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

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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 axis are less well understood. The pituitary gonadotropins, 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 mRNA expression were investigated to assess the utility of Lh beta-subunit (*lhb*) as a biomarker of estrogen exposure in juvenile coho salmon (*Oncorhynchus kisutch*). First, a controlled 72-hour exposure to 17α-ethynylestradiol (EE2) and 17β-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 EE2 L^{-1} had 17-fold and 215-fold higher *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 L^{-1} 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.

Keywords: Endocrine disrupting compound; pituitary; gonadotropin; luteinizing hormone; follicle-stimulating hormone; vitellogenin; wastewater effluent

52

53 **1. Introduction**:

54 It is now well established that some chemicals in the environment are capable of
55 disrupting normal endocrine function in humans and wildlife such as fish (Hotchkiss et al., 2008;
56 León-Olea et al., 2014). These endocrine disrupting compounds (EDCs), including certain
57 pharmaceuticals, pesticides, and a variety of industrial compounds, can act to mimic or block
58 endogenous hormones by interfering with their synthesis, availability, or action (Crisp et al.,
59 1998). EDC exposure has been associated with reduced fertility (Jobling et al., 2002), sex
60 reversal (Jobling et al., 1998), and reproductive failure (Kidd et al., 2007; Nash et al., 2004) in a
61 variety of aquatic organisms.

62 Municipal wastewater treatment plant (WWTP) effluents are one of the primary sources
63 of EDCs into the aquatic environment. Fish collected downstream of some WWTPs exhibit
64 symptoms of endocrine disruption and altered reproductive function including reduced gonad
65 size, delayed maturation, and decreased steroidogenesis (Folmar et al., 2001; Vajda et al., 2011,
66 2008; Woodling et al., 2006). In addition, feminization of male fish has been reported
67 downstream of WWTPs including observations of intersex gonads or reduced primary and
68 secondary male sex characteristics (Jobling et al., 1998; Purdom et al., 1994; Vajda et al., 2011,
69 2008). Chemical analyses identified natural estrogens [estradiol (E2) and estrone (E1)] and
70 synthetic estrogens [17α-ethynylestradiol (EE2)] as the most likely feminizing agents in
71 wastewater effluents (Desbrow et al., 1998; Rodgers-Gray et al., 2000). In addition, various
72 anthropogenic compounds such as alkylphenols [nonylphenols (NP) and octylphenols (OP)] and
73 bisphenol A (BPA) have also been identified as estrogen receptor (ER) agonists or antagonists
74 and are present in wastewater effluent (Snyder et al., 2001).

75 Early studies on the effects of WWTP effluent exposure on fish reproduction found
76 elevated expression of vitellogenin (Vtg) in male fish exposed to WWTP effluent (Folmar et al.,
77 1996; Harries et al., 1997; Purdom et al., 1994). Vtg is an egg yolk protein precursor that is
78 induced in maturing female fish in response to rising plasma E2 levels. Vtg synthesis can also be
79 stimulated in male or juvenile fish of both sexes in response to exogenous estrogen exposure.
80 Indeed, Vtg can be increased thousands fold in male fish in response to estrogens making it a
81 widely used biomarker of estrogen exposure (Sumpter and Jobling, 1995; Thorpe et al., 2000).

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^{-1} 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^{-1} 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 examine the effects of WWTP effluents on pituitary Gths in 14-18 month old juvenile coho salmon (*Oncorhynchus kisutch*) and to evaluate the utility of *lhb* as a potential biomarker of estrogen exposure. 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 (14-18 month old, 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 17β-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 eight WWTPs were selected to include a range of treatment processes (secondary and tertiary), which vary in their removal efficiency of steroid estrogens. 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).

2. Materials and methods

2.1 Chemicals for exposure and water chemistry

17α-ethynylestradiol (CAS #: 57-63-6; purity > 99%), 17β-trenbolone (CAS #: 10161-33-8; purity > 99%), methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), heptafluorobutyrylimidazole (HFBI), iodotrimethylsilane (ITS), and resublimed iodine were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). Fluoxetine hydrochloride (CAS #: 56296-78-7; purity > 98%) was obtained from Spectrum chemicals (Gardena, CA). Norfluoxetine-oxalate was obtained from Cerilliant® Analytical Reference Standards (CAS #: 107674-50-0; Sigma-Aldrich, St. Louis, MO). Sertraline hydrochloride (CAS #: 79559-97-0; purity > 98%) was obtained from Toronto Research Chemicals (Toronto, Ontario, CA). Citalopram hydrobromide (CAS #: 59729-32-7; purity > 98%) was obtained from TCI America (Portland, OR). [2,4,16,16]d4-17α-ethynylestradiol (EE2-d4, CAS #: 350820-06-3), [16,16,17]d3-estradiol (E2-d3, CAS #: 79037-37-9), [2,4,16,16]d4-estrone (E1-d4, CAS #: 53866-34-5) and [16α-Hydroxy-17β-estradiol]d2-estriol (E3-d2, CAS #: 53866-32-3), all > 97% purity, were purchased from C/D/N isotopes (Pointe-Claire, Quebec, CA). Pentadeuterated fluoxetine (fluoxetine-d5, CAS #: 1173020-43-3, > 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.

172 *2.2 Fish maintenance*

173 Coho salmon eyed embryos were obtained from the Issaquah Hatchery in mid-December
174 and incubated in Heath trays at 8 °C at the Northwest Fisheries Science Center hatchery facilities
175 (Seattle, WA). After ponding, fish were reared in recirculated 10 – 10.5 °C fresh water under a
176 simulated natural photoperiod. Lighting above the tanks was continually adjusted to match the
177 natural photoperiod of Seattle, WA (47.6˚N). Fish were fed a standard ration of BioDiet
178 commercial feed (Bio-Oregon, Longview, WA) according to Bio-Oregon's feed rate guidelines.
179 On January 31, 2012, 300 coho salmon parr (~14 month old, ~20 g body weight) were
180 transferred to Pacific Northwest National Laboratory – Marine Science Laboratory (PNNL-MSL,
181 Sequim, WA). Fish were initially maintained in circular 1400 L fiberglass tanks. The holding
182 tanks were maintained using a single-pass flow through system using fresh water obtained from
183 MSL's artesian well (440 ft depth), which was pre-aerated and added to tanks at a minimum flow
184 rate of 12 L/min. After three weeks of acclimation, groups of fish were transferred to smaller,
185 370 L circular fiberglass tanks (water inflow rate of 4 L/min). Water temperature, dissolved
186 oxygen, and pH were monitored weekly throughout the acclimation and exposures with values
187 ranging from 11.8 – 12.5 °C for temperature, 8.0 – 9.4 mg L^{-1} for dissolved oxygen, and 7.75 –
188 8.05 for pH. Fish were maintained under a simulated natural photoperiod regime for Sequim,
189 WA (48.1˚N) with artificial dusk and dawn and fed a standard ration of Bio-Oregon® soft moist
190 pellets. All fish were maintained according to the guidelines established by the Institutional
191 Animal Care and Use Committee of PNNL-MSL.
192
193 *2.3 EE2 and TREN Exposures*

194 Controlled exposures to EE2 and TREN were performed for comparison with WWTP
195 effluent exposures. Seventy two-hour exposures to EE2 or TREN were conducted using a
196 continuous flow-through protocol similar to previous studies (Schultz et al., 2013). Exposure
197 water was prepared using a concentrated aqueous stock solution that was slowly added to the
198 exposure tanks using a peristaltic pump. No organic co-solvents were used. All exposure tanks
199 were allowed to equilibrate with the dosing system for three days prior to the addition of fish.
200 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, 14-month old, 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 EE2 L^{-1} (0.0074 or 0.037 nM EE2) or 20 or 200 ng TREN L^{-1} (0.074 or 0.740 nM 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.

2.4 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^{-1} 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.

230 Exposures were conducted during April and May when fish were undergoing
231 smoltification. At this stage, fish are sexually immature with ovaries containing perinucleolar
232 stage oocytes (Campbell et al., 2006) and testes containing only Type A (undifferentiated)
233 spermatogonia (Schulz et al., 2010). Juvenile 16- to 17-month old, mixed-sex coho salmon were
234 randomly assigned to 0%, 20%, or 100% WWTP effluent treatment (2 tanks/treatment; 6
235 fish/tank) and exposed under semi-static conditions with a 60% tank water replacement
236 conducted daily in accordance with the EPA's *Methods for Measuring the Acute Toxicity of*
237 *Effluents and Receiving Waters to Freshwater and Marine Organisms* (U.S. EPA, 2002).
238 Exposure tanks were filled with 110 L of 0%, 20%, or 100% effluent. Every 24 hours,
239 approximately 66 L of exposure water was removed and replaced by new exposure water
240 previously chilled to 12 °C. Fish were fed up to the day before the experiment, and then were
241 fasted during the exposure. After 72 hours, fish were euthanized in buffered MS-222 and
242 decapitated. Fork length, body weight, gonad weight and liver weight, were recorded and GSI
243 and HSI were calculated as described above. The pituitary gland and a small piece (~ 50 mg) of
244 liver were dissected and placed in 0.75 mL RNA*later*® (Life Technologies, Carlsbad, CA).
245 Tissues were stored in RNA*later*® at room temperature for up to a week and then stored at -20
246 °C or -80 °C until RNA isolation. When possible, bile was collected from the gallbladder with a
247 clean 1-mL syringe tipped with a 23 G needle. Bile was stored at -80 °C until analysis.
248
249 *2.5 Exposure chemical analyses*
250 Exposure water from the control exposure was analyzed for the presence of EE2 or
251 TREN. Separate aliquots were analyzed for TREN and EE2. WWTP effluents were analyzed for
252 natural and synthetic estrogens including E1, E2, E3 (estriol) and EE2. Exposure water (100 mL)
253 or WWTP effluent samples (850-900 mL) were fortified with deuterated internal standards (E2-
254 d_3 for E2; EE2-d_4 for EE2; E1-d4 for E1), spiked with NaCl (1 or 10 g; previously baked at 500
255 °C for 12 hrs) and then extracted with either 15 or 150 mL of methyl-tert-butyl ether (MTBE).
256 The ether extracts were evaporated under N2 and the residue mixed with the appropriate
257 derivatizing agent. For estrogens, 25 µL of MSTFA containing ITS (1000:4 v/v) as a catalyst
258 was used and heated at 70 °C for 30 min prior to analysis (Schultz et al., 2001). For TREN, 25
259 µL of MSTFA containing re-sublimed I_2 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, 442, 504 and 506 that were used for E1, E1-d4, E2, E2-d_3, EE2, EE2-d_4, TREN, E3 and E3-d2 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 N_2) 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 N_2 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.

2.6 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 2.5 ng/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 N_2 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

290 an incubator for 2 h at 40 °C to ensure complete enzymatic hydrolysis of the glucuronide and
291 sulfate conjugated metabolites of the EDCs. Glacial acetic acid (300 µL) was added and the final
292 hydrolyzed bile mixture was loaded on to a solid phase extraction (SPE) cartridge packed with
293 60 mg of polymeric reversed-phase sorbent (Strata X from Phenomenex Inc, Torrance, CA,
294 USA) that had been previously conditioned with 2 mL of methanol and 2 mL of water. The
295 cartridge was then washed with 1.5 mL of water, followed by 1.5 mL of methanol/water (60/40,
296 v/v), and was dried under vacuum for 30 min. The analytes were eluted with 1.5 mL of methanol
297 into vial containing 30 µL of 2.5 ng/µL BPA-d4 solution, used as recovery standard (Rec-std).
298 An aliquot of the final methanolic extract was diluted 10 times in methanol prior to analyses.
299
300 *2.7 Bile analyses by liquid chromatography/tandem mass spectrometry (LC-MS/MS)*
301 Final bile extracts were analyzed by liquid chromatography (LC, Acquity system, Waters
302 Co., Milford, MA, USA) coupled with a triple quadrupole tandem mass spectrometer (MS/MS,
303 QTRAP 5500, AB Sciex, Framingham, MA, USA). For each sample, 10 µL of diluted extract
304 was injected into the LC-MS/MS. The LC was equipped with a 0.2 µm pre-filter followed by a
305 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)
306 reversed-phase column. Water (solvent A) and methanol (solvent B) were used as the mobile-
307 phase. The total analysis time was 26 min using a linear gradient, as follows (solvent A/solvent
308 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
309 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
310 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.
311 The column temperature was maintained at 45 ºC. Electrospray ionization (ESI) mode was used
312 for the ionization of all analytes. The MS/MS was operated in negative ion mode and the
313 analytes were detected via multiple-reaction monitoring (MRM). The ion source was kept at 700
314 °C and the capillary voltage was -4.5 kV. Declustering potential and entrance potential were set
315 at -60 V and -10 V, respectively. Other details on the MRM parameters are given in the Table 2.
316 The analytes were quantified by S-std and based on the calibration curve of each analyte. The
317 recovery of each S-std was calculated by the Rec-std.
318
319 *2.8 Gene expression analyses*

320 Tissues were homogenized using either a Mini Beadbeater-96 (BioSpec Products Inc,
321 Bartlesville, OK) or a TissueLyzer II homogenizer (QIAGEN, Valencia, CA). Total pituitary
322 RNA and total liver RNA from male fish were isolated using RNeasy Plus Mini Kit® (QIAGEN)
323 following the manufacturer's instructions for isolation of total RNA from animal tissues. Total
324 liver RNA was isolated from female fish using Tri-Reagent (Molecular Research Center,
325 Cincinnati, OH) according to the manufacturer's instructions. RNA degradation was observed in
326 some liver samples from the EE2 and TREN exposure experiment due to issues with freezer
327 temperature. Therefore, samples from subsequent experiments were collected in RNA*later*® to
328 reduce RNA degradation. RNA samples were run on 1% agarose gels to check for RNA quality.
329 Only samples with distinct 28S and 18S ribosomal RNA bands at an approximately 2:1 ratio
330 were used for qPCR analysis. High quality liver RNA samples were DNase treated using
331 Ambion® TURBO DNA-*free*™ (Life Technologies). Briefly, a 23 µL reaction was composed of
332 20 µL (3000 ng) total RNA, 2 µL DNase I Buffer and 1 µL TURBO DNase and incubated at 37
333 °C for 1 h. DNase treatment was then inactivated with 2 µL inactivation reagent and vortexed at
334 room temperature. RNA yield was quantified with a NanoDrop ND-1000 (Thermo Fisher
335 Scientific, Waltham, MA). DNA-free samples (125 ng pituitary RNA or 250 ng liver RNA) were
336 reverse transcribed with SuperScriptII (Life Technologies) in 10 µL reactions as described in
337 Kitano et al. (2010). Quantitative real-time RT PCR (qPCR) assays were designed and
338 performed as previously described (Harding et al., 2013). Briefly, qPCR assays were run using
339 an ABI 7900HT Fast Real-Time PCR System (Life Technologies) in 384-well plates using
340 standard cycling conditions. Reactions were 12.5 µL each and consisted of 1X Power SYBR
341 Green PCR Master Mix (Life Technologies), 150 nM of the forward and reverse primer, and 0.5
342 ng cDNA template. Four concentrations of standard curve samples generated from a serial
343 dilution of cDNA (from pooled RNA) ranging from 0.05 to 5 ng cDNA were included in each
344 plate in triplicate. When possible, samples of a given tissue from all exposure dates were run on
345 a single 384-well qPCR plate to eliminate plate-to-plate variation between qPCR runs. In every
346 case, all control and treated samples from a given exposure date were analyzed on a single plate.
347 No template controls showed no detectable amplification over 40 cycles of PCR. For *vtg* and
348 *fshb*, no amplification controls showed no detectable amplification within 35 cycles of PCR. For
349 *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*). *Eef1a* was selected as the housekeeping gene because it is stably expressed in both tissues and is not significantly affected by hormone treatment in this or previous studies (Harding et al., 2013; Luckenbach et al., 2010). Pituitary and liver *eef1a* mRNA levels were not significantly different in treated animals compared to controls (Supplemental Figure 1). 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).

2.9 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 at a threshold of $p < 0.05$ and were removed from analyses. Data were log-transformed where necessary to meet parametric test criteria. Initial analyses, using a 2-way ANOVA, found no evidence of a sex effect, therefore male and female samples were pooled for subsequent analyses. 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 ± 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).

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

3. Results:

3.1 Control EE2 and TREN Exposures

3.1.1 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^{-1} for EE2 and 20 and 200 ng L^{-1} for TREN. Mean EE2 exposure levels in duplicate treatment tanks were 2.89 and 2.11 ng L^{-1} for the 2 ng L^{-1} nominal treatment and 11.65 and 11.89 ng L^{-1} for the 10 ng L^{-1} nominal treatment. Mean TREN exposure levels in duplicate tanks were 27.4 and 15.0 ng L^{-1} for the 20 ng L^{-1} nominal concentration and 192.6 and 175.5 ng L^{-1} for the 200 ng L^{-1} nominal concentration. Hereafter, nominal concentrations (2 and 10 ng L^{-1}) and TREN (20 and 200 ng L^{-1}) will be used to indicate treatment exposure levels.

3.1.2 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 2). No mortalities were observed over the 72-hour exposure.

3.1.3 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^{-1}, 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^{-1} EE2 for 72 hours (Figure 1a). However, liver *vtg* mRNA levels were not affected by exposure to 2 ng EE2 L^{-1} or either TREN dose.

3.2 WWTP Effluent Exposures:

3.2.1 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

410 estrogens, E2 was detected in every effluent that was analyzed with levels reaching up to 2.6 ng
411 L^{-1} in effluent from site H. Low levels of E1 were detected in two of the effluents, but were
412 below the level of quantitation (~ 0.5 ng L^{-1}) in both cases. E3 was not detected in any effluent
413 that was analyzed. EE2 was detected in 2 effluents with up to 0.8 ng L^{-1} in site G effluent. SSRIs
414 were much more prevalent in WWTP effluent samples; all measured SSRIs were detected in
415 100% of the effluent samples. Average concentrations of SSRIs were 18.5 ng L^{-1}, 73.3 ng L^{-1},
416 75.7 ng L^{-1} and 504.6 ng L^{-1} for NFLX, SER, FLX, and CIT, respectively. FLX and SER were
417 present in WWTP effluent at similar concentrations, while CIT was, on average, an order of
418 magnitude higher in concentration. Combined, the total SSRI load ranged from a low of 77.5 ng
419 L^{-1} at site H to 1672.7 ng L^{-1} at site G.
420
421 *3.2.2 Survival and morphometric data*
422 Mean (± SEM) fish fork-length (138 ± 1 mm), body weight (30.9 ± 0.5 g), GSI (0.349 ±
423 0.019 for females and 0.027 ± 0.005 for males), and HSI (0.866 ± 0.009 for females and 0.868 ±
424 0.023 for males) were not significantly different between tanks, so duplicate tanks were
425 subsequently pooled. No mortalities were observed in response to effluent from WWTP A, B, C,
426 D, F, or G over the 72-hour exposure. However, in the 100% WWTP E effluent exposure, 92%
427 mortality was observed in the first 24 hours. This was attributed to residual chlorine exposure.
428 After the water change at 24 hours, new fish were added to the tanks. Of these, there was 50%
429 mortality within 24 hours of being added to the tanks, again presumably due to residual chlorine
430 exposure. Therefore, the remaining fish from the 100% WWTP E effluent exposure were
431 exposed to effluent for 48 hours, rather than the 72 hours used in other tests.
432
433 *3.2.3 Tissue mRNA levels*
434 Fish exposed to effluent from sites A, B, D, E, and F had significantly higher *lhb* mRNA
435 than their respective controls (Figure 2). Fish exposed to 20% and 100% effluent from WWTP B
436 had 9- and 42-fold higher *lhb* mRNA levels relative to controls, respectively. Similarly, fish
437 exposed to 20% and 100% effluent from WWTP F had 16- and 491-fold increases in *lhb* mRNA
438 levels as compared to controls. The mean level of *lhb* induction in fish exposed to 20% and
439 100% WWTP F are similar in magnitude to those observed in fish exposed to 2 and 10 ng EE2

440 L^{-1} in the control exposure. Although *fshb* mRNA levels were unaffected by EE2 and TREN
441 exposure, *fshb* mRNA levels increased in a dose-dependent manner when exposed to effluent
442 from WWTP B (Figure 3B). In contrast, liver *vtg* mRNA levels were not significantly altered by
443 any of the WWTP effluent exposures (Figure 4).
444
445 *3.2.4 Chemical analyses in bile*
446 Bile samples were analyzed for natural and synthetic steroidal estrogens, and the weakly
447 estrogenic chemical, bisphenol-A (BPA), which is a component of polycarbonate plastic and
448 epoxy resins and commonly found in WWTP effluent and the aquatic environment (Table 5).
449 BPA was the most commonly detected chemical in bile from fish used in this study with
450 concentrations ranging from 44.5 ng mL^{-1} to 3900 ng mL^{-1}. Natural steroidal estrogens, E1 and
451 E2, were also frequently detected in bile samples from effluent exposed animals, but were not
452 detected in any of the control bile samples. Mean E1 and E2 levels were as high as 71.7 and 50.7
453 ng mL^{-1}, respectively, in bile from WWTP F. EE2 was detected in bile from fish exposed to
454 effluent from WWTP sites C, D, and F, with concentrations of > 10 ng mL^{-1} in bile from fish
455 exposed to WWTP C and F effluents. Considering steroidal estrogens (reported as EEQ) and
456 BPA together, bile from fish exposed to WWTP H effluent showed the least amount of
457 estrogenic chemicals. In contrast, bile from fish exposed to WWTP F effluent showed the highest
458 occurrence and highest concentrations of estrogenic chemicals, including E1, E2, E3, and BPA.
459 Bile from fish exposed to WWTP B effluent had the highest levels and highest occurrence of
460 EE2.
461
462 **4. Discussion:**
463 In this study, juvenile coho salmon, a native species in Puget Sound, were exposed to
464 eight different WWTP effluents from the Puget Sound region and effects on pituitary *fshb* and
465 *lhb,* and hepatic *vtg* mRNAs were evaluated. The advantage of using juvenile salmon is that both
466 sexes are prepubertal, have low endogenous steroid levels (Campbell et al., 2006; Patiño and
467 Schreck, 1986; Sower et al., 1992), and low or non-detectable expression of both *lhb* and *vtg*.
468 However, both of these gene transcripts can be increased with exogenous estrogens in juvenile
469 males and females (Crim et al., 1981; Thorpe et al., 2000). We found that acute (72-hour)

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* 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.

4.1 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^{-1}, 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^{-1} EE2 exposure compared to control and TREN-exposed fish. In agreement with these results, immature coho salmon exposed to 12 ng EE2 L^{-1} 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 EE2 L^{-1} 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. Previous work in juvenile brown trout (*Salmo trutto*), showed that hepatic vtg mRNA levels are significantly induced 24 hours after a 6-hour 320 ng E2 L^{-1} exposure (Knudsen et al., 2011). These results indicate that *vtg* mRNA levels can be rapidly induced in juvenile salmonids in response to high levels of E2. In immature rainbow trout, exposure to levels as low as 1.0 ng EE2 L^{-1} or 14 ng E2 L^{-1} elevated *vtg* after 14 days (Thomas-Jones et al., 2003). Additionally the hepatic *vtg* mRNA levels were induced in immature rainbow trout exposed to 9.7 ng E2 L^{-1} after 4 days, but not after 2 days (Thomas-Jones et al., 2003). These results suggest that hepatic *vtg* can also be induced in response to low estrogens, but in a time-dependent manner. Indeed, hepatic *vtg* mRNA levels increased for up to 2 weeks in male rainbow trout injected once with E2 (Pakdel et al., 1991). Unfortunately, in salmonids there is

530 little information on Vtg protein or mRNA induction in response to both short term (< 14 days)
531 and low dose (< 5 ng L^{-1}) EE2 exposure experiments, raising the possibility that 72-hour
532 exposure duration is too short to observe *vtg* induction at low exposure levels. In fathead
533 minnow, lowest-observed-effect concentrations (LOEC) of 2.2 and 5 ng EE2 L^{-1} have been
534 reported for *vtg* mRNA induction at 48 hours (Biales et al., 2007; Flick et al., 2014; Reddy et al.,
535 2015). Similarly, in fathead minnow embryo and larvae exposed to 0, 18, or 1800 ng L^{-1} E1, *vtg*
536 mRNA levels were significantly induced at 6 days, but not at 3 days of exposure although the
537 other estrogen responsive genes (estrogen receptor α and cytochrome P450-aromatase-B) were
538 already significantly induced at 3 days (Cavallin et al., 2015). Cavallin and colleagues suggested
539 that the delayed induction of *vtg* may reflect the time required to upregulate a functional estrogen
540 receptor in the liver. These data suggest that the 3-day exposure used in the current study may be
541 insufficient to observe *vtg* induction in juvenile coho salmon in response to low concentrations of
542 estrogens in exposure water.
543
544 *4.2 WWTP effluent exposures*
545 In the current study, effluents collected from WWTPs in South Puget Sound were
546 analyzed for the presence of steroidal estrogens and SSRIs. Due to the method of effluent
547 sampling (grab samples rather than time-weighted sampling), it is not possible to draw firm
548 conclusions about the effect of alternate wastewater treatment technologies on the removal of
549 steroids or pharmaceuticals from WWTP effluents. However, steroidal estrogen and SSRI levels
550 are still useful for understanding the biological responses of exposed animals. In addition, all
551 sample collections occurred at the same time of day, and within a narrow seasonal time frame
552 limiting time-dependent differences between WWTP effluent collections. Additionally, previous
553 studies have shown that trickling filter WWTPs or trickling filters with activated sludge
554 treatment have lower removal efficiencies of pharmaceuticals and personal care products
555 including steroidal estrogens than tertiary treatment processes (Bartelt-Hunt et al., 2009; Janex-
556 Habibi et al., 2009; Kasprzyk-Hordern et al., 2009). Measured concentrations of steroidal
557 estrogens were in the low ng L^{-1} range (<3 ng L^{-1}) for all WWTP effluents at the beginning of the
558 semi-static exposure and may have decreased over time due to degradation. Of the steroidal
559 estrogens, E2 was the most prevalent and was detected in every effluent. E2 was also found in

560 the highest concentration (2.6 ng L^{-1}) of any of the measured steroidal estrogens. In general, the
561 levels of steroidal estrogens in effluent observed in this study are low compared to previous
562 studies. For example, analyses of two WWTP effluents from Puget Sound identified estrone
563 concentrations of 4.5 to 58 ng L^{-1} (Meador et al., 2016). Elsewhere in North America, natural
564 estrogens have been measured in wastewater effluents with concentrations ranging from 6-14
565 (E1), <5 (E2) and <10-33 ng L^{-1} (E3) (Huang and Sedlak, 2001; Lee and Peart, 1998; Snyder et
566 al., 1999). Reported EE2 concentrations in WWTP effluents can vary widely, but also typically
567 fall in the low ng L^{-1} range (Young et al., 2004). In the United States, WWTP effluents had EE2
568 concentrations ranging from <0.05-2.42 ng L^{-1} (Huang and Sedlak, 2001; Snyder et al., 1999),
569 however EE2 has been measured in streams at levels up to 273 ng L^{-1}, with median measured
570 concentrations below 10 ng L^{-1} (Kolpin et al., 2002). Similarly, in Canadian WWTP effluents,
571 EE2 concentrations reached 42 ng L^{-1}, with median concentration of 9 ng L^{-1} (Ternes et al.,
572 1999).
573 We also measured a variety of SSRIs in the effluents due to their high occurrence in
574 WWTP effluents (Hedgespeth et al., 2012; Meador et al., 2016; Verlicchi et al., 2012) due to
575 incomplete removal during wastewater treatment (Lubliner et al., 2010). In addition, SSRIs
576 bioaccumulate in tissues of fish exposed to WWTP effluent (Lajeunesse et al., 2011; Schultz et
577 al., 2010) and are capable of altering Gth mRNA levels in fish. For example, a 2-week exposure
578 of male zebrafish to CIT, decreased mRNA levels of GnRH 3 (*gnrhr3*), *fshb*, and *lhb* (Prasad et
579 al., 2015). These data suggest that SSRIs could accumulate in fish brain tissues and potentially
580 alter the brain-pituitary axis, including Gth mRNA expression.
581 In the present study, all of the SSRIs measured were detected in WWTP effluents
582 typically in the 10 to 100 ng L^{-1} range. These levels are consistent with concentrations of SSRIs
583 previously measured in WWTP effluents from Puget Sound and are in the 95th to 99th percentile
584 relative to what is seen elsewhere in the United States (Meador et al., 2016). CIT was present at
585 the highest concentration in effluent from all WWTP effluents, except WWTP H. This is in
586 agreement with a previous study that found measured CIT concentrations were, on average, an
587 order of magnitude greater than SER or FLX in two effluent impacted streams (Schultz et al.,
588 2010). The total SSRI concentrations were lowest in effluent from WWTP H (77.5 ng L^{-1}),
589 intermediate (480 – 770 ng L^{-1}) for WWTP A, B, C, D, and F effluents, and highest in WWTP G

effluent (1672.7 ng L^{-1}). 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^{-1} EEQ respectively (see Table 5). These EEQ levels were, in large part, due to relatively high (>10 ng mL^{-1}) 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.

5. Conclusion:

Pituitary *lhb* mRNA levels were significantly increased in juvenile coho salmon during an acute (72-hour) low EE2 (2 ng L^{-1}) 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 EE2 L^{-1} 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. Further work is needed to determine whether or to what extent altered Gth expression may affect reproductive function in EDC-exposed fish.

Conflict of Interest:

No competing financial interest is declared.

Author contributions:

LBH contributed to the design of the study, performed sampling of fish after the exposure, conducted RNA isolations and gene expression analyses, performed all statistical analyses of data, wrote the manuscript and generated all figures. IRS conducted the waterborne exposures and water chemical analyses, advised on the experimental design and set-up, wrote methods sections associated with the exposure chemical analyses, and provided valuable comments to the other sections of the manuscript. DAMS conducted the bile chemical analyses and wrote methods sections associated with the bile chemical analyses. DAMS and GMY assisted with data interpretation and manuscript revision. DR contributed to the design of the study, WWTP selection, and effluent collection. SIH and SB assisted with RNA isolations and manuscript preparation. BVP provided assistance with manuscript preparation and revisions. PS contributed to the study design, assisted with sampling of fish and data interpretation, and assisted LBH in writing and editing the manuscript. All co-authors have contributed to reviewing the manuscript and have approved the final submitted manuscript.

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710 **References:**

711 Antonopoulou, E., Swanson, P., Mayer, I., Borg, B., 1999. Feedback control of gonadotropins in
712 Atlantic salmon, *Salmo salar*, male parr. II. Aromatase inhibitor and androgen effects.
713 Gen. Comp. Endocrinol. 114, 142–150.
714 Aroua, S., Weltzien, F.-A., Belle, N.L., Dufour, S., 2007. Development of real-time RT-PCR
715 assays for eel gonadotropins and their application to the comparison of in vivo and in
716 vitro effects of sex steroids. Gen. Comp. Endocrinol. 153, 333–343.
717 Barber, L., Lee, K., Swackhamer, D., Schoenfuss, H., 2007. Reproductive responses of male
718 fathead minnows exposed to wastewater treatment plant effluent, effluent treated with
719 XAD8 resin, and an environmentally relevant mixture of alkylphenol compounds. Aquat.
720 Toxicol. 82, 36–46.
721 Barber, L.B., Brown, G.K., Nettesheim, T.G., Murphy, E.W., Bartell, S.E., Schoenfuss, H.L.,
722 2011. Effects of biologically-active chemical mixtures on fish in a wastewater-impacted
723 urban stream. Sci. Total Environ. 409, 4720–4728.
724 Bartelt-Hunt, S.L., Snow, D.D., Damon, T., Shockley, J., Hoagland, K., 2009. The occurrence of
725 illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in
726 Nebraska. Environ. Pollut. 157, 786–791
727 Biales, A.D., Bencic, D.C., Flick, R.W., Lazorchak, J., Lattier, D.L., 2007. Quantification and
728 associated variability of induced vitellogenin gene transcripts in fathead minnow
729 (*Pimephales promelas*) by quantitative real-time polymerase chain reaction assay.
730 Environ. Toxicol. Chem. 26(2): 287–296.
731 Borg, B., Antonopoulou, E., Mayer, I., Andersson, E., Berglund, I., Swanson, P., 1998. Effects
732 of gonadectomy and androgen treatments on pituitary and plasma levels of gonadotropins
733 in mature male Atlantic salmon, *Salmo salar*, parr–positive feedback control of both
734 gonadotropins. Biol. Reprod. 58, 814–820.
735 Breton, B., Govoroun, M., Mikolajczyk, T., 1998. GTH I and GTH II secretion profiles during
736 the reproductive cycle in female rainbow trout: Relationship with pituitary
737 responsiveness to GnRH-A stimulation. Gen. Comp. Endocrinol. 111, 38–50.
738 Breton, B., Sambroni, È., Govoroun, M., Weil, C., 1997. Effects of steroids on GTH I and GTH
739 II secretion and pituitary concentration in the immature rainbow trout *Onchorhynchus*
740 *mykiss*. Comptes Rendus Académie Sci.-Ser. III-Sci. Vie 320, 783–789.
741 Brown, K.H., Schultz, I.R., Nagler, J.J., 2007. Reduced embryonic survival in rainbow trout
742 resulting from paternal exposure to the environmental estrogen 17α-ethynylestradiol
743 during late sexual maturation. Reproduction 134, 659–666.
744 Campbell, B., Dickey, J.T., Young, G., Pierce, A.L., Fukada, H., Swanson, P., 2006.
745 Previtellogenic oocyte growth in salmon: Relationships among body growth, plasma
746 insulin-like growth factor-1, estradiol-17β, follicle-stimulating hormone and expression
747 of ovarian genes for insulin-like growth factors, steroidogenic-acute regulatory protein
748 and receptors for gonadotropins, growth hormone, and somatolactin. Biol. Reprod. 75,
749 34–44.
750 Cavaco, J.E.B., van Baal, J., van Dijk, W., Hassing, G.A.M., Goos, H.J.T., Schulz, R.W., 2001.
751 Steroid hormones stimulate gonadotrophs in juvenile male African catfish (*Clarias*
752 *gariepinus*). Biol. Reprod. 64, 1358–1365.
753 Cavallin, J.E., Schroeder, A.L., Jensen, K.M., Villeneuve, D.L., Blackwell, B.R., Carlson, K.,
754 Kahl, M.D., LaLone, C.A., Randolph, E.C., Ankley, G.T., 2015. Evaluation of whole-

mount in situ hybridization as a tool for pathway-based toxicological research with early-life stage fathead minnows. Aquat. Toxicol. 169, 19–26.
Crim, L.W., Peter, R.E., Billard, R., 1981. Onset of gonadotropic hormone accumulation in the immature trout pituitary gland in response to estrogen or aromatizable androgen steroid hormones. Gen. Comp. Endocrinol. 44, 374–381.
Crisp, T.M., Clegg, E.D., Cooper, R.L., Wood, W.P., Anderson, D.G., Baetcke, K.P., Hoffmann, J.L., Morrow, M.S., Rodier, D.J., Schaeffer, J.E., Touart, L.W., Zeeman, M.G., Patel, Y.M., 1998. Environmental endocrine disruption: An effects assessment and analysis*. Environ. Health Perspect. 106, 11–56.
Da Silva, D.A.M., Buzitis, J., Reichert, W.L., West, J.E., O'Neill, S.M., Johnson, L.L., Collier, T.K., Ylitalo, G.M., 2013. Endocrine disrupting chemicals in fish bile: A rapid method of analysis using English sole (*Parophrys vetulus*) from Puget Sound, WA, USA. Chemosphere 92, 1550–1556.
Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ. Sci. Technol. 32, 1549–1558.
Dickey, J.T., Swanson, P., 1998. Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). J. Mol. Endocrinol. 21, 291–306.
Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental oestrogen in fish. J. Mol. Endocrinol. 37, 121–134.
Flick, R.W., Bencic, D.C., See, M.J., Biales, A.D., 2014. Sensitivity of the vitellogenin assay to diagnose exposure of fathead minnows to 17α-ethynylestradiol. Aquat. Toxicol. 152, 353–360.
Folmar, L.C., Denslow, N.D., Kroll, K.J., Orlando, E.F., Enblom, J., Marcino, J., Metcalfe, C.D., Guillette Jr, L.J., 2001. Altered serum sex steroids and vitellogenin induction in walleye (*Stizostedion vitreum*) collected near a metropolitan sewage treatment plant. Arch. Environ. Contam. Toxicol. 40, 392–398.
Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J., Guillette Jr, L.J., 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104, 1096–1101.
Gibson, R., Smith, M.D., Spary, C.J., Tyler, C.R., Hill, E.M., 2005. Mixtures of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents. Environ. Sci. Technol. 39, 2461–2471.
Golshan, M., Hatef, A., Zare, A., Socha, M., Milla, S., Gosiewski, G., Fontaine, P., Sokołowska-Mikołajczyk, M., Habibi, H.R., Alavi, S.M.H., 2014. Alternations in neuroendocrine and endocrine regulation of reproduction in male goldfish (*Carassius auratus*) following an acute and chronic exposure to vinclozolin, in vivo. Aquat. Toxicol. 155, 73–83.
Gomez, J.M., Weil, C., Ollitrault, M., Le Bail, P.-Y., Breton, B., Le Gac, F., 1999. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 113, 413–428.
Harding, L.B., Schultz, I.R., Goetz, G.W., Luckenbach, J.A., Young, G., Goetz, F.W., Swanson, P., 2013. High-throughput sequencing and pathway analysis reveal alteration of the

799 pituitary transcriptome by 17α-ethynylestradiol (EE2) in female coho salmon,
800 *Oncorhynchus kisutch*. Aquat. Toxicol. 142-143, 146–163.
801 Harries, J.E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J.P., Tyler, C.R., 1999.
802 Estrogenic potency of effluent from two sewage treatment works in the United Kingdom.
803 Environ. Toxicol. Chem. 18, 932–937.
804 Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylor, T.,
805 Zaman, N., 1997. Estrogenic activity in five United Kingdom rivers detected by
806 measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16, 534–
807 542.
808 Harris, C.A., Santos, E.M., Janbakhsh, A., Pottinger, T.G., Tyler, C.R., Sumpter, J.P., 2001.
809 Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female
810 rainbow trout. Environ. Sci. Technol. 35, 2909–2916.
811 Hedgespeth, M.L., Sapozhnikova, Y., Pennington, P., Clum, A., Fairey, A., Wirth, E., 2012.
812 Pharmaceuticals and personal care products (PPCPs) in treated wastewater discharges
813 into Charleston Harbor, South Carolina. Sci. Total Environ. 437, 1–9.
814 Hotchkiss, A.K., Rider, C.V., Blystone, C.R., Wilson, V.S., Hartig, P.C., Ankley, G.T., Foster,
815 P.M., Gray, C.L., Gray, L.E., 2008. Fifteen years after "Wingspread" – Environmental
816 endocrine disrupters and human and wildlife health: where we are today and where we
817 need to go. Toxicol. Sci. 105, 235–259.
818 Huang, C.-H., Sedlak, D.L., 2001. Analysis of estrogenic hormones in municipal wastewater
819 effluent and surface water using enzyme-linked immunosorbent assay and gas
820 chromatography/tandem mass spectrometry. Environ. Toxicol. Chem. 20, 133–139.
821 Ings, J.S., Servos, M.R., Vijayan, M.M., 2011. Hepatic transcriptomics and protein expression in
822 rainbow trout exposed to municipal wastewater effluent. Environ. Sci. Technol. 45,
823 2368–2376.
824 Janex-Habibi, M.-L., Huyard, A., Esperanza, M., Bruchet, A., 2009. Reduction of endocrine
825 disruptor emissions in the environment: The benefit of wastewater treatment. Water Res.
826 43, 1565–1576.
827 Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G.,
828 Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P., 2002. Wild
829 intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Reprod. 67, 515–524.
830 Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual
831 disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.
832 Johns, S.M., Denslow, N.D., Kane, M.D., Watanabe, K.H., Orlando, E.F., Sepúlveda, M.S.,
833 2009. Effects of estrogens and antiestrogens on gene expression of fathead minnow
834 (*Pimephales promelas*) early life stages. Environ. Toxicol. 26, 195–206.
835 Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals,
836 personal care products, endocrine disruptors and illicit drugs during wastewater treatment
837 and its impact on the quality of receiving waters. Water Res. 43, 363–380
838 Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick,
839 R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc.
840 Natl. Acad. Sci. 104, 8897–8901.
841 Kitano, J., Lema, S.C., Luckenbach, J.A., Mori, S., Kawagishi, Y., Kusakabe, M., Swanson, P.,
842 Peichel, C.L., 2010. Adaptive divergence in the thyroid hormone signaling pathway in
843 the stickleback radiation. Curr. Biol. 20, 2124–2130.

Kobayashi, M., Sohn, Y.C., Yoshiura, Y., Aida, K., 2000. Effects of sex steroids on the mRNA levels of gonadotropin subunits in juvenile and ovariectomized goldfish *Carassius auratus*. Fish. Sci. 66, 223–231.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999−2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Lajeunesse, A., Gagnon, C., Gagné, F., Louis, S., Čejka, P., Sauvé, S., 2011. Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – Evidence of biological effects. Chemosphere 83, 564–571.

Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.-E., Förlin, L., 1999. Ethinyloestradiol - an undesired fish contraceptive? Aquat. Toxicol. 45, 91–97.

Le Dréan, Y., Lazennec, G., Kern, L., Saligaut, D., Pakdel, F., Valotaire, Y., 1995. Characterization of an estrogen-responsive element implicated in regulation of the rainbow trout estrogen receptor gene. J. Mol. Endocrinol. 15, 37–47.

Lee, H.B., Peart, T.E., 1998. Determination of 17β-estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. J. AOAC Int. 81, 1209–1216.

León-Olea, M., Martyniuk, C.J., Orlando, E.F., Ottinger, M.A., Rosenfeld, C.S., Wolstenholme, J.T., Trudeau, V.L., 2014. Current concepts in neuroendocrine disruption. Gen. Comp. Endocrinol. 203, 158–173.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437.

Liu, D., Xiong, F., Hew, C.L., 1995. Functional analysis of estrogen-responsive elements in Chinook salmon (*Oncorhynchus tschawytscha*) gonadotropin IIβ subunit gene. Endocrinology 136, 3486–3493.

Lubliner, B., Redding, M., Ragsdale, D., 2010. Pharmaceuticals and personal care products in municipal wastewater and their removal by nutrient treatment technologies. Publication 10-03-004. Washington State Department of Ecology, Olympia, WA, USA.

Luckenbach, J.A., Dickey, J.T., Swanson, P., 2010. Regulation of pituitary GnRH receptor and gonadotropin subunits by IGF1 and GnRH in prepubertal male coho salmon. Gen. Comp. Endocrinol. 167, 387–396.

Maeng, S., Jung, Y., Choi, E., Jeon, J.-K., Kim, S., Gen, K., Sohn, Y.C., 2005. Expression of gonadotropin subunit genes following 4-nonylphenol exposure in masu salmon: Effects on transcript levels and promoter activities via estrogen receptor alpha. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 142, 383–390.

Marchand, P., Le Bizec, B., Gade, C., Monteau, F., André, F., 2000. Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry. J. Chromatogr. A 867, 219–233.

McGowan, V., 2015. Water Quality Program Permit Writer's Manual. Washington State Department of Ecology, Olympia, WA. Publication No: 92–109. https://fortress.wa.gov/ecy/publications/SummaryPages/92109.html

888 Meador J.P., Yeh, A., Young, G., Gallagher E.P., 2016. Contaminants of emerging concern in a
889 large temperate estuary. Environ. Pollut. 213, 254–267.
890 Melo, M.C., van Dijk, P., Andersson, E., Nilsen, T.O., Fjelldal, P.G., Male, R., Nijenhuis, W.,
891 Bogerd, J., de França, L.R., Taranger, G.L., Schulz, R.W., 2015. Androgens directly
892 stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar*). Gen.
893 Comp. Endocrinol. 211, 52–61.
894 Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-
895 Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the
896 pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health
897 Perspect. 112, 1725–1733.
898 Patiño, R., Schreck, C.B., 1986. Sexual dimorphism of plasma sex steroid levels in juvenile coho
899 salmon, *Oncorhynchus kisutch*, during smoltification. Gen. Comp. Endocrinol. 61, 127–
900 133.
901 Pettersson, M., Adolfsson-Erici, M., Parkkonen, J., Förlin, L., Asplund, L., 2006. Fish bile used
902 to detect estrogenic substances in treated sewage water. Sci. Total Environ. 366, 174–
903 186.
904 Prasad, P., Ogawa, S., Parhar, I.S., 2015. Serotonin reuptake inhibitor citalopram inhibits GnRH
905 synthesis and spermatogenesis in the male zebrafish. Biol. Reprod. 93, 102–102.
906 Prat, F., Sumpter, J.P., Tyler, C.R., 1996. Validation of radioimmunoassays for two salmon
907 gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the
908 reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol.
909 Reprod. 54, 1375–1382.
910 Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994.
911 Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 8, 275–285.
912 Reddy, T.V., Flick, R., Lazorchak, J.M., Smith, M.E., Wiechman, B., Lattier, D.L., 2015.
913 Experimental paradigm for in-laboratory proxy aquatic studies under conditions of static,
914 non–flow-through chemical exposures. Environ. Toxicol. Chem.
915 Rhee, J.-S., Kim, R.-O., Seo, J.S., Kang, H.S., Park, C.-B., Soyano, K., Lee, J., Lee, Y.-M., Lee,
916 J.-S., 2010. Bisphenol A modulates expression of gonadotropin subunit genes in the
917 hermaphroditic fish, *Kryptolebias marmoratus*. Comp. Biochem. Physiol. Part C Toxicol.
918 Pharmacol. 152, 456–466.
919 Rodgers-Gray, T.P., Jobling, S., Morris, S., Kelly, C., Kirby, S., Janbakhsh, A., Harries, J.E.,
920 Waldock, M.J., Sumpter, J.P., Tyler, C.R., 2000. Long-term temporal changes in the
921 estrogenic composition of treated sewage effluent and its biological effects on fish.
922 Environ. Sci. Technol. 34, 1521–1528.
923 Rosenfeld, H., Levavi-Sivan, B., Gur, G., Melamed, P., Meiri, I., Yaron, Z., Elizur, A., 2001.
924 Characterization of tilapia FSHβ gene and analysis of its 5' flanking region. Comp.
925 Biochem. Physiol. B Biochem. Mol. Biol. 129, 389–398.
926 Saligaut, C., Linard, B., Mañanós, E.L., Kah, O., Breton, B., Govoroun, M., 1998. Release of
927 pituitary gonadotropins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*):
928 Modulation by estradiol and catecholamines. Gen. Comp. Endocrinol. 109, 302–309.
929 Schultz, I.R., Nagler, J.J., Swanson, P., Wunschel, D., Skillman, A.D., Burnett, V., Smith, D.,
930 Barry, R., 2013. Toxicokinetic, toxicodynamic, and toxicoproteomic aspects of short-
931 term exposure to trenbolone in female fish. Toxicol. Sci. 136, 413–429.

Schultz, I.R., Orner, G., Merdink, J.L., Skillman, A.D., 2001. Dose-response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17α-ethynylestradiol. Aquat. Toxicol. 51, 305–318.

Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O., Vajda, A.M., 2010. Antidepressant pharmaceuticals in two US effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ. Sci. Technol. 44, 1918–1925.

Schulz, R.W., França L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Snyder, S.A., Keith, T.L., Verbrugge, D.A., Snyder, E.M., Gross, T.S., Kannan, K., Giesy, J.P., 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. Environ. Sci. Technol. 33, 2814–2820.

Snyder, S.A., Villeneuve, D.L., Snyder, E.M., Giesy, J.P., 2001. Identification and quantification of estrogen receptor agonists in wastewater effluents. Environ. Sci. Technol. 35, 3620–3625.

Sohn, Y.C., Suetake, H., Yoshiura, Y., Kobayashi, M., Aida, K., 1998. Structural and expression analyses of gonadotropin Iβ subunit genes in goldfish (*Carassius auratus*). Gene 222, 257–267.

Sohn, Y.C., Yoshiura, Y., Suetake, H., Kobayashi, M., Aida, K., 1999. Nucleotide sequence of gonadotropin IIβ subunit gene in goldfish. Fish. Sci. 65, 800–801.

Sower, S.A., Karlson, K.H., Fawcett, R.S., 1982. Changes in plasma thyroxine, estradiol-17β, and 17α,20β-dihydroxy-4-pregnen-3-one during smolitification of coho salmon. Gen. Comp. Endocrinol. 85, 278–285.

Stanford, B.D., Weinberg, H.S., 2007. Isotope dilution for quantitation of steroid estrogens and nonylphenols by gas chromatography with tandem mass spectrometry in septic, soil, and groundwater matrices. J. Chromatogr. A 1176, 26–36.

Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, 173–178.

Swanson, P., Dickey, J.T., Campbell, B., 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. 28, 53–59.

Swanson, P., Suzuki, K., Kawauchi, H., Dickhoff, W.W., 1991. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol. Reprod. 44, 29–38.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D., Servos, M., 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants—I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81–90.

Thomas-Jones, E., Thorpe, K., Harrison, N., Thomas, G., Morris, C., Hutchinson, T., Woodhead, S., Tyler, C.R., 2003. Dynamics of estrogen biomarker responses in rainbow trout exposed to 17β-estradiol and 17α-ethynylestradiol. Environ. Toxicol. Chem. 22, 3001–3008.

Thorpe, K.L., Hutchinson, T.H., Hetheridge, M.J., Sumpter, J.P., Tyler, C.R., 2000. Development of an in vivo screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 19 (11), 2812–2820.

U.S. Environmental Protection Agency, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, 4th ed. National Center for Environmental Publications, Cincinnati, OH.

977 Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Bolden, A.M., Schoenfuss, H.L., Norris,
978 D.O., 2011. Demasculinization of male fish by wastewater treatment plant effluent.
979 Aquat. Toxicol. 103, 213–221.
980 Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O., 2008.
981 Reproductive disruption in fish downstream from an estrogenic wastewater effluent.
982 Environ. Sci. Technol. 42, 3407–3414.
983 Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in
984 urban wastewater: Removal, mass load and environmental risk after a secondary
985 treatment—A review. Sci. Total Environ. 429, 123–155.
986 Vermeirssen, E.L.M., Körner, O., Schönenberger, R., Burkhardt-Holm, P., 2005.
987 Characterization of environmental estrogens in river water using a three pronged
988 approach: Active and passive water sampling and the analysis of accumulated estrogens
989 in the Bile of Caged Fish. Environ. Sci. Technol. 39, 8191–8198.
990 Vischer, H.F., 2003. Cloning and spatiotemporal expression of the follicle-stimulating hormone
991 subunit complementary DNA in the African catfish (*Clarias gariepinus*). Biol. Reprod.
992 68, 1324–1332.
993 Wille, S.M.R., 2008. Quantitative analysis of new generation antidepressants using gas
994 chromatography-mass spectrometry applications in clinical and forensic toxicology
995 (Ph.D. dissertation). Ghent University.
996 Wille, S.M.R., Van Hee, P., Neels, H.M., Van Peteghem, C.H., Lambert, W.E., 2007.
997 Comparison of electron and chemical ionization modes by validation of a quantitative gas
998 chromatographic–mass spectrometric assay of new generation antidepressants and their
999 active metabolites in plasma. J. Chromatogr. A 1176, 236–245.
1000 Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O., Vajda, A.M., 2006. Intersex and
1001 other reproductive disruption of fish in wastewater effluent dominated Colorado streams.
1002 Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 144, 10–15.
1003 Xiong, F., Liu, D., Le Dréan, Y., Elsholtz, H.P., Hew, C.L., 1994. Differential recruitment of
1004 steroid hormone response elements may dictate the expression of the pituitary
1005 gonadotropin IIβ subunit gene during salmon maturation. Mol. Endocrinol. 8, 782–793.
1006 Yadetie, F., Male, R., 2002. Effects of 4-nonylphenol on gene expression of pituitary hormones
1007 in juvenile Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 58, 113–129.
1008 Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation
1009 of fish gonadotropins. Int. Rev. Cytol. 225, 131–185.
1010 Young, W.F., Whitehouse, P., Johnson, L., Sorokin, N., 2004. Proposed predicted-no-effect-
1011 concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters
1012 (EA R&D Technical Report No. EA5098), P2-T04/1.
1013
1014

1015 Table 1. Types of waste water treatment processes at each collection site. Effluent was collected
1016 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

1017 [a]Acute and chronic mixing zones: the area near the outfall where water quality standards for acute and chronic
1018 aquatic life criteria may exceed standards as authorized in a national pollutant discharge elimination system
1019 (NPDES) permit (McGowan, 2015).
1020 [b]Acute and chronic dilution factors: the lowest dilution achieved at the edge of the acute and chronic mixing zones
1021 (McGowan, 2015).

Table 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 3: Steroid levels (E1 = estrone, E2 = 17b-estradiol, E3 = estriol, EE2 = 17α-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^{-1})	E2 (ng L^{-1})	E3 (ng L^{-1})	EE2 (ng L^{-1})
A	0.863	trace	0.65	ND	ND
B	0.845	ND	1.89	ND	ND
C	0.854	ND	trace	ND	ND
D	0.885	ND	0.92	ND	ND
E	NA	NA	NA	NA	NA
F	0.872	ND	trace	ND	trace
G	0.844	ND	0.95	ND	0.83
H	0.836	trace	2.60	ND	ND

1038 Table 4: Selective serotonin reuptake inhibitor (SSRI) levels in undiluted WWTP effluent
1039 measured by GC-MS. (FLX = fluoxetine, NF = norfluoxetine, SER = sertraline, CIT =
1040 citalopram, ND = not detected, NA = not assayed).

WWTP	Sample volume (L)	FLX (ng L^{-1})	NF (ng L^{-1})	SER (ng L^{-1})	CIT (ng L^{-1})	Total SSRIs (ng L^{-1})
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

1041

Table 5: Mean detected concentration (ng mL^{-1}) and percent of samples above the limit of detection for steroidal estrogens [estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-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^{-1}	**% BPA**	**E1** **ng** mL^{-1}	**% E1**	**E2** **ng** mL^{-1}	**% E2**	**E3** **ng** mL^{-1}	**% E3**	**EE2** **ng** mL^{-1}	**% 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.

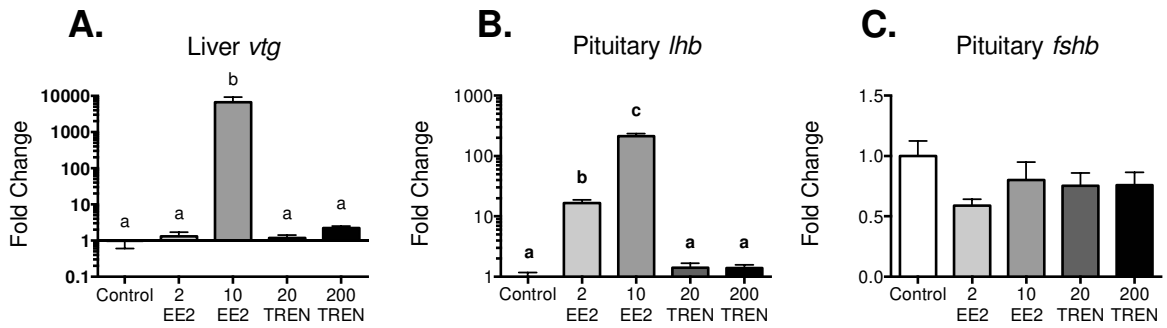


Figure 1. Relative levels of mRNAs for liver *vtg*, pituitary *lhb*, and pituitary *fshb* in juvenile coho salmon exposed to water containing 0, 2, or 10 ng L^{-1} EE2 or 20 or 200 ng L^{-1} TREN for 72 hrs. qPCR data were normalized to *eef1a* levels and then divided by control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean ± 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.

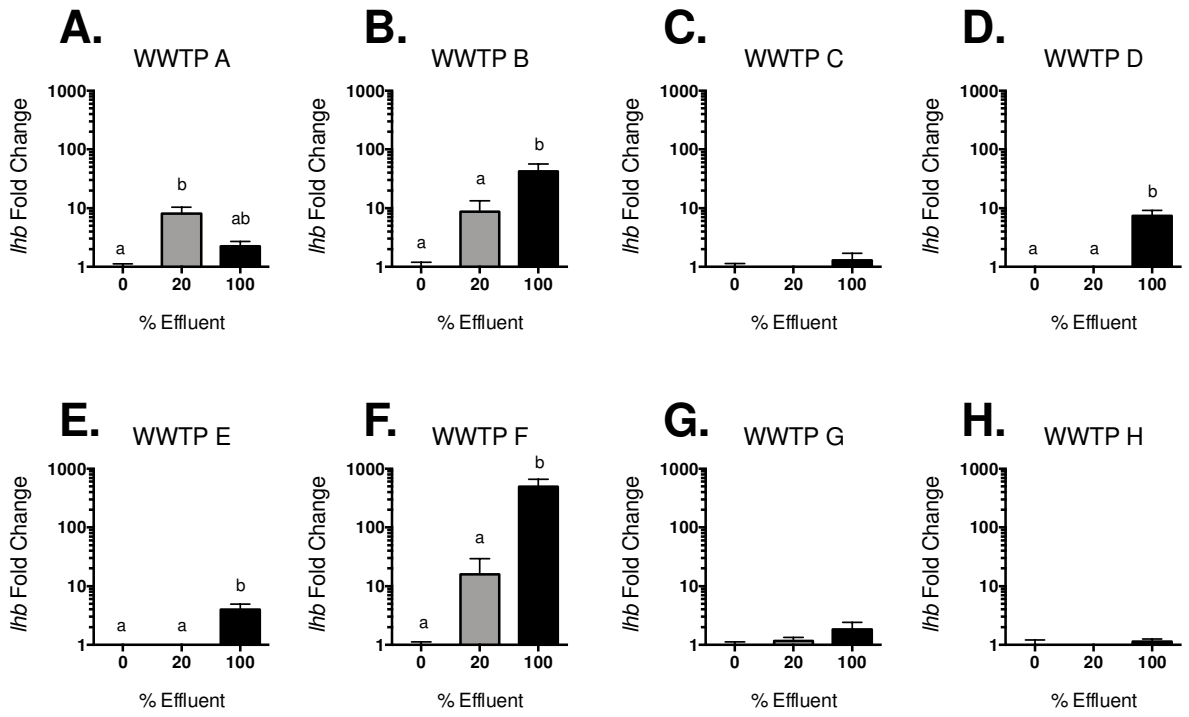


Figure 2. Relative levels of pituitary luteinizing hormone beta subunit (*lhb*) in juvenile coho salmon exposed to WWTP effluent for 72 hrs. The qPCR data were normalized to *eef1a* levels and then divided by the control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean ± 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).

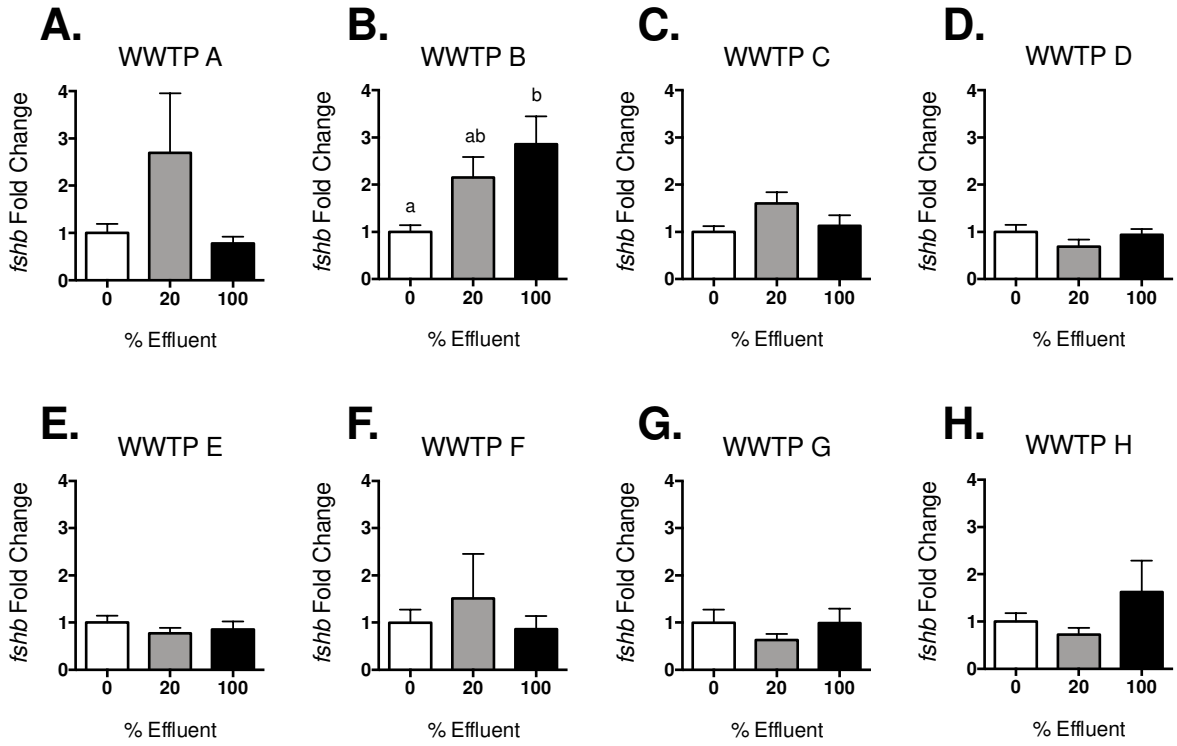


Figure 3. Relative levels of pituitary follicle stimulating hormone beta subunit (*fshb*) mRNA in juvenile coho salmon exposed to WWTP effluent for 72 hrs. qPCR data were normalized to *eef1a* levels and then divided by control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean ± 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).

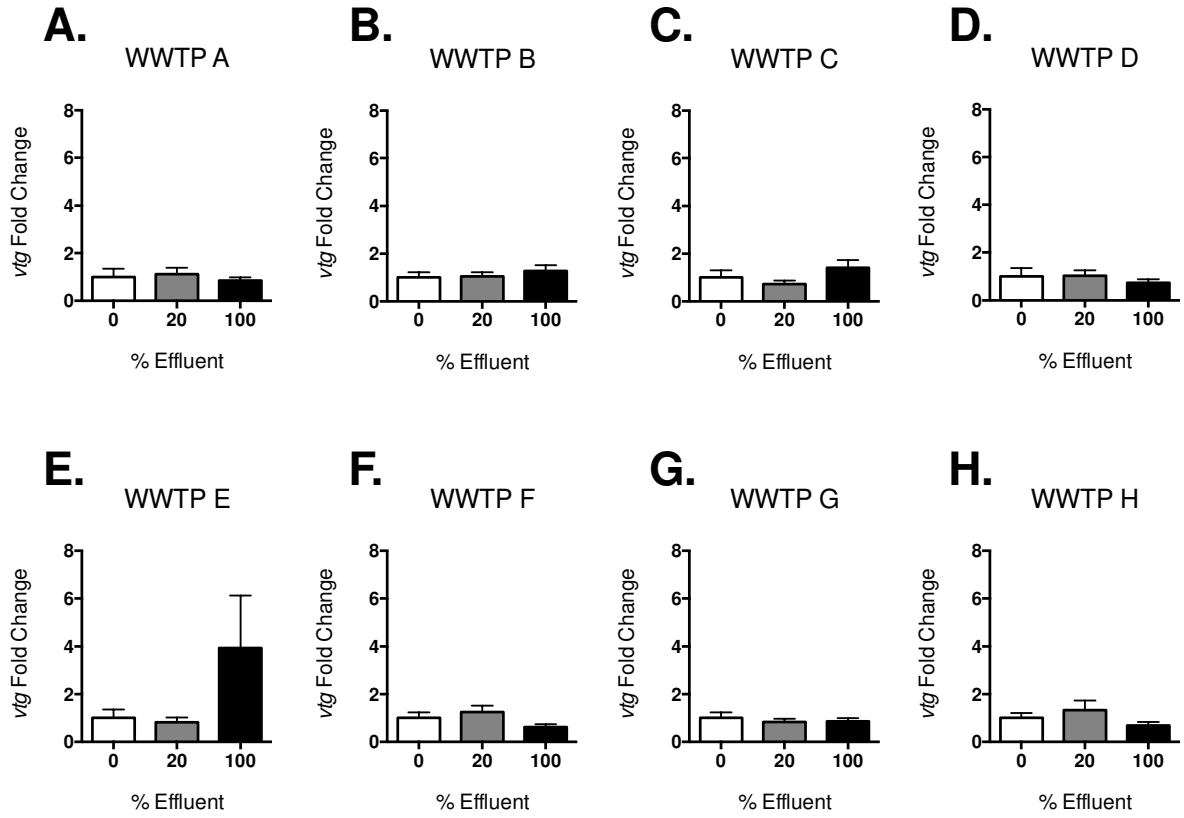
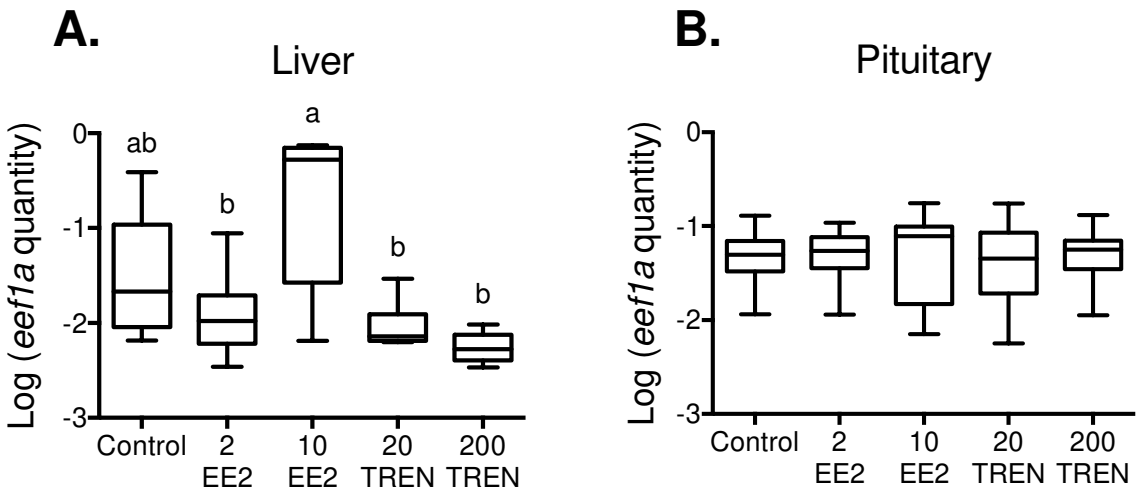
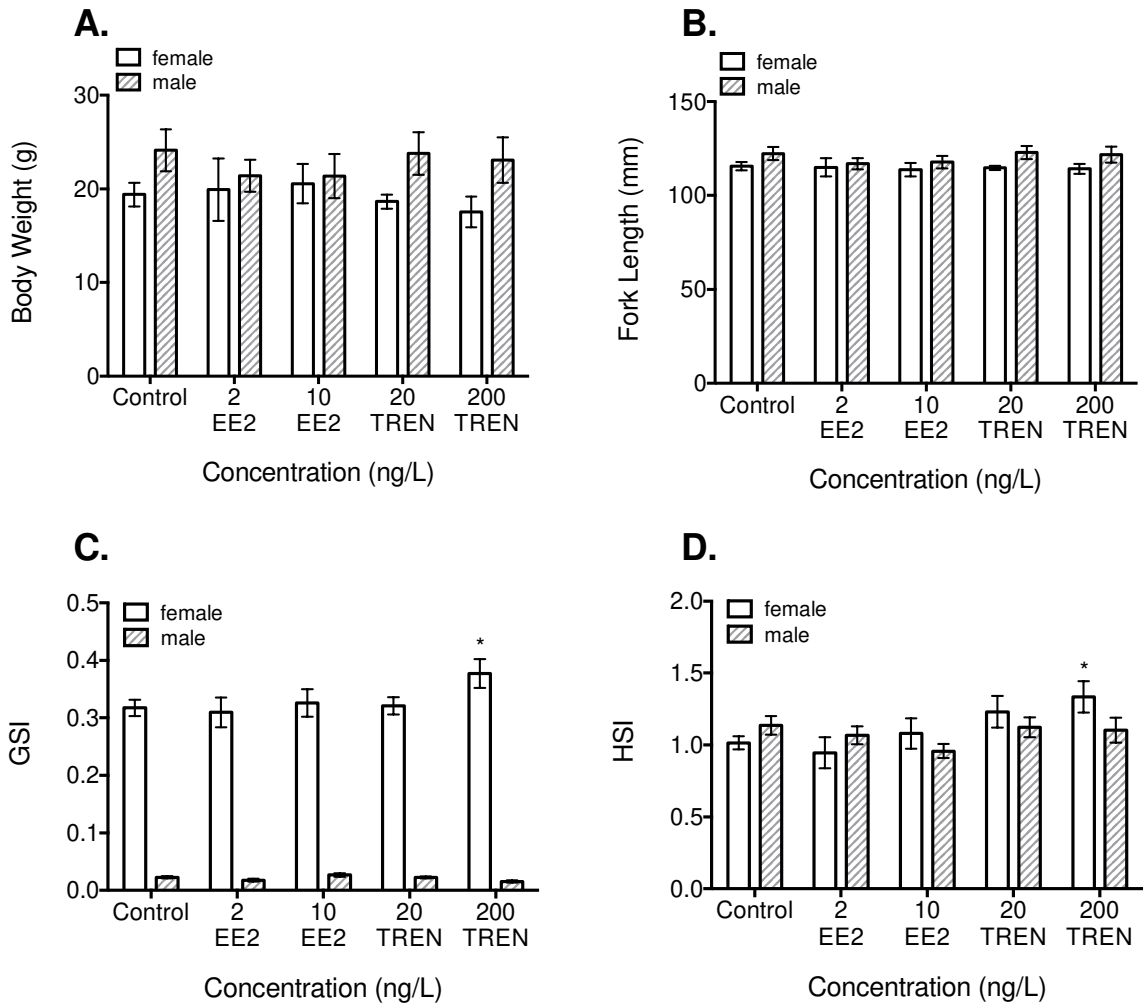


Figure 4. Relative levels of vitellogenin (*vtg*) mRNA in juvenile coho salmon exposed to WWTP effluent for 72 hrs. qPCR data were normalized to *eef1a* levels and the divided by the control mean mRNA level for improved visualization of relative expression. The data are expressed as the mean ± SEM (n = 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 1: Log levels of mRNA for *eef1a* measured by qPCR in (A) liver or (B) pituitary of fish exposed to 0 ng L^{-1}, 2 ngEE2 L^{-1}, 10 ng EE2 L^{-1}, 20 ng TREN L^{-1} or 200 ng TREN L^{-1} 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 ± SEM (n = 5 – 8 for liver and 20 for pituitary).



Supplemental Figure 2: 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 ± SEM (A and B; $n = 10$; C – D; $n = 2 – 9$).