

Sex steroid feedback regulation of pituitary gonadotropins during early secondary oocyte growth in coho salmon (*Oncorhynchus kisutch*) – a potential target of endocrine disruption.

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Abstract

Sex steroid feedback regulation of pituitary gonadotropins during early secondary oocyte growth in coho salmon (*Oncorhynchus kisutch*) – a potential target of endocrine disruption.

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In teleost fish as in other vertebrate species, the pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), are fundamentally involved in regulating physiological processes critical to the production of a fertilizable egg, including steroidogenesis, folliculogenesis, vitellogenesis, final oocyte maturation, and ovulation. Despite the importance of pituitary gonadotropins (Gths) in regulating fish reproduction, the complex network of factors regulating Gth synthesis and release in fishes is still not fully understood. Further, in the growing field of endocrine disruption, only limited data are available on impacts of endocrine disrupting chemicals (EDCs) on the gonadotropins or pituitary function. The research described in this dissertation examines the role of sex steroids in regulating Fsh and Lh during early stages of oogenesis in salmon and determines if chemicals or natural steroids in the environment originating from waste-water treatment plants (WWTPs) could disrupt reproduction via altered GtH expression. The study described in the first chapter demonstrates that of estrogen

differentially regulates Fsh and Lh in female coho salmon during the transition from late primary to early secondary oocyte growth, which corresponds to the onset of puberty in fish. In chapter 2, I report the results of the first transcriptome-wide gene expression analysis of the pituitary gland in prepubertal salmon exposed to water containing ethynylestradiol (EE2), a synthetic estrogen used in human contraceptives that enters the aquatic ecosystem via WWTP discharges. Lastly, the work presented in chapter 3 provides evidence that pituitary Gths, primarily Lh, are differentially expressed in coho salmon exposed to WWTP effluents, likely due to presence of estrogenic chemicals. This research demonstrates that natural and synthetic estrogens differentially regulate Fsh and Lh as well as numerous genes involved in pathways associated with Gth synthesis and secretion, steroid action, cell development and circadian rhythms. By combining basic and applied research, this work has provided valuable information on sex steroid regulation of Gths during early oogenesis that will be useful for predicting and interpreting results of endocrine disruption research. Further, the results of this work suggest that one mechanism of endocrine disruption in fish could be via disrupted gonadotropin synthesis.

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DEDICATION

I dedicate this dissertation to the memory of Karl Ullman, the founder of the Pacific Crest Leadership School (PCLS). The PCLS was a summer camp in which small groups of middle-school students hiked from the Washington-Oregon border to Glacier Peak, in three 4-week backpacking trips completed over three consecutive summers. The mission of the PCLS was “... to inspire and train young leaders to lead modern society into harmony with nature.” When Karl came to my 6th grade science class to recruit applicants for his second cohort of PCLS campers, I was mesmerized by his photographs and the adventures that he described. I had no idea the challenge that awaited me, but Karl’s belief in me, and my subsequent belief in myself carried me through. During our time in the woods, I developed a deep appreciation of nature and our role as stewards of the environment. At the end of our first summer trip, we painted self-portraits and Karl asked us what we wanted to do when we grew up. I said that I thought I would like to be a botanist and study plants. That was a defining moment – when I began to consider a life as a scientist. That moment with Karl was the first step down the path that led me here today.

Unfortunately, Karl was diagnosed with cancer before the third summer of our program and died about a year later. Although our time together was relatively brief, Karl had an immeasurable impact on me and I am forever indebted to him. Karl’s enthusiasm for life and nature knew no bounds and he lit that fire in me as well. If I have one hope for my career going forward, it is that my work will help to “lead modern society into harmony with nature.”

REVIEW OF CURRENT KNOWLEDGE AND STUDY OBJECTIVES

Introduction

Reproduction is the creation of new individuals that allow for the continuation of a species. In sexual reproduction, male and female haploid gametes, sperm and ova respectively, fuse to form a diploid zygote (Lombardi, 1998). Oogenesis is the development of a female germ cell, or oogonium, into a fertilizable ovum capable of supporting embryonic development (Bobe and Labbé, 2010; Lubzens et al., 2010). It involves the progression of the oogonium through a series of developmental stages including oogonial proliferation, primary oocyte growth, secondary oocyte growth (vitellogenesis), final oocyte maturation, and ovulation (Figure 1.1).

In fish, as in other vertebrates, oogenesis is controlled by the brain-pituitary-ovarian axis (Figure 1.2). The brain receives and integrates environmental and internal signals from organs and upon appropriate stimulation, gonadotropin-releasing hormone (GnRH) is released from hypothalamic neurons that directly innervate the pituitary gland. In response, the pituitary synthesizes and releases the pituitary gonadotropins (Gths), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which promote oocyte growth, maturation, and production of steroid hormones (Levavi-Sivan et al., 2010; Swanson et al., 2003). Gonadal steroid hormones and other ovarian factors further stimulate oogenesis and feedback on the brain and pituitary to regulate Fsh and Lh production. In this way, the pituitary Gths are central regulators of reproduction and are involved in most aspects of ovarian development and function (Swanson et al., 2003; Levavi-Sivan et al. 2010; Lubzens et al., 2010).

Stages of Salmon Oogenesis

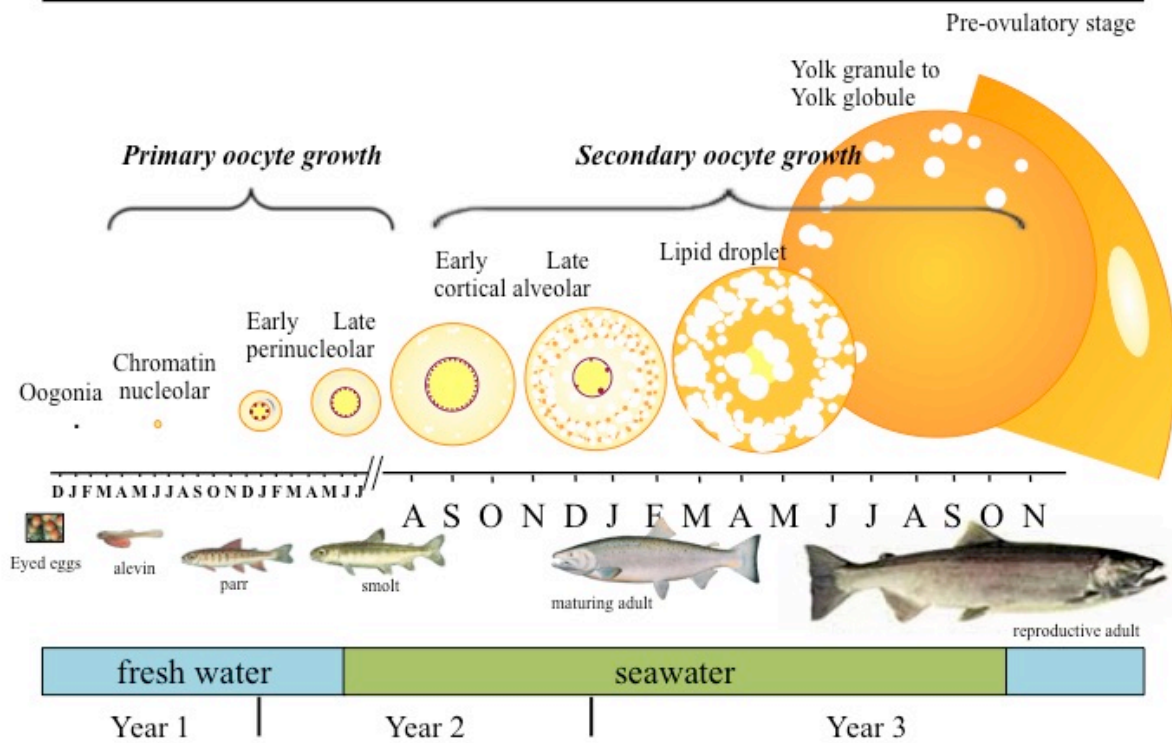


Figure 1.1. Stages of oogenesis in coho salmon relative to life history stage, age, and habitat. Modified from Forsgren (2010), I. Nakamura (unpubl.), P. Swanson (unpubl.), and Campbell et al., 2006.

Brain-Pituitary-Ovary Axis in Salmon

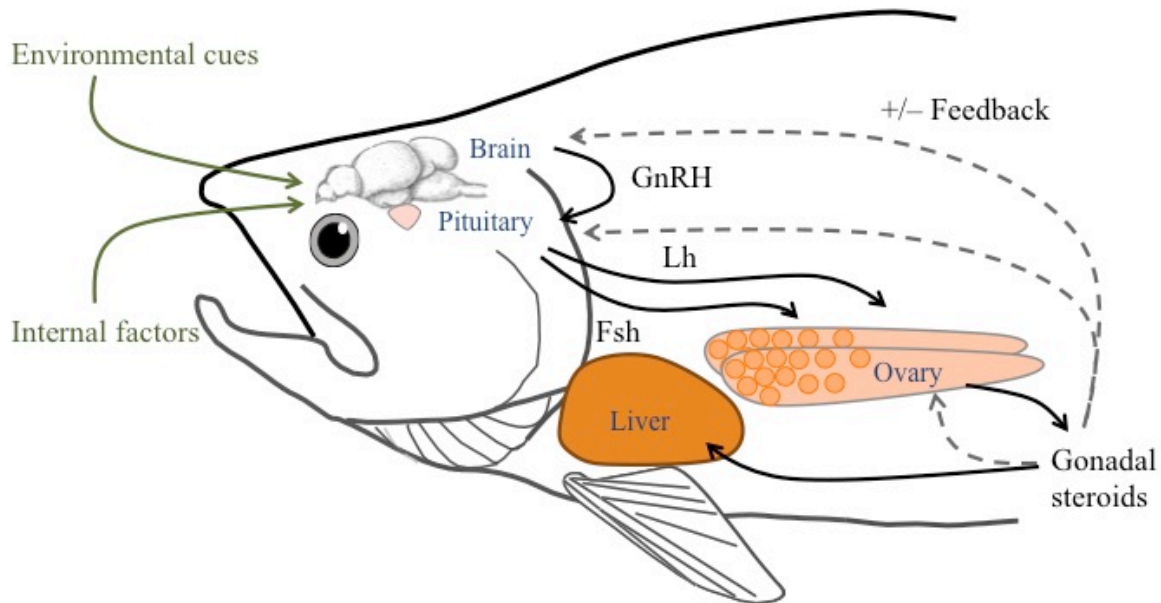


Figure 1.2. Simplified schematic of control of reproduction in salmon via the Brain-Pituitary-Ovary axis. Modified from J. Dickey (unpubl.) and A. Luckenbach (unpubl.).

The Pituitary Gonadotropins

The Gths are heterodimeric glycoprotein hormones composed of a common alpha subunit and distinct beta subunits that confer hormone-specific activity. The Gths are involved in all aspects of ovarian development and function including steroidogenesis, oogenesis, final oocyte maturation, and ovulation (Swanson et al., 2003). Initially, evidence suggested that a single Gth controlled reproduction in fish. However the presence of two distinct Gths, follicle-stimulating hormone (Fsh, formerly Gth I) and luteinizing hormone (Lh, formerly Gth II or maturational Gth) was established in the late 1980s in salmon (Suzuki et al., 1988a; Swanson et al., 1991, 1989) and later in all classes of bony fishes. Since the identification of two distinct Gths, studies

have revealed that Fsh and Lh exhibit distinct expression profiles and biological functions and are independently regulated (reviewed by Levavi-Sivan et al., 2010; Swanson et al., 2003; Yaron et al., 2003).

Fsh and Lh expression profiles during oogenesis

In salmonids, Fsh is the predominant Gth throughout early gametogenesis. In female coho salmon (*Oncorhynchus kisutch*) that spawn in late fall at age 3, an initial increase in plasma and pituitary Fsh levels is observed a year before spawning, during the transition from primary to secondary oocyte growth (Campbell et al., 2006). Plasma Fsh levels continue to increase throughout vitellogenesis and decline prior to ovulation (Figure 1.3, Swanson et al., 1989; 1991). Similarly, in rainbow trout (*O. mykiss*), plasma Fsh levels begin to rise about one year before spawning during the period of initial ovarian growth. Plasma Fsh levels peak during vitellogenesis and return to basal levels by the end of vitellogenesis (Breton et al., 1998; Gomez et al., 1999; Prat et al., 1996). Unlike semelparous salmonids, in rainbow trout, plasma Fsh levels rise again prior to ovulation and remain elevated after ovulation (Breton et al., 1998; Gomez et al., 1999; Prat et al., 1996). The high Fsh levels observed in trout after ovulation are thought to be involved in recruitment of the next group of follicles (Breton et al., 1998; Tyler et al., 1997). Plasma Lh levels, in contrast, remain very low or undetectable in both salmon and trout until late vitellogenesis, peaking at final oocyte maturation and ovulation (Breton et al., 1998; Davies et al., 1999; Gomez et al., 1999; Oppen-Berntsen et al., 1994; Prat et al., 1996; Slater et al., 1994).

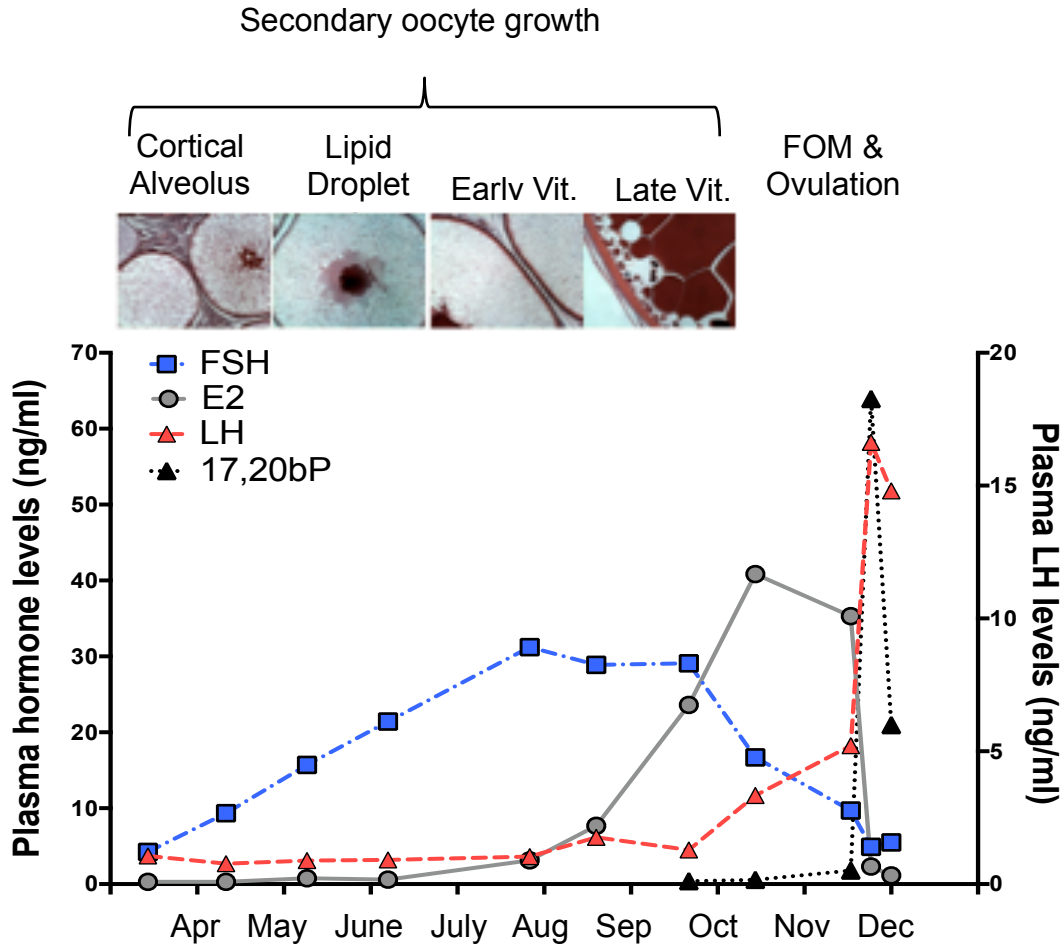


Figure 1.3: Mean plasma levels of Fsh, Lh, E2, and 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β P) in female coho salmon during secondary oocyte growth and final oocyte maturation (FOM) and ovulation. Standard errors are omitted for clarity of graphics. From Swanson, P. (1991) Salmon gonadotropins: Reconciling old and new ideas. In, "Reproductive Physiology of Fish" (A.P. Scott and J.P. Sumpter, eds.), pp.2-7. Univ. of East Anglia, Norwich, England

Investigations of plasma Gth levels in non-salmonid fish are limited to a few species due to a lack of specific Fsh and Lh immunoassays in many fishes. European sea bass (*Dicentrarchus labrax*) exhibit plasma Gth profiles similar to salmonids with Fsh peaking during early vitellogenesis and declining at oocyte maturation and ovulation (Molés et al., 2012) and Lh peaking during mid-spawning (Navas et al., 1998). In mature female tilapia (*Oreochromis*

niloticus), on the other hand, plasma Fsh and Lh have similar plasma profiles throughout the reproductive cycle. Both Fsh and Lh increase on the second day of the reproductive cycle, remain stable or decline slightly until day 8 of the cycle, and then increase again before spawning, with Lh reaching higher levels than Fsh prior to spawning (Aizen et al., 2007). In the absence of immunoassays, mRNA levels also provide information about seasonal Gth profiles. In Atlantic salmon (*Salmo salar*) and rainbow trout, Gth mRNA levels are relatively well correlated with plasma Gth levels. Steady state *lhb* mRNA levels peak at ovulation and *fshb* mRNA levels peak earlier during vitellogenesis and again at ovulation in iteroparous species (Andersson et al., 2013; Gomez et al., 1999; Oppen-Berntsen et al., 1994). In Atlantic cod (*Gadus morhua*), *fshb* mRNA levels begin to rise 6 months prior to spawning, peak 2 months before spawning and then decline precipitously after spawning while *lhb* mRNA levels begin to rise later and peak at spawning, like the pattern observed in salmonids (Mittelholzer et al., 2009). Similarly, in Japanese eel (*Anguilla japonica*) injected with salmon Gth to induce ovarian maturation, *fshb* mRNA levels were high in previtellogenic fish and decreased throughout vitellogenesis while *lhb* mRNA levels remained low until late vitellogenesis (Suetake et al., 2002). In the sea bass, *fshb* and *lhb* exhibit similar mRNA profiles, which may reflect maintenance of multiple batches of gametes at a given time (Mateos et al., 2003). In contrast to other fishes, female red seabream (*Pagrus major*) have low *fshb* and high *lhb* steady state mRNA levels throughout sexual maturation (early vitellogenesis through spawning) (Gen et al., 2002). Although it is still unknown whether Fsh and Lh plasma levels are well correlated with steady state mRNA levels in this species, these data suggest that Lh could be the primary Gth controlling oogenesis in red seabream, with little or no role of Fsh.

Biological functions of Fsh and Lh in oogenesis

In keeping with their differential expression profiles, it has been proposed that Fsh regulates early oocyte growth and vitellogenesis, while Lh is primarily involved in final oocyte maturation and ovulation, particularly in salmonids and synchronous spawners. Although Fsh and Lh are equipotent at stimulating estradiol-17 β (E2) production from ovarian fragments *in vitro* (Suzuki et al., 1988b; Swanson et al., 1989), Fsh is the predominant Gth present in the circulation when plasma E2 is increasing throughout secondary oocyte growth (Campbell et al., 2006; Oppen-Berntsen et al., 1994; Swanson et al., 1991). Additionally, studies of ovarian Gth receptor mRNAs in coho salmon, Atlantic salmon, zebrafish (*Danio rerio*) and tilapia have shown that Fsh receptor (*fshr*) transcripts are maximally expressed during vitellogenesis, whereas Lh receptor (*lhr*) mRNA levels peak during final oocyte maturation (Guzmán et al., 2014; Kwok et al., 2005; Oba et al., 2001). These data are consistent with earlier receptor autoradiography studies localizing Gth receptors in coho salmon (Miwa et al., 1994) and suggest that *in vivo*, Fsh is primarily responsible for stimulating E2 production and subsequent stimulation of vitellogenin production.

Several studies have also directly implicated Fsh in early oocyte development and vitellogenesis. Vitellogenic rainbow trout injected with Fsh had two-fold higher vitellogenin uptake into the oocytes than in control- or Lh-injected trout (Tyler et al., 1991). Similar results were obtained *in vitro*, with Fsh treatment increasing vitellogenin uptake into oocytes in a dose-dependent manner, versus no effect of Lh treatment (Tyler et al., 1991). In vitellogenic rainbow trout subjected to unilateral ovariectomy, a second population of primary oocytes was recruited into the vitellogenic growth phase and grew at a faster rate than oocytes from control fish. This

increased rate of development was associated with higher plasma Fsh levels, with no difference in plasma E2 levels (Tyler et al., 1997). These results suggest that higher plasma Fsh contributed to increased oocyte growth and vitellogenesis (Tyler et al., 1997). In addition, a series of studies in previtellogenic coho salmon demonstrated that Fsh regulates expression of genes involved in ovarian follicle growth (Guzmán et al., 2014; Luckenbach et al., 2013, 2011). Most recently, Fsh-deficient zebrafish were found to have significantly delayed ovary and testis development and puberty onset (Zhang et al., 2015b). Further, deletion of the zebrafish Fsh receptor gene (*fshr*) caused oocyte development to arrest completely in early primary oocyte growth (Zhang et al., 2015a). These data suggest that Fsh has a primary role in early ovarian growth and vitellogenesis.

In contrast, plasma Lh is generally low or undetectable in salmonids until final oocyte maturation and ovulation (Davies et al., 1999; Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991). Coincident with the rise in plasma Lh, steroidogenesis shifts from primarily E2 production to production of the maturation inducing hormone, $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17,20\beta$ -P). While Fsh and Lh are equipotent in stimulating E2 production, Lh greatly exceeds Fsh in its ability to stimulate gonadal $17,20\beta$ -P production (Planas et al., 2000; Suzuki et al., 1988b; Swanson et al., 1991; Yaron et al., 2003). The transition from E2 production to $17,20\beta$ -P production may be dependent on the presence of the Lh receptor (*Lhcgr*), which is expressed predominantly in granulosa cells (Miwa et al., 1994) during final oocyte maturation (Rosenfeld et al., 2007). Induction of $17,20\beta$ -P production by Lh is thought to be a critical step in achievement of oocyte and sperm maturation (Lubzens et al., 2010; Nagahama, 1994). This hypothesis is supported by results that Lh but not Fsh was able to induce germinal vesicle breakdown (GVBD) in coho salmon oocytes (Planas et al., 2000) and ovulation (Crespo et al.,

2013). Further, previous studies suggest that Lh may inhibit aromatase in preovulatory stages, and this may be a key regulator of the shift from E2 to 17,20 β -P production (Maestro et al., 1997; Lubzens et al., 2010; Sire et al., 1981). Finally, *lhb*- and *lhcr*-deficient zebrafish showed normal ovarian growth, but oocytes failed to undergo final oocyte maturation or ovulation (Chu et al., 2014; Zhang et al., 2015a, 2015b). Thus, current evidence suggests that Fsh regulates early gametogenesis and vitellogenesis, while Lh regulates late gametogenesis and gamete maturation. Because of their primary roles in gametogenesis, precise regulation of Gths is critical to the control of reproduction.

Regulation of Fsh and Lh

Pituitary Gth synthesis and release are regulated by a complex network of neuroendocrine, paracrine and endocrine signals from the brain, pituitary and gonad, respectively (Levavi-Sivan et al., 2010; Yaron et al., 2003). Based on the differential expression of Fsh and Lh throughout gametogenesis in salmonids and other fish, it is clear that the Gths are differentially regulated throughout development. In addition, the factors regulating Fsh and Lh expression can differ depending on the species, season, and stage of development. In the following sections, the current understanding of hypothalamic, pituitary and gonadal factors regulating Gth synthesis and secretion will be reviewed.

Hypothalamic factors regulating Fsh and Lh

The primary factor stimulating Gth secretion from the pituitary is hypothalamic gonadotropin-releasing hormone (GnRH). In mammals, changes in GnRH pulse frequency can differentially regulate secretion of Fsh and Lh with slow pulses favoring Fsh and fast pulses

favoring Lh (Bernard et al., 2010). Although it is assumed that GnRH is released in a pulsatile manner in all vertebrates, this has not been definitively established in fish, and pulsatile GnRH treatment is not necessary to induce spawning (Levavi-Sivan et al., 2010). In fish, GnRH has been shown to stimulate secretion of both Gths *in vivo* (Breton et al., 1998; Khan and Thomas, 1994; Mateos et al., 2002) and from pituitary cells *in vitro* (Ando et al., 2004; Dickey and Swanson, 2000; Kim et al., 2011; Luckenbach et al., 2010; Swanson et al., 1989). In addition to its role in Gth secretion, GnRH increases steady state mRNA levels of *fshb* (Ando et al., 2004; Dickey and Swanson, 2000; Golan et al., 2014) and *lhb* (Kitahashi et al., 1998; Melamed et al., 1996; Yamaguchi et al., 2005). Recent studies in fish have implicated kisspeptin (Kiss) and neurokinin B (NKB, encoded by *tac3* gene) in regulating GnRH and subsequent Gth secretion, as in mammals (Oakley et al., 2009). In support of this, Kiss 2 treatment increased *gnrhr1* and *fshb* mRNA levels *in vitro* in striped bass (Zmora et al., 2015) and NKB treatment resulted in rapid stimulation of Fsh and Lh release in tilapia (*Oreochromis niloticus*) both *in vivo* and *in vitro* (Biran et al., 2014).

In contrast to GnRH, dopamine (DA) is the primary negative regulator of Gth release in fish. DA inhibits Gth secretion, both by directly inhibiting release of Gths from the pituitary, and by inhibiting GnRH release (Zohar et al., 2010). Although the inhibitory role of DA has been shown predominantly for its effects on Lh, DA has been shown to inhibit secretion of Fsh and Lh in rainbow trout (Vacher et al., 2002).

Pituitary factors regulating Fsh and Lh

Within the pituitary, the activin-inhibin-follistatin system is an important pathway regulating Gth expression (reviewed in Ge, 2000). Activins and inhibins are structurally related,

dimeric proteins of the transforming growth factor- β (TGF- β) superfamily. Activins are homo- or heterodimers of two β -subunits producing activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$), or activin AB ($\beta_A\beta_B$). Inhibins are heterodimers of a unique α -subunit and one of two β -subunits (A or B) forming inhibin A ($\alpha\beta_A$) or inhibin B ($\alpha\beta_B$). Activin stimulates Fsh synthesis in the pituitary by increasing *fshb* promoter activity in mammals and fish (Ge et al., 2003; Huang et al., 2001). Through different modes of action, inhibin and a structurally unrelated protein, follistatin, can antagonize actions of activin, and other TGF- β family members. Gonadally derived inhibin binds to activin receptors via the common β -subunits thereby antagonizing activin action by competing for activin receptor binding. Locally produced follistatin, on the other hand, antagonizes activin by binding to activin and preventing it from binding to its receptors (reviewed in Ge, 2000). Although originally isolated from the ovary, activin and follistatin are locally produced within the pituitary where they regulate Fsh expression as well as Lh expression in some species. Studies in European eel (*Anguilla anguilla*), goldfish (*Carassius auratus*) and zebrafish have demonstrated that activin acts in an autocrine/paracrine fashion to increase *fshb* and decrease *lhb* mRNA levels in the pituitary, and these effects are antagonized by follistatin (Aroua et al., 2012; Lau and Ge, 2005; Lin and Ge, 2009). Studies in other species have shown activin increases *fshb* mRNAs but has no effect (coho salmon: Davies et al., 1999) or increases *lhb* (tilapia: Yaron et al., 2001). In addition, studies indicate that activin and follistatin may be important mediators of GnRH-induction of *fshb* mRNA levels. In L β T-2 cells, GnRH-stimulated activation of the goldfish *fshb* gene promoter was abolished when activin signaling was disrupted (Lau et al., 2012).

Gonadal factors regulating Fsh and Lh

In addition to hypothalamic and local pituitary control of Gths, gonadal factors including sex steroids and gonadal peptides (e.g., inhibin) feedback on the brain and pituitary to regulate Gth expression (Gharib et al., 1990). Gonadal steroids can both positively and negatively feedback on Fsh and Lh synthesis, depending on the species, season, and stage of development. Pituitary Lh content and *lhb* mRNA levels increase during late gametogenesis, concomitant with rises in gonadal steroids (Gomez et al., 1999). Additionally, treatment with E2 or testosterone (T) increase *lhb* steady state mRNA levels and pituitary Lh content in early stages of gametogenesis in salmonids (Borg et al., 1998; Breton et al., 1997; Crim et al., 1981; Dickey and Swanson, 1998; Forsgren, 2010; Melo et al., 2015) and other fishes (Jeng et al., 2007; Schmitz et al., 2005; Yaron et al., 2003). In rematuring Atlantic salmon male parr, T treatment increased pituitary Lh content and this effect was reduced when T was coadministered with an aromatase inhibitor, suggesting that the positive feedback effects of T are aromatase-dependent (Antonopoulou et al., 1999). Positive feedback of E2 on Lh is thought to act primarily through increased transcription of *lhb*. In support of this, estrogen response elements (EREs) have been found in the *lhb* promoter of Chinook salmon (*O. tshawytscha*) (Le Dréan et al., 1996; Liu et al., 1995; Wang et al., 2009; Xiong et al., 1994) and goldfish (Sohn et al., 1999). In addition, estrogen receptor alpha (*esr1*) and *esr2a* (formerly ER γ or ER β 2) mRNAs colocalize with *lhb* mRNA in pituitary gonadotrophs of Mediterranean sea bass (Muriach et al., 2008a, 2008b) and ricefield eels (*Monopterus albus*) (Zhang et al., 2014). Therefore, it is reasonable to expect that estrogens could directly regulate *lhb* transcription. Indeed, results from pituitary culture experiments demonstrated that E2 and/or T increase *lhb* mRNA levels, Lh cell content, and Lh release *in vitro* in various fishes (Ando et al., 2004; Aroua et al., 2007; Huang et al., 1997; Huggard-Nelson et al., 2002; Lin and Ge, 2009; Rebers et al., 2000; Zhang et al., 2014). Despite increases in

pituitary Lh content, increases in plasma Lh have not been observed in response to E2 treatment alone, suggesting that other factors are involved in Lh release *in vivo* (Breton et al., 1997; Dickey and Swanson, 1998; Saligaut et al., 1998). However, long-term treatment of immature yearling rainbow trout with T induced an increase in plasma Lh (Crim and Evans, 1983). Whether the increase is due to T and/or E2, as a result of aromatization of T, is not known.

Despite considerable research on the effects of E2 and T on Lh expression, relatively little is known about the effects of non-aromatizable androgens on Lh synthesis or secretion. Current data on the effects of androgens on Lh synthesis and secretion is contradictory. Studies have found that non-aromatizable androgens cause no change in *lhb* mRNA levels or pituitary Lh content (Aroua et al., 2007; Cavaco et al., 2001; Kobayashi et al., 2000), a slight increase in *lhb* mRNA or Lh levels (Antonopoulou et al., 1999; Borg et al., 1998; Crim et al., 1981; Hellqvist et al., 2008; Melo et al., 2015), or a slight decrease in *lhb* mRNA levels (Setiawan et al., 2012; Sohn et al., 1998b). Generally, in male salmonids, treatment with non-aromatizable androgens increases *lhb* mRNA and pituitary Lh protein levels *in vivo*, but to a lesser extent than E2 or T (Antonopoulou et al., 1999; Borg et al., 1998; Crim et al., 1981; Melo et al., 2015). Similarly, *in vitro*, 11-KT treatment increased Lh release from pre-spawning male masu salmon (*O. masou*) pituitary cells (Ando et al., 2003). To date, there are no data on the effects of non-aromatizable androgens on Lh expression in female salmonids. As such, further study is needed to understand the role that androgens play in Lh regulation during oogenesis.

The role of gonadal steroids in Fsh regulation is less well studied, but the data suggest a negative feedback effect of E2 on Fsh synthesis and secretion. Plasma E2 begins to increase during primary oocyte growth and is positively correlated with plasma and pituitary Fsh levels in coho salmon (Campbell et al., 2006). Gonadectomy increases plasma Fsh in male and female

salmonids (Antonopoulou et al., 1999; Larsen and Swanson, 1997; Saligaut et al., 1998) while treatment with E2 or aromatizable androgen decreases pituitary Fsh content and plasma Fsh levels (Breton et al., 1997; Dickey and Swanson, 1998; Saligaut et al., 1998). Further, E2 treatment decreases *fshb* steady state mRNA levels in other fishes (Jeng et al., 2007; Kobayashi et al., 2000; Mateos et al., 2002; Muriach et al., 2014; Sohn et al., 1998b). *In vitro*, E2 treatment alone did not affect *fshb* steady state mRNA levels in masu salmon (Ando et al., 2004). However, ERE sites have been identified in the promoter of fish *fshb* genes (Rosenfeld et al., 2001; Sohn et al., 1998a; Vischer, 2003) and estrogen receptor transcripts colocalize with *fshb* mRNA in gonadotrophs of Mediterranean sea bass (Muriach et al., 2008a, 2008b). Additionally, E2 treatment decreased *fshb* mRNA levels in sea bass pituitary cell culture within 48 hours suggesting that E2 regulates Fsh directly in some species (Muriach et al., 2014).

Androgen regulation of Fsh is also poorly characterized, particularly in female fish. Although typically considered a male-specific hormone, plasma 11-KT levels also increase during early oogenesis in coho salmon (Forsgren, 2010) and in New Zealand shortfinned eel, *Anguilla australis* (Setiawan et al., 2012). 11-KT treatment was shown to reduce *fshb* mRNA levels in a variety of fishes (Kobayashi et al., 2000; Mateos et al., 2002; Setiawan et al., 2012; Sohn et al., 1998b; Yamaguchi et al., 2006, 2005). In sexually immature and maturing salmonids, on the other hand, androgens increase pituitary Fsh (Borg et al., 1998; Forsgren, 2010) and plasma Fsh (Borg et al., 1998; Breton et al., 1997). Additionally, putative androgen response elements (AREs) were identified in the *fshb* gene promoter of various fish (Chong et al., 2004; Muriach et al., 2014; Sohn et al., 1998b; Vischer, 2003) suggesting that androgens could regulate Fsh transcription through nuclear androgen receptors. However, *in vitro* treatment with non-

aromatizable androgens did not alter *fshb* mRNA levels in eel pituitaries (Aroua et al., 2007; Zhang et al., 2014).

Although sex steroid regulation of Fsh and Lh may be due in part to direct regulation of Gth synthesis or secretion, there is also evidence that sex steroids feedback on neuroendocrine and paracrine factors in the brain and pituitary to regulate Gth expression. Treatment with E2 or T increases GnRH content in the brain and pituitary of fish (Breton and Sambroni, 1996; Montero et al., 1995; Trudeau, 1997). However, nuclear estrogen receptors are not expressed in GnRH neurons in fish (Navas et al., 1995), suggesting that other cell types are involved in E2 regulation of GnRH. In mammals, there is increasing evidence that Kiss and NKB neurons mediate sex steroid feedback on GnRH secretion (Angell and Steiner, 2015; Popa et al., 2008). While data in fish are scarcer, there is increasing evidence that Kiss and NKB may be involved in steroid-responsiveness of GnRH neurons in fish as well. Studies using double-label *in situ* hybridization demonstrated that Kiss neurons co-express estrogen receptors in goldfish and medaka (*Oryzias latipes*) (Kanda et al., 2012; Mitani et al., 2010) and E2 treatment increased mRNA levels of *kiss1*, *kiss2*, *tac3a*, and *gnrh3* and the Kiss and NKB receptors (*kiss1ra*, *tac3ra*, *tac3rb*) in prepubertal zebrafish (Biran et al., 2012; Servili et al., 2011). These results indicate that sex steroids may feedback on the brain to regulate Kiss, NKB, and GnRH expression and subsequent Gth synthesis and release.

Sex steroids may also indirectly regulate Gth expression within the pituitary. In goldfish, E2 and T increased pituitary follistatin mRNA levels *in vitro* and pituitary follistatin mRNA levels varied over the reproductive cycle *in vivo*, potentially in response to changing steroid levels (Cheng et al., 2007). Although there are no data on effects of steroids on activin receptors in brain or pituitary of fish, in brains of male adult rats, castration increased activin type-II

receptor (*acvR2*) mRNA levels, while E2 treatment suppressed *acvR2* mRNA levels (Trudeau et al., 1996). These results suggest that sex steroids may alter activin-follistatin signaling thereby regulating Fsh and potentially Lh expression. As such, gonadal steroids may indirectly regulate Gth expression via neuroendocrine or paracrine factors.

Regulation of glycoprotein hormones alpha subunit

When investigating regulation of Gth synthesis and expression, it is also important to consider the glycoprotein hormones alpha subunit (*cga*), which encodes the alpha subunit for Fsh and Lh as well as thyroid-stimulating hormone (Tsh), and is necessary for synthesis of biologically active Fsh, Lh and Tsh proteins. Similar to *fshb* and *lhb*, *cga* mRNA levels are regulated by hypothalamic factors and gonadal feedback. Studies have shown that GnRH increases *cga* mRNA levels *in vivo* and *in vitro* in isolated pituitary cells (Levavi-Sivan et al., 2010; Yaron et al., 2003). In salmonids, which have two forms of *cga* (*cga1* and *cga2*), GnRH stimulates *cga* mRNA levels *in vitro* in coho salmon (Dickey and Swanson, 2000) and masu salmon (Ando et al., 2004) and *in vivo* in sockeye salmon (Kitahashi et al., 1998). In addition, sex steroids have been shown to regulate *cga* mRNA levels in a variety of species. E2 treatment increases *cga* mRNA levels *in vivo* (Huggard-Nelson et al., 2002; Schmitz et al., 2005) and *in vitro* (Ando et al., 2004; Huggard-Nelson et al., 2002; Rebers et al., 2000) in a variety of fish species. In masu salmon, the response of *cga* to GnRH and E2 *in vitro* was found to be dependent on sex and stage of development, with GnRH- and E2-stimulated *cga* expression only occurring in pituitary cells from female fish during late oogenesis (Ando et al., 2004). These results suggest that GnRH and sex steroids may act in sex- and stage-specific ways to increase the capacity for pituitary glycoprotein hormone synthesis in fish.

Endocrine disruption

In addition to endogenous hormones, there is increasing evidence that chemicals in the environment are capable of disrupting normal endocrine function of fish and wildlife (Hotchkiss et al., 2008; León-Olea et al., 2014). Endocrine disrupting chemical (EDC) refers to any exogenous compound that alters the normal endocrine functioning of an organism or its progeny. EDCs can act to mimic or block endogenous hormones by interfering with their biosynthesis, secretion, transport, receptor binding or elimination (Crisp et al., 1998). In fishes, early evidence of endocrine disruption came from masculinized female fish downstream of pulp and paper mill effluents (PPME) (Howell et al., 1980) and feminization of male fish downstream of wastewater treatment plants (WWTP) (Purdom et al., 1994). Female fish collected downstream of PPME have reduced ovary size, increased ovarian cell apoptosis (atresia), increased age to maturity, lower plasma steroid levels and decreased egg production (Janz et al., 2001; McMaster et al., 1991; Munkittrick et al., 1998, 1991). In extreme cases, females exposed to PPME are completely masculinized, displaying male secondary sex characteristics and reproductive behavior (Howell et al., 1980). In contrast, male fish collected downstream of WWTPs contain intersex gonads (presence of oocytes in testicular tissue) or reduced primary and secondary male sex characteristics and express the female specific egg-yolk protein precursor vitellogenin (Folmar et al., 1996; Jobling et al., 1998; Purdom et al., 1994; Vajda et al., 2011).

The identities of the causative agents of endocrine disruption in PPME have remained elusive (Hewitt et al., 2008) but chemical analyses have identified natural estrogens (E2 and estrone) and the synthetic estrogen, 17 α -ethynylestradiol (EE2) as the most likely feminizing agents in WWTP (Desbrow et al., 1998). In the United States, EE2 is present in the environment

at levels up to 273 ng/L, with median measured concentrations between 1 and 10 ng/L (Kolpin et al., 2002). Despite low environmental concentrations, EE2 is stable in the environment (Larsson et al., 1999) and is readily taken up and equilibrated in the blood of exposed fish, bioconcentrating 480- to 720-fold in fish plasma (Skillman et al., 2006). EE2 is also a potent ER agonist with equal or higher ER binding affinity compared to E2 in mammals and fish (Blair et al., 2000; Denny et al., 2005). As such, EE2 is an environmentally relevant xenoestrogen with the ability to activate estrogen receptor signaling pathways. Indeed, EE2 has been shown to increase vitellogenin (Vtg) expression thousands-fold *in vivo* (Brown et al., 2007; Jobling et al., 2003, 1996; Lange et al., 2012, 2008; Pawlowski et al., 2004; Schultz et al., 2001; Skillman et al., 2006; Thomas-Jones et al., 2003; Thorpe et al., 2003) and *in vitro* (Hultman et al., 2015; Kordes et al., 2002; Rankouhi, 2004; Scholz et al., 2004), making Vtg a popular biomarker of estrogen exposure. Fish exposed to EE2 or other estrogenic chemicals also exhibit decreased testis size and inhibited spermatogenesis in males (Jobling et al., 1996) and delayed oogenesis and increased follicular atresia in females (Kidd et al., 2007). Chronic exposure of fish to environmentally relevant concentrations of EE2 (5 ng/L) causes reproductive failure in zebrafish (Nash et al., 2004) and complete feminization and subsequent population collapse of fathead minnows in an experimental lake (Kidd et al., 2007).

Although paper mill and wastewater effluents are thought to be androgenic and estrogenic, respectively, exposed fish exhibit many similar phenotypes including reduced gonad size, delayed maturation, and decreased steroidogenesis (Folmar et al., 2001; Hewitt et al., 2008; Vajda et al., 2008; Woodling et al., 2006). Based on the role of Gths in gonad growth, development and steroidogenesis, and the role of sex steroids in regulating Gths, it is plausible that some of the effects of paper mill and wastewater effluent exposures are due in part to

changes in Gth regulation. Studies examining changes in Gth expression in response to xenoestrogens in fish have found increased *lhb* and decreased *fshb* mRNA (Johns et al., 2009; Maeng et al., 2005; Rhee et al., 2010; Yadetie and Male, 2002). Fathead minnow embryos exposed to 2, 10, or 50 ng EE2/L from fertilization until swim up (~7 days) had significantly elevated *lhb* mRNA levels (Johns et al., 2009). Alterations in plasma Gth levels have also been observed in response to EDC exposure (Brown et al., 2007; Golshan et al., 2014; Harris et al., 2001). Female rainbow trout exposed to 0.7, 8.3, or 85.6 µg of the estrogenic chemical 4-nonylphenol (NP)/L for 18 weeks during early secondary oocyte growth showed reduced *fshb* mRNA levels, pituitary Fsh content and plasma Fsh levels and increased plasma Lh and vitellogenin (Harris et al., 2001). In this study, *fshb* mRNA and plasma Fsh levels were the most sensitive endpoints assessed. Additionally, a few studies have shown that EDCs are able to alter Gth expression *in vitro*. Dispersed pituitary cells from female Prussian carp treated with 10 to 100 ng/mL of a polychlorinated biphenyl had significantly higher spontaneous Lh secretion compared to untreated cells (Socha et al., 2013). Similarly, in pituitary cells from mature medaka, mRNA levels for *lhb* and *fshb* were altered by all EDC treatments tested (Tse et al., 2013). These findings suggest that Gths may be a sensitive target of EDC action and may be involved in inhibited gonad growth and disrupted reproduction observed in exposed fish (Filby et al., 2006; Harris et al., 2001).

Experimental organism: *Oncorhynchus kisutch*

Coho salmon are an ideal experimental organism for studying gonadotropin regulation and endocrine disruption during early oogenesis. First, the reproductive physiology and endocrinology of coho salmon is well characterized. Second, as synchronous, semelparous

spawners, coho salmon have a single clutch of eggs that develop simultaneously. This reproductive strategy results in simple hormone profiles throughout oogenesis that facilitate interpretation of changes in GTH expression at a given stage. Third, and importantly, coho salmon are one of the few teleost species for which an Fsh radioimmunoassay is available (Davies et al., 1999; Prat et al., 1996; Swanson et al., 1989). Finally, coho salmon were selected for this study as ecologically relevant species in the Pacific Northwest that could potentially be impacted by EDCs in Puget Sound.

Objectives

In female salmonids, there is evidence that E2 negatively and positively regulates Fsh and Lh, respectively, during vitellogenesis. Although data in female fish are lacking, there is also evidence that non-aromatizable androgens can regulate GTH synthesis and secretion in males. However, effects of sex steroids on GTH regulation during early oogenesis, a critical stage in puberty, are still unclear. Further, the ability of EDCs to disrupt sex steroid regulation of GTHs is poorly understood. Therefore, for my dissertation, I conducted a series of studies to examine pituitary GTH regulation by sex steroids and effects of endocrine disrupting chemicals on this process during early gametogenesis in female salmonids. The overall goal of this work is to determine: 1) whether estrogens and/or androgens regulate pituitary GTH synthesis during primary or early secondary oocyte growth in salmonids; and 2) whether endocrine disrupting chemicals with putative sex steroid agonistic activity mimic endogenous sex steroids in regulating pituitary GTHs in previtellogenic coho salmon. Early oogenesis will be targeted due to: 1) the importance of early oogenesis in the onset of puberty and subsequent fertility; 2) the current gap in data on sex steroid regulation of GTHs at these stages; and 3) the increased

likelihood for salmon to be residing in or migrating through urban waterways and subsequently exposed to EDCs during these stages. Additionally, fish in early oogenesis have low endogenous steroid levels which facilitates examination of the effects of elevating steroid levels using sustained release implants.

As steps toward achieving the overall objectives of this work, experiments will be conducted to address the following specific objectives:

1. Investigate whether estrogens and/or androgens regulate pituitary Gths during primary or early secondary oocyte growth in coho salmon *in vivo*. Specifically, does E2 and/or a non-aromatizable androgen, 11-KT, positively or negatively regulate steady state levels of *fshb* and *lhb*, and pituitary Fsh and Lh during early secondary oocyte growth?
2. Use transcriptome and pathway analyses to characterize the broad effects of a xenoestrogen, EE2, on Gth regulation. Specifically, how does EE2 affect steady state mRNA levels and what signaling pathways are involved in Gth regulation in previtellogenic coho salmon exposed to a xenoestrogen *in vivo*?
3. Determine whether WWTP effluent from Puget Sound has the potential to disrupt pituitary Gth mRNA levels in juvenile coho salmon.

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CHAPTER 1: *In vivo* effects of estradiol-17 β and 11-ketotestosterone on pituitary gonadotropins in female coho salmon (*Onchorhynchus kisutch*) during the transition from primary to early secondary oocyte growth.

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Abstract:

In salmonids, the gonadotropins (Gths) are differentially expressed throughout development, with plasma levels of follicle-stimulating hormone (Fsh) being prominent during early to mid gametogenesis and plasma luteinizing hormone (Lh) remaining low until final gamete maturation. Considerable research has been done to investigate the differential regulation of Gths by hypothalamic and gonadal factors including sex steroids, but many questions remain: 1) the factors stimulating an initial increase in Fsh synthesis and release at the onset of puberty are not well defined; 2) the role of androgens in regulating Gths in females is largely unexplored; and 3) the effects of gonadal steroids on Gth synthesis and release are unclear due to species- and stage- specific differences. To evaluate the role of gonadal steroids on Gth expression in previtellogenic coho salmon, we implanted fish with estradiol-17 β (E2)-containing, 11-ketotestosterone (11-KT)-containing, or control cholesterol pellets and examined pituitary mRNA levels and Gth content 1, 3, 7, and 21 days after implantation. Plasma steroid levels in steroid-treated fish were approximately 10 ng/mL over the experiment. Our results suggest that Fsh and Lh are differentially regulated by E2, but not 11-KT, during late primary and early secondary oocyte growth in coho salmon. E2 rapidly increased *lhb* mRNAs and Lh protein, as well as *gnrhr1*. On the other hand, divergent effects of E2 were observed on Fsh regulation: E2 treatment decreased *fshb* mRNA but increased Fsh protein levels. Unfortunately, the sensitivity of the Fsh and Lh RIAs were insufficient to quantify plasma Fsh and Lh at this stage, so it is unknown if gonadal steroids alter Gth secretion.

Introduction:

The pituitary gonadotropins (Gths) are heterodimeric glycoprotein hormones composed of a common alpha subunit and distinct beta subunits that confer hormone-specific activity. In fish, as in other vertebrates, the pituitary gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), are involved in all aspects of ovarian development and function including steroid hormone production, oogenesis, final oocyte maturation and ovulation (Levavi-Sivan et al., 2010; Lubzens et al., 2010; Swanson et al., 2003). A complex network of endocrine signals including hypothalamic factors (e.g., gonadotropin-releasing hormone, kisspeptin, dopamine) autocrine and paracrine factors within the pituitary (e.g., activin) and gonadally derived sex steroids and peptides regulate Gth synthesis and secretion (Yaron et al., 2003; Levavi-Sivan, 2010). Gonadal steroid feedback alone can act to positively and negatively regulate Fsh and Lh synthesis and secretion, depending on the species, season, and stage of development.

In salmonids, Fsh is the predominant Gth throughout early oogenesis with important roles in the onset of secondary oocyte growth (Zhang et al., 2015a, 2015b), estradiol-17 β (E2) production (Lubzens et al., 2010; Suzuki et al., 1988; Swanson et al., 1991), and vitellogenesis (Lubzens et al., 2010; Tyler et al., 1991). In female coho salmon, plasma and pituitary Fsh significantly increase during the transition from primary to secondary oocyte growth concurrent with an increase in plasma E2, suggesting that increasing steroid levels may positively regulate Fsh expression at this stage (Campbell et al., 2006; Swanson et al., 1989). Plasma Fsh levels continue to increase throughout vitellogenesis and decline prior to ovulation when E2 levels peak (Swanson et al., 1989). Most studies suggest that E2 exerts negative feedback on Fsh during

vitellogenesis. In female salmonids, ovariectomy increased plasma Fsh (Saligaut et al., 1998) while treatment with E2 or aromatizable androgens decreases pituitary *fshb* mRNA, Fsh content and plasma Fsh levels (Breton et al., 1997; Dickey and Swanson, 1998; Harding et al., 2013; Saligaut et al., 1998).

In contrast to Fsh, plasma Lh levels (Breton et al., 1998; Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991) and Lh receptor (*lhgr*) mRNA levels (Guzmán et al., 2014; Kwok et al., 2005; Oba et al., 2001) remain low or undetectable in salmonids until late vitellogenesis, peaking at final oocyte maturation and ovulation. Consistent with its expression during late oogenesis, Lh has been shown to regulate production of maturation-inducing steroid (17,20 β -dihydroxy-4-pregnen-3-one or 17,20 β ,21-trihydroxy-4-pregnen-3-one) (Planas et al., 2000; Suzuki et al., 1988; Swanson et al., 1991; Yaron et al., 2003), oocyte maturation (Kagawa et al., 1998) and ovulation (Crespo et al., 2013). Despite low plasma Lh levels, E2 or testosterone (T) treatment increases *lhb* steady state mRNA levels and pituitary Lh content during secondary oocyte growth in salmonids (Borg et al., 1998; Breton et al., 1997; Crim et al., 1981; Dickey and Swanson, 1998; Melo et al., 2015) and other fishes (Jeng et al., 2007; Schmitz et al., 2005; Yaron et al., 2003).

These data suggest that E2 or aromatizable androgens negatively and positively regulate Fsh and Lh, respectively, during vitellogenesis and final oocyte maturation. However, ontogenetic studies of Fsh beta subunit (*fshb*) and *lhb* expression have revealed that *fshb* and *lhb* mRNAs are expressed prior to secondary oocyte growth (Chen and Ge, 2012; Golan et al., 2014; Shimizu et al., 2008; Yan et al., 2012). Interestingly, the spatial distribution of *fshb* mRNA changed significantly at the onset of secondary oocyte growth and *lhb* mRNA expression increased dramatically at this stage. These findings suggest that Gth expression is regulated

during primary oocyte growth and at the onset of puberty in zebrafish (Chen and Ge, 2012), but the factors regulating Gths at these stages remain unclear. In addition, though the non-aromatizable androgen, 11-ketotestosterone (11-KT), is typically considered a male-specific hormone, it is present in the plasma of a number of species during early oogenesis (Endo et al., 2011; Forsgren, 2010; Kortner et al., 2009; Lokman et al., 1998) and stimulates primary oocyte growth in various fishes (Forsgren and Young, 2012; Kortner et al., 2009). However, very little is known about whether 11-KT regulates Gth expression in female fish.

The aim of this study was to evaluate the effects of E2 and 11-KT on pituitary Gths in previtellogenic coho salmon (*Oncorhynchus kisutch*). Coho salmon were selected for this study because of their simple life history, synchronous ovarian development, and the availability of assays to measure Gth mRNA and protein levels (Campbell et al., 2006; Harding et al., 2013; Swanson et al., 1989). In addition, their reproductive cycle is well-described, including characterization of E2 regulation of pituitary gonadotropin synthesis and secretion during secondary oocyte growth (Dickey and Swanson, 1998). We examined Gth regulation during the transition from primary to secondary oocyte growth due to the scarcity of information on Gth regulation at this stage and the importance of this transition to pubertal development. Additionally, fish at this stage have low endogenous circulating sex steroid levels permitting investigation of the role of sex steroids on Gth regulation by increasing circulating sex steroid levels. To evaluate the role of gonadal steroids on Gth expression in previtellogenic coho salmon, we implanted fish with estradiol-17 β (E2)-containing, 11-ketotestosterone (11-KT)-containing, or control cholesterol pellets and examined pituitary mRNA levels and Gth content 1, 3, 7, and 21 days after implantation.

Materials and Methods:

Fish maintenance and genetic sexing: In early December 2011, coho salmon eyed-embryos were obtained from the Issaquah Hatchery (Issaquah, WA) and incubated in Heath trays at 8 °C at the Northwest Fisheries Science Center hatchery facilities (Seattle, WA). After ponding, fish were reared in recirculated 10–10.5 °C fresh water under a simulated natural photoperiod. Fish were fed a standard ration of BioDiet commercial feed (Bio-Oregon, Longview, WA) according to Bio-Oregon's guidelines. Fish were maintained and experiments conducted according to protocol (#4078-01) approved by the Institutional Animal Care and Use Committee of the University of Washington (Seattle, WA).

Prior to the experiment, fish were anesthetized in buffered tricaine methanesulfonate (0.05% MS-222; Argent Laboratories, Redmond, WA) and tagged with passive-integrated transponder (PIT) tags. Anal fin tissue was collected and analyzed for genetic sex using a molecular marker for the Y chromosome (Du et al., 1993; Forbes et al., 1994). Females identified through genotyping were isolated in a separate tank until use.

Chemicals: 11-Ketotestosterone was purchased from Steraloids Inc. (Newport, RI). Cholesterol and E2 were purchased from Sigma-Aldrich (St. Louis, MO). L-15, Hematoxylin, eosin, and diethyl ether were purchased from Thermo Fisher Scientific (Waltham, MA). Bouin's fixative was purchased from Ricca Chemicals (Arlington, TX).

Experimental design: Two consecutive experiments were conducted to investigate the effects of E2 and 11-KT on pituitary gonadotropin expression in juvenile female salmon during late primary oocyte growth (experiment 1) and early secondary oocyte growth (experiment 2). For

experiment 1, fish that had oocytes transitioning from the late perinucleolus stage (LPN) to the very early cortical alveolus stage (ECA) were used, while fish with oocytes transitioning from ECA to mid-CA stage were used for experiment 2. In September (experiment 1) and November (experiment 2), 19- and 23-month old female coho salmon were anesthetized as described above and implanted with cholesterol pellets containing E2, 11-KT, or blank (controls) as described previously (Monson et al., in prep). Briefly, cylindrical pellets of 30 mg cholesterol with 100 µg E2, 5 µg 11-KT, or no steroid (control) were manufactured using a custom-made pellet press. Prior to implantation, 11-KT pellets were incubated in L-15 media (Thermo Fisher Scientific, Waltham, MA) for 24 hours to temper initial release of 11-KT from the pellets. Control, E2-, or 11-KT-containing pellets were implanted into the peritoneal cavity of anaesthetized female fish, and fish were transferred into treatment tanks. Prior to implantation (time 0), 6 fish were sampled for initial analyses. One, 3, 7, or 21 days post-implantation, 6 fish per treatment were anesthetized in buffered 0.1% MS-222 until movement of the gill operculum ceased. Fork length and body weight were measured, blood was collected in heparinized syringes from the caudal vein, and fish were euthanized by decapitation. Pituitaries and ovaries were removed and ovaries were weighed. Gonadosomatic index (GSI) was calculated by paired ovary weight/body weight $\times 100$. One ovary was snap frozen in liquid nitrogen and stored at -80°C for gene expression analyses (described in Monson et al., in prep) and the other ovary was fixed in Bouin's fixative for histological analysis. Blood plasma was obtained after centrifugation at $1200 \times g$ for 15 minutes and stored at -20°C or less until analysis. Pituitary glands were snap frozen in liquid nitrogen for protein and RNA analyses.

Histological analysis: Fixed tissues were washed with 70% ethanol then dehydrated in increasing concentrations of ethanol and xylene washes, and embedded in paraffin wax. Sections of 5- μ m thickness were cut, mounted on microscope slides and stained with hemotoxylin and eosin. Oocytes were scored for stage using the criteria of Bromage and Cumaranatunga (1998), and Campbell et al. (2006).

Hormone assays: Circulating sex steroids were double extracted from 0.25 ml of plasma using diethyl ether (1.5 ml), dried down under a nitrogen stream, and then re-suspended in an appropriate buffer for subsequent steroid assays. 11-KT levels were measured by enzyme-linked immunoassay (Cuisset et al., 1994) with reagents from Cayman Chemicals, (Ann Arbor, MI) and antisera kindly donated by Dr. Rudiger Schulz (University of Utrecht). Estradiol-17 β was measured by radioimmunoassay (Fitzpatrick et al., 1986; Sower and Schreck, 1982). Pituitary Lh and Fsh protein content were measured by radioimmunoassay as described previously except phosphate buffered saline, pH 7.5 was used instead of a barbital buffer, pH 8.6 (Swanson et al., 1989). Pituitaries of some LPN and ECA stage fish (~26% and 4% of fish in experiment 1 and experiment 2, respectively) had Lh levels below the limit of detection (LOD) defined as 2 standard deviations above zero. These were assigned the minimum LOD (0.022 to 0.043 ng/mL) rather than zero for the purpose of statistical analyses of data. The interassay coefficients of variation for Fsh and Lh, were 5.07% and 3.21% respectively.

Pituitary protein and RNA isolation: Protein and total RNA were isolated from pituitary glands using the PARIS™ Kit (Life Technologies, Carlsbad, CA). Briefly, frozen pituitary glands were homogenized for 1 minute in 300 μ L ice cold 1X PBS using a TissueLyzer II homogenizer

(QIAGEN, Valencia, CA). The pituitary gland homogenate was centrifuged at 10,000 rpm for 10 minutes at 4 °C and the supernatant was retained. 50 µL of the pituitary homogenate supernatant was stored at -20 °C for protein analysis. The remaining supernatant was combined with an equal volume (250 µL) of 2X Lysis/Binding Solution and RNA was isolated according to the PARIS™ Kit Protocol. Isolated RNA was stored at -80 °C until gene expression analysis was conducted.

Pituitary quantitative real time RT-PCR (qPCR) analyses: Pituitary RNA samples were DNase treated using Ambion TURBO DNA-free (Life Technologies) as previously described (Harding et al., in review). Briefly, a 23-µL reaction composed of 20 µL (1000 ng) total RNA, 2 µL DNase I Buffer and 1 µL TURBO DNase was incubated at 37 °C for 1 hour. DNase treatment was terminated by adding 2 µL of inactivation reagent followed by vortex mixing at room temperature. RNA yield was quantified with a NanoDrop ND-1000 spectrometer (Thermo Fisher Scientific, Waltham, MA). DNA-free samples (125 ng RNA) were reverse transcribed with SuperScriptII (Life Technologies) in 10 µL reactions as described in Kitano et al. (2010). Quantitative real-time RT-PCR (qPCR) assays were designed and performed as previously described (Harding et al., 2013). Briefly, qPCR assays were run using an ABI 7900HT Fast Real-Time PCR System (Life Technologies) in 384-well plates using standard cycling conditions. Reactions were 12.5 µL each and consisted of 1X Power SYBR Green PCR Master Mix (Life Technologies), 150 nM of the forward and reverse primer, and 0.5 ng cDNA template. Four concentrations of standard curve samples generated from a serial dilution of cDNA (from pooled RNA) ranging from 0.05 to 5 ng cDNA were included in each plate in triplicate. All samples were run on a single 384-well qPCR plate to eliminate plate-to-plate variation between qPCR runs. Data were expressed relative to the housekeeping gene, ubiquitin (*ubiq*), which was stably

expressed across treatments (Supplemental Figure 2.S1). Quantitative PCR primer sequences are provided in Supplemental Table 2.S1.

Statistical analyses: Statistical analyses were performed using Prism 6 for Mac OSX (GraphPad Software, La Jolla, CA). Outliers were identified by Grubb's outlier test and were removed from analyses. Data were log-transformed where necessary to meet parametric test criteria. Data were analyzed by 2-way ANOVA followed by Dunnett's multiple comparison test with a significance threshold of $p < 0.05$ to determine significant differences relative to control at a given time point. Data are reported as mean \pm SEM.

Results:

Experiment 1: Late primary oocyte growth

Morphometrics, histological ovarian stage, and plasma sex steroid levels:

In experiment 1, fish fork length (19.2 ± 0.1 mm), body weight (85.0 ± 1.5 g), and GSI (0.279 ± 0.004 %) were not significantly affected by steroid treatment, but did change over time. Fork length (2-way ANOVA, $p_{interaction} = 0.5921$, $p_{time} = 0.0007$, $p_{hormone} = 0.9538$) and body weight (2-way ANOVA, $p_{interaction} = 0.8014$, $p_{time} < 0.0001$, $p_{hormone} = 0.7665$) increased over time while GSI decreased over time (2-way ANOVA, $p_{interaction} = 0.4154$, $p_{time} < 0.0115$, $p_{hormone} = 0.5392$). Hepatosomatic index averaged 1.345 ± 0.029 overall, but at day 21, HSI was significantly higher in E2-implanted fish (1.951 ± 0.082) relative to controls (1.282 ± 0.140). Ovarian stage ranged from LPN to ECA stage, and the percent of fish at each stage did not differ among treatments. However, the percentage of fish at ECA-stage increased from 22% at day 1 to 94% at day 21.

Plasma sex steroid levels:

To confirm that implants elevated levels of steroids, 11-KT and E2 were measured in the plasma of control and treated fish. In experiment 1, plasma E2 levels averaged 0.1 ± 0.006 ng/ml at time zero and did not change significantly over time in control fish (Figure 2.1A). Treatment with E2 raised plasma E2 levels to 22.7 ± 2.4 , 12.2 ± 1.7 , 8.5 ± 1.8 and 3.2 ± 0.4 ng/mL at 1, 3, 7 and 21 days after implantation, respectively, resulting in a time-weighted average (TWA) exposure of 6.0 ng E2/mL over the experiment. Additionally, 11-KT treatment caused a transient, significant 2-fold increase in plasma E2 at 1 and 3 days. Plasma 11-KT levels averaged 0.08 ± 0.02 ng/mL at time zero and decreased significantly over time, though the magnitude of the decrease was very small. (Figure 2.1A). Treatment with 11-KT raised plasma 11-KT levels to 17.8 ± 5.5 , 5.6 ± 0.9 , 3.4 ± 1.0 , and 1.0 ± 0.2 ng/mL at 1, 3, 7 and 21 days after implantation, respectively, resulting in a TWA exposure of 2.7 ng 11-KT/mL. Although plasma 11-KT levels in 11-KT treated fish decreased over the course of the experiment, levels remained significantly elevated above control levels at day 21. In addition, by 21 days plasma 11-KT levels were significantly higher in E2-implanted fish (0.3 ± 0.01) compared to control fish (0.03 ± 0.01).

Pituitary mRNA levels:

Pituitary mRNA levels for gonadotropin subunits (*cga2*, *fshb*, and *lhb*), *ara* and *gnrhr1* were significantly altered in fish implanted with E2-containing pellets. *Cga2* mRNA levels were increased 1.4- and 1.6-fold at 7 and 21 days following E2-implantation (Figure 2.2A). Transcripts for *lhb* were dramatically and rapidly upregulated following E2 treatment, which resulted in 70-, 45-, 145-, and 448-fold induction at 1, 3, 7, and 21 days of treatment (Figure

2.2A). Treatment with E2 also increased *ara* mRNA levels 1.6-fold at 21 days (Figure 2.3A) and *gnrhr1* mRNA levels 2.9-, 2.2-, and 2.7-fold at 1, 3, and 21 days, respectively (Figure 2.4A). In contrast, E2 treatment decreased *fshb* mRNA levels 2.5-fold lower than controls at 7 days post implantation (Figure 2.2A). Treatment with 11-KT had variable effects on *lhb* gene expression; levels increased 2.6-fold at day 1 and decreased 4-fold at day 7 (Figure 2.2A). 11-KT treatment in coho salmon during late primary oocyte growth did not affect mRNA levels for any other genes examined in this study. Treatment with 11-KT did not alter levels of transcripts for nuclear forms of estrogen and androgen receptors (Figure 2.3A).

Pituitary gonadotropin protein levels:

In late primary oocyte growth, pituitary Fsh content averaged 396.9 ± 41.7 ng/pituitary in control fish over the experimental period. Pituitary Fsh content was significantly increased 2.8-fold at 21 days in E2-implanted fish (Figure 2.5A). Treatment with 11-KT had no effect on pituitary Fsh content. Pituitary Lh content in control fish was approximately 200 times lower than Fsh and averaged 2.4 ± 0.5 ng/pituitary over the 3-week sampling period. Treatment with E2 increased pituitary Lh levels in a time-dependent fashion (Figure 2.5A). Pituitary Lh content was 1.8-, 15.5-, 47.6-, and 358.7-fold higher in E2-treated compared to control fish at 1, 3, 7, and 21 days, respectively. Treatment with 11-KT caused a transient 2.2-fold decrease in pituitary Lh content at day 7.

Experiment 2: Early secondary oocyte growth:

Morphometrics and histological ovarian stage

In experiment 2, fish fork length (21.7 ± 0.01 mm), body weight (118.8 ± 0.15 g), GSI (0.325 ± 0.001 %), and HSI (1.119 ± 0.002 %) were not significantly different between steroid treatments. Body weight increased over time (2-way ANOVA, $p_{interaction} = 0.8045$, $p_{time} < 0.0001$, $p_{hormone} = 0.7979$), but no other factors were significantly different across time. Ovarian stage ranged from ECA to mid-CA and the proportion of fish at each stage was not different among treatments.

Plasma sex steroid levels:

In experiment 2, plasma E2 levels averaged 0.2 ± 0.02 ng/mL at time 0, double those in control fish from experiment 1, and levels did not change significantly over time in control fish. Treatment with E2-containing implants significantly raised plasma E2 levels above those in controls to 29.0 ± 3.1 , 16.8 ± 1.9 , 10.5 ± 2.3 and 4.4 ± 0.8 ng/mL at 1, 3, 7 and 21 days after implantation, resulting in a TWA exposure level of 7.9 ng E2/mL (Figure 2.1B). In contrast, plasma E2 levels in 11-KT treated fish were similar to controls at all time points except 21 days when levels were slightly lower than both control and E2-treated fish. Plasma 11-KT levels in control fish averaged 0.105 ± 0.059 ng/mL over the course of the study but showed a slight decreasing trend. Treatment of fish with 11-KT elevated plasma 11-KT levels to 53.1 ± 6.8 , 22.7 ± 3.6 , 6.5 ± 0.6 , and 0.7 ± 0.1 ng/mL at 1, 3, 7 and 21 days after implantation, respectively, resulting in a TWA exposure level of 6.4 ng 11-KT/mL (Figure 2.1B). Plasma 11-KT levels in 11-KT treated fish decreased 9-fold over the course of the experiment, but remained significantly elevated above control levels at day 21.

Pituitary mRNA levels

In experiment 2, mRNA levels for gonadotropin beta subunits, *lhb* and *fshb*, were significantly altered in response to E2 treatment, while transcript levels for both alpha subunits (*cga1* and *cga2*) were unchanged (Figure 2.2B). *Lhb* mRNA levels were dramatically and rapidly upregulated following E2 treatment, with E2-implants resulting in 48-, 263-, 403-, and 309-fold induction at 1, 3, 7, and 21 days of treatment. In contrast, *fshb* mRNA levels were 2.3- and 3.3-fold lower in E2-implanted fish 7 and 21 days after E2-implantation. In addition, treatment with E2 decreased *esr2b* mRNA levels 1.6- and 1.5-fold at 1 and 7 days, respectively, (Figure 2.3B) and increased *gnrhr1* mRNA levels 2.5-, 3.6-, 2.7-, and 2.1-fold at 1, 3, 7, and 21 days, respectively (Figure 2.4B).

In experiment 2, 11-KT treatment had variable effects on *lhb* gene expression but no effect on *fshb*, *cga1*, *cga2*, and *gnrhr1* transcripts (Figure 2.2B). *Lhb* mRNA levels were increased 4.8-fold at day 3 and suppressed 2.8-fold at day 21 after 11-KT implantation (Figure 2.2B). In addition, 11-KT treatment transiently suppressed steroid receptor mRNA levels. Transcript levels of *esr2a*, *esr2b* and *arb*, were 1.6-, 1.5-, and 1.7-fold lower, respectively, in 11-KT treated fish compared to control fish at day 7 (Figure 2.3B).

Pituitary gonadotropin protein levels:

In ECA- to MCA-stage coho salmon, pituitary Fsh content averaged 922.4 ± 43.5 ng/pituitary in control fish over the duration of the experiment, nearly twice that of fish in early primary growth (experiment 1). Treatment with E2 increased pituitary Fsh content 1.9- and 2.0-fold at 7 and 21 days, respectively (Figure 2.5B) while 11-KT treatment had no effect. Over the course of the experiment, pituitary Lh content in control fish averaged 1.6 ± 0.2 ng/pituitary and was similar to that of control fish in late primary oocyte growth (experiment 1). Treatment with

E2 increased pituitary Lh content relative to control fish in a time-dependent fashion with levels increasing 2.3-, 13.5, 72.4, and 214-fold at 1, 3, 7, and 21 days, respectively (Figure 2.5B). As with Fsh, 11-KT treatment had no effect on pituitary Lh levels.

Discussion:

The aim of this study was to investigate the role of sex steroids in regulating pituitary Gths during early oogenesis in coho salmon. Pituitary Gths, particularly Fsh, have been shown to be important during the transition from primary to secondary oocyte growth. However, the factors regulating Fsh and Lh at this stage remain unclear. In this study, endogenous plasma E2 and 11-KT levels were increased in gonad-intact juvenile coho salmon for up to three weeks using cholesterol implants, and changes in pituitary Lh and Fsh protein, and mRNAs for GtH subunits, steroid receptors and *gnrhr1* were determined. Our results suggest that during late primary and early secondary oocyte growth, Fsh and Lh are differentially regulated by E2, but not by 11-KT. E2 rapidly increased *lhb* mRNAs and Lh protein, as well as *gnrhr1*. On the other hand, divergent effects of E2 were observed on Fsh regulation: E2 treatment decreased *fshb* mRNA but increased Fsh protein levels. In addition, we found evidence that the responsiveness of pituitary cells to gonadal steroid feedback may change during the transition from primary to secondary oocyte growth.

Plasma steroid levels

In this study, plasma E2 levels were low (0.08 ng/ml) in control fish, but increased approximately two-fold from September (experiment 1) to November (experiment 2) when fish transitioned from the LPN- to MCA-stage, similar to results from previous studies (Campbell et

al., 2006; Forsgren, 2010). Although plasma 11-KT levels in control fish were slightly lower than those for E2, levels also increased during the transition from late primary to early secondary growth. Traditionally, 11-KT has been considered a male specific hormone in salmonids (Lokman et al., 2002). However, several studies have shown detectable 11-KT by immunoassay in female juvenile coho salmon (Forsgren, 2010) and adult salmonids (Slater et al., 1994; Lokman et al., 2002). The levels of 11-KT in juvenile female coho salmon cannot be explained by cross-reactivity with testosterone in the 11-KT assay (3.7%; see Guzmán et al., 2015) because testosterone levels at this stage are less than 0.1 ng/mL. The results from this study are consistent with previous plasma 11-KT levels measured in LPN-stage and ECA-stage coho salmon (Forsgren, 2010) and in immature female Atlantic cod (Kortner et al., 2009). Interestingly, these results suggest that plasma 11-KT levels are nearly as high as plasma E2 levels in immature coho salmon.

Treatment with E2-containing implants increased plasma E2 levels approximately 100-fold above controls. Plasma E2 levels in the 5 to 25 ng/mL range, as occurred in the E2-treated fish in this experiment, are similar to plasma E2 levels observed in female Chinook salmon during vitellogenesis (Slater et al., 1994). In 11-KT-implanted fish, plasma 11-KT levels were elevated approximately 100-fold. The TWA plasma 11-KT levels achieved in this study are similar to plasma 11-KT levels observed in maturing female Chinook salmon (Slater et al., 1994) and in New Zealand freshwater eels (Lokman et al., 1998), but were on average, an order of magnitude higher than plasma 11-KT levels reported by Lokman et al. (2002) in various salmonids. Overall, we believe the E2 and 11-KT treatments that were achieved in this study reflect high physiologically relevant levels, albeit levels that would occur much later in oogenesis.

Regulation of Lh by gonadal steroids

E2 rapidly increased both *lhb* transcript and Lh protein in the pituitary of females in both late primary and early secondary oocyte growth stage. These results are consistent with previous studies showing that treatment with E2 or aromatizable androgens (testosterone) increase *lhb* steady state mRNA levels and pituitary Lh content in immature and maturing salmonids of both sexes (Borg et al., 1998; Breton et al., 1997; Crim et al., 1981; Dickey and Swanson, 1998; Forsgren, 2010; Melo et al., 2015) and other fishes (Jeng et al., 2007; Schmitz et al., 2005; Yaron et al., 2003). Positive feedback of E2 on Lh synthesis likely occurs primarily through increased transcription of *lhb*. In support of this, estrogen response elements (EREs) have been identified in the *lhb* promoter of Chinook salmon (Le Dréan et al., 1996; Liu et al., 1995; Wang et al., 2009; Xiong et al., 1994) and goldfish (Sohn et al., 1999). In addition, mRNAs for estrogen receptor 1 (*esr1*) and 2a (*esr2a*; formerly ER γ or ER β 2) colocalize with *lhb* mRNA in pituitary gonadotrophs of sea bass (Muriach et al., 2008a, 2008b) and ricefield eels (Zhang et al., 2014). Unfortunately, plasma Lh was below the detection level of the assay in this experiment. However, despite increases in pituitary *lhb* mRNA levels and Lh content in this study, increased plasma Lh levels were not predicted. In previous studies, increases in plasma Lh have not been observed in response to E2 treatment alone, suggesting that other factors (GnRH release and/or dopamine inhibition) are required for Lh release *in vivo* (Breton et al., 1997; Dickey and Swanson, 1998; Saligaut et al., 1998).

11-KT-treatment had weak, transient and variable effects on Lh expression. In both stages, 11-KT treatment caused an initial increase followed by a decrease in *lhb* steady state mRNA levels. In contrast, 11-KT caused a transient decrease in Lh mRNA levels at day 7

compared to controls at day 7, but these were not significantly different from initial (time 0). Therefore, any changes in *lhb* transcripts did not appear to alter the pool of immunoreactive Lh protein, which would include free Lhb as well as the Lh heterodimer. Previous studies indicate that non-aromatizable androgens have little or no effect on *lhb* mRNA levels, pituitary Lh content or plasma Lh levels in immature fish (Antonopoulou et al., 1999; Borg et al., 1998; Cavaco et al., 2001; Crim et al., 1981; Kobayashi et al., 2000; Melo et al., 2015; Setiawan et al., 2012). However, data on the role of 11-KT in regulating Lh in immature female salmonids is very limited. Results from the current study suggest that the role of 11-KT in regulating Lh is relatively insignificant as compared to E2 in female coho salmon during late primary oocyte growth or early secondary oocyte growth.

Regulation of Fsh by gonadal steroids

E2 treatment significantly decreased steady state mRNA levels of *fshb* at day 7 in LPN-stage fish and at day 7 and 21 in ECA-stage fish. These results suggest that E2 exerts negative feedback on *fshb* mRNA levels by decreasing *fshb* transcription or increasing *fshb* turnover in coho salmon during late primary and early secondary oocyte growth. This finding is consistent with suppressive effects of E2 on *fshb* expression in mammals (Bernard et al., 2010) and in previtellogenic female Japanese eel and goldfish (Jeng et al., 2007; Sohn et al., 1998). In contrast, E2 treatment significantly increased pituitary Fsh content at day 21 in fish at late primary oocyte growth and at days 7 and 21 in fish during the transition to early secondary oocyte growth. This is similar to previous results by Forsgren (2010) who showed that pituitary Fsh increased in LPN- and ECA-stage coho salmon implanted with E2-containing pellets for up to 20 days (Forsgren, 2010). However, this conflicts with most published studies showing a negative

feedback effect of E2 on pituitary Fsh levels in fish at mid to late stages of gametogenesis. For example, gonadectomy increases plasma Fsh in male and female salmonids (Antonopoulou et al., 1999; Kobayashi et al., 2000; Larsen and Swanson, 1997; Saligaut et al., 1998) while treatment with E2 or aromatizable androgens decreases pituitary Fsh content and plasma Fsh levels (Breton et al., 1997; Dickey and Swanson, 1998; Kobayashi et al., 2000; Saligaut et al., 1998).

Previous studies in trout showed that, *fshb* mRNA levels, pituitary Fsh content and plasma Fsh levels are highly correlated (Gomez et al., 1999), suggesting that at least of a portion of the pool of pituitary Fsh is constitutively secreted, as in mammals (Bernard et al, 2010; Farnworth, 1995), with regulation of Fsh occurring primarily at the transcriptional level (Bernard et al., 2010; Farnworth, 1995; Saligaut et al., 1998; Swanson et al., 2003). However, in this study using fish at very early stages of oogenesis, *fshb* mRNA levels and pituitary Fsh content were not well correlated in E2-treated fish. This finding suggests that E2 may either exert differential effects on transcription versus translation and/or suppress Fsh secretion, resulting in an accumulation of stored protein. Unfortunately, the sensitivity of the Fsh RIA was insufficient to detect plasma Fsh at this stage so it is not known if E2 suppressed secretion of Fsh. Additionally, E2 had a delayed effect on Fsh and *fshb* mRNA levels with significant differences only after 7 to 21 days, while E2 had immediate effects on Lh and *lhb* mRNA levels. These results suggest that E2 regulates Fsh and Lh by different mechanisms. These include potential indirect effects on Fsh and *fshb* mRNA levels via other gonadal (activins, inhibins) or hypothalamic (Kiss, NKB) factors.

In contrast to E2, 11-KT had no significant effects on *fshb* mRNA levels or Fsh pituitary content. This differs from previous studies in sexually immature and maturing salmonids. In ECA-stage coho salmon implanted with 11-KT-containing cholesterol pellet, pituitary Fsh

content was significantly increased after 10 days (Forsgren, 2010). This could be due to increases in plasma E2 induced by the 11KT treatment since plasma levels of E2 were substantially increased in that study. Additionally, in immature male Atlantic salmon, treatment with non-aromatizable androgens increased pituitary and plasma Fsh levels (Borg et al., 1998).

Regulation of Gths via neuroendocrine factors

Gonadal steroid hormones may also feedback on Gth expression via regulation of hypothalamic factors including GnRH or GnRH signaling (Bernard et al., 2010). In addition to the role of GnRH in Gth release, GnRH is known to increase *fshb* and *lhb* mRNA levels in fish both *in vivo* and *in vitro* (Ando et al., 2004; Dickey and Swanson, 2000; Golan et al., 2014; Kitahashi et al., 1998; Melamed et al., 1996; Yamaguchi et al., 2005). In the present study, E2 treatment significantly increased steady state mRNA levels of *gnrhr1* during late primary and early secondary oocyte growth. This is consistent with previous studies showing an increase in *gnrhr1* mRNA levels in response to estrogen treatment in coho salmon and tilapia (Harding et al., 2013; Levavi-Sivan et al., 2006; Zohar et al., 2010). Estrogen-induced up-regulation of GnRH receptors may sensitize the pituitary to GnRH stimulation as has been shown in mammals (Reeves et al., 1971). Treatment with E2 or T has also been shown to increase GnRH content in the brain and pituitary of fish (Breton and Sambroni, 1996; Montero et al., 1995; Trudeau, 1997). However, as in mammals, nuclear estrogen receptors are not expressed in GnRH neurons of fish, suggesting that other cell types are involved in GnRH response to E2 (Navas et al., 1995).

Recent studies in fish, like those in mammals, have implicated kisspeptin (Kiss) and neurokinin B (NKB, encoded by *tac3* gene) in regulation of GnRH and subsequent Gth secretion, (Oakley et al., 2009). Kiss 2 treatment increased *gnrhr1* and *fshb* mRNA levels *in vitro* in striped

bass (Zmora et al., 2015) and NKB treatment resulted in rapid stimulation of Fsh and Lh release in tilapia (*Oreochromis niloticus*) both *in vivo* and *in vitro* (Biran et al., 2014). Additionally, there is increasing evidence that Kiss and NKB neurons mediate sex steroid feedback on GnRH secretion (Angell and Steiner, 2015; Popa et al., 2008). In prepubertal zebrafish, E2 treatment increased mRNA levels of *kiss1*, *kiss2*, *tac3a*, and *gnrh3* and the Kiss and NKB receptors (*kiss1ra*, *tac3ra*, *tac3rb*) (Biran et al., 2012; Servili et al., 2011). Therefore, it is possible that the *in vivo* E2 treatment used in the current study of coho salmon may have regulated Gth expression via a variety of neuroendocrine factors.

Sex steroid hormone receptors in the pituitary

In this study, transcript levels of estrogen (*esr2a* and *esr2b*) and androgen receptors (*ara* and *arb*) were low but detectable in pituitaries of control, E2- and 11-KT implanted fish. Steady state mRNA levels for *esr1a* and *esr1b* were not measured in this study because previous work in our lab has shown that they are expressed at very low levels or are undetectable by qPCR and Illumina high-throughput sequencing in coho salmon pituitaries during early secondary oocyte growth (unpublished observations and Harding et al., 2013). Studies examining steroid receptor transcripts in the pituitary gland are limited. However, all 4 estrogen receptor isoforms (*esr1a*, *esr1b*, *esr2a*, *esr2b*) have been measured in the pituitary of juvenile male rainbow trout (*O. mykiss*) (Nagler et al., 2007). Further, colocalization studies in sea bass (*Dicentrarchus labrax*) using *in situ* hybridization have shown that *esr1*, *esr2a*, and *esr2b* are expressed in Lh cells and *esr1* and to a lesser extent *esr2a* and *esr2b* are expressed in Fsh cells, suggesting that steroid hormones may directly regulate gonadotropin expression in gonadotrophs (Muriach et al., 2008a, 2008b).

In addition, steroid hormones may auto-regulate steroid hormone receptors in the pituitary to control steroid feedback effects on the pituitary. Most studies on the regulation of steroid hormone receptors by steroid hormones have focused on the liver and gonad. However, previous studies in coho salmon and goldfish indicate that *esr2b* mRNA levels are downregulated in the pituitary and hypothalamus, respectively, in response to estrogen exposure (Harding et al., 2013; Marlatt et al., 2008). In the current study, E2 treatment increased *ara* mRNA levels at day 21, but *arb* and estrogen receptor mRNA levels were unaffected by steroid treatment during late primary oocyte growth in coho salmon. However, in experiment 2, when fish were transitioning into early secondary oocyte growth, E2 and 11KT had weak, but significant negative effects on pituitary transcripts for both estrogen and androgen receptors, suggesting gonadal steroids exert negative feedback on steroid receptors in the pituitary during secondary oocyte growth. These results suggest that the responsiveness of pituitary cells to gonadal steroid feedback changes during the transition from primary to secondary oocyte growth, but more research is needed to investigate this hypothesis.

Conclusion:

This study demonstrates that E2, but not 11-KT, differentially regulates Fsh and Lh synthesis during late primary and early secondary oocyte growth in coho salmon. E2 had strong, rapid positive feedback effects on pituitary Lh synthesis increasing both *lhb* mRNA and Lh protein. The rapid effects are consistent with direct effects of E2 on the *lhb* promoter that contains multiple EREs. In contrast, E2 had divergent effects on pituitary Fsh, reducing *fshb* mRNA levels but increasing Fsh protein in the pituitary. The kinetics of the effects of E2 on Fsh mRNA versus protein levels suggest they may be indirect, mediated via the brain, gonad and/or

paracrine factors in the pituitary. The accumulation of Fsh protein in response to E2 may be due to inhibitory effects of E2 on Fsh secretion, as shown in previous studies in salmon at later stages of vitellogenesis. However, this hypothesis requires further investigation using Fsh assays with greater sensitivity to detect plasma Fsh at these early stages of oogenesis. Additional studies are also required to identify factors stimulating Fsh production during early oogenesis in salmon.

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Supplemental Table 2.S1: Primer sequences used in RT-qPCR for quantification of pituitary transcripts. Primers were designed with MacVector software using the Primer3 algorithm.

Targeted gene	Forward primer	Reverse primer
<i>Ara</i>	CCCTATCCCAACTCACCATGCA	GCCACCCTCAAACCTGGCATCTG
<i>Arb</i>	CCCTATCCCAACTCCCCGTACT	GCCACCCTCAAACCTGGCATCTG
<i>Esr2a</i> ₁	CTGACCCCAGAACAGCTGATC	TCGGCCAGGTTGGTAAGTG
<i>Esr2b</i> ₁	CCCAAGCGGGTCCTAGCT	TCCTCATGTCCTTCTGGAGGAA
<i>Fshb</i> ₂	GAAACGACGGACCTGAACTATC	AGCCTTCCAGGTAGACCTTCTC
<i>Cgal</i>	AGTATTCTCTAACCCGGGCG	GGGACCAACATGGCTTTCTTA
<i>Cga2</i>	AACATCATGCAGTGTACAGGCT	GGTAATAACAGGTGCTGCAGTG
<i>Gnrhr1</i> ₂	ACCATCACAGTCCCAGAAAAGT	AAGCAGGAATAGACAGGCAAAG
<i>Lhb</i> ₂	TTCATGTAGGCACCTTGATCTC	CTCCAGAGACACAGTCTGGTTG
<i>Ubiquitin</i> ₃	TCAACAGCGTCTGATCTTCG	AAGACGCAGCACAAAGATGAA

₁ Primer sequences previously reported in Nagler et al., 2007

₂ Primer sequences previously reported in Harding et al., 2013

₃ Primer sequences previously reported in Kim et al., 2015

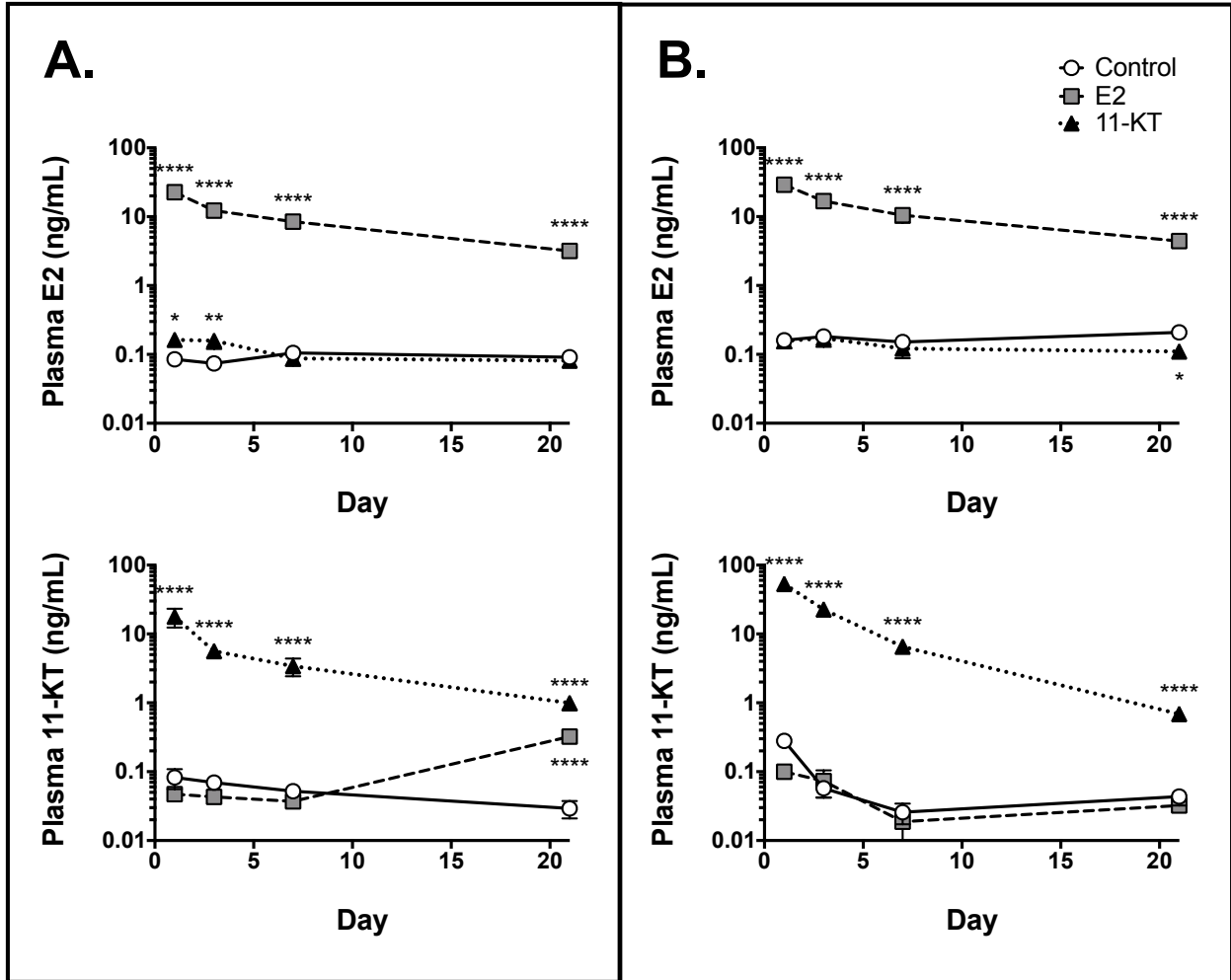


Figure 2.1: Plasma E2 and 11-KT levels from female coho salmon with LPN-ECA (A) or ECA-MCA (B) ovarian follicles. Fish were treated with implants containing vehicle (control), E2, or 11-KT (dose) for up to 21 days. Data are mean \pm SEM of N=5-6. Significant differences between treatment and controls at each time point are shown with asterisks. * P <0.005, ** P <0.01, *** P <0.001

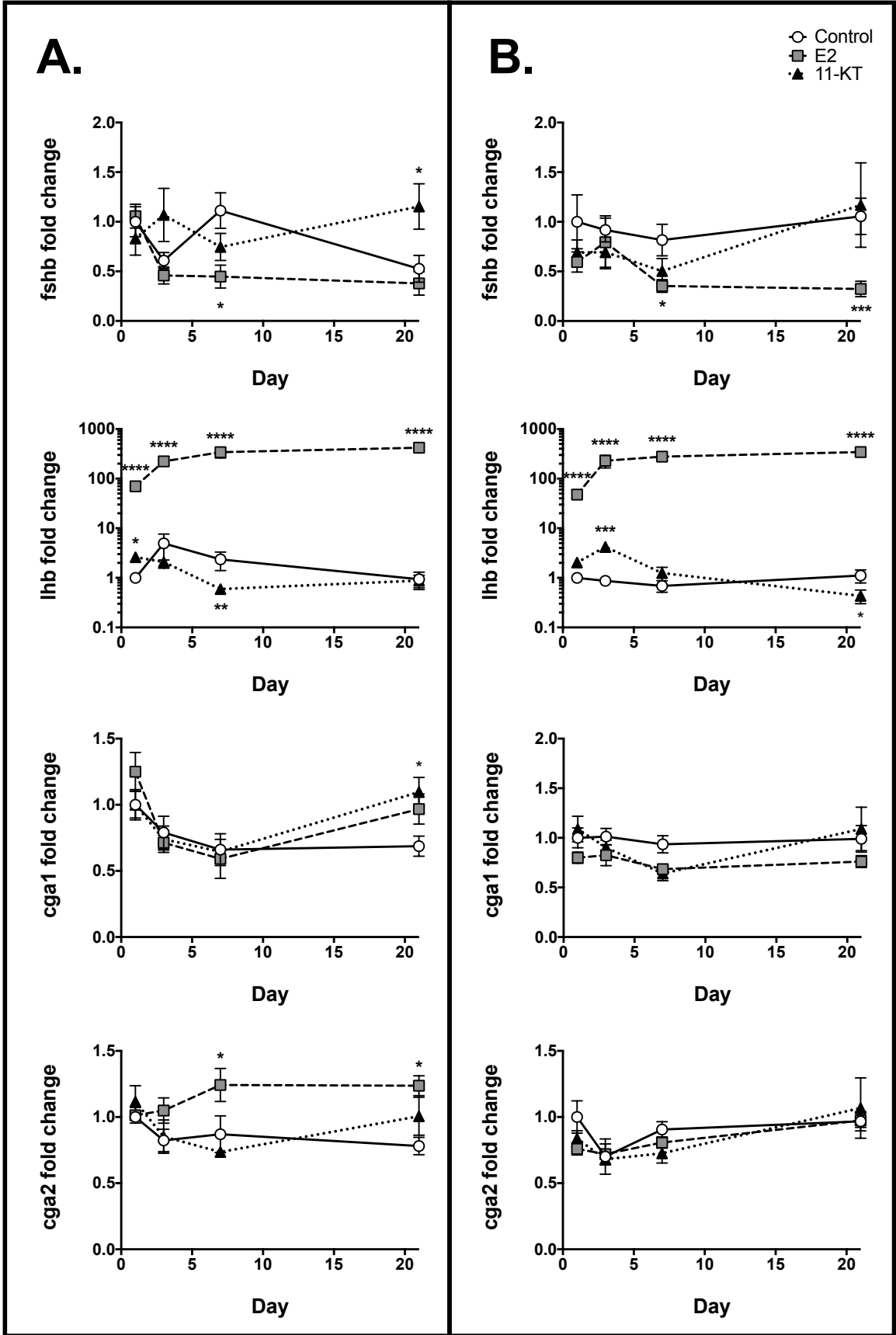


Figure 2.2. Pituitary gonadotropin subunit mRNA levels in female coho salmon with LPN-ECA (A) or ECA-MCA stage (B) ovarian follicles. Fish were treated with implants containing vehicle (control), E2, or 11-KT (dose) for up to 21 days. Data are mean \pm SEM of N=6. Significant differences between treatment and controls at each time point are shown with asterisks.

* $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$

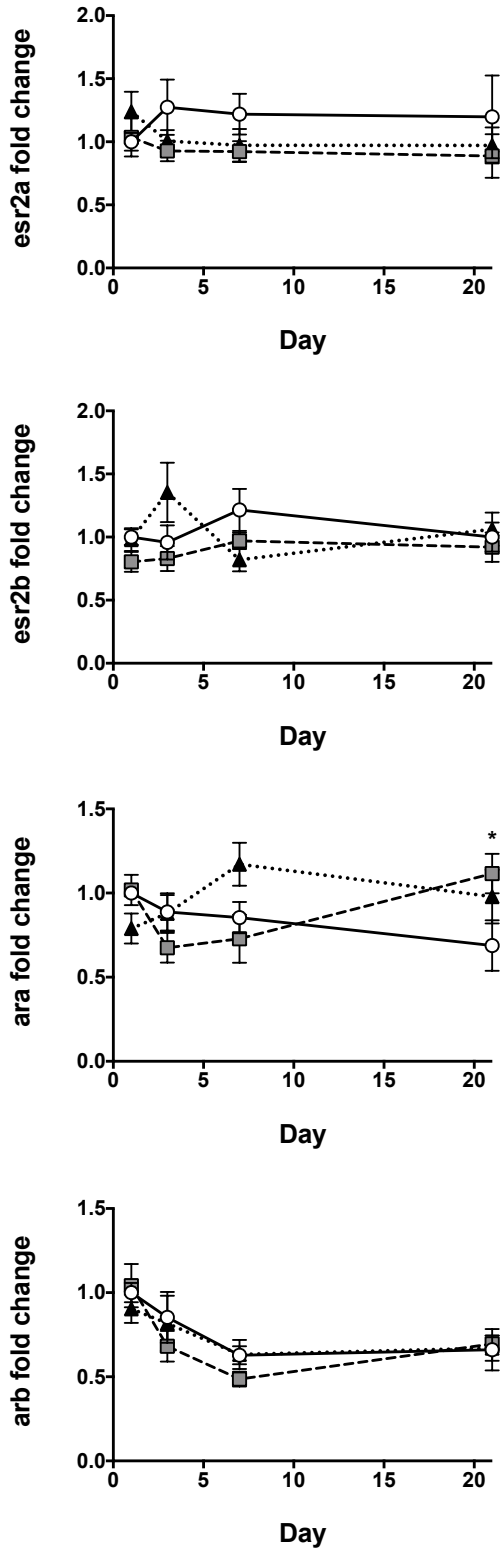
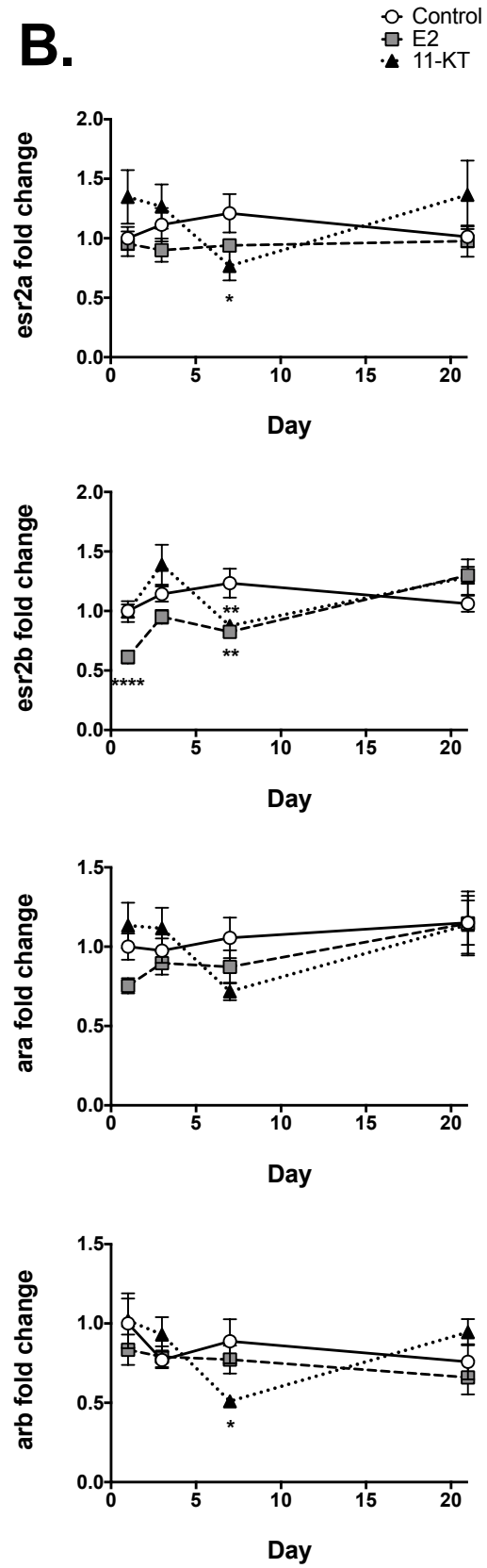
A.**B.**

Figure 2.3. Pituitary nuclear forms of estrogen and androgen receptor mRNA levels in female coho salmon with LPN-ECA (A) or ECA-MCA stage (B) ovarian follicles. Fish were treated with implants containing vehicle (control), E2, or 11-KT (dose) for up to 21 days. Data are mean \pm SEM of N=6. Significant differences between treatment and controls at each time point are shown with asterisks. * P <0.005, ** P <0.01, *** P <0.001

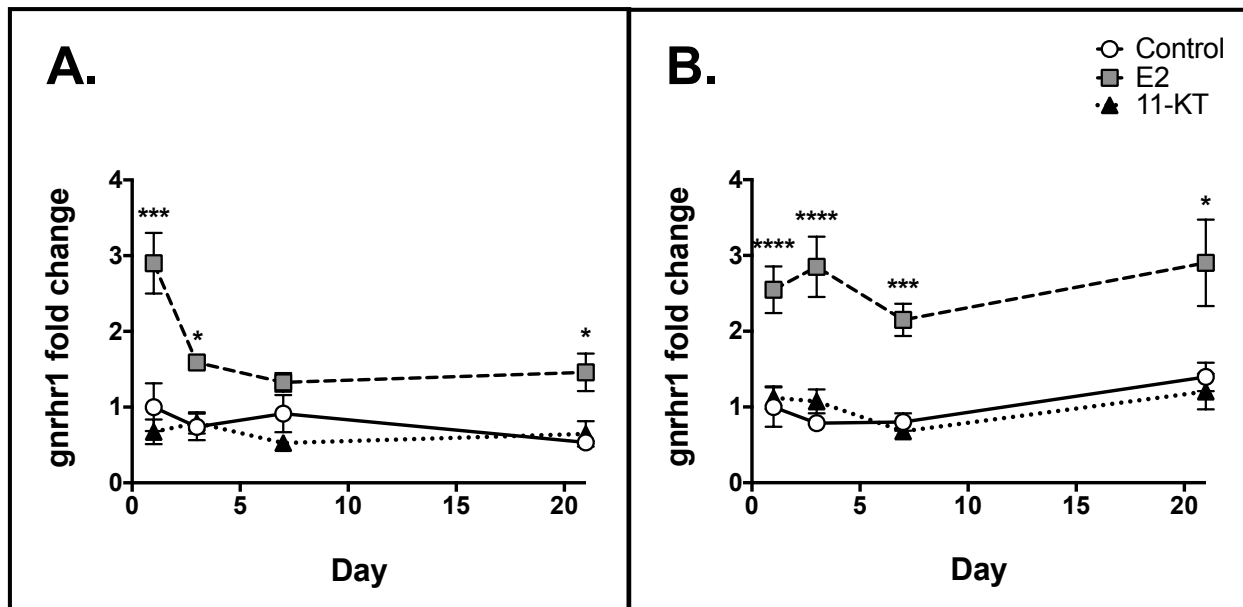


Figure 2.4. Pituitary gonadotropin releasing hormone receptor (*gnrhr1*) mRNA levels in female coho salmon with LPN-ECA (A) or ECA-MCA stage (B) ovarian follicles. Fish were treated with implants containing vehicle (control), E2, or 11-KT (dose) for up to 21 days. Data are mean \pm SEM of N=6. Significant differences between treatment and controls at each time point are shown with asterisks. * $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$

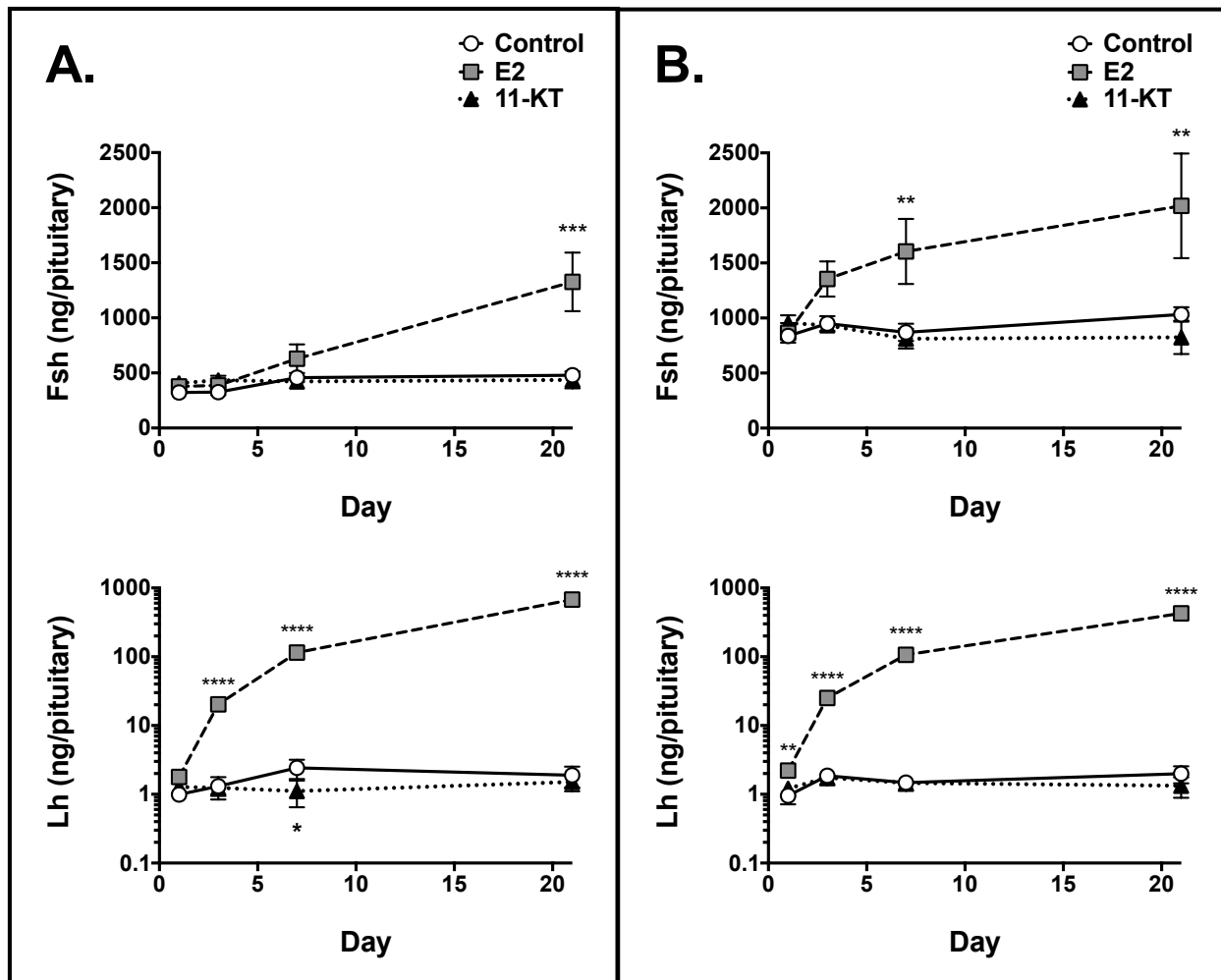


Figure 2.5: Pituitary Fsh and Lh protein content from female coho salmon with LPN-ECA (A) or ECA-MCA stage (B) ovarian follicles. Fish were treated with implants containing vehicle (control), E2, or 11-KT (dose) for up to 21 days. Data are mean \pm SEM of N=6. Significant differences between treatment and controls at each time point are shown with asterisks. * $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$

Chapter 2: High-throughput sequencing and pathway analysis reveal alteration of the pituitary transcriptome by 17 α -ethynylestradiol (EE2) in female coho salmon, *Oncorhynchus kisutch*.

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Supplemental tables and files associated with this chapter can be accessed online via the published article.

Note: Due to the use of mammalian pathway analysis software, human gene and protein nomenclature will be used throughout this chapter. Genes for which human orthologs do not exist will be presented in a similar style (e.g., for vitellogenin, *VTG* will be used to denote the vitellogenin gene or mRNA and VTG will denote the vitellogenin protein).

Abstract:

Considerable research has been done on the effects of endocrine disrupting chemicals (EDCs) on reproduction and gene expression in the brain, liver and gonads of teleost fish, but information on impacts to the pituitary gland are still limited despite its central role in regulating reproduction. The aim of this study was to further our understanding of the potential effects of natural and synthetic estrogens on the brain-pituitary-gonad axis in fish by determining the effects of 17 α -ethynylestradiol (EE2) on the pituitary transcriptome. We exposed sub-adult coho salmon (*Oncorhynchus kisutch*) to 0 or 12 ng EE2/L for up to 6 weeks and effects on the pituitary transcriptome of females were assessed using high-throughput Illumina® sequencing, RNA-Seq and pathway analysis. After 1 or 6 weeks, 218 and 670 contiguous sequences (contigs) respectively, were differentially expressed in pituitaries of EE2-exposed fish relative to control. Two of the most highly up- and down-regulated contigs were luteinizing hormone β subunit (241-fold and 395-fold at 1 and 6 weeks, respectively) and follicle stimulating hormone β subunit (-3.4-fold at 6 weeks). Additional contigs related to gonadotropin synthesis and release

were differentially expressed in EE2-exposed fish relative to controls. These included contigs involved in gonadotropin releasing hormone (GNRH) and transforming growth factor- β signaling. There was an over-representation of significantly affected contigs in 33 and 18 canonical pathways at 1 and 6 weeks, respectively, including circadian rhythm signaling, calcium signaling, peroxisome proliferator-activated receptor (PPAR) signaling, PPAR α /retinoid x receptor α activation, and netrin signaling. Network analysis identified potential interactions between genes involved in circadian rhythm and GNRH signaling, suggesting possible effects of EE2 on timing of reproductive events.

Introduction

In the past twenty years, the identification of environmental contaminants capable of disrupting normal endocrine function in wildlife and humans has become an area of great concern (reviewed by Hotchkiss et al., 2008). In fishes, early evidence of endocrine disruption came from expression of the female specific egg-yolk protein precursor vitellogenin (Harries et al., 1997, 1996; Purdom et al., 1994) and observations of intersex gonads (Jobling et al., 1998) in male fish downstream of wastewater treatment plants (WWTP). Chemical analyses of WWTP effluent identified natural estrogens (17 β -estradiol (E2) and estrone) and the synthetic, pharmaceutical estrogen, 17 α -ethynylestradiol (EE2) as likely sources of these feminizing effects (Desbrow et al., 1998). In the United States, EE2, the active estrogenic ingredient in oral contraceptives, is present in surface waters at concentrations up to 273 ng/L with median concentrations above the level of detection around 10 ng/L (Kolpin et al., 2002). Despite low environmental concentrations, laboratory exposures have shown that these levels are capable of inducing hepatic vitellogenin expression in male fish (Jobling et al., 1996; Lange et al., 2012; Pawlowski

et al., 2004), and disruption of reproduction. Chronic exposure to environmentally relevant concentrations of EE2 (5 ng/L) has been shown to cause reproductive failure in zebrafish (Nash et al., 2004) and complete feminization and subsequent population collapse of fathead minnows in an experimental lake (Kidd et al., 2007). Due to the potential impacts of EDCs on reproductive success of individuals and fish populations, much work is currently being done to model the impacts of EDCs on the reproductive axis in fish.

In fish, as in other vertebrate animals, reproduction is controlled by the brain-pituitary-gonad (BPG) axis. The brain integrates environmental stimuli (photoperiod, temperature, food availability) and internal factors (metabolic status, hormone levels) to control release of the gonadotropins from the pituitary (Bromage et al., 1993). The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are released from the pituitary and act on the gonads to stimulate synthesis of sex steroids (estrogens and androgens) and induce gametogenesis and gamete release. In females, E2 is produced primarily in the ovaries and acts locally to regulate ovarian development and peripherally to regulate hepatic vitellogenin production as well as hormone synthesis in the pituitary and brain. (Yaron and Sivan, 2006)

Considerable information is now available on the effects of EE2 on gene expression in the brain (Martyniuk et al., 2010, 2007, 2006; Vosges et al., 2010), gonad (Garcia-Reyero et al., 2009; Hirakawa et al., 2012; Miller et al., 2012; Santos et al., 2007), and liver (Benninghoff and Williams, 2008; De Wit et al., 2010; Hoffmann et al., 2006; Hook et al., 2006), and various computational models of impacts of EDCs such as EE2 to the BPG axis have been produced (Kim et al., 2006; Li et al., 2011; Villeneuve et al., 2012, 2007; Watanabe et al., 2009). However, despite the pituitary gland's central role in the reproductive axis, relatively few data are available on how the pituitary is impacted by EDC exposure to inform these models. Some models have

considered effects on plasma LH, but model predictions are contradictory (Li et al., 2011; Watanabe et al., 2009). Other studies have incorporated more pituitary data into their model, but empirical data to validate model predictions is lacking (Villeneuve et al., 2012, 2007). In studies that have looked at the pituitary gland in response to EDC exposure, the pituitary was found to be highly responsive and sensitive. In adult fathead minnows exposed to E2, pituitary mRNA levels were more responsive than brain mRNA levels of target genes (Filby et al., 2006). Further, in female rainbow trout exposed to nonylphenol, plasma FSH and pituitary FSH mRNA levels were the most sensitive endpoints assessed (Harris et al., 2001).

The aim of this study was to determine the effects of EE2 on pituitary gland gene expression to further our understanding of potential impacts of estrogenic chemicals on the BPG axis in fish. To maximize discovery of estrogen responsive genes and molecular pathways in the pituitary gland, a transcriptomics approach was selected. Microarrays have been used for this purpose in other studies, but microarrays can only measure changes in transcript levels of genes present on the array. Unless expressly designed for such a purpose, available arrays may not include genes important to the reproductive axis or the pituitary (Villeneuve et al., 2007). By contrast, Illumina® sequencing is unbiased in its representation of transcripts, has a wider dynamic range than microarrays, and simultaneously generates species-specific sequence data for future use. Coho salmon (*Oncorhynchus kisutch*) were selected for this study as an ecologically relevant species in the Pacific Northwest and because they have a well-described reproductive cycle, including characterization of estrogen regulation of pituitary gonadotropin synthesis and secretion (Dickey and Swanson, 1998). We have focused on females because estrogens play an important role in normal ovarian development. As such, genes transcripts identified as EE2 responsive may provide new information on the role of estrogen in normal

reproductive development as well as how reproduction may be perturbed by xenoestrogen exposure. We focused our studies on sub-adult fish at early stages of previtellogenic ovarian growth because these stages are sensitive to environmental conditions and occur during periods when salmon are migrating through populated marine environments such as Puget Sound (Washington, USA). The results of this study support our current understanding of estrogen regulation of pituitary gonadotropin transcripts in sexually immature (sub-adult) salmonids and provide new insights into roles of estrogen in the pituitary and how estrogenic chemicals in the environment could disrupt pituitary function.

Materials and methods

Chemicals for exposure and water chemistry

17 α -ethynylestradiol (>99% purity), methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), and iodotrimethylsilane were obtained from Sigma-Aldrich Chemical Co. (EE2, St Louis, MO)., [2,4,16,16]d4- 17 α -Ethynylestradiol (EE2-d4) was purchased from C/D/N isotopes (Pointe-Claire, Quebec, CA). All other chemicals were of reagent grade or better and obtained from standard sources.

Fish maintenance

Fish from the Issaquah Hatchery stock were reared at the Northwest Fisheries Science Center hatchery facilities (Seattle, WA) in recirculated 10 - 10.5°C fresh water under a simulated natural photoperiod. Fish were fed a standard ration of BioDiet commercial feed (Bio-Oregon, Longview, WA) according to Bio-Oregon's feed rate guidelines. On 1 July 2010, approximately 450 coho salmon (16 months old; 30-50g body weight) were transferred to Battelle Pacific

Northwest National Laboratory, Marine Science Laboratory (MSL, Sequim, WA). Fish were maintained in circular 1400 L fiberglass tanks. The holding tanks were maintained using a single-pass flow through system initially using fresh water obtained from MSL's artesian well (440 ft depth), which was pre-aerated and added to tanks at a minimum flow rate of 12 L/min. After 7 d acclimation, the salinity in the holding tanks was gradually raised by mixing fresh water with sand-filtered (approximately 20 µm cutoff) seawater from Sequim Bay, WA. Over a 14 d period, the proportion of seawater was gradually increased until 100% of the incoming tank water was seawater. A failure to fully adapt to saltwater can result in stunting, poor growth and mortality in coho salmon (Duan et al., 1995). As a result, mortalities occurring over the course of the study were assumed to be a result of failure to acclimate to seawater or stunting, and fish below a conservative size criterion (body weight < 50 g) were removed from the study.

Two months after saltwater acclimation, fish were transferred from large holding tanks into smaller, 370-L circular fiberglass tanks. At this time, fish were anesthetized in buffered tricaine methanesulfonate (0.05% MS-222; Argent Laboratories, Redmond, WA) and tagged with passive-integrated transponder (PIT) tags. Fin tissue was collected and analyzed for genetic sex using a molecular marker for the Y chromosome (Campbell et al., 2006; Du et al., 1993). Genetic sex identification allowed for random assignment of equal numbers of male and female fish to treatment tanks for a balanced experimental design. Water temperature, dissolved oxygen, pH and salinity were monitored weekly throughout the study with values ranging from 11.4 – 12.5°C for temperature, 8.0 – 9.4 mg/L for dissolved oxygen, 7.75 – 7.90 for pH, and 29.5-31.75 ppt for salinity. All fish were maintained under a simulated natural photoperiod regime with artificial dusk and dawn and fed a standard ration of Bio-Oregon® soft moist pellets. All fish

were maintained according to the guidelines established by the Institutional Animal Care and Use Committee of Battelle.

Waterborne EE2 exposure

The waterborne exposure was initiated in September, 2 months following seawater acclimation when fish were considered to be sub-adults and in previtellogenic stages of secondary oocyte growth. Concentrated EE2 aqueous stock solution was prepared in a 19 L Pyrex glass bottle filled with 18 L of deionized water at a nominal EE2 concentration of 240 µg/L. Initially, 432 µl of a 10 mg/ml (w/v) EE2 solution in methanol was added to the empty bottle. The methanol was evaporated under a stream of N₂ and then the deionized water added and stirred overnight in darkness. During subsequent exposures, the EE2 stock bottle was wrapped in black plastic to prevent photodegradation. This stock solution was slowly added to exposure tanks with a peristaltic pump at a flow rate of 7 – 9 ml/h to achieve the nominal concentration of 12 to 15 ng/L. This concentration was selected because it is within the range of environmentally observed levels of EE2. Fish were randomly assigned to tanks (2 tanks/treatment, 10 females and 10 males/tank) and exposed continuously to control or EE2-treated seawater for 46 d. Water and stock solution in-flow rates for all tanks in each treatment were monitored daily to verify continuous exposure throughout the treatment. Control and EE2 exposure water was analyzed at four different times during the experiment. There was no EE2 detected in control tanks and the time-weighted average concentrations for EE2 exposure tanks were 17.7 and 16.4 ng EE2/L.

Chemical analysis

The exposure water was analyzed for EE2 by GC-MS (Agilent 6890 GC / 5973 inert MS). Water samples (0.05 – 0.1 L in volume) were extracted with 4 – 8 mL of methyl-tert-butyl-ether (MTBE) per sample. The MTBE fraction was removed, evaporated under N₂ and subsequently derivatized with MSTFA as described in Schultz et al. (2001) except that EE2-d₄ was added as an internal standard in the extractions. The GC-MS was operated in selected ion monitoring mode with m/z 429 and m/z 425 ions used for EE2-d₄ and EE2 quantification. Recovery of EE2 from fortified water standards typically exceeded 95%.

Sample collection

At 1 week and ~6 weeks (46 d), 5 females and 5 males per tank were euthanized in buffered MS-222 and decapitated. Fork length (nearest mm), body weight (nearest 0.1 g), gonad weight and liver weight (nearest mg), were recorded. Gonad and liver weight were used to calculate gonadosomatic index (GSI) and hepatosomatic index (HSI) respectively, according to the following equation: (tissue mass/body mass) X 100. Blood was collected in heparinized syringes from the caudal vein and plasma was obtained after centrifugation at 1000 x g for 15 min. The pituitary gland and small pieces (~ 50 mg) of liver and one gonad were flash frozen in liquid nitrogen for RNA isolation. All frozen tissues and plasma were stored at -80 °C. A small piece of the other gonad was fixed in 0.5 mL of HistoChoice™ Tissue Fixative (AMRESCO, Solon, OH) for 72 h followed by Bouin's fixative for 24 h. Although only female fish were analyzed for this study, male tissues were collected for future analyses.

Radioimmunoassay and histology

Circulating plasma levels of E2 were measured by radioimmunoassay (RIA) using the ImmuChem™ Double Antibody 17β-Estradiol ¹²⁵I RIA Kit (MP Biomedicals LLC, Solon, OH) according to the manufacturer's instructions. This kit has previously been validated for quantification of plasma E2 levels in coho salmon plasma (Campbell et al., 2006). Fixed ovaries were processed for histology and staged as described in Campbell et al. (2006).

Library preparation and sequencing

Pituitary glands were homogenized using a TissueLyzer II homogenizer (QIAGEN, Valencia, CA). Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA yield was quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). Equal quantities of total pituitary RNA from three (1 week) or four individuals (6 weeks) per treatment were submitted to the University of Washington's High-Throughput Genomics Unit for quality checking, library preparation, and 36 bp single end Illumina® sequencing. Individual samples were barcoded and run 2 per lane on the Illumina® HiSeq2000 platform. Since there was no treatment effect on ovarian stage as assessed by histology (Fig. 3.1B, 2-way ANOVA, treatment $p > 0.05$), individuals at the early to mid cortical alveolus stage (predominant stages in the groups) were selected for Illumina sequencing to avoid confounding effects of ovarian stage on transcript levels.

Bioinformatics

Illumina sequences were quality trimmed (limit = 0.05, maximum ambiguous nucleotides = 2, adapters removed, minimum length = 20) using CLC Genomics Workbench v. 4.9 (CLC Bio,

Cambridge, MA). Trimmed reads from control (n=7) and EE2 exposed (n=7) individuals were *de novo* assembled using the transcriptome assembler Trinity (Grabherr et al., 2011). Trinity v.20120518 was run using the kmer method meryl and a minimum contig length of 200 bp. The resulting contiguous sequences (contigs) were annotated using BLASTX (Altschul et al., 1990) against the NCBI non-redundant protein database (www.ncbi.nlm.nih.gov). Annotated sequences with an e-value \leq E-05 were retained.

To examine differential transcript levels between control and EE2-exposed salmon pituitary libraries, RNA-Seq was performed using RSEM v.1.1.19 (Li and Dewey, 2011). RSEM was optimized using the library fragment length mean and standard deviation. Trinity and RSEM are designed to assemble and quantify high-throughput RNA sequence data to the level of transcript isoforms. However, paired-end sequences are recommended for the accurate identification of splice variants and isoforms (Li and Dewey, 2011). Therefore, contigs and annotations were collapsed into gene-level contigs using unique chrysalis IDs. Gene-level count estimates generated by RSEM were analyzed for differentially expressed genes using DESeq (Anders and Huber, 2010). Contigs with a *p*-value \leq 0.05 were considered significantly altered. Statistics were not corrected for false discovery rate because it can increase the rate of Type II ‘false negative’ errors, particularly when variability is high and sample size is low. For an exploratory study such as this where comprehensive identification of differentially expressed transcripts improves downstream analyses (pathway analysis, network analysis and gene hunting), alternative screening methods have been recommended (Cole et al., 2003). Therefore, instead of adjusting statistics for false discovery rate correction, we have focused our analysis on differentially expressed genes involved in pathways identified as significantly “enriched” by EE2 exposure as determined by the Fisher’s exact test ($p < 0.05$), similar to the method previously used

by Martinovic et al. (2009). Further, to reduce the chance that sequencing errors or differences in sequencing depth could contribute to misidentification of differential expression of contigs with very low read count, contigs with a baseMean ≤ 10 for either treatment were not analyzed. Clustering analysis was performed on differentially expressed contigs at each time point using the cluster::agnes package in R with the Spearman method (Maechler et al., 2012). Data were log2 transformed and centered to improve visualization of relative expression.

Pathway and network analyses were conducted using Ingenuity® Systems Pathway Analysis (IPA) software (Redwood City, CA). To analyze by IPA, annotated contigs were mapped to zebrafish and human orthologs using BLASTN against the Ensembl *Danio rerio* gene database (v.Zv9.66) and the *Homo sapiens* transcript database (v.GRCh37.66) with an e-value $\leq 1.00E-05$. The coho salmon contigs were mapped first to zebrafish as it was expected that there would be higher degree of sequence similarity between coho salmon and zebrafish. However, some zebrafish genes have not been mapped to mammalian orthologs, so any remaining contigs were mapped to human orthologs. To ensure that functions and pathways relevant to endocrine function of the pituitary would be identified if affected, we confirmed whether pituitary hormones and hormone receptors had mapped correctly. We found that 5 transcripts did not map to IPA (*GNRH*, GNRH receptor 1 (*GNRHRI*), FSH β subunit (*FSHB*), prolactin (*PRL*), and kisspeptin 1 (*KISS1*)) and mapped them manually before uploading to IPA. If multiple contigs mapped to the same gene ID, duplicates were resolved by selecting the contig with the lowest *p*-value. Human and zebrafish orthologs were then compared to the Ingenuity® Knowledge Base (www.ingenuity.com) and significantly altered pathways and biological functions were determined using the Fisher Exact Test ($p < 0.05$). Up to 25 gene networks were generated, maximizing the connectivity of significantly altered genes based on known functional

interactions (Calvano et al., 2005). Due to the use of mammalian pathway analysis software, human gene and protein nomenclature will be used throughout this paper. Genes for which human orthologs do not exist will be presented in a similar style (for vitellogenin, *VTG* will be used to denote the vitellogenin gene or mRNA and VTG will denote the vitellogenin protein).

Quantitative real time RT-PCR (qPCR)

To compare RNA-Seq and qPCR platforms for quantification of mRNA levels, transcripts encoding a subset of genes involved in the reproductive axis (*LHB* [LH β subunit], *FSHB*, *TSHB* [thyroid stimulating hormone β subunit], *CGA* [glycoprotein hormones, α polypeptide], and *GNRHR1*) and genes identified as estrogen-responsive in mammals (*CYP19A1B* [brain aromatase], *EGR1* [early growth response protein 1], *ESR2B* [estrogen receptor 2B, previously ERbeta-1], *ESR2A* [estrogen receptor 2A, previously ERbeta-2], *FOS* [proto-oncogene c-Fos], and *PRL*) were measured by qPCR in the same pituitary RNA samples sent for Illumina® sequencing. Additionally, expression of vitellogenin (*VTG*), a classical biomarker of estrogen exposure in fish was measured in liver tissue to confirm that fish responded to the EE2 exposure. RNA samples for qPCR were DNase treated using Ambion® TURBO DNA-free™ (Life Technologies, Carlsbad, CA). A 12 μ L reaction was composed of 10 μ L (1500 ng) total pituitary RNA, 1 μ L DNase I Buffer and 1 μ L TURBO DNase and incubated at 37°C for 1 h. DNA-free samples were reverse transcribed with SuperScriptII (Life Technologies) as described in Kitano et al. (2010). qPCR primers (Supplemental Table 3.S1) were obtained from the literature or designed according to coho salmon sequence data using MacVector Software v.12.5.1 (Accelrys, San Diego, CA) and purchased from Integrated DNA Technologies (Coralville, IA). qPCR methods were similar to Luckenbach et al., 2011, but

adapted to a 384-well platform. Assays were run using an ABI 7900HT Fast Real-Time PCR System (Life Technologies) in 384-well plates using standard cycling conditions. Reactions were 12.5 μ L each and consisted of 1X Power SYBR Green PCR Master Mix (Life Technologies), 150 nM of the forward and reverse primer, and 0.5 ng cDNA template. Four concentrations of standard curve samples generated from a serial dilution of cDNA (from pooled pituitary RNA) ranging from 0.05 to 5 ng cDNA were included in each plate in triplicate. No amplification controls included in each plate showed no detectable amplification over 40 cycles of PCR. No template controls showed no detectable amplification over 40 cycles of PCR except for the *VTG* assay, which had a non-specific signal indicated by melt-curve analysis (no amplification at less than 35 cycles). Data were expressed relative to eukaryotic elongation factor-1 α (*EEF1A*), which was stably expressed across treatments in pituitary and liver (Supplemental Fig. 3.S1). To improve visualization of qPCR results relative to RNA-Seq, the mean value of the control treatments were set to 1 at each time point.

Statistical analyses

All statistical analyses were performed using Prism 5 for Mac OSX (GraphPad Software, La Jolla, CA). For morphometric data and plasma E2 levels, differences between fish in duplicate tanks were analyzed by Student's unpaired t-test. Because no significant differences were found ($p > 0.05$), duplicate tanks were pooled for further analyses. Significant differences in morphometric data and measures of reproductive stage between control and EE2 treated fish were determined by 2-way ANOVA followed by Bonferroni post-hoc test using a p -value threshold of $p < 0.05$. For qPCR data, values that did not amplify within 40 cycles were assigned the lowest detectable value. Mean levels of expression for each transcript were compared

between control and EE2 treatments at each time point using Student's unpaired t-test with a significance threshold of $p < 0.05$. Data were log-transformed where necessary to meet parametric test criteria. Data in the figures are mean \pm SEM.

Results

Morphometrics and reproductive stage

Fish fork length (control, 193 ± 3 mm; EE2, 195 ± 3 mm), weight (control, 85.0 ± 4.3 g; EE2, 87.6 ± 4.7 g), GSI (control, 0.346 ± 0.013 ; EE2, 0.333 ± 0.019), and HSI (control, 1.281 ± 0.051 ; EE2, 1.408 ± 0.067) were not significantly altered by EE2 exposure at either time point (Supplemental Fig. 3.S2). Plasma E2 levels were not significantly different between control and EE2-treated fish at either time point (Fig. 3.1A). Ovarian stage was variable among individuals, ranging from the late perinucleolar to late cortical alveolus stage at 1 and 6 weeks but ovarian stage did not differ between EE2 and control treatments or between time points (Fig. 3.1B). Although there were no significant effects of EE2-exposure on plasma E2, GSI, HSI, or ovarian stage, mRNA levels of liver *VTG* were significantly up-regulated (96,890-fold and 888,636-fold at 1 and 6 weeks, respectively) in EE2-exposed individuals suggesting that the treatment was sufficient to alter patterns of hepatic gene expression as expected from previous studies in many other fish species (Fig. 3.1C).

Illumina sequencing and backbone construction

Illumina sequencing of the pituitary transcriptome resulted in 440,706,890 and 604,745,974 reads for control and EE2-exposed libraries, respectively, with an average length of 36 bp. Greater than 99.8% of reads were retained after quality trimming, leaving 440,192,475

and 604,149,475 trimmed reads with an average length of 34.9 bp (Supplemental Table 3.S2). Sequence data from this experiment are available at NCBI (BioProject: PRJNA186554). De novo assembly generated 133,880 contigs ranging from 201 bp to 16,166 bp with a mean length of 599 bp. A total of 40,456 contigs were annotated. Contigs were collapsed into gene-level contigs, resulting in 40,293 annotated gene-level contigs included in our backbone.

RNA-Seq analysis and qPCR

RNA-Seq analysis identified 218 contigs that were differentially expressed (DESeq $p < 0.05$) after 1 week of EE2 exposure. At 6 weeks, the number of significantly altered contigs was more than tripled with 670 regulated contigs (DESeq $p < 0.05$). The majority of differentially expressed contigs were induced by EE2 with 137 (63%) and 395 (59%) contigs up-regulated in the EE2-exposed individuals at 1 and 6 weeks, respectively. Cluster analysis of significantly altered contigs indicated a distinct difference between control and EE2 exposed individuals at both 1 and 6 weeks (Fig. 3.2). Full lists of EE2-regulated contigs are available in Supplemental Files, S1 and S2.

Our comparison of RNA-Seq and qPCR mRNA level quantification showed good agreement in the direction and magnitude of fold change between platforms (Supplemental Fig. 3.S3). At 1 week, *ESR2B* assessed by RNA-Seq was significantly down-regulated, but was non-significantly down-regulated when quantified with qPCR. This was the only gene analyzed by qPCR for which significant expression changes found by RNA-Seq, were not supported by qPCR.

Functional and pathway analyses

To investigate biological functions, pathways and gene networks in the pituitary affected by EE2 exposure, zebrafish and human orthologs were used to mine the Ingenuity® Knowledge Base. Of the 40,293 contigs, 30,670 (76%) blasted to zebrafish or human gene orthologs and mapped to IPA. Duplicate mappings were removed, leaving 11,319 unique genes included in the pathway analysis, 121 and 402 of which were differentially expressed (DESeq $p < 0.05$) at 1 week and 6 weeks, respectively. Tables 3.1 and 3.2 show a summary of the results of the pathway analyses for coho salmon exposed to EE2 for 1 or 6 weeks compared to control fish. To improve readability, only the most significantly affected contigs (fold change ≥ 1.8 , DESeq $p < 0.01$) and putative pathways (Fisher's exact test $p < 0.05$) are included in the tables (full contig lists in Supplemental files S1, S2).

Endocrine systems

To determine the effect of EE2 on basic pituitary endocrine function, we investigated alterations to pituitary hormone gene expression. Overall, contigs for four of the eleven pituitary hormone transcripts had altered levels in response to EE2 exposure (Fig. 3.3). Transcripts of both gonadotropin β subunits were affected by EE2 exposure and *LHB* was the most significantly altered transcript at both time points. At 1 week, EE2-exposure resulted in a 241-fold induction of *LHB*. At 6 weeks, this increased to 395-fold higher pituitary expression of *LHB* in EE2 exposed individuals relative to control. Additionally, at 6 weeks *FSHB* was significantly down-regulated (-3.4-fold, $p < 0.001$) and somatolactin α (*SLA*) and somatolactin β (*SLB*) were up-regulated (1.7-fold, $p = 0.047$ and 2.7-fold, $p = 0.002$ respectively).

In addition to the gonadotropin β subunits, a number of contigs with reported roles in the fish reproductive axis (Villeneuve et al., 2012) had altered expression in response to EE2

exposure. At 1 week, gonadotropin releasing hormone receptor 1 (*GNRHR1*, 2.3-fold, $p < 0.01$) and retinoid x receptor β (*RXR β* , 3.8-fold, $p < 0.01$) were significantly up-regulated in EE2-exposed fish pituitaries relative to control. Induction of *GNRHR1* was increased at 6 weeks (3.5-fold, $p < 0.0001$) and transforming growth factor β -1 (*TGF β 1*, 1.6-fold, $p = 0.03$) and apolipoprotein B (*APOB*, 3.6-fold, $p < 0.001$) were significantly up-regulated, while matrix metalloproteinase 21 (*MMP21*) was down-regulated (-1.7-fold, $p = 0.04$).

Estrogen responsive transcripts

Contigs significantly altered by EE2 exposure also included genes previously reported to be responsive to estrogens. Comparison of EE2-responsive contigs in our data that mapped to IPA (n = 482) to lists of genes previously reported to be estrogen-responsive in mammals (n = 1,641 curated by Ingenuity Systems Inc.) or fish (Benninghoff and Williams, 2008; Garcia-Reyero et al., 2009; Martyniuk et al., 2007, 2006; Santos et al., 2007) identified a total of 72 estrogen-responsive genes that had altered expression at 1 week (n = 12), 6 weeks (n = 50) or both (n = 10) (Fig. 3.4). Expression of some classical estrogen responsive genes, such as growth regulation by estrogen in breast cancer 1 (*GREB1*), was increased by EE2 exposure at 1 and 6 weeks (2.7- and 2.4-fold respectively). However, levels of contigs annotated as classical estrogen responsive transcripts (*EGR1*, estrogen receptor α (*ESR1*), *FOS*, *PRL*) were not significantly different between control and EE2-exposed fish. Interestingly, levels of 31 contigs which were not identified as estrogen responsive in the IPA Knowledgebase® or in various studies in fish were significantly altered after both 1 and 6 weeks of EE2 exposure (Fig. 3.4). These included contigs with putative roles in circadian rhythm signaling (PER1 [Period 1], PER2 [Period 2], NPAS2 [neuronal PAS domain-containing protein 2], DBP [D site of albumin promoter binding

protein], and RORB [nuclear receptor ROR- β], brain and nervous system development (TIMP2 [metalloproteinase inhibitor 2], HSPG2 [basement membrane-specific heparan sulfate proteoglycan core protein], TRIM3 [tripartite motif-containing protein 3], ACSL3 [long-chain-fatty-acid-CoA ligase 3]), and histone acetylation (MEAF6 [chromatin modification protein MEAF6], HCFC1 [host cell factor 1]).

Biological functions

Functional analysis identified 77 and 75 biological functions that were potentially altered by EE2 exposure at 1 week and 6 weeks respectively ($p < 0.05$, Supplemental Tables 3.S3 and 3.S4). Biological functions that were affected by EE2 exposure were consistent across time points, with 93% of the affected functions being conserved between 1 and 6 weeks. The most significantly affected functions at 1 week included behavior, nervous system development and function, gene expression, reproductive system disease, reproductive system development and function, and lipid metabolism. However, basic cell organization processes dominated the most strongly altered functions at 6 weeks including molecular transport, cellular assembly and organization, cellular function and maintenance, cell signaling, cell cycle, cell morphology and organ morphology.

Canonical pathways

Pathway analysis identified 33 and 18 putative pathways altered by EE2 exposure at 1 and 6 weeks respectively ($p < 0.05$, Supplemental Tables 3.S5 and 3.S6). Only 5 pathways were consistently altered at both 1 and 6 weeks, including calcium signaling, circadian rhythm signaling, pregnenolone biosynthesis, netrin signaling, and inhibition of angiogenesis by TSP1

(thrombospondin 1). Circadian rhythm signaling and calcium signaling were among the most strongly affected pathways at both time points (Tables 3.1B and 3.2B). Additional putative pathways altered by EE2 exposure at 1 week were aryl hydrocarbon receptor signaling, GNRH signaling, transforming growth factor β (TGF- β) signaling, peroxisome proliferator-activated receptor (PPAR) signaling, retinoic acid receptor (RAR) activation glucocorticoid receptor signaling and androgen signaling. Interestingly, estrogen receptor signaling was not significantly altered ($p=0.051$). Following 6 weeks of EE2 exposure, alteration of pathways involved in basic cell organization and function was more apparent, including actin cytoskeleton signaling, cell cycle control of chromosome replication, and role of BRCA1 (breast cancer type 1 susceptibility protein) in DNA damage response (Table 3.2B). Although additional GNRH pathway signaling transcripts were differentially regulated at 6 weeks, the GNRH signaling pathway was not significantly over-represented at this time ($p=0.06$).

Gene networks

Up to 25 gene networks were generated by maximizing the connectivity of significantly altered contigs based on known functional interactions. There were 13 gene networks generated from the 1 week dataset. A full list of the networks, their genes and the associated biological functions are available in Supplemental Table 3.S7. At 1 week, a network was generated from 18 molecules involved in behavior, nervous system development and function, and connective tissue development and function. This network illustrates a possible interaction between molecules in circadian rhythm signaling and GNRH signaling (Fig. 3.5). Period 1 (*PER1*, -1.7-fold change at 1 week) and GNRHR (2.3-fold change at 1 week) were highly connected to other molecules in the network.

At 6 weeks, 25 networks were generated from differentially expressed contigs in the dataset (Supplemental Table 3.S8). At 6 weeks, the interaction between GNRH signaling molecules and circadian rhythm signaling molecules was not seen in any of the networks generated even though RNA-Seq revealed differential expression of a number of contigs in these pathways. Instead, the gonadotropin β subunits from the GNRH signaling pathway were closely associated with 25 molecules involved in cell morphology, digestive system development and function, and endocrine system development and function (Fig. 3.6). This network illustrates the relationship of LHB and FSHB with inhibin and nuclear factor NF-kappa- β signaling molecules. Circadian rhythm signaling molecules were more closely associated with the TGF- β complex in a network involving 20 molecules associated with behavior, nervous system development and function and reproductive system development and function (Supplemental Fig. 3.S4). TGFB1 was also the central molecule in the highest scoring network at 6 weeks (Supplemental Fig. 3.S5). This network illustrates the relationships of TGFB1 with 28 molecules involved in cellular development, growth and proliferation, suggesting that basic cell processes are potentially more affected after 6 weeks versus 1 week of EE2 exposure.

Discussion

The effects of natural and synthetic estrogenic chemicals on the liver and reproductive system have been well studied in fish. However, there is surprisingly little information on the effects of EDCs on the pituitary gland. Here, we report the results of the first transcriptome-wide gene expression analysis of the pituitary gland in EE2-exposed animals. Female sub-adult coho salmon were exposed to an environmentally relevant concentration of EE2 and effects on the pituitary transcriptome were assessed using high-throughput Illumina® sequencing and RNA-

Seq analysis. With our analysis parameters, levels of 218 and 670 contigs were regulated in the pituitary of coho salmon exposed to EE2 for 1 or 6 weeks, respectively. This is consistent with the numbers of gene transcripts found to be altered by estrogen treatment in a number of tissues including the brain (Martyniuk et al., 2007), gonad (Garcia-Reyero et al., 2009; Santos et al., 2007), and liver (De Wit et al., 2010; Hook et al., 2006) in other species of fish.

Regulated contigs included a number of genes with established roles in reproductive function in all vertebrates, including the gonadotropin hormone subunits. In fact, *LHB* was the most dramatically altered transcript at both time points, indicating that it is highly sensitive to estrogen exposure in sexually immature salmon. Functional analysis of regulated contigs identified potential alteration of pathways involved in gonadotropin regulation (GNRH signaling, TGF- β signaling), pathways previously reported to be estrogen responsive (calcium signaling, lipid metabolism) and pathways not previously identified as disrupted by estrogens in fish (circadian rhythm signaling). Network analysis identified potential interactions between genes involved in circadian rhythm and GNRH signaling in response to EE2 treatment, suggesting possible novel mechanisms of estrogen action in the pituitary gland.

The response of the pituitary transcriptome to EE2 depended on duration of exposure. The main effects after 1- week exposure were on expression of contigs involved in activation of transcription factors and signaling pathways. After 6 weeks, contigs involved in signaling pathways were still affected, and basic cell processes (cell cycle and cytoskeleton signaling) were altered as well.

Bioinformatics work flow in a non-model organism

Here we have presented a bioinformatics workflow for transcriptome-wide gene expression and pathway analysis in a non-model species. Single end, short-read Illumina sequencing was selected based on its superior estimation of gene abundances (Li and Dewey, 2011) and Trinity and RSEM were chosen for their improved handling of transcriptomic over genomic data (Grabherr et al., 2011; Li and Dewey, 2011; reviewed in Martin and Wang, 2011). In preliminary analyses, we found that contigs generated by genomic de novo assemblers frequently omitted highly expressed mRNA sequences (data not shown). DESeq and edgeR were both tested for determination of differentially expressed contigs, and DESeq was generally more conservative in the number of significant contigs it identified. Additionally, DESeq has been shown to better identify differentially expressed genes across a wider range of expression levels than edgeR (Anders and Huber, 2010). IPA was selected for pathway analysis for its extensive Ingenuity® Knowledgebase of annotations and functional pathways. Although these pathways are based on data from the mammalian literature, the data provide useful information for hypothesis generation. IPA software has been used for this purpose in a number of studies investigating effects of EDCs on fish tissue transcriptomes (Garcia-Reyero et al., 2011; Lam et al., 2008; Miller et al., 2012; Wang et al., 2010).

The coho salmon pituitary backbone, generated by de novo assembly of Illumina® sequences, included 40,293 annotated contigs. This backbone may include multiple contigs representing different portions of the same gene. However, 11,319 contigs mapped to unique human orthologs, indicating at least this number of unique genes present in the backbone. This is similar to the number of unique transcripts (~12,000) reported in the mouse pituitary (Brinkmeier et al., 2009) and far exceeds the few previous pituitary sequencing efforts in fish (Cao et al., 2011; Ma et al., 2011). Our backbone included at least one variant of each pituitary hormone

transcript indicating good breadth of coverage; and rare transcripts, such as some hormone receptors were present, indicating good depth of coverage. Furthermore, *LHB*, which is quite low in sub-adult salmonids and at the level of detection for our standard qPCR protocol (median CT value = 37), was revealed in control fish pituitaries by RNA-Seq.

Effects of EE2 on phenotypic endpoints of the female reproductive axis

A number of phenotypic endpoints have been used to examine effects of natural and synthetic estrogens on reproduction in female fish, including behavior, fecundity, gonadal size and stage. In the present study we did not observe significant effects of EE2 on ovary mass, GSI, stage of ovarian follicles, or plasma E2 levels in coho salmon at the primary oocyte growth stage. This is in contrast to other studies where exposure to similar concentrations of EE2 has been shown to alter ovarian histology (Miles-Richardson et al., 1999; Van den Belt et al., 2002, 2001) and decrease GSI (Parrott and Blunt, 2005; Pawlowski et al., 2004; Van den Belt et al., 2002, 2001) and plasma steroid concentrations (Flores-Valverde et al., 2010; Hoffmann et al., 2006) in a number of fish species. Most of these studies have been carried out in adult fish and discrepancies may be due to differing sensitivities to EE2 based on species or stage of maturity. Previous studies in juvenile rainbow trout found that long-term exposures to estrogens did not result in detectable changes in GSI (Thorpe et al., 2000) or gonadal stage (Ackermann et al., 2002). Further, ovarian stage was variable among individuals in the present study and changes in gonad growth may be obscured by the small gonad to body size ratio in juvenile fish (Thorpe et al., 2000).

Despite a lack of effect on ovarian development and plasma sex steroid concentrations, *VTG* mRNA was up-regulated 96,890- and 887,862-fold in response to EE2 treatment for 1 and

6 weeks, respectively. This is similar to the magnitude of *VTG* mRNA increase reported in female juvenile rainbow trout exposed to 26 ng EE2/L for 14 days (Thomas-Jones et al., 2003). As such, *VTG* induction in the current study indicated that the exposure was sufficient to elicit gene expression changes and that the fish were responding to the EE2 treatment.

Effects of EE2 on pituitary hormones

In the current study, two of the most highly up- and down-regulated contigs in the pituitary after EE2 exposure were *LHB* and *FSHB*, subunits of the two major pituitary hormones controlling reproduction in fish. For *LHB*, pituitary transcript levels increased 241- and 395-fold in EE2 exposed fish at 1 and 6 weeks, while a 3.4-fold reduction in *FSHB* mRNA occurred at 6 weeks. This result is not surprising because in many vertebrates estrogens affect gonadotropin synthesis and release both positively and negatively, depending on the stage of reproduction (reviewed by Yaron et al., 2003). In agreement with our results, estrogens generally have a negative feedback effect on FSH synthesis and a positive feedback effect on LH synthesis in immature fish.

Numerous studies have shown that estrogens and aromatizable androgens elevate pituitary LH content and *LHB* mRNA in immature fish (reviewed by Melamed et al., 1998). In a previous study in vitellogenic coho salmon, 7 d of E2 treatment increased *LHB* mRNA expression and pituitary LH protein levels (Dickey and Swanson, 1998). *LHB* was also up-regulated in juvenile fish in response to xenoestrogens including EE2 (Johns et al., 2009) and 4-nonylphenol (Maeng et al., 2005; Yadetie and Male, 2002). In the case of FSH, previous studies showed that E2 treatment decreased plasma and pituitary FSH levels in coho salmon (Dickey and Swanson, 1998). Decreases in *FSHB* mRNA were not observed in juvenile or sub-adult female

fish in response to short-term (up to 7d) estrogen treatment (Dickey and Swanson, 1998; Maeng et al., 2005; Yadetie and Male, 2002). However, longer term exposure (18 weeks) to 4-nonylphenol decreased pituitary *FSHB* mRNA and FSH protein levels in maturing female rainbow trout (Harris et al., 2001). Similarly, in the present study, reduction in pituitary *FSHB* mRNA levels only occurred after the prolonged exposure to EE2 suggesting that estrogen regulation of *FSHB* transcription is gradual or involves more indirect modes of action, taking several weeks for significant differences to appear.

The actions of EE2 on steady state levels of *LHB* mRNA could be either direct effects on transcription or indirect through alterations in gonadal factors or neurohormones such as GNRH that regulate LH synthesis (reviewed by Yaron et al., 2003). In the current study, EE2 exposure significantly altered mRNA levels for genes involved in GNRH signaling at 1 week including up regulation of *GNRHRI* and *JUN* at both 1 and 6 weeks. The *LHB* promoter in Chinook salmon has a proximal estrogen response element necessary for GNRH stimulation of *LHB* transcription via binding to transcription factor AP-1 (*JUN*). Stimulation of Chinook salmon *LHB* transcription by *JUN* was enhanced by *ESR1*, indicating a synergistic action of GNRH and estrogen on *LHB* transcription (Melamed et al., 2006; reviewed by Wang et al., 2009). Thus it is possible that increased abundance of *GNRHRI* and *JUN* mRNAs in response to EE2 may contribute to the strong induction of *LHB*.

TGF- β signaling is another important pathway in the regulation of gonadotropins in fish and may mediate the observed effects of EE2 on *FSHB* and *LHB*. In the current study, contig levels for several TGF- β signaling molecules were altered in response to EE2. Levels of *BMPRI1B* (bone morphogenetic protein receptor type-1B), a receptor involved in increasing *FSHB* promoter activity in mice (Ho and Bernard, 2009), were decreased in pituitary glands of

coho salmon exposed to EE2 for 1 week. Additionally, expression of ZFYVE9 (zinc finger FYVE domain-containing protein 9) was down-regulated at 6 weeks. ZFYVE9 is involved in recruiting Smads to the TGF- β receptor complex and promoting TGF- β signaling and activin receptor-mediated signaling (reviewed by Welt et al., 2002). Recently, it was shown that the Chinook salmon and goldfish *FSHB* promoters contain Smad response elements that mediate activin stimulation of the *FSHB* gene (Wang et al. 2009; Lau et al., 2012); however no work has been done to address how estrogens may modulate Smad signaling.

We did not observe effects of EE2 on other important TGF- β signaling molecules involved in gonadotropin regulation, such as activin inhibin, and follistatin (FST) (Aroua et al., 2012). Although IPA network analysis suggested an interaction between altered levels of *FSHB* and *LHB* with inhibin (Fig. 3.6), read counts for inhibin subunits were below our threshold for inclusion in relative expression analyses. Further investigation of estrogen action on TGF- β signaling molecules identified in this study may help elucidate the mechanism of negative estrogen feedback on *FSHB* transcription.

Regardless of the mechanisms by which EE2 alters gonadotropin transcript levels, disruption of gonadotropin synthesis could have a significant impact on salmon reproduction. Previous studies in maturing coho salmon suggest that while estrogen-induced increases in *LHB* transcription may lead to increased LHB protein in the pituitary, this does not typically result in detectable plasma LH levels (Dickey and Swanson, 1998). Although we did not measure plasma FSH levels in the current study due to insufficient amounts of plasma, previous studies showed that treatment of maturing coho salmon with estrogen reduced plasma FSH (Dickey and Swanson, 1998). Given the importance of FSH in regulating ovarian gene expression during

secondary oocyte growth (Luckenbach et al., 2013, 2011), prolonged exposure to EE2 and subsequent reductions in FSH could negatively impact ovarian growth and development.

The only other pituitary hormones that had altered transcript levels in response to EE2 were *SLA* and *SLB*, which were up-regulated at 6 weeks. This is consistent with the stimulatory effect of estrogen on *SL* mRNA levels in masu salmon (Onuma et al., 2005) and correlations in plasma levels of SL and estradiol during oogenesis in coho salmon (Rand-Weaver et al., 1992). Although estrogens have been shown to affect seawater tolerance in juvenile salmonids (McCormick et al., 2005), mRNA levels of pituitary hormones associated with osmoregulation (PRL, GH, TSHB, ACTH) were not affected by EE2 exposure in the current study.

Effects of EE2 on known estrogen-regulated pathways/processes

Estrogens are well known regulators of cell proliferation (reviewed by Spady et al., 1999), cell death (reviewed by Seilicovich, 2010), and cell cycle (reviewed by Sutherland et al., 1983) in a variety of cell types including mammal pituitary cells (Zárate et al., 2010). In the current study, EE2-exposure resulted in up-regulation of contigs involved in cell cycle (*CCNF* [cyclin F], *HCFC1*, and *RFC5* [replication factor C subunit 15]) and cell proliferation, cell death, and tissue morphology (*DKK3* [dickkopf-related protein 3], *GATA2* [endothelial transcription factor GATA-2], *KLF2* [krueppel-like factor 2], *PPP3CA* [serine/threonine-protein phosphatase 2B catalytic subunit α isoform], and *TIMP2*) at both 1 and 6 weeks suggesting estrogens may act to control these cell processes in pituitary glands of fish as well.

In fishes, the role of estrogens in cellular maintenance, cell turnover or remodeling in the pituitary has not been reported. However, in the rainbow trout, increased plasma E2 and pituitary *LHB* mRNA levels during late vitellogenesis are associated with increased number and

size of LH producing gonadotrophs (Naito et al., 1991). Further, in juvenile male African catfish, aromatizable androgens and estrogens stimulated gonadotroph growth and development of mature-type gonadotrophs (Cavaco et al., 2001). These results suggest that in the current study, growth, proliferation, and maturation of gonadotrophs may be occurring in response to EE2 and the strong EE2-induction of *LHB*. In support of this, EE2 exposure up-regulated contigs with putative roles in netrin signaling at 1 week (*ABLIM3* [actin-binding LIM protein 3] and *PPP3CA*) and 6 weeks (*PPP3CA*, *RYR1* [ryanodine receptor 1], *RYR3* [ryanodine receptor 3], *UNC5D* [netrin receptor UNC5D]). Netrin signaling is involved in cell adhesion and tissue morphogenesis in a variety of tissues and directs cell and axon migration in neural development (Sun et al., 2011). As such, netrin signaling may be involved in pituitary gland remodeling or perhaps in directing innervation of new gonadotroph cells.

Expression of contigs with putative roles in calcium signaling was significantly altered in pituitaries of EE2-exposed salmon at both 1 and 6 weeks. Calcium signaling has been implicated in estrogen-regulated cell death (Pretorius and Bornman, 2005) and cell proliferation (Watson et al., 2010) in several species and has previously been identified as a target of estrogen treatment in fish (Kirk et al., 2003; Martyniuk et al., 2010; Zhang et al., 2009). Calcium signaling also mediates actions of GNRH on gonadotropin release (Jobin and Chang, 1992). We hypothesize that estrogen-regulated calcium signaling may alter a variety of cellular processes in the pituitary, including hormone secretion.

Previous studies on the effects of EE2 on the reproductive axis in fish have identified disruption of steroidogenesis and lipid metabolism pathways (Flores-Valverde et al., 2010; Hoffmann et al., 2006; Hogan et al., 2010). Estrogens have also been shown to alter steroidogenic enzymes in the brain of fish (Arukwe, 2005; Lyssimachou and Arukwe, 2007). In

agreement with this, expression of contigs involved in lipid metabolism was altered at both time points. Contig levels for retinoid x receptor, β (*RXR β*) and apolipoprotein B (*APOB*) were significantly increased at 1 and 6 weeks, respectively. *RXR β* and *APOB* have previously been identified as important to reproductive function in the liver and ovary of fish for their roles in steroid metabolism and cholesterol uptake (Villeneuve et al., 2012). In addition, contigs with putative roles in PPAR signaling were altered in response to EE2 exposure including up-regulation of *PPARD* (PPAR delta). PPAR signaling is also involved in lipid metabolism and provides further evidence for alteration of lipid metabolism in the pituitary of EE2-exposed fish (Lee et al., 2003). It is unclear what function these genes have in pituitary glands or how altered lipid metabolism in the pituitary may affect reproduction. However, disruption of lipid metabolism in the pituitary provides additional evidence that this process is a sensitive target of estrogen exposure in a variety of tissues.

Effects of EE2 on expression of genes in circadian rhythm

In addition to alteration of known-estrogen responsive genes, transcript levels of 31 genes were consistently regulated by EE2 treatment in salmon pituitaries, but these genes were not identified as EE2- or E2-regulated in the IPA Knowledgebase® or in various fish studies. Interestingly, a considerable number of these were circadian clock genes (*PER1* and *PER2*) or genes related to the circadian rhythm signaling pathway (*DBP*, *NPAS2*, and *RORB*). In fact, circadian rhythm signaling was the first and second most significantly altered pathway in pituitaries of EE2 exposed salmon at 1 and 6 weeks respectively. Interestingly, 2 other circadian clock genes, *CLOCK* (circadian locomotor output cycles protein kaput) and *CRY2A*

(cryptochrome 2a), have previously been identified as significantly altered by EE2 exposure in fish testis (Garcia-Reyero et al., 2009) and liver (De Wit et al., 2010), respectively.

Circadian rhythm signaling is most often studied in the context of the master clock, where clock genes in the retina and suprachiasmatic nucleus (SCN) of the brain control daily cycles in response to light. Clock genes are present in a variety of tissues where they control the rate of cell turnover and gene expression (reviewed by Zhang and Kay, 2010). The presence of a circadian clock in the pituitary was described in mice (Guillaumond et al., 2012). Further, the presence of rhythmic expression of *PER2* in the SCN and pituitary gland was recently reported in fish (Watanabe et al., 2012).

Interestingly, in the present study, circadian rhythm signaling molecules were grouped in a gene network with GNRH signaling molecules following 1 week of EE2 exposure (Fig. 3.5). In mammals, circadian clocks have also been implicated in the pulsatile timing of GNRH release (reviewed by Chappell, 2005). In teleosts, GNRH is released into the pituitary via direct innervation, precluding measurement of pulsatility of GNRH release. In lieu of direct measurement, the pulsatile release of gonadotropin hormones has provided evidence that GNRH may be released in a pulsatile nature in fish (Zohar et al., 1986). Recent studies in medaka showed that GNRH1 neurons exhibited time-of-day dependent firing activity that corresponded to changes in gonadotropin release (Karigo et al., 2012).

Although clock genes are not widely recognized as being estrogen responsive, there is evidence in mammals that expression of core clock genes in the rodent uterus (Nakamura et al., 2008) and SCN are altered in response to estrogen (Nakamura et al., 2005, 2001). Additionally, estrogen regulation of clock genes has been implicated in control of ovulation timing (de la Iglesia and Schwartz, 2006) and *CLOCK* mutant rodents have irregular estrous cycles and a lack

of LH surge (Dolatshad et al., 2006; Miller et al., 2004). These previous reports and the current results suggest a role of estrogen in regulating the interaction between GnRH signaling and circadian rhythms in the pituitary to achieve appropriate reproductive timing and, on the other hand, the potential for xenoestrogens to disrupt normal reproductive development and/or timing. We aim to test this hypothesis in future investigations.

Conclusion

In this study we have shown that exposure to low levels of one xenoestrogen, EE2, had a profound affect on the pituitary transcriptome in female salmon in previtellogenic stages of ovarian growth, altering the expression of hundreds of genes involved in multiple pathways. Since EE2 acts as an estrogen receptor agonist, these results provide insight into potential normal actions of estrogens as well as modes for disruption of pituitary function by xenoestrogens. Gonadotropin subunits and genes involved in gonadotropin synthesis and/or release were among the most strongly affected genes, suggesting that estrogenic chemicals could alter reproduction at multiple points in the gonadotropin signaling pathway. Because this experiment was conducted *in vivo*, alterations in gonadotropin transcript levels could be a result of estrogen action on the brain, the pituitary, and/or feedback from the gonads. Thus, further research is needed to elucidate the mechanism of estrogen regulation of pituitary gonadotropins. Although EE2 exposure for up to 6 weeks altered pituitary gonadotropin transcript levels in sub-adult salmon, no significant affects on ovarian development were found in this study. It remains to be seen how longer term exposure to exogenous estrogens might affect pituitary gonadotropin protein levels and secretion, as well as downstream effects on gonadal development and fertility.

EE2 exposure also affected transcript levels of genes known to be involved in cell turnover, calcium signaling, and steroid metabolism in mammalian species. While we have no direct evidence for the functions of many of these genes in fish, the results of the pathway analysis suggest estrogens may be involved in regulating a wide range of pituitary functions including pituitary gland development, maturation, and hormone release. Most surprisingly, exposure to EE2 altered expression of circadian rhythm signaling molecules. In a seasonally spawning fish, such as salmon, timing is critical for successful reproduction. As such, investigations into the role of estrogen in regulating expression of pituitary circadian clock genes and downstream effects on pituitary cell function and ultimately seasonal timing of spawning will be important in determining impacts of EDCs on salmon reproduction and recruitment.

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Table 3.1: Pituitary contigs and putative pathways that were significantly regulated after 1 week EE2 exposure in female juvenile coho salmon. (A) The most significantly affected contigs at 1 week EE2 exposure identified by DESeq (base mean >10, fold change ≥ 1.8 , p-value <0.01) that were mapped to Ingenuity® Knowledge Base are listed. Shading indicates genes previously reported to be important to the reproductive axis in fish (Villeneuve et al., 2011). (B) List of significantly over-represented putative pathways following 1 week EE2-exposure, the associated p-value (Fisher’s exact test $P < 0.05$), and the ratio of contigs significantly altered by EE2 relative to control over the total number of genes in that pathway in the Ingenuity® Knowledge Base. Gene titles and canonical pathway names are from Ingenuity® Systems.

A. Top contigs (base mean > 10, fold change ≥ 1.8 , p<0.01)			B. Top Putative Pathways		
Gene Title (up-regulated)	Fold Change	P-value	Ingenuity Canonical Pathway	P-Value	Ratio
luteinizing hormone beta polypeptide	241.2	4.14E-10	Circadian Rhythm Signaling	2.24E-06	5/37
TIMP metalloproteinase inhibitor 2	8.4	1.40E-07	LXR/RXR Activation	1.45E-05	7/136
Fanconi anemia, complementation group A	7.9	6.33E-09	VDR/RXR Activation	1.74E-03	4/81
metastasis associated 1	5.2	6.64E-03	Aryl Hydrocarbon Receptor Signaling	2.24E-03	5/161
sodium channel, voltage-gated, type IV, alpha subunit	4.0	5.58E-03	Calcium Signaling	6.31E-03	5/211
heat shock 70kDa protein 4	3.9	9.09E-03	GNRH Signaling	9.33E-03	4/147
retinoid X receptor, beta	3.8	4.18E-03	D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	9.77E-03	2/35
protein phosphatase 3, catalytic subunit, alpha isozyme	3.8	5.38E-03	D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	9.77E-03	2/35
LON peptidase N-terminal domain and ring finger 3	3.4	2.17E-03	Diphthamide Biosynthesis	1.32E-02	1/13
KIAA1731	3.1	6.71E-07	Huntington's Disease Signaling	1.41E-02	5/237
phosphatidylinositol transfer protein, cytoplasmic 1	3.1	7.75E-03	Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.015	3/89
G protein-coupled receptor 6	2.7	4.88E-03	TGF- β Signaling	0.017	3/89
growth regulation by estrogen in breast cancer 1	2.6	9.79E-05	Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	0.017	2/52
zinc finger protein 200	2.5	5.39E-03	TR/RXR Activation	0.018	3/96
transducer of ERBB2, 1	2.5	6.31E-03	Inhibition of Angiogenesis by TSP1	0.021	2/39
heparan sulfate proteoglycan 2	2.5	5.20E-03	Stearate Biosynthesis I (Animals)	0.023	2/50
neuromedin U receptor 2	2.4	7.29E-03	PPAR Signaling	0.024	3/105
replication factor C (activator 1) 5, 36.5kDa	2.4	5.12E-03	PPAR α /RXR α Activation	0.024	4/184
microtubule associated monooxygenase, calponin and LIM domain containing 2	2.3	1.63E-04	Netrin Signaling	0.027	2/57
MAM domain containing 2	2.3	1.03E-03	D-myo-inositol-5-phosphate Metabolism	0.027	2/52
gonadotropin-releasing hormone receptor	2.3	1.61E-03	RAR Activation	0.027	4/189
ubiquitin specific peptidase 54	2.3	4.56E-03	Glucocorticoid Receptor Signaling	0.029	5/294
Kruppel-like factor 2 (lung)	2.2	6.21E-03	3-phosphoinositide Degradation	0.030	2/54
D site of albumin promoter (albumin D-box) binding protein	1.9	9.22E-03	Role of NFAT in Cardiac Hypertrophy	0.031	4/207
host cell factor C1 (VP16-accessory protein)	1.8	7.84E-03	Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	0.035	2/45
Gene Title (down-regulated)	Fold Change	P-value	Androgen Signaling	0.036	3/143
transmembrane protein 164	-1.8	4.61E-03	EGF Signaling	0.036	2/52
angiopoietin-like 7	-1.9	8.96E-03	nNOS Signaling in Neurons	0.038	2/52
aldo-keto reductase family 1, member B1 (aldose reductase)	-2.0	6.51E-03	Glycogen Biosynthesis II (from UDP-D-Glucose)	0.039	1/12
chromosome 1 open reading frame 51	-2.0	3.14E-03	Pregnenolone Biosynthesis	0.039	1/13
MYST/Esal-associated factor 6	-2.1	1.51E-03	CD28 Signaling in T Helper Cells	0.041	3/132
clusterin	-2.7	1.55E-04	Hereditary Breast Cancer Signaling	0.041	3/128
basic helix-loop-helix family, member e41	-3.0	1.85E-05	3-phosphoinositide Biosynthesis	0.045	2/71
myotubularin related protein 8	-4.3	5.81E-04	Nur77 Signaling in T Lymphocytes	0.050	2/63
			Estrogen Receptor Signaling	0.051	3/136
			PI3K Signaling in B Lymphocytes	0.052	3/140

Table 3.2: (A) Most significantly altered pituitary contigs and (B) putative pathways affected after 6 weeks EE2 exposure in juvenile coho salmon. For details, see Table 1 caption

A. Top Contigs (base mean > 10, fold change ≥1.8, p<0.01)			A. Top Contigs (base mean > 10, fold change ≤ -1.8, p<0.001)		
Gene Title (up-regulated)	Fold Change	P-value	Gene Title (down-regulated)	Fold Change	P-value
luteinizing hormone beta polypeptide	395.1	5.44E-143	aldehyde oxidase 3	-3.8	3.33E-03
myosin, heavy chain 11, smooth muscle nucleolar complex associated 3 homolog (S. cerevisiae)	31.6	2.31E-05	DnaJ (Hsp40) homolog, subfamily B, member 5	-3.5	8.96E-03
LON peptidase N-terminal domain and ring finger 3	3.8	2.26E-03	follicle stimulating hormone, beta polypeptide	-3.4	2.69E-04
apolipoprotein B (including Ag(x) antigen)	3.7	1.12E-04	transmembrane protein 45A	-2.9	4.36E-04
tumor necrosis factor receptor superfamily, member 14	3.6	2.41E-04	TBC1 domain family, member 8B (with GRAM domain)	-2.9	3.90E-03
gonadotropin-releasing hormone receptor	3.5	5.40E-03	Williams-Beuren syndrome chromosome region 17	-2.7	4.75E-04
cryptochrome 2 (photolyase-like)	3.5	1.34E-08	BRCA1 associated RING domain 1	-2.5	8.68E-03
neuronal PAS domain protein 2	3.2	2.72E-03	peroxisomal proliferator-activated receptor A interacting complex 285	-2.4	3.11E-03
glycerol-3-phosphate dehydrogenase 1-like	3.1	1.16E-03	netrin G2	-2.1	8.46E-03
keratin 17	3.0	2.83E-03	crystallin, gamma B	-2.1	8.16E-03
tripartite motif containing 3	2.8	4.81E-03	replication protein A3, 14kDa	-2.1	1.55E-04
chromosome 17 open reading frame 28	2.7	3.59E-08	xanthine dehydrogenase	-2.0	2.35E-03
WWC family member 3	2.6	4.41E-03	nuclear receptor subfamily 2, group E, member 3	-2.0	7.06E-03
spectrin repeat containing, nuclear envelope 1	2.5	7.55E-04	claudin 6	-1.9	5.30E-03
adrenoceptor beta 2, surface	2.5	2.62E-03	Kruppel-like factor 11	-1.9	2.65E-03
fibroblast growth factor 12	2.5	7.26E-04	MYST/Esa1-associated factor 6	-1.8	2.19E-04
chordin	2.5	4.66E-03	transient receptor potential cation channel, subfamily V, member 1	-1.8	2.86E-03
growth regulation by estrogen in breast cancer 1	2.4	9.78E-03	prickle homolog 2 (Drosophila)	-1.8	4.18E-03
RecQ protein-like 4	2.4	2.11E-06	nudix (nucleoside diphosphate linked moiety X)-type motif 15	-1.8	3.32E-03
collagen, type VI, alpha 1	2.4	1.63E-04	HEAT repeat containing 1	-1.8	1.42E-03
SH2B adaptor protein 2	2.4	1.14E-06	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)	-1.8	5.27E-03
D site of albumin promoter (albumin D-box) binding protein	2.3	4.26E-03	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	-1.8	7.71E-03
cyclin-dependent kinase-like 1 (CDC2-related kinase)	2.3	3.92E-06	nebulin	-1.8	8.28E-03
sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	2.2	1.95E-03			
phosphatidylinositol-specific phospholipase C, X domain containing 1	2.2	2.96E-03			
thymus, brain and testes associated	2.2	1.98E-04			
vitronectin	2.2	5.83E-04			
ryanodine receptor 1 (skeletal)	2.2	5.41E-03			
G protein-coupled receptor 6	2.2	7.39E-03			
NDRG family member 2	2.1	5.42E-04			
spermatogenesis associated 18	2.1	3.70E-04			
fibronectin type III domain containing 3A	2.1	1.59E-03			
ClpX caseinolytic peptidase X homolog (E. coli)	2.1	5.59E-03			
microtubule associated monooxygenase, calponin and LIM domain containing 2	2.0	1.00E-03			
ryanodine receptor 3	2.0	5.01E-04			
cyclin F	2.0	1.91E-03			
dickkopf 3 homolog (Xenopus laevis)	2.0	8.56E-05			
tumor protein p53 inducible nuclear protein 2	2.0	1.46E-04			
calcium channel, voltage-dependent, alpha 2/delta subunit 2	2.0	1.99E-03			
plasminogen activator, urokinase	2.0	2.45E-03			
LON peptidase N-terminal domain and ring finger 1	1.9	9.70E-04			
CASK interacting protein 2	1.9	9.57E-04			
MAM domain containing 2	1.9	1.14E-03			
RAR-related orphan receptor B	1.9	9.89E-03			
myomesin 1, 185kDa	1.8	3.34E-04			
plexin A2	1.8	2.56E-03			
GATA binding protein 2	1.8	1.07E-03			
nuclear receptor corepressor 2	1.8	4.06E-03			
Neurofascin	1.8	8.39E-03			
glutaryl-CoA dehydrogenase	1.8	4.68E-03			
potassium voltage-gated channel, shaker-related subfamily, member 7	1.8	3.67E-03			
host cell factor C1 (VP16-accessory protein)	1.8	9.86E-03			
retinal pigment epithelium-specific protein 65kDa	1.8	4.66E-03			
	1.8	5.16E-03			

B. Top Putative Pathways		
Ingenuity Canonical Pathway	P-Value	Ratio
Calcium Signaling	7.59E-04	12/211
Circadian Rhythm Signaling	8.13E-04	5/37
Actin Cytoskeleton Signaling	1.18E-03	13/238
nNOS Signaling in Skeletal Muscle Cells	4.27E-03	3/31
Pregnenolone Biosynthesis	7.24E-03	2/13
Netrin Signaling	1.15E-02	4/57
Histidine Degradation VI	1.66E-02	2/20
IL-15 Production	2.24E-02	3/31
Cell Cycle Control of Chromosomal Replication	2.24E-02	3/31
L-cysteine Degradation III	2.29E-02	1/9
Glutaryl-CoA Degradation	0.025	2/24
Neuropathic Pain Signaling In Dorsal Horn Neurons	0.026	6/108
Ubiquinol-10 Biosynthesis (Eukaryotic)	0.029	2/29
Triacylglycerol Biosynthesis	0.038	3/46
Inhibition of Angiogenesis by TSP1	0.042	3/39
Cysteine Biosynthesis/Homocysteine Degradation	0.045	1/8
Putrescine Biosynthesis III	0.045	1/4
Role of BRCA1 in DNA Damage Response	0.047	4/65
Mismatch Repair in Eukaryotes	0.050	2/24

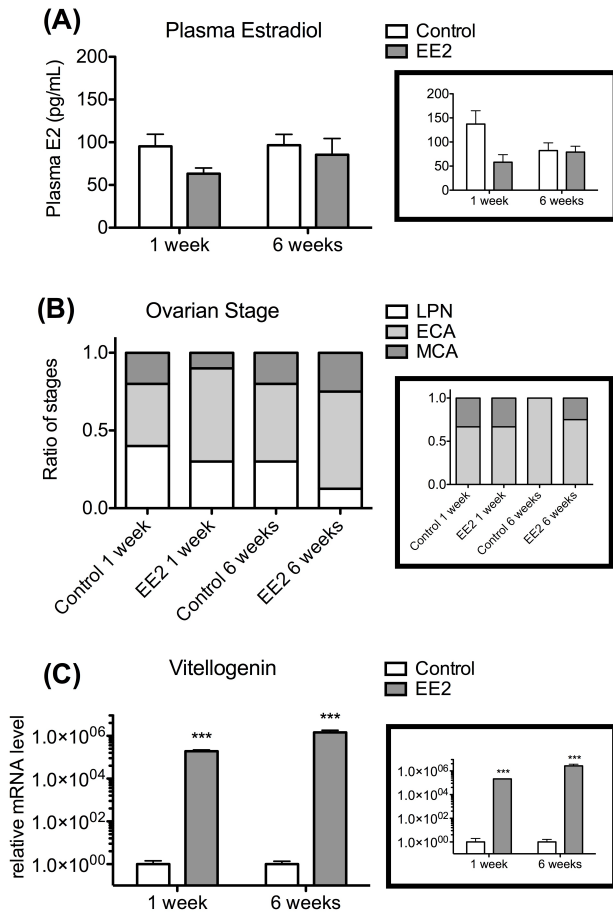


Figure 3.1. Reproductive endpoints of control and EE2-exposed female juvenile coho salmon. The data are expressed as mean \pm SEM ($n = 8 - 10$). Data from individuals used for Illumina sequencing are provided in the insets ($n = 3 - 4$). (A) Plasma estradiol (E2) (pg/mL), (B) ratio of individuals at late perinucleolar (LPN) early cortical alveolar (ECA), or mid to late cortical alveolar (MCA), and (C) liver *VTG* (vitellogenin) mRNA levels measured by qPCR and normalized to the housekeeping gene *EEF1A* (eukaryotic elongation factor 1 α). Plasma E2 and hepatic *VTG* mRNA data were log-transformed where necessary to meet parametric test criteria. Significant differences were determined by 2-way ANOVA followed by Bonferroni post-hoc test *** Signifies significance at $p < 0.001$ compared to control.

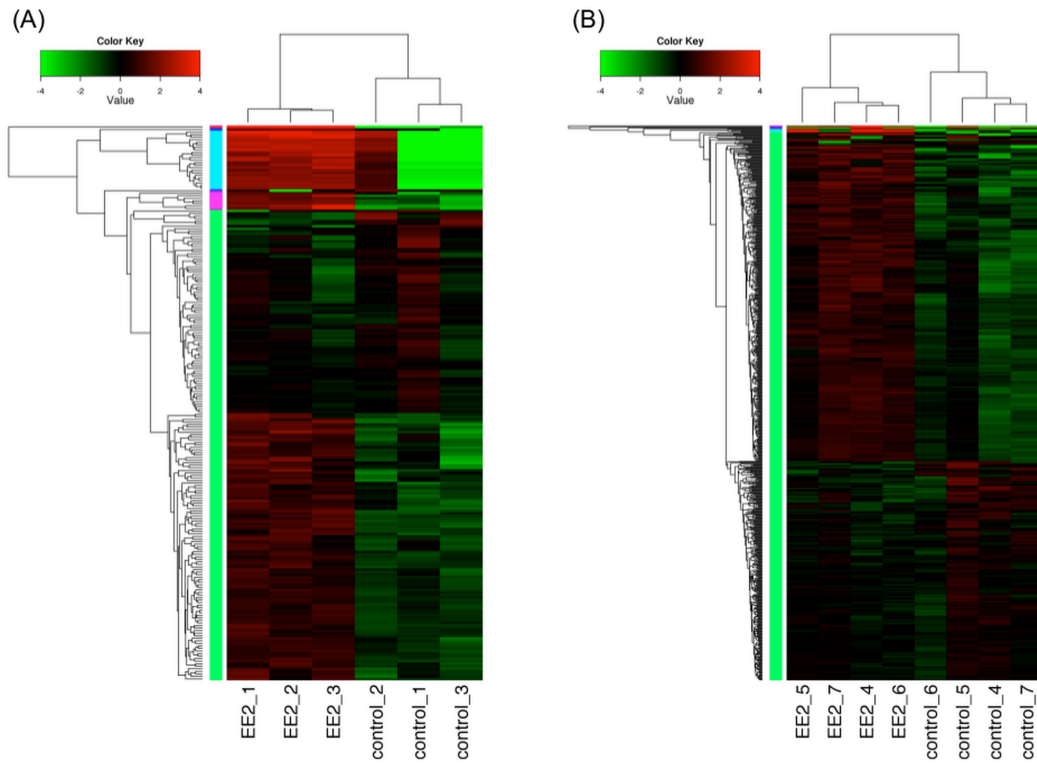


Figure 3.2. Clustering analysis of differentially expressed contigs ($p < 0.05$, baseMean > 10) across individual control and EE2-exposed fish at (A) 1 week and (B) 6 weeks. Relative expression of contigs (rows) is displayed across individuals (columns) with red representing up-regulation and green representing down-regulation. Each column represents data from a single individual.

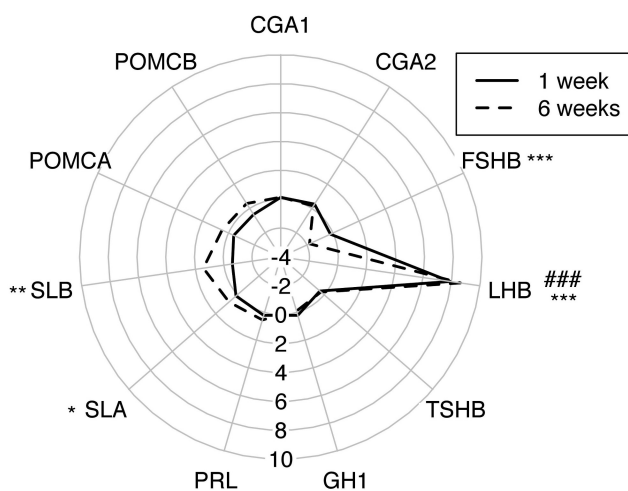


Figure 3.3. Log₂ fold-change of contig levels annotated as pituitary hormone transcripts in response to EE₂-exposure at 1 week (solid line) and 6 weeks (dashed line) as measured by RNA-Seq. Significant differences were determined using DESeq with a p -value threshold of $p < 0.05$. Significant differences are indicated by ### $p < 0.001$ at 1 week, *** $p < 0.001$ at 6 weeks, ** $p < 0.01$ at 6 weeks, * $p < 0.05$ at 6 weeks. CGA1: glycoprotein hormones α 1 subunit; CGA2: glycoprotein hormones α 2 subunit; FSHB: follicle stimulating hormone β subunit; LHB: luteinizing hormone β subunit; TSHB: thyroid stimulating hormone β subunit; GH1: growth hormone 1; PRL: prolactin; SLA: somatolactin α ; SLB: somatolactin β ; POMCA: proopiomelanocortin a; POMCB: proopiomelanocortin b.

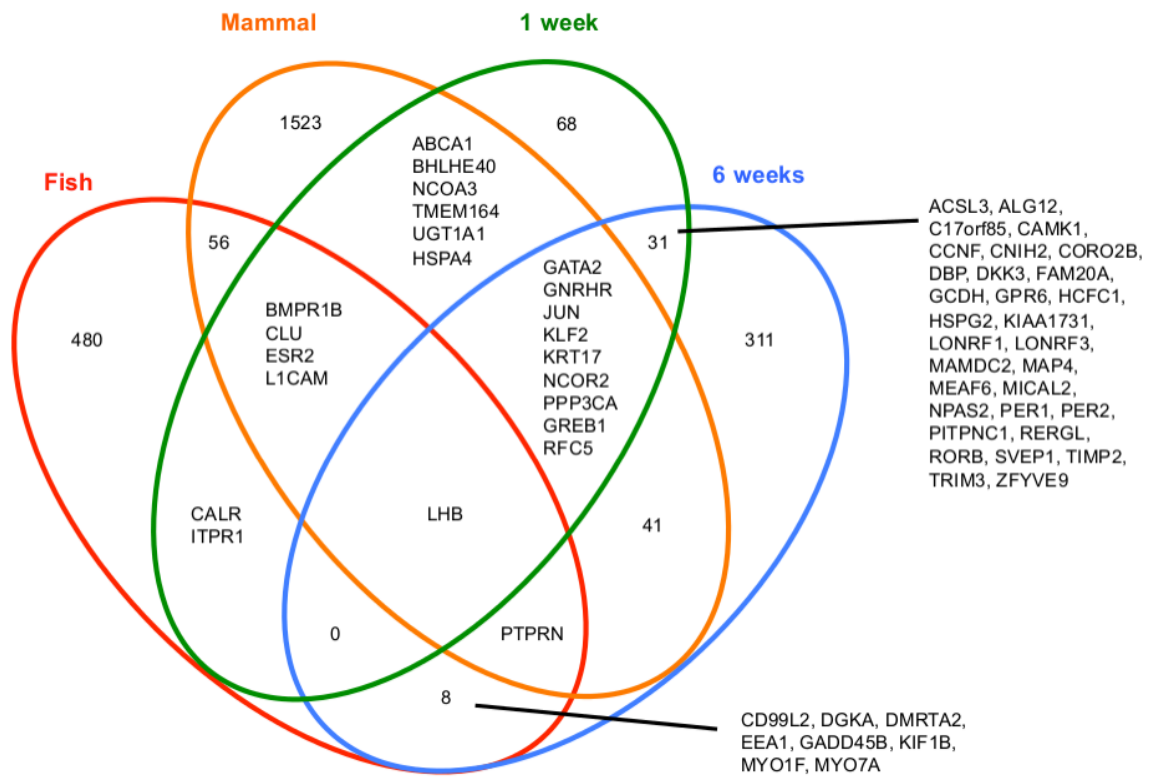
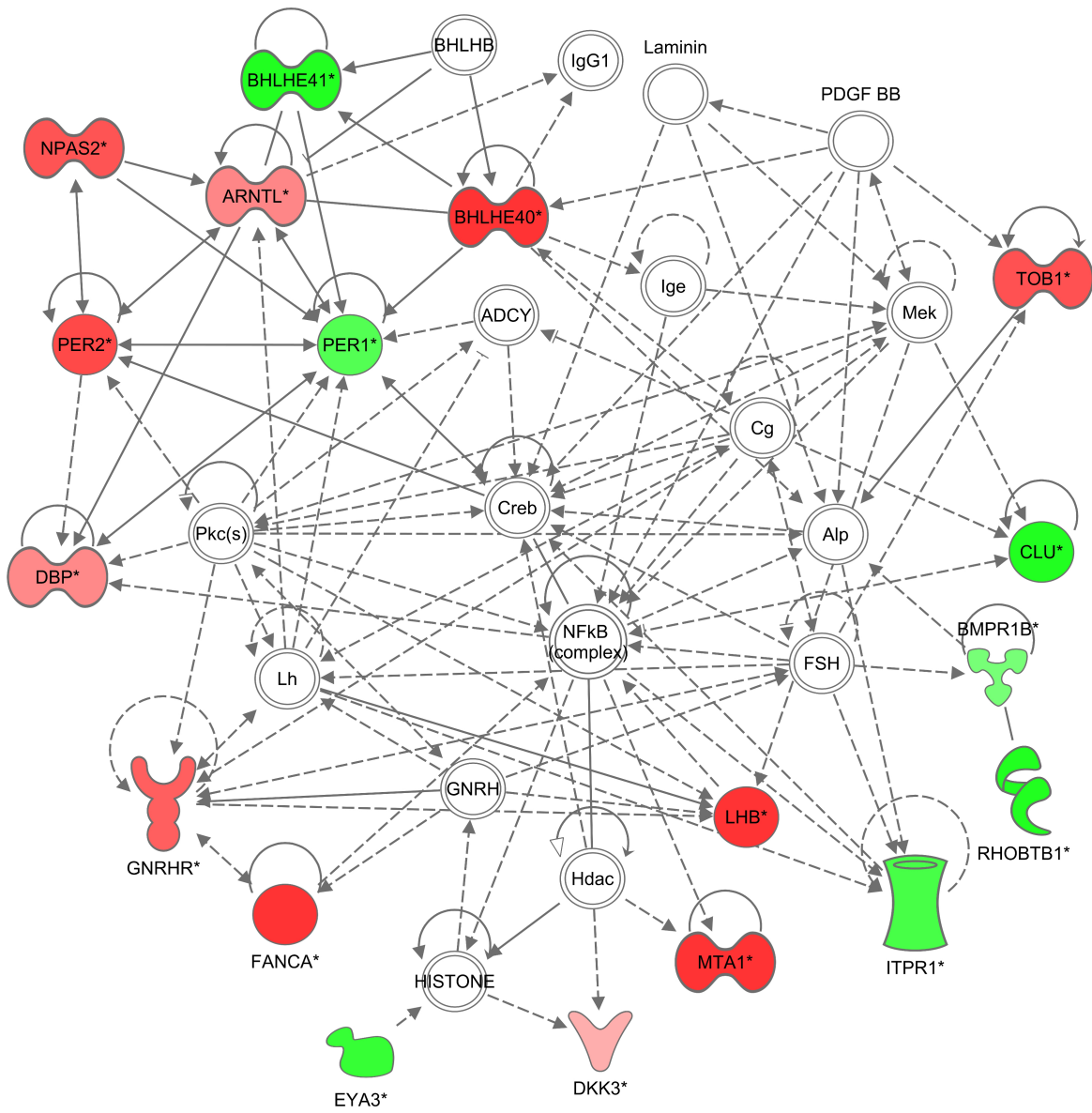
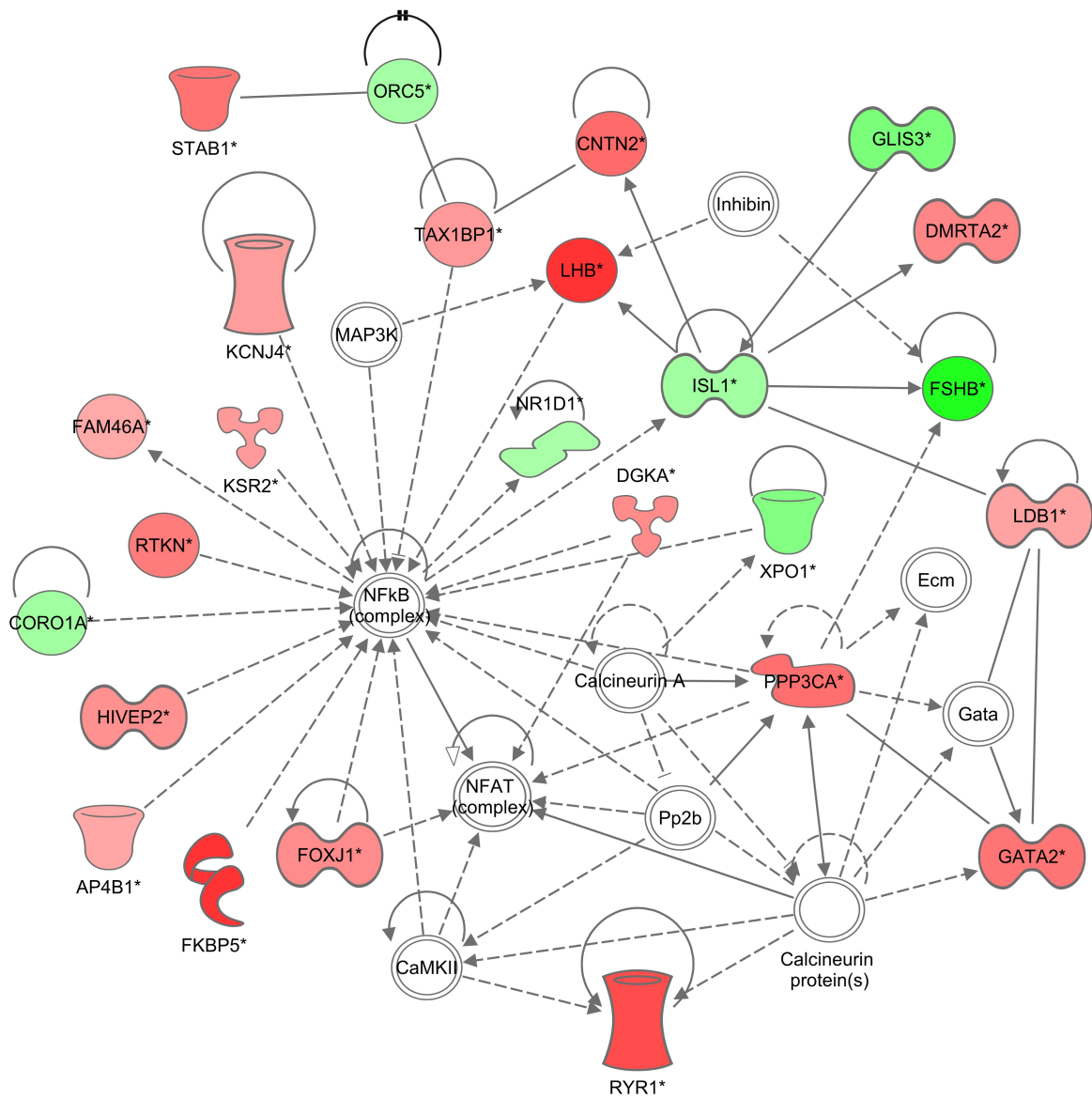


Figure 3.4. Comparison of zebrafish and human orthologs of contigs that were significantly altered (DESeq $p < 0.05$) in coho salmon pituitary glands at 1 week or 6 weeks with genes previously reported to be estrogen-responsive in mammals (curated by Ingenuity Systems Inc.) or fish (list compiled from Martyniuk et al., 2006; 2007; Santos et al., 2007; Benninghoff et al., 2008; Garcia-Reyero et al., 2009). ABCA1: ATP-binding cassette, sub-family A (ABC1), member 1; ACSL3: acyl-CoA synthetase long-chain family member 3; ALG12: asparagine-linked glycosylation 12, alpha-1,6-mannosyltransferase homolog; BHLHE40: basic helix-loop-helix family, member e40; BMPR1B: bone morphogenetic protein receptor, type IB; C17orf85: chromosome 17 open reading frame 28; CAMK1: calcium/calmodulin-dependent protein kinase I; CCNF: cyclin F; CD99L2: CD99 molecule-like 2; CLU: clusterin; CNIH2: cornichon homolog 2; CORO2B: coronin, actin binding protein, 2B; DBP: D site of albumin promoter (albumin D-box) binding protein; DGKA: diacylglycerol kinase, alpha 80kDa; DKK3: dickkopf 3 homolog; DMRTA2: DMRT-like family A2; EEA1: early endosome antigen 1; ESR2: estrogen receptor 2 (ER beta); FAM20A: family with sequence similarity 20, member A; GADD45B: growth arrest and DNA-damage-inducible, beta; GATA2: GATA binding protein 2; GCDH: glutaryl-CoA dehydrogenase; GNRHR: gonadotropin-releasing hormone receptor; GPR6: G protein-coupled receptor 6; GREB1: growth regulation by estrogen in breast cancer 1; HCFC1: host cell factor C1 (VP16-accessory protein); HSPA4: heat shock 70kDa protein 4; HSPG2: heparan sulfate proteoglycan 2; JUN: jun proto-oncogene; KIF1B: kinesin family member 1B; KLF2: Kruppel-like factor 2; KRT17: keratin 17; L1CAM: L1 cell adhesion molecule; LONRF1: LON peptidase N-terminal domain and ring finger 1; LONRF3: LON peptidase N-terminal domain and ring finger 3; MAMDC2: MAM domain containing 2; MAP4: MAM domain containing 2; MEAF6: MYST/Esa1-associated factor 6; MICAL2: microtubule associated monooxygenase, calponin and LIM domain containing 2; MYO1F: myosin IF; MYO7A: myosin VIIA; NCOA3: nuclear receptor coactivator 3; NCOR2: nuclear receptor corepressor 2; NPAS2: neuronal PAS domain protein 2; TMEM164: transmembrane protein 164; UGT1A1: UDP glucuronosyltransferase 1 family, polypeptide A1; PPP3CA: protein phosphatase 3, catalytic subunit, alpha isozyme; RFC5: replication factor C (activator 1) 5, 36.5kDa; PER1: period homolog 1; PER2: period homolog 2; PITPNC1: phosphatidylinositol transfer protein, cytoplasmic 1; RERGL: RERG/RAS-like; RORB: RAR-related orphan receptor B; SVEP1: sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1; TIMP2: TIMP metalloproteinase inhibitor 2; TRIM3: tripartite motif containing 3; ZFYVE9: zinc finger, FYVE domain containing 9















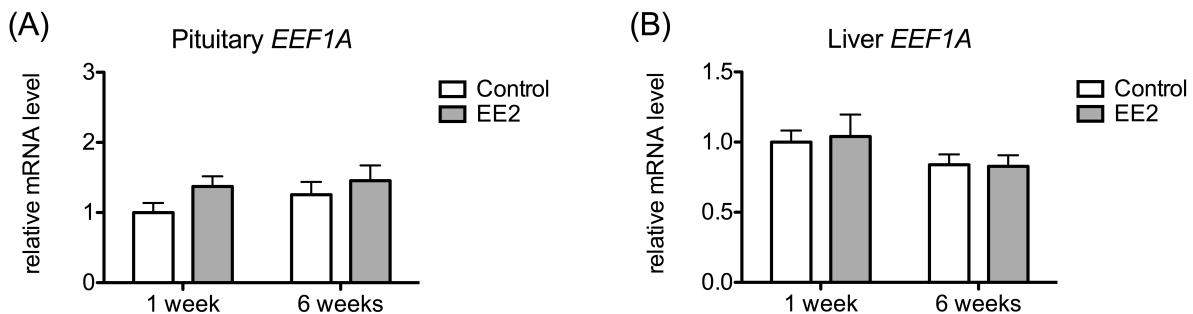
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Figure 3.5. Gene network generated from 18 molecules involved in behavior, nervous system development and function, and connective tissue development and function that were differentially expressed at 1 week. This network illustrates interaction between molecules in the circadian rhythm signaling pathway and the GNRH signaling pathway. An asterisk at the end of a molecule symbol indicates the contig for coho salmon ortholog was significantly altered after 1 week EE2-exposure ($p < 0.05$). Molecules are shown as red (up regulated) or green (down regulated) with intensity indicating the relative magnitude of regulation. √ Cytokine/Growth Factor, ⚙ Enzyme, ⚡ G-Protein Coupled Receptor, ⚡ Ion Channel, ⚡ Kinase, ⚡ Ligand-dependent Nuclear Receptor, ⚡ Phosphatase, ⚡ Transcription Regulator, ⚡ Transmembrane Receptor, ⚡ Transporter, ⚡ Complex, ⚡ Other. ADCY: adenylyl cyclase; Alp: alkaline phosphatase; ARNTL: aryl hydrocarbon receptor nuclear translocator-like; BHLHB: class B basic helix-loop-helix protein; BHLHE40: basic helix-loop-helix family, member e40; BHLHE41: basic helix-loop-helix family, member e41; BMPR1B: bone morphogenetic protein receptor, type IB; Cg: choriogonadotropin; CLU: clusterin; Creb: cyclic AMP-responsive element-binding protein; DBP: D site of albumin promoter (albumin D-box) binding protein; DKK3: dickkopf 3 homolog; EYA3: eyes absent homolog 3; FANCA: Fanconi anemia, complementation group A; Fsh: follicle stimulating hormone; GNRH: gonadotropin-releasing hormone; GNRHR: GNRH receptor; Hdac: histone deacetylase; Ige: immunoglobulin E; IgG1: immunoglobulin G; ITPR1: inositol 1,4,5-trisphosphate receptor, type 1; Lh: luteinizing hormone; LHB: LH beta subunit; Mek: MAPK/ERK kinase 1; MTA1: metastasis associated 1; NPAS2: neuronal PAS domain protein 2; PDGF BB: Platelet-derived growth factor subunit B dimer; PER1: period homolog 1; PER2: period homolog 2; Pkc(s): protein kinase C; RHOBTB1: Rho-related BTB domain containing 1; TOB1: transducer of ERBB2, 1

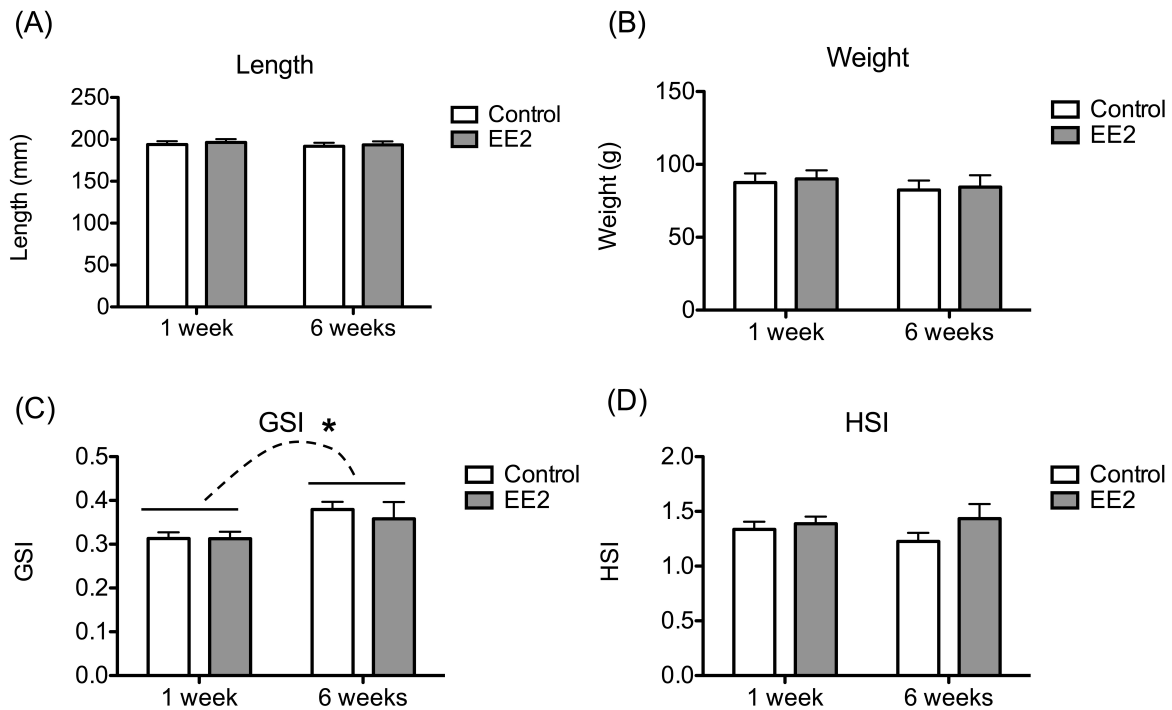


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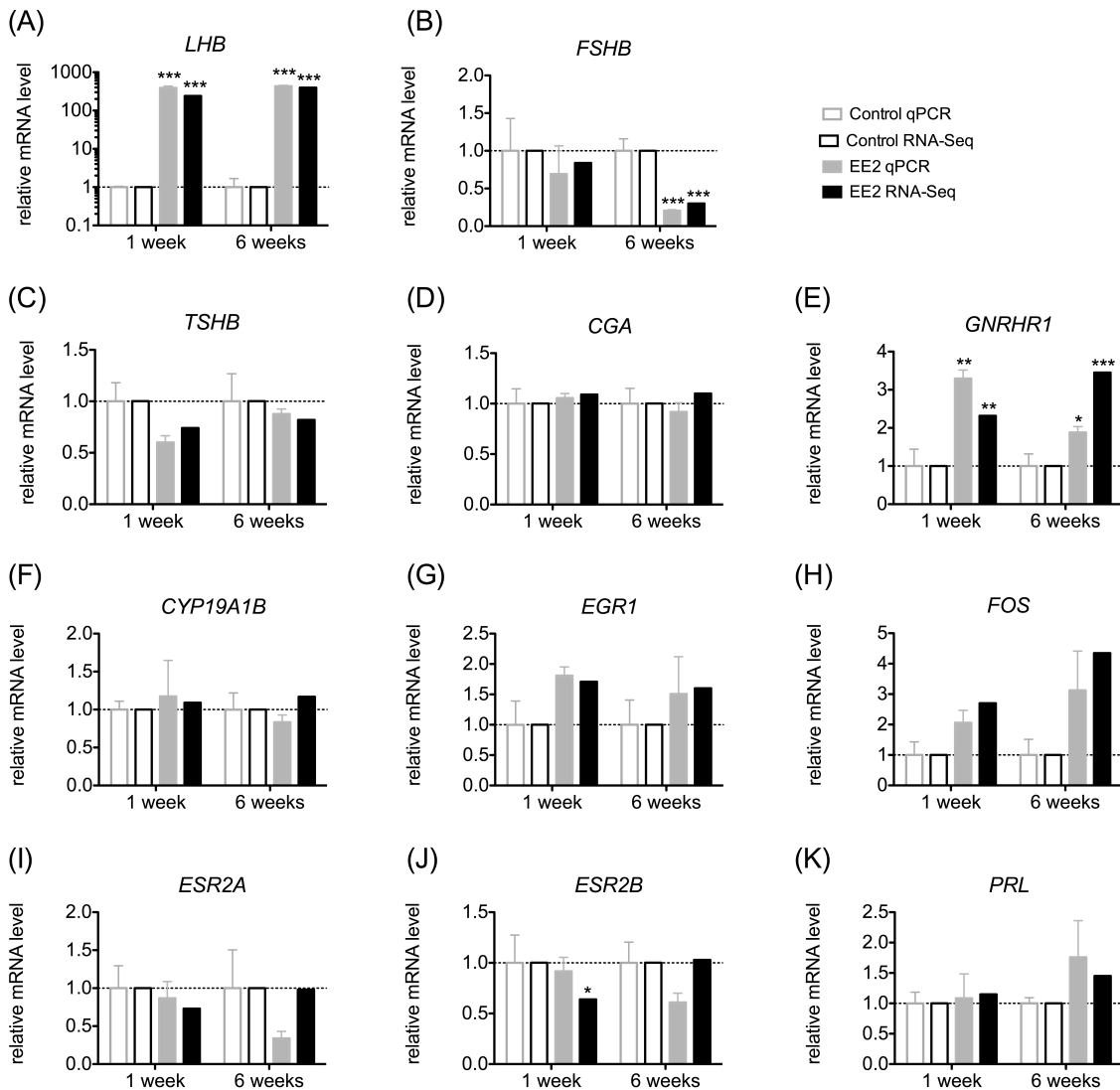
Figure 3.6. Gene network generated from 25 molecules associated with cell morphology, digestive system development and function, and endocrine system development and function that had significantly altered contig levels in EE2 exposed animals at 6 weeks. According to the network, LHB and FSHB expression levels may be influenced by inhibin after 6 weeks of EE2 exposure. An asterisk at the end of a molecule symbol indicates the coho salmon ortholog contig was significantly altered after 6 weeks EE2-exposure ($p < 0.05$). Molecules are shown as red (up-regulated) or green (down-regulated) with intensity indicating the relative magnitude of regulation.  Cytokine/Growth Factor,  Enzyme,  G-Protein Coupled Receptor,  Ion Channel,  Kinase,  Ligand-dependent Nuclear Receptor,  Phosphatase,  Transcription Regulator,  Transmembrane Receptor,  Transporter,  Complex,  Other. AP4B1: adaptor-related protein complex 4, beta 1 subunit; CaMKII: Ca⁺/calmodulin-dependent protein kinase II; CNTN2: contactin 2 (axonal); CORO1A: coronin, actin binding protein, 1A; DGKA: diacylglycerol kinase, alpha 80kDa; DMRTA2: DMRT-like family A2; Ecm: extracellular matrix proteins; FAM46A: family with sequence similarity 46, member A; FKBP5: FK506 binding protein 5; FOXJ1: forkhead box J1; FSHB: follicle stimulating hormone, beta subunit; Gata: GATA transcription factors; GATA2: GATA binding protein 2; GLIS3: GLIS family zinc finger 3; HIVP2: human immunodeficiency virus type I enhancer binding protein 2; ISL1: ISL LIM homeobox 1; KCNJ4: potassium inwardly-rectifying channel, subfamily J, member 4; KSR2: kinase suppressor of ras 2; LDB1: LIM domain binding 1; LHB: luteinizing hormone beta polypeptide; MAP3K: mitogen-activated protein kinase kinase kinase; NR1D1: nuclear receptor subfamily 1, group D, member 1; ORC5: origin recognition complex, subunit 5; Pp2b: protein phosphatase 2B; PPP3CA: protein phosphatase 3, catalytic subunit, alpha isozyme; RTKN: rhotekin; RYR1: ryanodine receptor 1 (skeletal); STAB1: stabilin 1; TAX1BP1: Tax1 (human T-cell leukemia virus type I) binding protein 1; XPO1: exportin 1 (CRM1 homolog, yeast)



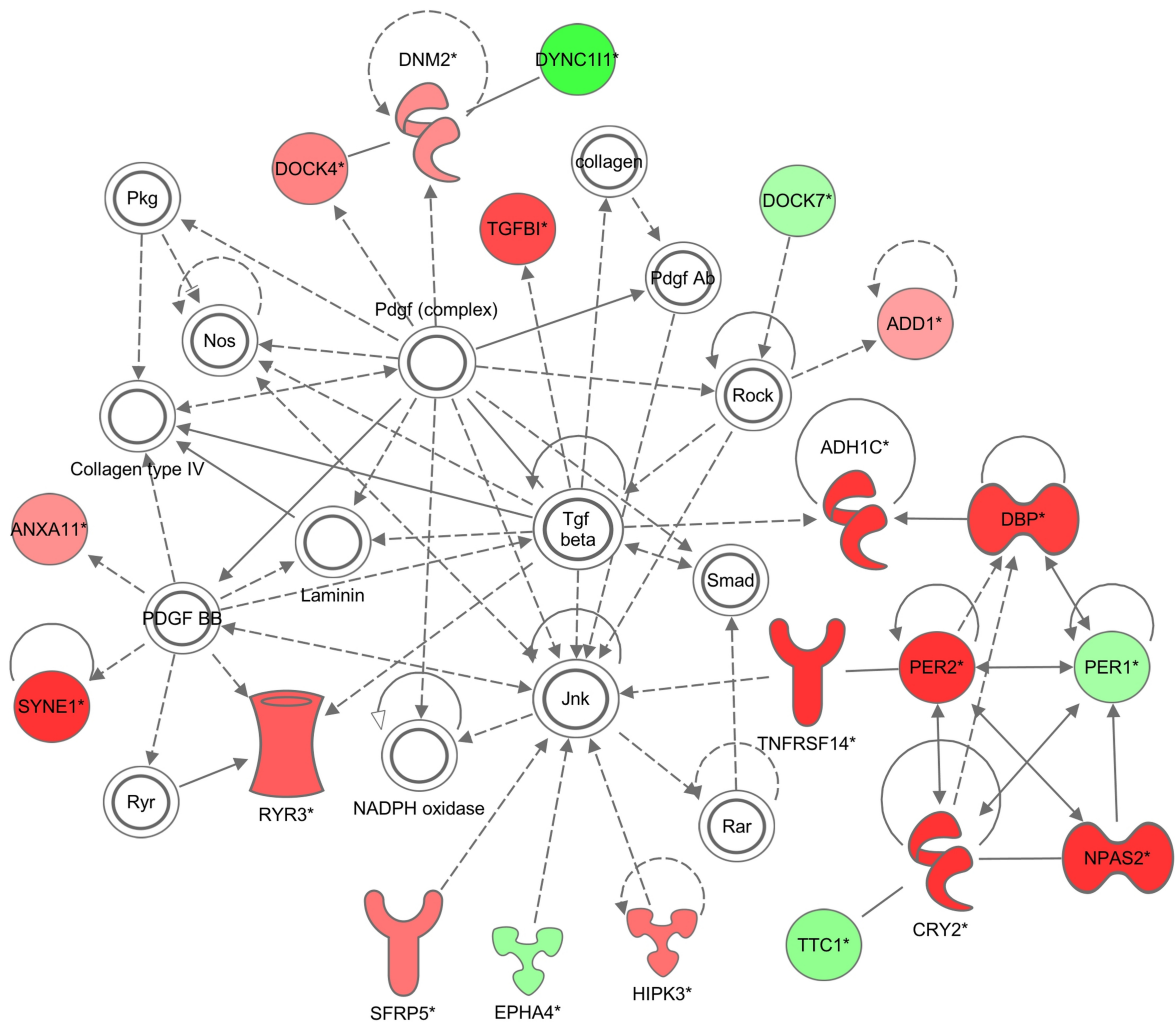
Supplemental Figure 3.S1: Levels of mRNA for *EEF1A* measured by qPCR in (A) pituitary or (B) liver at 1 and 6 weeks. *EEF1A* was used to normalize levels of genes in Supplemental Figure 3.S3. No significant differences were observed between control and EE2-exposed samples at either 1 or 6 weeks. (Student's unpaired t-test, $p > 0.05$). The data are expressed as mean \pm SEM ($n = 8 - 10$).















Supplemental Figure 3.S2: Morphometric data of control (white bars) and EE2-exposed (grey bars) fish at 1 and 6 weeks. There were no significant effects of treatment on (A) length, (B) weight, (C) gonadosomatic index or (D) hepatosomatic index (2-way ANOVA, $p > 0.05$). The data are expressed as mean \pm SEM ($n = 8 - 10$). There was a significant effect of time on the gonadosomatic index (2-way ANOVA, $p=0.0145$), but there were no significant effects on any of the other parameters measured. Significant differences are indicated, $*p < 0.05$.

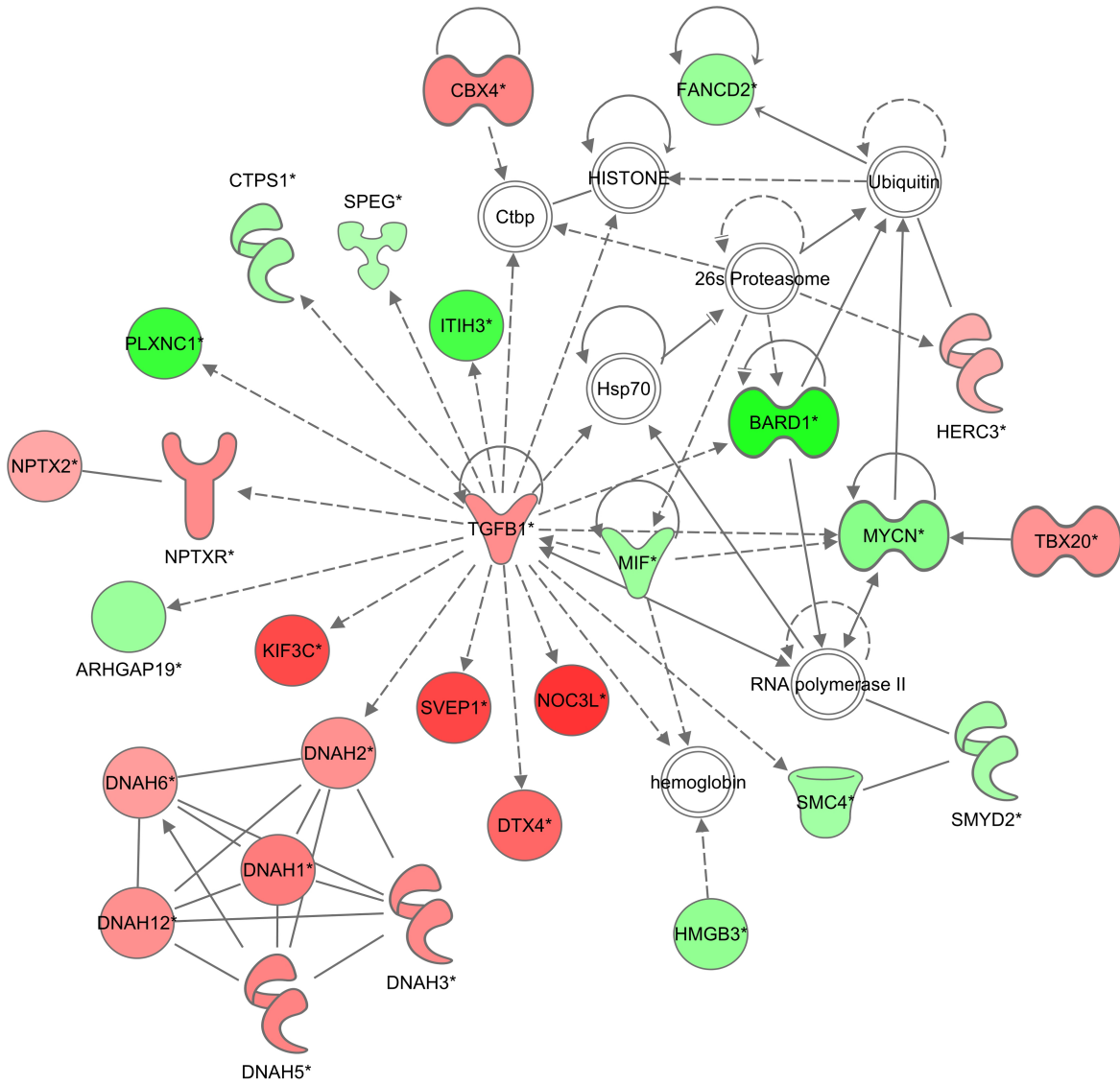


Supplemental Figure 3.S3: Relative expression of transcripts in pituitary glands of control and EE2-exposed female juvenile coho salmon at 1 and 6 weeks as measured by qPCR (grey) and RNA-Seq (black). (A) luteinizing hormone β subunit; (B) follicle stimulating hormone β subunit; (C) thyroid stimulating hormone β subunit; (D) glycoprotein hormone α subunit; (E) GNRH receptor 1 (F) brain aromatase, (G) proto-oncogene c-fos; (H) early growth response protein 1; (I) estrogen receptor 2A; (J) estrogen receptor 2B; and (K) prolactin. The data are expressed as mean \pm SEM ($n = 3 - 4$). Significant differences from the control at each time point as determined by Student's unpaired t-test are indicated, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.















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Supplemental Figure 3.S4: A gene network generated from 20 molecules that had significantly altered transcript levels following 6 weeks of EE2 exposure. Molecules were involved in behavior, nervous system development and function, and reproductive system development and function. The network illustrates that at 6 weeks, circadian rhythm signaling molecules may be interacting with genes involved in the TGF- β signaling pathway. Molecules are shown as red (up-regulated) or green (down-regulated) with intensity indicating the magnitude of regulation. An asterisk at the end of a molecule symbol indicates the transcript was significantly altered in our dataset after 6 weeks EE2-exposure (DESeq, $p < 0.05$).  Cytokine/Growth Factor,  Enzyme,  G-Protein Coupled Receptor,  Ion Channel,  Kinase,  Ligand-dependent Nuclear Receptor,  Phosphatase,  Transcription Regulator,  Transmembrane Receptor,  Transporter,  Complex,  Other. ADD1: adducin 1 (alpha); ADH1C: alcohol dehydrogenase 1C (class I), gamma polypeptide; ANXA11: annexin A11; CRY2: cryptochrome 2; DOCK4: dedicator of cytokinesis 4; DOCK7: dedicator of cytokinesis 7; DBP: D site of albumin promoter (albumin D-box) binding protein; DNM2: dynamin 2; DYNC1I1: dynein, cytoplasmic 1, intermediate chain 1; EPHA4: EPH receptor A4; HIPK3: homeodomain interacting protein kinase 3; Jnk: c-Jun N-terminal kinases; Nos: nitric oxide synthase; NPAS2: neuronal PAS domain protein 2; Pdgf Ab: platelet-derived growth factor AB; PDGF BB: platelet-derived growth factor BB; PER1: period homolog 1; PER2: period homolog 2; Pkg: protein kinase G; Rar: retinoic acid receptor; Rock: rho kinase; Ryr: ryanodine receptor; RYR3: ryanodine receptor 3; SFRP5: secreted frizzled-related protein 5; SYNE1: spectrin repeat containing, nuclear envelope 1; TGFBI: transforming growth factor, beta-induced, 68kDa; TNFRSF14: tumor necrosis factor receptor superfamily, member 14; TTC1: tetratricopeptide repeat domain 1



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Supplemental Figure 3.S5: Gene network generated from 28 molecules that had significantly altered contig levels following 6 weeks of EE2 exposure. The network illustrates the interaction between TGFB1 (1.6-fold, $p=0.03$) with molecules involved in cellular development, cellular growth and proliferation and tumor morphology at 6 weeks. An asterisk at the end of a molecule symbol indicates the transcript was significantly altered in our dataset after 6 weeks EE2-exposure ($p<0.05$). Molecules are shown as red (up-regulated) or green (down-regulated) with intensity indicating the relative magnitude of regulation.  Cytokine/Growth Factor,  Enzyme,  G-Protein Coupled Receptor,  Ion Channel,  Kinase,  Ligand-dependent Nuclear Receptor,  Phosphatase,  Transcription Regulator,  Transmembrane Receptor,  Transporter,  Complex,  Other. ARHGAP19: Rho GTPase activating protein 19; BARD1: BRCA1 associated RING domain 1; CBX4: chromobox homolog 4; Ctbp: C-terminal binding protein; CTPS1: CTP synthase 1; DNAH1: dynein, axonemal, heavy chain 1; DNAH2: dynein, axonemal, heavy chain 2; DNAH3: dynein, axonemal, heavy chain 3; DNAH5: dynein, axonemal, heavy chain 5; DNAH6: dynein, axonemal, heavy chain 6; DNAH12: dynein, axonemal, heavy chain 12; DTX4: deltex homolog 4; FANCD2: Fanconi anemia, complementation group D2; HERC3: HECT and RLD domain containing E3 ubiquitin protein ligase 3; HMGB3: high mobility group box 3; Hsp70: heat shock 70kDa protein ; ITIH3: inter-alpha-trypsin inhibitor heavy chain 3; KIF3C: kinesin family member 3C; MIF: macrophage migration inhibitory; MYCN: v-myc myelocytomatosis viral related oncogene; NOC3L: nucleolar complex associated 3 homolog; NPTX2: neuronal pentraxin II; NPTXR: neuronal pentraxin receptor; PLXNC1: plexin C1; SMC4: structural maintenance of chromosomes 4; SMYD2: SET and MYND domain containing 2; SPEG: SPEG complex locus; SVEP1: sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1; TBX20: T-box 20; TGFB1: transforming growth factor, beta 1

Chapter 3: Wastewater treatment plant effluent alters pituitary gland gonadotropin mRNA levels in juvenile coho salmon (*Oncorhynchus kisutch*)

A version of this manuscript has been submitted to Aquatic Toxicology for publication.

Coauthors: Irvin R. Schultz, Denis A. M. da Silva, Gina M. Ylitalo, Dave Ragsdale, Stephanie I. Harris, Stephanie Bailey, Barry V. Pepich, and Penny Swanson

Abstract:

It is well known that endocrine disrupting compounds (EDCs) present in wastewater treatment plant (WWTP) effluents interfere with reproduction in fish, including altered gonad development and induction of vitellogenin (Vtg), a female-specific egg yolk protein precursor produced in the liver. As a result, studies have focused on the effects of EDC exposure on the gonad and liver. However, impacts of environmental EDC exposure at higher levels of the hypothalamic-pituitary-gonad (HPG) axis are less well understood. The pituitary gonadotropins (Gths), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) are involved in all aspects of gonad development and are subject to feedback from gonadal steroids making them a likely target of endocrine disruption. In this study, the effects of WWTP effluent exposure on pituitary gonadotropin (Gth) mRNA expression were investigated in juvenile coho salmon (*Oncorhynchus kisutch*). First, a controlled 72-hour exposure to 17 α -ethynylestradiol (EE2) and trenbolone (TREN) was performed to evaluate the response of juvenile coho salmon to EDC exposure. Second, juvenile coho salmon were exposed to 0, 20 or 100% effluent from eight WWTPs from the Puget Sound, WA region for 72 hours. Juvenile coho salmon exposed to 2 and 10 ng/L EE2 had 17-fold and 215-fold higher Lh beta-subunit (*lhb*) mRNA levels relative to control fish. Hepatic *vtg* mRNA levels were dramatically increased 6,670-fold, but only in response to 10 ng EE2 and Fsh beta-subunit (*fshb*) mRNA levels were not altered by any of the

treatments. In the WWTP effluent exposures, *lhb* mRNA levels were significantly elevated in fish exposed to five of the WWTP effluents. In contrast, transcript levels of *vtg* were not affected by any of the WWTP effluent exposures. Mean levels of natural and synthetic estrogens in fish bile were consistent with pituitary *lhb* expression, suggesting that the observed *lhb* induction may be due to estrogenic activity of the WWTP effluents. These results suggest that *lhb* gene expression may be a sensitive index of acute exposure to estrogenic chemicals in juvenile coho salmon. Further work is needed to determine the kinetics and specificity of *lhb* induction to evaluate its utility as a potential indicator of estrogen exposure in immature fish.

Introduction:

It is now well established that some chemicals in the environment are capable of disrupting normal endocrine function in humans, wildlife, and fish (Hotchkiss et al., 2008; León-Olea et al., 2014). These endocrine disrupting compounds (EDCs), including certain pharmaceuticals, pesticides, and a variety of industrial compounds, can act to mimic or block endogenous hormones by interfering with their synthesis, availability, or action (Crisp et al., 1998). EDC exposure has been associated with reduced fertility (Jobling et al., 2002), sex reversal (Jobling et al., 1998), and reproductive failure (Kidd et al., 2007; Nash et al., 2004) in a variety of aquatic organisms.

Municipal wastewater treatment plant (WWTP) effluents are one of the primary sources of EDCs in the aquatic environment. Fish collected downstream of some WWTPs exhibit symptoms of endocrine disruption and altered reproductive function including reduced gonad size, delayed maturation, and decreased steroidogenesis (Folmar et al., 2001; Vajda et al., 2011, 2008; Woodling et al., 2006). In addition, feminization of male fish has been reported

downstream of WWTPs including observations of intersex gonads or reduced primary and secondary male sex characteristics (Jobling et al., 1998; Purdom et al., 1994; Vajda et al., 2011, 2008). Chemical analyses identified natural estrogens [estradiol (E2) and estrone (E1)] and synthetic estrogens [17 α -ethynylestradiol (EE2)] as the most likely feminizing agents in wastewater effluents (Desbrow et al., 1998; Rodgers-Gray et al., 2000). In addition, various anthropogenic compounds such as alkylphenols [nonylphenols (NP) and octylphenols (OP)] and bisphenol A (BPA) have also been identified as estrogen receptor (ER) agonists or antagonists and are present in wastewater effluent (Snyder et al., 2001).

Early studies on the effects of WWTP effluent exposure on fish reproduction found elevated expression of vitellogenin (Vtg) in male fish exposed to WWTP effluent (Folmar et al., 1996; Harries et al., 1997; Purdom et al., 1994). Vtg is an egg yolk protein precursor that is induced in maturing female fish in response to rising plasma E2 levels. Vtg synthesis can also be stimulated in male or juvenile fish of both sexes in response to exogenous estrogen exposure. Indeed, Vtg can be increased thousands fold in male fish in response to estrogens making it a widely used biomarker of estrogen exposure (Sumpter and Jobling, 1995). As such, many studies of endocrine disruption or estrogen exposure have focused on the gonad and liver. However, reproduction is controlled by the hypothalamic-pituitary-gonad (HPG) axis and EDCs may exert their effects at higher levels of the HPG axis.

The pituitary gonadotropins (Gths), follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), are heterodimeric glycoprotein hormones composed of a common alpha subunit and a hormone-specific beta subunit. The Gths are involved in all aspects of gonad development and function including steroidogenesis, gametogenesis, final gamete maturation, and gamete release (Levavi-Sivan et al., 2010; Swanson et al., 2003). The Gths are synthesized and released

in response to a variety of factors from the brain, primarily gonadotropin-releasing hormone (GnRH) released from the hypothalamic neurons that directly innervate the fish pituitary gland. In addition, the Gths are regulated by positive and negative feedback from the gonad via steroid hormones and other gonadal peptides. For example, when immature trout or salmon are treated with estrogen or aromatizable androgens, pituitary and plasma Fsh levels decrease while pituitary Lh beta subunit (*lhb*) mRNA levels and pituitary Lh content increase (Breton et al., 1997; Dickey and Swanson, 1998; Saligaut et al., 1998), suggesting estrogens play an important role in regulating both gonadotropins. In support of this, estrogen response elements (EREs) have been identified in the *lhb* (Le Dréan et al., 1995; Liu et al., 1995; Sohn et al., 1999; Xiong et al., 1994) and Fsh beta subunit (*fshb*) (Rosenfeld et al., 2001; Sohn et al., 1998; Vischer, 2003) gene promoters of various fish species. Therefore, it is possible that Gths may be susceptible to endocrine disruption by estrogenic contaminants such as EE2 or WWTP effluent.

In salmonids, pituitary *fshb* and *lhb* mRNA and plasma Fsh and Lh expression profiles are well characterized (Breton et al., 1998; Campbell et al., 2006; Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991). In male and female coho salmon, pituitary *fshb* mRNA levels, pituitary Fsh content, and plasma Fsh levels begin to increase about one year prior to spawning (Campbell et al., 2006). In contrast, *lhb* mRNA levels and pituitary Lh content increase during late gametogenesis and final gamete maturation in response to increasing levels of estradiol or aromatizable androgens (Breton et al., 1998; Gomez et al., 1999; Prat et al., 1996; Swanson et al., 1991). However, similar to the case of *Vtg*, increased expression of *lhb* mRNA and accumulation of Lh protein content in the pituitary of immature fish can be induced in response to estrogen treatment. Studies have shown that mRNA levels for *lhb* are induced in response to EE2 or other estrogenic contaminants (Harding et al., 2013; Harris et al., 2001; Johns et al., 2009; Maeng et al.,

2005; Rhee et al., 2010; Yadetie and Male, 2002). Using high-throughput sequencing and RNA-Seq, we previously demonstrated that waterborne exposure of previtellogenic coho salmon to 12 ng/L EE2 for up to 6 weeks had widespread effects on the pituitary transcriptome and dramatically altered Gth mRNA levels. At 6 weeks, *lhb* was induced 395-fold and was the most significantly altered transcript, while *fshb* was downregulated -3.5 fold (Harding et al., 2013). Alterations in plasma Gth levels have also been observed in response to EDC exposure (Brown et al., 2007; Golshan et al., 2014; Harris et al., 2001). Female rainbow trout (*Oncorhynchus mykiss*) exposed to 4-nonylphenol at 0.7, 8.3, or 85.6 µg/L for 18 weeks during early secondary oocyte growth showed reduced *fshb* mRNA levels, pituitary Fsh content and plasma Fsh levels and increased plasma Lh and Vtg (Harris et al., 2001). These findings suggest that Gths may be sensitive targets of EDC exposure and may be involved in inhibited gonad growth and altered reproduction associated with endocrine disruption (Filby et al., 2006; Harris et al., 2001).

The aim of this study was to evaluate the effects of WWTP effluents on pituitary Gths in 1+ age juvenile coho salmon (*Oncorhynchus kisutch*). Based on the strong induction of *lhb* mRNA levels in response to EE2 and other ER agonists, we hypothesized that *lhb* mRNA levels would be increased in response to WWTP effluents containing estrogenic chemicals. Juvenile coho salmon were selected for this study because: 1) coho salmon are ecologically relevant species in the Pacific Northwest region of North America; 2) presmolts or smolts (1⁺ age, early gametogenesis) have low to non-detectable basal expression of hepatic *vtg* and pituitary *lhb* in both male and female fish at this stage; 3) primary oocyte growth and early stages of spermatogenesis occur at this life history stage and may be affected by contaminants in WWTP effluent; 4) the low body size (< 50 g body mass) at this stage makes them more practicable for waterborne exposure studies; and 5) salmon presmolts and smolts are residing in or migrating

through urban waterways and potentially exposed to EDCs during these stages. To evaluate the response of juvenile coho salmon to EDC exposure, we first conducted a controlled 72-hr exposure to EE2 and 17 β -trenbolone (TREN; a synthetic androgen used in cattle production). TREN and EE2 were selected as a model androgen and estrogen, respectively. Second, we exposed coho salmon to 100% effluent, 20% effluent, or control water for 72 hrs. Effluent from various WWTPs were selected to include a range of treatment processes (secondary and tertiary). In addition to pituitary Gth mRNA levels, we measured hepatic *vtg* mRNA levels as a positive control of estrogen exposure. Where possible, chemical analyses on exposure effluents and bile from exposed fish were conducted to quantify exposure to a variety of contaminants with estrogenic activity. Analysis of several selective serotonin reuptake inhibitors (SSRIs) in effluents was also performed because of high occurrence in WWTP effluent and in Puget Sound estuary waters (Hedgespeth et al., 2012; Meador et al., 2016; Verlicchi et al., 2012) and reported effects on Gth levels in fish (Prasad et al. 2015).

Materials and methods

Chemicals for exposure and water chemistry

17 α -ethynylestradiol (> 99% purity), trenbolone (> 99% purity), methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), heptafluorobutyrylimidazole (HFBI), iodotrimethylsilane (ITS), and resublimed iodine were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). Fluoxetine hydrochloride (> 98% purity) was obtained from Spectrum chemicals (Gardena, CA). Norfluoxetine-oxalate was obtained from Cerilliant[®] Analytical Reference Standards (Sigma-Aldrich, St. Louis, MO). Sertraline hydrochloride (> 98% purity) was obtained from Toronto Research Chemicals (Toronto, Ontario, CA). Citalopram hydrobromide (> 98% purity) was

obtained from TCI America (Portland, OR). [2,4,16,16]d4-17 α -Ethinylestradiol (EE2-d4), [16,16,17]d3-Estradiol (E2-d3), [2,4,16,16]d4-estrone (E1-d4) and [16 α -Hydroxy-17 β -estradiol]d2-estriol (E3-d2), all > 97% purity, were purchased from C/D/N isotopes (Pointe-Claire, Quebec, CA). Pentadeuterated fluoxetine (fluoxetine-d5; > 99% ring labeled) was obtained from Isotec (Sigma-Aldrich, St. Louis, MO). All other chemicals were of reagent grade or better and were obtained from standard sources.

Fish maintenance

Coho salmon eyed embryos were obtained from the Issaquah Hatchery in mid-December and incubated in Heath trays at 8 °C at the Northwest Fisheries Science Center hatchery facilities (Seattle, WA). After ponding, fish were reared in recirculated 10 – 10.5 °C fresh water under a simulated natural photoperiod. Fish were fed a standard ration of BioDiet commercial feed (Bio-Oregon, Longview, WA) according to Bio-Oregon's feed rate guidelines. On January 31, 2012, 300 1+ age coho salmon parr (~20 g body weight) were transferred to Pacific Northwest National Laboratory – Marine Science Laboratory (PNNL-MSL, Sequim, WA). Fish were initially maintained in circular 1400 L fiberglass tanks. The holding tanks were maintained using a single-pass flow through system using fresh water obtained from MSL's artesian well (440 ft depth), which was pre-aerated and added to tanks at a minimum flow rate of 12 L/min. After three weeks of acclimation, groups of fish were transferred to smaller, 370-L circular fiberglass tanks (water inflow rate of 4 L/min). Water temperature, dissolved oxygen, and pH were monitored weekly throughout the acclimation and exposures with values ranging from 11.8 – 12.5 °C for temperature, 8.0 – 9.4 mg/L for dissolved oxygen, and 7.75 – 8.05 for pH. Fish were maintained under a simulated natural photoperiod regime with artificial dusk and dawn and fed a

standard ration of Bio-Oregon® soft moist pellets. All fish were maintained according to the guidelines established by the Institutional Animal Care and Use Committee of PNNL-MSL.

EE2 and TREN Exposures

Controlled exposures to EE2 and TREN were performed for comparison with WWTP effluent exposures. Seventy two-hour exposures to EE2 or TREN were conducted using a continuous flow-through protocol similar to previous studies (Schultz et al., 2013). Exposure water was prepared using a concentrated aqueous stock solution that was slowly added to the exposure tanks using a peristaltic pump. No organic co-solvents were used. All exposure tanks were allowed to equilibrate with the dosing system for three days prior to the addition of fish. Nominal exposure values were adjusted based on daily monitoring of water and stock solution in-flow rates. Water samples from each tank were removed and analyzed for EE2 or TREN at the start and end of the exposure.

In February 2012, juvenile, mixed sex coho salmon were randomly assigned to treatment tanks (2 tanks/treatment; 10 fish/tank) and exposed to nominal concentrations of 0, 2 or 10 ng/L EE2 or 20 or 200 ng/L TREN fresh water for 72 hours. At the end of 72 hours, fish were anesthetized in buffered tricaine methanesulfonate (0.05% MS-222; Argent Laboratories, Redmond, WA) and euthanized by decapitation. Fork length (nearest mm), body weight (nearest 0.1 g), gonad weight and liver weight (nearest mg) were recorded. Gonad and liver weights were used to calculate gonadosomatic index (GSI) and hepatosomatic index (HSI) respectively, according to the following equation: (tissue mass/body mass) X 100. The pituitary gland and a small piece (~50 mg) of liver were dissected and flash frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

WWTP Effluent Exposures

In April and May, effluent grab samples were collected from eight WWTPs around Puget Sound (WWTP A – H), which were selected based on differences in treatment processes and effluent dilution factors (Table 1). Approximately 200 gallons (750 L) of effluent were collected from each WWTP in 55-gallon (208-L) Teflon[®]-lined drums. Wherever possible, samples were collected at treatment points after final filtration, but prior to chlorine disinfection. For WWTP E some mortality due to residual chlorine was observed. Therefore, for sites F-H, if samples were collected post chlorination, a minimum volume of anhydrous sodium thiosulfate solution was added to attain a concentration of 6.7 mg/L in order to reduce the free available chlorine (FAC) based on guidance in EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (U.S. EPA, 2002). The effluent samples were refrigerated and transported to PNNL-MSL for exposure studies. Drums of effluent were stored in fiberglass tanks with running well water to maintain a temperature of ~12 °C. Effluents were chilled overnight with aeration to remove residual chlorine if present and exposures were initiated the following morning.

Exposures were conducted during April and May when fish were undergoing smoltification. At this stage, fish are sexually immature with ovaries containing perinucleolar stage oocytes and testes containing only Type A spermatogonia. Juvenile 1+ age coho salmon were exposed to 0%, 20%, or 100% WWTP effluent under semi-static conditions with a 60% tank water replacement conducted daily in accordance with the EPA's *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (U.S. EPA, 2002). Exposure tanks were filled with 110 L of 0%, 20%, or 100% effluent. Every 24

hours, approximately 66 L of exposure water was removed and replaced by new exposure water previously chilled to 12 °C. Fish were fed up to the day before the experiment, and then were fasted during the exposure. After 72 hours, fish were euthanized in buffered MS-222 and decapitated. Fork length, body weight, gonad weight and liver weight, were recorded and GSI and HSI were calculated as described above. The pituitary gland and a small piece (~ 50 mg) of liver were dissected and placed in 0.75 mL RNAlater[®] (Life Technologies, Carlsbad, CA). Tissues were stored in RNAlater[®] at room temperature for up to a week and then stored at -20 °C or -80 °C until RNA isolation. When possible, bile was collected from the gallbladder with a clean 1-mL syringe tipped with a 23 G needle. Bile was stored at -80 °C until analysis.

Exposure chemical analyses

Exposure water from the control exposure was analyzed for the presence of EE2 or TREN. Separate aliquots were analyzed for TREN and EE2. WWTP effluents were analyzed for natural and synthetic estrogens including E1, E2, and EE2. Exposure water (100 mL) or WWTP effluent samples (850-900 mL) were fortified with deuterated internal standards (E2-d₃ for E2; EE2-d₄ for EE2; E1-d₄ for E1), spiked with NaCl (1 or 10 g; previously baked at 500 °C for 12 hrs) and then extracted with either 15 or 150 mL of methyl-tert-butyl ether (MTBE). The ether extracts were evaporated under N₂ and the residue mixed with the appropriate derivatizing agent. For estrogens, 25 µL of MSTFA containing ITS (1000:4 v/v) as a catalyst was used and heated at 70 °C for 30 min prior to analysis (Schultz et al., 2001). For TREN, 25 µL of MSTFA containing re-sublimed I₂ as the catalyst was used (Marchand et al., 2000). The steroid-trimethylsilyl derivatives were then quantified using a gas chromatography-mass spectrometer (GC-MS) system (Agilent 6890 GC, 5973inert MS). The analytes were separated on a DB-5 column (J&W

30m, 0.25 mm I.D., 0.25 μm film thickness) with splitless injection at 90 °C with other oven conditions as described by Stanford and Weinberg (2007). The MS was operated in electron impact mode with selective ion monitoring (SIM) made for m/z 342, 346, 416, 419, 425, 429, and 442 that were used for E1, E1-d₄, E2, E2-d₃, EE2, EE2-d₄, and TREN quantification, respectively.

WWTP effluents were also analyzed for the presence of selective serotonin reuptake inhibitors (SSRIs) including fluoxetine (FLX), norfluoxetine (NFLX), citalopram (CIT), and sertraline (SER). Analyses of SSRIs were conducted using GC-MS using the method described by Wille (2008) and Wille et al. (2007). In brief, 0.8 L water samples were spiked with an internal standard (IS; d₅-fluoxetine) adjusted to ~ pH 12 with 2 N NaOH and then extracted once with an excess volume of MTBE. The MTBE extract was evaporated to dryness (under N₂) and the residue derivatized with 50 μL heptafluorobutyrylimidazole (HFBI) at 80 °C for 30 min. Afterwards, the samples were cooled, mixed first with 400 μL deionized water to terminate the reaction, then mixed with 600 μL toluene, vortexed and centrifuged. The toluene layer containing the HFBI derivatives was removed, volume reduced to 125 μL under N₂ and then injected onto the GC-MS. Quantification was done using selected-ion monitoring with monitored ions (m/z): 117, 344 (FLX), 117, 340 (NFLX), 122, 349 (d₅-fluoxetine [IS]) 274, 501 (SER) and 58 (CIT). The retention times and spectra of all analytes were determined or confirmed from authentic standards.

Bile sample preparation, enzymatic hydrolysis and solid-phase extraction (SPE)

This method was modified from da Silva et al. (2013). Briefly, approximately 30 μL of bile was diluted with 200 μL of water, followed by the addition of 30 μL of 2.5ng/ μL mixture of

surrogate standard (S-std) containing BPA-d16, E1-d4, E2-d4, EE2-d4 and NP-d5. Acetone (2 mL) was added and the sample was kept in -20 °C for 45 min for partial protein precipitation. The sample was then centrifuged at 4,000 rpm for 10 min and supernatant was transferred to a new glass tube. The acetone was evaporated under N₂ flow at 35 °C and 1 mL of 1 M acetate buffer pH 5 was added containing 2,000 U of β-glucuronidase/sulfatase. The mixture was kept in an incubator for 2 h at 40 °C to ensure complete enzymatic hydrolysis of the glucuronide and sulfate conjugated metabolites of the EDCs. Glacial acetic acid (300 μL) was added and the final hydrolyzed bile mixture was loaded on to a solid phase extraction (SPE) cartridge packed with 60 mg of polymeric reversed-phase sorbent (Strata X from Phenomenex Inc, Torrance, CA, USA) that had been previously conditioned with 2 mL of methanol and 2 mL of water. The cartridge was then washed with 1.5 mL of water, followed by 1.5 mL of methanol/water (60/40, v/v), and was dried under vacuum for 30 min. The analytes were eluted with 1.5 mL of methanol into vial containing 30 μL of 2.5 ng/μL BPA-d4 solution, used as recovery standard (Rec-std). An aliquot of the final methanolic extract was diluted 10 times in methanol prior to analyses.

Bile analyses by liquid chromatography/tandem mass spectrometry (LC-MS/MS)

Final bile extracts were analyzed by liquid chromatography (LC, Acquity system, Waters Co., Milford, MA, USA) coupled with a triple quadrupole tandem mass spectrometer (MS/MS, QTRAP 5500, AB Sciex, Framingham, MA, USA). For each sample, 10 μL of diluted extract was injected into the LC-MS/MS. The LC was equipped with a 0.2 μm pre-filter followed by a 2.1 x 5.0 mm (1.7 μm particle size) C18 guard column and a 2.1 x 150 mm (1.7 μm particle size) reversed-phase column. Water (solvent A) and methanol (solvent B) were used as the mobile-phase. The total analysis time was 26 min using a linear gradient, as follows (solvent A/solvent

B): initial gradient was 60/40 at 0.2 mL/min; 14 min to 20/80 at 0.2 mL/min; 1 min to 100% B at 0.2 mL/min; 0.1 min to increase the flow up to 0.35 mL/min and held for 4.9 min; 0.1 min to reduce flow to 0.30 mL/min; 0.9 min to initial gradient 60/40 at 0.3 mL/min and held for 5 min. The column temperature was maintained at 45 °C. Electrospray ionization (ESI) mode was used for the ionization of all analytes. The MS/MS was operated in negative ion mode and the analytes were detected via multiple-reaction monitoring (MRM). The ion source was kept at 700 °C and the capillary voltage was -4.5 kV. Declustering potential and entrance potential were set at -60 V and -10 V, respectively. Other details on the MRM parameters are given in the Table 2. The analytes were quantified by S-std and based on the calibration curve of each analyte. The recovery of each S-std was calculated by the Rec-std.

Gene expression analyses

Tissues were homogenized using either a Mini Beadbeater-96 (BioSpec Products Inc, Bartlesville, OK) or a TissueLyzer II homogenizer (QIAGEN, Valencia, CA). Total pituitary RNA and total liver RNA from male fish were isolated using RNeasy Plus Mini Kit[®] (QIAGEN) following the manufacturer's instructions for isolation of total RNA from animal tissues. Total liver RNA was isolated from female fish using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RNA degradation was observed in some liver samples from the EE2 and TREN exposure experiment due to issues with freezer temperature. Therefore, samples from subsequent experiments were collected in RNAlater[®] to reduce RNA degradation. RNA samples were run on 1% agarose gels to check for RNA quality. Only samples with distinct 28S and 18S ribosomal RNA bands at an approximately 2:1 ratio were used for qPCR analysis. High quality liver RNA samples were DNase treated using

Ambion® TURBO DNA-*free*[™] (Life Technologies). Briefly, a 23 µL reaction was composed of 20 µL (3000 ng) total RNA, 2 µL DNase I Buffer and 1 µL TURBO DNase and incubated at 37 °C for 1 h. DNase treatment was then inactivated with 2 µL inactivation reagent and vortexed at room temperature. RNA yield was quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). DNA-free samples (125 ng pituitary RNA or 250 ng liver RNA) were reverse transcribed with SuperScriptII (Life Technologies) in 10 µL reactions as described in Kitano et al. (2010). Quantitative real-time RT PCR (qPCR) assays were designed and performed as previously described (Harding et al., 2013). Briefly, qPCR assays were run using an ABI 7900HT Fast Real-Time PCR System (Life Technologies) in 384-well plates using standard cycling conditions. Reactions were 12.5 µL each and consisted of 1X Power SYBR Green PCR Master Mix (Life Technologies), 150 nM of the forward and reverse primer, and 0.5 ng cDNA template. Four concentrations of standard curve samples generated from a serial dilution of cDNA (from pooled RNA) ranging from 0.05 to 5 ng cDNA were included in each plate in triplicate. When possible, samples of a given tissue from all exposure dates were run on a single 384-well qPCR plate to eliminate plate-to-plate variation between qPCR runs. In every case, all control and treated samples from a given exposure date were analyzed on a single plate. No template controls showed no detectable amplification over 40 cycles of PCR. For *vtg* and *fshb*, no amplification controls showed no detectable amplification within 35 cycles of PCR. For *lhb*, no amplification controls showed no detectable amplification within 34 cycles of PCR, any samples amplifying after Ct 34 were considered below the level of quantitation. Samples that were below the level of quantitation were assigned the lowest measurable value. Data were expressed relative to the housekeeping gene, eukaryotic elongation factor-1 a (*eef1a*). Pituitary and liver *eef1a* mRNA levels were not significantly different in treated animals compared to

controls (Supplemental Figure 4.S1). To improve visualization, normalized qPCR values were divided by their control mean to set the control mean value to one. qPCR primers were previously reported (Harding et al., 2013).

Statistical analyses

Statistical analyses were performed using Prism 6 for Mac OSX (GraphPad Software, La Jolla, CA). Differences in morphometric data between duplicate tanks were analyzed by 2-way ANOVA (data not shown). Because no significant differences were found ($p > 0.05$); duplicate tanks were pooled in further analyses. For qPCR data, outliers were identified by Grubb's outlier test and were removed from analyses. Data were log-transformed where necessary to meet parametric test criteria. Significant differences between treatments were determined by One-way ANOVA followed by Tukey's multiple comparison test with a significance threshold of $p < 0.05$. Data are expressed as mean \pm SEM. Total SSRI concentrations and combined estradiol equivalent (EEQ) were calculated from water and bile analytical chemistry data using the calculations below. Total SSRI concentration was calculated by summing the individual SSRI and SSRI metabolites.

$$\text{Total SSRI} = [\text{FLX}] + [\text{NF}] + [\text{SER}] + [\text{CIT}]$$

EEQ was calculated using the equation by Young et al., (2004).

$$\text{EEQ} = (\text{E1} \times 0.3) + (\text{E2} \times 1) + (\text{EE2} \times 10)$$

Results:

Control EE2 and TREN Exposures

Chemical Analyses

Levels of EE2 and TREN in exposure water for respective chemicals were measured in each of the duplicate exposure tanks at the start and end of the 72-hour exposure. Exposures were designed to be approximately 2 and 10 ng/L for EE2 and 20 and 200 ng/L for TREN. Mean EE2 exposure levels in duplicate treatment tanks were 2.89 and 2.11 ng/L for the 2 ng/L nominal treatment and 11.65 and 11.89 ng/L for the 10 ng/L nominal treatment. Mean TREN exposure levels in duplicate tanks were 27.4 and 15.0 ng/L for the 20 ng/L nominal concentration and 192.6 and 175.5 ng/L for the 200 ng/L nominal concentration. Hereafter, nominal concentrations (2 and 10 ng/L) and TREN (20 and 200 ng/L) will be used to indicate treatment exposure levels.

Survival and morphometric data

Fish fork length (118 ± 1 mm), body weight (20.9 ± 0.7 g), GSI (0.336 ± 0.010 for females and 0.022 ± 0.001 for males) and HSI (1.151 ± 0.049 for females and 1.070 ± 0.029 for males) were not significantly affected by EE2 or TREN exposure (Supplemental Figure 4.S2). No mortalities were observed over the 72-hour exposure.

Tissue mRNA levels

In the control EE2 and TREN exposure, pituitary *lhb* mRNA levels were significantly increased 17-fold and 215-fold relative to controls in fish exposed to 2 or 10 ng EE2/L, respectively (Figure 1b). TREN, on the other hand, did not significantly affect pituitary *lhb* mRNA levels in exposed fish. Pituitary *fshb* mRNA levels were not significantly affected by either the EE2 or TREN exposure (Figure 1c). Liver *vtg* mRNA levels were increased 6,670-fold in fish exposed to 10 ng/L EE2 for 72 hours (Figure 1a). However, liver *vtg* mRNA levels were not affected by exposure to 2 ng/L EE2 or either TREN dose.

WWTP Effluent Exposures:

Chemical Analyses in Effluent

Samples of undiluted effluent from all sites except E were analyzed for estrogens, 3 selective serotonin reuptake inhibitors (SSRIs) and one SSRI metabolite, NFLX (Tables 3 and 4). WWTP E effluent was not analyzed due to acute lethality during the exposure. For the estrogens, E2 was detected in every effluent that was analyzed with levels reaching up to 2.6 ng/L in effluent from site H. E1 was detected in two of the effluents, but was below the level of quantitation (~ 0.5 ng/L) in both cases. EE2 was detected in 2 effluents with up to 0.8 ng/L in site G effluent. SSRIs were much more prevalent in WWTP effluent samples; all measured SSRIs were detected in 100% of the effluent samples. Average concentrations of SSRIs were 18.5 ng/L, 73.3 ng/L, 75.7 ng/L and 504.6 ng/L for NFLX, SER, FLX, and CIT, respectively. FLX and SER were present in WWTP effluent at similar concentrations, while CIT was, on average, an order of magnitude higher in concentration. Combined, the total SSRI load ranged from a low of 77.5 ng/L at site H to 1672.7 ng/L at site G.

Survival and morphometric data

Mean (\pm SEM) fish fork-length, body weight, GSI, and HSI were 138 ± 1 mm, 30.9 ± 0.5 g, 0.349 ± 0.019 (females) and 0.027 ± 0.005 (males), and 0.866 ± 0.009 (females) and 0.868 ± 0.023 (males). No mortalities were observed in response to effluent from WWTP A, B, C, D, F, or G over the 72-hour exposure. However, in the 100% WWTP E effluent exposure, 92% mortality was observed in the first 24 hours. This was attributed to residual chlorine exposure. After the water change at 24 hours, new fish were added to the tanks. Of these, there was 50%

mortality within 24 hours of being added to the tanks, again presumably due to residual chlorine exposure. Therefore, the remaining fish from the 100% WWTP E effluent exposure, were exposed to effluent for 48 hours, rather than the 72 hours used in other tests.

Tissue mRNA levels

Fish exposed to effluent from sites A, B, D, E, and F had significantly higher *lhb* mRNA than their respective controls (Figure 2). Fish exposed to 20% and 100% effluent from WWTP B had 9- and 42-fold higher *lhb* mRNA levels relative to controls, respectively. Similarly, fish exposed to 20% and 100% effluent from WWTP F had 16- and 491-fold increases in *lhb* mRNA levels as compared to controls. The mean level of *lhb* induction in fish exposed to 20% and 100% WWTP F are similar in magnitude to those observed in fish exposed to 2 and 10 ng/L EE2 in the control exposure. Although *fshb* mRNA levels were unaffected by EE2 and TREN exposure, *fshb* mRNA levels increased in a dose-dependent manner when exposed to effluent from WWTP B (Figure 3B). In contrast, liver *vtg* mRNA levels were not significantly altered by any of the WWTP effluent exposures (Figure 4).

Chemical analyses in bile

Bile samples were analyzed for natural and synthetic steroidal estrogens, and the weakly estrogenic chemical, bisphenol-A (BPA), which is a component of polycarbonate plastic and epoxy resins and commonly found in WWTP effluent and the aquatic environment (Table 5). BPA was the most commonly detected chemical in bile from fish used in this study with concentrations ranging from 44.5 ng/mL to 3900 ng/mL. Natural steroidal estrogens, E1 and E2, were also frequently detected in bile samples from effluent exposed animals, but were not

detected in any of the control bile samples. Mean E1 and E2 levels were as high as 71.7 and 50.7 ng/mL, respectively, in bile from WWTP F. EE2 was detected in bile from fish exposed to effluent from WWTP sites C, D, and F, with concentrations of > 10 ng/mL in bile from fish exposed to WWTP C and F effluents. Considering steroidal estrogens (reported as EEQ) and BPA together, bile from fish exposed to WWTP H effluent showed the least amount of estrogenic chemicals. In contrast, bile from fish exposed to WWTP F effluent showed the highest occurrence and highest concentrations of estrogenic chemicals, including E1, E2, E3, and BPA. Bile from fish exposed to WWTP B effluent had the highest levels and highest occurrence of EE2.

Discussion:

In this study, juvenile coho salmon, a native species in Puget Sound, were exposed to eight different WWTP effluents from the Puget Sound region and effects on pituitary *fshb* and *lhb*, and hepatic *vtg* mRNAs were evaluated. The advantage of using juvenile salmon is that both sexes are prepubertal, have low endogenous steroid levels, and low or non-detectable expression of both *lhb* and *vtg*. However, both of these gene transcripts can be increased with exogenous estrogens in juvenile males and females (Crim et al., 1981; Thorpe et al., 2000). We found that acute (3-day) exposure of juvenile salmon to effluents from five WWTPs increased expression of pituitary *lhb*, but none of the effluents altered hepatic *vtg* expression and only one increased *fshb*. Fish exposed to effluents that induced the highest *lhb* levels also had the highest levels of estrogenic chemicals in bile. Acute exposures of juvenile coho salmon to low concentrations of EE2, a synthetic estrogen commonly found in WWTP effluent, indicated that pituitary *lhb* transcripts increased at lower exposure concentrations of EE2 than hepatic *vtg*, while exposure to

an androgen, TREN, had no effect on any of the measured mRNAs. To our knowledge this is the first time that altered *lhb* or *fshb* mRNA levels have been reported in fish exposed to WWTP effluent. Further, our results suggest that *lhb* gene expression may be a sensitive indicator of acute exposure to estrogenic chemicals in juvenile coho salmon.

EE2 and TREN exposure

In the current study, a controlled 72-hour exposure experiment demonstrated that pituitary *fshb* mRNA levels were not significantly altered in juvenile coho salmon exposed to EE2 or TREN. Previous studies examining the effects of sex steroids on Fsh regulation have shown that E2 suppresses *fshb* mRNA levels, pituitary Fsh content and plasma Fsh levels (Breton et al., 1997; Dickey and Swanson, 1998; Kobayashi et al., 2000; Saligaut et al., 1998). Further, female rainbow trout exposed to the estrogenic contaminant, 4-nonylphenol (NP), for 18 weeks had significantly decreased pituitary *fshb* mRNA levels, pituitary Fsh content and plasma Fsh levels (Harris et al., 2001). Harris and colleagues found that plasma Fsh and pituitary *fshb* mRNA levels were the most sensitive endpoint assessed, being downregulated at the lowest NP concentrations tested. However, in coho salmon exposed to 12 ng EE2/L, significant declines in *fshb* mRNA levels were observed at 6 weeks, but not after 1 week (Harding et al., 2013). These results suggest that while *fshb* mRNA levels may be suppressed by exposure to estrogenic chemicals, longer duration exposures may be necessary to observe significant decreases in *fshb* mRNA levels.

In the EE2 and TREN exposure experiment, levels of pituitary *lhb* mRNA were significantly induced by 2 and 10 ng/L EE2 exposure compared to control and TREN-exposed fish. In agreement with these results, immature coho salmon exposed to 12 ng/L EE2 for 1 or 6

weeks increased *lhb* mRNA levels 241- and 395-fold, respectively, relative to controls (Harding et al., 2013). Fathead minnow (*Pimephales promelas*) embryos exposed to 2, 10, or 50 ng/L EE2 from fertilization until swim up (~7 days) had significantly elevated *lhb* mRNA levels (Johns et al., 2009). In other studies, weak estrogens increased *lhb* mRNA in exposed fish (Johns et al., 2009; Maeng et al., 2005; Rhee et al., 2010; Yadetie and Male, 2002). These data suggest that *lhb* mRNA levels are increased in immature fish in response to exogenous estrogens even at environmentally relevant concentrations.

While *lhb* mRNA levels were significantly increased in response to EE2 exposure, *lhb* mRNA levels were not altered by exposure to TREN, a nonaromatizable androgen. This is in agreement with previous studies that showed no change in *lhb* mRNA levels or pituitary Lh content in fish treated with nonaromatizable androgens (Aroua et al., 2007; Cavaco et al., 2001; Kobayashi et al., 2000). In salmonids, treatment with nonaromatizable androgens has been shown to cause small increases in *lhb* mRNA or Lh protein levels *in vivo*, but to a much lesser extent than E2 or testosterone (Antonopoulou et al., 1999; Borg et al., 1998; Crim et al., 1981; Melo et al., 2015). Additionally, in castrated Atlantic salmon (*Salmo salar*) parr, testosterone treatment dramatically increased pituitary Lh content and this effect was significantly attenuated by treatment with testosterone combined with an aromatase inhibitor (Antonopoulou et al., 1999). Therefore, our results and the results of previous studies suggest that positive regulation of *lhb* by androgens is aromatase dependent and nonaromatizable androgens have little, if any effect on *lhb* induction in salmonids.

In contrast to the *lhb* response, only the high concentration of EE2 resulted in elevated *vtg* expression in the liver. In other studies of immature rainbow trout, exposure to levels as low as 1.0 ng/L EE2 elevated *vtg*, but in this study the exposure time was 14 days (Thomas-Jones et

al., 2003). Unfortunately, in salmonids there is little information on Vtg protein or mRNA induction in experiments lasting less than 14 days, raising the possibility that 72-hour exposure duration is too short to observe *vtg* induction at low exposure levels. In fathead minnow, lowest-observed-effect concentrations (LOEC) of 2.2 and 5 ng/L EE2 have been reported for *vtg* mRNA induction at 48 hours (Biales et al., 2007; Flick et al., 2014; Reddy et al., 2015). Similarly, in fathead minnow embryo and larvae exposed to 0, 18, or 1800 ng/L E1, *vtg* mRNA levels were significantly induced at 6 days, but not at 3 days of exposure although the other estrogen responsive genes (estrogen receptor α and cytochrome P450-aromatase-B) were already significantly induced at 3 days (Cavallin et al., 2015). Cavallin and colleagues suggested that the delayed induction of *vtg* may reflect the time required to upregulate a functional estrogen receptor in the liver. These data suggest that the 3-day exposure used in the current study may be insufficient to observe *vtg* induction in juvenile coho salmon in response to low concentrations of estrogens in exposure water.

WWTP effluent exposures

In the current study, effluents collected from WWTPs in South Puget Sound were analyzed for the presence of steroidal estrogens and SSRIs. Due to the method of effluent sampling (grab samples rather than time-weighted sampling), it is not possible to draw firm conclusions about the effect of alternate wastewater treatment technologies on the removal of steroids or pharmaceuticals from WWTP effluents. However, steroidal estrogen and SSRI levels are still useful for understanding the biological responses of exposed animals. In addition, all sample collections occurred at the same time of day, and within a narrow seasonal time frame limiting time-dependent differences between WWTP effluent collections. Additionally, previous

studies have shown that trickling filter WWTPs or trickling filters with activated sludge treatment have lower removal efficiencies of pharmaceuticals and personal care products including steroidal estrogens than tertiary treatment processes (Bartelt-Hunt et al., 2009; Kasprzyk-Hordern et al., 2009). Measured concentrations of steroidal estrogens were in the low ng/L range (<3 ng/L) for all WWTP effluents. Among these, E2 was the most prevalent and was detected in every effluent. E2 was also found in the highest concentration (2.6 ng/L) of any of the measured steroidal estrogens. In general, the levels of steroidal estrogens in effluent observed in this study are low compared to previous studies. For example, analyses of two WWTP effluents from Puget Sound identified estrone concentrations of 4.5 to 58 ng/L (Meador et al., 2016). Elsewhere in North America, natural estrogens have been measured in wastewater effluents with concentrations ranging from 6-14 (E1), <5 (E2) and <10-33 ng/L (estriol, E3) (Huang and Sedlak, 2001; Lee and Peart, 1998; Snyder et al., 1999). Reported EE2 concentrations in WWTP effluents can vary widely, but also typically fall in the low ng/L range (Young et al., 2004). In the United States, WWTP effluents had EE2 concentrations ranging from <0.05-2.42 ng/L (Huang and Sedlak, 2001; Snyder et al., 1999), however EE2 has been measured in streams at levels up to 273 ng/L, with median measured concentrations below 10 ng/L (Kolpin et al., 2002). Similarly, in Canadian WWTP effluents, EE2 concentrations reached 42 ng/L, with median concentration of 9 ng/L (Ternes et al., 1999).

We also measured a variety of SSRIs in the effluents due to their high occurrence in WWTP effluents (Hedgespeth et al., 2012; Meador et al., 2016; Verlicchi et al., 2012) due to incomplete removal during wastewater treatment (Lubliner et al., 2010). In addition, SSRIs bioaccumulate in tissues of fish exposed to WWTP effluent (Lajeunesse et al., 2011; Schultz et al., 2010) and are capable of altering Gth mRNA levels in fish. For example, a 2-week exposure

of male zebrafish to CIT, decreased mRNA levels of GnRH 3 (*gnrhr3*), *fshb*, and *lhb* (Prasad et al., 2015). These data suggest that SSRIs could accumulate in fish brain tissues and potentially alter the brain-pituitary axis, including Gth mRNA expression.

In the present study, all of the SSRIs measured were detected in WWTP effluents typically in the 10 to 100 ng/L range. These levels are consistent with concentrations of SSRIs previously measured in WWTP effluents from Puget Sound and are in the 95th to 99th percentile relative to what is seen elsewhere in the United States (Meador et al., 2016). CIT was present at the highest concentration in effluent from all WWTP effluents, except WWTP H. This is in agreement with a previous study that found measured CIT concentrations were, on average, an order of magnitude greater than SER or FLX in two effluent impacted streams (Schultz et al., 2010). The total SSRI concentrations were lowest in effluent from WWTP H (77.5 ng/L), intermediate (480 – 770 ng/L) for WWTP A, B, C, D, and F effluents, and highest in WWTP G effluent (1672.7 ng/L). There was no clear relationship between CIT or total SSRI concentrations in the effluents and any of the biological measurements. This may reflect varying kinetics of accumulation or bioconcentration for different SSRIs. Previous work suggests that particular SSRIs are selectively taken up in fish tissues. The main SSRIs measured in white sucker (*Catostomus commersonii*) brain tissues were FLX, SER and their metabolites (NFLX and N-desmethylsertraline), despite higher environmental levels of CIT (Schultz et al., 2010). Additionally, previously reported effects of SSRIs on Gth mRNA levels in fish were observed after 2 weeks of exposure (Prasad et al. 2015) whereas in the current study juvenile coho were only exposed to SSRIs via WWTP effluent for 3 days. Therefore, a longer exposure period may be required to observe effects of SSRIs on Gth expression.

In the WWTP effluent exposures, *lhb* mRNA levels were significantly elevated in fish exposed to 100% effluent from five of the eight WWTPs. Fish exposed to effluent from WWTP F, had the highest *lhb* induction. While in most cases, fish would not be expected to be exposed to 100% effluent, the acute dilution factor for several of the WWTPs is between 2 and 4 resulting in effluent concentrations of 25 to 50% (Table 1). In addition, it is expected that fish may be exposed for much longer than 72 hours resulting in increased *lhb* induction over time.

The primary known factor stimulating *lhb* transcription in fish is E2 (Yaron et al., 2003). Based on the estrogenic nature of most WWTP effluents, it is tempting to speculate that the increased *lhb* mRNA levels observed in effluent exposed fish are due to estrogenic activity of those effluents. However, we were unable to demonstrate relationships between effluent steroid concentrations and *lhb* mRNA levels because of limited ability to measure steroidal estrogens in effluent samples using the methods we employed. It is possible that additional estrogenic chemicals are present in the effluents that were not measured. Additionally, the possibility that other non-estrogenic chemicals are able to induce *lhb* mRNA levels cannot be ruled out.

To further investigate the hypothesis that *lhb* mRNA levels are increased in effluent exposed fish due to exogenous estrogens, we measured steroidal estrogens in bile of fish exposed to wastewater effluents where possible. Analysis of bile fluid can provide an indication of the internal exposure level to estrogenic chemicals including steroidal estrogens, NP, and BPA (Larsson et al., 1999; Pettersson et al., 2006; Vermeirssen et al., 2005). Additionally, due to the accumulation of some estrogenic chemicals, bile analysis can be used to measure estrogenic chemicals that are below the limit of detection in WWTP effluent (Gibson et al., 2005). In fish exposed to WWTP effluent, the highest steroid hormone levels were detected in effluent B and effluent F with 161 and 187 ng/L EEQ respectively (see Table 5). These EEQ levels were, in

large part, due to relatively high (>10 ng/mL) bile concentrations of EE2. Interestingly, these results correspond well with the *lhb* induction we observed in fish exposed to effluents from WWTP B and F. Additionally, bile EEQ concentrations were highest in WWTP F, a trickling filter treatment plant which is known to be less efficient in removing pharmaceuticals including steroidal estrogens (Bartelt-Hunt et al., 2009; Kasprzyk-Hordern et al., 2009). These data support the hypothesis that induction of *lhb* expression in effluent-exposed juvenile coho is due to exposure to estrogenic chemicals in WWTP effluent.

Consistent with the lack of change observed in the control EE2 exposure, *fshb* mRNA levels were generally unaffected in fish exposed to WWTP effluent. However, *fshb* mRNA levels were significantly increased following exposure to WWTP B effluent. As mentioned above, E2 and estrogen mimics such as NP tend to suppress *fshb* expression or Fsh protein via negative feedback, but these effects generally occur after long-term exposure or treatment with estrogens. Given the exposure used was only 3 days, it is possible that the lack of negative effect on *fshb* was due to duration of exposure. The factors in WWTP B effluent that caused an increase in steady state levels in *fshb* are unknown, but could act by either antagonizing factors that reduce *fshb* levels, or stimulating those that increase *fshb* levels such as, gonadotropin-releasing hormone (GnRH) and kisspeptin from the brain and activin from the ovary (Levavi-Sivan et al., 2010; Yaron et al., 2003). Since Fsh plays a key role in regulating early stages of gametogenesis (Levavi-Sivan et al., 2010; Swanson et al., 2003), prolonged disruption of *fshb* production might impact plasma Fsh levels and ultimately alter age or seasonal timing of gonad growth, fecundity and gamete quality. Further research is needed to determine what chemicals present in WWTP effluents may elevate *fshb* levels, and whether prolonged exposure results in downstream effects on plasma Fsh and gametogenesis.

In the current study, liver *vtg* mRNA levels were not significantly elevated in fish exposed to effluents from any of the WWTPs tested. This is consistent with the lack of *vtg* induction observed in the low EE2 exposure in our control EE2 and TREN exposure. However, this is in contrast to previous studies that have shown *vtg* induction in fish exposed to WWTP or in wild fish sampled downstream of WWTPs (Barber et al., 2007, 2011; Folmar et al., 2001, 1996; Harries et al., 1999, 1997; Ings et al., 2011). These results suggest that the effluents tested had low estrogenic activity that was insufficient to stimulate *vtg* mRNA levels within the 72-hr period of exposure. Indeed, the steroidal estrogen levels measured in effluents in the current study are lower, on average, than previously reported levels (Huang and Sedlak, 2001; Lee and Peart, 1998; Snyder et al., 1999; Ternes et al., 1999). Further, the current experiment was shorter in duration than previous studies showing *vtg* mRNA induction in response to WWTP effluent exposure (14-28 days; Barber et al., 2007, 2011; Folmar et al., 2001, 1996; Harries et al., 1999, 1997; Ings et al., 2011). Therefore, longer exposure duration may be necessary to induce *vtg* mRNA levels in response to estrogenic WWTP effluent. Because of the static renewal system we used and our limited ability to store large volumes of chilled WWTP effluent required for exposures, it was not possible to increase the duration of exposure in the current study.

Conclusion:

Pituitary *lhb* mRNA levels were significantly increased in juvenile coho salmon during an acute (72-hour) low EE2 (2 ng/L) exposure. Similarly, *lhb* mRNA levels were induced 42-fold and 491-fold respectively above control in immature coho salmon exposed to 100% effluent from WWTP B and F for 72 hrs. Interestingly, fish exposed to 100% effluent from WWTP B and F also had the highest EEQ levels in their bile. These results suggest that *lhb* mRNA induction is

a sensitive indicator of exposure to EDCs with estrogenic activity in juvenile coho salmon. However, due to the complex mixture of WWTP effluent, it is possible that other chemicals (other steroid hormones, pharmaceuticals, industrial compounds) may be contributing to the induction of *lhb* mRNA levels. In contrast, hepatic *vtg* transcripts were not significantly altered in response to 72-hour exposure to 2 ng/L EE2 or any of the WWTP effluents tested. As a result, further work on the kinetics and specificity of the pituitary *lhb* response compared to hepatic *vtg* in this species and life history stage is needed. The results of this study emphasize the importance of measuring multiple biological endpoints to detect endocrine disruption, and raise the possibility that one mechanism whereby WWTP effluents may alter reproductive function in fish is via disrupted gonadotropin synthesis.

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Table 4.1. Types of waste water treatment processes at each collection site. Effluent was collected after the final filtration step at all WWTPs.

WWTP Site	Site Treatment Type	Waterbody Type	Acute Mixing Zone ^a	Chronic Mixing Zone ^a	Acute Dilution Factor ^b	Chronic Dilution Factor ^b
A	Activated sludge with ultraviolet (UV) disinfection	Marine	30 ft plus horizontal length of diffuser downstream, 10 ft upstream, 25% of river width	300 ft plus horizontal length of diffuser downstream, 100 ft upstream, 25% of river width	1.9	13.6
B	Oxidation ditch with chlorine disinfection	Fresh	10 ft. upstream, 30 ft. downstream, 26.75 ft width	100 ft. upstream, 300 ft. downstream, 26.75 ft width	2.62	11.35
C	Secondary sequencing batch reactor with UV disinfection	Marine	30 ft downstream	300 ft downstream, 17.7 ft from left bank.	3.9	19.7
D	Activated sludge with biological (Bardenpho process) nutrient removal and UV disinfection	Marine	21.4 ft from the ends of the diffuser and 21.5 feet from the centerline of the diffuser pipe	213.5 ft from the last discharge point at both ends of the diffuser section and 215 feet from the centerline of the diffuser section	22	22
E	Reclaimed water (Class A Reuse) from Collection Site D	No discharge to surface waters	NA	NA	NA	NA
F	Trickling filter with chlorine disinfection	Marine	27 ft x 67 ft	271 ft x 670 ft	53	88
G	STEP collection followed by secondary treatment (SBRs), and coagulation and flocculation with filtration to meet Class A reclaimed water requirements and chlorine disinfection	Fresh	19.7 feet wide, extends 30.15 feet downstream and 10.0 feet upstream.	19.7 feet wide, extends 301.5 feet downstream and 100.0 feet upstream	6.5	20
H	Activated sludge with membrane filtration and chlorine disinfection	No discharge to surface waters	NA	NA	NA	NA

^aAcute and chronic mixing zones: the area near the outfall where water quality standards for acute and chronic aquatic life criteria may exceed standards as authorized in a national pollutant discharge elimination system (NPDES) permit (McGowan, 2015).

^bAcute and chronic dilution factors: the lowest dilution achieved at the edge of the acute and chronic mixing zones (McGowan, 2015).

Table 4.2: Individual multiple-reaction monitoring (MRM) parameters of target EDCs and deuterated standards

EDCs	ions (m/z)		CE (V)	CXP (V)
	Precursor	Product ^a		
E3	287.04	171.0	-50	-13
		(145.1)	-52	-9
BPA	226.91	212.1	-24	-7
		(133.1)	-34	-7
E1	269.16	144.9	-50	-5
		(142.8)	-64	-11
E2	271.06	145.1	-50	-7
		(182.9)	-54	-7
EE2	295.08	144.9	-54	-7
		(143.0)	-62	-11
OP	205.05	105.9	-26	-17
		(177.0)	-26	-13
NP	219.10	105.9	-28	-3
		(82.8)	-22	-13
BPA-d16	242.06	142.2	-36	-9
		(224.1)	-26	-9
BPA-d4	231.04	216.0	-24	-9
		(135.0)	-40	-15
E1-d4	273.04	147.1	-52	-7
		(145.1)	-68	-7
E2-d4	274.90	147.0	-54	-7
		(187.1)	-56	-7
EE2-d4	299.08	147.1	-48	-7
		(145.1)	-72	-7
NP-d5	224.10	110.6	-28	-9
		(110.0)	-28	-5

^a Product ions in parenthesis were used for helping identifying the analytes only. CE = collision energy; CXP = collision cell exit potential.

Table 4.3: Steroid levels (E1 = estrone, E2 = 17 β -estradiol, EE2 = 17 α -ethynylestradiol) in 100% WWTP effluent measured by GC-MS. NA = not assayed, nd = not detected, trace = detected, but below quantifiable detection by GC-MS.

WWTP	Sample volume (L)	E1 (ng/L)	E2 (ng/L)	EE2 (ng/L)
A	0.863	trace	0.65	ND
B	0.845	ND	1.89	ND
C	0.854	ND	trace	ND
D	0.885	ND	0.92	ND
E	NA	NA	NA	NA
F	0.872	ND	trace	trace
G	0.844	ND	0.95	0.83
H	0.836	trace	2.60	ND

Table 4.4: Selective serotonin reuptake inhibitor (SSRI) levels in undiluted WWTP effluent measured by GC-MS. (FLX = fluoxetine, NF = norfluoxetine, SER = sertraline, CIT = citalopram, ND = not detected, NA = not assayed).

WWTP	Sample volume (L)	FLX (ng/L)	NF (ng/L)	SER (ng/L)	CIT (ng/L)	Total SSRIs (ng/L)
A	0.804	37.9	4.7	44.8	467.0	554.5
B	0.861	26.5	4.1	43.1	408.3	482.1
C	0.86	51.6	7.7	52.9	389.1	501.3
D	0.857	91.1	5.9	71.6	600.6	769.2
E	NA	NA	NA	NA	NA	NA
F	0.843	67.2	56.4	186.5	336.6	646.8
G	0.822	209.0	47.1	87.6	1328.9	1672.7
H	0.837	46.3	3.5	26.2	1.5	77.5

Table 4.5: Mean detected concentration (ng/mL) and percent of samples above the limit of detection for steroidal estrogens [estrone (E1), 17 β -estradiol (E2), estriol (E3), and 17 α -ethynylestradiol (EE2)] and bisphenol A (BPA) in bile from control and WWTP effluent-exposed juvenile fish (mixed sex) as measured by LC-MS/MS. (ND = not detected, NA = not assayed).

Treatment	N	BPA ng/mL	% BPA	E1 ng/mL	% E1	E2 ng/mL	% E2	E3 ng/mL	% E3	EE2 ng/mL	% EE2	EEQ
Composite control	9	76.5	22	ND	0	0	0	ND	0	ND	0	0
A	6	114	100	8.3	100	5.5	67	ND	0	ND	0	8.0
B	7	152	100	5.2	71	6.1	71	ND	0	15.3	86	161
C	3	1120	100	2.3	33	2.0	33	1.6	33	2.9	33	31.7
D	2	2050	100	ND	0	ND	0	ND	0	ND	0	0
E*	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
F	3	3900	100	71.7	100	50.7	100	44.0	100	11.5	67	187
G*	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
H	3	44.5	33	ND	0	ND	0	ND	0	ND	0	0

* Not analyzed because bile was not collected or bile volume was not adequate.

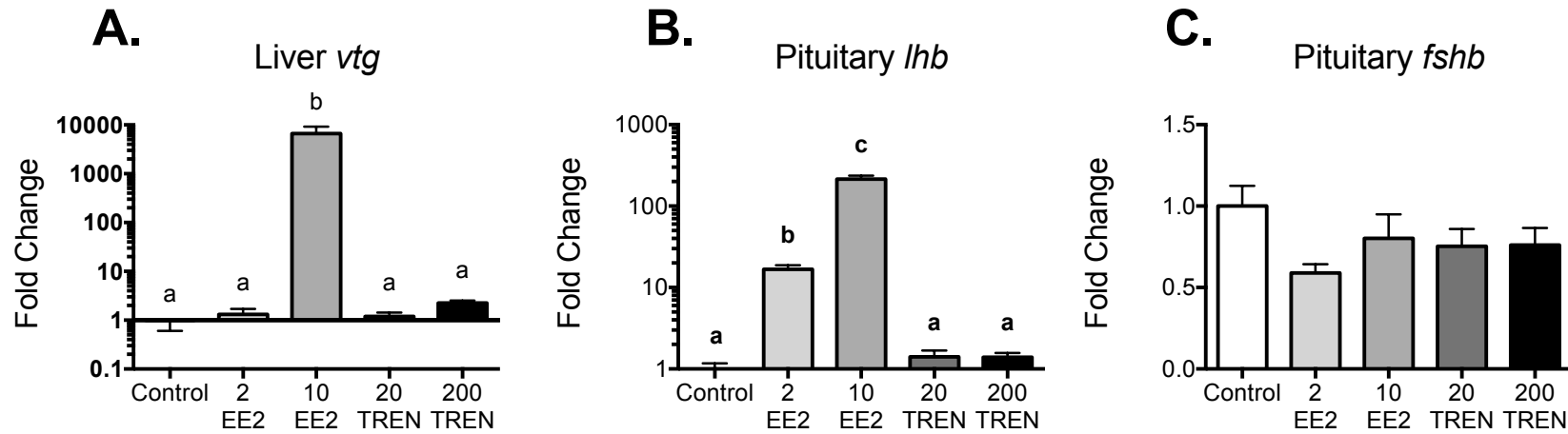


Figure 4.1. Relative levels of mRNAs for liver *vtg*, pituitary *lhb*, and pituitary *fshb* in juvenile coho salmon exposed to water containing 0, 2, or 10 ng/L EE2 or 20 or 200 ng/L TREN for 72 hrs. qPCR data were normalized to *eef1a* levels and then divided by control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean \pm SEM ($n = 5-8$ for liver, 19-20 for pituitary). Data were log transformed when necessary to conform to normality and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate significant differences between treatments.

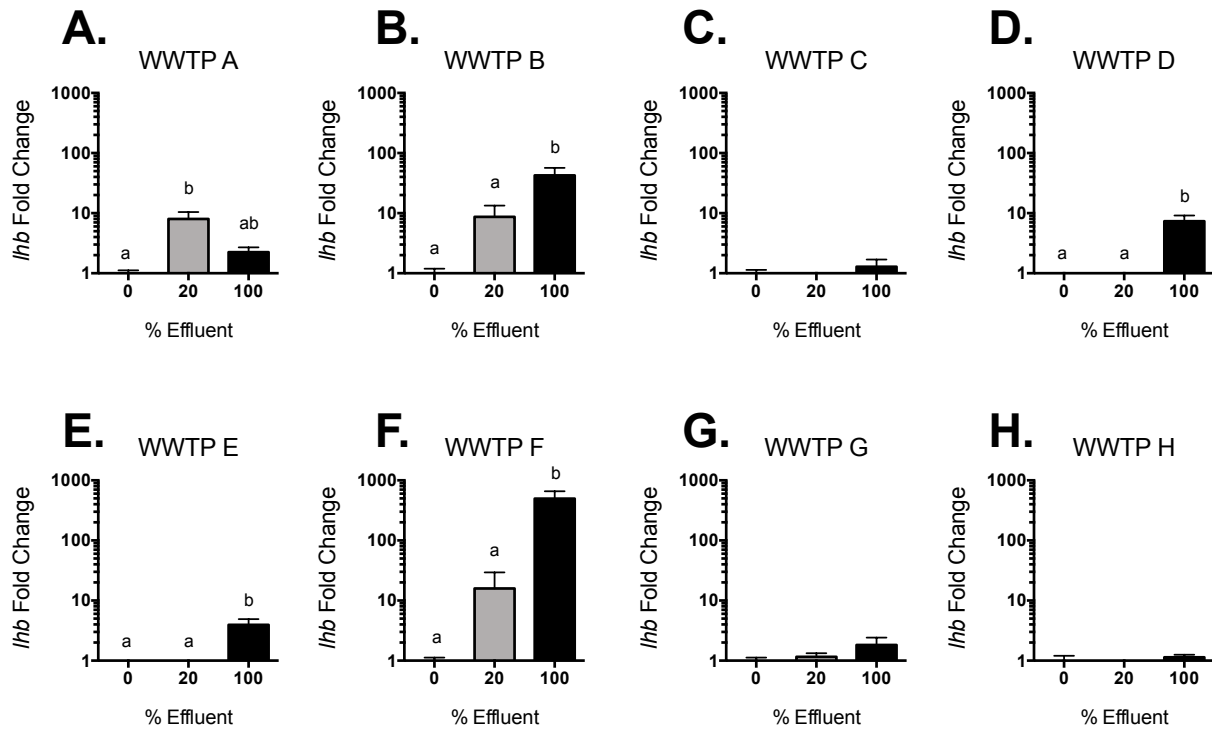


Figure 4.2. Relative levels of pituitary luteinizing hormone beta subunit (*lhb*) in juvenile coho salmon exposed to WWTP effluent for 72 hrs. The qPCR data were normalized to *ee1a* levels and then divided by the control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean \pm SEM ($n = 6-12$). Data were log transformed to conform to normality and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate significant differences between treatments ($p < 0.05$).

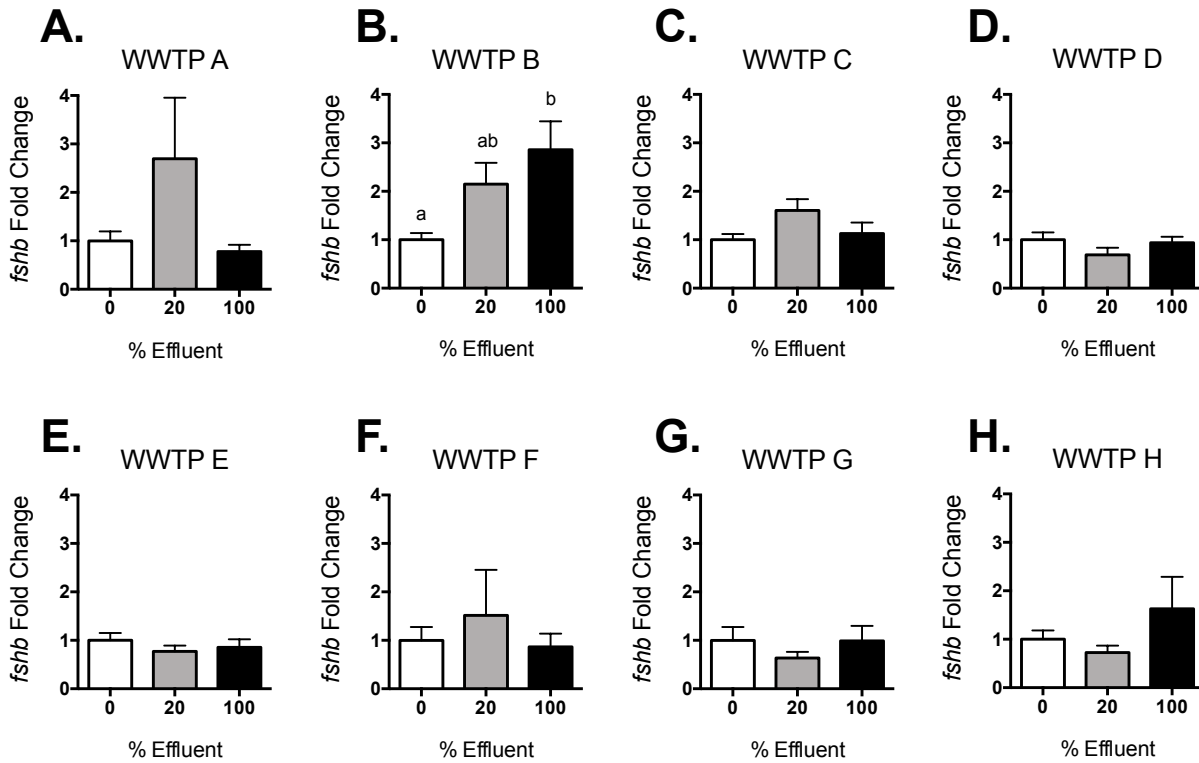


Figure 4.3. Relative levels of pituitary follicle stimulating hormone beta subunit (*fshb*) mRNA in juvenile coho salmon exposed to WWTP effluent for 72 hrs. qPCR data were normalized to *eef1a* levels and then divided by control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean \pm SEM ($n = 6-12$). Data were log transformed when necessary to conform to normality and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate significant differences between treatments ($p < 0.05$).

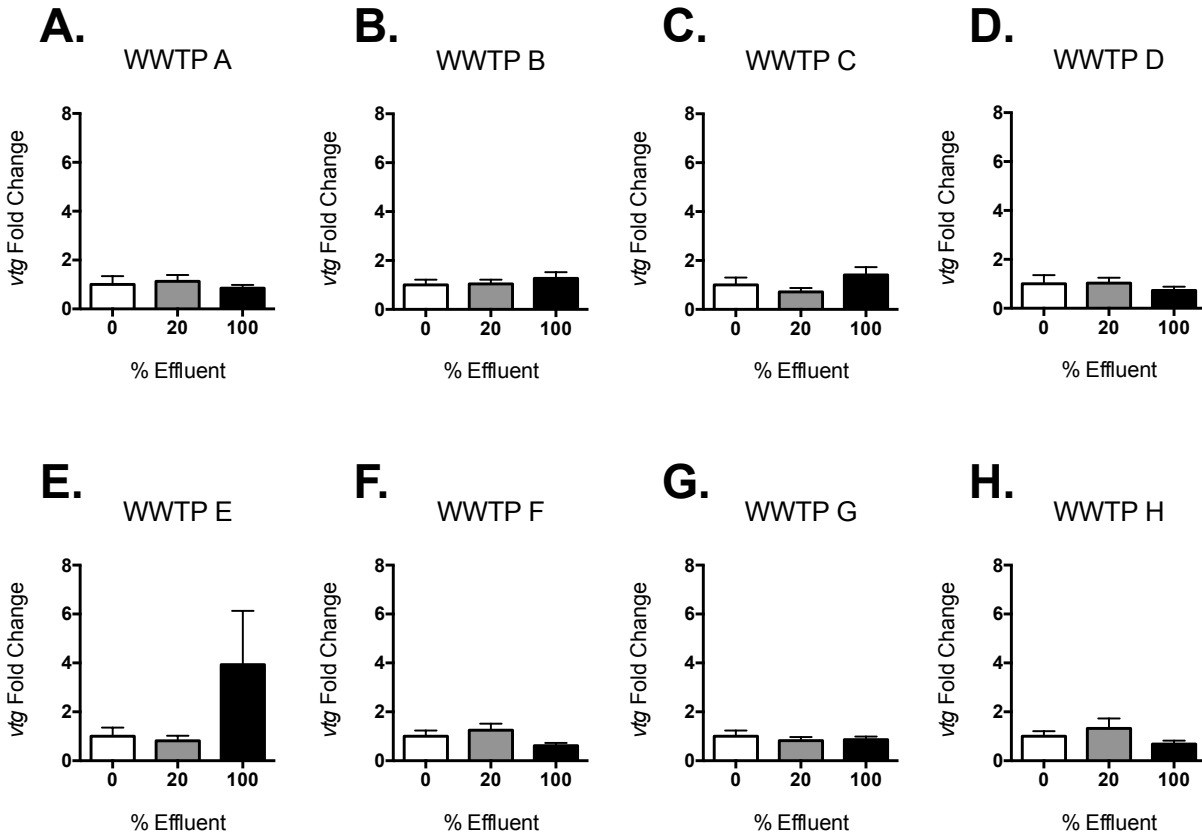
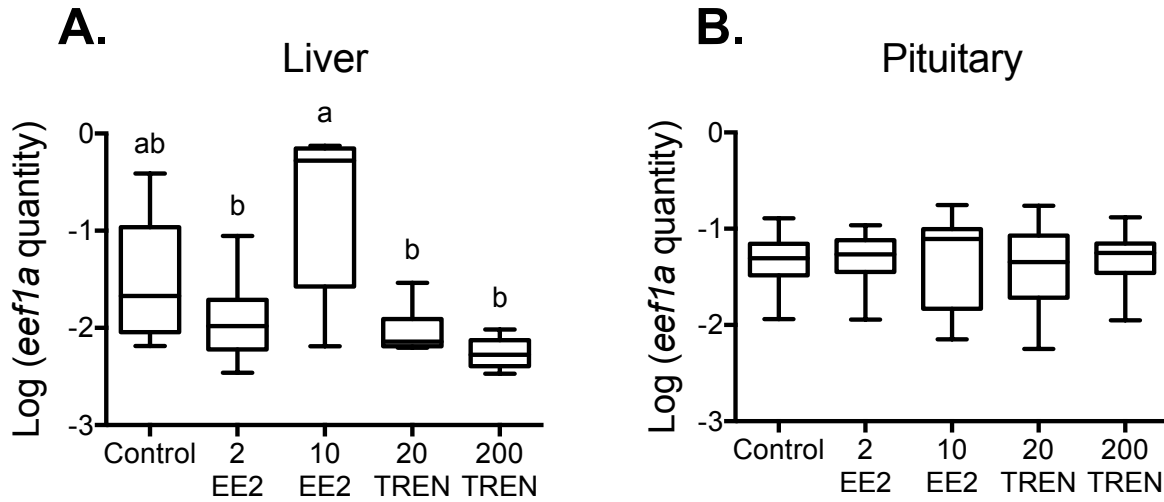
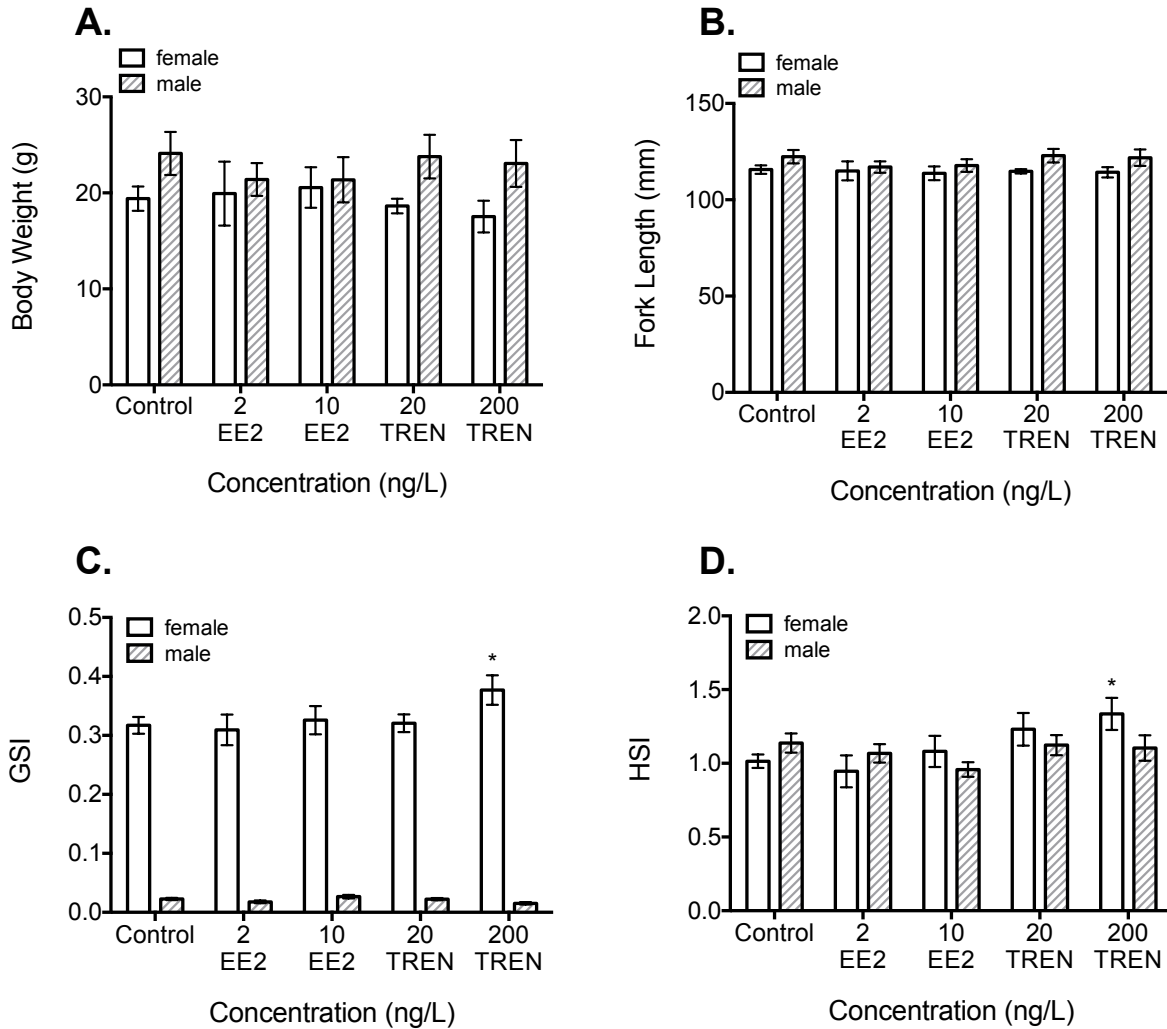


Figure 4.4. Relative levels of vitellogenin (*vtg*) mRNA in juvenile coho salmon exposed to WWTP effluent for 72 hrs. qPCR data were normalized to *eef1a* levels and the divided by the control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean \pm SEM ($n = 6-12$). Data were log transformed to conform to normality and were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Different letters indicate significant differences between treatments ($p < 0.05$).



Supplemental Figure 4.S1: Log levels of mRNA for *eef1a* measured by qPCR in (A) liver or (B) pituitary of fish exposed to 0 ng/L, 2 ng/L EE2, 10 ng/L EE2, 20 ng/L TREN or 200 ng/L TREN for 72 hrs. *eef1a* levels were used to normalize hepatic *vtg* and pituitary *lhb* and *fshb* mRNA levels. No significant differences were observed between control and EE2 or TREN-exposed samples. (1-way ANOVA followed by Tukey's multiple comparisons test). The data are expressed as mean \pm SEM ($n = 5 - 8$ for liver and 20 for pituitary).



Supplemental Figure 4.S2: Morphometric data from control EE2 and TREN exposure. Body weight (A) and fork length (B) did not significantly differ between tanks or treatments (2-way ANOVA, $p > 0.05$). Gonadosomatic index (GSI) significantly differed between male (C) and female (D) fish (2-way ANOVA, $p < 0.0001$), but did not significantly differ between tanks (2-way ANOVA, $p > 0.05$). Hepatosomatic index (HSI) was not significantly different based on sex or tank. The data are expressed as mean \pm SEM (A and B; $n = 10$; C – D; $n = 2 - 9$).

SYNTHESIS AND CONCLUSIONS

The overall aims of my dissertation research were to investigate sex steroid regulation of pituitary Gths during early stages of oogenesis in salmon and to determine the extent to which sex steroid regulation of Gths could be disrupted by chemicals or natural steroids in the environment. My motivation for this research was to contribute to our basic understanding of the role of sex steroids in regulating Fsh and Lh at early stages of oogenesis, to provide a basis on which to predict effects of EDCs and/or interpret results from endocrine disruption research, and to aid in the development of ecologically relevant models for studying EDCs in Puget Sound. My rationale for choosing coho salmon at this stage of reproduction was that: 1) the onset of secondary oocyte growth is a critical stage of development, representing the onset of puberty in fish; 2) the factors regulating an increase in plasma Fsh at this stage remain unknown; and 3) this stage coincides with the timing of juvenile outward migration when they are likely to transit through urban waterways and potentially be exposed to EDCs.

At the outset of my research (Chapter 1), I investigated effects of natural sex steroids, E2 and the nonaromatizable androgen, 11-KT, on Gth subunit transcripts and protein in the pituitary in vivo. My hypothesis was that E2 would increase pituitary *lhb* mRNA and pituitary Lh levels and decrease pituitary *fshb* mRNA and Fsh levels during early oogenesis. Further, I hypothesized that 11-KT would increase pituitary and plasma Fsh levels. I examined sex steroid regulation during late primary and early secondary oocyte growth because I hypothesized that sex steroid feedback would change at this stage when Gths normally begin to rise. I found that in coho salmon during late primary and early secondary oocyte growth, E2 treatment caused rapid and robust increase in pituitary *lhb* mRNA levels and Lh content (Chapter 1). The rapid response of *lhb* to E2 stimulation suggests that these effects are due, at least in part, to direct stimulation of

lhb transcription via EREs in the *lhb* promoter. This hypothesis is supported by previous studies showing E2-induced upregulation of *lhb* in primary pituitary cells of masu salmon (Ando et al., 2004) and other fishes (Aroua et al., 2007; Huang et al., 1997; Huggard-Nelson et al., 2002; Lin and Ge, 2009; Rebers et al., 2000; Zhang et al., 2014). In addition, estrogen-stimulation of *lhb* appears to be time-dependent with *lhb* mRNA and pituitary Lh levels increasing over the duration of the experiment (Chapter 1 and 2), suggesting that the capacity for *lhb* production increases over time due to recruitment of transcriptional or translational cofactors or proliferation of Lh gonadotrophs. E2 treatment also caused modest increases in *cga2* mRNA levels during late primary oocyte growth, which may contribute to increased synthesis of Lh heterodimer. Although plasma Lh levels were too low to detect in this study, other studies have shown that Lh is not released in response to E2 stimulation alone (Breton et al., 1997; Dickey and Swanson, 1998; Saligaut et al., 1998).

In contrast, E2 had differential effects on pituitary *fshb* mRNA and Fsh content: E2 decreased *fshb* mRNA level and increased Fsh pituitary content (Chapter 1). The divergent effects of E2 on *fshb* mRNA and Fsh protein levels indicate that regulation of Fsh is complex and may involve regulation of Fsh synthesis, release, clearance, or a combination of these processes. However, discrepancies between *fshb* mRNA levels and pituitary Fsh content have been observed previously. In female rainbow trout, *fshb* mRNA and plasma Fsh levels decrease during the transition from vitellogenesis into oocyte maturation, whereas pituitary Fsh content continues to increase (Gomez et al., 1999). The accumulation of pituitary Fsh observed in trout as well as in my study in coho salmon may reflect suppression of Fsh release in response to elevated E2 levels. This hypothesis requires further investigation using an Fsh assay with greater sensitivity that would allow measurement of plasma Fsh during early stages of oogenesis. In

addition, the effects of estrogens on Fsh synthesis were protracted, with alterations of *fshb* mRNA levels occurring after 7 days of treatment or longer (Chapter 1 and 2), and increased pituitary Fsh levels after 21 days (Chapter 1). The delayed response of Fsh to estrogens may reflect indirect mechanisms of action of E2 or EE2 via the brain, autocrine/paracrine factors in the pituitary, gonadal factors such as inhibin and/or some interaction of these factors.

The results of this study did not support my hypothesis regarding androgen effects on Fsh levels. Treatment with 11-KT did not affect pituitary *fshb* mRNA or Fsh levels. The lack of effect on pituitary Fsh levels was in contrast to previous work in coho salmon that found increased pituitary Fsh levels after 10 or 20 days of 11-KT treatment in ECA stage fish (Forsgren, 2010). The plasma 11-KT levels achieved by Forsgren (2010) were roughly an order of magnitude lower than the concentrations used here, therefore the lack of effect may be due to differences in 11-KT concentrations achieved by the implants. Further, regulation of Fsh and Lh synthesis by 11-KT cannot be ruled out as we were unable to measure plasma Fsh and Lh levels in this study.

The study described in Chapter 2 was aimed at examining broader effects of estrogen on the pituitary, revealing signaling pathways involved in estrogen regulation of Gths, and testing potential impacts of environmentally relevant concentrations of one EDC (EE2) on coho salmon at early stages of oogenesis. I hypothesized that waterborne EE2 exposure would increase *lhb* mRNA levels and decrease *fshb* mRNA levels, as observed in Chapter 1, and would alter expression of genes involved in Gth signaling. For this study, I used high-throughput sequencing and pathway analysis to examine transcriptome-wide changes in pituitary gene expression in response to EE2 treatment (Chapter 2). I selected EE2 because it is a known ER agonist and thus, the results of this study provide additional information on potential normal mechanisms of

estrogenic regulation of Fsh and Lh as well as insight into modes of endocrine disruption in the pituitary gland. I conducted a waterborne exposure because this is a natural route of exposure in the environment and constant low levels could be maintained over time. Consistent with the effect of E2-implants at this stage, waterborne EE2 exposure resulted in dramatic induction of *lhb* mRNA levels and a delayed decrease in *fshb* mRNA levels. In addition, EE2 exposure altered mRNA levels of genes involved in steroid receptor signaling pathways (ER signaling, AR signaling, glucocorticoid receptor signaling), including reduction of *esr2* mRNA levels. This is consistent with suppression of *esr2* transcripts by E2 in ECA-stage fish (Chapter 1). EE2 exposure also altered mRNA levels of genes involved in Gth synthesis and secretion including genes involved in GnRH, calcium and TGF- β signaling pathways. Estrogen treatment consistently upregulated *gnrhr1* mRNA levels in previtellogenic coho salmon (Chapters 1 and 2). It is possible that E2- or EE2-induced expression of GnRHR may act to sensitize the pituitary to GnRH stimulation as has been shown in mammals (Reeves et al., 1971). However, given the stimulatory effect of GnRH on *fshb* mRNA levels and Fsh secretion (Ando et al., 2004; Breton et al., 1998; Dickey and Swanson, 2000; Golan et al., 2014; Khan and Thomas, 1994), this is not consistent with the observed suppression of *fshb* mRNA levels by E2 (Chapters 1 and 2). However, since I did not measure brain or pituitary GnRH, the role of GnRH signaling in E2- or EE2-induced suppression of *fshb* is unclear. Further, it is unknown whether the observed increase in *gnrhr1* mRNA levels occurred in Lh cells, Fsh cells, or both. Future studies using double-label *in situ* hybridization to examine *gnrhr1* mRNA levels in Fsh and Lh gonadotrophs in response to estrogen treatment could help to clarify these results.

One of the most intriguing findings from my studies of EE2 effects on the pituitary transcriptome was the potential effects of estrogen on circadian rhythms. Pathway analysis

revealed significant connections between GnRH signaling and circadian rhythm signaling in the pituitary gland in response to EE2 treatment. Recent studies have identified the presence of an autonomous circadian clock in the pituitary gland of mice that may be involved in regulating timing of hormone production or release (Becquet et al., 2014; Guillaumond et al., 2012). Further, E2 has been shown to alter the expression of clock genes in a variety of tissues in mammals (Gery et al., 2007; Nakamura et al., 2008, 2005, 2001). It is interesting to speculate, then, that E2 or exogenous estrogens could regulate an autonomous pituitary circadian clock that could in turn regulate hormone production or release. I attempted to investigate the role of E2 in regulating a pituitary circadian clock *in vitro* using primary pituitary cell culture, but my results were inconclusive (data not shown). This may be either because the experimental culture system was not optimized for such studies, or that the network of intercellular communication within the pituitary needs to be maintained as the effects of E2 could be indirect via other cell types in the pituitary (GnRH neurons, folliculostellate cells, etc.). Recent studies by Golan et al. (2016a, 2016b) have shown that Lh cells form extensive networks via close cell-cell contacts, whereas, Fsh cells communicate with a network of stellate cells that may play a paracrine role in Fsh regulation. This is an exciting area of basic biological research on effects of sex steroids on circadian rhythms of transcription, translation, and secretion of pituitary hormones. Further, this may have implications for the effects of xenoestrogens on reproductive timing. This novel finding highlights the value of transcriptome approaches as it provides a basis to develop hypotheses that could be tested with empirical studies that go beyond gene expression.

Finally, our transcriptome analysis revealed that *fshb* and *lhb* were among the most significantly altered transcripts in response to EE2 exposure. Indeed, *lhb* was hundreds fold upregulated in response to EE2 and was the single most significantly upregulated gene. This

finding suggests that pituitary gonadotropin mRNA levels are altered in response to environmentally relevant xenoestrogen exposure and that changes in gonadotropin expression may be one mechanism by which EDCs can disrupt reproductive function. Additionally, these results suggest that pituitary *lhb* mRNA may be a useful marker of estrogen exposure in juvenile fish.

The goals of the study described in Chapter 3 were to explore the utility of juvenile coho salmon as an ecologically relevant bioassay animal for testing estrogenic activity in effluent from wastewater treatment plants (WWTPs) in the Puget Sound Region, and to evaluate pituitary *lhb* mRNA levels as a marker of estrogen exposure. Based on the rapid and robust increase in *lhb* in response to E2 and EE2 observed in the studies in Chapters 1 and 2, I hypothesized that acute exposure of juvenile coho salmon to natural estrogens and/or xenoestrogens present in WWTP effluent would rapidly increase *lhb* mRNA levels. Therefore, I compared the response of pituitary *lhb* mRNA levels and a standard marker of estrogen exposure, liver *vtg* mRNA levels in coho salmon in response to waterborne EE2 or WWTP effluent exposure. An acute exposure was used to be consistent with standardized EDC screening methods used by the US Environmental Protection Agency, and also because it was not practical to transport large volumes of effluent needed for longer exposures in a static renewal system. My results showed that, pituitary *lhb* mRNA levels were induced at lower EE2 concentrations than hepatic *vtg*. Further, I showed that effluent from 5 of the 8 WWTPs induced *lhb* and this was related to estrogenic equivalents of chemicals measured in bile from exposed fish. In contrast, there was no effect of WWTP effluents on hepatic *vtg* or pituitary *fshb* expression. The lack of effect on *fshb* was not surprising in relation to my other studies, due to the short duration of exposure. The results of this study suggest that *lhb* may be a more sensitive indicator of estrogen exposure than the commonly used

vtg. However more research is needed to compare the kinetics and sensitivity of the *lhb* and *vtg* response. Moreover, these results suggest that pituitary *lhb* mRNA levels may be disrupted by EDCs entering Puget Sound via WWTP effluent. Although WWTP effluents will become more diluted in Puget Sound, fish may be chronically exposed enhancing the observed effects. Therefore, one mechanism whereby WWTP effluents may alter reproductive function in fish is via disrupted Gth synthesis.

Despite the importance of pituitary Gths in regulating reproduction, the pituitary gland has been largely ignored in the context of endocrine disruption. The results of my dissertation research suggest that *lhb* and *fshb* are highly regulated in response to E2 and exogenous estrogens, with *lhb* being a rapid and sensitive marker of estrogen exposure. Further, it is possible that exposure of fish to exogenous estrogens could result in disrupted reproductive development if altered Gth expression resulted in changes in plasma Gths or expression of gonadal Gth receptors. For example, chronic exposure of fish to xenoestrogens could decrease Fsh synthesis and release with potential consequences for gonadal development, fecundity and egg quality. Inhibition of Fsh-signaling has been associated with delayed reproductive development and reproductive failure in zebrafish (Chu et al., 2014; Zhang et al., 2015a, 2015b). Additionally, it is possible that xenoestrogen exposure could alter the timing of Lh synthesis or release, potentially affecting reproductive timing in exposed fish.

In summary, this body of work has improved understanding of Gth regulation during a critical stage of ovarian development. Further, valuable information for predicting and interpreting results of endocrine disruption research has been generated. In addition to the work presented here, I have begun investigating the role of sex steroids in Gth regulation using rainbow primary trout pituitary cell culture. Pituitary gland or cell culture systems allow for

investigating the effects of sex steroid feedback at the level of the pituitary gland, without the potentially confounding effects of sex steroid feedback on the brain. My preliminary work indicates that E2 directly stimulates *lhb* mRNA levels and Lh protein at the level of the pituitary (data not shown). Moreover, these results suggest that *lhb* mRNA levels in primary pituitary cell cultures could be a useful system for screening chemicals with estrogenic activity. However, as discussed above, the effects of E2 on Fsh regulation are delayed and may be indirect via paracrine factors. As such, it may not be possible to maintain healthy pituitary cells in culture long enough to observe E2 regulation of Fsh. Alternatively, pituitary cell dispersal may inhibit the ability of pituitary cells to communicate effectively with one another in autocrine or paracrine fashions. To overcome this, pituitary gland cultures might be a better tool, but the time limit during which whole pituitary gland cultures remain viable may limit the feasibility of this method. Ultimately, future work examining endocrine disruption in juvenile fish should examine the long-term impacts of EDC exposure on Gth expression and reproductive timing and fertility.

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