (primarily early mature) in mummichog are most LH-responsive and presumably will have the highest expression of LH receptors, which can be confirmed with the gene expression data of LH receptors from this study. E₂ has been shown to reduce the action of hCG in gonadal incubations of goldfish ovaries (Post, 1994). It is worthwhile to further examine how mummichog ovaries can consistently respond to hCG with continuously high levels of E₂ produced until late maturation.

The current study is the first to examine gene expression changes in the steroidogenic enzymes and receptors within the ovary (whole tissue and isolated

follicles), liver and brain, across the ovarian development cycle in mummichog (from reproductively regressed to ovulating). This study showed differences in expression trends between whole ovary tissue and isolated follicles (separated per group), which demonstrated different regulation patterns of these steroidogenic enzymes and receptors between isolated follicles and whole ovary tissue. The expression results from isolated follicles provide an accurate representation of steroidogenic receptor and enzyme activity from specific follicular stages (cortical alveolus, vitellogenic, early mature, late mature, and ovulated); because mummichog are group-synchronous spawners, ovarian sections from regressed, vitellogenic, early mature, fully mature and ovulating fish containing follicular mixes (>75% stage-dominant, ~25% other) provide a less precise representation of follicular-specific activity. Therefore, the focus of the discussion will be on the isolated follicles (by 100% stage). Stage 5 (ovulated follicles) were excluded from analysis because expression levels were non-detectable.

Gene expression of enzymes in the steroidogenic pathway (StAR, CYP11, CYP17, and CYP19a1) were measured and characterized across stages in the maturing ovary. The steroidogenic pathway is involved in the enzymatic synthesis of sex steroids from cholesterol. StAR (aids in the transport of cholesterol across the mitochondrial membrane) continues to increase in expression throughout the ovarian development cycle and peaks during the later stages. These trends in StAR expression parallel the increasing pattern of E₂ throughout the stages of maturation. StAR expression in the current study is similar to StAR expression in rainbow trout (Sreenivasulu and Senthilkumaran, 2009), but differs from zebrafish StAR expression as there are no significant changes in expression across all stages in zebrafish (Ings and Kraak, 2006). StAR seems to be

associated with ovarian development and E₂ production. It is possible that increases in StAR expression can also be triggered by LH-mediated signaling pathways. As the transport of cholesterol is a rate-determining step for steroidogenesis and StAR is the putative key rate-limiting mediator in acute regulation of steroidogenesis (Nagahama and Yamashita, 2008; Young *et al.*, 2005), it emphasizes the importance of StAR throughout oocyte maturation and its possible role in maintaining high levels of steroidogenic precursors, such as cholesterol, pregnenolone, and progesterone.

CYP11 (converts cholesterol to pregnenolone) expression increases throughout the ovarian development cycle, peaks in expression during early maturation, and drops in expression during late maturation. These trends in CYP11 expression parallel the increasing pattern of E₂ throughout the stages of ovarian development discussed previously. CYP11 expression is also similar to other teleosts (Kumar *et al.*, 2000) as CYP11 in catfish increased from ovarian recrudescence to vitellogenesis (elevations in CYP11 expression corresponded to E₂ production patterns); however, the current study differs from zebrafish expression as CYP11 expression decreased throughout the cycle in zebrafish (Ings and Van Der Kraak, 2006). CYP11 is a critical enzyme involved in the synthesis of major sex hormones, as it catalyzes the enzymatic reaction following the rate-limiting step in steroidogenesis (Kumar *et al.*, 2000). In the current study, CYP11 follows E₂ production patterns as it is associated with oocyte maturation, suggesting CYP11 expression may mediate production of final steroidogenic hormones.

CYP17 (P450 C17: converts pregnenolone to 17 α -hydroxypregnenolone and then to dehydroepiandrosterone, or converts progesterone to 17 α -hydroxyprogesterone and then androstenedione) peaks in cortical alveolus staged follicles and remains equally and

lowly expressed from vitellogenesis to late maturation. CYP17 trends in mummichog are similar to those in zebrafish, where CYP17 expression decreases throughout the ovarian development cycle (Ings and Van Der Kraak, 2006); however, CYP17 expression trends in the current study differ from catfish expression trends as CYP17 expression in catfish spiked within spawning ovaries and dropped during regressed and pre-spawning stages (Sreenivasulu and Senthilkumaran, 2009). In the current study, CYP17 in mummichog does not appear to parallel E₂ levels. CYP17 was described as a critical enzyme involved in the initiation of maturational events in fish (Senthilkumaran *et al.*, 2004). Thus, the lack of increase in the expression of CYP17 throughout recrudescence in mummichog, may be explained by the lack of an E₂ to MIS switch at maturation, as the expression patterns of CYP17 were previously suggested to have a role in the switch (Clelland and Peng, 2009).

CYP19a1 (P450 aromatase: converts T to E₂) had no significant differences in expression between the cortical alveolus stage and early maturation stage, and demonstrated a decline in expression during late maturation. CYP19a1 expression in the current study differs from other teleosts. CYP19a1 is suspected to be an estrogen-responsive gene; CYP19a1 expression correlates with the onset of ovarian recrudescence and vitellogenesis in catfish, closely mirroring E₂ production trends (Kumar *et al.*, 2000). CYP19a1 expression in zebrafish decreases as the ovarian development cycle progresses (Ings and Van Der Kraak, 2006), and dramatically declines in expression after vitellogenesis in catfish (Kumar *et al.*, 2000) and rainbow trout (Gohin *et al.*, 2010). In catfish, CYP19a1 had the most dramatic increase in expression during recrudescence and vitellogenesis, when compared to the other P450 enzymes (Kumar *et al.*, 2000),

suggesting CYP19a1 is potentially the most crucial enzyme involved in regulating hormones (such as E₂) for ovarian recrudescence and vitellogenesis. This holds true in the current study on mummichog as the expression pattern parallels the increasing pattern of E₂ throughout the stages of ovarian development until early maturation; however, the drop off in CYP19a1 expression at late maturation does not follow E₂ patterns and warrants further studies to determine what may be causing the drop-off in CYP19a1.

P450 aromatase is also expressed in the brain (CYP19b), which showed no significant differences in expression across ovarian development. Because P450 aromatase is a key enzyme associated with E₂ regulation and ovarian development, it was important to assess the expression in brain, in addition to the ovary. P450 aromatase was similarly expressed between ovary and brain, with the exception that brain expression of CYP19b was continuous throughout ovarian development, including ovulation. This indicates regulation of local brain E₂ production is independent of gonadal steroidogenesis, as CYP19b is related to estrogen-dependent neurogenesis, which is continuous through adulthood in fish (Forlano *et al.*, 2001; Pellegrini *et al.*, 2007).

This is the first study to measure gene expression of estrogen receptors (ESR1, ESR2a, and ESR2b) across stages in maturing ovary of mummichog. E₂ signaling pathways involve binding to the different subunits of estrogen receptors in target organs to stimulate biological activity (Hall *et al.*, 2001; Nagler *et al.*, 2012). The pattern of ER expression should correlate with E₂ biological function and regulation; however, ERs could be down-regulated in some tissue as E₂ increases to regulate E₂ effects. For example, ERs in goldfish ovary, liver and brain demonstrated differential patterns of all ER-subtype expression during ovarian development, potentially altering ER-mediated

processes across tissues (Marlatt *et al.*, 2008). RT-PCR was applied to determine the tissue distribution of ERs in mummichog and found that all ER subtypes were primarily found in the gonad or liver of males and females, but sex differences in mRNA levels were gene- and tissue-specific (Greytak and Callard, 2007).

Follicular expression of ESR1 paralleled E₂ trends, as ESR1 expression had no significant differences between the cortical alveoli and early mature stages, and then significantly decreased in expression during late maturation. ESR1 is the only subtype that has been linked to biological function, i.e. vitellogenin synthesis in liver and E₂ regulation in ovary (Chakraborty *et al.*, 2011). ESR1 trends are generally similar to Greytak and Callard (2007)'s study which demonstrated an increase in ovary ESR1 expression in reproductively active female mummichog, compared to reproductively inactive female mummichog. Mummichog ESR1 expression in the current study is also similar to other teleosts, as Nagler *et al.* (2012) demonstrated low and relatively unchanging ESR1 expression in the ovary of rainbow trout. ESR2a expression in the current study remained stable across the stages in ovary, whereas ESR2b expression peaked in the cortical alveolus stage and decreased throughout the cycle. ESR2a expression patterns generally follow Greytak and Callard (2007)'s study as they demonstrated no changes in expression between reproductively active or inactive gonadal ESR2a expression; however, ESR2a expression patterns from the current study do not follow patterns in other teleosts such as rainbow trout. Mummichog ESR2b expression patterns from the current study do not follow Greytak and Callard (2007)'s study as they demonstrated no changes in expression between reproductively active or inactive gonadal ESR2b expression; however, mummichog ESR2b expression patterns from the current

study are similar to other teleosts (Nagler *et al.*, 2012). Nagler *et al.* (2012) also showed that ESR2a was expressed much higher than ESR2b, with both ER β subtypes having expression which peaked during earlier stages in follicular development and gradually decreased until spawning began. In the current study, the various estrogen receptor subtypes other than ESR1, are expressed differently throughout the cycle, in differing tissues, and are not always associated with the pattern of E₂. The function of ER β subtypes has not been determined, but ER β subtypes can be further examined in various tissues to determine whether their expression across the ovarian development cycle correlates to endocrine mediators, such as gonadotropins, steroids, or intracellular regulators.

ESR1 and VTG1 expression in the liver were associated with E₂ regulation, in which ESR1 expression was highest during ovulation, and VTG1 expression peaked during ovulation. Vitellogenin (a female-specific lipoprotein) is synthesized in the liver under the stimulation of ovarian E₂ and is secreted into the blood and transported to the ovary where it is taken up into the oocytes to stimulate egg yolk protein production during development of the oocyte (Hoar *et al.*, 1983). It is expected that ESR1 expression in the liver will be associated with VTG1 expression, as ESR1 is linked to E₂ function. Hepatic ESR1 expression in mummichog in the current study differed from other teleosts, as ESR1 expression in rainbow trout remained fairly static until the end of the spawning period, and then significantly increased in post-spawning fish (Nagler *et al.*, 2012). VTG1 expression, on the other hand, corresponds to other teleosts as VTG1 expression in zebrafish liver is tightly coupled to an increase in E₂ activity within maturing females (Levi *et al.*, 2009). In the current study, hepatic ESR2a peaked during the cortical

alveolus stage and decreased throughout the cycle, whereas ESR2b peaked during vitellogenesis and had low expression across all other stages. Neither ESR2a nor ESR2b patterns follow expression patterns in other teleosts. In rainbow trout, ESR2a had a significant peak in expression in regressed stages, and ESR2b showed a sharp decrease in regressed stages (Nagler *et al.*, 2012). These results from the current study suggest that ESR1 expression is closely related to E₂ levels and vitellogenin expression within mummichog; the relationships for estrogen receptors ERβ1 and ERβ2 with ovarian development are still unclear.

Gonadotropin receptors (FSHr and LHr) expression was measured and characterized across stages in the maturing ovary. Gonadotropins are regulated through the expression of their corresponding receptors; expression of the gonadotropin receptor genes vary with reproductive state, partly due to feedback of FSH, LH and steroids including E₂, T and MIS (Moles *et al.*, 2007). In the current study, follicular expression of FSHr peaked during vitellogenesis and had low expression in all other stages. These follicular FSHr results corresponded to previous mummichog work (Ohkubo *et al.*, 2013), which demonstrated a peak in follicular FSHr expression during vitellogenesis as well. The follicular FSHr results from the current study also corresponded with other teleosts, as FSHr expression is expected to peak near vitellogenesis and drop prior to maturation (Clelland and Peng, 2009). In zebrafish, FSHr increased from the primary growth stage to vitellogenesis, where it peaked (Kwok *et al.*, 2005). FSH is primarily involved in regulating gonadal steroidogenesis in the early ovarian developmental stages, especially during vitellogenesis (Ohkubo *et al.*, 2013; Swanson *et al.*, 1991). FSH's dominant role in vitellogenesis is to stimulate E₂ production and release from ovarian

follicles (Nagahama and Yamashita, 2008); it also influences the increase in P450 aromatase expression in vitellogenic follicles, as noted in brown trout (Montserrat *et al.*, 2004). These expression trends from the current study suggest that FSHr correlates to FSH is presumed actions on steroidogenesis during follicular growth and vitellogenesis; however, FSHr is not associated with the increasing E₂ levels in maturing mummichog follicles. It may be that once stimulated, E₂ levels are maintained by other factors including intracellular receptors, cAMP (cyclic adenosine monophosphate) / pKA (protein kinase A) pathway factors and calcium channels involved in intracellular signal transduction pathways (Matagne *et al.*, 2005; Menuet *et al.*, 2005).

Follicular LHr expression patterns exhibited low expression during the cortical alveolus stage, a peak in expression during vitellogenesis to early mature stages, and no significant differences in expression between vitellogenesis to late maturation stages. The follicular LHr results from the current study did not correspond to previous mummichog work, as Ohkubo *et al.* (2013) demonstrated a peak in follicular LHr expression during early maturation and low expression during vitellogenesis, and late maturation. A reason for the discrepancy could be due to methodological differences as cortical alveolus staged follicles were included in a separate analysis in Ohkubo *et al.* (2013)'s study.

Alternatively, the follicular LHr expression trends from the current study did correspond to other teleosts. In zebrafish, LHr expression was low during the early stages of the ovarian developmental cycle, but was significantly higher during vitellogenesis, and peaked prior to oocyte maturation (Kwok *et al.*, 2005). LH primarily regulates the final stages of ovarian development and spawning (Breton *et al.*, 1998; Tyler *et al.*, 1997), partially due to its ability to stimulate MIS production from ovarian follicles (Clelland

and Peng, 2009; Nagahama and Yamashita, 2008). The surge in LH prior to maturation is what is believed to cause the steroidogenic shift in E₂ to MIS commonly found in teleosts (Nagahama and Yamashita, 2008), as LH is said to inhibit the production of E₂ prior to maturation (Planas *et al.*, 2000); however, mummichog continue to produce increasing amounts of E₂ with a high expression of LHR in maturing follicles. In the current study, LHR expression is associated with hCG stimulation activity of *in vitro* hormone production, with the exception that LHR is highest in vitellogenic and early maturation follicles, whereas hCG stimulation caused the largest increase in hormone production within early maturation and late maturation follicles. This could indicate that the increase in gonadotropin receptors is required before the increase in gonadotropin occurs. It would be beneficial to analyze FSH and LH levels across maturation in mummichog in further studies to determine whether gonadotropin levels correlate with the patterns of their receptors. The present study suggests that similar to other species, LHR expression correlates to MIS levels and LH stimulation of maturation and this relationship is independent of an E₂ decline.

Basal and hCG-treated ovarian follicles, grouped by stage of ovarian development, were exposed to 50-250 nM of EE₂ for 24 hours, *in vitro*. The experiment sought to examine the effect of EE₂ on the *in vitro* production of T and MIS, and on the gene expression of CYP19a1 and LHR. The goal was to provide insight on whether tolerance exhibited by mummichog *in vivo* to exogenous estrogens may be partly attributable to a lack of response to EE₂ at the level of the ovary. The rationale for the experimental design (concentration range and exposure time) was from previous *in vitro* exposures of fish ovarian and liver cells to EE₂. Hepatic cells in rainbow trout

demonstrated a 20-30% increase in expression change of VTG1 to 1-10 nM of EE₂ *in vitro* after 24 hours (Hultman *et al.*, 2015); zebrafish hepatocytes demonstrated a 1- to 10-fold increase in ER and VTG1 expression with 100 nM of EE₂ *in vitro* for 24 hours (Eide *et al.*, 2014); and zebrafish ovarian cells demonstrated approximately a 10-fold increase in LHr expression with 5 μM EE₂ *in vitro* for 24 hours (Liu *et al.*, 2013).

Adult female mummichog responses are generally resistant to *in vivo* EE₂ exposure compared to other fish species. For example, egg production is not decreased at nominal concentrations of 100 ng/L, or 84.1 ± 6.0% of nominal, for 28 days (Bosker *et al.*, 2016), female plasma E₂ levels were not affected after a 14-day exposure to 250 ng/L of EE₂ (Doyle *et al.*, 2013), and male and female plasma T levels were not affected after a 14-day exposure to 500 ng/L (nominal) of EE₂, or 247.9 ± 12.3 (average) ng/L (Hogan *et al.*, 2010). There was no effect of EE₂ in the current study on either T or MIS production (E₂ was not measured due to high cross-reactivity to EE₂ in the EIA analysis), or gene expression (CYP19a1 and LHr) at any follicular stage. The results validate the hypothesis that mummichog are more tolerant to EE₂ than other teleosts. Liu *et al.* (2013) conducted a 24 h EE₂ *in vitro* exposure at 5 μM on zebra fish ovarian cells, causing an approximate 10-fold increase in LHr expression; the ED50 (median effective dose) for EE₂ was 17.9 pM at 1.5 hours, which was similar to the ED50 of E₂ (exposed at 5 nM).

hCG stimulation increased both hormone production and CYP19a1 expression in incubated follicles. That hCG stimulation did not increase LHr expression at any stage of maturation (cortical alveolus – late mature) was unexpected, as MIS levels did increase with hCG stimulation. In Liu *et al.*'s (2013) study, the use of hCG resulted in an increase in LHr expression in zebrafish; there was a 5-fold increase in LHr expression with only

hCG stimulation, and an 8-fold increase in LHr expression with a combination of hCG and 5 μ M EE₂. Expression patterns of other steroidogenic enzymes can be explored to provide insight on this discrepancy, such as 20 β -HSD, which should increase in expression after the induction of LH or hCG during oocyte maturation (Senthilkumaran *et al.*, 2002). The hormone and gene expression results after EE₂ *in vitro* exposure adds weight to the idea that mummichog ovarian follicles are resistant to exogenous estrogen, and the previous work in the current study suggests high levels of E₂ in maturing follicles may cause this lack of sensitivity. It would be beneficial to further explore the extent of EE₂, *in vitro*, on mummichog ovarian follicles, with a broader concentration range and a number of incubation time periods. The explanation for the lack of increase in LHr expression with hCG also needs to be further explored as it could be contributing to the lack of response to EE₂ in mummichog.

2.6 Conclusions

The current study provides confirmation of a continuously increasing production of E₂ in maturing follicles, supplemented by new molecular analysis of the gene expression of key steroidogenic enzymes and hormone/gonadotropin receptors across the ovarian steroidogenic pathway in mummichog. These differences exhibited in mummichog ovarian development, along with no effect of exposure to EE₂ on maturing follicles, support the hypothesis that high estrogen levels during oocyte maturation may be responsible for reduced sensitivity in mummichog to environmental estrogens. I.e. the exposure of mummichog *in vivo* to EE₂ does not manifest in molecular or biochemical changes in the ovary, which in other fish is associated with cessation in egg production or altered physiological steroid levels, as the ovary is adapted to continuous levels of E₂ during maturation.

Appendix A: Supplementary Material

Adjusting Normalizing Gene Calculation

When no gene was found to be steadily expressed across stages, such as in experiment 1C (EE₂ *in vitro* exposure), the gene that was most steadily expressed was adjusted through a calculation (Ings and Van Der Kraak, 2006) to have constant expression. The calculation is as follows:

$$\text{Adjusted Value} = \frac{\text{Individual Input Value}}{\text{Mean Group Input Value} \div \text{Mean of Reference Group Input Value}}$$

Reference group refers to the treatment group chosen to which to adjust all other treatment groups. For instance, stage 1 was chosen as the reference group.

Stage 1 Mean = 0.0318

Stage 2 Mean = 0.0038

Stage 2 individual value example (fish #10) = 0.0042

Final adjusted value example (fish #10) = 0.0355

Standard Curve Equation Values

Table S2.1 Slope and y-intercept from standard curves of all primers used in this study.

Enzyme/Receptor	Gene	Tissue	Slope	Y-Intercept
P450 Aromatase Brain	CYP19b	Brain	-3.3379	25.396
Elongation Factor 1 α	EF1 α	Brain	-3.7111	18.86
Estrogen Receptor α	ESR1	Liver	-3.5421	24.344
Estrogen Receptor β 1	ESR2a	Liver	-3.7906	27.974
Estrogen Receptor β 2	ESR2b	Liver	-3.2274	26.309
Vitellogenin	VTG1	Liver	-3.7368	14.545
18s RNA	18sRNA	Liver	-3.7313	9.4068
Estrogen Receptor α	ESR1	Ovary	-3.1784	28.321
Estrogen Receptor β 1	ESR2a	Ovary	-3.4627	27.244
Estrogen Receptor β 2	ESR2b	Ovary	-3.3403	26.485
Follicle Stimulating Hormone Receptor	FSHr	Ovary	-3.3691	26.8
Luteinizing Hormone Receptor	LHr	Ovary	-3.5261	26.57
Steroidogenic Acute Regulatory Protein	StAR	Ovary	-3.1238	26.84
P450 Side Chain Cleavage	CYP11a1	Ovary	-3.2424	24.798
P450 C17	CYP17a1	Ovary	-3.0117	27.577
P450 Aromatase	CYP19a1	Ovary	-3.4627	24.672
β -Actin	Actin	Ovary	-3.2588	16.477

Normalizing Genes

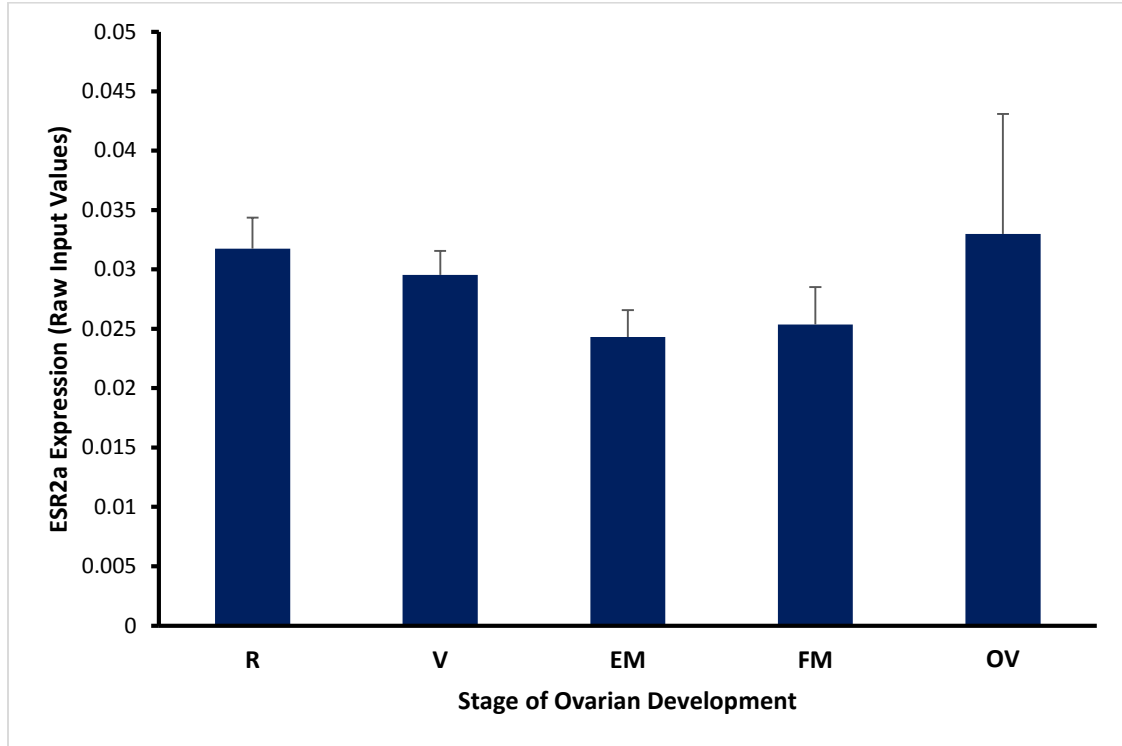


Figure S2.1. Expression (raw input values) of ESR2a in mummichog whole ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (O) follicles. Values represent the mean \pm S.E.M. (N = 9, 10, 14, 14, 4 for stages 1-5, respectively) as determined by qPCR. There were no significant changes across expression. ($p = 0.298$, ANOVA followed by Tukey post-hoc test).

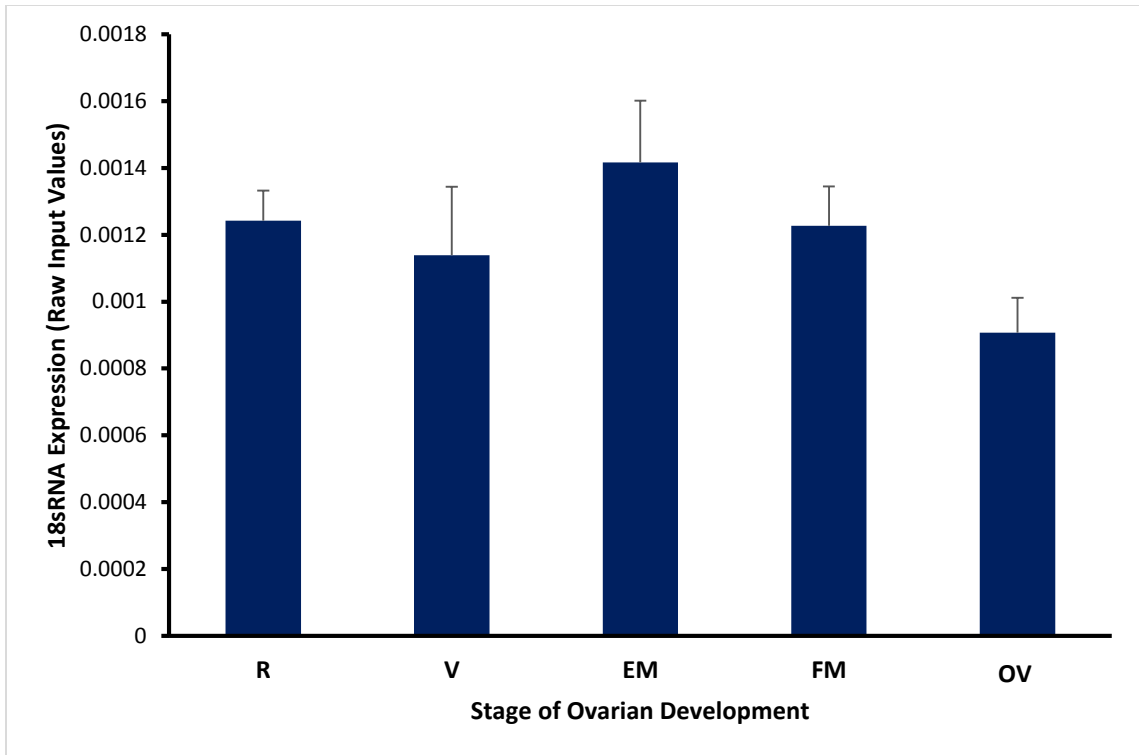


Figure S2.2. Expression (raw input values) of 18sRNA in liver tissue from mummichog with ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Values represent the mean \pm S.E.M. N = 12, 10, 14, 14, 4 for stages 1-5, respectively, as determined by qPCR. There were no significant changes across expression. ($p = 0.547$, ANOVA followed by Tukey post-hoc test).

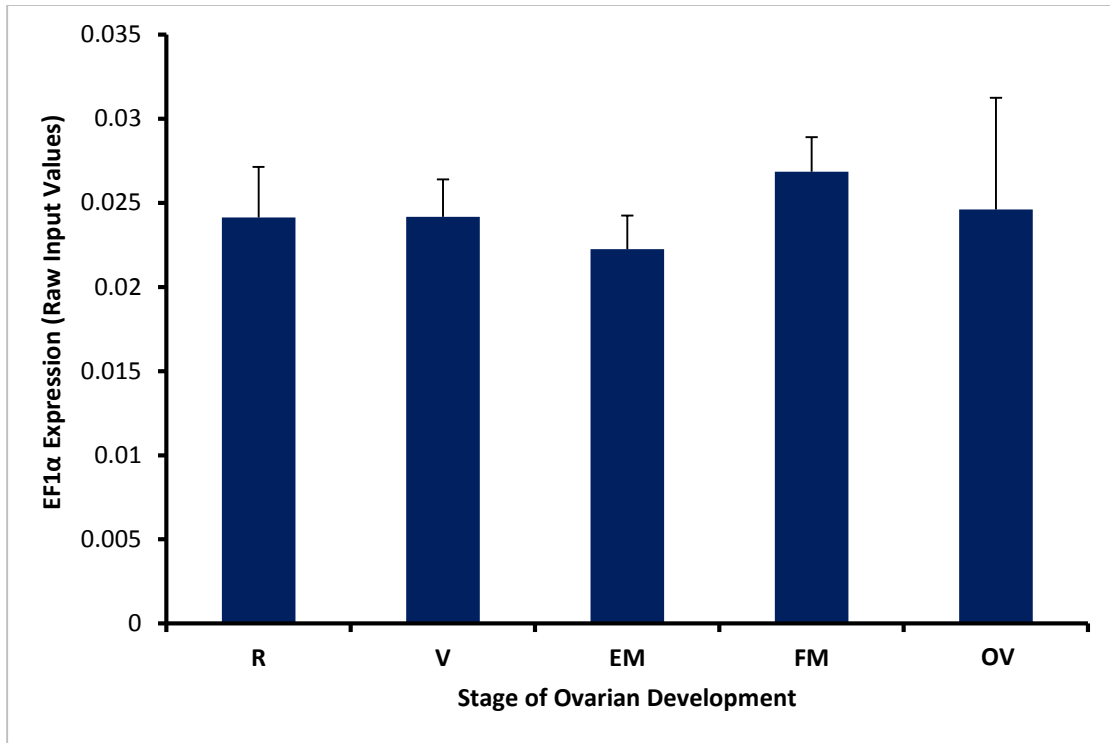


Figure S2.3. Expression (raw input values) of EF1 α in brains from mummichog with ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Values represent the mean \pm S.E.M. N = 12, 11, 15, 14, 4 for stages 1-5, respectively, as determined by qPCR. There were no significant changes across expression. ($p = 0.726$, ANOVA followed by Tukey post-hoc test).

Additional Methods FlowCharts and Diagrams

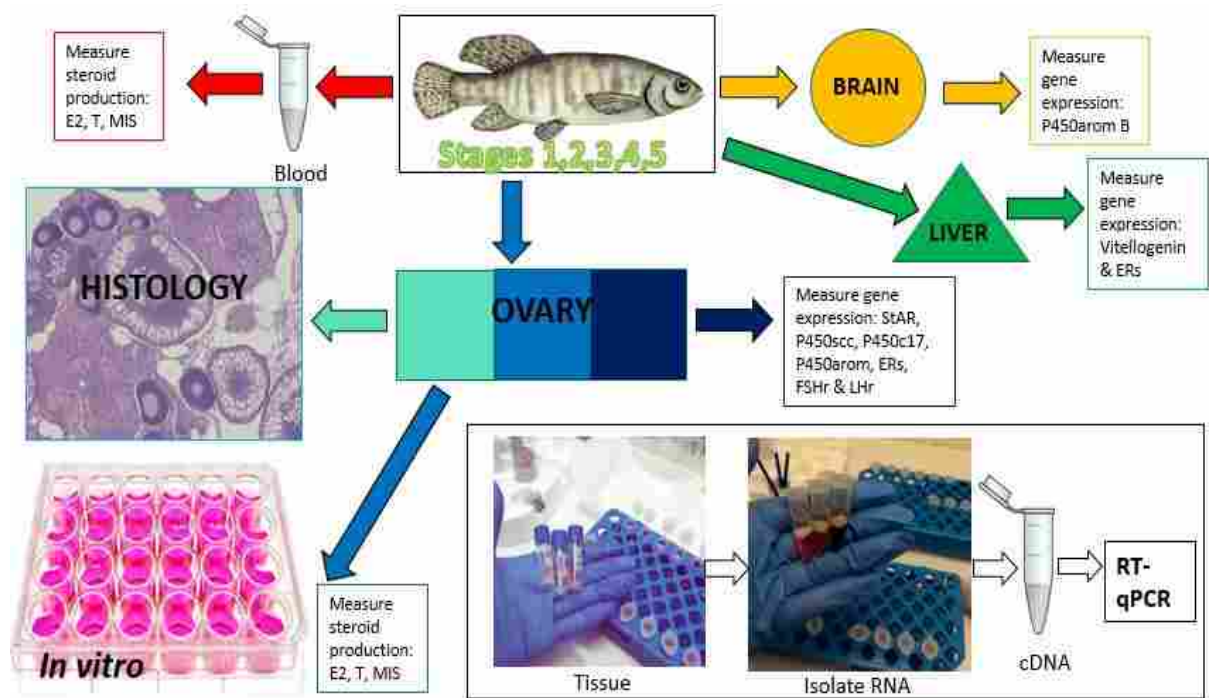


Figure S2.4. Simplified flowchart of experimental protocols undertaken for hormone analysis (plasma and *in vitro*), histology, and gene expression of whole tissue (ovary, liver, and brain), explained in sections 2.3.2 of the methods.

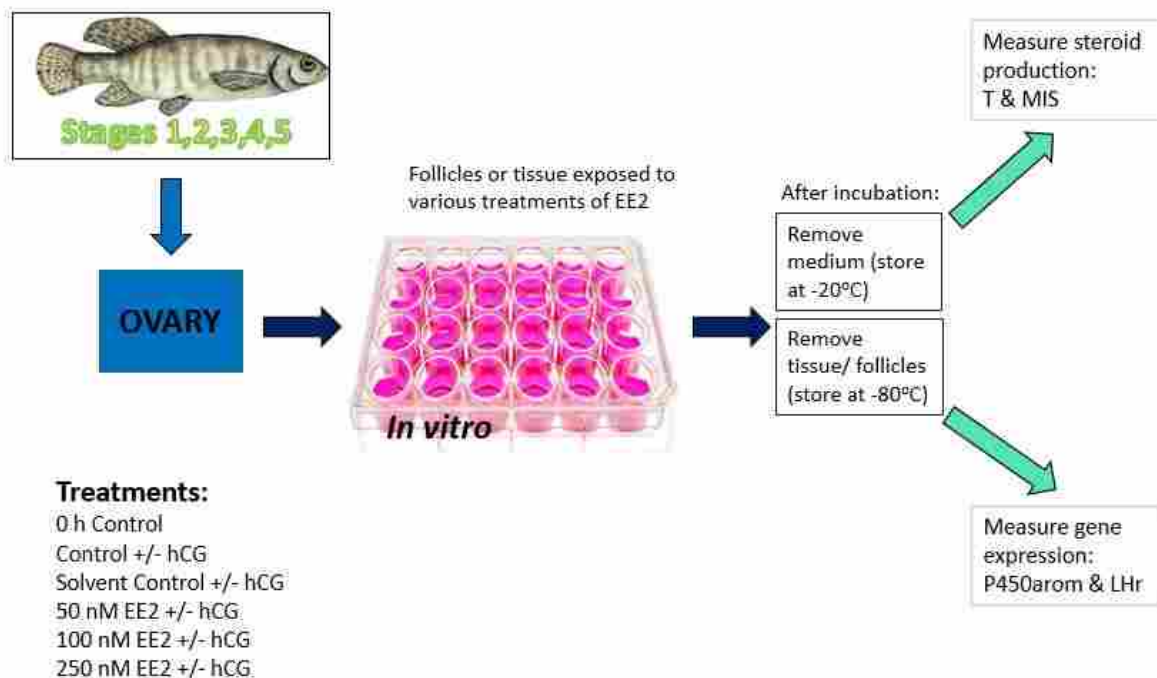


Figure S2.5. Simplified flowchart of experimental protocols undertaken for hormone analysis and gene expression of separated ovarian follicles after a 24-hour EE₂ *in vitro* exposure, explained in sections 2.3.4 of the methods.

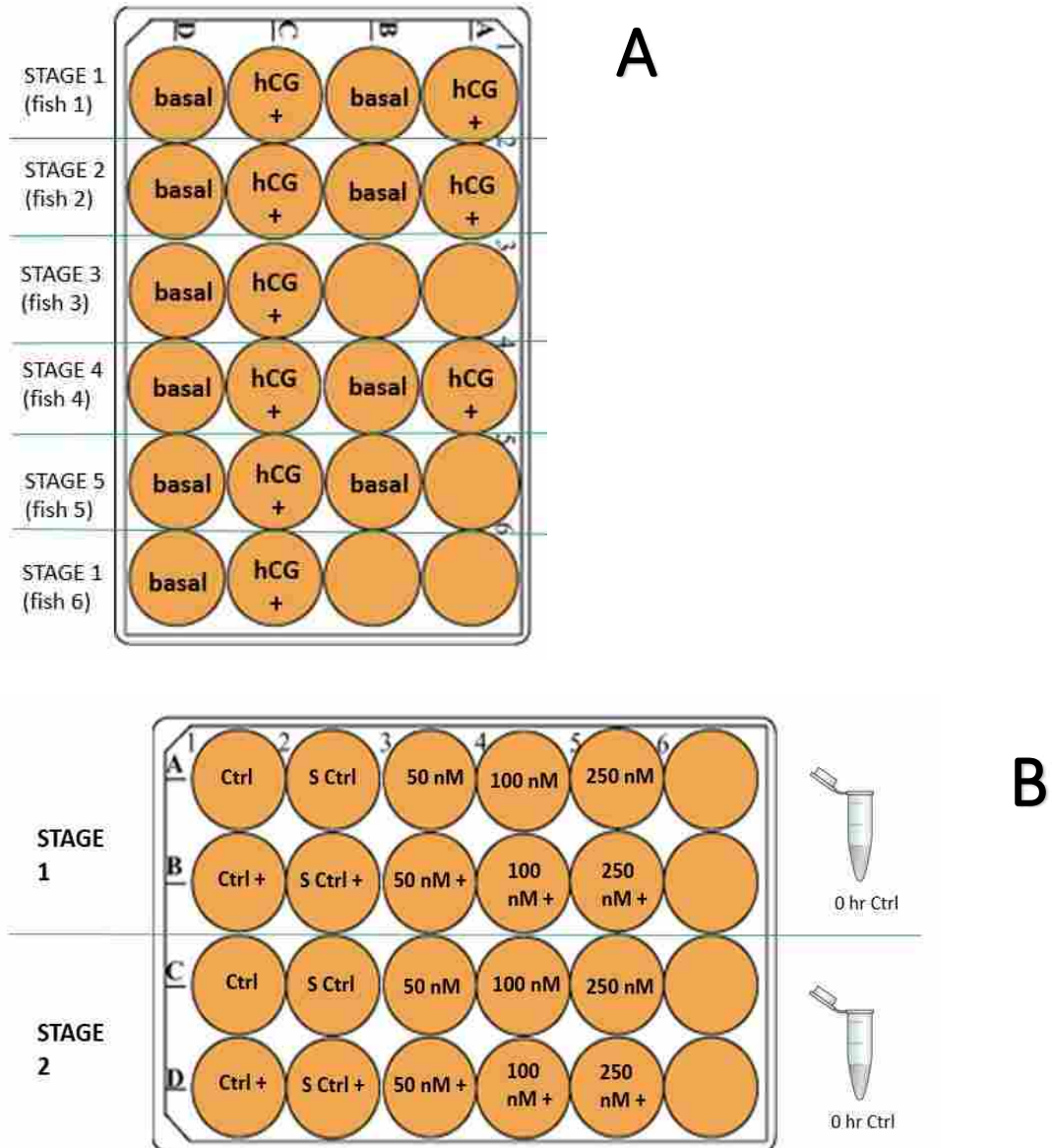


Figure S2.6. Example *in vitro* plate setups for hormone analysis of basal and hCG (human chorionic gonadotropin)-treated follicles across ovarian development (A), explained in methods section 2.3.2 ii, and for hormone and gene analysis of the EE₂ *in vitro* exposure of ovarian follicles across ovarian development (B), explained in methods section 2.3.4 ii.

Brain Gene Expression Analysis

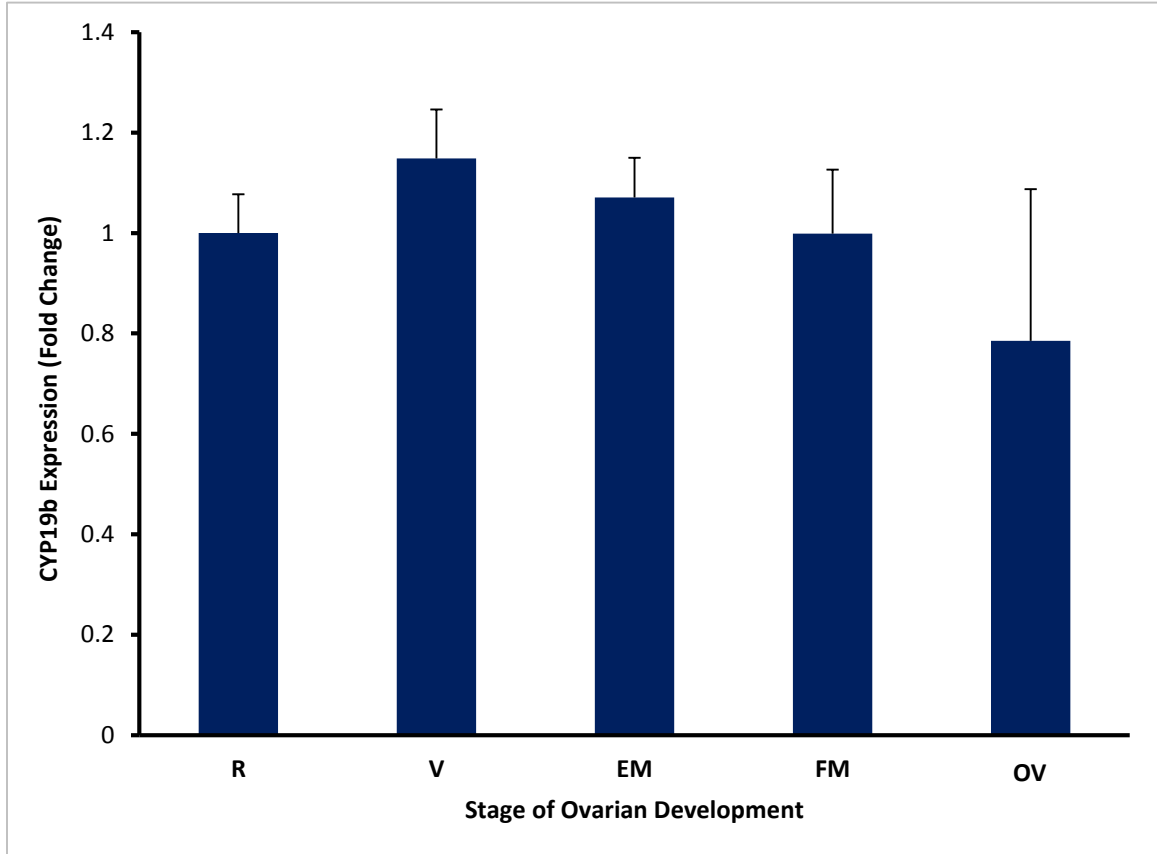


Figure S2.7. Expression of CYP19b in brains from mummichog with ovaries dominated by cortical alveoli (regressed: R), vitellogenic (V), early mature (EM), late mature (fully mature: FM) or ovulated (OV) follicles. Values represent the Mean \pm S.E.M. N = 12, 11, 15, 14, 4 for stages 1-5, respectively, as determined by qPCR. Data were normalized to EF1 α and displayed as the fold change relative to the R stage. There were no significant changes across expression. ($p = 0.256$, ANOVA followed by Tukey post-hoc test).

Table S2.2. P-Values from statistical tests performed on T (testosterone) and MIS (maturation inducing steroid) hormone data from 24-hour *in vitro* ovarian follicles at different stages of development [stage 2 (vitellogenic), stage 3 (early mature), stage 4 (late mature)] incubations with EE₂ treatments (control, solvent control, 50 nM EE₂, 100 nM EE₂, 250 nM EE₂) between groups (basal or hCG-stimulated).

Hormone/ Stage	2-way ANOVA (Stage & Basal Treatments)	1-way ANOVA (Stages from Basal only)	1-way ANOVA (Basal EE ₂ Treatments)	1-way ANOpVA (hCG+ EE ₂ Treatments)	1-way ANOVA (Fold Increase)
T	0.822	0.00			
2			0.114	0.513	0.088
3			0.093	0.363	0.281
4			0.669	0.574	0.939
MIS	0.779	0.00			
2			0.884	0.439	0.208
3			0.258	0.688	0.842
4			0.976	0.981	0.834

Table S2.3. P-Values from statistical tests performed on CYP19a1 (P450 aromatase) and LHr (luteinizing hormone receptor) gene expression data from 24-hour *in vitro* ovarian follicles at different stages of development [stage 1 (cortical alveolus), stage 2 (vitellogenic), stage 3 (early mature), stage 4 (late mature)] incubations with EE₂ treatments (basal or hCG-stimulated: control, solvent control, 50 nM EE₂, 100 nM EE₂, 250 nM EE₂).

Gene	Stage	1-way ANOVA among Treatments within Stage
CYP19a1	1	0.00
	2	0.03
	3	0.00
	4	0.00
LHr	1	0.21
	2	0.71
	3	0.09
	4	0.43

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Chapter 3: General Discussion & Integration

3.1 Summary

This thesis further develops the estuarine mummichog as a model species for understanding the reproductive physiology of teleosts. It has been confirmed that, similar to other work in mummichog (Lin *et al.*, 1987), 17β -estradiol (E_2) is produced by the ovarian follicles from the regressed (cortical alveolus) stages to late maturational stages, distinguishing this species from many other teleosts, in which E_2 levels rise from cortical alveolus to completion of vitellogenesis and drop prior to maturation (Clelland and Peng, 2009). In most teleost fish, there is a marked shift from estrogens (such as E_2) to progestogens (such as maturation inducing steroid; MIS) as follicles progress to early maturation. However, in mummichog there is no shift detected, as levels of E_2 are consistently higher in all stages of follicular development. Mummichog have also been noted to be more tolerant to exogenous estrogen exposure, such as 17α -ethinyl estradiol (EE_2), compared to commonly studied teleosts; most freshwater species will respond to environmentally-relevant concentrations (Armstrong *et al.*, 2016), whereas mummichog respond to much higher concentrations (if at all) (Bosker *et al.*, 2016). The differences in ovarian physiology and EDC (endocrine disrupting compound) responses amongst species led to the hypothesis that provides the foundation for this thesis, which is that continuous high levels of E_2 produced in the maturing mummichog ovary may be a reason for the species' increased tolerance to EE_2 exposure.

Plasma (from blood), ovary, liver and brain tissue, and ovarian follicles, were collected from maturing mummichog and grouped into five stages of maturation; follicular steroid production and/or gene expression in hormone signaling and steroidogenic pathways were assessed by stage. Plasma and follicular production of E_2

increased as the ovarian development cycle progressed, and dropped after maturation; MIS, however, was equally expressed during development and early maturation stages, and increased in late maturation and ovulation stages. T (testosterone) levels corresponded with E₂ trends, whereas MIS peaked during late maturational stages. This study also demonstrated that the steroidogenic response of ovarian follicles to hCG (human chorionic gonadotropin) is highly dependent on the stage of maturation; early mature, followed by late mature ovarian follicles, were most responsive to hCG. Because hCG mimics LH (luteinizing hormone) bioactivity, as it binds to gonadal LH receptors (LHr) and stimulates ovarian development and maturation (Mananos *et al.*, 2009), the present study suggests that maturing follicles (primarily early mature) in mummichog are the most LH-responsive. This study suggests that T and E₂ production correlates with vitellogenesis and final maturation in mummichog, whereas MIS correlates only with final maturation, with no E₂ drop prior to maturation.

The current study is the first to examine changes in gene expression of steroidogenic enzymes and receptors within the ovary (whole tissue and isolated follicles), liver and brain, across the ovarian development cycle (from reproductively regressed to ovulating) in mummichog. StAR (steroidogenic acute regulatory protein; aids in the transport of cholesterol across the mitochondrial membrane), CYP11 (P450 side-chain-cleavage; converts cholesterol to pregnenolone), and LHr (luteinizing hormone receptor; surge in LH prior to maturation is believed to be the main mediator causing the steroidogenic shift in E₂ to MIS) (Nagahama and Yamashita, 2008) follow similar expression patterns as found in other teleosts. StAR and CYP11 follow the general pattern of increasing E₂ throughout the stages of maturation, whereas LHr shows

an increase in expression, primarily with maturation. LH is said to inhibit the production of E₂ prior to maturation (Planas *et al.*, 2000); however, mummichog continue to produce increasing amounts of E₂ with a high expression of LHR in maturing follicles.

Differences between mummichog and other teleosts in maturational gene expression include the expression of P450c17 (catalyzes the hydroxylation of pregnenolone to yield 17 α -hydroxypregnenolone and then dehydroepiandrostone, or the hydroxylation of progesterone to yield 17 α -hydroxyprogesterone and then androstenedione), which drops earlier in the maturation cycle; follicle stimulating hormone receptor (FSHR; key gonadotropin responsible for ovarian development), which has low expression in the cortical alveolus stage; P450 aromatase (converts T to E₂), or CYP19a1, which is evenly expressed through all stages prior to dropping at late maturation; and estrogen receptor α (ESR1; the only estrogen receptor subtype that has been linked to vitellogenin synthesis in liver and E₂ regulation (Chakraborty *et al.*, 2011)), which has no significant differences in expression over the ovarian development stages, prior to dropping at late maturation. CYP19a1 and ESR1 follow the general increasing pattern of E₂ throughout the stages of maturation, whereas CYP17 and FSHR do not. The lack of increase in the expression of CYP17 throughout recrudescence in mummichog may be explained by the lack of an E₂ to MIS switch at maturation, as the expression patterns of CYP17 were previously suggested to have a role in the switch (Clelland and Peng, 2009). CYP19a1 is suspected to be an estrogen-responsive gene; the drop off in CYP19a1 expression at late maturation does not follow E₂ patterns and warrants further studies to determine what may be causing the decrease in CYP19a1. CYP19b (P450 aromatase in brain) on the other hand, showed no significant differences

in expression across ovarian development, suggesting regulation of local brain E₂ production is independent of gonadal steroidogenesis. The results from this study suggest that ESR1 expression is closely related to E₂ levels and vitellogenin expression within mummichog; however, the relationships for estrogen receptors ER2a and ER2b with ovarian development are still unclear.

To determine EE₂ effects on maturing follicles, grouped follicles were exposed to 50-250 nM of EE₂ for 24 hours, *in vitro*, with and without hCG stimulation. There was no effect on either T or MIS production (E₂ was not measured due to high cross-reactivity to EE₂ in the EIA analysis), or gene expression (CYP19a1 and LHr) at any follicular stage. The results validate the hypothesis that mummichog are more tolerant to EE₂ than other teleosts, as previous studies demonstrated an increase in LHr expression in zebrafish ovarian cells after EE₂ *in vitro* exposure (Liu *et al.*, 2013). hCG stimulation increased both hormone production and CYP19a1 expression in incubated follicles. It was unexpected, however, that hCG stimulation did not increase LHr expression at either stage of maturation (cortical alveolus – late mature), as MIS levels did increase with hCG stimulation and Liu *et al.*'s (2013) study demonstrated hCG caused an increase in LHr expression. The hormone and gene expression results after EE₂ *in vitro* exposure adds weight to the idea that mummichog ovarian follicles are resistant to exogenous estrogen.

Overall, the current study provided confirmation of a continuously increasing production of E₂ in maturing follicles, supplemented by new molecular analysis of key steroidogenic enzymes and hormone/gonadotropin receptors across the ovarian steroidogenic pathway in mummichog. These results, along with no effect of exposure to EE₂ on maturing follicles, support the hypothesis that high estrogen levels during oocyte

maturation may be responsible for reduced sensitivity in mummichog to environmental estrogens. However, the mechanism behind the correlation of high estrogen levels during oocyte maturation and reduced sensitivity to exogenous estrogens need to be explored further.

3.2 Future Directions

It has been demonstrated that mummichog are fairly resistant to exogenous estrogens such as EE₂, when compared to commonly-studied teleost species. Growth and reproductive processes in freshwater teleosts, such as fathead minnow (*Pimephales promelas*), zebrafish (*Danio reiro*), and Chinese rare minnow (*Gobiocypris rarus*), are negatively impacted by exogenous estrogens at environmentally-relevant EE₂ concentrations (ex. 0.1-10 ng/L) (Armstrong *et al.*, 2016; Kidd *et al.*, 2007; Lin and Janz, 2006; Parrott and Blunt, 2005; Zha *et al.*, 2008). However, mummichog respond to much higher concentrations at 100 ng/L or higher (if at all) (Bosker *et al.*, 2016; Gillio Meina *et al.*, 2013; Hogan *et al.*, 2010). Currently, the reason for this species-specific sensitivity with EE₂ is unknown. This thesis aimed to determine if continuously high levels of E₂ present in maturing mummichog ovarian follicles were partially responsible for the decreased sensitivity to EE₂; results from this thesis have supported this hypothesis. In order to further investigate this suggestion, similar experiments from this thesis can be conducted on teleosts that are generally more tolerant to EE₂ exposure, such as sheepshead minnow (*Cyprinodon variegates*; Zillioux *et al.*, 2001), in order to determine if similar patterns of E₂ regulation exist during ovarian development.

The experimental work on EE₂ *in vitro* exposure of mummichog ovarian follicles paves the way for more *in vitro* analysis on the mechanisms of EE₂ action. *In vitro*

exposures of staged ovarian follicles to EE₂ have not been completed on any fish species. The concentrations used in the current study were chosen after a thorough literature research on previous EE₂ *in vitro* exposures conducted on hepatic or ovarian tissue/cells. The lack of effect of EE₂ on P450 aromatase (CYP19a1), LHr gene expression, or on T and MIS hormone production suggests that mummichog ovarian follicles are resistant to EE₂ exposure at 50-250 nM. It would be beneficial to assess the effect of EE₂ at a broader concentration range (e.g., 10 pM – 10 uM), as this range is commonly found in EDC *in vitro* exposures (Beitel *et al.*, 2015; Eide *et al.*, 2014; Hultman *et al.*, 2015; Liu *et al.*, 2013), or to run a concentration-response curve prior to choosing a new concentration range. It would also be beneficial to assess the effect of EE₂ *in vitro* in mummichog follicles across a time course from 0 – 24 hours, at much shorter and intermediary intervals, to determine if there are any molecular effects occurring at a shorter time period than 24 hours. This is because EDCs can act on a molecular level within minutes (membrane receptors) or hours (nuclear receptors) (Thomas, 2008) and as well that effects can be transient (Shanle and Xu, 2011; Vandenberg *et al.*, 2012). In the current study, expression of CYP19a1 decreased after the 24-hour incubation, and although LHr expression did not decrease after the 24-hour incubation, it is important to determine what is happening to CYP19a1 expression before the 24-hour time period, as changes in expression can occur rapidly and are transient. Noting these changes in expression prior to the 24-hour incubation time point can be vital in determining the rate of perturbation on CYP19a1 expression across stages of follicular development. Perhaps there is an important piece missing with changes in LHr expression as well, which might provide insight on why LHr expression does not decrease after 24 hours of incubation, or increase

with hCG stimulation. A positive control could also be added to this study to confirm the effect of EE₂ on the measured endpoints. Because the mechanism of EE₂ on mummichog ovarian follicles is not yet known, zebrafish follicles could be used as a positive control as it was demonstrated that EE₂ has a negative effect on LHr expression in zebrafish ovarian cells (Liu *et al.*, 2013).

As previous EE₂ *in vitro* exposures have been completed on hepatic cells (Beitel *et al.*, 2015; Eide *et al.*, 2014), it would also be interesting to conduct an EE₂ *in vitro* exposure on liver tissue sampled at various stages of ovarian development, while assessing vitellogenin and ER α expression changes. Brain sections could also be dissected at various stages of ovarian development, to assess P450 aromatase in the brain, as well as gonadotropin receptors and/or estrogen receptors. The data could be analyzed alongside gene expression data from the current study measured in liver and brain at different stages of ovarian development. This research could then be utilized to compile a better understanding of how EE₂ is affecting, or not affecting, mummichog through the HPG axis.

In the current study, key receptors and enzymes in the steroidogenic pathway in mummichog were characterized by molecular analysis for the first time. This work is important to support further development of the mummichog model for EDC bioassay work. Estrogen receptors, gonadotropin receptors, vitellogenin, StAR and P450 enzymes were characterized across ovarian development in mummichog; however, there are still some important genes in the steroidogenic pathway which could be characterized, such as 20 β -HSD (20 β -hydroxysteroid dehydrogenase), which catalyzes the conversion of 17 α -hydroxy progesterone to produce MIS (Young *et al.*, 2005). As high levels of MIS

coincide with high levels of E₂ in mummichog, it would be interesting to see how 20 β -HSD (20 β -hydroxysteroid dehydrogenase) is expressed across maturation. The current study, along with previous work, emphasizes the importance of developing a thorough understanding of comparative endocrinology and physiology among model teleost species used in EDC studies. There are other endpoints which can be measured across the ovarian development cycle in order to develop a full working model of mummichog; these endpoints include furthering the molecular characterization of steroidogenic enzymes, signaling pathways, and also more hormones and gonadotropins, etc. (Table 3.1). Measuring the levels of FSH and LH through EIA (enzymatic immunoassay) analysis will be beneficial to see what is happening to these gonadotropins and whether their levels correspond with receptor expression. Since LHR showed no increase in expression after hCG stimulation in the current study, an analysis of the gonadotropin LH with EE₂ exposure across ovarian development may provide more insight into this question. Filling in the knowledge gaps for mummichog physiology, as well as expanding on EE₂ exposure studies, will bring us closer to determining the mechanisms of tolerance of estrogenic EDCs in mummichog.

Table 3.1. Gap analysis chart of endpoints for developing the mummichog model across the ovarian development cycle. Endpoints highlighted in green have been completed in this study; endpoints highlight in pink have not been completed by any study.

Characterization of Hormones, Gonadotropins, etc.	Molecular Characterization of Signaling Pathways	Molecular Characterization of Steroidogenic Enzymes
E ₂	ER α	StAR
T	ER β 1	P450scc
MIS	ER β 2	P450c17
FSH	VTG1	P450arom
LH	FSHr	3 β -HSD
VTG	LHr	11 β -HSD
Progesterone	G-protein coupled receptor	17 β -HSD
Intracellular cAMP levels		20 β -HSD
PKA levels		

Further studies need to be completed in order to determine the mechanistic reason for why higher E₂ levels during oocyte development may cause reduced sensitivity to exogenous estrogens, such as EE₂. A possible mechanism behind this correlation could be associated with estrogen receptors. Ligand binding to ERs is responsible for regulating the basic biology of estrogen-sensitive tissues; the use of selective agonists or antagonists can modulate this biology (Rich *et al.*, 2002). Therefore, binding activity is an important factor to consider. ER competitive binding assays (assess a chemical's ability to bind to an ER) between E₂ and EE₂ have been completed in other species and could be a valuable asset to understanding the mechanistic effects of EDCs in mummichog (Blair *et al.*, 2000). This could determine whether exogenous estrogens, such as EE₂, are capable of acting through the same binding mechanisms as endogenous E₂. Eventually this would demonstrate whether E₂ has a higher binding affinity to ERs than EE₂ in mummichog,

and thus is displacing EE₂ from ERs more so than in other fish. Further studies could also be completed on the binding affinity of E₂ and EE₂ to the different ER subtypes within various tissues, to get a more thorough understanding of the mechanisms.

Throughout this thesis, nuclear estrogen receptors were examined thoroughly. More work could be completed on membrane receptors and explore the pKA (protein kinase)/cAMP (cyclic AMP) pathway further. Ligand binding results in the exchange of GDP for GTP on the Galphas protein; its dissociation from the BY subunit complex stimulates the enzyme adenyl cyclase (AC) to catalyze the cyclization of ATP to generate cAMP and pyro-phosphate (Serezani *et al.*, 2008). Intracellular levels of cAMP are tightly regulated by AC and the enzyme phosphodiesterase (PDE) (Serezani *et al.*, 2008). An increase in intracellular cAMP levels is an important intracellular signaling mechanism involved in the regulation of gene expression (Chen *et al.*, 1999); and elevation in intracellular cAMP levels in the ovary is also important in the regulation of steroid production, prior to GVBD in synergism with E₂ (Lu *et al.*, 2017). This leads to the question of whether or not there is an increase in cAMP levels in maturing mummichog follicles prior to maturation. A thorough characterization of the enzymes in this pathway and intracellular cAMP levels can provide insight on whether the link between reduced sensitivity to exogenous estrogens and high levels of E₂ in maturing oocytes is correlated to a component of this pathway.

There is also a possibility that the high E₂ levels present in maturing mummichog oocytes could be a result of adaptation to the environment or due to its asynchronous spawning nature. In asynchronous spawners, the pre-vitellogenic, vitellogenic and maturation phases almost completely overlap, with vitellogenesis and maturation

occurring simultaneously; however, in synchronous spawners, the phases are clear with little or no overlap, and in group-synchronous spawners, phases are less clear than synchronous spawners and there is little overlap (Wootton and Smith, 2015). These different patterns in oocyte dynamics can be caused by either radically different control mechanisms for clear distinct phases, or a shift in temporal patterns for non-distinct phases (Wootton and Smith, 2015). It would be interesting to determine what selective factors cause these dynamic changes in oocyte development and maturation, through evolutionary analysis of the species *mummichog*.

3.3 Integrative Nature of this Study

This study expanded the current understanding of reproductive physiology in *mummichog* by linking endpoints across various biological levels. These include the whole organism (e.g., hormone levels from blood (plasma)), organ (e.g., gonad size, liver size, characterization of ovarian stage through histological analysis), physiological (e.g., hormonal production from ovarian tissue and follicles) and molecular (e.g., gene expression of various steroidogenic enzymes and receptors in different tissues) levels. The endocrine system is one of the key signalling integrators in organisms; a thorough assessment across these biological levels enhances our understanding for *mummichog* and general fish reproductive physiology. This study also analyzed the effects of EE₂ on *mummichog* ovarian follicles across maturation, which furthers our interpretation of EE₂ effects under various reproductive circumstances (e.g., at different stages of ovarian development).

With the results from this study, we can develop a better understanding of mechanisms of action of EDCs and link these results to the field of toxicology. We can

utilize the results gained from this study to support the development of toxicological tools that investigate the mechanisms of action of EDCs, including those compounds that, similar to EE₂, have estrogenic mechanisms of action. The knowledge of the mechanism of action of certain substances can also supplement other studies which have addressed these issues on a larger ecological scale. Through integration, the work from this study can be utilized to determine the effect of EDCs (such as EE₂) at various biological levels across species, the mechanisms of action or tolerance of EE₂ across species, and ultimately can lead to improving risk assessment of pollutants (such as exogenous estrogens) over a large scale, and protection of Canadian water systems.

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