

# **Xenoestrogen effects on fish: molecular to physiological approaches**

**by**

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## Abstract

Estrogen hormones, or compounds that mimic them (xenoestrogens), are found in surface waters worldwide mainly due to discharges to the environment from anthropogenic sources, such as sewage treatment plant (STP) effluents. Fish are adversely affected by these xenoestrogens in the areas of reproduction, growth, immune function and offspring fitness. To identify and explore the effects of xenoestrogens on salmonids, a series of toxicological studies were performed using comprehensive panels of assays evaluating immune, osmoregulatory, hematological, metabolic, reproductive and endocrine-related parameters in response to xenoestrogen- and STP effluent-exposures. Most studies included a post-treatment depuration phase to determine recovery timeframes of altered parameters.

In male or juvenile fish, xenoestrogens are known to induce vitellogenesis, the mature female-related process of making eggs under estrogen receptor (ER) control that is initiated by the hepatic production of egg proteins, vitellogenin (VTG) and vitelline envelope proteins (VEPs). In three studies, induction of liver somatic index (LSI), plasma VTG protein or hepatic gene transcripts (ERs, VTG and VEPs) were found due to exposure to a tertiary-treated STP effluent, a synthetic wastewater, and estrogen hormones. In Rainbow trout exposed to xenoestrogens, LSI and transcripts recovered to baseline values after treatments ceased, while VTG protein concentrations remained elevated through recovery phases. Thus, gene expression alterations were only useful as indicators of existing exposure, while VTG protein levels were indicative of both present and prior exposure. Xenoestrogens affected several immune system functions, including reduced leukocyte counts, potentially increasing susceptibility to pathogens. Isolated leukocytes from head kidney and peripheral blood contained ER $\alpha$  and ER $\beta$

transcripts, although only ER $\alpha$ 1 and ER $\alpha$ 2 were altered by xenoestrogen exposure, providing a direct mechanistic pathway for xenoestrogen modulation of the immune system. Reductions in burst swimming performance were found in Rainbow trout exposed to xenoestrogens, with potential mechanisms suggested involving altered osmoregulation (evidenced by reduced chloride ions) and decreased blood oxygen carrying capacity via a reduction in red blood cell counts. Overall, most but not all altered parameters recovered from exposure to xenoestrogens, and the adverse effects of environmentally-relevant concentrations of xenoestrogens on many biological functions showed that xenoestrogens pose a hazard to wild juvenile fish.

**Keywords:** Fish; estrogen hormone; toxicology; gene expression; sewage treatment plant effluent; vitellogenin

## Dedication

*To Kyle and Kaylie,  
and my parents, Carol and Don.*

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## List of Acronyms and Abbreviations

d	Day
h	Hour
min	Minute
s	Second
SEM	Standard error of the mean
sd	Standard deviation
VTG	Vitellogenin
ER	Estrogen receptor
VEP	Vitelline envelope protein
E1	Estrone
E2	17 $\beta$ -estradiol
EE2	17 $\alpha$ -ethinylestradiol
STP	Sewage treatment plant
US or USA	United States of America
BC	British Columbia

## **Chapter 1.**

### **Introduction and Study Objectives**



## Endocrine Disrupting Compounds

Due to anthropogenic inputs, aquatic environments have been under considerable pressure from contaminant loadings. Reported environmental contaminants include hormones, metals, pesticides, pharmaceuticals, and other persistent organic pollutants. There is growing concern that some of these contaminants can interact with the endocrine systems of organisms, affecting processes under hormonal control. The endocrine system communicates actions both within and between cells to regulate and coordinate a multitude of biological functions, including growth, development, metabolism, homeostasis and reproduction. Disruption of this important system is likely to have impacts on overall organism fitness, with ramifications at the population level. Chemicals that disrupt the endocrine system are called endocrine disrupting compounds (EDCs); they alter or interact with the endocrine system to adversely affect normal function. Numerous chemicals have been identified as EDCs from many classes of compounds, including dioxins, metals, antibiotics, polycyclic aromatic hydrocarbons (PAHs), pesticides, plasticizers and pharmaceuticals. EDCs are commonly identified with the hormone that they interfere with. Main classification groups include estrogen (those interfering with  $17\beta$ -estradiol (E2), the main vertebrate estrogen hormone), androgen (interfering with testosterone) and thyroid (interfering with thyroid hormones). Compounds that are applied exogenously and/or mimic E2 are often called xenoestrogens.

## **Xenoestrogens**

Xenoestrogens vary in their chemical structures, uses and properties. They interact with estrogen receptors or induce effects typically regarded as estrogen-responsive or female-related. The main classes of xenoestrogens include hormones, surfactants/detergents, pesticides, and plastic-related compounds (e.g. bisphenol A and phthalates). A list of xenoestrogens and their relative potency to E2 shows there are several orders of dynamic range between the compounds (Table 1-1). Only 17 $\alpha$ -ethinylestradiol (EE2; the birth control pill hormone) is more potent than E2. The other natural estrogen hormones, estrone (E1) and estriol (E3), are less potent than E2 but more potent than other synthetic compounds (Table 1-1). The estrogenicity of mixtures is attributable to the additive concentrations of the constituent xenoestrogens adjusted by their relative potency to E2 (Thorpe et al., 2003; Brian et al., 2005; Thorpe et al., 2006).

## **Effects on Salmonid Reproduction**

Rainbow trout (*Oncorhynchus mykiss*) are a freshwater fish found in northern hemisphere aquatic environments, including Britain, Europe, Russia, Northeast Asia, and North America. Rainbow trout are a commonly used toxicological organism and a model species for Pacific salmon (also Oncorhynchids), such as Chinook (*O. tshawytscha*), Coho (*O. kisutch*), and Sockeye salmon (*O. nerka*). Rainbow trout and Pacific salmon are environmentally, culturally, economically and socially important in British Columbia (BC), Canada. Xenoestrogens have been shown to adversely affect

salmonid reproduction, a key process controlled by the endocrine system, and E2 in particular, in adult female fish. In adult male Rainbow trout exposed to EE2, fish exhibited reduced sex-steroid levels, testis mass and success of offspring in achieving the eyed egg stage (Schultz et al., 2003), as well as inhibited testicular growth and spermatogenesis (Jobling et al., 1996). Exposure of Chinook to xenoestrogens during the early life stage affected gender determination, resulting in genetic males with intersex gonadal tissue (Afonso et al., 2002). Xenoestrogenic effects on reproduction have been well-studied in other fish species as well (e.g. fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*)), showing alterations in male:female sex ratios, nest holding, courtship and mating behaviour, and reduced fecundity (Kristensen et al., 2005; Parrott & Blunt, 2005; Martinovic et al., 2007; Hyndman et al., 2010; Dammann et al., 2011).

## **Vitellogenesis**

As a part of the reproductive process, adult female fish produce egg components in the liver, which are transported via blood to the ovary where they are assembled into eggs as the fish matures (Mommensen & Walsh, 1988). These hepatic components are a precursor to egg yolk, called vitellogenin (VTG), and structural parts of the egg chorion, known as vitelline envelope proteins (VEPs). This process, known as vitellogenesis, is under the control of E2. When E2 enters a hepatocyte, it interacts with estrogen receptor (ER) initiating ER dimerization. The ER complex enters the nucleus, interacts with estrogen response elements (EREs), and initiates transcription of VTG, VEPs and ERs, which are then translated into proteins (Aruwke & Goksoyr, 2003). In many fish

species, measurement of hepatic or plasma VTG protein is commonly performed as a biomarker of vitellogenesis and its response in Rainbow trout has been described by concentration-response curves that are dependent on the potency of the xenoestrogen (Thorpe et al., 2003). Since vitellogenesis is inducible in immature or male fish, VTG protein has been used to gauge the extent of xenoestrogen exposure. However, juvenile or male fish lack ovarian tissue to store this protein and so it remains in circulation until nephretic elimination occurs (Herman & Kincaid, 1988), which can take weeks or months (Hemmer et al., 2002).

## **Effects of Xenoestrogens on Other Biological Systems**

The effects of xenoestrogens on biological systems other than reproduction have been reported in the literature but to a much lesser extent. The alteration of numerous biological systems in salmonids is likely possible due to the presence of ERs in many tissues (evidenced by the presence of transcripts but proteins have not yet been characterized (Nagler et al., 2007)). Two areas have been studied that highlight the ability of xenoestrogens to alter non-reproductive functions in salmonids: immune defense and migration ability. In studies on immune function, Rainbow trout exposed to E2 or nonylphenol (NP) showed reduced survival following challenge with a pathogen (Wenger et al., 2011; Shelley et al., 2012), thus, xenoestrogens can increase susceptibility to disease. It is known that xenoestrogens are immunomodulators but the mechanisms are largely unknown and the extent of immune alterations in fish is also largely unknown (Iwanowicz & Ottinger, 2009). In a study on migration ability, Atlantic salmon (*Salmo salar*) injected with E2 or NP were released into a river and their arrival

at a downstream migration point was measured (Madsen et al., 2004). Xenoestrogen-treated fish were delayed by approximately one week in reaching the migration point and had significantly increased mortality compared to control fish. The authors proposed that it was increased predation upon the E2- and NP-treated fish that led to these results; however, this remains speculative.

## **Sewage Treatment Plant Effluents**

Sanitation is an essential factor of human civilization to prevent disease and ensure clean environments. The treatment of domestic, agricultural and/or industrial wastewaters is carried out by sewage treatment plants (STPs), whose effluents represent point discharges to the environment. STPs consist of modular processes, with each addition of a process adding special capabilities for nutrient, microorganism or chemical removal. Primary treatment, the base level, includes screening of incoming waste (influent) and primary clarifiers (i.e. settling tanks or ponds) prior to discharge. At the next level, secondary treatment builds upon primary treatment and includes other processes to remove nutrients and microorganisms, including lagoons or biological nutrient removal. Tertiary treatment, the highest level, builds upon secondary treatment by utilizing additional processes, such as ozonation or chlorination/dechlorination, to disinfect the effluent prior to discharge. In Canada, municipalities may operate primary, secondary or tertiary STPs, and with the recent release of the federal government's Canada Wide Strategy for the Management of Municipal Wastewater Effluent, the new minimum treatment level for all STPs will be secondary treatment (CCME, 2012).

Compared to historical human waste, modern STP influents contain pharmaceutical and personal care products, leachate from plastics and pesticides (i.e. bioactive chemicals), some of which are EDCs. Even with treatment, some of these chemicals are not degraded or eliminated and are discharged in effluents. Other chemicals are greatly reduced in concentration compared to influents, yet remain at levels in effluents that can cause impacts to aquatic organisms. Two classes of xenoestrogens that can actually increase in concentration with increased STP processing include estrogen hormones and alkylphenols. Estrogen hormones are excreted in urine and feces from vertebrates in their parent form, as oxidative metabolites, or conjugated to sulfate or glucuronide moieties (Larsson et al., 1999; Andersen et al., 2003; Khanal et al., 2007; Shrestha et al., 2013). STP processes can break the conjugate bonds, thereby re-releasing the estrogen hormones (Andersen et al., 2003; Khanal et al., 2007; Suarez et al., 2008). Similarly, alkylphenols, such as NP, are released by degradation of alkylphenol ethoxylates (one type of surfactant). Thus, it is a paradox that increasing treatment does not always result in increased removal of chemicals. One area of active research in the field of sewage treatment relates to investigations of treatment plant processes that are effective in removing or degrading xenoestrogens and other toxicants of concern.

Approximately 20 years ago, it was found that European wastewaters caused feminization in fish downstream of STPs (Harries et al., 1996; Jobling et al., 1998). Subsequently, it has been determined that STP effluents can be estrogenic due to the presence of natural and synthetic estrogen hormones as well as chemicals that mimic estrogen hormones, thus, these effluents are considered to be endocrine disrupting mixtures. Since estrogenic STP effluents have resulted in feminized fish in other

jurisdictions (Jobling et al., 1998; Jobling et al., 2002), it is possible that this may be occurring within Canada. In a preliminary study, secondary- and tertiary-treated STP effluents from six confidential sites in British Columbia (BC) were evaluated for estrogen hormone concentrations and Furtula et al. (2012) found estrone (E1) in three STP effluents (Table 1-2), indicating that local aquatic organisms are exposed to xenoestrogens and their potentially adverse effects.

## **Molecular to Physiological Approaches**

Recently, the field of toxicology has embraced genomic tools and the use of molecular-level endpoints by evaluating gene transcript responses to contaminant exposure. Currently, genomics approaches offer the advantage of generating immense amounts of information relatively quickly. These measures can offer insights into mechanisms of action and molecular-level targets, pointing to impacts on a diverse array of biological processes. However, these results are difficult to link to established adverse physiological or population outcomes, which are valuable as actual measurements of reduced fitness. Advances in research are needed to establish these linkages, i.e. to relate gene expression results to phenotype, otherwise known as phenotypic anchoring. The best approach to characterize chemical impacts would be to integrate endpoints from the molecular, biochemical, tissue, organism and population levels. This is difficult to achieve in practice due to the requirements for interdisciplinary expertise and the expense of such a comprehensive approach. However, using multi-level approaches is an important goal so that molecular data is utilized to its fullest

potential and ultimately such comprehensive research should result in complete understanding of how a chemical contaminant acts as a toxicant.

## **Research Objectives**

Six studies were undertaken with a common goal to advance knowledge of effects caused by estrogen hormones and STP effluents on salmonids, using approaches from the molecular to physiological levels.

The first objective was to characterize xenoestrogen impacts on immune function and swimming performance, two areas of research on non-reproductive effects that have been understudied, and to identify potential mechanisms by which any adverse effects occur.

The second objective was to use the well-characterized system of vitellogenesis to investigate the time course of induced estrogen-responsive parameters, and the return of those alterations to baseline levels, in xenoestrogen-exposed fish.

The third objective was to address several issues regarding STP processes and their effluents. Specifically, the presence and concentrations of estrogen hormones, the utility of various treatment processes in removing chemical contaminants, and potential impacts on salmonids were investigated.

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**Table 1-1** *Reported xenoestrogenic potencies in fish.*

Compound	Abbreviation	Potency (fold, compared to E2)	Reference
17 $\beta$ -estradiol	E2	1.0	
17 $\alpha$ -ethinylestradiol	EE2	28	Brian et al., 2005
		11 - 27	Thorpe et al., 2003
		31 - 33	Van den Belt et al., 2004
		100	Legler et al., 2002
		133	Metcalfe et al., 2001
estrone	E1	0.3 - 0.4	Thorpe et al., 2003
		0.5	Metcalfe et al., 2001
		0.5 - 0.8	Van den Belt et al., 2004
		~ 1	Dammann et al., 2011
		1	Legler et al., 2002
estriol	E3	0.005	Metcalfe et al., 2001
nonylphenol	NP	0.00356	Brian et al., 2005
		0.0006 - 0.0007	Van den Belt et al., 2004
bisphenol A	BPA	0.00016	Brian et al., 2005
		0.00068	Metcalfe et al., 2001
$\beta$ -sitosterol	-	0.00005	Tremblay & Van Der Kraak, 1998
di(2-ethylhexyl) phthalate	DEHP	$< 8 \times 10^{-7}$	Metcalfe et al., 2001

**Table 1-2 Estrogen hormone concentrations determined by gas chromatography mass spectrometry analysis in a preliminary study on six sewage treatment plant (STP) effluents from British Columbia, Canada (Furtula et al., 2012).**

Compound	Abbreviation	MDL ( $\mu\text{g/L}$ )	STP 1	STP 2	STP 3	STP 4	STP 5	STP 6
			Concentration ( $\mu\text{g/L}$ )					
Estrone	E1	0.005	<i>nd</i>	0.045	0.026	<i>nd</i>	<i>nd</i>	0.035
17 $\beta$ -Estradiol	E2	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Estriol	E3	0.02	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
17 $\alpha$ -Ethinylestradiol	EE2	0.02	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>

*nd* = not detected

## Chapter 2.

# Alteration of immune function endpoints and differential expression of estrogen receptor isoforms in leukocytes from 17 $\beta$ -estradiol exposed Rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

While the endocrine system is known to modulate immune function in vertebrates, the role of 17 $\beta$ -estradiol (E2) in cellular immune function of teleosts is poorly understood. The cellular and molecular responses of juvenile Rainbow trout (*Oncorhynchus mykiss*) to E2 treatment were evaluated by exposing fish to  $0.47 \pm 0.02$   $\mu\text{g/L}$  E2 (mean  $\pm$  SEM) for either 2 or 7 d, with a subsequent 14 d recovery period. After 2 and 7 d of exposure to E2, hematocrit was significantly lower than in control fish. Lipopolysaccharide-induced lymphocyte proliferation was elevated on day 2 and concanavalin A-induced lymphocyte proliferation was reduced following 7 d of E2 exposure. Four estrogen receptor (ER) transcripts were identified in purified trout head kidney leukocytes (HKL) and peripheral blood leukocytes (PBL). While the mRNA abundance of ER $\beta$ 1 and ER $\beta$ 2 was unaffected by treatment, ER $\alpha$ 1 was up-regulated in HKL and PBL following 7 d of E2 exposure. ER $\alpha$ 2 was up-regulated in HKL after 7 d of E2 exposure, but down-regulated in PBL after 2 and 7 d of treatment. All parameters that were altered during the E2 exposure period returned to baseline levels following the recovery period. This study reports the presence of the full repertoire of ERs in purified HKL for the first time, and demonstrates that ER $\alpha$  transcript abundance in leukocytes can be regulated by waterborne E2 exposure. It also demonstrated that physiologically-relevant concentrations of E2 can modulate several immune functions in salmonids, which may have widespread implications for xenoestrogen-associated immunotoxicity in feral fish populations inhabiting contaminated aquatic environments.

## **Keywords**

Immune system; Rainbow trout;  $17\beta$ -estradiol; lymphocyte proliferation; estrogen receptor; recovery



## Introduction

The immune system of teleosts provides a critical barrier function for protection against pathogens commonly found in the aquatic environment [1,37]. Its function can be up- or down-regulated by a variety of exogenous factors that include season and photoperiod, water quality parameters such as temperature and pH, as well as xenobiotic exposure [6]. In addition, hormones that guide the activity and development of normal physiological processes can also act as signaling molecules linking the perception of external conditions to internal responses. Thus, circulating hormone concentrations can be influenced by exogenous factors and hormones have been shown to modulate the activities and function of immune system components [22,74]. The direct and indirect effects of altered hormone status may provide the mechanistic underpinning of altered immune function caused by such factors.

The main vertebrate estrogen hormone, 17 $\beta$ -estradiol (E2), has known functions in fish reproduction, growth, behaviour, differentiation and development [30]. Classical genomic pathway signaling by E2 occurs following the interaction of E2 with intracellular estrogen receptors (ERs) and associated proteins located in the cytoplasm. Translocation of the E2-ER dimer complex into the nucleus enables its action as a transcription factor for genes containing an estrogen response element [41]. Although controversial [33], E2 signaling may also occur through the interaction of E2 with transmembrane receptors such as GPER (G-protein coupled estrogen receptor, previously known as GPR30), which triggers an intracellular signaling cascade with more rapid effects [53]. Regardless of the signaling mechanism, elevated E2 concentration is mainly associated with sexual maturity and spawning, as it initiates the transcription and

translation of a plethora of proteins related to gonadal maturation and the production of eggs in females [48].

In Rainbow trout, four forms of ER have been identified: ER $\alpha$ 1, ER $\alpha$ 2, ER $\beta$ 1 and ER $\beta$ 2 [45]. All four forms have been detected in a variety of tissues including the liver, gonads, brain and heart, as well as tissues associated with the immune system (e.g. head kidney and spleen). The head kidney and spleen consist of a variety of different cell types, so these findings are only suggestive of the presence of ERs in leukocytes themselves. Recent evidence has indicated that the four Rainbow trout ERs are found in leukocytes from peripheral blood [46,61], providing a signaling mechanism through which E2 may exert influence over cells of the immune system.

17 $\beta$ -estradiol is present throughout the lifecycle of both male and female fish, with plasma concentrations in the low pg/mL range in males and immature females, and in the low ng/mL concentration range in reproductively mature females [8,49]. Estrogens can also be found in the aquatic environment in the low ng/L range as a result of sewage treatment plant effluent discharges, sewage biosolids application as soil fertilizer and runoff from agricultural livestock waste, since endogenously produced estrogens, including E2 and the other estrogens estriol and estrone, are excreted by humans and agricultural animals [26,32]. Other compounds are capable of exerting estrogen-like effects in exposed organisms; synthetic estrogens such as those used in contraceptives (e.g. 17- $\alpha$ -ethinylestradiol (EE2)), naturally derived phytoestrogens (e.g. genistein), and several with more limited structural resemblance to endogenous estrogens (e.g. nonylphenol, bisphenol A, and some phthalate esters) [54]. While most xenoestrogens (except EE2) have a lower binding affinity for the ER, and thus a lower biological activity than endogenous E2 [54], some of these chemicals can be present in the aquatic

environment at concentrations high enough to exert physiological effects [32]. For example, sewage effluents, which consist of a complex mixture of substances, including estrogen hormones and xenoestrogens, have been associated with reproductive abnormalities [29] and immune system dysfunction [55] in exposed fish.

Chemicals that alter fish immune function and decrease pathogen resistance have important implications to fish survival and population growth [9,62]. Host resistance assays provide perhaps the most poignant evidence of the biological implications of chemical-associated immunotoxicity, since the major endpoint is survivability following exposure to a pathogen [31]. Exposure to E2 (either as an implant or through aquatic exposures) has been shown to alter susceptibility to parasitic [68] and bacterial infections [71]. Exposure to E2, therefore, is believed to affect components of the immune system that have consequences for disease survivability in individuals, and may have significant implications at the population level.

While it is clear that E2 causes immunosuppression at the organism level, considerable uncertainty exists regarding the mechanisms of E2 effects on fish immune function at the cellular level. In the current study, the immunomodulatory effects of aquatic E2 exposure in juvenile Rainbow trout were examined using a panel of immune function assays and quantification of gene expression to gain insight into responses to estrogen hormone at both the cellular and molecular levels. Groups of fish were exposed to E2 for either 2 or 7 days, followed by a 14 d depuration period to assess the ability of fish to recover once E2 exposure ceased.

## **Materials and Methods**

### ***Fish Source and Maintenance***

Rainbow trout, (mass at the time of sampling =  $31.9 \pm 0.7$  g, mean  $\pm$  SEM) were obtained from Sun Valley Trout Hatchery (Mission, BC, Canada) and maintained in fiberglass tanks with continuously flowing well water at  $15 \pm 1^\circ\text{C}$  and a 16:8 h (light:dark) photoperiod. Prior to exposure, fish were fed a daily ration with commercial trout feed (Ewos, Surrey, BC, Canada) at 2% of their body weight per day. All experimental procedures performed with fish were done under an Animal Care Permit issued by the Simon Fraser University Animal Care Committee, and in accordance with Canadian Council on Animal Care guidelines.

### ***Chemicals***

Charcoal-stripped fetal bovine serum (FBS) was obtained from Invitrogen (Gibco, Portland, OR, USA). Unless noted, all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

### ***Experimental Design and Sampling***

During the experiment, Rainbow trout were individually housed in 68 L glass aquaria (n = 10 to 15 aquaria per treatment group per sampling day) held in temperature controlled rooms ( $15 \pm 1^\circ\text{C}$ ) and were exposed to either a nominal concentration of zero (control) or  $1 \mu\text{g/L}$   $17\beta$ -estradiol (E2) in well water for either 2 or 7 d. Anhydrous ethanol

(100.0%; Commercial Alcohols, Tiverton, ON, Canada) was used as a carrier for the addition of E2 to the treatment tanks and an equivalent amount of anhydrous ethanol was added to the control tanks, such that the solvent concentration in all the tanks was 0.001% v/v. Fish that were not sampled at 7 d were kept for a 14 d recovery period following E2 exposures. At the end of the 7 d exposure, these fish were transferred to new, clean aquaria containing well water and no E2. Exposures were conducted as static renewal, with water and chemical being refreshed (100% renewal) every 3-4 d throughout both the exposure and recovery periods. Water quality parameters including pH, dissolved oxygen, conductivity and temperature were monitored regularly. Composite water samples from the control or E2-treatment tanks were collected for E2 analysis at the beginning of the experiment and before and after each water change. Analysis for E2 was performed using the exact method of Furtula *et al.* [20].

At each sampling time point, individual fish were euthanized with sodium bicarbonate-buffered 0.1 g/L tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA), and then weighed. Blood was aseptically collected by caudal venipuncture into a heparinized syringe and immediately transferred to a tube containing an equal volume of sterile supplemented HBSS (sHBSS; 15 mM HEPES, 5% FBS, 10 U/mL heparin and 1% penicillin/streptomycin). Additional blood was collected following severing of the caudal peduncle into heparinized capillary tubes, which were centrifuged in order to determine hematocrit and to collect plasma samples. The head kidney was excised and placed into tubes containing 1 mL of sHBSS. All samples were stored on ice.

### ***Hematocrit and Plasma 17 $\beta$ -Estradiol***

Following centrifugation of the hematocrit tubes, hematocrit was determined based on the measured total height of the packed red blood cell column divided by the total height of the blood column. Hematocrit tubes were then cut to collect plasma, which was stored temporarily on ice until transfer for storage at -80°C. Plasma E2 was measured using a commercially available E2 ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) following manufacturer instructions.

### ***White Blood Cell Purification***

In order to obtain single cell suspensions, head kidney samples were gently disrupted on a 75  $\mu$ m nylon mesh and washed through with 2 mL of sHBSS. Purified white blood cells were then isolated from both the head kidney and blood using a hypotonic lysis method [12]. Briefly, 9 mL of sterile cold water was added to the cell suspension for 30 s to induce lysis, which was then stopped by the addition of 1 mL of 10 x PBS (0.1 M). Gross debris was removed with a sterile Pasteur pipette, samples were centrifuged at 400 x *g* for 5 min at 4°C and cells were washed twice with 5 mL of sHBSS. Cells were resuspended in 2 mL of sHBSS for counting and assessment of viability by trypan blue exclusion. Cells were then adjusted to either 1 x 10<sup>7</sup> viable cells/mL for head kidney leukocytes (HKL) or 5 x 10<sup>6</sup> viable cells/mL for peripheral blood leukocytes (PBL).

## ***Phagocytosis and Phagocytic Capacity***

Head kidney leukocytes were used in assays to measure phagocytosis and respiratory burst activity. Phagocytosis was assessed using flow cytometry where the ability of HKL to take up fluorescent beads is measured [10,60]. Briefly,  $1 \times 10^6$  viable cells were incubated at room temperature in either 1 mL of sRPMI (live cells) or 1 mL of 1% paraformaldehyde in 0.01 M PBS (fixed cells, control), in duplicate. Cells were centrifuged at  $500 \times g$  for 5 min at  $18^\circ\text{C}$ , resuspended in 100  $\mu\text{L}$  of sRPMI and transferred to round-bottom 96-well culture plates. Fluorescent latex beads (1  $\mu\text{m}$  diameter) were added at a ratio of 100:1 (beads:cells) and samples incubated at  $18^\circ\text{C}$  for 18 h. Cells were then layered over a cushion containing 3% BSA in 0.01 M PBS and centrifuged at  $100 \times g$  for 10 min at  $4^\circ\text{C}$  before being resuspended in 0.01 M PBS.

On the flow cytometer (Becton Dickinson FACSAria, Franklin Lakes, New Jersey, USA), a gate was set up that excluded non-phagocytosed beads and debris and 10,000 cell events were collected for this gate for each tube. Cells that had taken up beads were identified by creating a gate of bead positive cells based on the FITC histogram. The percent of cells that phagocytosed beads was calculated as percent bead positive live cells minus percent bead positive fixed cells. A phagocytic capacity (PC) based on mean fluorescence intensity was also calculated as an approximation of the number of beads per cells, where PC is the mean fluorescence intensity of bead positive live cells divided by the mean fluorescence intensity of a single bead suspension.

### ***Respiratory Burst***

Respiratory burst was measured with the nitroblue tetrazolium (NBT) method using 96-well flat-bottomed microplates [59]. This assay is based on the reduction of the soluble NBT dye to insoluble formazan by reactive oxygen species. An aliquot of 100  $\mu$ L of HKL was added to quadruplicate wells and incubated at room temperature for 2 h. Non-adherent cells were removed by gently washing twice with 0.01 M PBS. Half of the cells received 100  $\mu$ L of RPMI with 1 mg/mL NBT (unstimulated cells) and the other half received 100  $\mu$ L of medium containing both 1 mg/mL NBT and 1  $\mu$ g/mL phorbol myristate acetate (PMA) to induce the respiratory burst (stimulated cells). Cells were incubated at room temperature for 30 min before media was removed and cells were fixed by submerging plates in 70% methanol three times. Plates were air dried before adding 120  $\mu$ L/well of KOH and 140  $\mu$ L/well of DMSO to solubilize the formazan. Absorbance was analyzed on a Bio-Tek® PowerWave 340 microplate reader (Bio-Tek, Winooski, VT, USA) at 630 nm. Respiratory burst activity was calculated as an index of the OD of stimulated cells divided by the OD of unstimulated cells.

### ***B and T Cell Proliferation***

Peripheral blood leukocytes were used in an assay of B and T cell mitogen-induced proliferation. Mitogenesis was assessed by flow cytometry by comparing the proliferation of unstimulated cells to that of leukocytes following stimulation with *Escherichia coli* lipopolysaccharide (LPS) to stimulate B cell proliferation or concanavalin A (Con A) to stimulate T cell proliferation [57,58]. Briefly,  $5 \times 10^5$  cells from each fish were added to 6 wells of a round-bottomed 96-well microplate. Duplicate wells received



either 100  $\mu$ L of sRPMI, 100  $\mu$ L of sRPMI containing 100  $\mu$ g/mL LPS, or 100  $\mu$ L of sRPMI containing 10  $\mu$ g/mL Con A. Plates were incubated in a humidified chamber for 96 h at 18°C before cells were transferred to glass 5 mL tubes with 0.8 mL 0.01 M PBS (total volume 1 mL) for analysis by flow cytometry.

A total of 10,000 events per sample were collected and proliferation was determined based on shifts in forward and side scatter that occur as cells begin to divide and become more complex [57,58]. Previously, this flow cytometric method has been shown to have good positive correlation with results from  $^3\text{H}$ -thymidine labeling that is frequently used to measure lymphoproliferation [57]. Gates (resting leukocyte gate and proliferating leukocyte cell gate) were defined based on a sample of unstimulated cells and the same gates were used throughout the experiment [61]. Proliferation of viable cells was determined as a stimulation index (SI), where  $\text{SI} = \% \text{ stimulated cells in proliferating cell gate} / \% \text{ unstimulated cells in proliferating cell gate}$ .

## ***Evaluation of Gene Expression***

### **RNA Extraction and cDNA Preparation**

For purified HKL and PBL, total RNA extraction was performed using RNeasy Mini Spin kits (Qiagen, Mississauga, ON, Canada). To obtain  $1 \times 10^6$  leukocytes per sample, an appropriate volume of cells in sHBSS, was placed in 1.5 ml tubes and spun at  $400 \times g$  for 5 min to pellet the cells. The medium was removed and 1 ml of Buffer RLT (from the Qiagen RNeasy kit) containing 10  $\mu$ l of  $\beta$ -mercaptoethanol was added per tube. Samples were then vortexed for 1 min to lyse the cells and stored at -80°C until extraction. To extract total RNA, leukocytes were thawed, vortexed for 1 min, mixed 1:1

with 70% ethanol, loaded onto the spin column and washed as per the manufacturer's protocol, including an on-column DNase treatment (Qiagen). Immediately after extraction, samples were placed on ice and their total RNA concentrations were determined using a Nanodrop® 1000 instrument (Thermo Fisher Scientific, Toronto, ON, Canada).

Total RNA (1 µg) was transcribed into cDNA using a QuantiTect reverse transcription kit (Qiagen) following manufacturer's instructions, which included a genomic DNA wipeout buffer, and the resultant cDNAs were diluted 1:10 with nuclease-free water.

### **Quantitative Polymerase Chain Reaction (QPCR)**

Gene expression of the ERs in HKL and PBL was analyzed with QPCR in E2-treated fish compared to control fish at each time point, while the set of four immune genes were analyzed only at day 2 and 7. Sequences for ER primers were obtained from Nagler *et al.* [45]. The primers for four immune-related genes (TNFα, IL1β, CXCR4, CCR7; see Table 2-1) were obtained from the Pacific Environmental Science Centre (Environment Canada, North Vancouver, BC, Canada). The reference gene, ribosomal protein L8, primer sequences were obtained from Dr. Nik Veldhoen and Dr. Caren Helbing (University of Victoria, Victoria, BC, Canada). Primers were synthesized by AlphaDNA (Montreal, QC, Canada) and the sequences used are provided in Table 2-1.

Set-up of QPCR reactions occurred in triplicate, on ice, and utilized Master Mixes that contained the following per 15 µl total volume reaction: 50% iTaq™ SYBR® Green

Supermix With ROX (Bio-Rad, Mississauga, ON, Canada); 20 pmoles forward primer; 20 pmoles reverse primer; 2  $\mu$ l of diluted cDNA; and nuclease-free water (Ambion, Austin, TX, USA) up to the final volume. Plates were run on Stratagene MX3000P™ QPCR instruments (Agilent, Cedar Creek, TX, USA) with the program: 95°C for 1 min and 40 cycles of 95°C for 15 s, 58°C for 25 s, 72°C for 35 s. Amplification of the correct product for each gene and tissue was verified using agarose gels, melting curves and sequencing. No template controls (NTC) were performed in triplicate for each primer pair for each tissue and time point analyzed.

Standard curves for each primer pair in each tissue were prepared and used to calculate the absolute quantity (copy number) of the transcript in each sample. Criteria for the standard curve included:  $R^2$  values > 0.99 (range was 0.992-0.999), baseline threshold levels < 0.020 (calculated as 10X the background noise level based on cycles 5 – 9), and amplification efficiencies between 80 – 120 % (actual values were 80 – 108%). The invariant ribosomal protein L8 was used as a reference gene to normalize the copy number results to account for technical variations [18].

## **ER Sequencing**

Confirmatory QPCR reactions for the 4 ERs and L8 were run on 1.5% agarose gels to visualize products for each gene target. The other 4 immune-related genes have been cloned and sequenced previously as a part of a Rainbow trout microarray [73]. Bands of the correct size were excised, placed in 1.5 ml tubes, and ‘freeze and squeezed’ (3 cycles of 10 min in a dry-ice 100% ethanol bath, followed by 10 min in a 37°C water bath) to re-release the target amplicon, which was cloned in duplicate using

Topo®-TA 2.1 cloning kits (Invitrogen, Streetsville, ON, Canada). The DNA Technologies Laboratory at the National Research Council Plant Biotechnology Institute (Saskatoon, SK, Canada) sequenced the duplicate clones in both directions utilizing Sanger methodology and an ABI 3730xl sequencer (Applied Biosystems Inc., Streetsville, ON, Canada). Comparison of HKL and PBL ER sequences to National Centre for Biotechnology Information nucleotide collection database occurred using BLASTN (Basic Local Alignment Search Tool-Nucleotide), specifically with Rainbow trout (organism #8022).

### ***Statistics***

Data for hematocrit and the immunological assays were first normalized to percent of control for each day in order to minimize any potential differences due to sampling day. Grubbs' test was used to detect and remove outliers (GraphPad Software Inc., La Jolla, CA, USA). Data were tested for equal variance and normal distribution of residuals before being analyzed using the fit model platform in JMP (v8.0.2, SAS Institute Inc, Cary, NC, USA) using a 2-way ANOVA, followed by post-hoc Bonferroni (non-QPCR data) or student t-tests (QPCR data) when differences were detected ( $p < 0.05$ ). For the four immune genes evaluated using QPCR, Bartlett's test found that variances were not equal so Welch's t-test ( $p < 0.05$ ) was used to compare control to E2-treated fish within each time point.

## Results

### ***Basic Water Quality Parameters and Water 17 $\beta$ -Estradiol Concentration***

Water quality parameters were monitored regularly throughout the experiment and were: pH =  $7.7 \pm 0.3$ , dissolved oxygen =  $9.7 \pm 0.9$  mg/L, conductivity =  $479 \pm 9$   $\mu$ S and temperature =  $15.0 \pm 1.0$  °C. The average water concentration of E2 measured in the control treatment tanks was not detectable ( $< 0.005$   $\mu$ g/L in all samples) and in the E2 treatment tanks was  $0.47 \pm 0.02$   $\mu$ g/L (mean  $\pm$  SEM) with a range of 0.40 – 0.51  $\mu$ g/L. The concentration of E2 in the treatment tanks during the recovery period was also non-detectable ( $< 0.005$   $\mu$ g/L in all samples).

### ***Plasma 17 $\beta$ -Estradiol Concentration and Hematocrit***

Plasma E2 concentrations were significantly elevated in E2-treated fish after both 2 and 7 d of treatment compared to control fish (Fig. 2-1), but returned to control levels following the 14 d depuration period (Fig. 2-1). Following E2 treatment, hematocrit was decreased on both day 2 and day 7, with recovery to control levels by 14 d post-exposure (Fig. 2-2).

### ***Phagocytosis and Respiratory Burst Activity***

Phagocytosis and respiratory burst assays were conducted on HKL, which had a viability of  $95.3 \pm 0.3\%$  (mean  $\pm$  SEM) at the beginning of the assay. The percent of

phagocytic cells, phagocytic capacity and respiratory burst were all unaffected by E2 treatment (data provided as supplemental information, Table S2-1).

### ***B and T cell Proliferation***

B and T cell proliferation were assessed using PBL, which had a viability of 98.0 ± 0.2% (mean ± SEM) at the beginning of the assay. LPS-stimulated B cell proliferation was transiently increased on day 2 of E2 exposure, with proliferation returning to control levels by day 7 of exposure (Fig. 2-3). Concanavalin A-stimulated T cell proliferation decreased, with effects being significant by day 7 of E2 exposure and recovery to control levels by 14 d post-exposure (Fig. 2-4).

### ***Evaluation of Gene Expression***

#### **QPCR Analysis**

All four ER transcripts were detected in both HKL and PBL, with varying levels of expression (Fig. 2-5). The PBL expressed greater levels of both ER $\alpha$ 1 and ER $\alpha$ 2 compared to HKL, while HKL expressed greater levels of both ER $\beta$ 1 and ER $\beta$ 2. The most prevalent transcript for both HKL and PBL was ER $\alpha$ 1 and the least expressed transcript was ER $\beta$ 1 (HKL = ER $\alpha$ 1 > ER $\beta$ 2 > ER $\alpha$ 2 > ER $\beta$ 1; PBL = ER $\alpha$ 1 > ER $\alpha$ 2 > ER $\beta$ 2 > ER $\beta$ 1).

Exposure to E2 had no effect on the mRNA abundance of ER $\beta$ 1 or ER $\beta$ 2 in either HKL or PBL (Supplemental Data, Table S2-2). However, treatment with E2 for 7 d

resulted in up-regulation of ER $\alpha$ 1 expression in both HKL (Fig. 2-6A) and PBL (Fig. 2-6B). ER $\alpha$ 2 in HKL was also up-regulated after 7 d of E2 treatment, but was down-regulated in PBL after both 2 and 7 d of exposure. Transcript abundance for all ERs returned to control levels following the 14 d depuration period.

For the four immune-related genes analyzed by QPCR (TNF $\alpha$ , IL1 $\beta$ , CXCR4, CCR7), all were present in the HKL and PBL at the day 2 and day 7 sampling time points. However, there were no differences in expression between the E2-exposed fish and the control fish (Supplemental data, Table S2-3).

### **ER Sequencing**

BLASTN results of ER $\alpha$ 1 and ER $\alpha$ 2 from HKL and PBL (Table 2-2) each had 99% similarity to the sequences reported by Nagler *et al.* [45], including the original ER $\alpha$ 1 sequence GenBank IDs: AJ242740 and AJ242741 [47] and ER $\alpha$ 2 sequence GenBank ID: DQ177438 [45]. For both HKL and PBL, the same mismatch was present in ER $\alpha$ 1 and ER $\alpha$ 2. The mismatch in ER $\alpha$ 1 (nucleotide A instead of G) leads to an amino acid change (aspartate (D) instead of glycine (G)). The mismatch in ER $\alpha$ 2 did not alter the amino acid sequence. ER $\beta$ 1 and ER $\beta$ 2 from HKL and PBL each had 100% similarity to the sequences reported by Nagler *et al.* [45], including ER $\beta$ 1 sequence GenBank ID: DQ177439, and ER $\beta$ 2 sequence GenBank ID: DQ248229 (Table 2-2).

## Discussion

There is widespread recognition of the relationship between the endocrine system and the function of the immune system in vertebrates. Field studies have found that reproductively mature and spawning fish are more susceptible to disease, particularly ectoparasites [13,51]. During reproductive periods, circulating concentrations of a number of hormones are elevated, including cortisol, androgens and estrogens. All of these hormones have the potential to exert immunomodulatory effects, such that it remains difficult to establish which hormone, if any, are involved in increased disease susceptibility in wild populations. While the effects of cortisol on overall immunocompetence is recognized [39,52], the relationship between circulating E2 concentrations and immune system function is not as well established and existing literature regarding effects at the cellular level is often contradictory. Although the E2 in this study was exogenously delivered through aquatic exposure to juvenile Rainbow trout, the resulting plasma E2 concentration (7.5 – 10.8 ng/mL) was within the range observed in mature adult females (1.5 to 45 ng/mL reported by Tyler *et al.* [66]), suggesting that the effects observed in this study may have physiological relevance.

Often used as a general health indicator in fish, hematocrit declined following E2 treatment, with effects beginning within 2 d of exposure and recovery to control levels following 14 d of depuration. Similar decreases in hematocrit have been reported by others in studies of salmon [24] and trout [56]. A decrease in hematocrit can reflect a decrease in red blood cell volume or an increase in plasma volume (hemodilution). While there is no information on the effects of E2 on erythropoiesis in fish, estradiol benzoate slowed the maturation of new red blood cells in channel catfish [21]. Elevated E2 in birds caused hemodilution by increasing plasma osmolality due to production of



yolk precursors in the liver and their release to the blood, resulting in water movement into the blood to compensate [67]. Plasma vitellogenin levels are often increased in fish following E2 exposure [65], suggesting that, as in birds, hemodilution may also occur in fish.

E2 had no effect on phagocytosis or respiratory burst. There are mixed findings in the literature regarding the effects of E2 on these immune function endpoints. E2 has generally been implicated in reducing phagocytosis, both *in vitro* and *in vivo* [69,70,75]. However, this may be species-specific since one *in vitro* study of the effects of hormones on phagocytosis found that E2 was suppressive in one species of fish (common carp, *Cyprinus carpio*), but not another (tilapia, *Oreochromis* spp.) [34]. The effects on respiratory burst activity also vary, with E2 shown to stimulate [64], suppress [70] or have no effect [69,75] on respiratory burst activity. Some of the disparate observations may be due to differences in exposure protocols and pharmacokinetics among studies.

Few studies have examined the influence of E2 on B and T cell function in fish. In the present study, LPS-stimulated lymphocyte proliferation was found to be transiently elevated after 2 d of E2 exposure, while Con A-stimulated lymphocyte proliferation was decreased after 7 d. Similarly, following E2 treatment, Wang and Belosovic [74] found that stimulating lymphocytes with phorbol myristate acetate and a calcium ionophore (which would activate both B and T cells) suppressed lymphocyte proliferation in goldfish, both *in vitro* and *in vivo*. Cook [11] found that E2 increased B cell proliferation in Rainbow trout, however a more recent *in vitro* study found that E2 exposure for 4 d suppressed B cell proliferation at high concentrations (> 272 µg/L) but had no effect at lower concentrations [61] similar to the plasma concentrations measured in the current study. While the findings by Shelley *et al.* [61] initially seem contradictory, it is plausible

that E2 exposure may not cause effects after 4 d since in the current study B cell proliferation returned to control levels sometime between 2 and 7 d of E2 exposure.

The detection and characterization of ER transcripts in isolated leukocytes was performed in this study using QPCR (Fig. 2-5) and represents the first report of the full complement of Rainbow trout ER mRNA in purified HKL. In terms of transcript abundance, our results for HKL ( $ER\alpha1 > ER\beta2 > ER\alpha2 > ER\beta1$ ) differ from those reported for the Rainbow trout anterior kidney tissue ER mRNA abundance ( $ER\beta2 > ER\alpha1 > ER\beta1 > ER\alpha2$ ) [45], likely due to their use of whole tissue versus our use of isolated leukocytes. For PBL, all ER transcripts were detected, which differs from Iwanowicz and Ottinger [27], who found only  $ER\alpha$  in channel catfish PBL. The abundance of ER transcripts in our study ( $ER\alpha1 > ER\alpha2 > ER\beta2 > ER\beta1$ ) is similar to that previously published for Rainbow trout, except  $ER\alpha1$  and  $ER\alpha2$  are reversed ( $ER\alpha2 > ER\alpha1 > ER\beta2 > ER\beta1$ ) [61], which may be related to developmental stage. In the present study with Rainbow trout PBL,  $ER\alpha$  was most abundant, consistent with both the previous studies of PBL in Rainbow trout [61] and humans [50].

Previously it was shown that E2 did not differentially regulate ER transcript abundance in Rainbow trout PBL [61]. However, that study was conducted *in vitro* with PBL derived from immature adult fish, used much higher E2 concentrations (~ 2 mg/L) and only assessed ER transcript abundance in activated (LPS-stimulated) PBL after 4 d of E2 exposure. In our current *in vivo* study, exposure to E2 had direct effects on HKL and PBL  $ER\alpha1$  and  $ER\alpha2$  gene expression but not  $ER\beta1$  or  $ER\beta2$  (Fig. 2-6). There have been reports of ER transcript induction in liver tissue by xenoestrogens [19,25], including E2 treatment [4].

While differential regulation of ER $\alpha$  (but not ER $\beta$ ) by E2 treatment has been reported in human macrophages [44], this is the first report of E2 treatment altering ER $\alpha$ 1 or ER $\alpha$ 2 gene expression in isolated fish leukocytes. This supports the hypothesis that leukocytes expressing ERs are likely direct targets of estrogens [27]. Therefore, E2-induced immunomodulation observed at the cellular level may be mediated via differential expression of ER $\alpha$ 1 and ER $\alpha$ 2. The ERs are nuclear transcription factors that alter the expression of genes containing estrogen-responsive elements (EREs), which have now been found in upstream regions of some cytokine genes [27]. Several microarray studies of E2-treated fish have also demonstrated that a number of immune system-related genes are differentially regulated [4,72], and it can be hypothesized that E2-mediated changes in ER $\alpha$  mRNA abundance may underlie at least some of those alterations in gene expression. A future study utilizing an ER antagonist would be useful in determining whether the effects of E2 are a result of direct interaction with the ER or are caused indirectly due to alterations outside of the immune system that lead to immunomodulation.

In an effort to further evaluate the genomic basis of immune function alteration in a teleost, QPCR was used to detect and characterize target genes that have been shown in previous studies involving E2 exposure [28,35]. Jin *et al.* [28] showed that, while TNF $\alpha$  and IL1 $\beta$  were up-regulated by exposure to 12.5  $\mu$ g/L E2 in newly hatched zebrafish (*Danio rerio*), exposure to 0.5  $\mu$ g/L E2 had no significant induction, which was comparable to the present results in Rainbow trout exposed to 0.47  $\mu$ g/L E2 (Table S2-3). Similarly, Liarte *et al.* [35] found that TNF $\alpha$  and IL-1 $\beta$  were up-regulated following *in vitro* exposures to 5  $\mu$ g/L E2 in gilthead seabream (*Sparus aurata*) macrophages; however, this concentration is 10 fold higher than the E2 exposure in this study. Jin *et*

*al.* [28] also evaluated chemokine receptor gene target(s) similar to CXCR4 and CCR7, and found no significant increases in transcript abundance following E2 treatment, which is consistent with the present results. Future studies would benefit from examining the responses of these and other immune-related genes during an activated immune response (e.g. following mitogen stimulation).

Some of the variation in outcomes, particularly in cellular assays, following E2 exposures in fish can be attributed to differences in assay methodologies and sensitivities, as well as small sample sizes [5]. Differences among studies that may constrain comparisons also include toxicokinetic factors (uptake/absorption, distribution, metabolism, elimination) and the exposure route (*in vitro*, or *in vivo* via injection/implants, feeding or waterborne exposures) which influence the amount and form of E2 available to exert effects [27,54]. This could be overcome to some extent by comparing results based on plasma E2 concentrations; however many of the available studies do not report this. In addition, various species of fish have different ER repertoires which may be differentially expressed among cell or tissue types, resulting in the potential for an inter-species divergence of responses [27,45]. Life stage also influences which ERs are expressed at least in some tissues [16] and, although not currently demonstrated in leukocytes, this differential expression might also explain the variability in responses to E2 in the immune system. In addition, as reported here, E2 itself has been found to auto-regulate the transcription of ERs [7,38], which may change responses over time as long as E2 levels remain elevated.

Widespread reports of the effects of endocrine disrupting chemicals (EDCs) on fish have highlighted concerns about the estrogenic or anti-androgenic properties of municipal and/or industrial effluents. Much of the research has examined reproductive

outcomes, with reports of reproductive abnormalities in wild fish populations living in polluted environments downstream of sewage treatment plant [3,29] and pulp mill effluent discharges [40]. Laboratory studies have further confirmed the field findings (reviewed by [15]) and uncovered some of the mechanisms underlying these effects [2,17,63]. However, the presence of ER transcripts in leukocytes and the effects of E2 on immune function suggest that the immune system may also be a vulnerable target for estrogenic EDCs [9,42]. Several recent studies have noted immunotoxicity in fish following exposure to xenoestrogens, such as those in sewage effluent [36,55] or nonylphenol [23,61]. While the estrogens and xenoestrogens present in sewage or other effluents may be degraded in the environment, their continual discharge may result in their 'pseudo-persistence' in impacted aquatic habitats [14] and the effects of these low-level, chronic exposures on immune system function are largely unknown. In addition, short-term pulse exposures to EDCs, such as in the current study, have been linked to long-term immunotoxicological impairment when the exposures occur during sensitive, hormone-mediated developmental stages [43]. Additional research is needed in this area to fully understand the implications of EDC- and hormone-mediated immunomodulation in fish.

## **Conclusion**

This study indicates that estrogen, comparable to physiological levels in adult fish, can affect components of the immune response in teleosts. The full complement of ERs is reported for the first time in purified HKLs and confirms previous reports of ER transcript presence in Rainbow trout PBLs. Similar to what is observed in other tissues,

the ERs in both HKL and PBL are susceptible to auto-regulation by E2, which may contribute to the changes observed in immune function at the cellular level. Additionally, the altered endpoints measured here during E2 exposure appear to be transient, with cellular function and changes in ER transcript levels returning to normal levels within 2 weeks of the withdrawal of E2 exposure. Future research on the immunological effects of estrogens and xenoestrogens (EDCs) will help to clarify the implication of real-world exposures in aquatic environments influenced by urban and industrial activities.

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## Tables

**Table 2-1 Genes and primer sequences used in QPCR analysis.**

Gene Name	Gene Bank ID	Forward Primer	Reverse Primer
Tumor necrosis factor $\alpha$ (TNF $\alpha$ )	AJ277604	TGCGATGACACCTGAAGA	CCTGGCTGTAAACGAAGA
Interleukin 1 $\beta$ (IL1 $\beta$ )	AJ223954	AGGGTCTGGATCTGGAGGT	TCTGCTGGCTGATGGACTT
Chemokine (C-X-C motif) receptor 4 (CXCR4)	AJ001039	CAGCTCGGAAGAATCAGG	GTGGCAAACACTATGTCAGG
Chemokine (C-C motif) receptor 7 (CCR7)	AJ003159	ATCTGCCGTTAGGGCTTTC	TGCGGTTGTTCTGGTTACTC
Ribosomal protein L8 (L8)	AY957563	CAGGGGACAGAGAAAAGGTG	TGAGCTTTCTTGCCACAG

**Table 2-2 Blast results (# of nucleotides matching and % match) of estrogen receptor (ER) sequences isolated from head kidney leukocytes (HKL) and peripheral blood leukocytes (PBL).**

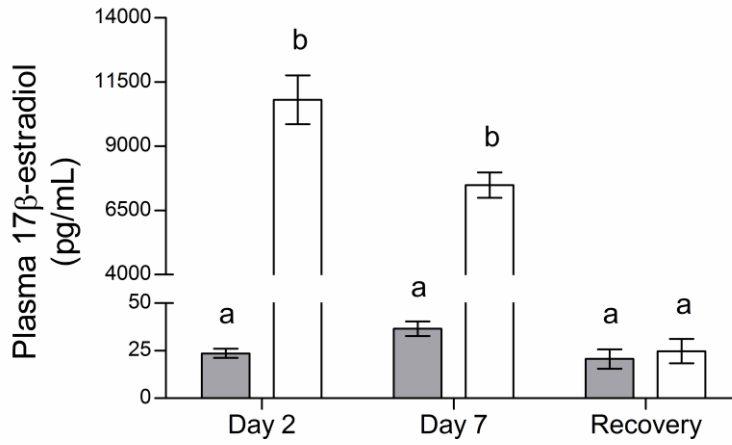
Target Gene	# of Nucleotides Matching	% Match	Accession # of Top Hit
HKL			
ER $\alpha$ 1	106 / 107 <sup>a</sup>	99	AJ242741 <sup>c</sup>
ER $\alpha$ 2	82 / 83 <sup>b</sup>	99	DQ177439
ER $\beta$ 1	133 / 133	100	DQ177439
ER $\beta$ 2	125 / 125	100	DQ248229
PBL			
ER $\alpha$ 1	106 / 107 <sup>a</sup>	99	AJ242741 <sup>c</sup>
ER $\alpha$ 2	82 / 83 <sup>b</sup>	99	DQ177439
ER $\beta$ 1	133 / 133	100	DQ177439
ER $\beta$ 2	125 / 125	100	DQ248229

<sup>a</sup> Mismatch at position #101 (G) compared to AJ242741; HKL or PBL sequence has (A).

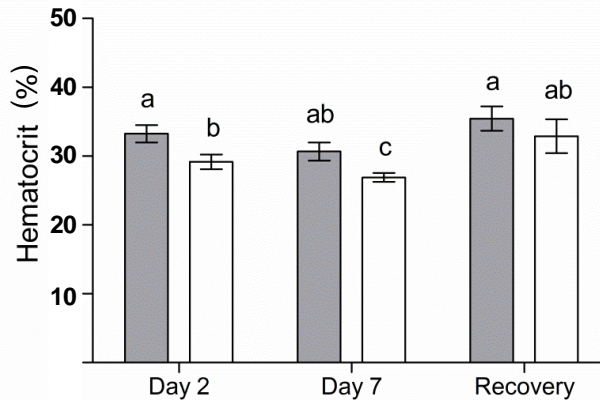
<sup>b</sup> Mismatch at position #1485 (T) compared to DQ177438; HKL or PBL sequence has (C).

<sup>c</sup> Same E-score (5e-50) as AF099079 and AJ242740.

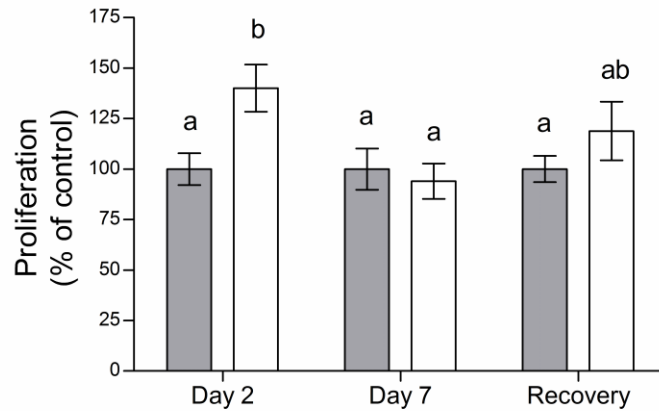
## Figures



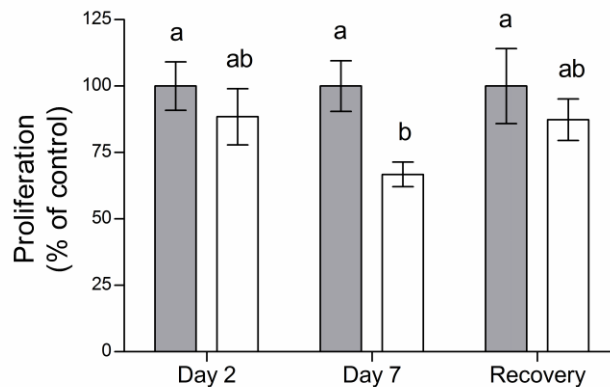
**Figure 2-1** Plasma 17β-estradiol (E2) was elevated in juvenile Rainbow trout treated with 0.47 μg/L E2 (white bars) after both 2 and 7 d of exposure, but returned to control (grey bars) levels after a 14 d recovery period. Data are shown as mean ± SEM, and n = 9 – 15. Differences between treatments over time were detected using a 2-way ANOVA ( $p < 0.05$ ), and treatments not connected by the same letter are significantly different.



**Figure 2-2** Hematocrit was decreased in juvenile Rainbow trout after both 2 and 7 d of exposure to 0.47 μg/L 17β-estradiol (white bars), but returned to control (grey bars) levels after a 14 d recovery period. Data are shown as mean ± SEM, and n = 9 – 15. Differences between treatments over time were detected using a 2-way ANOVA ( $p < 0.05$ ), and treatments not connected by the same letter are significantly different.

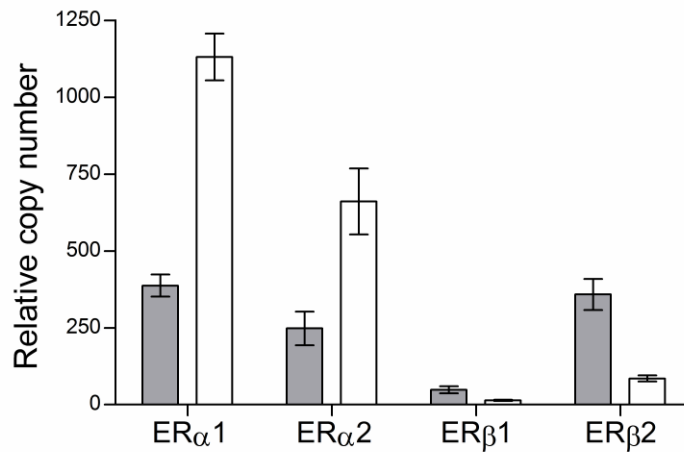


**Figure 2-3** *Lipopolysaccharide-stimulated lymphocyte proliferation was increased in peripheral blood leukocytes from juvenile Rainbow trout following 2 d of exposure to 0.47 µg/L 17β-estradiol (white bars), but returned to control (grey bars) levels by 7 d of treatment and after a 14 d recovery period. Data were normalized to percent of control on each day and are expressed in percent as mean ± SEM, and n = 10 – 15. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments not connected by the same letter are significantly different.*

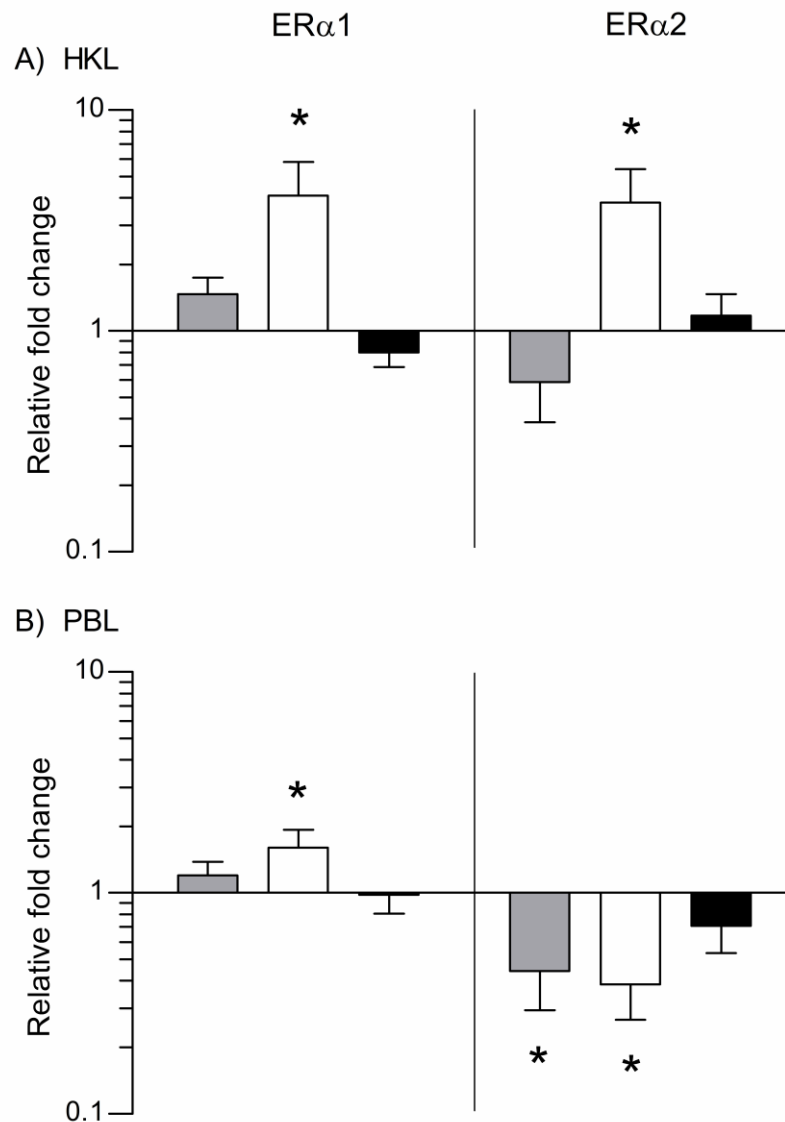


**Figure 2-4** *Concanavalin A-stimulated lymphocyte proliferation was decreased in peripheral blood leukocytes from juvenile Rainbow trout following 7 d of exposure to 0.47 µg/L 17β-estradiol (white bars), but returned to control (grey bars) levels after a 14 d recovery period. Data were normalized to percent of control on each day and are expressed in percent as mean ± SEM, and n = 9 – 15. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments not connected by the same letter are significantly different.*





**Figure 2-5** *Four forms of estrogen receptor (ER; ER $\alpha$ 1, ER $\alpha$ 2, ER $\beta$ 1 and ER $\beta$ 2) mRNA were detected in both head kidney leukocytes (grey bars) and peripheral blood leukocytes (white bars) isolated from juvenile Rainbow trout. Data are shown as mean L8-normalized copy number  $\pm$  SEM and n = 28 – 34 individual control group fish for each gene in each tissue.*



**Figure 2-6** Abundance of estrogen receptors (ER) ER $\alpha$ 1 and ER $\alpha$ 2 mRNA was differentially regulated in A) head kidney leukocytes (HKL) and B) peripheral blood leukocytes (PBL) isolated from juvenile Rainbow trout exposed to 0.47  $\mu$ g/L 17 $\beta$ -estradiol (E2) for either 2 d (grey bars) or 7 d (white bars), but returned to control levels after a 14 d recovery period (black bars). Data are shown as fold change  $\pm$  SEM in L8-normalized ER transcript abundance relative to the control (where a fold change of 1 = same as control), and  $n = 8 - 11$ . Differences between treatments over time were detected using a 2-way ANOVA ( $p < 0.05$ ), and treatments that were significantly different than the control are indicated by \*.

## Supplementary Information

**Table S2-1** *Percent phagocytic cells, phagocytic capacity and respiratory burst were unaffected by 17 $\beta$ -estradiol (E2) treatment. Fish were treated with 0.47  $\mu$ g/L 17 $\beta$ -estradiol for up to 7 days, followed by a 14 day recovery period. Data was normalized to percent of control activity on each day and is expressed in percent as mean  $\pm$  SEM. Data was analyzed using a 2-way ANOVA, and no significant differences were detected ( $p > 0.05$ ).*

Assay	Day 2		Day 7		Recovery	
	control	E2	control	E2	control	E2
Phagocytic Cells (%)	100.0 $\pm$ 15.4	84.1 $\pm$ 16.3	100.0 $\pm$ 15.2	102.7 $\pm$ 18.9	100.0 $\pm$ 11.3	76.5 $\pm$ 4.9
(n)	11	11	15	15	8	6
Phagocytic Capacity	100.0 $\pm$ 0.9	100.8 $\pm$ 0.4	100.0 $\pm$ 0.8	101.3 $\pm$ 0.7	100 $\pm$ 0.7	99.3 $\pm$ 1.3
(n)	11	11	15	15	8	6
Respiratory Burst	100.0 $\pm$ 13.9	95.7 $\pm$ 11.2	100.0 $\pm$ 17.3	96.6 $\pm$ 18.9	100.0 $\pm$ 9.4	79.4 $\pm$ 7.2
(n)	10	11	12	10	6	5

**Table S2-2** *Transcript abundance of estrogen receptor  $\beta$ 1 (ER $\beta$ 1) and ER $\beta$ 2 in head kidney (HKL) or peripheral blood leukocytes (PBL) was unaffected by 17 $\beta$ -estradiol (E2) treatment. Fish were treated with 0.47  $\mu$ g/L 17 $\beta$ -estradiol for up to 7 days, followed by a 14 day recovery period. Data is shown as L8-normalized copy number  $\pm$  SEM, and n = 10-15 per treatment group per time point. Data was analyzed using a 2-way ANOVA and no significant differences were detected ( $p > 0.05$ ).*

	Day 2		Day 7		Recovery	
	control	E2	control	E2	control	E2
<b>HK</b>						
ER $\beta$ 1	69.9 $\pm$ 25.8	63.7 $\pm$ 19.7	49.6 $\pm$ 18.3	76.2 $\pm$ 24.7	21.9 $\pm$ 3.0	71.5 $\pm$ 26.8
ER $\beta$ 2	410.5 $\pm$ 110.4	205.2 $\pm$ 67.9	222.0 $\pm$ 30.5	540.4 $\pm$ 206.6	447.1 $\pm$ 85.4	636.8 $\pm$ 151.7
<b>PBL</b>						
ER $\beta$ 1	14.8 $\pm$ 3.9	13.2 $\pm$ 2.9	15.3 $\pm$ 4.6	8.3 $\pm$ 1.8	12.3 $\pm$ 2.4	40.6 $\pm$ 22.9
ER $\beta$ 2	95.9 $\pm$ 20.6	84.3 $\pm$ 6.6	80.1 $\pm$ 17.1	72.9 $\pm$ 11.9	78.5 $\pm$ 13.3	42.3 $\pm$ 10.6

**Table S2-3** *Transcription of four immune system-related genes in Rainbow trout head kidney (HKL) or peripheral blood leukocytes (PBL) is unaffected by exposure to 0.47 µg/L 17β-estradiol for 2 or 7 d. Data is expressed as mean L8 normalized copy number ± SEM and n = 10 – 12.*

Gene	Tissue	Day	Control	17β-Estradiol	Fold change	p-value (Welch's t-test)
Tumor necrosis factor α (TNFα)	HKL	2	4836 ± 777	8006 ± 2903	1.7	0.30
		7	2811 ± 496	2740 ± 575	-1.0	0.93
	PBL	2	1974 ± 913	867 ± 156	-2.3	0.24
		7	1348 ± 559	363 ± 57	-3.7	0.15
Interleukin 1β (IL1β)	HKL	2	17372 ± 4828	23830 ± 7975	1.4	0.50
		7	20549 ± 6717	36402 ± 12985	1.8	0.28
	PBL	2	6773 ± 3711	1645 ± 390	-4.1	0.18
		7	1642 ± 896	191 ± 32	-8.6	0.19
Chemokine (C-X-C motif) receptor 4 (CXCR4)	HKL	2	3051046 ± 338752	2799715 ± 891581	-1.1	0.79
		7	1713142 ± 320363	1431502 ± 345010	-1.2	0.56
	PBL	2	768585 ± 156309	549871 ± 51704	-1.4	0.20
		7	941930 ± 356926	287109 ± 64291	-3.3	0.16
Chemokine (C-C motif) receptor 7 (CCR7)	HKL	2	527504 ± 113871	555262 ± 175119	1.1	0.90
		7	383434 ± 121355	591784 ± 142973	1.5	0.28
	PBL	2	267209 ± 57420	200520 ± 24923	-1.3	0.30
		7	186753 ± 35947	139350 ± 24818	-1.3	0.34

## Chapter 3.

# **Alterations in Rainbow trout (*Oncorhynchus mykiss*) burst swimming following exposure to xenoestrogens**

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## Abstract

Adverse impacts of xenoestrogen exposure have been widely documented in fish, yet there has been limited investigation of xenoestrogen effects on swimming ability. In this study, juvenile Rainbow trout (*Oncorhynchus mykiss*) were exposed to 17 $\beta$ -estradiol (E2; natural estrogen hormone) and 17 $\alpha$ -ethinylestradiol (EE2; a potent synthetic estrogen hormone) to evaluate potential effects on burst swimming performance. Burst swimming speed ( $U_{burst}$ ) was reduced in fish exposed for 4 d to 0.5 and 1  $\mu\text{g L}^{-1}$  E2 and 0.5 and 1  $\mu\text{g L}^{-1}$  EE2 in each of six successive burst swimming assays. Using linear regression analysis,  $\Delta U_{burst}$  was not different between control and xenoestrogen-exposed groups and mean  $\Delta U_{burst}$  for all groups was -2.1% per swim. A practice swim (2 d prior to exposure) in control fish elevated  $U_{burst}$  but this training effect was not evident in the 1  $\mu\text{g L}^{-1}$  EE2-exposed fish. Several potential estrogen-mediated mechanisms for  $U_{burst}$  reductions were investigated, including effects on metabolic products, osmoregulation, and the oxygen carrying capacity of the blood. Prior to burst swimming trials, fish exposed to E2 and EE2 for 4 d had significantly reduced numbers of erythrocytes and lower plasma glucose concentrations. After six repeated burst swimming trials, plasma glucose, lactate and creatinine concentrations were not significantly different between any treatment group, however, plasma Cl<sup>-</sup> concentrations were significantly reduced in E2 and EE2-treated fish. In summary, E2 and EE2 exposure altered oxygen carrying capacity (erythrocytes) and an osmoregulatory-related parameter ([Cl<sup>-</sup>]), effects that may underlie reductions in burst swimming speed, which will have implications for fish fitness in the wild.

## **Keywords**

Rainbow trout;  $17\beta$ -estradiol;  $17\alpha$ -ethinylestradiol; burst swimming; osmoregulation; erythrocytes



## Introduction

Estrogenic substances are frequently detected and reported in aquatic habitats worldwide. When applied exogenously, these xenoestrogens (varying widely in their structure and origin, including pesticides, plasticizers, pharmaceuticals, and hormones), all induce estrogenic-responses in vertebrates similar to  $17\beta$ -estradiol (E2). Both natural (E2) and synthetic ( $17\alpha$ -ethinylestradiol (EE2); the birth control pill hormone) estrogens have been measured in surface waters at concentrations ranging from 0.5 – 831 ng L<sup>-1</sup> (Kolpin *et al.*, 2002; Kolodziej *et al.*, 2004; Sousa *et al.*, 2010; Jeffries *et al.*, 2010; Lee *et al.*, 2011; Barber *et al.*, 2013). The main contributors of these hormones to the aquatic environment are human and animal wastes, since natural and synthetic estrogen hormones are excreted in urine and feces (Kolodziej *et al.*, 2004; Liu *et al.*, 2009). E2 and EE2 are environmental contaminants of significant concern due to their potent, bioactive nature and ability to cause adverse effects in many species of aquatic organisms. Since these hormones primarily relate to vertebrate reproduction, piscine endocrine and reproductive systems have been well-studied in relation to estrogenic contaminants. In this regard, effects found in fish include reduced embryonic survival, feminization of males, decreased egg fecundity, development of abnormal gonads, and altered sex-steroid levels (Folmar *et al.*, 1996; Jobling *et al.*, 1998; Batty & Lim, 1999; Kristensen *et al.*, 2005; Parrott & Blunt, 2005; Scott *et al.*, 2006; Brown *et al.*, 2007; Hyndman *et al.*, 2010).

In salmonids, xenoestrogens are known to adversely impact other physiological systems in juvenile and male fish in addition to the endocrine and reproductive systems, including growth, osmoregulation, the stress response, and the immune system (Jobling

*et al.*, 1996; Schultz *et al.*, 2003; Arsenault *et al.*, 2004; Lerner *et al.*, 2007; Wenger *et al.*, 2011; Hanson *et al.*, 2012; Shelley *et al.*, 2013). Others have shown xenoestrogen impacts on smoltification and seawater tolerance (Arsenault *et al.*, 2004; McCormick *et al.* 2005; Lerner *et al.*, 2007), as well as migration ability (Madsen *et al.*, 2004; Bangsgaard *et al.*, 2006). For example, an ecologically relevant reduction in locomotory performance in Atlantic salmon *Salmo salar* was exhibited as a delayed downstream migration and increased mortality post-injection with the xenoestrogens E2 and nonylphenol (Madsen *et al.*, 2004). These xenoestrogen-induced effects could lead to overall reductions in fish fitness and population-level impacts.

Activities that rely on optimal swimming performance include migration, spawning, maintaining position in a current, prey capture (Nikl & Farrell, 1993), and predator avoidance (Reidy *et al.*, 1995). Swim performance has typically been a measurement used to compare exercise capacities, physiological differences and ecological requirements between fish species (Jain *et al.*, 1998; Kieffer, 2000). Since optimal swimming integrates several physiological systems, effects on any subcomponent may result in alterations of performance. Therefore, swim performance has been used to detect the sublethal effects of environmental stressors, temperature, and xenobiotics (Jain *et al.*, 1998; Jain & Farrell, 2003; MacNutt *et al.*, 2004; Tierney *et al.*, 2007; Farrell, 2008; Goertzen *et al.*, 2011).

Swimming activity is usually classified as sustained, prolonged or burst swimming (Jobling, 1992; Hammer, 1995; Kieffer, 2000). Sustained swimming is moderated swimming behaviour that lasts longer than 200 min (such as low-speed foraging, holding position and long-distance migration), uses aerobic metabolism and does not result in muscular fatigue. Prolonged swimming is of shorter duration (20 s to

200 min) but occurs at accelerated swimming speeds that utilize aerobic metabolism followed by anaerobic metabolism until fatigue occurs. Burst swimming involves rapid, sprint activity (for predator avoidance or advancement over rapids) that lasts for 20 s or less; it is fuelled by anaerobic metabolism and results in fatigue of the fish. Several exercise protocols have been established to evaluate swim performance in fish (Hammer, 1995) usually measuring the swimming speed achieved in an interval of time (min to h) after experiencing steady or incremental water velocities. A measurement of maximum sustained swimming speed is  $U_{\max}$  (Sepulveda & Dickson, 2000). A more common measure of prolonged swimming is 'critical swimming speed' or  $U_{\text{crit}}$  (Brauner *et al.*, 1994; Reidy *et al.*, 1995; MacNutt *et al.*, 2004; Kennedy & Farrell, 2006; Farrell, 2008; Li *et al.*, 2009). Burst swimming activity tests or constant acceleration protocols measure 'burst swimming speed' or  $U_{\text{burst}}$  (Reidy *et al.*, 1995; Pedersen *et al.*, 2004; Farrell, 2008; Pedersen *et al.*, 2008; Nadeau *et al.*, 2009).

It has been suggested that predator avoidance is an ability that may be impacted by xenoestrogen exposure (Madsen *et al.*, 2004). Since reductions in swimming ability directly relate to increased predation (Handeland *et al.*, 1996),  $U_{\text{burst}}$  and the ability of fish to repeatedly perform burst swimming were investigated following exposure to xenoestrogens. Repeat testing appears to be a useful tool for identifying contaminant effects in fish; for example, caged fish held in contaminated environments could not repeat initial swim performances, whereas those held in uncontaminated waters were able to swim well in repeat trials (McKenzie *et al.*, 2007). In other studies, fish swim performance has been evaluated using repeated swimming protocols measuring  $U_{\text{crit}}$  or  $U_{\text{burst}}$  with at least two separate swim trials using the same fish (Farrell *et al.*, 1998; Jain *et al.*, 1998; McFarlane & McDonald 2002; Pedersen *et al.*, 2004; McKenzie *et al.*, 2007;

Farrell, 2008; Pedersen *et al.*, 2008). Repeat swimming evaluates the recovery ability of fish (restoration of energy stores and removal of lactate) from the first test to perform subsequent bouts of further exercise (Kieffer, 2000). Since escaping from predators and advancing past a sequence of rapids can involve multiple burst swimming episodes (Kieffer, 2000), repeat swimming ability is an ecologically relevant endpoint and an evaluation of physiological fitness.

In this study, the burst swimming ability of juvenile Rainbow trout *Oncorhynchus mykiss* was evaluated following exposure to 0, 0.5 and 1  $\mu\text{g L}^{-1}$  of E2 or EE2, which are the two most potent estrogen hormones found in the environment (Thorpe *et al.*, 2003; Van den Belt *et al.*, 2004). To begin to identify potential mechanisms of any swimming effects, parameters relating to swimming performance were evaluated pre- and post-exercise and after a recovery period.

## **Materials and Methods**

### ***Chemicals***

Ferric nitrate, mercuric thiocyanate, 17 $\beta$ -estradiol (E2; CAS 50-28-2) and 17 $\alpha$ -ethinylestradiol (EE2; CAS 57-63-6) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Creatinine, L-lactate and glucose assay kits were supplied by Cayman Chemical Company (Ann Arbor, Michigan, USA). All other supplies were purchased from VWR Canada (Mississauga, ON, Canada), unless otherwise noted.

## ***Fish Source and Maintenance***

Juvenile Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) of mass  $17.2 \pm 0.3$  g and length  $11.5 \pm 0.1$  cm (mean  $\pm$  SEM) were obtained from Miracle Springs Hatchery (Mission, BC, Canada). Fish were maintained in a 400 L tank supplied with aerated, flow-through, dechlorinated municipal water (dissolved oxygen > 90%,  $\sim 10$  mg L<sup>-1</sup> CaCO<sub>3</sub> hardness, pH  $\sim 7.0$ ,  $8 \pm 1$  °C) for at least 2 weeks prior to experiments. Trout were fed to satiation daily, but prior to use, fish were fasted for approximately 20 h. Experimental protocols and fish use were approved by the Simon Fraser University Animal Care Committee (Burnaby, BC, Canada).

## ***Exposures and Swim Tests***

Three experiments were conducted to evaluate the burst swimming performance of Rainbow trout exposed to estrogen hormones (overview in Supplementary information Figure S3-1). Fish (n = 12 per tank) were exposed to 0.001% (v/v) ethanol solvent control, or 0.5 or 1  $\mu\text{g L}^{-1}$  of E2 or EE2 (dissolved in ethanol, final concentration of 0.001%) in duplicate fiberglass tanks containing 180 L of aerated, dechlorinated municipal water.

In the first experiment, fish were exposed to ethanol solvent control, 1  $\mu\text{g L}^{-1}$  E2 or 1  $\mu\text{g L}^{-1}$  EE2 for 4 d and then transferred to the swim tunnel (Mackinnon & Farrell, 1992; Goulding *et al.*, 2013). Fish were swum in batches of 12 or 24 (i.e. duplicate tanks of 12 treated fish were combined) and each treatment had 4 swim tests each. After a 20 min acclimation period with a water velocity of 10 cm s<sup>-1</sup>, fish were subjected to a burst swimming speed test (Farrell, 2008; Nendick *et al.*, 2009). The acceleration speed was

in  $5 \text{ cm s}^{-1}$  increments for each 1 min interval (Farrell, 2008). Water in the swim tunnel was maintained at a constant level and flow standard curves (relating voltages to water velocities) were prepared daily using a current meter (Forestry Suppliers Inc., Jackson, MI, USA). The burst acceleration swimming speed test was initiated at  $20 \text{ cm s}^{-1}$  and terminated after all fish were exhausted, which was typically around  $100 \text{ cm s}^{-1}$ . A fish was considered exhausted when it was inactive on the rear net for at least several seconds and unable to resume swimming after mechanical prodding. Exhausted fish were immediately removed and, under mild anaesthesia ( $50 \text{ mg L}^{-1}$  buffered MS-222), mass ( $M$ ) and fork length ( $L_F$ ) measurements were taken. This same protocol was used in all swim tests described in this study. Fish were returned to their tank and exposure was continued. After 24 h, fish were tested again in the burst swimming protocol as above and then monitored for mortalities for 48 h. Six random fish per treatment were sampled for plasma after the first and second swim tests. To obtain plasma, blood from caudal puncture was collected in heparinized glass capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN, USA) and then centrifuged for 3 min in a microcapillary centrifuge (International Equipment Company, Chattanooga, TN, USA). Hematocrit was determined and then separated plasma placed in 1.5 ml centrifuge tubes and stored on dry ice until transfer to  $-80 \text{ }^\circ\text{C}$  for further analysis.

In the second experiment, the recovery of fish was evaluated by using successive burst swimming trials. Fish were exposed to the same treatments as in the first experiment, with the addition of lower concentrations to evaluate differences in concentration responses:  $0.5 \text{ } \mu\text{g L}^{-1}$  E2 and  $0.5 \text{ } \mu\text{g L}^{-1}$  EE2. Fish were swum in batches of 24 (i.e. 2 tanks of 12 exposed fish were combined) and each treatment had 2 swim tests. The burst swimming protocol used above was repeated six times in an 8 h period,

with a 40 min recovery period between each swim (Jain & Farrell, 2003). After each swim test, measurements ( $M$  and  $L_F$ ) were taken under mild anaesthesia, and after the sixth swim test, fish were placed into clean, aerated dechlorinated water and monitored for 48 h. Whole blood was collected and stored in Dacie's fluid (1% formaldehyde, 0.1 M trisodium citrate, 0.1% (w v<sup>-1</sup>) Brilliant cresyl blue and deionized water up to 1 L filtered prior to use to remove large debris (Blaxhall & Daisley, 1973)) in microfuge tubes at a ratio of 1:50 (Blaxhall & Daisley, 1973) from 6 random fish exposed for 4 d to each treatment: -ve CON, 0.5 and 1 µg L<sup>-1</sup> E2, and 0.5 and 1 µg L<sup>-1</sup> EE2. Six random fish per treatment were sampled for plasma, as described in the first experiment, after 4 d of treatment (pre-exercise), six swim tests, and 48 h of recovery.

To provide perspective on the magnitude of any alterations in metabolic parameters found altered by xenoestrogen exposure and/or burst swim tests, a positive control group (+ve CON) was used that was subjected to a general 'handling' stress. Solvent control fish exposed for 4 d to 0.001% (v/v) ethanol were stressed following the protocol of Vijayan & Moon (1992). In brief, fish were chased around a tank with a net, and occasionally caught and scooped into a net, for a 3-min duration. These fish were not subjected to a burst swimming protocol. Six random fish from this group were sampled for plasma, as described above.

In a third experiment, the impact of a prior swim on  $U_{burst}$  was determined to evaluate the possibility of a 'training effect' (i.e. fish learning from their first swim; Kieffer, 2000) and any impacts on training caused by EE2, the most potent xenoestrogen used in this study. One set of fish ( $n = 72$ ) were subject to the burst swimming protocol two days prior to dosing and then returned to their holding tank and allowed to recover with feeding. Fish were then divided into 3 groups: (1) d0 = fish ( $n = 24$ ) that were not

exposed but tested in a burst swimming protocol, (2) d4 CON = fish (n = 24) exposed only to 0.001% ethanol as a solvent control for 4 d and then tested in a burst swimming protocol, and (3) d4 EE2 = fish (n = 24) exposed to 1  $\mu\text{g L}^{-1}$  EE2 for 4 d and then tested in a burst swimming protocol.

### ***Plasma Assays***

Plasma samples were analyzed in duplicate for glucose, creatinine, L-lactate, VTG protein (Biosense Laboratories, Abraxis, Warminster, PA) and cortisol (Neogen Corp., Lansing, MI) concentrations as per the respective manufacturer's protocols. Concentrations that were below the detection limit of a kit were set to the detection limit for statistical purposes.

Chloride ion ( $\text{Cl}^-$ ) concentrations were measured in triplicate using a method adapted from Ewing et al. (1994). Working solutions of 0.69 mM ferric nitrate  $\text{Fe}(\text{NO}_3)_3$  (prepared in deionized water) and 0.34 mM mercuric thiocyanate  $\text{Hg}(\text{SCN})_2$  (prepared in methanol) were prepared separately each day in glass vials and combined just prior to plating. Some precipitate settled and pipetting of it was avoided. Plasma samples were diluted 100-fold in deionized water in microfuge tubes. In 96-well plates, each well contained 100  $\mu\text{l}$  of ferric nitrate/mercury isothiocyanate working solution and 50  $\mu\text{l}$  of standard or diluted sample. Standards were made from 2 mM sodium chloride and ranged from 0 to 2 mM. Plates were incubated at room temperature for 5 min while ferric thiocyanate formed, and then read at a wavelength of 480 nm.



All plates were read on a Bio-tek® PowerWave Reader (Bio-Tek, Winooski, VT) at the appropriate wavelength as specified in the manufacturers' protocols. Standard curves were run concurrently on each plate with samples ( $R^2 > 0.95$ ) and CVs between duplicates  $< 20\%$  were considered acceptable.

### ***Blood Cell Counts***

Whole blood samples were enumerated within 3 weeks using a Neubauer haemocytometer after further dilution (up to 6-fold) in Dacie's fluid. For red blood cells (RBCs), a total area of  $0.2 \text{ mm}^2$  was counted. For white blood cells (WBCs), a total area of  $4 \text{ mm}^2$  was counted. Cell counting was performed by following generic standards (Bastidas, 2013).

### ***Calculations and Statistics***

Condition factor ( $K$ ) was calculated using the equation:  $M \text{ (g)} \times 100 \times (L_F \text{ (cm)})^{-3}$ . All statistical analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., La Jolla, CA), with statistical significance denoted by  $P < 0.05$ . Data were tested for normal distribution using Shapiro-Wilk's test for normality. If data were normally distributed, then the analysis for treatment effects was performed using a one-way ANOVA followed by Bonferroni's multiple test correction. If data were non-parametric, or too few points were available for an analysis for normality with Shapiro-Wilk, Kruskal-Wallis tests were used followed by Dunn's post-hoc test. Within each experiment, data from all swim tests for a treatment were combined for

statistical analyses. For experiment 2, a two way ANOVA was used to evaluate treatment and burst trial (parameters in Supplementary Table S3-2). To determine exact *P* values, some parameters were analyzed by Mann-Whitney U tests (described where appropriate). Hematocrit data were log transformed before statistical analysis. All data presented are mean ± standard error of the mean (SEM), with the number of fish (*n*) noted.

## **Results**

### ***Mortality and K***

Fish appeared to be generally healthy in all exposure groups and there were no mortalities during the 48 h monitoring period post-burst swimming tests. Condition factor (*K*) was not significantly different between exposure groups at any timepoint (Supplementary Table S3-1).

### ***VTG Protein***

Mean VTG protein levels for the –ve CON exposure group were  $1.1 \times 10^{-4}$  mg mL<sup>-1</sup> ( $6.3 \times 10^{-4}$  μM), while 0.5 and 1 μg L<sup>-1</sup> E2 treatment groups had 0.88 mg mL<sup>-1</sup> (4.9 μM) and 1.89 mg mL<sup>-1</sup> (10.5 μM), respectively, and 0.5 and 1 μg L<sup>-1</sup> EE2 treatments had 1.94 mg mL<sup>-1</sup> (10.8 μM) and 3.05 mg mL<sup>-1</sup> (17.0 μM), respectively. VTG concentrations were significantly higher in 1 μg L<sup>-1</sup> E2 (*P* < 0.05), 0.5 μg L<sup>-1</sup> EE2 (*P* < 0.05), and 1 μg L<sup>-1</sup> EE2 (*P* < 0.001) exposure groups compared to the –ve CON treatment (Figure 3-1A).

## **Cortisol**

Within any group, there was a large range of plasma cortisol concentrations (Figure 3-1B). Mean (and range) cortisol concentrations for the –ve CON exposure group was 4.6 ng mL<sup>-1</sup> (2.1 – 9.4), while 0.5 and 1 µg L<sup>-1</sup> E2 exposure groups were 5.8 ng mL<sup>-1</sup> (1.7 – 13.6) and 34.4 ng mL<sup>-1</sup> (3.9 – 80.0), respectively, and the 0.5 and 1 µg L<sup>-1</sup> EE2 exposure groups were 13.8 ng mL<sup>-1</sup> (2.4 – 31.4) and 18.3 ng mL<sup>-1</sup> (2.3 – 76.6), respectively. The +ve CON had a mean (and range) plasma cortisol concentration of 53.5 ng mL<sup>-1</sup> (27.8 – 105.0). The only significantly elevated plasma cortisol level was the +ve CON compared to the –ve CON ( $P < 0.01$ ).

## **Swimming Performance, Metabolic Products and Chloride Ions**

In the first experiment, burst swimming performance (measured as  $U_{burst}$ ) was significantly reduced in fish from both the 1 µg L<sup>-1</sup> E2 and 1 µg L<sup>-1</sup> EE2 treatment groups in the second swim ( $P < 0.01$  and  $P < 0.05$ , respectively; Table 3-1A). To evaluate a relationship between metabolism and  $U_{burst}$ , metabolic products were measured, including plasma glucose (Table 3-1B), creatinine (Table 3-1C) and lactate (Table 3-1D) concentrations. None of these parameters in exposed fish were significantly different from fish in the control group ( $P > 0.05$ ). Plasma Cl<sup>-</sup> concentrations were significantly different between the –ve CON and 1 µg L<sup>-1</sup> E2 and 1 µg L<sup>-1</sup> EE2 exposure groups after the first swim 1 ( $P < 0.001$  for both xenoestrogen treatments (Table 3-1E)) but not after the second swim ( $P = 0.54$ ).

In the second experiment, Rainbow trout were subjected to six repeat burst swimming trials, enabling further investigation into reductions in swim performance seen in the first experiment. In all swim trials, xenoestrogen exposure groups had significantly lower burst swimming speeds ( $U_{burst}$ ) compared to the -ve CON treatment (treatment  $P < 0.0001$  and swim  $P < 0.0001$ ; Figure 3-2). The order of reduced  $U_{burst}$  by treatment was:  $0.5 \mu\text{g L}^{-1} \text{E2} < 1 \mu\text{g L}^{-1} \text{E2} \leq 0.5 \mu\text{g L}^{-1} \text{EE2} < 1 \mu\text{g L}^{-1} \text{EE2}$ . The  $1 \mu\text{g L}^{-1} \text{E2}$  and  $0.5 \mu\text{g L}^{-1} \text{EE2}$  groups were approximately the same in this respect, indicating that EE2 was approximately twice as potent as E2 in its effects on swim performance. The decreasing trend over six burst swimming trials was best described by linear regression for each treatment ( $R^2$  of 0.95 – 0.99, Figure 3-2). The slopes of the lines for the xenoestrogen groups did not differ significantly from the -ve CON group ( $P > 0.05$ ); therefore, the ability to perform burst swimming assays repeatedly was not different between the -ve CON and xenoestrogen treatments and the initial reduction in  $U_{burst}$  at the first swim was most important in defining the effects of xenoestrogen exposure. The mean decrease in  $U_{burst}$  per swim was determined to be  $0.14 \text{ bl s}^{-1}$  or -2.1% using data from all treatments.

The potential contributions of metabolism-related parameters to the burst swimming speed declines (Figure 3-2) found in the second experiment were investigated by measuring plasma glucose, creatinine and lactate at various timepoints (Table 3-2). After 4 d of exposure, but before burst swimming trials, plasma glucose concentrations were significantly lower in fish from all xenoestrogen exposed groups compared to the -ve CON (Table 3-2A). After 6 consecutive swims or a 48 h recovery period, glucose concentrations were no longer significantly different in any xenoestrogen treatment compared to -ve CON. Plasma creatinine and lactate concentrations were not significantly different between xenoestrogen treatment groups and the -ve CON at any

timepoint in the second experiment (Table 3-2B and 3-2C, respectively). Plasma Cl<sup>-</sup> concentrations were significantly lower in fish from all xenoestrogen treatment groups compared to the -ve CON after six burst swimming assays and after the 48 h recovery period (Table 3-2D).

### ***Training Effect***

The third experiment evaluated whether an initial swim trial (timepoint called d-2) affected subsequent swims in an unexposed group (d0), a control group (d4 CON), and an EE2-exposed group (d4 EE2) (Figure 3-3). There was a significant increase in  $U_{burst}$  between the baseline swim (d-2) and d0 group, as well as d4 CON. There was, however, no significant difference between the d-2 group and 4 d exposed  $1 \mu\text{g L}^{-1}$  EE2 (d4 EE2), thus, the training effect seen in the other two groups (d0 and d4 CON) was absent in the EE2 group.

### ***Hematocrit and Blood Cell Counts***

Oxygen carrying capacity may be a reason why burst swimming speed was affected in xenoestrogen exposed fish, and therefore, hematocrit and RBC counts were evaluated. Hematocrit did not change in most treatments, except for the  $1 \mu\text{g L}^{-1}$  EE2 group, which was significantly reduced after treatment for 4 d (pre-exercise), and in the  $1 \mu\text{g L}^{-1}$  E2 group, which was significantly reduced after six burst swimming tests in the second experiment (Table 3-3). Compared to controls, 4 d of xenoestrogen treatment (pre-exercise) reduced RBC counts by 49% and 48% in  $0.5 \mu\text{g L}^{-1}$  E2 and  $1 \mu\text{g L}^{-1}$  E2

(respectively), and by 39% and 52% in 0.5  $\mu\text{g L}^{-1}$  EE2 and 1  $\mu\text{g L}^{-1}$  EE2, respectively (Table 3-3). Similar to RBCs, WBC counts were also decreased in all groups (exposed for 4 d) by 66%, 65%, 64% and 77% in 0.5  $\mu\text{g L}^{-1}$  E2, 1  $\mu\text{g L}^{-1}$  E2, 0.5  $\mu\text{g L}^{-1}$  EE2 and 1  $\mu\text{g L}^{-1}$  EE2 treatments, respectively (Table 3-3).

## Discussion

### ***Burst Swimming Ability***

Evidence regarding the impact of xenoestrogens on salmonid swimming abilities has mainly come from studies showing impaired smoltification or migration following exposure (Fairchild *et al.*, 1999; Madsen *et al.*, 2004; McCormick *et al.*, 2005; Bangsgaard *et al.*, 2006). The present study examined the direct effects of estrogen hormones (E2 and EE2) on the burst swimming ability of juvenile Rainbow trout; at 0.5 and 1  $\mu\text{g L}^{-1}$  E2 or EE2 showed reduced burst swimming ability compared to controls. The speculation that increased predation may have affected E2- and nonylphenol-injected Atlantic salmon arrival at a downstream migration point (Madsen *et al.*, 2004) is supported by the present results, since predator escape often involves burst swimming (Kieffer, 2000). Initial  $U_{\text{burst}}$  values were consistently lower in xenoestrogen-exposed fish compared to -ve CON fish, decreases that appear to be reflective of the two xenoestrogen potencies since EE2 is a more potent estrogen than E2 (Thorpe *et al.*, 2003; Van den Belt *et al.*, 2004; Brian *et al.*, 2005). The potency of xenoestrogens is primarily determined by comparisons to E2 and assigned a potency value  $> 1$  if more potent, or  $< 1$  if less potent. Reported potencies for EE2 (11-33) using VTG induction as

the endpoint (Thorpe *et al.*, 2003; Van den Belt *et al.*, 2004; Brian *et al.*, 2005) indicate that  $U_{burst}$  is a less sensitive endpoint for potency assessment (EE2 was 2-fold more effective at reducing  $U_{burst}$ ), which is not surprising considering it is a locomotory endpoint and not a directly-related reproductive or endocrine endpoint.

The ability to consistently perform repeated burst swimming tests was not significantly different between groups and the mean % decrease per swim across all groups was -2.1%. Others have reported reductions between -7 and -17% for repeated swims in untreated fish (MacNutt *et al.*, 2004; Pedersen *et al.*, 2008). The lack of a significant reduction in repeat swim performances of xenoestrogen-exposed fish suggest that initial reductions in  $U_{burst}$  were the most important difference between treated and control fish and the factor that caused these reductions was present prior to or during the first round of exhaustive exercise.

### ***Metabolic Products***

The mechanism of xenoestrogen impact on swimming ability could relate to altered energy homeostasis before or during exercise. Xenoestrogens may potentially divert resources for the precocious production of estrogen-responsive parameters (Vijayan *et al.*, 2001), thereby affecting the mobilization or utilization of energy for swimming. Gene expression studies support this concept since estrogens are known to alter mitochondrial function and energy metabolism (Filby *et al.*, 2007; Hook *et al.*, 2007; Santos *et al.*, 2007; Williams *et al.*, 2007). Plasma lactate, creatinine and glucose concentrations were measured to determine if alterations in their levels indicated altered energetics due to xenoestrogen exposure.

Lactate production in the muscle results in increased plasma lactate concentrations post-exercise, which return to baseline levels as hepatic lactate dehydrogenase converts it to pyruvate (Milligan, 1996; Kieffer, 2000). In gluconeogenesis, pyruvate is converted to glucose, a process that can be depressed with E2 treatment (Washburn *et al.*, 1993). If E2 and EE2 fish in the present study had an impaired ability to process lactate into glucose, it would likely have altered performance after lactate build-up from the first swim and in subsequent swim trials. However, plasma lactate concentrations were not different between treatments and impaired swimming performance was evident during the first swim (not only in later swim trials).

Creatinine is produced in muscle from the high-energy substrate phosphocreatine. Following its exit from muscle, it remains in plasma until nephritic elimination occurs. Creatinine concentrations were 2 to 3 fold higher (43 to 81  $\mu\text{M}$ ) pre-exercise and 48 h post-exercise compared to those reported as resting levels in Rainbow trout (24 to 27  $\mu\text{M}$  [Manera & Britti, 2006]). Creatinine concentrations were not significantly altered by xenoestrogen treatment pre- or post-exercise (Tables 3-1 and 3-2).

Plasma glucose concentrations were significantly lower in E2 and EE2 treated fish pre-exercise. These results are similar to Rainbow trout with E2 implants (Washburn *et al.*, 1993) and may be due to estrogen-related energy demands from increased protein synthesis (Vijayan *et al.*, 2001) or inhibition of gluconeogenesis (Washburn *et al.*, 1993). Post-exercise and after 48 h recovery, plasma glucose concentrations were not different between controls and treatment groups making it unlikely that this was a factor in reduced swimming speeds.



## **Osmoregulation**

Xenoestrogens are known to disrupt osmoregulation in fish (Vijayan *et al.*, 2001; McCormick *et al.*, 2005; Lerner *et al.*, 2012). In this study, plasma Cl<sup>-</sup> concentrations were not altered by xenoestrogen treatment pre-exercise (Table 3-1E), similar to the gilthead sea bream *Sparus auratus* injected with E2 (Carrera *et al.*, 2007). Exercise can also disrupt osmoregulation by altering ion and water balances in fish (Nendick *et al.*, 2009). In the -ve CON group, plasma Cl<sup>-</sup> concentrations increased significantly between pre- and post-exercise (Table 3-2), which has been reported elsewhere (Nendick *et al.*, 2009). However, in E2 and EE2-treated fish, plasma Cl<sup>-</sup> concentrations were significantly reduced after the first swim in the first experiment and after six repeated trials in the second experiment (Tables 3-1 and 3-2). Exercise typically results in a net water loss or decrease in plasma volume (Brauner *et al.*, 1994; Nendick *et al.*, 2009). Plasma volume and hematocrit are inversely proportional (Brauner *et al.*, 1994), but since hematocrit did not decrease post-exercise, the significantly reduced Cl<sup>-</sup> concentrations were not a result of altered plasma volume and potentially due to a net ion loss. Since plasma Cl<sup>-</sup> concentrations were not significantly altered pre-exercise in E2 and EE2-exposed fish, xenoestrogen effects occurred only with exercise and continued into the 48 h recovery period. Xenoestrogens, therefore, impaired the ability of fish to maintain osmoregulatory homeostasis, which may have adversely impacted their swimming performance.

## ***Stress and Cortisol Hormone***

Plasma cortisol concentrations are elevated in response to stress, including post-exhaustive exercise, and can play a role in altered swim performance if induced prior to swim trials (Milligan, 1996). In fish, plasma cortisol concentrations in the 30 to 300 ng mL<sup>-1</sup> range are typically found in response to acute stressors. The highest cortisol concentration was found in the E2-exposed group (34.5 ng mL<sup>-1</sup>); however, this was not a significant increase, results similar to others (Fuzzen *et al.*, 2011; Osachoff *et al.*, 2013). Cortisol concentrations in net-chased fish from the +ve CON group were significantly elevated (53.5 ng mL<sup>-1</sup>) and approximately 40% of the values reported under similar conditions (Vijayan & Moon 1992), indicating stressed fish.

## ***Oxygen Carrying Capacity***

The representative measurement of blood oxygen carrying capacity in this study was RBC counts, which were significantly reduced in all xenoestrogen treatments pre-exercise. Reduced RBC counts were also found in Chinook salmon *Oncorhynchus tshawytscha* exposed to 1 µg L<sup>-1</sup> E2 for 7 d (unpublished results; Chapter 7). Reduced concentrations of RBCs in xenoestrogen-exposed fish would impact oxygen delivery to tissues during exercise and thus be a limiting factor in swim performance since oxygen consumption increases with exercise (Milligan, 1996). Xenoestrogen-induced reductions in circulating RBCs may be caused by a redistribution or elimination of RBCs. Carp *Cyprinus carpio* injected with EE2 exhibited altered RBC distributions, with increases in immature RBCs, lowered total RBC counts, and reduced hemoglobin content and mean corpuscular hemoglobin concentration, all of which would affect blood oxygen carrying

capacity (Schwaiger *et al.*, 2000). These results suggest that xenoestrogens possibly enhance mature RBC turnover due to increased RBC fragility (Schwaiger *et al.*, 2000), resulting in an increased splenic RBC content. In this regard, hypertrophic spleens have been seen with xenoestrogen (E2 or EE2) treatment in fish (Herman & Kincaid, 1988; Schwaiger *et al.*, 2000). Since the spleen contains estrogen receptors, E2 (or xenoestrogens) likely directly modulates its function in fish (Nagler *et al.*, 2007; Shved *et al.*, 2009).

## ***Training***

Fish have the ability either to learn or to become physiologically conditioned for improved swim performance as a result of training, which can affect repeat swimming performance (Hammer, 1995; Davison, 1997). There are different protocols used depending on the intent of the training. One type involves lengthy swim training for physiological conditioning, for example, Rainbow trout that were trained for 18 h per d for a month had improved maximum cardiac performance and increased muscular aerobic enzymes (Farrell *et al.*, 1991). Another type of training involves conducting a single practice swim prior to a study commencing to teach the fish how to swim in an apparatus (Jain *et al.*, 1997; Lee *et al.*, 2003; Farrell, 2008). This type of swim training was evaluated in this study by performing a practice swim with fish before treatment. Control fish increased swimming performance 6 d after one practice swim, while fish exposed to  $1 \mu\text{g L}^{-1}$  EE2 did not respond similarly (Figure 3-3). Either the EE2 group did not learn from the practice swim or, as described above, their stamina was reduced by the xenoestrogen as a result of either reduced RBC levels or an osmoregulatory

perturbation. This impairment has ecological relevance for groups of wild fish that move into xenoestrogen-contaminated waters that are then challenged by an action that requires burst swimming, such as escaping predators, passing quickly through shallow waters, advancing through turbulent waters, or capturing prey (Reidy *et al.*, 1995; Kieffer, 2000). These fish may not perform well and that may have consequences for their survival.

### ***Environmental Relevance***

The waterborne concentrations of E2 and EE2 used in this study were generally, but not always, higher than those found in many environmental situations, even considering that a xenoestrogenic mixture is additive in terms of its estrogenic effects (Brian *et al.*, 2005; Thorpe *et al.*, 2006). The 0.5 µg L<sup>-1</sup> E2 or EE2 treatments were approximately 0.6 to 6 fold higher than reported US surface water levels (maximums of 0.831 µg L<sup>-1</sup> EE2 and 0.093 µg L<sup>-1</sup> E2 by Kolpin *et al.* (2002)) and about 500 fold higher than Albertan (Canada) river maximum E2 concentrations (Jeffries *et al.*, 2010), British river concentrations (Williams *et al.*, 2012), surface waters in an US agricultural region (Kolodziej *et al.*, 2004), and Minnesota (US) streams (Lee *et al.*, 2011). However, in the environment, water concentrations do not account for all xenoestrogens available to fish from other sources, which can include particulates, and interaction with sediment or absorption from food can be important routes of exposure. For example, E2 can bioaccumulate in biofilm (Writer *et al.*, 2011) and fish to a limited degree (Maunder *et al.*, 2007). Thus, it is possible that fish in some environments, particularly those immediately downstream of sewage treatment plants where pseudo-persistent conditions exist, can

experience sufficient amount of estrogenicity such that swim performance is affected. However, further research is needed to determine the no observed effect and lowest observed effect concentrations of E2 and EE2, and other xenoestrogens, on fish burst swimming ability. In the future, it may be advantageous to correlate reductions in swimming performance with plasma xenoestrogen concentrations instead of waterborne concentrations so that results are transferrable to feral fish; plasma xenoestrogen concentrations reflect the availability of chemicals from all environmental sources (water, sediment and food).

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## Tables

**Table 3-1** *Burst swimming speed ( $U_{burst}$ ; Body lengths per second (Bl/s)) in Rainbow trout exposed to solvent control (-ve Control), 1  $\mu\text{g/L}$  17 $\beta$ -estradiol (E2) or 1  $\mu\text{g/L}$  17 $\alpha$ -ethinylestradiol (EE2) in the first experiment, including plasma glucose, chloride, L-lactate and creatinine concentrations for each treatment. Results shown as mean  $\pm$  SEM ( $n = 39-54$  for burst swimming;  $n = 6$  for plasma assays), with significant results in bold and denoted by: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .*

Treatment	Post- $U_{burst}$ Swim #1	Post- $U_{burst}$ Swim #2
(A).	$U_{burst}$ (Bl/s)	
-ve Control	7.53 $\pm$ 0.14	7.66 $\pm$ 0.16
1 $\mu\text{g/L}$ E2	7.36 $\pm$ 0.13	<b>7.06 <math>\pm</math> 0.12 **</b>
1 $\mu\text{g/L}$ EE2	7.36 $\pm$ 0.11	<b>7.10 <math>\pm</math> 0.17 *</b>
(B).	Plasma Glucose Concentration (mM)	
-ve Control	34.8 $\pm$ 2.0	42.7 $\pm$ 2.3
1 $\mu\text{g/L}$ E2	29.8 $\pm$ 1.2	52.9 $\pm$ 2.5
1 $\mu\text{g/L}$ EE2	33.0 $\pm$ 2.2	38.9 $\pm$ 3.2
(C).	Plasma Creatinine Concentration ( $\mu\text{M}$ )	
-ve Control	39.9 $\pm$ 8.4	42.8 $\pm$ 1.2
1 $\mu\text{g/L}$ E2	51.6 $\pm$ 5.7	37.6 $\pm$ 2.9
1 $\mu\text{g/L}$ EE2	67.3 $\pm$ 12.0	37.9 $\pm$ 3.1
(D).	Plasma L-Lactate Concentration (mM)	
-ve Control	7.7 $\pm$ 0.7	5.3 $\pm$ 0.1
1 $\mu\text{g/L}$ E2	8.6 $\pm$ 0.5	5.5 $\pm$ 0.1
1 $\mu\text{g/L}$ EE2	8.0 $\pm$ 0.6	5.5 $\pm$ 0.1
(E).	Plasma Chloride Concentration (mM)	
-ve Control	119.3 $\pm$ 1.1	129.8 $\pm$ 2.3
1 $\mu\text{g/L}$ E2	<b>98.3 <math>\pm</math> 1.5 ***</b>	120.9 $\pm$ 5.4
1 $\mu\text{g/L}$ EE2	<b>105.5 <math>\pm</math> 2.2 ***</b>	121.3 $\pm$ 9.7

**Table 3-2 Plasma glucose, chloride, L-lactate and creatinine concentrations for Rainbow trout exposed to solvent control (-ve Control), 0.5 and 1 µg/L 17β-estradiol (E2), 0.5 and 1 µg/L 17α-ethinylestradiol (EE2), and net-chased positive Control (+ve Control) in the second experiment. Results shown as mean ± SEM (n = 6), with significant results in bold and denoted by: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.**

Treatment	4 d Dosed	Post- $U_{burst}$ Swim #6	48 h Recovery
(A). Plasma Glucose Concentration (mM)			
-ve Control	30.2 ± 2.3	23.4 ± 3.5	37.3 ± 1.4
0.5 µg/L E2	<b>19.0 ± 1.2 ***</b>	27.7 ± 1.7	39.6 ± 0.9
1 µg/L E2	<b>20.5 ± 2.9 *</b>	27.4 ± 3.5	40.1 ± 3.9
0.5 µg/L EE2	22.7 ± 0.8 <sup>#</sup>	28.6 ± 2.0	34.3 ± 1.8
1 µg/L EE2	<b>21.2 ± 1.8 *</b>	17.8 ± 3.0	37.9 ± 2.7
+ve Control (net-chased)	<b>39.0 ± 3.7 *</b>		
(B). Plasma Creatinine Concentration (µM)			
Control	68.1 ± 10.0	83.8 ± 25.1	44.9 ± 3.7
0.5 µg/L E2	81.3 ± 6.9	145.5 ± 28.7	55.8 ± 1.2
1 µg/L E2	43.5 ± 8.8	114.0 ± 32.8	48.5 ± 5.0
0.5 µg/L EE2	55.2 ± 5.3	132.9 ± 42.5	61.3 ± 8.4
1 µg/L EE2	48.2 ± 6.5	106.5 ± 26.5	54.7 ± 4.0
+ve Control (net-chased)	54.9 ± 5.0		
(C). Plasma L-Lactate Concentration (mM)			
Control	3.3 ± 0.7	3.6 ± 0.8	1.6 ± 0.1
0.5 µg/L E2	2.6 ± 0.6	4.8 ± 1.1	1.7 ± 0.2
1 µg/L E2	3.8 ± 0.6	2.9 ± 0.3	1.5 ± 0.2
0.5 µg/L EE2	3.3 ± 0.6	3.1 ± 0.4	1.7 ± 0.4
1 µg/L EE2	2.2 ± 0.3	3.2 ± 0.4	1.8 ± 0.4
+ve Control (net-chased)	<b>11.3 ± 0.8 *</b>		
(D). Plasma Chloride Concentration (mM)			
Control	120.3 ± 2.2	133.3 ± 2.0 <sup>®</sup>	154.7 ± 6.3
0.5 µg/L E2	<b>144.1 ± 3.6 *</b>	<b>98.6 ± 1.7 **</b>	<b>134.6 ± 4.3 *</b>
1 µg/L E2	121.4 ± 3.7	<b>120.1 ± 2.4 *</b>	<b>105.0 ± 3.6 ***</b>
0.5 µg/L EE2	125.7 ± 4.1	<b>118.2 ± 3.6 *</b>	<b>121.5 ± 3.7 ***</b>
1 µg/L EE2	121.7 ± 2.7	<b>117.1 ± 1.6 **</b>	<b>127.2 ± 4.1 ***</b>
+ve Control (net-chased)	141.9 ± 15.2		

<sup>#</sup> Significant with Mann-Whitney U-test, p = 0.0022.

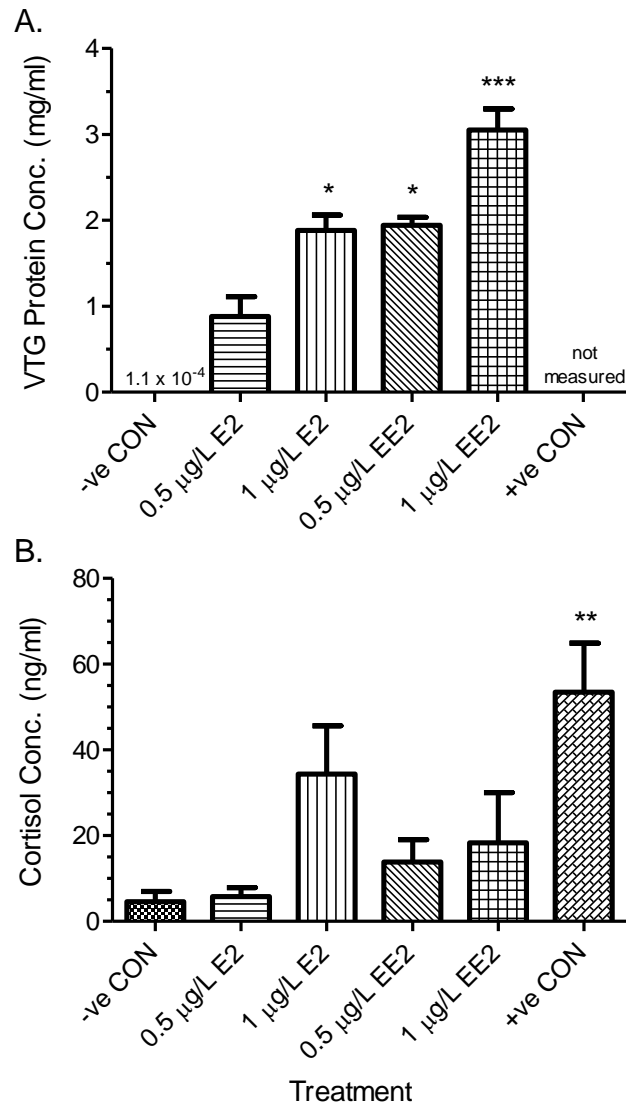
<sup>®</sup> Sig. different from 4 d dosed Control (Kruskal-Wallis followed by Dunn's post-hoc test, P < 0.01) .

**Table 3-3** White and red blood cell counts and hematocrit for Rainbow trout exposed to solvent control (-ve Control), 0.5 and 1 µg/L 17β-estradiol (E2), and 0.5 and 1 µg/L 17α-ethinylestradiol (EE2). Results shown as mean ± SEM (n = 6), with significant results in bold and denoted by: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

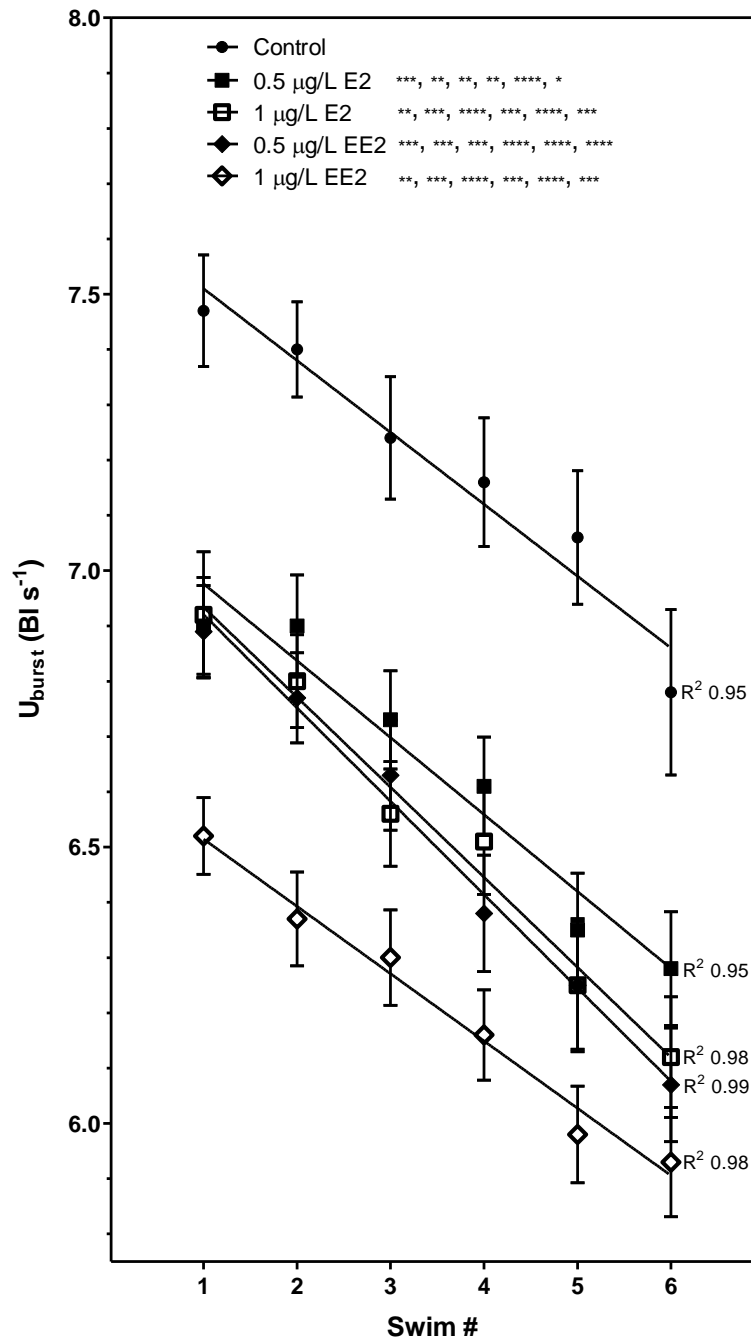
Treatment	White blood cells (cells/ml)	Red blood cells (cells/ml)	Hematocrit		
	4 d Dosed	4 d Dosed	4 d Dosed	Post- <i>U</i> <sub>burst</sub> Swim #6	48 h Recovery
-ve Control	2.01 x 10 <sup>7</sup> ± 1.9 x 10 <sup>6</sup>	6.98 x 10 <sup>8</sup> ± 7.2 x 10 <sup>7</sup>	35.8 ± 0.9 %	40.6 ± 1.6 %	29.8 ± 0.9 %
0.5 µg/L E2	<b>6.85 x 10<sup>6</sup> ± 8.1 x 10<sup>5</sup> *</b>	<b>3.58 x 10<sup>8</sup> ± 3.8 x 10<sup>7</sup> **</b>	33.2 ± 4.5 %	36.6 ± 1.8 %	32.4 ± 1.0 %
1 µg/L E2	<b>7.12 x 10<sup>6</sup> ± 1.6 x 10<sup>5</sup> *</b>	<b>3.60 x 10<sup>8</sup> ± 9.6 x 10<sup>7</sup> *</b>	32.2 ± 1.4 %	<b>34.0 ± 1.5 % *</b>	32.4 ± 0.6 %
0.5 µg/L EE2	<b>7.17 x 10<sup>6</sup> ± 9.4 x 10<sup>5</sup> *</b>	<b>4.28 x 10<sup>8</sup> ± 6.6 x 10<sup>7</sup> *</b>	34.6 ± 2.5 %	36.2 ± 1.1 %	32.9 ± 1.2 %
1 µg/L EE2	<b>4.63 x 10<sup>6</sup> ± 8.9 x 10<sup>5</sup> ***</b>	<b>3.35 x 10<sup>8</sup> ± 5.3 x 10<sup>7</sup> *</b>	<b>30.8 ± 1.3 % *</b>	37.2 ± 1.3 %	31.1 ± 1.2 %



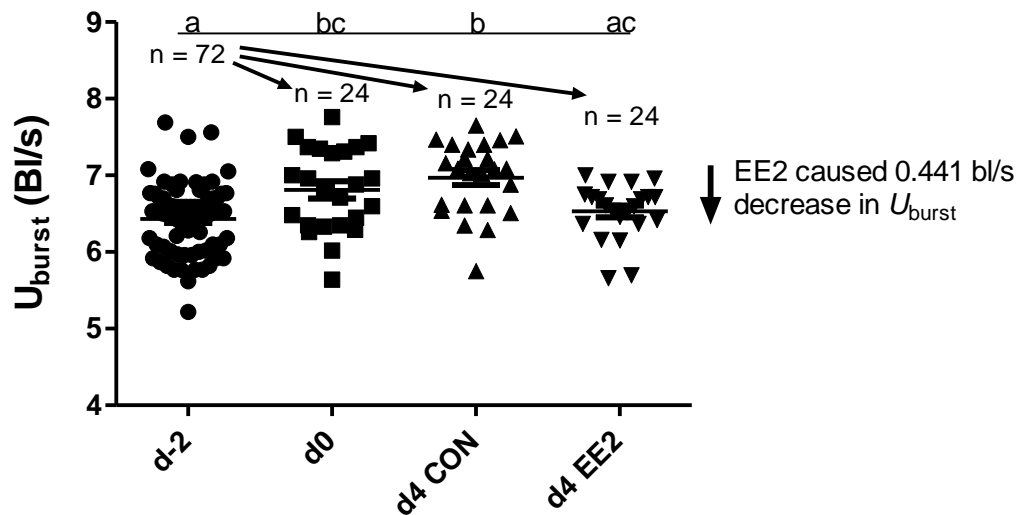
## Figures



**Figure 3-1** Plasma concentrations of (A.) vitellogenin (VTG) protein, and (B.) cortisol, for Rainbow trout exposed to solvent control (-ve Control), 0.5 and 1 µg/L E2, 0.5 and 1 µg/L EE2, and net-chased positive Control (+ve Control) in the second experiment. Results shown as mean ± SEM (n = 6); \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.



**Figure 3-2** Repeated burst swimming speed measured as  $U_{burst}$  (Body lengths per second ( $Bl s^{-1}$ )) in Rainbow trout exposed to solvent control (-ve Control),  $0.5$  and  $1 \mu g L^{-1}$  E2, and  $0.5$  and  $1 \mu g L^{-1}$  EE2. Significance listed in legend for -ve Control vs treatment for each swim as determined using 2-way ANOVA followed by Bonferroni's multiple test correction (\*  $p < 0.05$ ; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ;  $n = 40 - 48$ ).



**Figure 3-3** One set of Rainbow trout ( $n = 72$ ) were evaluated for their burst swimming speed ( $U_{burst}$  measured as Body lengths per second (Bl/s)) at day -2 (d-2) and then returned to their holding tank for recovery. Two days later, they were split into 3 groups: (1)  $n = 24$  fish were evaluated for  $U_{burst}$  (d0); (2)  $n = 24$  fish were treated for 4 d with ethanol solvent and then evaluated for  $U_{burst}$  (d4 CON); (3)  $n = 24$  fish were treated for 4 d with  $1 \mu\text{g/L}$   $17\alpha$ -ethinylestradiol (EE2) and then evaluated for  $U_{burst}$  (d4 EE2). Treatments that do not share a letter were significantly different ( $p < 0.05$ ) using Kruskal-Wallis followed by Dunn's post-hoc test.

## Supplementary Information

**Table S3-1** *Fish condition factors (K) for each treatment at each timepoint (mean  $\pm$  SEM; all comparisons made to Control within each timepoint were non-significant).*

Treatment	Condition Factor (K)				
	d0, n=12-24	4 d Dosed, n=6	Post-U <sub>max</sub> Swim #1, n=48	Post-U <sub>max</sub> Swim #6, n=48	48 h Recovery, n=6
Control	1.12 $\pm$ 0.03	1.11 $\pm$ 0.02	1.08 $\pm$ 0.01	1.07 $\pm$ 0.01	1.08 $\pm$ 0.03
0.5 $\mu$ g/L E2	1.16 $\pm$ 0.02	1.08 $\pm$ 0.08	1.05 $\pm$ 0.01	1.05 $\pm$ 0.02	1.07 $\pm$ 0.03
1 $\mu$ g/L E2	1.13 $\pm$ 0.01	1.12 $\pm$ 0.02	1.07 $\pm$ 0.01	1.02 $\pm$ 0.02	1.12 $\pm$ 0.03
0.5 $\mu$ g/L EE2	1.10 $\pm$ 0.02	1.10 $\pm$ 0.08	1.11 $\pm$ 0.01	1.08 $\pm$ 0.01	1.08 $\pm$ 0.02
1 $\mu$ g/L EE2	1.10 $\pm$ 0.01	1.09 $\pm$ 0.03	1.09 $\pm$ 0.01	1.06 $\pm$ 0.01	1.12 $\pm$ 0.03
+ve CON (net stressed)	1.12 $\pm$ 0.05	1.10 $\pm$ 0.02			

**Table S3-2** *Two-way ANOVA parameters for analysis of six repeat burst swimming assays in the second experiment.*


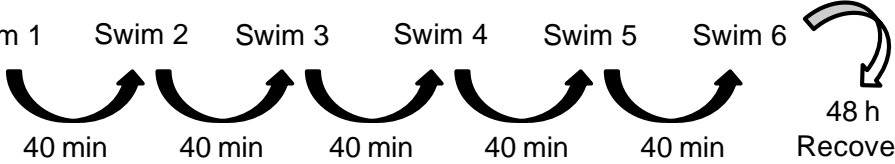
Source of Variation	% of total variation	P value
Interaction	0.38	0.9973
Treatment	17.23	< 0.0001
Swim	10.36	< 0.0001

Source of Variation	P value summary	Significant?
Interaction	ns	No
Treatment	****	Yes
Swim	****	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	20	2.952	0.1476	0.339
Treatment	4	134	33.51	76.98
Swim	5	80.56	16.11	37.01
Residual	1287	560.3	0.4353	

Experiment	Time-point (d)	n per replicate (# of replicates)	Swim test profile
1	4	12 or 24 (3)	Swim 1    Swim 2  24 h
2	4	24 (2)	Swim 1    Swim 2    Swim 3    Swim 4    Swim 5    Swim 6  40 min    40 min    40 min    40 min    40 min    40 min    48 h Recovery
3	-2	24 (3)	Swim 1 $\longrightarrow$ Holding tank
	0	24 (1)	Holding tank $\longrightarrow$ Swim 2
		24 (-)	Holding tank $\longrightarrow$ Treatment tank with solvent control
		24 (-)	Holding tank $\longrightarrow$ Treatment tank with 1 $\mu\text{g/L}$ EE2
	4	24 (1)	Treatment tank with solvent control $\longrightarrow$ Swim 2
		24 (1)	Treatment tank with 1 $\mu\text{g/L}$ EE2 $\longrightarrow$ Swim 2

**Figure S3-1** Overview of swim performance tests in three experiments. Fish were treated in replicate tanks ( $n = 12$ ) and combined for swim tests ( $n = 24$  per replicate) in Experiments 2 and 3 (in Experiment 1,  $n = 12$  or 24 was used for swim tests). (-) indicates fish not placed in swim test at this timepoint.

## Chapter 4.

# Induction and recovery of estrogenic effects following short-term 17 $\beta$ -estradiol exposure in juvenile Rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

Estrogenic compounds found in the aquatic environment include natural and synthetic estrogen hormones as well as other less potent estrogenic xenobiotics. In this study, a comprehensive approach was used to examine effects on fish endocrine system endpoints during a short-term xenoestrogen exposure as well as after post-exposure recovery. Rainbow trout (*Oncorhynchus mykiss*) were exposed to an aqueous 17 $\beta$ -estradiol (E2) concentration of 0.473  $\mu\text{g l}^{-1}$  for 2 and 7 d, followed by a 14 d recovery period. At d2 and d7, plasma E2 concentrations in treated fish were 458-fold and 205-fold higher than in control fish, and 23-fold and 16-fold higher than the exposure water concentration. E2 treatment resulted in significant increases in hepatosomatic index (HSI), plasma vitellogenin (VTG) protein concentrations, and liver VTG and estrogen receptor alpha mRNA levels. All of these parameters, with the exception of plasma VTG protein, returned to baseline values during the recovery period. Plasma cortisol concentrations were unaffected by treatment. This research shows varied time frames of the estrogen-responsive molecular, biochemical, and tissue-level alterations in juvenile Rainbow trout treated with aqueous E2, and their persistence. These results have implications for feral Rainbow trout exposed to xenoestrogens and indicate the importance of evaluating a comprehensive suite of endpoints in assessing the impact of this type of environmental contaminant.

## Keywords

17 $\beta$ -estradiol; Rainbow trout; recovery; liver; plasma; vitellogenin



## Introduction

The main vertebrate estrogen hormone, 17 $\beta$ -estradiol (E2), plays a role in fish reproduction, behaviour, development, growth, and differentiation (Kime, 1998). In teleosts, levels of endogenous E2 are lowest in juveniles and highest in adult females (Pavlidis et al. 1994; Campbell et al. 2006). E2 coordinates egg production in adult female fish by communicating actions between the brain, ovary and liver (Kime, 1998; Henley et al. 2009). Circulating E2 actions are mediated via nuclear estrogen receptors (ERs) that are well characterized as two distinctly different sub-types: ER $\alpha$  and ER $\beta$  (Iwanowicz & Ottinger, 2009). In numerous Rainbow trout tissues, both ER $\alpha$  and ER $\beta$  have been shown to have two gene transcript (mRNA) isoforms: ER $\alpha$ 1 and ER $\alpha$ 2; ER $\beta$ 1 and ER $\beta$ 2 (Nagler et al. 2007). In addition to this classical nuclear action, membrane-bound ERs provide another, less well-studied, mode of action for this hormone (Iwanowicz & Ottinger, 2009). E2 regulates gene expression in target genes with promoters containing estrogen response elements (EREs), which includes the genes for ERs, enabling estrogen-dependent processes (Henley et al. 2009).

Although E2 is a natural hormone, it can cause endocrine disruption when applied exogenously (i.e. as a xenoestrogen) in fish (e.g. Japanese medaka (*Oryzias latipes*), Metcalfe et al. 2001; summer flounder (*Paralichthys dentatus*), Zarogian et al. 2001; and chinook salmon (*Oncorhynchus tshawytscha*), Afonso et al. 2002). Xenoestrogens other than E2 can vary widely in chemical structure and class (e.g. pesticides: DDT (Zarogian et al. 2001); synthetic hormones: 17 $\alpha$ -ethinylestradiol (EE2, Metcalfe et al. 2001; Schultz et al. 2003; Kristensen et al. 2005); and plasticizers: bisphenol A (Stoker et al. 2008)). As endocrine disruptors, they can interact with ERs as agonists or antagonists, thereby altering estrogen-related events including the

feminization of males (Batty & Lim, 1999), reduced embryonic survival (Brown et al. 2007), decreased egg fecundity (Parrott & Blunt, 2005), development of abnormal gonads (Jobling et al. 1998; Rodgers-Gray et al. 2001; Afonso et al. 2002; Parrott & Blunt, 2005), and altered sex-steroid levels (Folmar et al. 1996; Scott et al. 2006).

The main biomarker of xenoestrogen exposure is the production of the egg yolk precursor protein vitellogenin (VTG), which normally occurs during egg production in mature oviparous females (Arukwe & Goksoyr, 2003). Xenoestrogen exposure has been shown to cause juveniles and/or males to express VTG protein in several species of fish, including Rainbow trout (*Oncorhynchus mykiss*, Jobling et al. 1996; Thomas-Jones et al. 2003; Thorpe et al. 2003; Brown et al. 2007), sheepshead minnow (*Cyprinodon variegatus*, Bowman et al. 2000; Folmar et al. 2000), fathead minnow (*Pimephales promelas*, Panter et al. 1998; Parrott & Blunt, 2005), and zebrafish (*Danio rerio*, Van den Belt et al. 2004). Xenoestrogens also alter estrogen-responsive gene expression in fish, including those involved in the female process of producing eggs, such as VTG and vitelline envelope proteins (Arukwe & Goksoyr, 2003; Thomas-Jones et al. 2003; Garcia-Reyero et al. 2004; Filby et al. 2007a; Williams et al., 2007; Benninghoff & Williams, 2008) and ERs (Sabo-Attwood et al. 2004; Hook et al. 2006; Filby et al. 2006; Filby et al. 2007a; Benninghoff & Williams, 2008).

The endocrine system of *Oncorhynchus* species has been relatively well studied; however, little information is available about the timing and permanence of perturbations caused by xenoestrogens. Biomarkers (like VTG protein) can demonstrate alterations in estrogen-responsive endpoints but little is known about the ability of salmonids to recover after acute xenoestrogen exposure. There is some information in other fish species regarding the clearance of xenoestrogens and the return of altered parameters

to baseline levels, in particular for aqueous E2 or EE2-exposed fish including plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*) and sand goby (*Pomatoschistus minutus*; Craft et al. 2004); fathead minnow (Panter et al. 2000; Schmid et al. 2002; Thorpe et al. 2007; Ekman et al. 2008; Hyndman et al. 2010); zebrafish (Fenske et al. 2005); and sheepshead minnow (Hemmer et al. 2002). Studies have evaluated recovery post-E2 injection in Rainbow trout (Le Guellec et al. 1988; Pakdel et al. 1991; Celius et al. 2000; among others) but at present, no studies evaluating the recovery ability of Rainbow trout (or a salmonid) after *in vivo* waterborne E2 exposures exist. This may be due to the difficulties in studying Rainbow trout/salmonids (compared to other well-studied teleosts), such as long maturity time, housing requirements due to large adult fish size, and end-of-life (or semelparous) spawning. Additionally, estrogenicity studies often evaluate gonad histology or sex-specific behaviour/characteristics, both of which are difficult to assess in adult salmonids.

The onset of xenoestrogen effects, as well as their duration, magnitude, and recovery should be evaluated using a suite of endpoints (i.e. beyond the biomarker of VTG protein), an approach that is rarely attempted in any species of fish. The present study examined a comprehensive set of molecular, biochemical and tissue-level measurements in order to more fully characterize the induction and recovery of estrogenic responses in E2-treated fish. Immature Rainbow trout were exposed to nominal  $1 \mu\text{g l}^{-1}$  waterborne levels of E2 for 2 and 7 d, followed by a 14 d depuration period. At each timepoint, plasma cortisol concentrations and estrogen-responsive endpoints (hepatosomatic index (HSI), plasma E2 and VTG concentrations, and liver VTG and ER mRNA levels) were evaluated.

## Materials and Methods

### ***Exposures and Tissue Collection***

Exposures to E2 were conducted as static renewal in 68 L glass aquaria maintained at  $15 \pm 1$  °C under a 16:8 h light:dark photoperiod. Individual juvenile Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) of mass  $31.9 \pm 0.7$  g (mean  $\pm$  SEM) from Sun Valley Trout Hatchery (Mission, BC) were randomly placed into aquaria (i.e. one fish per aquarium) washed with 5% (v v<sup>-1</sup>) glacial acetic acid and rinsed with well water to remove trace metals or contaminants. Treatments consisted of well water with either 0.001% (v/v) anhydrous 100% ethanol (Commercial Alcohols, Brampton, ON) for the solvent control, or 1  $\mu\text{g l}^{-1}$  17 $\beta$ -estradiol (E2; CAS = 50-28-2; Sigma-Aldrich, Oakville, ON) dissolved in anhydrous 100% ethanol (final solvent concentration of 0.001% (v v<sup>-1</sup>)). Fish were exposed to the treatments for 2 or 7 d (n = 12 and n = 15, respectively). Fish exposed for 7 d had a 100% water renewal at day 4. After 7 d, fish were transferred to clean aquaria (still maintaining one fish per tank) containing well water only for a 14 d depuration period (sampled on day 21, the recovery timepoint, n = 10). Water quality (dissolved oxygen, pH, conductivity and temperature) was measured every 1 to 3 d, as well as before and after each water change. The number of fish (n) sampled per treatment group was 12 for day 2 (d2); 15 for day 7 (d7) and 10 for day 21 (d21; recovery timepoint).

Fish were euthanized in 100 mg l<sup>-1</sup> buffered MS-222 (tricaine methanesulfonate; Syndel Labs, Vancouver, BC), blotted dry, and then weighed. Fish tissues were dissected and blood withdrawn within 5 min of capture to minimize the potential for a physiological stress response. Peripheral blood and head kidney were collected for procedures described in Shelley et al. (2012). Whole blood was collected in heparinized

capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN) and spun for 3 min in a microcapillary centrifuge (International Equipment Company, Chattanooga, TN). Plasma from each capillary tube was placed separately into 0.2 ml Axygen plastic tubes (VWR, Mississauga, ON) and temporarily stored on dry ice until transfer to -80 °C. The liver was dissected out and weighed for HSI calculations (liver weight x body weight<sup>-1</sup> x 100), then each liver was stored separately in 1.5 ml Axygen tubes (VWR, Mississauga, ON) containing 1 ml of RNALater® (Ambion, Austin, TX) at -80 °C for transcriptomics analysis.

### ***Plasma Analysis***

Cortisol concentrations were measured in plasma samples using a cortisol ELISA kit (enzyme-linked immunosorbent assay; Neogen Corporation, Lansing, MI). Measurements of plasma E2 concentrations were performed using an E2 ELISA kit (Cayman Chemical Company, Ann Arbor, MI). Plasma concentrations of vitellogenin (VTG) were determined using a Rainbow trout VTG ELISA kit (Biosense® Laboratories, Abraxis, Warminster, PA). Samples in which VTG was not detected were set to the detection limit of the kit (3.125 ng ml<sup>-1</sup>) for statistical purposes. The mean CVs between duplicates were 6.4%, 8.9%, and 2.8% (respectively for plasma cortisol, E2 and VTG concentrations) and the R<sup>2</sup> values for standard curves were > 0.98 for all assays. All ELISA plates were read on a Bio-tek® PowerWave Reader (Bio-Tek, Winooski, VT) at the appropriate wavelength as specified in the manufacturers' protocols. All samples were analyzed in duplicate (triplicate for cortisol).

## ***Water Chemistry***

Composite 1 L water samples were collected from glass aquaria, as fresh preparations of solutions on d0 and d4, and 'old' solutions on d2, d4, d7 and d21. Samples were preserved with 1% (v/v) formaldehyde and stored in the dark at 4 °C until analysis by gas chromatography mass spectrometry for sterol compounds occurred as per Furtula et al. (2012). Only the d0 E2 treatment was sampled in duplicate (results averaged in Table 4-1).

## ***RNA Extractions***

Total RNA extractions from liver were performed using RNeasy Mini Spin kits (Qiagen, Mississauga, ON), including an on-column DNase treatment. Weighed livers ( $30 \pm 2$  mg) were placed in 1.5 ml Eppendorf safe-lock tubes (Thermo Fisher Scientific, Toronto, ON) containing 1 ml of Qiazol (Qiagen, Mississauga, ON) and two 3 mm stainless steel beads (Retsch, Newtown, PA). They were homogenized using a Mixer Mill 300 (Qiagen, Mississauga, ON) bead mill homogenizer for 3 cycles of 2 min each at 25 Hz (with 180° rotation of the tube holders after each cycle). Elution occurred using 2 x 35 µl aliquots of RNase-free water. RNA concentrations were determined using a Nanodrop® 1000 instrument (Thermo Fisher Scientific, Toronto, ON).

## ***cDNA Preparation***

Using a QuantiTect reverse transcription kit (Qiagen, Mississauga, ON), 1 µg of total RNA was transcribed into cDNA (complementary deoxyribonucleic acid) with a 60

min synthesis step. To evaluate gene expression, liver cDNAs were diluted 15 fold in nuclease-free water.

## **QPCR**

Sequences for ER primers were acquired from Nagler et al. (2007). Ribosomal protein L8 reference gene primer sequences (*forward (f)* CAGGGGACAGAGAAAAGGTG, *reverse (r)* TGAGCTTTCTTGCCACAG) were acquired from Dr. Nik Veldhoen and Dr. Caren Helbing, University of Victoria, Victoria, BC. VTG primers were from a Rainbow trout microarray (Wiseman et al. 2007): *f* ATGAGAGCAGTAGTACTTG, *r* TCTTGCACACTCCCTGAGC. Primers were synthesized by AlphaDNA (Montreal, QC).

QPCR analysis evaluated hepatic VTG and ER gene expression alterations caused by exposure to E2, as compared to control fish, at each timepoint (d2, d7 and d21 (recovery)). Triplicate QPCR reactions were set-up on ice in 0.2 ml Axygen chimney 96-well plates (VWR, Mississauga, ON), sealed with Microamp® optical adhesive films (ABI, Foster City, CA). Two µl of diluted cDNA was used per well. Master Mixes for each primer pair contained the following per 15 µl total volume reaction: 50% iTaq™ SYBR® Green Supermix With ROX (Bio-Rad, Mississauga, ON); 20 pmoles forward primer; 20 pmoles reverse primer; and nuclease-free water (Ambion, Austin, TX). Triplicate no template controls (NTC) were included for each primer pair for each timepoint analyzed. Plates were run with the following program in Stratagene MX3000P™ QPCR instruments (Agilent, Cedar Creek, TX): 95 °C for 1 min and 40

cycles of 95 °C for 15 sec, 58 °C for 25 sec, 72 °C for 35 sec. Verification of the correct product for each gene occurred utilizing agarose gels, melting curves and sequencing.

Seven-point standard curves ( $2 - 2 \times 10^5$  copy number) for each primer pair were prepared and used to calculate the absolute quantity (copy number) of the target transcript in each cDNA. Copy number reported as mRNA transcript levels was chosen to visualize the QPCR results of the expression of the hepatic gene targets over time. Criteria for acceptable standard curves included:  $R^2 > 0.99$  (range was 0.992-0.999); efficiency (E) 80 - 120% (range was 80% - 108%); at least a 2 cycle at threshold ( $C_t$ ) difference from the NTC  $C_t$  value; threshold  $< 0.020$  (threshold calculated as 10X the background noise level based on cycles 5 - 9). The invariant ribosomal protein L8 (statistically proven to be invariant using ANOVA followed by Tukey's post-hoc test,  $P$ -values  $> 0.05$ ) was used to normalize the transcript results to account for technical variations (Filby & Tyler, 2007b). L8 transcript levels were (mean  $\pm$  SEM): liver  $1.6 \times 10^5 \pm 1.4 \times 10^4$ , PBL  $3.2 \times 10^5 \pm 4.5 \times 10^4$ , and HKL  $1.4 \times 10^5 \pm 2.7 \times 10^4$ .

### ***Statistical Analysis***

GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., La Jolla, CA), with statistical significance denoted by  $P < 0.05$ , was used to perform all statistical tests. Grubbs' test was used to detect and remove outliers (GraphPad Software Inc.). Normality of data was analyzed using the Shapiro-Wilk's test. For normally distributed parameters, ANOVA with Tukey's post-hoc test was conducted to test for differences between the control and E2 treatment. Otherwise, non-parametric data (plasma cortisol



concentrations and VTG mRNA levels) were analyzed using Kruskal-Wallis and Dunn's multiple comparison tests.

## **Results**

### ***Aqueous E2 Concentrations and Water Quality***

For the duration of the experiment in the control treatment (Table 4-1), 17 $\beta$ -estradiol (E2) concentrations were found to be < 0.005  $\mu\text{g l}^{-1}$ , which was the limit of quantification (LOQ). The measured concentration of E2 in the exposed group ranged from 0.397 to 0.509  $\mu\text{g l}^{-1}$ , with a mean  $\pm$  SEM concentration of 0.473  $\pm$  0.021  $\mu\text{g l}^{-1}$ , which was 47% of the target concentration of 1  $\mu\text{g l}^{-1}$  (Table 4-1). There was a decrease in E2 over time from d0 to d4 (0.509  $\mu\text{g l}^{-1}$  to 0.397  $\mu\text{g l}^{-1}$ ) but the same trend was not seen between d4 and d7 (0.465  $\mu\text{g l}^{-1}$  to 0.503  $\mu\text{g l}^{-1}$ ). This demonstrates that the waterborne E2 concentrations were stable in the aquaria over time.

Water quality parameters measured in both controls and exposure treatment aquaria were acceptable for the maintenance of fish: dissolved oxygen was 9.7  $\pm$  0.9 mg  $\text{l}^{-1}$ , pH was 7.7  $\pm$  0.3, conductivity was 479  $\pm$  9  $\mu\text{S}$ , and temperature was 15.0  $\pm$  1.0  $^{\circ}\text{C}$ . There were no fish mortalities during the experiment.

### ***HSI, Plasma Hormones and VTG Protein***

During the E2 exposure period (timepoints d2 and/or d7), HSI (Fig. 4-1A;  $P < 0.001$ ), plasma E2 concentrations (Fig. 4-1B;  $P < 0.001$ ), plasma VTG protein

concentrations (Fig. 4-1C;  $P < 0.001$ ), and liver VTG mRNA levels (Fig. 4-1D;  $P < 0.001$ ) increased significantly in E2-treated fish compared to controls. HSI, plasma E2, plasma VTG, and liver VTG mRNA were 1.3, 458, 1403 and 760-fold higher (respectively) at d2, and 1.8, 205, 88985 and 955-fold higher (respectively) at d7 in treated fish compared to control fish. HSI, plasma E2 concentrations, and liver VTG mRNA returned to control values in E2-treated fish at the recovery (d21) timepoint, however, plasma VTG protein concentrations remained significantly elevated ( $P < 0.001$ ).

Plasma cortisol concentrations were not significantly different ( $P > 0.05$ ) between E2-exposed and control fish at any time point (Fig. 4-1E). However, plasma cortisol levels at the recovery (d21) timepoint were significantly higher in both control and E2-treated fish compared to d2 E2-treated fish ( $P < 0.01$ ).

### ***ER Transcripts***

All four ER transcripts (ER $\alpha$ 1, ER $\beta$ 2, ER $\beta$ 1 and ER $\alpha$ 2) were detected in liver at every timepoint for both control and E2-treated Rainbow trout (Fig. 4-2). In control fish, ER $\alpha$ 2 was the least expressed transcript while ER $\alpha$ 1 was the highest expressed transcript (ER $\alpha$ 1 > ER $\beta$ 2 > ER $\beta$ 1 > ER $\alpha$ 2). Levels of expression of ER transcripts were consistent for control fish across the timepoints. ER $\alpha$ 1 was significantly elevated in E2-treated fish at d2 ( $P < 0.001$ ) and d7 ( $P < 0.001$ ) compared to control fish (Fig. 4-2). ER $\alpha$ 2 was only significantly elevated in E2-treated fish at d7 ( $P < 0.001$ ). ER $\beta$ 1 and ER $\beta$ 2 were not significantly altered ( $P > 0.05$ ) by the E2 treatment at any timepoint.

## Discussion

Due to the lack of studies utilizing a suite of estrogen-responsive parameters, and the limited information available on the permanence of xenoestrogen effects in fish, the current study was undertaken to evaluate the induction and subsequent recovery of a comprehensive set of estrogenic endpoints following a short-term exposure of Rainbow trout to E2.

Measured water concentrations of E2 in exposure aquaria (Table 4-1) were lower than the nominal  $1 \mu\text{g l}^{-1}$ , results similar to other reports (Hemmer et al. 2002; Maunder et al. 2007), possibly due to non-specific binding in the test system. E2 was accumulated by the E2-treated fish: plasma E2 concentrations were  $10.8 \text{ ng ml}^{-1}$  at d2 and  $7.5 \text{ ng ml}^{-1}$  at d7 (Fig. 4-1B), which were 458-fold and 205-fold higher, respectively, than in control fish. Although E2 was exogenously applied, the resulting plasma E2 concentrations were consistent with those typically observed in mature, ovulating female Rainbow trout (Pavlidis et al. 1994; Tyler et al. 1996), suggesting that the findings in this study might be indicative of the response to endogenous E2 in mature females.

Few studies have reported the magnitude of E2 bioconcentration upon exposure to this hormone in water (Cakmak et al. 2006). In the present study, the Rainbow trout plasma E2 concentrations were 23-fold higher at d2 and 16-fold higher at d7 than the aqueous treatments. This was similar to the ratio of E2 found in the plasma of tench (*Tinca tinca*) to water of 26.3 (after 6 hours) as reported by Scott et al. (2005). Maunder et al. (2007) found very similar results as well, with plasma E2 levels ~ 50 fold higher (after 6 hours or 6 d, compared to aqueous treatments) in three-spined stickleback (*Gasterosteus aculeatus*). These values are much lower than a legacy contaminant like DDT (bioconcentration factor (BCF) =  $3 \times 10^4$  (Lai et al. 2002)), but on par with another

xenoestrogen: bisphenol A (BCF = 5 to 68 (Staples et al. 1998)). Thus, E2 may not be a compound of great concern with regards to bioconcentration or persistence, but it can be an important factor for aquatic life in environmental situations enduring chronic discharges, termed pseudo-persistence (Sumpter & Johnson, 2008), such as fish downstream of sewage treatment works (Batty & Lim, 1999; Jobling et al. 1998).

Cortisol is a glucocorticoid steroid hormone that increases in concentration in plasma by stimulation of the hypothalamic-pituitary-interrenal axis during a physiological stress response (Barton, 2002). A range of 30 to 300 ng ml<sup>-1</sup> of plasma cortisol indicates an acute stress response in fish (Barton, 2002). In this study, concentrations of plasma cortisol (Fig. 4-1E) were all below 10 ng ml<sup>-1</sup> during the treatment phase and below 30 ng ml<sup>-1</sup> in the recovery phase, which indicates that plasma cortisol concentrations were not elevated above levels indicating acute stress (Barton, 2002). Plasma cortisol concentration was unaffected by E2 exposure, similar to the findings in zebrafish by Fuzzen et al. (2011), who showed that 0.1 µg l<sup>-1</sup> E2 treatment did not alter plasma cortisol concentrations. In contrast, a study with Atlantic salmon exposed to 2 µg l<sup>-1</sup> of E2 for 21 days found plasma cortisol levels were elevated approximately 4-fold (Lerner et al. 2007). However, the E2 treatment in that study increased plasma cortisol from ~ 1 ng ml<sup>-1</sup> in the solvent control to ~ 4 ng ml<sup>-1</sup> in the E2 treatment, which are levels still well below the range indicating acute physiological stress and very similar to the levels found in the current study. In addition to its role as a primary-level biological responder to stress, elevation of cortisol is known to result in secondary responses, one of which includes suppression of fish immune system function (Barton, 2002). In the related publication to this study, plasma cortisol concentrations were not altered by E2 treatment

and so cortisol can be eliminated as a causal factor in the reduced immune parameters found (Shelley et al. 2012).

Hepatosomatic index (HSI) has been shown to increase due to xenoestrogen exposure (E2 (Persson et al. 1997); EE2 (Parrott & Blunt, 2005; Schultz et al. 2003)) and this effect is attributed to increased liver weight (Cakmak et al. 2006; Arukwe & Goksoyr, 2003). Reasons for acute liver weight increases include increased RNA content, lipid deposition, and VTG protein concentration (Arukwe & Goksoyr, 2003; Cakmak et al. 2006). HSI increases at d2 and d7 were likely due to hepatocytes producing copious amounts of VTG protein (Fig. 4-1A and 4-1C), as well as increased mRNA levels (Fig. 4-1D and 4-2). Plasma VTG protein concentrations in this study (Fig. 4-1C) were similar to those reported by Thorpe et al. (2003) for a comparable level of E2 treatment of Rainbow trout although their exposure design was flow-through for 14 d.

The evaluation of hepatic ER transcripts was performed in this study using QPCR (Fig. 4-2). The present study matched the results of Nagler et al. (2007) very closely with respect to the abundance of transcripts in liver. In that study, the relative transcript abundance was  $ER\beta_2 > ER\alpha_1 > ER\beta_1 > ER\alpha_2$  (untreated fish), while in the present study, the most abundant transcript in control fish was  $ER\alpha_1$  followed by  $ER\beta_2$ . There have been reports of ER transcript induction in liver tissue by xenoestrogens (Hook et al. 2006; Filby et al. 2007a), including E2 treatment specifically (Benninghoff & Williams, 2008). Consistent with those reports, E2 induced some ER transcripts significantly:  $ER\alpha_1$  was elevated at d2, and both  $ER\alpha_1$  and  $ER\alpha_2$  were elevated at d7. Neither  $ER\beta$  transcript was altered by E2 at d2 or d7 (Fig. 4-2). Induction of  $ER\alpha$ , but not  $ER\beta$ , was also reported in liver for largemouth bass injected with E2 (Sabo-Attwood et al. 2004), and fathead minnow exposed to  $100 \text{ ng l}^{-1}$  of E2 (Filby & Tyler, 2005) or 10

ng l<sup>-1</sup> of EE2 (Filby et al. 2006). ER $\beta$  appears to have a distinct hepatic physiological role compared to ER $\alpha$  in that it appears not to be regulated by E2, thus ER $\beta$  may not be involved in bony fish vitellogenesis (see discussion in Filby & Tyler, 2005).

This research evaluated the recovery of parameters once E2 treatment was withdrawn. Hepatocytes ceased VTG production by d21 as evidenced primarily by the HSI (Fig. 4-1A) and VTG mRNA transcription (Fig. 4-1D) returning to normal/control levels; plasma E2 concentrations were no longer significantly elevated at d21, reducing the VTG synthesis stimulus (Fig. 4-1B). Gene expression of VTG, ER $\alpha$ 1 and ER $\alpha$ 2 (Fig. 4-1D and Fig. 4-2, respectively) returned to baseline levels; therefore, exposure to 0.473  $\mu$ g l<sup>-1</sup> E2 did not cause permanent alterations to the expression of these genes. The only parameter that had not recovered after 14 d of depuration was plasma VTG protein concentration (Fig. 4-1C). Ovaries in mature females are responsible for the removal of VTG protein from plasma. The lack of ovaries in males and immature ovaries in juvenile females are likely key factors in the persistence of VTG protein in plasma in these fish since, with nowhere to deposit the VTG, it will likely remain in circulation until it can be eliminated by the kidneys (Bowman et al. 2000; Arukwe & Goksoyr, 2003). Elevated levels of VTG protein in juvenile or male fish can be detrimental to their health and has been associated with renal failure (Herman & Kincaid, 1988; Schwaiger *et al.*, 2000) or male reproductive impairment (Kramer et al. 1998; Thorpe et al. 2007). Thorpe *et al.* (2007) linked plasma VTG protein concentrations > 1 mg ml<sup>-1</sup> in fathead minnow to higher mortality in males, reduced total egg production in females and reduced spawning events. By d7 in the present study, plasma VTG levels were much greater than 1 mg ml<sup>-1</sup> (Fig. 4-1C; d7: 57.5 mg ml<sup>-1</sup>, and d21: 37.0 mg ml<sup>-1</sup>), thus the juvenile Rainbow trout could have been negatively impacted; however, there were no mortalities in the present

study, likely due to its short term nature. In addition to VTG protein toxicity, E2 or xenoestrogen exposure at this life stage could potentially misdirect limited resources away from areas such as growth, resulting in negative fitness (Kristensen et al. 2005) or performance repercussions (Madsen et al. 2004).

The E2 concentration utilized in the present study was < 1 - 2 orders of magnitude higher than levels measured in the aquatic environment (maximum 0.093  $\mu\text{g l}^{-1}$  found by Kolpin et al. 2002; maximum of 0.001  $\mu\text{g l}^{-1}$  found by Jeffries et al., 2010), and in sewage treatment plant (STP) effluents and/or studies evaluating compounds utilizing E2-equivalents (Larsson et al. 1999; Spengler et al., 2001; Aerni et al., 2004; Vermeirssen et al., 2005; Sousa et al., 2010; Vajda et al. 2011; Williams et al., 2012). It was one order of magnitude higher than estrone (the oxidized, ketone form of E2 that can have equivalent potency to E2 (Dammann et al. 2011)) measured in sewage effluents (Ternes et al. 1999; Vajda et al. 2011; Furtula et al., 2012) and some surface waters (Lee et al., 2011). Possibly, the additive levels (Thorpe et al., 2006; Correia et al., 2007) of all xenoestrogens in an STP effluent, or combined uptake from water and other aquatic matrices (e.g. partitioning from sediments to fish), could result in an equivalent level of E2 accumulated by fish in the present study. Therefore, it is possible that feral Rainbow trout exposed to such a level of E2, or lower levels, would respond and recover similarly (dependent upon no further exposure).

VTG protein may not be an adequate biomarker for indicating the xenoestrogen exposure timeframe, since, as shown in this study and others, it remains in circulation long after its production stimulus has been removed (Thomas-Jones et al. 2003). In the present study, there was a 35.7% decrease in VTG protein concentrations between d7 (57.5  $\text{mg ml}^{-1}$ ) and d21 (37.0  $\text{mg ml}^{-1}$ ) (i.e. with 14 d of recovery time). Plasma VTG

protein has been shown to have an exponential clearance rate in sheepshead minnow, with a half-life of 13.8 d for a 16 d exposure to  $0.71 \mu\text{g l}^{-1}$  E2 (Hemmer et al. 2002). In that study, plasma VTG protein peaked 4 d after the end of E2 exposure, and returned to baseline levels by 96 d. Using the clearance rate calculated by Hemmer et al. (2002), VTG concentrations in the present study would only return to baseline values 5 months following exposure. Similar results would be obtained when using the half-life of flounder VTG clearance determined by Craft et al. (2004). It has been suggested that VTG mRNA/transcript may be a better temporal marker of acute xenoestrogen exposure since the return of gene expression to baseline levels is faster than the return of VTG protein to baseline values (Bowman et al. 2000; Hemmer et al. 2002; Thomas-Jones et al. 2003). Hemmer et al. (2002) showed that induction of VTG gene expression returned to baseline levels within 8 d of exposure cessation. In the present study, VTG mRNA levels returned to baseline values after 14 d of recovery (Fig. 4-1D), in contrast to plasma VTG protein concentrations (Fig. 4-1C). Our results support the idea of using VTG mRNA as an indicator of recent xenoestrogen exposure, while VTG protein may be used as a biomarker of xenoestrogen exposure long after its occurrence. Future studies are needed that examine the specific timing and speed of recovering parameters, as well as the VTG mRNA and protein clearance rates in Rainbow trout, as have been determined in other fish species (Hemmer et al. 2002; Craft et al. 2004).

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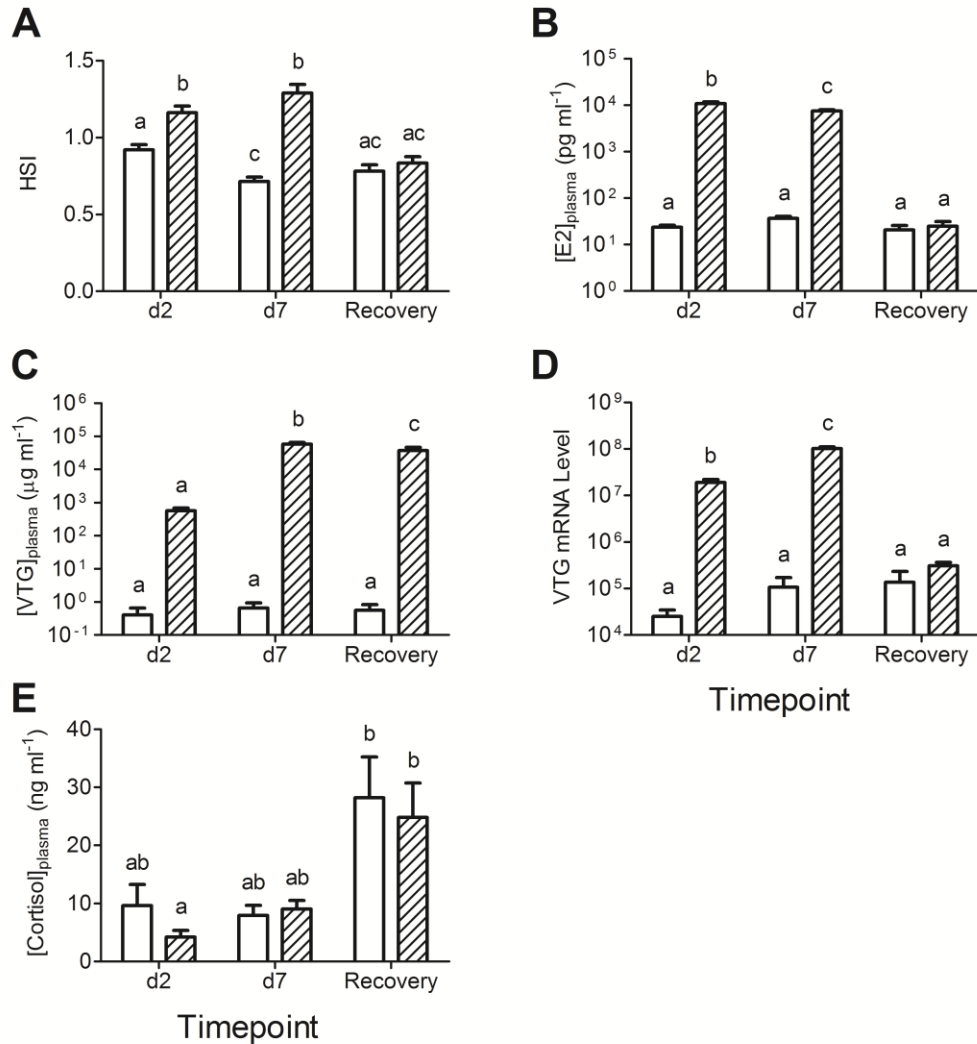
## Tables

**Table 4-1** *The concentrations of 17β-estradiol (E2) in composite samples taken from exposure aquaria at each stage of the experiment (New indicates freshly prepared solutions; Old indicates solutions aged for the period of time indicated). The E2 mean ± SEM concentration over the duration of the experiment was 0.473 ± 0.021 μg l<sup>-1</sup>. Limit of Quantification (LOQ) for the chemical analysis was 0.005 μg l<sup>-1</sup>.*

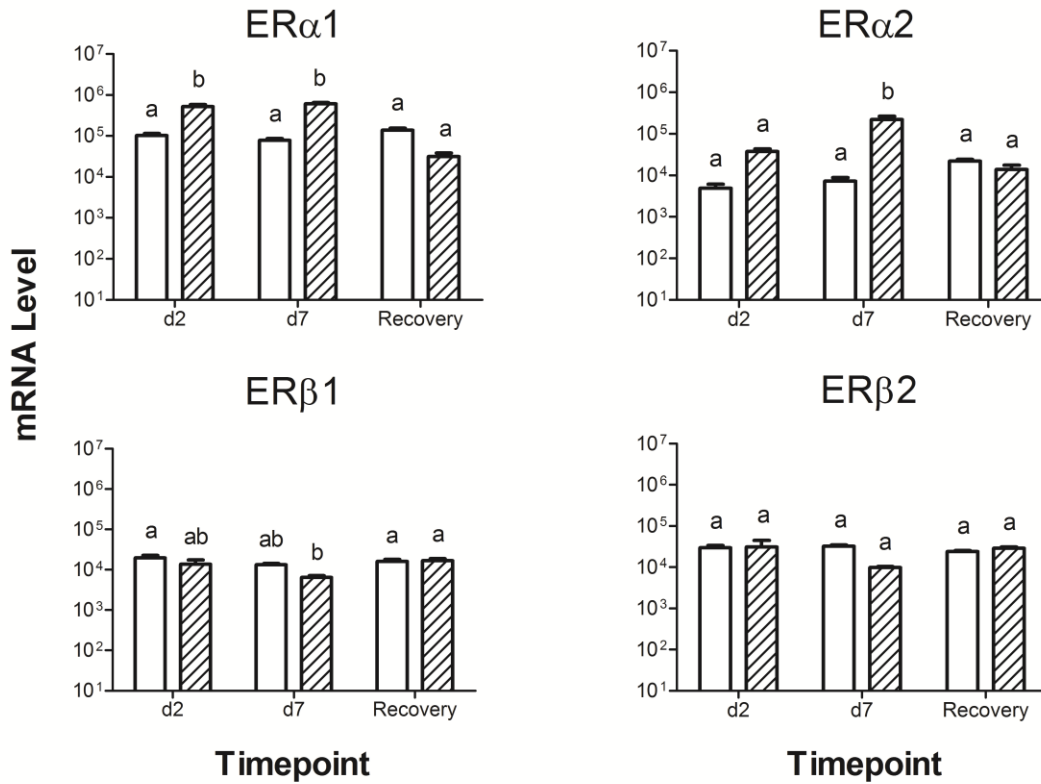
Treatment	Timepoint (d)	Type of Solution	Concentration of E2 (μg l <sup>-1</sup> )	% of Nominal	Mean (μg l <sup>-1</sup> )	SEM
Control	0	New	< 0.005		< 0.005	
	2	Old	< 0.005			
	4	Old	< 0.005			
	4	New	< 0.005			
	7	Old	< 0.005			
	7	New	< 0.005			
	21	Old	< 0.005			
1 μg l <sup>-1</sup> E2	0	New	0.509	50.9%	0.473	0.021
	2	Old	0.494	49.4%		
	4	Old	0.397	39.7%		
	4	New	0.465	46.5%		
	7	Old	0.503	50.3%		
	7 *	New	< 0.005			
	21 *	Old	< 0.005			

\* Recovery solutions (same well water as control treatment).

## Figures



**Figure 4-1** Exposure to 17 $\beta$ -estradiol (E2) for 2 or 7 days resulted in elevation of (A) Hepato-somatic index (HSI), (B) plasma 17 $\beta$ -estradiol (E2), (C) plasma vitellogenin (VTG) protein and (D) liver VTG mRNA, but had no effect on (E) plasma cortisol in Rainbow trout. All parameters returned to control levels following a 14 day post-exposure recovery period, except plasma VTG. Data from control fish are represented by open bars and E2 treated fish are hatched bars. Results are presented as mean  $\pm$  SEM, and different letters denote significant differences ( $P < 0.05$ ;  $n = 10 - 15$ , except  $n = 6$  for plasma VTG concentrations).



**Figure 4-2** *Hepatic Estrogen Receptor  $\alpha$ 1 (ER $\alpha$ 1),  $\alpha$ 2 (ER $\alpha$ 2),  $\beta$ 1 (ER $\beta$ 1), and  $\beta$ 2 (ER $\beta$ 2) mRNA is present and differentially regulated in ER $\alpha$ 1 and ER $\alpha$ 2, but not ER $\beta$ 1 or ER $\beta$ 2, following 2 and/or 7 days of 17 $\beta$ -estradiol (E2) exposure. All inductions returned to control levels in the 14 day post-exposure Recovery period. Data from control fish are represented by open bars and E2 treated fish are hatched bars. Results are presented as L8 normalized expression of mRNA transcript levels and bars represent mean  $\pm$  SEM. Different letters denote significant differences ( $p < 0.05$ ;  $n = 10 - 12$ ).*

## Chapter 5.

# Time course of hepatic gene expression and plasma vitellogenin protein concentrations in estrone-exposed juvenile Rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

Estrone (E1) is a natural estrogen hormone found in sewage effluents and surface waters and because of its endocrine disrupting effects in fish, it is a contaminant of emerging concern. In this study, molecular-level endpoints of E1-induced endocrine disruption were investigated in the liver, including: (1) the transcriptome, and (2) the time course of effects on vitellogenesis, an inducible process of producing egg components. Juvenile Rainbow trout (*Oncorhynchus mykiss*) were exposed to an environmentally relevant concentration of E1 (24 ng/L E1 [0.1 nM]) for 7 d and then placed in clean water for a 9 d recovery period. Using a reference Rainbow trout transcriptome, RNA sequencing (RNASeq) showed that 115 transcripts were altered by E1 after 4 d of treatment. The UniProt identification and GO values classified the transcripts as representing numerous biological processes, including immune, metabolic, transcription/DNA replication, apoptosis, cellular differentiation, clotting, endocrine, and structural functions. The time course of E1-inducible responses relating to vitellogenesis was examined in fish sampled daily during the two phases of exposure. Hepatic gene expression alterations evaluated by quantitative polymerase chain reaction (QPCR) were found during the exposure period for vitellogenin (*VTG*), vitelline envelope proteins (*VEPs*)  $\alpha$ ,  $\beta$  and  $\gamma$ , and estrogen receptor  $\alpha 1$  (*ER $\alpha 1$* ) transcripts. *ER $\alpha 1$*  was the only transcript induced each day during the treatment phase, thus it was a good indicator of E1 exposure. Gradual increases occurred in *VEP $\beta$*  and *VEP $\gamma$*  transcripts, peaking at d7. *VTG* transcript was only elevated at d4, making it less sensitive than *VEPs* to this low-level E1 treatment. Inductions of *ER $\alpha 1$* , *VEP $\alpha$* , *VEP $\beta$*  and *VEP $\gamma$*  transcripts ceased immediately upon exposure termination, 1 d into the recovery phase. Plasma *VTG* protein concentrations were not immediately elevated but peaked significantly at d14, 7

d into the recovery phase. Thus, elevated vitellogenesis-related gene expression and protein production occurred slowly but steadily at this concentration of E1, confirming the sequence of events for transcripts and VTG protein responses to xenoestrogen exposure.

## **Keywords**

Estrone; QPCR; RNASeq; Rainbow trout; vitellogenin; vitelline envelope proteins, recovery

## Introduction

Contaminants are released into surface waters from anthropogenic activities and may cause harmful effects in exposed organisms. Exogenous exposure to estrogen hormones (e.g.  $17\beta$ -estradiol [E2], the main vertebrate estrogen hormone) and chemicals that act like estrogens (xenoestrogens) in aquatic environments are of particular concern because of their bioactive nature and ability to cause feminization of male and juvenile organisms. The estrogen hormone estrone (E1) is the oxidized form of E2 and is commonly found in aquatic environments, with reported environmental concentrations ranging from 0.1 – 40 ng/L (Belfroid et al., 1999; Ternes et al., 1999; Kolpin et al., 2002; Aerni et al., 2004; Kim et al., 2007; Jeffries et al., 2010; Sousa et al., 2010; Lee et al., 2011; Furtula et al., 2012a; Williams et al., 2012; Barber et al., 2013). E1 is equipotent to, or slightly less potent (up to 2.5-fold), than E2 (Metcalf et al., 2001; Thorpe et al., 2003a; Van den Belt et al., 2004; Aerni et al., 2004; Dammann et al., 2011). Since E1 is excreted in the urine and feces of vertebrates (Khanal et al., 2007), it is found associated with agricultural operations (Lange et al., 2002; Kolodziej et al., 2004; Soto et al., 2004; Jeffries et al., 2010; Furtula et al., 2012a) and sewage treatment plant (STP) discharges. Reported concentrations of E1 in STP effluents range from 0.2 – 50 ng/L (Johnson et al., 2005; Lishman et al., 2006; Vajda et al., 2011; Furtula et al., 2012b; Williams et al., 2012). STP effluents are mixtures of estrogenic chemicals including surfactants (e.g. alkylphenol ethoxylates), plastic-related compounds (e.g. phthalates), and natural and synthetic estrogen hormones. Research has focused mainly on surfactants and  $17\alpha$ -ethinylestradiol (EE2) as contributors to the feminization of fish downstream of STP discharges (Jobling et al., 1998; Vajda et al., 2008), and, only

recently, E1 has been recognized as being an important contributor to these effects (Dammann et al., 2011).

E1 can feminize fish and have subsequent developmental and reproductive repercussions, which makes it an endocrine disrupting compound (EDC). E1 can inhibit testicular growth (Panter et al., 1998), induce intersex, skew female:male sex ratios (Metcalfe et al., 2001), reduce spawning frequency, increase male mortality (Thorpe et al., 2003b), inhibit egg production (Thorpe et al., 2007), and decrease reproductive success (Dammann et al., 2011). To provide insights into the mechanisms behind xenoestrogen actions, several studies examined the alterations in gene expression caused by xenoestrogen exposures (Larkin et al., 2007; Gunnarsson et al., 2007; Moens et al., 2007; Garcia-Reyero et al., 2008; Levi et al., 2009; Shelley et al., 2012). To date, however, there is a distinct lack of research regarding the effects of E1 on gene expression in fish. The present study evaluated the effects of E1 on the Rainbow trout hepatic transcriptome using RNA sequencing (RNASeq), a technique used to discover novel gene targets, identify biological processes affected, and potentially provide insights into mechanisms of action.

Vitellogenin (VTG) gene transcription and protein induction have been commonly used as biomarkers of xenoestrogen exposure since VTG is a biomarker of vitellogenesis, a process that normally occurs in maturing females when producing eggs (Sumpter & Jobling, 1995). Vitellogenesis can be initiated in hepatocytes by xenoestrogens binding to estrogen receptors (ERs) resulting in the transcription of estrogen-responsive genes and the production of egg proteins: VTG, a precursor to egg yolk, and vitelline envelope proteins (VEPs), structural parts of the egg envelope (Mommensen & Walsh, 1988; Arukwe & Goksoyr, 2003). In adult females, the egg



proteins are transported to the ovary for inclusion as components in egg formation (Mommensen & Walsh, 1988; Arukwe & Goksoyr, 2003); however, in male or juvenile fish, these egg proteins remain in circulation longer than in females until they are cleared by the kidney (Hemmer et al., 2002). Since xenoestrogens induce vitellogenesis in both male and juvenile fish, a model system has been developed for evaluating potentially estrogenic chemicals by measuring estrogen-responsive gene transcripts or proteins associated with egg production (Sumpter & Jobling, 1995; Arukwe & Goksoyr, 2003). This model system of vitellogenesis was evaluated in the present study using Rainbow trout (*Oncorhynchus mykiss*) exposed to an environmentally-relevant concentration of E1, to specifically characterize the time course of these responses. The time course included a recovery period to evaluate the return of altered parameters to baseline levels. This knowledge may aid with selecting transcripts that could be biomarkers of E1 exposure and further characterize the use of VTG protein as a biomarker so that temporal trends are identified and can indicate appropriate sampling timeframes.

## **Materials and Methods**

### ***Estrone Exposures and Sampling***

Exposures were conducted as static renewal in 38 L glass aquaria maintained at  $15 \pm 1$  °C under a 16:8 h light:dark photoperiod. Water quality parameters (dissolved oxygen, pH, conductivity and temperature) were measured daily, as well as before and after each water change. Juvenile Rainbow trout (*Oncorhynchus mykiss*) (mass  $3.8 \pm 1.1$  g, mean  $\pm$  SEM) were obtained from Sun Valley Trout Hatchery (Mission, BC, Canada) and housed according to Environment Canada method EPS1/RM/09

(Environment Canada, 1990). On day 0 (d0), 6 fish were randomly placed into each of 64 aquaria, with 32 containing solvent control (CON) and 32 containing E1 treatments. In the solvent control, fish were exposed to well water containing 0.001% (v/v) anhydrous 100% ethanol (Commercial Alcohols, Brampton, ON, Canada). In the E1 exposure, fish were exposed to well water containing 27 ng/L E1 (CAS = 53-16-7; Sigma-Aldrich, Oakville, ON, Canada) in anhydrous 100% ethanol (final solvent concentration of 0.001% (v/v)). On d0, 1 L each of prepared aqueous CON and E1 treatments was collected from 2 aquaria (i.e. duplicates), prior to the introduction of fish. These samples were analyzed in duplicate for E1 concentrations using the gas chromatography mass spectrometry method of Furtula et al. (2012b). Fish were exposed to CON or E1 treatments for up to 7 days (d), with 100% water renewal every second day. After 7 d, fish were transferred to clean aquaria containing well water only for a depuration period of 9 d. During this period, a 100% water renewal occurred every second day. Fish were fed 2.5% of their body weight of commercial feed pellet (Skretting, Vancouver, BC, Canada) 2 h prior to a water renewal.

Each d for 16 d, fish in 2 tanks per treatment (n = 12) were euthanized in 150 mg/L buffered MS-222 (tricaine methanesulfonate; Syndel Labs, Vancouver, BC, Canada), blotted dry, and then measured for mass and fork length. Whole blood was collected from the caudal vasculature into heparinized capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN, USA) and spun for 3 min in a microcapillary centrifuge (International Equipment Company, Chattanooga, TN, USA). Plasma from each fish was separated from red blood cells and stored in a 1.5 ml tube (VWR, Mississauga, ON, Canada) at -80 °C until analyzed for VTG protein. The liver was collected, weighed, and

stored in a 1.5 ml tube containing 1.2 ml of RNALater® (Ambion, Life Technologies Inc., Burlington, ON, Canada) at -80 °C for total RNA extraction.

### ***Plasma Analysis***

VTG protein concentrations in plasma were determined using a Rainbow trout VTG ELISA kit (Biosense® Laboratories, Abraxis, Warminster, PA, USA). Samples in which VTG was not detected were set to the detection limit of the kit for statistical purposes. Plates were read on a Bio-tek® PowerWave Reader (Bio-Tek, Winooski, VT, USA) using parameters specified in the manufacturer's protocol.

### ***Transcriptomics Evaluation***

#### **RNA Isolation**

Hepatic total RNA was extracted from all liver samples (30 mg per fish) for QPCR and RNA-Seq analysis using RNeasy mini extraction kits (Qiagen, Mississauga, ON, Canada). The manufacturer's protocol was followed except two 35 µl elutions were used in the final step, and the optional on-column DNase step was included. Total RNA concentration was determined via  $A_{260}/A_{280}$  concentration analysis on a Nanodrop 1000 (Thermo Fisher Scientific, Mississauga, ON, Canada). RNA quality was evaluated using the Bio-Rad Experion Electrophoresis Station and the RNA StdSens Analysis Kit as per manufacturer's protocols (Bio-Rad, Mississauga, ON, Canada).

## QPCR

Preparation of cDNA from 1 µg of total RNA occurred using a QuantiTect Reverse Transcription kit (Qiagen), with a 60 min synthesis time. A working dilution of cDNA was prepared for each sample by diluting stock cDNA 15-fold in nuclease-free water (Ambion). QPCR analysis was performed on both treatments from each day (for 16 days). Samples were set-up in triplicate on ice in 96-well Axygen chimney plates (VWR), sealed with MicroAmp® optical adhesive film (Applied Biosystems, Life Technologies, Burlington, ON, Canada), and run on Stratagene Mx3000P instruments (Agilent, Mississauga, ON, Canada) as described in Osachoff et al. (2013) and Shelley et al. (2013). In brief, each well contained 50% iTaq SYBR green Supermix (Bio-Rad, Mississauga, ON, Canada), nuclease-free water (Ambion), and 20 pmoles of each primer (primer sequences and gene transcript details in Table 5-1). *VTG*, *VEPα*, *VEPβ*, and *VEPγ* were used to evaluate egg-protein related induction by E1. All four known isoforms of Rainbow trout ER transcripts were evaluated (*ERα1*, *ERα2*, *ERβ1*, *ERβ2*; Nagler, 2007). Each amplicon was verified as the correct product by agarose gel electrophoresis (band size), cloning and sequencing in TopoTA kits (Invitrogen, Life Technologies, Burlington, ON, Canada), and confirmation of a unique, characteristic temperature for a single peak on the dissociation curve. The same criteria for standard curves and absolute quantification calculations, including normalization to ribosomal protein *L8* (Filby & Tyler, 2007a), were used as described previously (Shelley et al., 2013; Osachoff et al., 2013).

## **RNA Seq - Transcriptome Mapping and Differential Gene Expression Analysis**

Six fish RNA samples (3 CON-treated and 3 E1-treated fish) from d4 were selected for RNA-Seq analysis. The timepoint was chosen based on the QPCR results at d4 that were suggestive of an altered transcriptome and thereby deemed the earliest appropriate candidate timepoint for evaluation of widespread alterations. Sequencing libraries were generated using the TruSeq™ RNA Sample Prep Kit (Illumina, San Diego, CA, USA), which includes a unique hexamer oligonucleotide tag for each library; libraries were quantified using the Illumina KAPA Library Quant Kit and normalized to 10 pM for cluster generation and sequencing, as per the manufacturers' protocols. The six libraries were sequenced in a multiplex format in a single lane using an Illumina GA Ix sequencer.

TopHat 2.0.6 and Bowtie 2.0.2 (Langmead et al., 2009; Trapnell et al., 2010) were used to map Illumina 75 bp paired-end reads against the Rainbow trout reference transcriptome previously generated (Salem et al., 2010). Bam format output files were pipelined into Cufflinks 2.0.2 for assembling a comprehensive transcript set and computing transcript abundance (Roberts et al., 2011). Isoform and transcript sequencing depth was estimated using BEDTools 2.17.0 (Quinlan et al., 2010).

### ***Calculations and Statistics***

Condition factor (CF) was calculated as:  $\text{mass} \times 100 / \text{fork length}^3$ . Liver somatic index (LSI) was calculated as  $\text{liver weight} / \text{mass} \times 100$ . Differential gene expression analysis and statistical testing for RNASeq results were performed using R Bioconductor

package, DESeq (Anders et al., 2010). Significance level was denoted by Bonferroni-corrected p-values ( $p < 0.05$ ). For all other parameters, statistical analyses were conducted using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., La Jolla, CA), with statistical significance denoted by  $p < 0.05$ . The normality of data were analyzed using the Shapiro-Wilk's test. Grubbs' test was used to detect and remove outliers (GraphPad Software Inc., La Jolla, CA). Data were normally distributed for CF and LSI parameters, thus they were analyzed using one-way ANOVA with Bonferroni's multiple test correction. Plasma VTG protein concentrations and QPCR data were non-parametric and were analyzed using Kruskal-Wallis tests, followed by Mann-Whitney U tests to identify significant differences between CON and E1 within each timepoint. All values were reported as mean  $\pm$  standard error of the mean (SEM).

## Results

### ***Estrone Water Concentrations and Water Quality***

The measured concentration of E1 on d0 in the E1 treatment tank was 24 ng/L (89% of the nominal value). The E1 concentration was below the limit of quantification ( $< 5$  ng/L) in the solvent control (CON) treatment tank.

Measured water quality parameters were within Environment Canada (1990) guidelines and consistent between CON and E1 tanks throughout the experiment. For CON, pH was  $7.78 \pm 0.03$ ; dissolved oxygen was  $9.5 \pm 0.3$  mg/L; conductivity was  $464 \pm 4$   $\mu$ S; and temperature was  $15.1 \pm 0.3$  °C. For E1, pH was  $7.80 \pm 0.03$ ; dissolved

oxygen was  $9.6 \pm 0.3$  mg/L; conductivity was  $467 \pm 4$   $\mu$ S; and temperature was  $15.1 \pm 0.3$  °C. There were no fish mortalities during the study.

### ***CF, LSI and Plasma Vitellogenin Protein***

CF was not significantly different between CON ( $0.960 \pm 0.007$ ) and E1 ( $0.959 \pm 0.007$ ) treatments at any timepoint. LSI was also not significantly different between CON ( $0.641 \pm 0.025$ ) and E1 ( $0.637 \pm 0.026$ ) treatments at any timepoint. CF and LSI values for E1 treated fish, shown as % of control for their respective days, were included in Supplementary Figure S5-1.

Plasma VTG protein concentrations were significantly different between CON and E1 at several timepoints (Figure 5-1). During the exposure phase, VTG protein was increased at d5 and d6 in E1-exposed fish compared to fish in their respective CON treatments, which had no measurable plasma VTG. During the recovery phase, plasma VTG protein concentrations were elevated in the E1-treated fish at d11, d12, d14 (peak) and d15.

### ***Quantitative Polymerase Chain Reaction***

Five genes relating to vitellogenesis were altered by E1 exposure (Figure 5-2). The vitelline envelope proteins (*VEP $\alpha$* , *VEP $\beta$*  and *VEP $\gamma$* ) were all significantly elevated for d4 – d8 (*VEP $\beta$*  d3 as well). The expression of *VTG* transcripts was highly variable and only significantly elevated at d4 of the exposure phase. Only one gene, *ER $\alpha$ 1*, was significantly elevated at all timepoints of the exposure phase (d1 – d7). *ER $\alpha$ 2*, *ER $\beta$ 1* and *ER $\beta$ 2* were not significantly altered at any timepoint (data not shown). *VTG* was the only

gene transcript to be significantly increased during the recovery phase (d12 and d13; Figure 5-2).

### ***RNASeq***

RNASeq was performed as a single lane multiplex, yielding a total of 15.9 million reads. Using TopHat and Bowtie, the 75 bp paired-end reads were mapped against a reference Rainbow trout transcriptome (Salem et al., 2010). Of the total reads from each sample, 83.7% were successfully aligned to one of the 161,818 reference contigs. In total, 115 gene transcripts were found by RNASeq to be significantly altered ( $p < 0.05$ ) by E1 exposure at d4 when compared to transcripts from CON fish. Of these, 75 were down-regulated and 40 were up-regulated. Many transcripts were unidentifiable at the current time as their BLAST results found 'unnamed protein product'. However, for 39 transcripts, associated biological processes altered by E1 were determined, with UniProt identification and GO values (The UniProt Consortium, 2012; Table 5-2). These biological processes included apoptotic signalling, cellular differentiation, clotting, vitellogenesis, the innate and acquired immune systems, carbohydrate and lipid metabolism, and the cytoskeleton (Table 5-2). The complete list of altered gene transcripts found by RNASeq analysis is shown in Supplementary information Table S5-1.

Several transcripts were also identified in duplicate: DNA-damage-inducible transcript 4 (*DDT*), phosvitinless vitellogenin (*VTG*), and complement factor H1 (*CFH*) (Table 5-2). This is a direct result of utilizing RNASeq for an organism whose genome has not yet been sequenced and annotated. Each pair of duplicates was highly similar in the magnitude and direction of their fold change.



## Discussion

E1 has become a chemical of significant environmental concern due to its prevalence in surface waters worldwide (Kolpin et al., 2002; Barber et al., 2013) and reports of its endocrine disrupting effects in fish (Panter et al., 1998; Metcalfe et al., 2001; Thorpe et al., 2003b; Thorpe et al., 2007; Dammann et al., 2011). To address the limited knowledge available regarding E1 effects at the molecular and biochemical levels, the present study investigated several such endpoints following E1-exposure, and revealed temporal trends in vitellogenesis-related parameters and alterations of hepatic transcripts representing numerous biological functions.

In mature females, vitellogenesis is characterized by initial alterations in hepatic gene and protein expression, followed by protein transport in blood to the ovary for incorporation into the egg (Mommensen & Walsh, 1988; Arukwe & Goksoyr, 2003). The process of vitellogenesis can also be induced in juvenile and male Rainbow trout exposed to xenoestrogens (Celius et al., 2000; Thorpe et al., 2003b; Osachoff et al., 2013). Xenoestrogens are known to cause increased LSI (Schultz et al., 2003; Parrott & Blunt, 2005; Osachoff et al., 2013), due to increases in lipid deposition and hepatic mRNA and VTG protein production (Arukwe & Goksoyr, 2003; Cakmak, 2006). In the present study, exposure to 24 ng/L E1 (0.1 nM) altered estrogen-responsive parameters, including gene and VTG protein expression, but did not affect LSI. Thus, the induced estrogen response was not sufficient to increase liver weight through such accumulations, possibly reflecting the low dose of E1.

VTG protein has been used as a biomarker of xenoestrogen exposure in studies with feral or caged Rainbow trout to identify aquatic environments contaminated with estrogenic substances (Harries et al., 1996; Harries et al., 1997; Harries et al., 1999; Orrego et al., 2006; Ings et al., 2011). Since the concentration of E1 used in this study has been reported in surface waters, the identification and characterization of the time course of VTG protein inductions has relevance for the aquatic environment (Kolpin et al., 2002; Kolodziej et al., 2004; Soto et al., 2004; Jeffries et al., 2010; Furtula et al., 2012a). Plasma VTG protein concentrations were significantly elevated in E1-treated fish, with a magnitude of induction similar to those reported by others (Thorpe et al., 2003a). Concentrations of VTG protein peaked at d14, which was well into the recovery phase (7 d post-exposure). This delayed induction of VTG protein post-exposure indicates that when collecting fish in studies of potentially contaminated habitats, the time frame for sampling to detect VTG protein can be one week post-exposure to a low concentration of a moderately potent xenoestrogen such as E1. This information can be used to guide sampling timeframes for biomarkers in feral fish.

Plasma VTG protein concentrations returned to near baseline values after 7 d of recovery; therefore, the time frame to characterize VTG protein induction from a low concentration of E1 exposure was short. In studies with > 20-fold higher concentrations of E2, VTG protein clearance lasted weeks to months (Hemmer et al., 2002; Osachoff et al., 2013) and, in those studies, fish appeared to not recover from xenoestrogen exposure due to the length of VTG protein elevations. This study shows that VTG protein inductions can be slow to occur in Rainbow trout, and, once they reached a maximum, they appear to recover quickly (i.e. within days) from this concentration of E1. This is important information that indicates that when VTG protein inductions are found,

they may be attributed to relatively recent exposure, although the exact timing of the exposure cannot be determined from this endpoint alone.

Feral fish species may be exposed intermittently to xenoestrogens through pulses (e.g. rain events) or movement in and out of contaminated areas. The results presented here indicate that fish exposed for 7 d to 24 ng/L E1 can recover from xenoestrogen exposure at this concentration of E1 in approximately 7 d; however, exposures are always site-dependent. In some aquatic environments, xenoestrogens are chronically discharged from point sources such as STP effluents, which would not allow time for resident fish to recover but may only transiently affect migrating fish, with unknown implications for their fitness.

Vitellogenesis-related gene transcripts were evaluated daily to characterize their induction and recovery. The process of vitellogenesis is under estrogen receptor (ER) control and there are four known isoforms of hepatic ERs in Rainbow trout (Nagler et al., 2007). However, only *ER $\alpha$ 1* was responsive to E1 in the present study (Figure 5-2). Thus, the actions of E1 appear to be mediated by *ER $\alpha$ 1* and not the other ERs. This is supported by other studies with similar results using other estrogen hormones (Filby et al., 2007b; Osachoff et al., 2013; Shelley et al., 2013). The daily evaluation of egg-protein related mRNAs (*VTG* and *VEPs*) identified a 3 to 4 day delay in induction of these transcripts post-initiation of xenoestrogen treatment; however, *ER $\alpha$ 1* was significantly elevated at all timepoints during the exposure phase (Figure 5-2). Thus, *ER $\alpha$ 1* was a good indicator of E1 exposure. *VEP $\beta$*  and *VEP $\gamma$*  were also good indicators of E1 treatment since their responses gradually increased with E1 exposure (peaking at d7). When exposure ceased, *ER $\alpha$ 1* and all three *VEP* gene expression inductions were terminated (i.e. at d8, they returned to baseline levels). *VTG* transcript response was

quite different from the other estrogen-responsive genes because it was also elevated in the recovery phase (Figure 5-2). The rationale for the two phases of *VTG* transcript expression induction (d4 and d12 to d13) is not clear. In other studies, *VTG* and *VEPs* have been differentially responsive to estrogen hormones or estrogenic effluents; *VTG* is somewhat less sensitive to xenoestrogenic induction compared to *VEPs* (Arukwe et al., 1997; Celius et al., 2000; Thomas-Jones et al., 2003). The significant induction in *VTG* transcript levels were followed one day later by significant elevations in plasma *VTG* protein concentrations (Figures 5-1 and 5-2). These results support the current paradigm regarding these estrogen-responsive events; gene expression alterations occur first and are followed by protein production. The 24 h delay likely reflects the low E1 concentration and potency, as more potent xenoestrogens (i.e. EE2), or higher concentrations of E1, may initiate protein induction on the same day as transcript inductions (i.e. within hours of exposure) (Hemmer et al., 2002). The alterations of vitellogenesis-related transcripts (*ER $\alpha$ 1*, *VEP $\beta$*  and *VEP $\gamma$*  in particular) suggest that they are useful biomarkers for indicating current or immediate xenoestrogen exposure.

E1 induced alterations of 39 known gene transcripts within the hepatic transcriptome, which were identified as representative of a diverse array of biological processes (Table 5-2). Estrogen hormones are known to modulate the immune system (Wenger et al., 2011; Shelley et al., 2012; Shelley et al., 2013) and the alteration of 8 immune-related transcripts (Table 5-2) supported the findings from other studies in this regard (Benninghoff and Williams, 2008; Shelley et al., 2012; Wenger et al., 2012). Gene transcripts mainly involved in the acute phase immune response were altered, including mannose-binding lectin 2 C-type (*MBL2C*), complement factor H1 (*CFH*), serum amyloid A (*SAA*), ferritin (*FRIM*) and hepcidin (*HAMP*). *CFH* was significantly

elevated by E1-treatment in the present study, which was contrary to Wenger et al. (2012) who found E2 decreased *CFH* expression in Rainbow trout infected with bacteria. *HAMP* is a hepatic antimicrobial peptide of the innate immune system. As shown in marine medaka (*Oryzias melastigma*), *HAMP* was induced during bacterial challenge (Bo et al., 2011). Since *HAMP* was repressed in the E1-treated Rainbow trout, these fish may have a lowered ability to respond to bacterial infection, which would be similar to other studies reporting increased susceptibility to bacterial infection in xenoestrogen-treated Rainbow trout (Wenger et al., 2011; Shelley et al., 2012). Some significantly altered transcripts reflected commonly acknowledged medical issues related to human females. For example, low iron levels and anemia are a common issue, particularly due to estrogen hormone therapies. Anemia has been reported in carp exposed to xenoestrogens (Schwaiger et al., 2000). In the present study, ferritin (*FRIM*) and hepcidin (*HAMP*) were significantly repressed and they are involved in fish iron homeostasis. Possibly in a related issue, an increase in blood clots (or risk thereof) is a known issue for humans taking estrogen hormones therapeutically. In the present study, Coagulation Factor X (*FA10*) was induced in the E1-treated fish.

VTG protein is a phospholipoglycoprotein of considerable size and it is a relatively expensive protein to produce (Arukwe & Goksoyr, 2003). Producing copious amounts of VTG protein requires hepatic resources (Vijayan et al., 2001). Hepatic glycogen levels and lipid profiles are known to change with xenoestrogen exposure to provide the building blocks for VTG protein (Cakmak et al., 2006). As Cakmak et al. (2006) determined, E2 treatment led to an increased hepatic fatty acid composition. In the present study, the transcripts related to fatty acid metabolism were down-regulated (Acyl-CoA-binding protein (*ACBP*), Acyl-CoA desaturase (*ACOD*), Apolipoprotein A-IV

(*APOA4*), likely to enable the availability of fatty acids. Phosphoenolpyruvate carboxykinase (*PEPCK*) was also repressed by E1 treatment, similar to another study (Larkin et al., 2007). Gluconeogenesis was potentially down-regulated to enable cellular machinery to break down hepatic glycogen to provide glucose as a response to E1-treatment (Washburn et al., 1993; Cakmak et al., 2006).

The implications of altering *DDT4* (apoptotic signalling) and glycogen synthase kinase binding proteins (*GBP* and *GBP3*; cellular differentiation) are difficult to interpret given that they are singular representations of more complex pathways. Both apoptosis and cellular differentiation are known xenoestrogen-related issues in diseases such as cancer.

The characterization of phosvitinless *VTG* (both *Danio rerio* and *Pagrus major*, Table S5-1) will need further investigation as there are no reports of phosvitinless *VTG* in Rainbow trout. However, there are many reports of this *VTG* in other fish species (Sawaguchi et al., 2006). This may be an artifact due to the utilization of the Salem et al. (2010) transcriptome but it also could be the first report of a phosvitinless *VTG* transcript in Rainbow trout. The possibility of *VTG* variants was previously suggested in a related Oncorhynchid, Chinook salmon (Osachoff, 2008). QPCR analysis was performed in this study using primers designed to the lipovitellin area of the protein; thus, future research needs to discriminate between *VTG* variants and thereby elucidate potential differential expression of phosvitin-containing versus phosvitinless *VTG*.

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## Tables

**Table 5-1** *Quantitative polymerase chain reaction (QPCR) primer sequences, GenBank accession numbers, and literature sources.*

Gene transcript	Abbreviations	Accession #	Forward primer sequence	Reverse primer sequence	Primer Sequence Citation
vitellogenin	VTG	AJ011691	ATGAGAGCAGTAGTACTTG	TCTTGACACTCCCTGAGC	Osachoff et al. (2013)
vitelline envelope protein $\alpha$	VEPA / VEP $\alpha$	AF231706	CAACAGATACCCTACACCAA	TAATCAGTGTCTCAACGGAAG	
vitelline envelope protein $\beta$	VEPB / VEP $\beta$	AF231707	CTTCTGTCTTTGCCATCTAC	TGGTCAGGTAACGGTCAT	
vitelline envelope protein $\gamma$	VEPG / VEP $\gamma$	AF231708	AGACTGCCCTATGACTGGA	TATCTGGGATTGGCGTTTA	
estrogen receptor $\alpha$ 1	ERA1 / ER $\alpha$ 1	AJ242741	CCCTGCTGGTGACAGAGAGAA	ATCCTCCACCACCATTGAGACT	Nagler et al. (2007)
estrogen receptor $\alpha$ 2	ERA2 / ER $\alpha$ 2	DQ177438	GTGGCACTGCTGGTGACAAC	ACCACCGAAGCTGCTGTTCT	Nagler et al. (2007)
estrogen receptor $\beta$ 1	ERB1 / ER $\beta$ 1	DQ177439	CCCAAGCGGGTCCTAGCT	TCCTCATGTCCTTCTGGAGGAA	Nagler et al. (2007)
estrogen receptor $\beta$ 2	ERB2 / ER $\beta$ 2	DQ248229	CTGACCCCAGAACAGCTGATC	TCGGCCAGGTTGGTAAGTG	Nagler et al. (2007)
ribosomal protein L8	L8	AY957563	CAGGGGACAGAGAAAAGGTG	TGAGCTTTCTTGCCACAG	Shelley et al. (2013) or Osachoff et al. (2013)

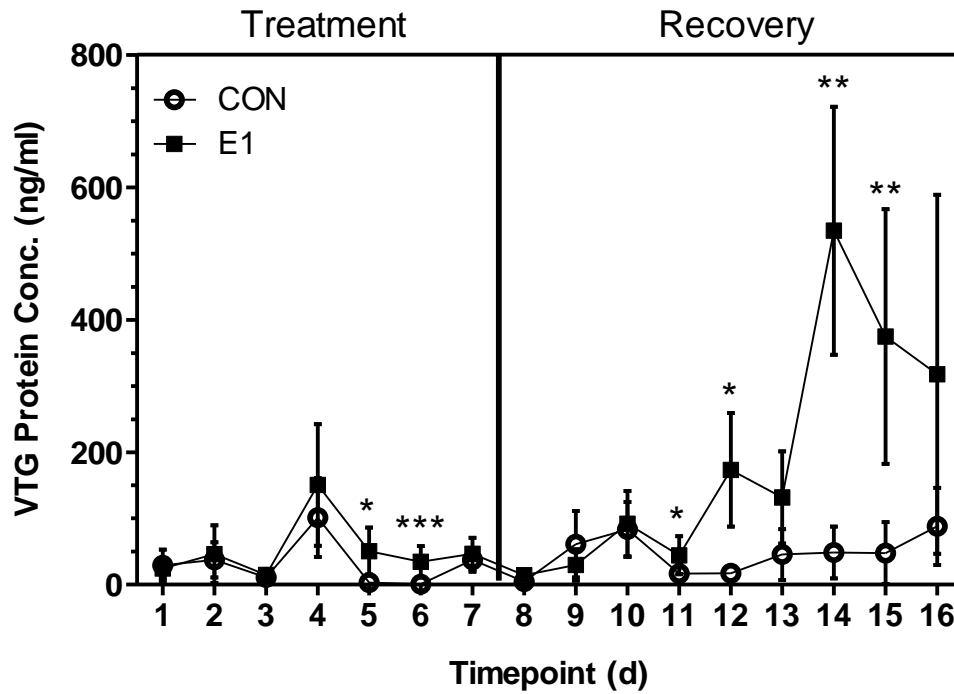
**Table 5-2** *Significant gene transcripts altered after 4 days in E1-treated Rainbow trout (compared to CON) determined with RNASeq analysis.*



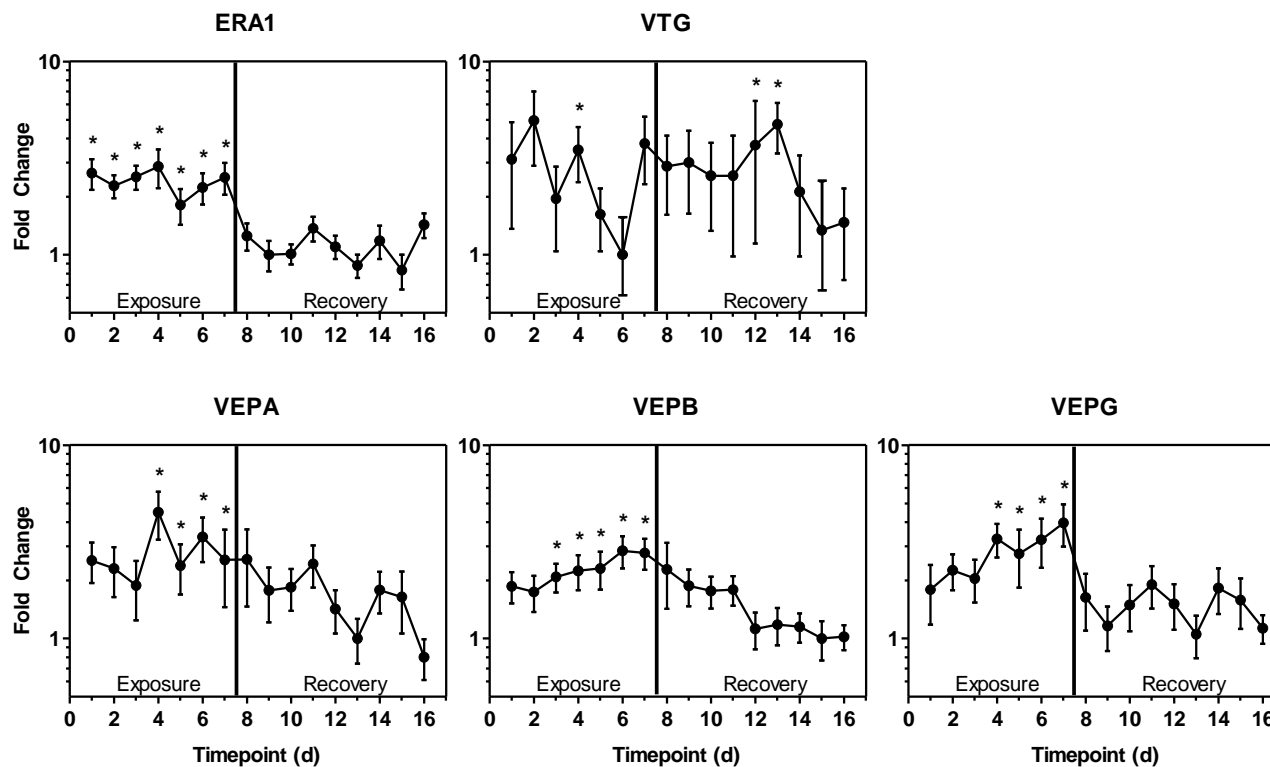
Gene Class, Function	Gene Transcript	Abbrev.	Accession #	GI
Apoptosis				
signalling	DNA-damage-inducible transcript 4	DDT4	ACO14087	225716482
signalling	DNA-damage-inducible transcript 4	DDT4	NP_956401	41056183
Cellular				
differentiation	Glycogen synthase kinase-3 binding protein	GBP3	ACO07805	225703918
differentiation	Glycogen synthase kinase binding protein	GBP	NP_001117918	185134290
Clotting				
blood coagulation	Coagulation factor X	FA10	ACN11213	223648910
DNA-related				
integration	Transposable element 1 Tcb1transposase	TCB1	ACN11475	223649434
integration	Transposase	TPase	CAB51372	5579035
transcription	cAMP responsive element modulator	CREM	EAW85920	119606326
transcription	Transcription factor CP2-like protein 1	TF2L1	ACN11169	223648822
reverse transcription	Pol-like protein	ORF2	BAC82623	34392581
Endocrine				
vitellogenesis	Phosvitin-less vitellogenin	VTG	AAG30407	11118642
vitellogenesis	Phosvitin-less vitellogenin	VTG	BAE43872	74149758
Immune				
innate system (complement)	Mannose-binding lectin 2, C-type	MBL2C	NP_001117900	185133386
inflammation or metabolism	Prostaglandin D synthase	PTGDS	ABY21331	162949438
acute phase response	Serum amyloid A	SAA	NP_001117908	185133765
unknown	Differentially regulated trout protein 1	DRTP1	NP_001118073	185132333
iron homeostasis, iron ion transport	Ferritin, middle subunit	FRIM	ACI69640	209737542
innate system or iron homeostasis	Hepcidin	HAMP	NP_001134321	213512933
innate system (complement)	Complement factor H1	CFH	NP_001117899	185133340
innate system (complement)	Complement factor H1	CFH	ABY55261	164422327
antigen presentation	H-2 class II histocompatibility antigen, A-B alpha chain	HA2B	ACO08490	225705288
Metabolism				
fatty acid/lipid	Acyl-CoA-binding protein	ACBP	ACI68322	209734906
amino acid	Kynurenine formamidase	KFase	NP_001134570	213511606
fatty acid/lipid	Acyl-CoA desaturase	ACOD	ACN11041	223648566
carbohydrate binding	Galectin-9	LEG9	ACI67845	209733952
gluconeogenesis	Phosphoenolpyruvate carboxykinase	PEPCK	ACN58684	224587565
lipoprotein	Apolipoprotein A-IV	APOA4	ACI68623	209735508
Vitamin D	Cytochrome P450 24A1	CYP24A	ACN11270	223649024
amino acid	Methylmalonate-semialdehyde dehydrogenase	MMSA	ACI34202	209155940
lipoprotein	Type-4 ice-structuring protein (antifreeze protein 4)	AFP4	ACI66601	209731464
Structural				
cytoskeleton	LIM domain and actin binding 1	LIMA-1	NP_001072345	118403487

Abbrev.	Fold Change	UniProt ID	Biological Function	Molecular Function					
DDT4	9.05	<a href="#">C1BY14</a>	<a href="#">negative regulation of signal transduction</a>	GO:0009968	<a href="#">apoptotic process</a>	GO:0006915			
DDT4	11.03	<a href="#">Q7T346</a>	<a href="#">negative regulation of signal transduction</a>	GO:0009968	<a href="#">apoptotic process</a>	GO:0006915			
GBP3	3.80	<a href="#">C1BFK2</a>	<a href="#">cell differentiation</a>	GO:0030154	<a href="#">Wnt receptor signaling pathway</a>	GO:0016055	<a href="#">protein serine/threonine kinase activity</a>	GO:0004674	<a href="#">tau-protein kinase activity</a>
GBP	10.81	<a href="#">C1BFK2</a>	<a href="#">cell differentiation</a>	GO:0030154	<a href="#">Wnt receptor signaling pathway</a>	GO:0016055	<a href="#">protein serine/threonine kinase activity</a>	GO:0004674	<a href="#">tau-protein kinase activity</a>
FA10	7.49	<a href="#">C0HAZ4</a>	<a href="#">blood coagulation</a>	GO:0007596	<a href="#">proteolysis</a>	GO:0006508	<a href="#">calcium ion binding</a>	GO:0005509	
TCB1	-22.12	<a href="#">B5X5A8</a>	<a href="#">DNA integration</a>	GO:0015074	<a href="#">transposition, DNA-mediated</a>	GO:0006313	<a href="#">DNA binding</a>	GO:0003677	<a href="#">transposase activity</a>
TPase	-14.15	<a href="#">Q9PW00</a>	<a href="#">DNA integration</a>	GO:0015074	<a href="#">transposition, DNA-mediated</a>	GO:0006313	<a href="#">DNA binding</a>	GO:0003677	<a href="#">transposase activity</a>
CREM	-6.68	<a href="#">Q03060</a>	<a href="#">transcription, DNA-dependent</a>	GO:0006351	<a href="#">cell differentiation</a>	GO:0030154	<a href="#">sequence-specific DNA binding</a>	GO:0003700	<a href="#">cAMP response element binding protein activity</a>
TF2L1	3.26	<a href="#">C0HAV0</a>	<a href="#">transcription, DNA-dependent</a>	GO:0006351			<a href="#">DNA binding</a>	GO:0003677	
ORF2	> 2815	<a href="#">Q76IK6</a>	<a href="#">RNA-dependent DNA replication</a>	GO:0006278			<a href="#">RNA binding</a>	GO:0003723	<a href="#">RNA-directed DNA polymerase activity</a>
VTG	3.20	<a href="#">Q9DFT9</a>	<a href="#">vitellogenesis</a>	GO:0007296	<a href="#">response to chemical stimulus</a>	GO:0042221	<a href="#">lipid transporter activity</a>	GO:0005319	
VTG	5.32	<a href="#">Q3V7A0</a>	<a href="#">vitellogenesis</a>	GO:0007296			<a href="#">lipid transporter activity</a>	GO:0005319	
MBL2C	-88.05	<a href="#">Q4LAN6</a>	<a href="#">carbohydrate binding</a>	GO:0030246					
PTGDS	-19.26	<a href="#">A9YVA4</a>	<a href="#">lipid metabolic process</a>	GO:0006629			<a href="#">small molecule binding</a>	GO:0036094	<a href="#">transporter activity</a>
SAA	-7.05	<a href="#">A6PZ09</a>	<a href="#">acute-phase response</a>	GO:0006953					GO:0005215
DRTP1	-3.84	<a href="#">Q9DFD5</a>	<a href="#">immune response</a>	GO:0006955					
FRIM	-12.35	<a href="#">P49947</a>	<a href="#">cellular iron ion homeostasis</a>	GO:0006879	<a href="#">iron ion transport</a>	GO:0006826	<a href="#">ferric iron binding</a>	GO:0008199	<a href="#">ferroxidase activity</a>
HAMP	-3.81	<a href="#">Q801Y3</a>	<a href="#">cellular iron ion homeostasis</a>	GO:0006879	<a href="#">defense response to bacterium</a>	GO:0042742	<a href="#">hormone activity</a>	GO:0005179	
CFH	4.53	<a href="#">Q4QZ18</a>	<a href="#">complement response</a>	GO:0006956					
CFH	5.07	<a href="#">B0FFJ7</a>	<a href="#">complement response</a>	GO:0006956					
HA2B	8.60	<a href="#">C1BHI7</a>	<a href="#">immune response</a>	GO:0006955	<a href="#">antigen processing and presentation</a>	GO:0002504			
ACBP	> -89	<a href="#">B5XBV2</a>	<a href="#">transport</a>	GO:0006810	<a href="#">triglyceride metabolic process</a>	GO:0006641	<a href="#">fatty-acyl-CoA binding</a>	GO:0000062	
KFase	-7.59	<a href="#">B5XB27</a>	<a href="#">tryptophan catabolic process to kynurenine</a>	GO:0019441			<a href="#">arylformamidase activity</a>	GO:0004061	
ACOD	-6.93	<a href="#">B5X1T0</a>	<a href="#">fatty acid biosynthetic process</a>	GO:0006633			<a href="#">iron ion binding</a>	GO:0005506	<a href="#">stearoyl-CoA 9-desaturase activity</a>
LEG9	-7.09	<a href="#">B5X9R4</a>					<a href="#">carbohydrate binding</a>	GO:0030246	
PEPCK	-4.33	<a href="#">B5X4B1</a>	<a href="#">gluconeogenesis</a>	GO:0006094			<a href="#">phosphoenolpyruvate carboxykinase activity</a>	GO:0004613	
APOA4	-3.01	<a href="#">B5XCQ3</a>	<a href="#">lipid transport</a>	GO:0006869	<a href="#">lipoprotein metabolic process</a>	GO:0042157	<a href="#">lipid binding</a>	GO:0008289	
CYP24A	-2.86	<a href="#">C0HB51</a>	<a href="#">vitamin D catabolic process</a>	GO:0042369	<a href="#">vitamin D receptor signaling pathway</a>	GO:0070561	<a href="#">1-alpha,25-dihydroxyvitamin D3 binding</a>	GO:0030342	<a href="#">iron ion binding</a>
MMSA	2.61	<a href="#">B5X4I4</a>	<a href="#">branched-chain amino acid catabolic process</a>	GO:0009083			<a href="#">fatty-acyl-CoA binding</a>	GO:0000062	<a href="#">methylmalonate-semialdehyde dehydrogenase activity</a>
AFP4	> 2815	<a href="#">B5XDA4</a>	<a href="#">lipid transport</a>	GO:0006869	<a href="#">lipoprotein metabolic process</a>	GO:0042157	<a href="#">lipid binding</a>	GO:0008289	
LIMA-1	> -89	<a href="#">Q7ZU52</a>					<a href="#">zinc ion binding</a>	GO:0008270	

## Figures

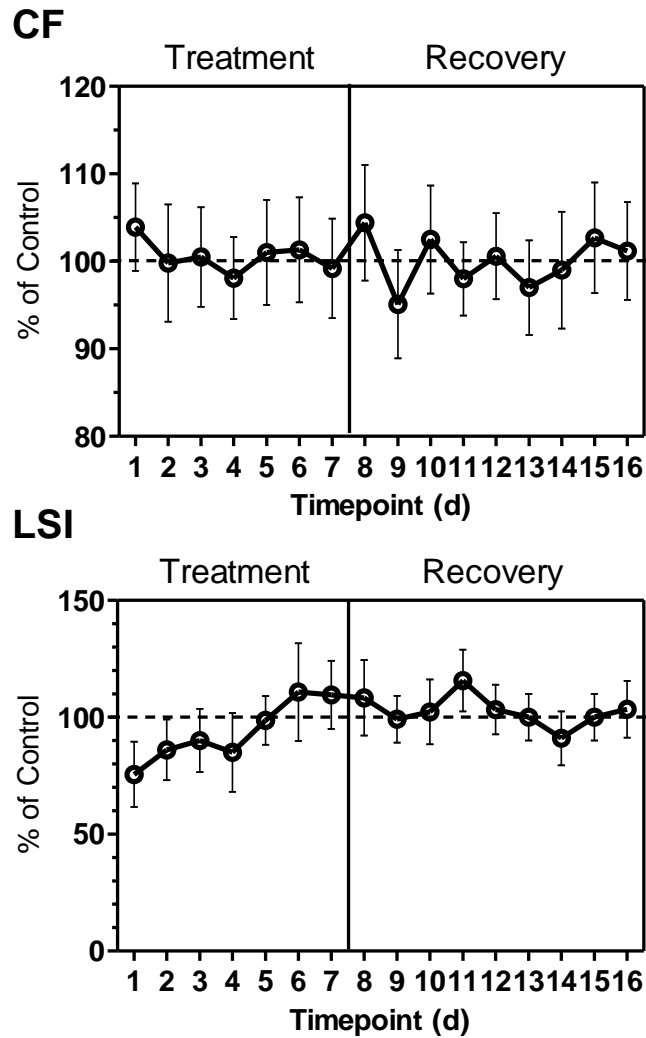


**Figure 5-1** Plasma vitellogenin (VTG) protein concentrations (mean  $\pm$  SEM; ng/ml) in the treatment phase (days 1 to 7) and the recovery phase (days 8 to 16) in juvenile Rainbow trout exposed to ethanol solvent control (CON; open-circles) or 24 ng/L of estrone (E1; black squares). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$



**Figure 5-2** Normalized gene expression levels from quantitative polymerase chain reaction (QPCR), shown as fold change of E1-treated versus control Rainbow trout, during the treatment phase (days 1 to 7) and the recovery phase (days 8 to 16). ERA1 = estrogen receptor  $\alpha$ 1, VTG = vitellogenin, VEPA = vitelline envelope protein  $\alpha$ , VEPB = vitelline envelope protein  $\beta$ , VEPG = vitelline envelope protein  $\gamma$ . Error bars shown are standard error of the mean ( $\pm$  SEM). \*  $p < 0.05$

## Supplementary Information



**Figure S5-1** Condition Factor (CF) and Liver Somatic Index (LSI) for E1-treated fish, each shown as % of Control (CON) for their respective day. No significant changes were found between CON and E1 at any timepoint using ANOVA, followed by Bonferroni's multiple test correction ( $p > 0.05$ ).

## Chapter 6.

# Evaluating the treatment of a synthetic wastewater containing a pharmaceutical cocktail: compound removal efficiency and effects on juvenile Rainbow trout

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## Abstract

Compounds that are pharmaceutical and personal care products (PPCPs) can evade degradation in sewage treatment plants (STPs) and can be chronically discharged into the environment, causing concern for aquatic organisms, wildlife, and humans that may be exposed to these bioactive chemicals. The ability of a common STP process, conventional activated sludge (CAS), to remove PPCPs (caffeine (CAF), di(2-ethylhexyl)phthalate (DEHP), estrone (E1), 17 $\alpha$ -ethinylestradiol (EE2), ibuprofen (IBPF), naproxen (NAP), 4-nonylphenol (NP), tonalide (TON), triclocarban (TCC) and triclosan (TCS)) from a synthetic wastewater was evaluated in this study. The removal of individual PPCPs by the laboratory-scale CAS treatment plant ranged from 40 – 99.6%. While the efficiency of removal for some compounds was high, remaining quantities have the potential to affect aquatic organisms even at low concentrations. Juvenile Rainbow trout (*Oncorhynchus mykiss*) were exposed to influent wastewater with methanol (IM, solvent control) or with PPCP cocktail (IC), or CAS-treated effluent wastewater with methanol (EM, treated control) or with PPCP cocktail (EC). Alterations in hepatic gene expression (evaluated using a quantitative nuclease protection plex assay) and plasma vitellogenin (VTG) protein concentrations occurred in exposed fish. Although there was partial PPCP removal by CAS treatment, the 20% lower VTG transcript levels and 83% lower plasma VTG protein concentration found in EC-exposed fish compared to IC-exposed fish were not statistically significant. Thus, effects on the fish found in the influent exposure were retained in the effluent exposure, even though typical percent removal levels were achieved. In summary, PPCP compound removal occurred in the CAS treatment process, however, remaining bioactive components had sufficient activity to alter estrogen-responsive endpoints in exposed fish.

## **Keywords**

Synthetic wastewater; conventional activated sludge; vitellogenin; Rainbow trout; gene expression



## Introduction

Pharmaceuticals and personal care products (PPCPs) are widely used for medicinal, domestic, agricultural, veterinary and industrial purposes. Wastes from some or all of these uses are collected and delivered as influent to municipal sewage treatment plants (STPs). STPs process a complex mixture of microorganisms and chemicals to generate a less toxic, nutrient- and microorganism-depleted effluent. Many PPCP compounds are eliminated from the wastewater stream during this treatment process, however, some exhibit limited reductions and are found in effluents and receiving environments (Onesios et al. 2009; Oulton et al. 2010). Examples of such compounds of emerging concern include tonalide, ibuprofen, naproxen, salicylic acid, carbamazepine, caffeine, triclosan, and estrogen hormones, all of which have been measured in North American STP effluents (Ternes et al., 1999; Metcalfe et al., 2003a; Brun et al., 2006; Barber et al., 2007; Carlson et al., 2013) and surface waters (Kolpin et al., 2002; Metcalfe et al., 2003b; Anderson et al., 2004; Jeffries et al., 2010; Lee et al., 2011; Barber et al., 2013). The presence of these compounds in the receiving environment is concerning due to their bioactive nature (Corcoran et al., 2010). They are often highly potent, and therefore, ultra-low concentrations may adversely affect unintended target organisms including wildlife and humans (Corcoran et al., 2010).

A widely recognized and international issue with STP effluents is that they can often be complex estrogenic mixtures with endocrine disrupting properties. The discharge of estrogenic sewage to the environment is considered a hazard to aquatic organisms (Corcoran et al., 2010) as studies have shown reproductive and other adverse effects (Jobling et al., 1998; Batty and Lim, 1999; Rodgers-Gray et al., 2001; Afonso et al., 2002; Vajda et al., 2008). The sources of estrogenic compounds in

effluents are solely anthropogenic and include natural and synthetic hormones, alkylphenol ethoxylates, plasticizers, phthalates and other organic compounds. Estrogen hormones are excreted from humans in their parent form, as oxidative metabolites, or conjugated to sulfate or glucuronide moieties (Larsson et al., 1999; Andersen et al., 2003; Khanal et al., 2007; Suarez et al., 2008; Shrestha et al., 2013). Interestingly, several conditions during the STP process can break conjugate bonds resulting in limited reductions or actual increases in parent hormone concentrations in final effluents (Andersen et al., 2003; Khanal et al., 2007; Suarez et al., 2008).

Since conventional treatment processes are not designed specifically for PPCP removal, additional treatment processes are needed, such as advanced oxidation or membrane filtration (Suarez et al., 2008; Omil et al., 2010; Oulton et al., 2010; Basile et al., 2013). Typically, treatment processes are implemented to reduce the concentrations of emerging contaminants substantially, such as by 90% (or the equivalent: 10 fold reduction or 1 log<sub>10</sub> amount; Oulton et al., 2010). While a target of 90% removal may be of substantial benefit, it may not be sufficient to protect aquatic life since residual contaminant concentrations may remain that cause adverse effects. A more effective strategy for the protection of aquatic life would be to design and implement treatment processes that achieve concentrations below specific chemical concentrations or water quality guidelines (if they exist). For example, in British Columbia, Canada, the water quality guideline for 17 $\alpha$ -ethinylestradiol (EE2) is 0.5 ng/L (BC Ministry of Environment, 2010). This strategy has not traditionally been utilized since most STPs have been operating for decades and the wealth of knowledge regarding contaminants is relatively new in this field, and also because water quality guidelines rarely exist for PPCPs. In Canada and Europe, in particular, advances are being made in this regard, as

regulations evolve to consider contaminants of emerging concern in relation to STP design and operation (CCME, 2012; Corominas et al., 2013).

One of the difficulties in identifying and developing effective wastewater treatment processes is the complex, concentrated nature of the influent wastewater (Furtula et al., 2012). Influent contains hundreds to thousands of chemicals. In order to evaluate treatment efficiencies for reductions in chemical concentrations between the influents, effluents, and various treatment processes, a comprehensive analysis of chemical composition must be performed. Several problems are associated with chemical analyses for these purposes: 1) analyses can be cost prohibitive, 2) chemicals may adsorb to solids or are present as metabolites in the wastewater, 3) some may co-elute during chemical analysis, and 4) resolution and sensitivity can be difficult to achieve due to matrix interferences. In the present study these issues were circumvented by using a synthetic wastewater and a specific mixture of pharmaceutical compounds of emerging concern (called the PPCP cocktail). The objectives of this study were to contribute to the growing knowledge of the ability of conventional activated sludge (CAS) to functionally reduce PCPP compounds and the effects of a mixture of PPCPs in fish, information useful for STP engineers and environmental risk assessors. A laboratory-scale CAS process, a widely-used STP type (Johnson & Sumpter, 2001; Johnson et al., 2005; Lishman et al., 2006), was used to treat the synthetic wastewater to assess the efficiency of PPCP concentration reductions. In addition, effects of the influents and the corresponding CAS-treated effluents on Rainbow trout (*Oncorhynchus mykiss*) molecular endpoints were examined.

## Materials and Methods

### *Synthetic Wastewater and Chemical Analysis*

Synthetic wastewater was prepared in the laboratory and contained non-pharmaceutical components (sources of carbon, nutrients and metals) to maintain the treatment biodegradation processes (Table 6-1A). The PPCP cocktail consisted of ten pharmaceuticals considered emerging contaminants of concern: caffeine (CAF), di(2-ethylhexyl)phthalate (DEHP), estrone (E1), 17 $\alpha$ -ethinylestradiol (EE2), ibuprofen (IBPF), naproxen (NAP), 4-nonylphenol (NP), tonalide (TON), triclocarban (TCC) and triclosan (TCS) (Table 6-1B). Target concentrations were chosen based on reported values for STP influents (see Appendix 1). All nutrients and pharmaceuticals were purchased from Sigma Aldrich (Oakville, ON, Canada), except tonalide, which was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Synthetic wastewater was processed using two laboratory-scale conventional activated sludge (CAS) wastewater treatment plants at the Department of Civil Engineering, University of British Columbia (UBC, Vancouver, BC, Canada). Each CAS plant was comprised of an aerobic bioreactor (fine bubble diffusers provided air) and two clarifiers. Activated sludge from a larger-scale pilot wastewater plant was seeded into laboratory-scale CAS plants once, approximately 2 months prior to the start of this study. One treatment plant was operated as a negative control and received only nutrients and methanol solvent (final concentration 0.005% v/v). This plant had synthetic wastewater influent plus methanol (influent methanol or IM treatment) and produced synthetic wastewater effluent containing methanol (effluent methanol EM treatment). A second treatment plant received synthetic wastewater influent as well as a PPCP cocktail prepared in methanol solvent (influent cocktail or IC treatment; final methanol

concentration of 0.005% v/v) and produced synthetic wastewater effluent containing PPCPs (effluent cocktail or EC treatment). The synthetic wastewater influents were prepared fresh each week and were added daily to the treatment systems. The treatment plants were pre-conditioned for approximately 3 solid retention times (SRTs) in accordance with conventional wastewater treatment systems. The PPCP mixture or methanol was added daily for 2 months prior to and during the collection of samples. Effluents were collected at a rate of 50 L per day per treatment plant.

Duplicate 1 L water samples were collected for each treatment type (IM, IC, EM, and EC treatments at 100% concentrations) and immediately analyzed for pharmaceutical concentrations using a gas chromatography mass spectrometry method (Samaras et al., 2011). Each control water sample was spiked with known amounts of each PPCP compound to obtain percent recovery data, which were applied to other water samples. Results are presented as the mean concentration of the duplicate samples, with the % removal of a compound calculated between the IC and EC treatment types using the calculation:  $(1 - (\text{concentration}_{\text{EC}}) / (\text{concentration}_{\text{IC}})) * 100$ .

### ***Fish Exposures***

Juvenile Rainbow trout (mass  $4.9 \pm 2.8$  g; fork length  $7.1 \pm 2.2$  cm) were obtained from Miracle Springs Trout Hatchery (Mission, BC, Canada) 10 weeks prior to initiation of exposures. Fish were maintained according to Environment Canada method EPS1/RM/09 (Environment Canada, 1990) in 200 L fibreglass tanks with flow-through well water at  $15 \pm 1$  °C under a 16:8 h light:dark photoperiod. Two sets of exposures were conducted 24 h apart to accommodate the production of appropriate volumes of

influent and effluent wastewater. In the first set, fish were exposed to (1) well water negative control (CON-1), (2) IM, and (3) IC. In the second set, fish were exposed to (1) well water negative control (CON-2), (2) EM, and (3) EC. Fish were randomly placed into triplicate 35 L glass aquaria containing each treatment, for a total of  $n = 15$  fish per treatment ( $n = 5$  fish per aquarium). Water quality parameters (conductivity, dissolved oxygen (DO), pH and temperature) were measured in each aquarium at experiment initiation and termination. Aquaria were checked daily for mortalities. After 4 d of exposure, fish were euthanized with buffered MS-222 (tricaine methanesulfonate; Syndel Labs, Vancouver, BC, Canada). Plasma was obtained by collecting blood from caudal vasculature in heparinized glass capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN, USA), which were spun in a micro-capillary centrifuge for 3 min (International Equipment Company, Chattanooga, TN, USA) and stored at  $-80\text{ }^{\circ}\text{C}$  until VTG protein analysis. Livers were collected and stored at  $-80\text{ }^{\circ}\text{C}$  in 1 ml of RNALater (Qiagen, Mississauga, ON, Canada) until analyzed for gene expression alterations.

### ***RNA Isolation and Gene Expression Analysis***

Hepatic total RNA was extracted using RNeasy mini extraction kits (Qiagen) including the on-column DNA digestion step. Each liver sample was thawed on ice (in RNALater), weighed ( $17.9 \pm 8.7$  mg), and homogenized in 700  $\mu\text{l}$  of Qiazol (Qiagen) in 1.5 ml Safe-lock tubes (Thermo Fisher Scientific, Toronto, ON, Canada), containing two 3 mm stainless steel beads (Retsch, Newtown, PA), on a TissueLyser machine (Qiagen) at 30 Hz for 6 min. Total RNA concentration was determined via  $A_{260}/A_{280}$  concentration analysis on a Nanodrop 1000 (Thermo Fisher Scientific). RNA integrity was evaluated

using the Experion Automated Electrophoresis System and an Experion RNA StdSens Analysis Kit (Bio-Rad, Mississauga, ON, Canada), according to the manufacturer's instructions. A portion of each total RNA sample (range was  $23.6 \pm 8.3 \mu\text{g}$ ) was sent to HTG Molecular Diagnostics, Inc. (Tucson, AZ, USA) for gene expression analysis using a quantitative nuclease protection assay (qNPA) ArrayPlate (Martel et al., 2002). For each treatment, 9 – 10 fish were analyzed, in duplicate, using a custom fish 47-gene plex (Table 6-2; full list provided in Supplementary Table S6-1). Gene transcripts were considered below detection or absent if there was no signal data (reported in relative light units (RLU)) for at least one third of samples. Normalizer gene candidates (*ribosomal protein S10* [S10], *acidic ribosomal protein* [ARP], *glyceraldehyde 3-phosphate dehydrogenase* [GAPDH], *hypoxanthine phosphoribosyltransferase* [HPRT1], *ribosomal protein L8* [L8], and *ribosomal protein L23A* [RPL23A]) were evaluated for statistical significance between treatments (described in section 2.5 below). The geometric mean of the normalizer gene transcripts not affected by treatment was used to normalize the gene expression data.

### **VTG Protein**

Protein vitellogenin (VTG) concentrations were determined in duplicate plasma samples ( $n = 9 - 11$ ), which were thawed on ice and kept cold, using a VTG ELISA kit (Biosense Laboratories, Abraxis, Warminster, PA, USA), as per the manufacturer's protocol. Microplates were read on a Bio-tek® PowerWave Reader (Bio-Tek, Winooski, VT, USA). Coefficients of variation (CVs) for the duplicate samples had a mean of 2.4% (range of 0.1 – 12.9%). The  $R^2$  values for the standard curves were all  $> 0.98$ .

Samples having VTG below detection were set to the detection limit of the kit for statistical purposes.

### ***Statistical Analysis***

GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Normality of data was checked using the Shapiro-Wilk's test. For parametric data, one-way ANOVAs were conducted, followed by Bonferroni's multiple test correction. If data were determined to be non-parametric, they were analyzed using a Kruskal-Wallis test, followed by Dunn's multiple test correction. Statistical significance was denoted by  $p < 0.05$ .

## **Results**

### ***Water Quality Parameters and Fish Mortalities***

IM and IC treatments resulted in immediate fish mortalities due to low DO levels ( $< 3$  mg/L). Therefore, a second set of exposures were performed with IM or IC diluted to 50% (w/w) in well water. The DO levels in the 50% IM and IC treatments ( $5.3 \pm 0.9$  and  $4.5 \pm 0.2$  mg/L, respectively) were initially lower than CON-1 DO levels ( $10.1 \pm 0.2$  mg/L) but no fish mortalities occurred in these treatments (Table 6-3). Plasma and liver samples from fish exposed to the 50% IM and IC treatments were collected and used in the gene expression and protein concentration analyses.



EM and EC DO levels ( $9.6 \pm 0.1$  and  $8.8 \pm 0.1$  mg/L, respectively) were very similar to CON-2 DO levels ( $10.0 \pm 0.2$  mg/L), yet there were 87% and 7% cumulative fish mortalities in these treatment groups over the 4-day exposure period. The EM treatment was omitted from molecular analyses because too few fish survived. There were no significant differences between treatments for final ( $t = 4$  d) water quality parameters (Table 6-3).

### ***Chemical Concentrations***

The achieved target IC pharmaceutical concentrations were 99.8 – 102.1% of nominal values for all compounds except NP, which was much higher at 143.7% (Table 6-1). All compounds were detected in the EC treatment (Table 6-1). The results for the % removed by the CAS process showed that there were two groups of compounds: (1) < 75% removed = DEHP, E1, EE2, IBPF, NAP; (2) > 90% removed = CAF, NP, TCC, TCS, TON. In the IM treatment (data not shown), CAF and DEHP were detected, with CAF measured at 10 ng/L (or 0.04% of the target concentration) and DEHP measured at 8520 ng/L (or 46% of the target concentration).

### ***Plex Gene Expression***

The influent data were normalized using *S10* and *GAPDH*, while the effluent data were normalized using *S10*, *GAPDH*, *ARP* and *RPL23A*. Five gene transcripts were below the detection limit: *estrogen receptor  $\alpha 2$* , *cytochrome p450 19b*, *thioredoxin*, *TATA box binding protein*, and *insulin-like growth factor 1*. In the IM treatment, four mRNAs

had significantly altered expression, while the IC and EC treatments each had seven significantly altered transcripts (Table 6-2). The significantly altered IM gene transcripts consisted of two endocrine mRNAs (*estrogen receptor  $\alpha$ 1 (ERA1)* and *high density lipoprotein binding protein (vigilin; HDLBP)*) and two mRNAs involved in oxidative stress (*peroxisome proliferator activated receptor  $\gamma$  (PPARG)* and *acyl-coenzyme A oxidase 1 (ACOX1)*). Both PPCP cocktail treatments (IC and EC) had similar gene expression profiles with five gene transcripts commonly impacted (Table 6-2): *ERA1*, *vitelline envelope protein  $\alpha$  (VEPA)*, *envelope protein  $\beta$  (VEPB)*, *vitelline envelope protein  $\gamma$  (VEPG)*, and *vitellogenin (VTG)*. IC treatment uniquely impacted *estrogen receptor  $\beta$ 1 (ERB1)* and *HDLBP*, while EC treatment uniquely impacted *cytochrome p450 1a1 (CYP1A1)* and *estrogen receptor  $\beta$ 2 (ERB2)*.

Normalized *VTG* transcript levels are shown for each treatment (Figure 6-1A). The change in *VTG* gene expression between: CON-1 and IM was -1.6 fold ( $p > 0.05$ ); CON-1 and IC was 30.1 fold ( $p < 0.01$ ); and CON-2 and EC was 24.0 fold ( $p < 0.001$ ). CON-1 and CON-2 did not significantly differ ( $p > 0.05$ ), so the *VTG* transcript levels in EC and IC treatments were compared directly and were found not significantly different ( $p > 0.05$ ). Since that was a comparison of 50% IC and 100% EC treatments, the same evaluation was performed using twice the amount of *VTG* transcript in the IC treatment (to approximate 100% IC levels), and the result remained non-significant ( $p > 0.05$ ). Based on the fold change values in EC and IC treatments (24.0 and 30.1 fold change, respectively), there was 20% less induction of *VTG* in the EC treatment compared to the IC treatment (not significant based on the above comparison of their transcript levels).

## ***VTG Protein***

Plasma VTG protein concentrations were significantly higher in fish from the IC ( $p < 0.001$ ; 90 fold) and EC ( $p < 0.01$ ; 15.4 fold) treatments compared to their respective control groups; concentrations in fish from CON-1, IM and CON-2 groups were not significantly different from each other ( $p > 0.05$ ; Figure 6-1B). To determine the difference in induction of VTG protein between EC and IC treatments, their VTG protein levels were compared directly and were found not significantly different ( $p > 0.05$ ). Since that was a comparison of 50% IC and 100% EC treatments, the same evaluation was performed using twice the amount of VTG protein in the IC treatment (to approximate 100% IC levels), and the result remained non-significant ( $p > 0.05$ ). Based on the respective fold change values in the EC and IC treatments (15.4 and 90.0 fold), there was 83% less induction of VTG protein (not significant,  $p > 0.05$  as calculated above) in the EC treatment compared to the IC treatment (i.e. due to the removal of compounds by the CAS process).

## **Discussion**

A synthetic wastewater with the addition of a PPCP cocktail was used to evaluate the pharmaceutical removal capacity of CAS treatment and the resulting biological impacts of influent and CAS-treated effluent using molecular and biochemical analyses of Rainbow trout tissues. Chemical analysis revealed that 40 to 99.6% of the compounds were eliminated by CAS treatment, however, corresponding reductions in gene and protein expression levels in EC compared to IC treatments were not seen. This demonstrated that the successful removal of the majority of PPCP compounds by

treatment may be of little toxicological consequence since even low concentrations of PPCPs in effluents can cause significant effects in fish at the molecular level.

The CAS treatment in the present study appeared to operate similarly to full-scale treatment plants. The percent removal values for eight compounds (CAF, E1, EE2, IBPF, NAP, NP, TON, TCC, TCS) used in this study (Table 6-1) were all within range of removal of other CAS STP values (Heidler et al., 2006; Oulton et al., 2010). In a study evaluating percent reduction values for seven Canadian CAS STPs, the median percent reduction of IBPF or NAP was 95% and 93%, respectively, which are higher than the 68.6% and 40.0% found in the present study (Lishman et al., 2006). However, the removal values of E1 and TCS in this study were similar to that study (Lishman et al., 2006). CAF removal (99.5%) was also equivalent to that found at the secondary effluent stage and final effluent from a Michigan tertiary treatment plant (Gao et al., 2012). For DEHP, removal of 61.7% was lower than 78% or 97% reported by Dargnat et al. (2009) and Marttinen et al. (2003), respectively. Even though there was a moderate to large reduction in each of the ten PPCP compounds in influent compared to effluents, measurable quantities still remained in effluents as reported in other studies (Ternes et al., 1999; Spengler et al., 2001; Metcalfe et al., 2003a; Brun et al., 2006; Heidler et al., 2006; Liebig et al., 2006; Barber et al., 2007; Dargnat et al., 2009). CAS is one of the widest used STP technologies in the world (Johnson & Sumpter, 2001), and these percent removal results, and remaining PPCP concentrations, give further evidence of the limited usefulness of CAS to treating wastewater containing PPCP compounds (Oulton et al., 2010). In addition, these results suggest that subsequent treatment of CAS effluent is needed, which could be provided by technologies such as advanced

oxidation or membrane filtration (Johnson & Sumpter, 2001; Westerhoff et al., 2005; Filby et al., 2010; Gerrity et al., 2010; Oulton et al., 2010; Yang et al., 2011).

VTG protein has been widely documented as a biomarker of exposure to xenoestrogens (Sumpter & Jobling, 1995). The levels in the C-1, IM and C-2 treatments were similar to concentrations in immature or male untreated or control fish in another study (Osachoff et al., 2013). When the VTG protein concentrations (Figure 6-1) were compared to literature values for individual xenoestrogens, they were lower than would be expected (Thorpe et al., 2003; Van Den Belt et al., 2004). For example, 6 ng/L EE2 alone has been shown to result in plasma VTG protein concentrations as high as 6000 µg/mL in trout (flow-through design, 14 d exposure), which is approximately 1000-fold higher than this study (Thorpe et al., 2003). However, these results may not be comparable due to the short duration of the present study. When the concentrations of individual estrogenic compounds in the IC and EC treatments were compared to known EC50 values (median effective concentration) for VTG protein induction, EE2, E1 and NP concentrations in the IC treatment were  $\geq$  EC50 values for VTG protein induction, while only EE2 was present at the EC50 value for VTG protein induction in the EC treatment (Thorpe et al., 2003; Sumpter et al., 2005). Thus, the VTG protein induction in the IC treatment was likely produced by the mixture of estrogenic compounds, while in the EC treatment, VTG protein induction was also likely a result of the presence of a mixture of compounds but probably driven by the concentration of EE2.

Significant inductions of *VTG* transcript and protein were seen in the IC and EC treatments (Figure 6-1) strongly indicating that both of the wastewaters were estrogenic. The VTG protein concentration in the IC treatment (8875 ng/ml) was approximately 15 fold greater than the VTG protein concentration in the EC treatment (597 ng/ml; Figure

6-1B). Looking at the estrogenic contents of the wastewaters, the IC treatment contained a mixture of four known estrogenic compounds: DEHP, E1, EE2 and NP (Table 6-1). The EC treatment was largely a mixture of three estrogenic compounds (DEHP, E1 and EE2) because NP was nearly completely removed (99.6%). The IC treatment contained estrogenic compounds at 2.6, 3.5, 4.0 and 248-fold higher concentrations (for DEHP, E1, EE2 and NP, respectively) than the EC treatment (Table 6-1). However, the estrogenicity of the treatments would have been a summation of all the estrogenic compounds and their related potencies (Thomas-Jones et al., 2003; Thorpe et al., 2003; described by concentration addition theory (Brian et al., 2005; Thorpe et al., 2006)), which would not be accounted for if only each compound's individual concentration was assessed. Thus, the finding that the IC treatment had 15 fold more VTG protein than the EC treatment was a better indicator of each mixture's estrogenicity than the individual chemical concentrations.

Gene transcripts were significantly altered in the IM, IC and EC treatments (Table 6-2). The IM treatment had four mRNAs with significant alterations, two of which were shared by the IC and/or EC treatments (endocrine gene transcripts: *ERA1* and *HDLBP*; Table 6-2), so possibly the presence of DEHP in the IM treatment had some impact on the fish. DEHP is a known xenoestrogen but much less potent than the other estrogenic substances used (Metcalf et al., 2001; Legler et al., 2002) and the levels in the IM treatment (8.5 µg/L) were well below the maximum concentration studied (5000 µg/L) in Japanese medaka (*Oryzias latipes*) that had no estrogenic effects (although gene expression endpoints were not evaluated in that study; Metcalfe et al., 2001). The other mRNAs altered in the IM treatment were *ACOX1* and *PPARG*, which are both in the fatty acid beta-oxidation pathway. The endocrine pathway predominantly affected by the

synthetic wastewater containing PPCPs was estrogen-related fish vitellogenesis, the process of producing eggs in adult females. All transcripts involved in this process, *ERA1*, *VEPA*, *VEPB*, *VEPG* and *VTG* (Arukwe & Goksoyr, 2003), were significantly elevated in the IC and EC treatments. Although there were some differences in the degree of estrogenicity of the IC and EC treatments, they were relatively small considering the quantity of xenoestrogens removed by CAS treatment. Typically, xenoestrogens induce higher hepatic *ERA* levels but not *ERB* mRNA levels (Sabotwood et al., 2004; Filby & Tyler, 2005; Filby et al., 2006; Shelley et al., 2013; Osachoff et al., 2013), which was confirmed in this study. *VEPs* are known to be sensitive indicators of xenoestrogen exposure and they may be differentially responsive to xenoestrogens (Arukwe, 1997; Celius et al., 2000; Ings et al., 2011). Rainbow trout exposed to EE2 at concentrations matching those evaluated in this study (1.5 ng/L and ~ 6 ng/L) induced the hepatic *VEPs* in the order of *VEPB* > *VEPA* > *VEPG* (Thomas-Jones et al., 2003) and at higher levels than *VTG* transcript. In this study, all three *VEPs* were induced by exposure to the IC and EC treatments (*VEPA* > *VEPG* > *VEPB*), albeit at lower levels than *VTG* transcript. Possibly these differences in *VEP* alterations reflect the presence of multiple xenoestrogens in the wastewater mixtures.

Targeting the complete absence of all PPCPs in STP effluents may not be a reasonable or achievable goal (Oulton et al., 2010). However, the goal of substantial reduction or near-zero for some compounds might be a necessity to protect wildlife and humans. Natural or synthetic estrogen hormones would fall into the category of compounds to strive for near-zero content in STP effluents since they are extremely potent and have well-documented adverse effects (Madsen et al., 2004; Werner et al., 2011; Shelley et al., 2012). Since it was found that exposure to 5-6 ng/L EE2, an

environmentally-relevant concentration in other parts of the world (Kolpin et al., 2002; Barber et al., 2013), in an experimental lake caused collapse of the fathead minnow fish population after two years, EE2 in particular has been acknowledged as a contaminant of significant concern (Kidd et al., 2007). As shown in this study, while % removal values appeared high (up to 99.6%; Table 6-1), the resulting effluent retained sufficient pharmaceutical content to cause effects, which were primarily estrogen-related. As suggested, individual chemical concentrations may or may not be measured and yet effluents may still exhibit biological effects, which ultimately are the most important indicators of complex mixture toxicity (Escher et al., 2011). Therefore, it is imperative that suitable treatment technologies be identified that remove all (> 99.9%) estrogen hormone content to severely limit the estrogenicity of an effluent in order to protect wild fish populations and other aquatic organisms (Filby et al., 2010).

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## Tables

**Table 6-1**

**A. List of nutrients and non-pharmaceuticals added to create a synthetic sewage. B. List of pharmaceutical cocktail compounds, including target concentrations and actual measured concentrations in Influent wastewater plus PPCP Cocktail (IC) and Effluent wastewater with PPCP Cocktail (EC), and the % Removed by the conventional activated sludge (CAS) process.**

A.		Influent Conc. (mg/L)	
Yeast extract		28	
Glucose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> )		42	
Starch (C <sub>6</sub> H <sub>10</sub> O <sub>5</sub> ) <sub>n</sub>		62	
Sodium acetate anhydrous (NaCH <sub>3</sub> COO)		180	
Monopotassium phosphate (KH <sub>2</sub> PO <sub>4</sub> )		21	
Sodium bicarbonate (NaHCO <sub>3</sub> )		122	
Ammonium chloride (NH <sub>4</sub> Cl)		100	
Ferric chloride (FeCl <sub>3</sub> )		6	
Magnesium chloride (MgCl <sub>2</sub> •6H <sub>2</sub> O)		24	
Calcium chloride (CaCl <sub>2</sub> •2H <sub>2</sub> O)		36	
Zinc sulphate (ZnSO <sub>4</sub> •7H <sub>2</sub> O)		0.2	

Pharmaceutical Cocktail	Abbreviation	Target Influent Cocktail (ng/L) <sup>1</sup>	Actual % Recovery of Spikes	Actual Influent Cocktail (IC) (ng/L)	% of Target	Effluent Cocktail (EC) (ng/L)	% Removed by CAS
Caffeine	CAF	45076	79	45579	(101.1%)	250	99.5%
Di(2-ethylhexyl)phthalate	DEHP	40000	85	40609	(101.5%)	15565	61.7%
Estrone	E1	70.0	95	70.3	(100.4%)	20	71.6%
17α-Ethinylestradiol	EE2	6.0	118	6.0	(100.3%)	1.5	75.1%
Ibuprofen	IBPF	27022	81	27600	(102.1%)	8667	68.6%
Naproxen	NAP	15025	80	15000	(99.8%)	9000	40.0%
4-Nonyphenol	NP	21580	75	31000	(143.7%)	125	99.6%
Tonalide	TON	1000	64	1017	(101.7%)	25	97.5%
Triclocarban	TCC	3005	62	3000	(99.8%)	73	97.6%
Triclosan	TCS	3005	73	3000	(99.8%)	250	91.7%

<sup>1</sup> Appendix 2 Figures show derivations of target influent cocktail concentrations.



**Table 6-2 HTG multi-plex genes, with the GenBank accession number of the probe, normalized hepatic expression results (fold change  $\pm$  SEM compared to the well water control) for Influent Methanol (IM), Influent Cocktail (IC) or Effluent Cocktail (EC) exposed Rainbow trout. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$**

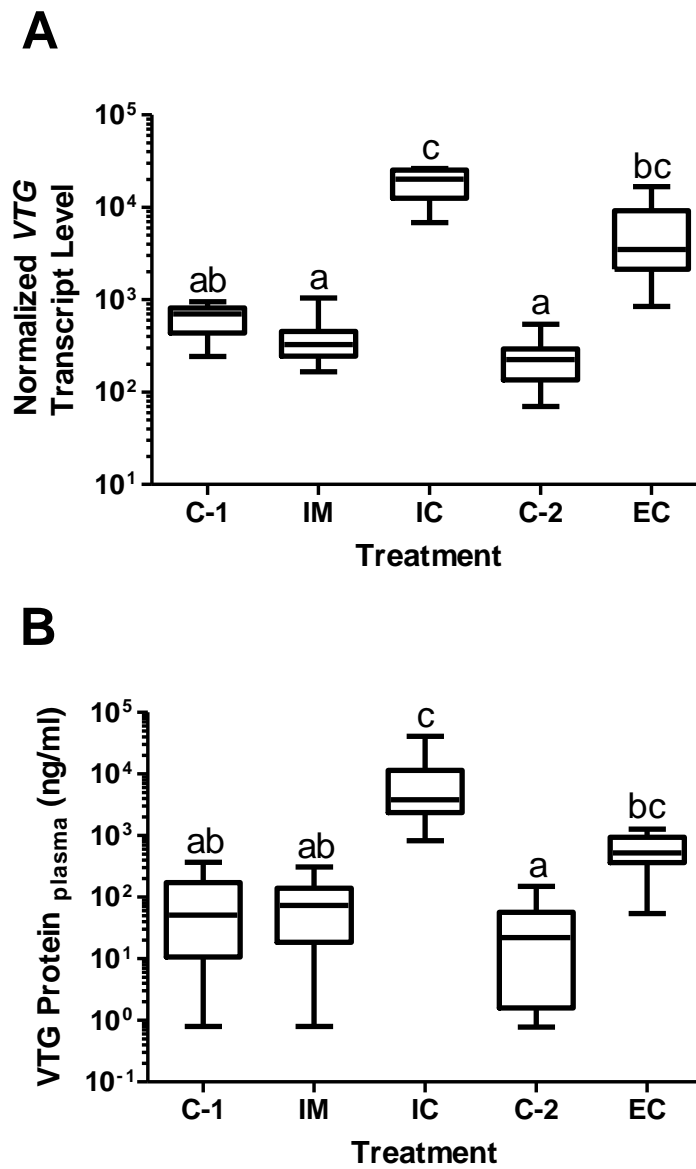
Class	Gene	Abbreviation	GenBank Accession #	IM	IC	EC
<i>Aryl Hydrocarbon</i>						
	Aryl Hydrocarbon Receptor $\alpha$	AHRA	NM_001124251	1.17 $\pm$ 0.05	1.01 $\pm$ 0.04	1.24 $\pm$ 0.08
	Aryl Hydrocarbon Receptor $\beta$	AHRB	NM_001124252	1.09 $\pm$ 0.08	-1.04 $\pm$ 0.07	-1.08 $\pm$ 0.06
	Aryl Hydrocarbon Nuclear Translocator	ARNT	NM_001124710	1.23 $\pm$ 0.08	1.19 $\pm$ 0.06	1.12 $\pm$ 0.05
<i>Biotransformation, Detoxification or Oxidative Stress</i>						
	Acyl-coenzyme A Oxidase 1	ACOX1	GU128074	<b>1.39 **</b> $\pm$ 0.09	1.27 $\pm$ 0.07	1.16 $\pm$ 0.09
	Catalase	CAT	FJ226371 / FJ226382	-1.08 $\pm$ 0.07	-1.22 $\pm$ 0.04	1.27 $\pm$ 0.14
	Cytochrome p450 1a1	CYP1A1	AF059711	1.08 $\pm$ 0.20	1.49 $\pm$ 0.22	<b>2.07 *</b> $\pm$ 0.34
	Cytochrome p450 2k1	CYP2K1	L11528	-1.01 $\pm$ 0.13	1.01 $\pm$ 0.13	1.04 $\pm$ 0.12
	Cytochrome p450 2k5	CYP2K5	AF151524	1.14 $\pm$ 0.09	-1.15 $\pm$ 0.07	1.20 $\pm$ 0.15
	Cytochrome p450 2m1	CYP2M1	U16657	1.04 $\pm$ 0.08	1.03 $\pm$ 0.07	1.07 $\pm$ 0.07
	Cytochrome p450 3a27	CYP3A27	U96077	1.02 $\pm$ 0.07	-1.04 $\pm$ 0.07	1.13 $\pm$ 0.10
	Metallothionein	MT1A	M18103	-1.33 $\pm$ 0.09	-1.09 $\pm$ 0.11	1.16 $\pm$ 0.12
	Peroxisome Proliferator Activated Receptor $\alpha$	PPAR $\alpha$	NM_001197211 / DQ294237	1.22 $\pm$ 0.10	1.34 $\pm$ 0.13	1.28 $\pm$ 0.12
	Peroxisome Proliferator Activated Receptor $\beta$	PPAR $\beta$	NM_001197207 / AJ416953	1.01 $\pm$ 0.07	-1.03 $\pm$ 0.08	1.10 $\pm$ 0.10
	Peroxisome Proliferator Activated Receptor $\gamma$	PPAR $\gamma$	NM_001197212 / AJ292962	<b>1.48 *</b> $\pm$ 0.15	1.43 $\pm$ 0.14	1.27 $\pm$ 0.16
	Pregnane X Receptor	PXR	NM_001124672	1.08 $\pm$ 0.08	1.18 $\pm$ 0.06	1.26 $\pm$ 0.10
	Retinoid X Receptor	RXR	AJ969439	1.12 $\pm$ 0.06	-1.01 $\pm$ 0.05	1.01 $\pm$ 0.06
	Super Oxide Dismutase	SOD	NM_001160614 / NM_001124	1.24 $\pm$ 0.07	1.10 $\pm$ 0.07	1.24 $\pm$ 0.13
<i>Endocrine</i>						
	Androgen Receptor $\alpha$	ARA	AB012095	1.03 $\pm$ 0.06	1.09 $\pm$ 0.07	-1.04 $\pm$ 0.07
	Androgen Receptor $\beta$	ARB	AB012096	1.07 $\pm$ 0.06	1.05 $\pm$ 0.04	1.22 $\pm$ 0.10
	Estrogen Receptor $\alpha$ 1	ERA1	AJ242741	<b>2.02 **</b> $\pm$ 0.19	<b>3.87 ****</b> $\pm$ 0.36	<b>2.79 ****</b> $\pm$ 0.28
	Estrogen Receptor $\beta$ 1	ERB1	DQ177438	-1.23 $\pm$ 0.10	<b>-1.53 *</b> $\pm$ 0.09	1.40 $\pm$ 0.13
	Estrogen Receptor $\beta$ 2	ERB2	DQ177439	1.14 $\pm$ 0.08	-1.06 $\pm$ 0.05	<b>1.36 *</b> $\pm$ 0.13
	High Density Lipoprotein Binding Protein (Vigilin)	HDLBP	NM_001173653	<b>1.34 **</b> $\pm$ 0.07	<b>1.42 ***</b> $\pm$ 0.05	1.00 $\pm$ 0.04
	3 $\beta$ -Hydroxy Steroid Dehydrogenase	HSD3B1	S72665	1.16 $\pm$ 0.11	1.18 $\pm$ 0.11	-1.03 $\pm$ 0.10
	Steroidogenic Factor 1	SF1	AB038151	1.15 $\pm$ 0.07	1.15 $\pm$ 0.06	1.14 $\pm$ 0.07
	Thyroid Hormone Receptor $\alpha$	THRA	AF302245	-1.04 $\pm$ 0.08	-1.05 $\pm$ 0.07	1.07 $\pm$ 0.12
	Thyroid Hormone Receptor $\beta$	THRB	AF302246	1.29 $\pm$ 0.08	1.12 $\pm$ 0.06	1.41 $\pm$ 0.15
	Vitellogenin Envelope Protein $\alpha$	VEPA	AF231706	1.11 $\pm$ 0.15	<b>11.45 ****</b> $\pm$ 1.29	<b>15.11 ****</b> $\pm$ 2.62
	Vitellogenin Envelope Protein $\beta$	VEPB	AF231707	1.57 $\pm$ 0.17	<b>1.70 *</b> $\pm$ 0.20	<b>2.24 ***</b> $\pm$ 0.18
	Vitellogenin Envelope Protein $\gamma$	VEPG	AF231708	1.73 $\pm$ 0.33	<b>3.68 ***</b> $\pm$ 0.63	<b>4.58 ***</b> $\pm$ 0.98
	Vitellogenin	VTG	AJ011691	-1.58 $\pm$ 0.11	<b>30.11 **</b> $\pm$ 3.66	<b>24.04 ***</b> $\pm$ 6.12
<i>Growth</i>						
	Insulin-like Growth Factor Binding Protein 1	IGFBP1	DQ190460	-1.05 $\pm$ 0.07	-1.34 $\pm$ 0.07	-1.02 $\pm$ 0.09
	Insulin-like Growth Factor Binding Protein 2	IGFBP2	DQ146968	1.18 $\pm$ 0.07	1.10 $\pm$ 0.05	1.17 $\pm$ 0.08
	Insulin-like Growth Factor Binding Protein 3	IGFBP3	DQ146966	1.15 $\pm$ 0.08	1.18 $\pm$ 0.06	1.25 $\pm$ 0.07
<i>Immune</i>						
	Hepcidin	HAMP	AF281354 / EL553897	1.19 $\pm$ 0.41	1.23 $\pm$ 0.39	-2.99 $\pm$ 0.14
	Signal Transducer/Activator of Transcription 3	STAT3	U60333	1.05 $\pm$ 0.11	-1.14 $\pm$ 0.08	-1.20 $\pm$ 0.08

**Table 6-3** Water quality parameters (dissolved oxygen (D.O.), conductivity, pH, and temperature), shown as mean  $\pm$  SEM of replicate aquaria, in each treatment at experiment initiation ( $t = 0$ ) and termination ( $t = 4$  d).

Treatment	Time	D.O. (mg/L)	Conductivity ( $\mu$ S)	pH	Temp. ( $^{\circ}$ C)
Well Water Control 1 (CON-1)	$t = 0$	10.1 $\pm$ 0.2	417 $\pm$ 6	7.57 $\pm$ 0.02	14.8 $\pm$ 0.3
	$t = 4$ d	9.6 $\pm$ 0.3	<i>nm</i>	7.91 $\pm$ 0.05	15.1 $\pm$ 0.1
Influent Methanol (IM)	$t = 0$	5.3 $\pm$ 0.9	543 $\pm$ 6	7.12 $\pm$ 0.03	14.9 $\pm$ 0.1
	$t = 4$ d	9.5 $\pm$ 0.1	<i>nm</i>	8.21 $\pm$ 0.04	14.8 $\pm$ 0.6
Influent Cocktail (IC)	$t = 0$	4.5 $\pm$ 0.2	567 $\pm$ 6	7.04 $\pm$ 0.04	14.8 $\pm$ 0.1
	$t = 4$ d	9.0 $\pm$ 0.4	<i>nm</i>	8.08 $\pm$ 0.07	15.2 $\pm$ 0.1
Well Water Control 2 (CON-2)	$t = 0$	10.0 $\pm$ 0.2	440 $\pm$ 10	7.58 $\pm$ 0.02	14.9 $\pm$ 0.3
	$t = 4$ d	8.4 $\pm$ 0.3	<i>nm</i>	7.61 $\pm$ 0.03	15.1 $\pm$ 0.1
Effluent Methanol (EM)	$t = 0$	9.6 $\pm$ 0.1	520 $\pm$ 0	6.94 $\pm$ 0.02	15.0 $\pm$ 0.0
	$t = 4$ d	9.7 $\pm$ 0.5	<i>nm</i>	7.24 $\pm$ 0.10	15.0 $\pm$ 0.2
Effluent Cocktail (EC)	$t = 0$	8.8 $\pm$ 0.1	510 $\pm$ 0	7.24 $\pm$ 0.01	15.0 $\pm$ 0.1
	$t = 4$ d	9.3 $\pm$ 0.4	<i>nm</i>	7.68 $\pm$ 0.08	15.1 $\pm$ 0.1

*nm* = not measured

## Figures



**Figure 6-1** *A. Normalized hepatic vitellogenin (VTG) transcript levels, or B. Plasma VTG protein concentrations were significantly elevated in Influent Cocktail (IC) and Effluent Cocktail (EC) treatments, but not in the Influent Methanol (IM) treatment, compared to Control (C-1 for IM/IC, or C-2 for EC) after 4 days of exposure in Rainbow trout. Different letters denote significant differences,  $p < 0.05$ .*

## Supplementary Information

**Table S6-1** *HTG 47-plex gene transcript info.*

#	Gene ID	Gene Name	Genbank Accession #	Biological System
1	ACOX1	Acyl-coenzyme A oxidase 1	GU138074	Oxidative stress
2	AHRA	Aryl hydrocarbon receptor alpha	NM_001124251	Biotransformation
3	AHRB	Aryl hydrocarbon receptor beta	NM_001124252	Biotransformation
4	ARA	Androgen receptor alpha	AB012095	Endocrine - androgen
5	ARB	Androgen receptor beta	AB012096	Endocrine - androgen
6	ARNT	Aryl hydrocarbon nuclear translocator	NM_001124710	Biotransformation
7	ARP	Ribosomal phosphoprotein P0	AY255630 / AY255631	Reference
8	CAT	Catalase	FJ226371 / FJ226382	Oxidative stress
9	CYP19B	Cytochrome P450, family 19, subfamily B	AJ311938	Endocrine
10	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	AF059711	Biotransformation
11	CYP2K1	Cytochrome P450, family 2, subfamily K, polypeptide 1	L11528	Biotransformation
12	CYP2K5	Cytochrome P450, family 2, subfamily K, polypeptide 5	AF151524	Biotransformation
13	CYP2M1	cytochrome P450, family 2, subfamily M, polypeptide 1	U16657	Biotransformation
14	CYP3A27	Cytochrome P450, family 3, subfamily A, polypeptide 27	U96077	Biotransformation
15	ERA1	Estrogen receptor alpha one	AJ242741	Endocrine - estrogen
16	ERA2	Estrogen receptor alpha two	DQ177438	Endocrine - estrogen
17	ERB1	Estrogen receptor beta one	DQ177439	Endocrine - estrogen
18	ERB2	Estrogen receptor beta two	DQ248229	Endocrine - estrogen
19	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	AF027130	Reference
20	HAMP	Hepcidin	AF281354 / EL553897	Immune
21	HDLBP	High density lipoprotein binding protein (vigilin)	NM_001173653	Endocrine
22	HPRT1	Hypoxanthine phosphoribosyltransferase 1	BT043501	Reference

#	Gene ID	Gene Name	Genbank Accession #	Biological System
23	HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	S72665	Endocrine
24	IGF-1	Insulin-like growth factor 1	NM_001124696 / M32792	Growth
25	IGFBP1	Insulin-like growth factor binding protein 1	DQ190460	Growth
26	IGFBP2	Insulin-like growth factor binding protein 2	DQ146968	Growth
27	IGFBP3	Insulin-like growth factor binding protein 3	DQ146966	Growth
28	L8	Ribosomal Protein L8	AY957563	Reference
29	MT1A	Metallothionein	M18103	Oxidative stress
30	PPARa	Peroxisome proliferator activated receptor alpha	NM_001197211 / DQ294237	Oxidative stress
31	PPARb	Peroxisome proliferator activated receptor beta1A	NM_001197207 / AJ416953	Oxidative stress
32	PPARg	Peroxisome proliferator activated receptor gamma	NM_001197212 / AJ292962	Oxidative stress
33	PXR	Nuclear receptor subfamily 1, group I, member 2 (nr1i2) (pregnane X receptor)	NM_001124672	Biotransformation
34	RPL23A	Ribosomal protein 23A (60S)	AF281334	Reference
35	RXR	Retinoid X Receptor	AJ969439	Oxidative stress
36	S10	Ribosomal Protein S10	NM_001160622	Reference
37	SF1	Steroidogenic Factor 1 (NR5A1)	AB038151	Endocrine
38	SOD	Superoxide dismutase	NM_001160614 / NM_001124329	Oxidative stress
39	STAT3	Signal transducer/activator of transcription	U60333	Immune
40	TBP	TATA box binding protein	NM_001173674	Reference
41	THRA	Thyroid hormone receptor alpha	AF302245	Endocrine - thyroid
42	THRB	Thyroid hormone receptor beta	AF302246	Endocrine - thyroid
43	TXN	Thioredoxin	CB493026	Oxidative stress
44	VEPA	Vitellogenin envelope protein alpha	AF231706	Endocrine - estrogen
45	VEPB	Vitellogenin envelope protein beta	AF231707	Endocrine - estrogen
46	VEPG	Vitellogenin envelope protein gamma	AF231708	Endocrine - estrogen
47	VTG	Vitellogenin	AJ011691	Endocrine - estrogen

## Chapter 7.

# **Tertiary-treated sewage effluents and estrogen hormones: effects on osmoregulatory, hematological and vitellogenic parameters in juvenile Chinook salmon**

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## Abstract

In British Columbia, Canada, limited knowledge exists regarding the profile and effects of estrogen hormones in local sewage treatment plant (STP) effluents. To address this, effluents from two tertiary-treated STPs were evaluated for (1) estrogen hormone constituents and concentrations using gas chromatography/mass spectrometry, and (2) effects on osmoregulatory, hematological and vitellogenic parameters in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Effluent from STP#1 contained no detectable estrogen hormones while STP#2 5% effluent contained  $2 \text{ ng L}^{-1}$  E1. Chinook were exposed to 0, 1, and 5% effluent or pure estrogen hormones ( $2 \text{ ng L}^{-1}$  estrone (E1),  $0.5 \text{ ng L}^{-1}$   $17\alpha$ -ethinylestradiol (EE2),  $1 \mu\text{g L}^{-1}$   $17\beta$ -estradiol (E2), and a mixture (containing all 3 at the same concentrations)) for 7 d (exposure phase) and then transferred to clean water for a 7 d depuration period (recovery phase). Both STP#2 5% effluent and  $2 \text{ ng L}^{-1}$  E1 groups caused significant changes at d7 for plasma cortisol, gill  $\text{Na}^+/\text{K}^+$  ATPase (NKA) activity, and hepatic vitelline envelope protein  $\gamma$  (VEPG) transcript in males.  $1 \mu\text{g L}^{-1}$  E2 and the estrogen hormone mixture treatment groups caused alterations at d7 including significantly decreased NKA activity, gonadal somatic index (GSI) in males, and red and white blood cell counts, while significantly increasing plasma glucose, liver somatic index (LSI), plasma vitellogenin (VTG) protein, and hepatic vitellogenesis-related transcripts (estrogen receptors (ERs), VTG and VEPs). After the recovery phase (d14), all parameters except VTG protein and vitellogenesis-related transcripts returned to baseline levels in the  $1 \mu\text{g L}^{-1}$  E2 and mixture groups. In summary, only one STP effluent contained estrogen hormones, which consisted solely of E1, and xenoestrogen exposure as single chemicals or as mixtures affected several important parameters related to Chinook salmon health during

the exposure phase, but most of these alterations returned to baseline values with 7 d of recovery.

## **Keywords**

Chinook; recovery; estrone; sewage treatment plant effluent; osmoregulation; hematology; vitellogenesis



## Introduction

Feminized fish have been found downstream of sewage treatment plant (STP) effluent discharges (Jobling et al., 1998; Vajda et al., 2008). Feminization occurs in response to STP effluents that contain natural and synthetic estrogen hormones excreted by humans and animals (Lange et al., 2002; Liu et al., 2009). Compounds other than hormones such as sterols (e.g.  $\beta$ -sitosterol and equol), alkylphenols and alkylphenol ethoxylates (e.g. nonylphenol), bisphenol A, and phthalates in STP effluents can also cause estrogenic effects. In general, however, these chemicals do not contribute much estrogenicity to STP effluents because of their very low potency compared to estrogen hormones (2 to 5 orders of magnitude lower) even though they can be present at much higher concentrations than estrogen hormones (Sumpter & Johnson, 2005). STPs exist as three main types (primary, secondary and tertiary treatment), each with different capabilities that build upon the microorganism and nutrient removal of the level previous. For example, tertiary treatment, the highest level, builds upon secondary STP processes by further eliminating bacteria, viruses, and substances using advanced technologies, such as ozonation or chlorination, to disinfect the effluent prior to discharge. Secondary treatment is very common worldwide (Johnson & Sumpter, 2001) but because tertiary treatment results in an effluent depleted of nutrients and microorganisms, tertiary STPs are often utilized in areas where the receiving water body is used as drinking or agricultural water.

There are three natural estrogen hormones: estrone (E1), 17 $\beta$ -estradiol (E2), and estriol (E3). E2 is the main vertebrate estrogen hormone (controlling reproduction, maturation, development, behaviour, and more) and is used for comparisons of the

potency of other estrogenic substances. E1 is an oxidized form of E2 that is approximately 2-fold less potent than E2 in fish (Thorpe et al., 2003; Van den Belt et al., 2004). E3 is the hormone associated with human pregnancy and is 200-fold less potent than E2 in fish (Metcalf et al., 2001). The most potent estrogen hormone is the synthetic compound 17 $\alpha$ -ethinylestradiol (EE2), which is used in contraceptives or hormone replacement therapies, and is approximately 11 to 27-fold more potent than E2 in fish (Van den Belt et al., 2004). Some, or all, of these hormones can be found in STP effluents, and the total estrogenicity of the effluent is the sum of its constituent xenoestrogens adjusted by their potency (Brian et al., 2005; Thorpe et al., 2006). Estrogen hormones have been reported downstream of wastewater discharges (Kolodziej et al., 2004; Jeffries et al., 2010; Williams et al., 2012; Barber et al., 2013). EE2 is a compound of particular concern due to reports of it in surface waters at levels up to 160-fold higher (Kolpin et al., 2002) than the concentration (5 ng L<sup>-1</sup>) found to cause the collapse of a fish population in experimental lakes (Kidd et al., 2007). A water quality guideline has been developed for EE2 in British Columbia (BC), Canada of 0.5 ng L<sup>-1</sup> mean in 30 days (0.75 ng L<sup>-1</sup> maximum of any one value) to protect aquatic life (BC MOE, 2010); no other water quality guidelines exist for estrogen hormones.

Exposure to exogenous estrogen hormones or compounds that mimic E2 (xenoestrogens) have been well documented to cause adverse effects in fish. The primary actions of estrogen hormones relate to reproduction, and xenoestrogen exposure has resulted in detrimental reproductive effects, including altered gonads, reduced spawning frequency and success, and altered sex hormone levels (Panter et al., 1998; Batty & Lim, 1999; Metcalf et al., 2001; Jobling et al., 2002; Kristensen et al., 2005; Hoger et al., 2006; Martinovic et al., 2007; Meier et al., 2007; Thorpe et al., 2007;

Dammann et al., 2011). The endocrine system is susceptible to xenoestrogen interference and the inopportune exposure of males, larval or juvenile fish to these compounds can initiate processes normally associated with females. In adult females, egg production involves gene expression and protein synthesis in the liver, transport of proteins via the bloodstream, and assembly of eggs in the ovary (Mommsen & Walsh, 1988). This process, called vitellogenesis, is under E2 control but subject to interference or induction by xenoestrogens. Other functions can be impaired by xenoestrogen exposure since E2 communicates with many physiological systems and cells. In this regard, estrogen receptors (ERs) have been found in many different fish tissues (Nagler et al., 2007; Shelley et al., 2013). Estrogen-related exposures have caused impairments or alterations in shoaling (Ward et al., 2006), aggression (Dammann et al., 2011), growth (Metcalf et al., 2001), osmoregulation (Arsenault et al., 2004; Madsen et al., 2004; McCormick, 2005), hematological parameters (Schwaiger et al., 2000), and immune defense (Wenger et al., 2011; Shelley et al., 2012). More research is needed to understand the effects of individual xenoestrogens or mixtures (including complex mixtures such as STP effluents), and the magnitude and duration of these effects.

Limited knowledge exists regarding the profile, concentrations, or effects on salmon of estrogen hormones in STP effluents from British Columbia (BC), Canada. To address this, a combination of chemical and toxicological assessments were performed on two local, tertiary-treated STP effluents and E1, E2, EE2 and a mixture of the three hormones. Juvenile Chinook (*Oncorhynchus tshawytscha*), a potentially exposed salmonid species, were exposed to effluents or estrogen hormones to characterize the induction and recovery of molecular, biochemical, tissue and organism-level parameters relating to osmoregulation, hematology, and vitellogenesis.

## Materials and Methods

### *Exposures*

Juvenile Chinook were obtained from Sun Valley Trout Hatchery (Mission, BC, Canada) and maintained in well water for 4 months (Environment Canada, 1990). STP effluents were collected 2 d prior to experiment initiation from two confidential locations in the interior of BC that had previously been shown to contain estrogen hormones (STP2 and STP3 from Furtula et al., 2012). Exposures were conducted as static with 100% renewal every 3.5 d in 68 L glass aquaria maintained at  $15 \pm 1$  °C under a 16:8 h light:dark photoperiod. Seven Chinook per aquarium (duplicate aquaria per treatment, or  $n = 14$  fish per treatment) of mass (mean  $\pm$  SEM)  $10.3 \pm 0.2$  g were randomly placed into aquaria. Water quality parameters were measured daily and after each water change. In all treatments, dissolved oxygen levels were  $> 8.5$  mg L<sup>-1</sup>, pH was 7.4 – 8.2, conductivity was 360 - 500  $\mu$ S, and temperature was 14.2 - 16.0 °C.

Exposures were conducted in two sets, one with sewage effluent treatments and one with corresponding levels of estrogen hormones found in the sewage effluents plus other estrogen hormone treatments (Figure 7-1). In the first set, fish were exposed to well water (negative control; CON-1) and 1% and 5% sewage effluent treatments ( $v v^{-1}$ , prepared in well water) for each STP site. In the second set, fish were exposed to well water containing 0.001% ( $v v^{-1}$ ) anhydrous 100% ethanol (solvent control; CON-2; Commercial Alcohols, Brampton, ON, Canada) and estrogen hormones dissolved in ethanol (final solvent concentration of 0.001%  $v v^{-1}$ ). The sole estrogen hormone treatment that matched one of the 5% STP effluents was 2 ng L<sup>-1</sup> estrone (E1). However, additional estrogen hormone treatments of interest were included: (1) 0.5 ng L<sup>-1</sup> 17 $\alpha$ -ethinylestradiol (EE2), which is a BC water quality guideline (BC MOE, 2010), (2) 1

$\mu\text{g L}^{-1}$  17 $\beta$ -estradiol (E2; a positive control), and (3) a mixture of 2 ng L<sup>-1</sup> E1, 0.5 ng L<sup>-1</sup> EE2 and 1  $\mu\text{g L}^{-1}$  E2 (called Mixture throughout the study). Estrogen hormones were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Each set of experiments had an exposure phase and a recovery phase, each followed by a saltwater challenge (SWC) (Figure 7-1). Fish were sampled at four timepoints: (1) after 7 d of treatment (d7), (2) after 7 d of treatment and a SWC (d8), (3) after 7 d of treatment, 7 d of recovery (d14), and (4) after 7 d of treatment, 7 d of recovery, and a SWC (d15). SWCs were conducted in 150 L tubs containing aerated saltwater (27 ppt). Fish from duplicate treatment aquaria were combined into one SWC tub (n = 14) and monitored for 24 h (Handeland et al., 1996; McCormick et al., 2005). A set of fish (n = 14) was also monitored in a 250 L tub containing well water (negative control).

### ***Tissue Collection***

Fish were euthanized in 100 mg L<sup>-1</sup> buffered MS-222 (tricaine methanesulfonate; Syndel Labs, Vancouver, BC, Canada), blotted dry, and weighed. Blood was withdrawn by caudal venipuncture within 5 min of capture to minimize the potential for a physiological stress response. Whole blood was collected in syringes and transferred to a 1.5 mL tube containing Drabkin's reagent (ratio of 1:125 for hemoglobin analysis, Sigma-Aldrich) or Dacie's buffer (ratio of 1:50, for red and white blood cell counts, Blaxhall and Daisley, 1973). Microcapillary tubes (Chase Scientific Glass, Inc., Rockwood, TN, USA) were filled from the syringe and spun for 3 min in a microcapillary centrifuge (International Equipment Company, Chattanooga, TN, USA). Hematocrit was

measured in duplicate for each fish and plasma was stored in 0.2 mL Axygen plastic tubes (VWR, Mississauga, ON, Canada) on dry ice until transfer to -80 °C. Livers were collected, weighed and stored separately in 1.5 mL tubes containing 1 mL of RNALater® (Ambion, Austin, TX, USA) at -80 °C for transcriptomics analysis. Sex was determined by phenotypic identification of the gonad, which was weighed. A piece of muscle tissue was removed and stored in a 1.5 mL tube containing 1 mL of RNALater® for genetic sex analysis. Gill filaments (10-15) were collected and placed in 0.5 mL tubes, in duplicate, containing 125 µl of SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3; McCormick, 1993) and two stainless steel 2 mm beads (Retsch, Newtown, PA, USA). Gills were stored at -80 °C until Na<sup>+</sup>/K<sup>+</sup> ATPase activity was evaluated. Post-SWC, only plasma, muscle and gill tissues were sampled.

### ***Water Chemistry***

Duplicate, composite 4 L well water, 5% and 100% effluent samples were collected from glass aquaria as fresh preparations on d0, prior to fish introduction. Samples were preserved with 1% (v/v) formaldehyde and stored at 4 °C until analysis for sterol compounds occurred (initiated within 1 d), in duplicate, using a gas chromatography mass spectrometry method as per Furtula et al. (2012). This method was also used to verify stock pure estrogen hormones concentrations.

### ***Plasma Parameters***

Analysis of plasma ion concentrations of sodium ( $\text{Na}^+$ ) and chloride ( $\text{Cl}^-$ ) occurred as described in Farrell et al. (2001). Cortisol concentrations were measured in plasma samples using a cortisol ELISA kit (enzyme-linked immunosorbent assay; Neogen Corporation, Lansing, MI, USA). Plasma concentrations of vitellogenin (VTG) were determined using a Rainbow trout VTG ELISA kit (Biosense® Laboratories, Abraxis, Warminster, PA). Samples in which VTG was not detected were set to the detection limit for statistical purposes. Glucose (colorimetric) and L-lactate concentrations were measured in plasma samples using kits from Cayman Chemical Company (Ann Arbor, Michigan, USA). For all assays, plasma samples were analyzed in duplicate and CV's < 20% were considered acceptable. All assay plates were read on a Bio-tek® PowerWave Reader (Bio-Tek, Winooski, VT) at the appropriate wavelength as specified in the manufacturers' protocols.

### ***Gill ATPase Activity***

Gill  $\text{Na}^+/\text{K}^+$  ATPase activity was evaluated using the protocol of McCormick (1993), with the exception that gill homogenates were prepared using a Mixer Mill 300 (Qiagen, Mississauga, ON, Canada) bead mill homogenizer with a program of 20 Hz for 30 seconds.

### ***Genetic Sex Determination***

Genetic sex of each fish was determined using a Y-linked growth hormone pseudogene (Afonso et al., 2002). Genomic DNA (gDNA) was extracted from muscle (10 mg) using the DNeasy Mini Spin kit (Qiagen, Mississauga, ON, Canada), with overnight tissue digestion. Polymerase Chain Reaction (PCR) components included 3 µl of gDNA and 34 µl of Master Mix containing (per reaction): 1.15 U Platinum® Taq DNA polymerase (Invitrogen, Burlington, ON, Canada), 4.9 µl of buffer, 20 pmoles of each primer (GH5 forward 5'-AGCCTGGATGACAATGACTC-3', GH6 reverse 5'-TACAGAGTGCAGTTGGCCT-3'; Afonso et al., 2002), 7.5 nmoles of dNTPs (Sigma-Aldrich), and 27.8 µl of nuclease-free water (Ambion). Positive controls were included from known males and females. Reactions were run on a PTC-200 MJ Research instrument (Bio-Rad, Mississauga, ON, Canada) with a program consisting of 30 cycles of 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 60 s. PCR products were analyzed on 1.2% agarose gels containing SYBR Safe (Invitrogen) and evaluated for the presence of the male-specific 240 bp band.

### ***RNA Extractions***

Total RNA extractions from liver were performed using RNeasy Mini Spin kits (Qiagen), including an on-column DNase treatment. Weighed livers ( $23 \pm 5$  mg) were placed in 1.5 mL Eppendorf safe-lock tubes (Thermo Fisher Scientific, Toronto, ON) containing 1 mL of Qiazol (Qiagen) and two 3 mm stainless steel beads (Retsch). Livers were homogenized using a Mixer Mill 300 (Qiagen) bead mill homogenizer for 3 cycles of 2 min each at 25 Hz (with 180° rotation of the tube holders after each cycle). Elution



occurred using 2 x 35 µl aliquots of RNase-free water. RNA concentrations were determined using a Nanodrop® 1000 instrument (Thermo Fisher Scientific, Toronto, ON).

### ***cDNA Preparation***

Using a QuantiTect reverse transcription kit (Qiagen), 1 µg of total RNA was transcribed into cDNA (complementary deoxyribonucleic acid) with a 60 min synthesis step. To evaluate gene expression, liver cDNAs were diluted 10-fold in nuclease-free water (Ambion).

### ***Quantitative Polymerase Chain Reaction***

Quantitative Polymerase Chain Reaction (QPCR) analysis evaluated hepatic gene expression alterations caused by exposure to STP effluents or estrogen hormones at d7 and d14. All conditions, programs, reagents and criteria for acceptable standard curves and reactions were described previously (Osachoff et al., 2013). Primer sequences for vitelline envelope protein  $\alpha$  (*VEPA*) were 5'-CAACAGATACCCTACACCAAA-3' and 5'-TAATCAGTGTCTCAACGGAAG-3', vitelline envelope protein  $\beta$  (*VEPB*) were 5'-CTTCTGTCTTTGCCATCTAC-3' and 5'-TGGTCAGGTAACGGTCAT-3', and vitelline envelope protein  $\gamma$  (*VEPG*) were 5'-AGACTGCCCTATGACTGGA-3' and 5'-TATCTGGGATTGGCGTTTA-3'. Primer sequences were previously published for estrogen receptor  $\alpha$ 1 (*ERA1*), estrogen receptor  $\alpha$ 2 (*ERA2*), estrogen receptor  $\beta$ 1 (*ERB1*), estrogen receptor  $\beta$ 2 (*ERB2*) (Nagler

et al., 2007) and vitellogenin (VTG) and ribosomal protein L8 (Osachoff et al., 2013). The invariant L8 (statistically proven to be invariant using ANOVA followed by Dunnett's post-hoc test, p-values > 0.05) was used to normalize the transcript results to account for technical variations (Filby & Tyler, 2007), similar to previous studies (Shelley et al., 2012; Osachoff et al., 2013).

### ***Calculations and Statistical Analyses***

Liver somatic index (LSI) was calculated as: liver weight x body weight<sup>-1</sup> x 100. Gonadal somatic index (GSI) was calculated as: gonad weight x body weight<sup>-1</sup> x 100. GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., La Jolla, CA, USA), with statistical significance denoted by  $P < 0.05$ , was used to perform all statistical tests. Grubbs' test was used to detect and remove outliers (GraphPad Software Inc.). Within a timepoint, treatments were compared to their respective control, unless otherwise noted. Normality of data was analyzed using the Shapiro-Wilk's test. For normally distributed parameters, ANOVA with Dunnett's post-hoc test was conducted to test for differences between the control and treatments. Otherwise, non-parametric data were analyzed using Kruskal-Wallis and Mann-Whitney U tests. Results are shown as mean  $\pm$  standard error of the mean (SEM).

## Results and Discussion

### *Hormone Concentrations*

Limited information is available on the profile and concentration of estrogen hormones in BC STP effluents. To address this, two BC STP effluents were evaluated using a sterol chemistry suite that included natural and synthetic estrogen hormones, zoosterols, and phytosterols (Furtula et al., 2012). Concentrations of some estrogen hormones (including E2, EE2, and E3) were below detection, but E1 was found at  $2 \pm 0.2 \text{ ng L}^{-1}$  in the 5% effluent from STP#2 (Table 7-1, with full suite listed in Supplementary Information Table S7-1). Other non-hormone estrogenic sterols were below detection (mestranol, equol, equilin, and norgestrel) except for  $\beta$ -sitosterol, which was  $188 \pm 10 \text{ ng L}^{-1}$  in STP#1 5% effluent, and  $74 \pm 9 \text{ ng L}^{-1}$  in STP#2 5% effluent (Table 7-1).  $\beta$ -sitosterol has been shown to be estrogenic to fish (Orrego et al., 2010) at 1000-fold higher concentrations than those observed in this study ( $75 - 150 \mu\text{g L}^{-1}$ ; Tremblay and Van Der Kraak, 1999). STP#1 did not contain any estrogen hormones, and so the only known estrogenic substance present was  $\beta$ -sitosterol (Table 7-1). In contrast, STP#2 5% effluent contained two estrogenic sterols:  $2 \text{ ng L}^{-1}$  E1 and  $74 \text{ ng L}^{-1}$   $\beta$ -sitosterol (Table 7-1). Using reported potencies for these compounds, 0.4 for E1 (Thorpe et al., 2003; Van den Belt et al., 2004) and  $5 \times 10^{-5}$  for  $\beta$ -sitosterol (Tremblay and Van Der Kraak, 1999), E2 equivalencies (EEq) were calculated to be  $0.8 \text{ ng L}^{-1}$  for E1 and  $0.0037 \text{ ng L}^{-1}$  for  $\beta$ -sitosterol. Thus, in STP#2, the main contributor (> 99.5%) to overall estrogenicity was E1. Hence, E1 was included as a pure estrogen hormone treatment in the second set of exposures, which allowed for the investigation of the estrogenic contribution to STP#2 5% effluent from  $2 \text{ ng L}^{-1}$  E1.

## ***Osmoregulation and Stress***

### **NKA Activity and Plasma Ions**

Xenoestrogens have been shown to affect osmoregulation, saltwater tolerance and preference, migration success, and smolt returns in Atlantic salmon (*Salmo salar*; Fairchild et al., 1999; Arsenault et al., 2004; Madsen et al., 2004; McCormick et al., 2005; Bangsgaard et al., 2006). These effects in anadromous Atlantic salmon can culminate in reductions in survival and fitness and have potential consequences for population success. Chinook salmon are also anadromous and so the effects of STP effluents and estrogen hormones on osmoregulatory parameters and performance were examined in two 24 h saltwater challenges (SWCs), one in the post-treatment phase (d8) and one in the post-recovery phase (d15). No mortalities were observed in fish subjected to SWCs. When fish enter saltwater environments, plasma ion concentrations generally increase (McCormick, 2001) and gill Na<sup>+</sup>/K<sup>+</sup> ATPase (NKA), as well as other transport proteins, aid in their excretion to maintain osmoregulatory homeostasis (McCormick, 1993). The activity of NKA reflects the ability of the fish to tolerate or adapt to saltwater (McCormick, 2001). NKA was evaluated in the present experiment pre- and post-SWC to identify effects of treatment (d7), the ability of fish to recover from treatments (d14), and the ability of the fish to tolerate saltwater after each of these phases (d8 and d15). Normally, gill NKA activity increases when transitioning from freshwater to saltwater, as occurred in control fish in the present study (Figure 7-2). Reduced NKA activity was seen in STP#2 5% effluent treated fish at d8, but increases in NKA activity were seen in the STP#2 1% and 5% groups after recovery at d14, and d15 for STP#2 5% (Figure 7-2). Otherwise, effluent groups were similar to control fish in terms of NKA activities (Figure 7-2A). Alterations were seen in NKA activity in Chinook exposed to estrogen hormones (Figure 7-2B). E1 and E2 NKA activities were elevated

at d7 but decreased at d8, which indicated that they did not respond to induction by saltwater as in the control group. E2 also decreased NKA activity in tilapia (*Oreochromis mossambicus*) (Vijayan et al., 2001) and in Atlantic salmon (Madsen et al., 2004; Lerner et al., 2007). Similar to the E2 group, the mixture group also exhibited significantly decreased NKA activity at d8 (Figure 7-2B) but at d14, the mixture group had significantly elevated NKA activity, which was not retained at d15 (Figure 7-2B). Reductions in NKA activity cause impaired saltwater tolerance as the ability to maintain plasma osmolality depends on this activity (Handeland et al., 1996). Atlantic salmon with reduced NKA activity had reduced SW preference indicating a delayed behaviour in entering estuarine environments (Lerner et al., 2007), increased mortality after release (Madsen et al., 2004), and poorer migration performance (Madsen et al., 2004; Bangsgaard et al., 2006). Fish not prepared to enter seawater have increased mortality, as shown in brown trout (*Salmo trutta*) with low NKA activity compared to Atlantic salmon (Urke et al., 2009). Although reductions in NKA activity were seen in some fish, corresponding plasma ion alterations were not seen (Figures 7-3 and 7-4). There were no significant changes in Na<sup>+</sup> or Cl<sup>-</sup> concentrations during this study when treatments within a timepoint were compared to their respective control. When Na<sup>+</sup> or Cl<sup>-</sup> concentrations were compared for each treatment between d7 and d8 or d14 and d15, the SWC caused significant increases in concentrations, which was expected since ions are absorbed from saltwater (McCormick, 2001). Since the alterations in NKA activities did not translate to altered plasma ion levels, the implications to osmoregulation are difficult to interpret.

## Plasma Cortisol Concentrations

Fish are considered physiologically stressed when cortisol concentrations are elevated above 30 ng mL<sup>-1</sup> (Barton, 2002). After 7 d of treatment (d7), in 1% and 5% STP#2 effluent groups, cortisol was significantly higher than in control fish indicating stress: 97 ng mL<sup>-1</sup> and 106 ng mL<sup>-1</sup>, respectively (Figure 7-5). In the 2 ng L<sup>-1</sup> E1 and 0.5 ng L<sup>-1</sup> EE2 exposure groups, cortisol concentrations were also significantly elevated (92 ng mL<sup>-1</sup> and 83 ng mL<sup>-1</sup>, respectively), however, the more potent estrogen hormone (and positive control) 1 µg L<sup>-1</sup> E2 did not increase it significantly (Figure 7-5), results similar to others (Osachoff et al., 2013). Exposure to STP effluent has been shown to cause a physiological stress response in Rainbow trout (*Oncorhynchus mykiss*) (Ings et al., 2012) and xenoestrogens can increase plasma cortisol in another salmonid species, Atlantic salmon (Lerner et al., 2007). Cortisol is also one of the hormones responsible for preparing smolts for saltwater entry (McCormick, 2001). However, the relationship between cortisol concentrations and seawater tolerance is not clear at this time since cortisol concentrations, alterations of NKA, and plasma ion concentrations do not show a consistent pattern.

## Plasma Glucose and Lactate Concentrations

Metabolic products, such as glucose and lactate, can be indicators of an organismal stress response and altered energy demands in xenoestrogen-treated fish (Washburn et al., 1993; Vijayan et al., 2001; Ings et al., 2012). Plasma glucose concentrations were significantly induced in 1 µg L<sup>-1</sup> E2 and the mixture groups at d7 (Figure 7-6). Atlantic salmon also had elevated plasma glucose upon exposure to 2 µg L<sup>-1</sup> E2 (Lerner et al., 2007). Potentially, glucose was released via glycogenolysis for

delivery to tissues as fuel for estrogen-related inductions, including synthesis of the energy-demanding VTG protein (Vijayan et al., 2001). Glucose was not significantly elevated at d14 in the E2 and mixture treatments; thus, the energy demand ended with the exposure cessation (Figure 7-6). Lactate is a product of anaerobic metabolism but can be converted back to glucose by the liver via gluconeogenesis. Lactate was elevated at d7 in the mixture group (Figure 7-6) and this may be explained by inhibition of gluconeogenesis (Washburn et al., 1993). Lactate was reduced in STP#2 5% at d14 (Figure 7-6) for some unknown reason.

### ***Hematology***

Several studies have reported adverse effects on fish immune systems by exposure to xenoestrogens, including an increased susceptibility to pathogens resulting in death (Wenger et al., 2011; Shelley et al., 2012). Estrogenic STP effluents could potentially be hazardous to fish in this manner and may even have enhanced toxicity due to simultaneous discharge of estrogenic substances and microorganisms. In the present study, white blood cells (WBCs) were evaluated as an indicator of immune system status. No reductions in WBCs were seen with STP effluent exposures (Table 7-2). Suggestive of an infection, WBCs were elevated at d14 (recovery) in the 2 ng L<sup>-1</sup> E1, 0.5 ng L<sup>-1</sup> EE2 and 1 µg L<sup>-1</sup> E2 exposure groups (Table 7-2). A significant reduction in WBCs was seen in 1 µg L<sup>-1</sup> E2 at d7, which was also reported for other xenoestrogens (Schwaiger et al., 2000). Thus, leukopenia could potentially be a factor in the vulnerability of xenoestrogen-treated fish to pathogens (Wenger et al., 2011; Shelley et

al., 2012). Since isolated leukocytes contain estrogen receptor (ER) transcripts, E2 has been postulated to play a direct role in WBC modulation (Shelley et al., 2013).

Xenoestrogens have been suggested to increase RBC fragility in common carp (*Cyprinus carpio*) leading to anemia (reduced RBCs; Schwaiger et al., 2000). RBC levels in fish from the 1  $\mu\text{g L}^{-1}$  E2 group at d7 were significantly lower than controls by 9% (Table 7-2). No other RBC-related hematological parameters were altered (Table 7-2), which contrasts with results in carp in which hemoglobin and mean corpuscular hemoglobin concentrations (MCHC) decreased correspondingly with RBC levels (Schwaiger et al., 2000). Reduced RBCs in the E2-exposed Chinook can reduce the oxygen carrying capacity of blood, potentially impacting oxygen delivery. Since chinook swim continuously to maintain their position or to travel, and require sprint swimming for some life functions including predator-avoidance and prey capture, such a limitation on oxygen could impair fish fitness (Kieffer, 2000).

Hematocrit is a measurement of the ratio of packed RBCs to plasma volume and is used to indicate changes in RBC volume (swelling/shrinkage or levels), osmoregulation, or as a general indicator of altered homeostasis. Although reduced RBCs were seen in E2-treated Chinook, hematocrit did not decrease correspondingly (Table 7-2), which was contrary to another study with the same E2 treatment in Rainbow trout; hematocrit decreased after 2 and 7 d of exposure (Shelley et al., 2013). The decrease in RBCs may have been balanced by another factor, such as a decrease in plasma volume. Hematocrit was significantly elevated in 2  $\text{ng L}^{-1}$  E1 and the mixture at d14 (Table 7-2). Increases in hematocrit can indicate increases in RBCs or decreases in plasma volume, but since no other hematological parameters changed, the reason for increased hematocrit in these treatments is not known.



## ***Vitellogenesis***

### **Sex of Fish**

Phenotypic sex was identified during the collection of gonad tissue while genetic sex was identified using a PCR method (Afonso et al., 2002). For 97.5% of fish (273 out of 280), the genetic and phenotypic sex identifications matched. For those few mismatches, phenotypic sex was used in data analyses. The proportion of females and males in each set of exposures was the same: 47% females, 53% males.

### **LSI, GSI and VTG protein**

LSI is known to increase with xenoestrogen exposure (Parrott & Blunt, 2005; Skillman et al., 2006; Carrera et al., 2007; Osachoff et al., 2013) due to increased hepatic gene expression and protein production, and alterations in hepatic lipid profiles (Arukwe & Goksoyr, 2003; Cakmak et al., 2006). Two treatments at d7 had significantly elevated LSI: 1  $\mu\text{g L}^{-1}$  E2 ( $P < 0.001$ ) and the mixture ( $P < 0.001$ ). LSI was also significantly elevated in 1  $\mu\text{g L}^{-1}$  E2-treated Rainbow trout (Osachoff et al., 2013). When fish sex was considered, the females in the E2 and mixture groups were significantly elevated vs females in CON-1, but the males were not (Figure 7-7). The opposite was seen in effects on the GSI. At d14, males in the E2 and mixture treatments had significantly reduced GSI but females did not (Figure 7-8). Thus, these treatments inhibited growth of the testes of juvenile Chinook. This has also been reported in testes of Rainbow trout exposed to 100  $\text{ng L}^{-1}$  EE2 for 62 d (Schultz et al., 2003), Rainbow trout exposed to various xenoestrogens for 21 d (Jobling et al., 1996), and fathead minnows exposed to 1  $\mu\text{g L}^{-1}$  E2 for 21 d (Panter et al., 1998). The sole treatment with elevated GSI was the E1 group (males) at d14 (Figure 7-8). In a similar study with Rainbow trout,

an inverse relationship was found with 15 ng L<sup>-1</sup> E1 having higher GSI than 54 ng L<sup>-1</sup> E1 (Dammann et al., 2011). Thus, it appears that E1 stimulates testes growth at low concentrations.

Vitellogenin (VTG) is a precursor egg protein evaluated frequently in studies of xenoestrogen effects on fish. Its presence (including magnitude and duration of induction) is commonly used as an indicator of xenoestrogen exposure (Sumpter & Jobling, 1995). In fish, the liver is the site of production of egg proteins, which are transported via the bloodstream to the ovary for uptake into eggs in mature females (Mommensen & Walsh, 1988; Arukwe & Goksoyr, 2003). Juvenile and male fish can be induced to produce egg proteins but lack ovaries; thus, proteins that originate in the liver, such as VTG, remain in circulation for weeks or months (Hemmer et al., 2002; Skillman et al., 2006; Osachoff et al., 2013). In this study, VTG protein concentrations were measured in the plasma of juvenile chinook and significant elevations were seen for 1 µg L<sup>-1</sup> E2 and mixture groups at d7 (Figure 7-9). These significant increases were retained even after 7 d of depuration (at d14) and likely would have remained for weeks or months until cleared (Hemmer et al., 2002; Osachoff et al., 2013). The STP effluents and the other estrogen hormone groups (2 ng L<sup>-1</sup> E1 and 0.5 ng L<sup>-1</sup> EE2) did not induce plasma VTG protein levels, suggesting that these treatments were not estrogenic. This may be attributable to the 7 d exposure design, since male Rainbow trout exposed for 10 d to 0.5 ng L<sup>-1</sup> EE2 had measureable levels of VTG protein, although not significantly elevated over controls (Purdom et al., 1994). However, the E1 and EE2 concentrations in this study (and STP#2 5% since it contained 2 ng L<sup>-1</sup> E1) were just below reported lowest observed effect concentrations (LOECs) of 3.3 ng L<sup>-1</sup> E1 and 1.0 ng L<sup>-1</sup> EE2 for VTG protein induction in 14 d treated Rainbow trout (Thorpe et al., 2003).

## Vitellogenesis Transcripts

In Oncorhynchids, vitellogenesis, which is susceptible to xenoestrogen interference, involves hepatic estrogen receptors (ERs) that induce hepatic egg protein transcripts called vitelline envelope proteins (VEPs) and VTG (Arukwe and Goksoyr, 2003; Darie et al., 2004; Nagler et al., 2007). In this study, the four known isoforms of ER transcripts (*ERA1*, *ERA2*, *ERAB1* and *ERB2*) and four known egg protein-related transcripts (*VEPA*, *VEPB*, *VEPG* and *VTG*) were evaluated at d7 and d14 in both sets of exposures (Figure 7-10). In addition, the response of gene transcripts in males and females at d7 were evaluated to identify sex-specific differences (Figure 7-11).

### **Treatment Phase (d7)**

At d7, for all of the STP effluent treatment groups, there were no significant alterations vs CON-1 for ERs or egg protein-related transcripts (Figure 7-10). However, when fish sex was considered, *VEPG* was significantly different in males only for STP#2 5% effluent (Figure 7-11). This was similar to 2 ng L<sup>-1</sup> E1, in which *VEPG* was also significantly elevated in males only (Figure 7-11). In a study with E2-treated rainbow trout, *VEPA* and *VEPB* were more sensitive (i.e. earlier responders) than *VEPG* (Thomas-Jones et al., 2003); thus, potentially *VEPG* can be identified as the *VEP* more responsive to E1. The males in the E1 group also had *VEPB* increase but this was not seen in the STP#2 5% effluent group (Figure 7-11), so not every alteration in the E1 group was seen in the STP#2 5% effluent.

At d7, only *VEPA* was elevated in 0.5 ng L<sup>-1</sup> EE2 (Figure 7-10) and it was significantly increased for both males and females (Figure 7-11). Chinook in this study

appear to be relatively insensitive to this concentration of EE2 since only one gene transcript was altered after 7 d of exposure. This likely reflects the short duration of this study because this concentration of EE2 has been shown to have profound effects in male fathead minnows (*Pimephales promelas*) exposed for 6 months, including reduced reproduction success and feminized males (Parrott & Blunt, 2005), as well as causing intersex gonads in Japanese medaka (*Oryzias latipes*) exposed for 100 d post-hatch (Metcalf et al., 2001).

For 1 µg L<sup>-1</sup> E2 and Mixture groups, *VTG*, *VEPA*, *VEPB*, *VEPG* were all increased significantly (Figure 7-10) in both males and females (Figure 7-11). *ERB1* was not altered in these treatments at all, but *ERA1* and *ERA2* were elevated, while *ERB2* was reduced (Figure 7-10). Both males and females were affected in E2 and mixture groups for *ERA1* and *ERB2*, but in *ERA2*, it was only the females that were significantly elevated and not the males (Figure 7-11). In a similar study, Rainbow trout exposed to 1 µg L<sup>-1</sup> E2 also had elevated hepatic *VTG*, *ERA1* and *ERA2*; however, *ERB2* was not significantly reduced (Osachoff et al., 2013), indicating a difference in response to xenoestrogens between salmonid species.

#### **Recovery Phase (d14)**

For STP effluents, E1 and EE2 groups, vitellogenesis-related gene expression alterations were apparent in Chinook at the recovery phase timepoint (d14) even though these transcripts were not altered after the exposure phase (d7). STP#1 1% and STP#2 1% and 5% all had reduced *ERA1* (Figure 7-10). *ERB1* and *ERB2* were elevated for the E1 group (Figure 7-11), and in the 0.5 ng L<sup>-1</sup> EE2 group, *VEPB*, *VEPG*, *ERA1*, *ERB1*,

*ERB2* were all increased (Figure 7-11). This indicates that gene expression alterations can be very slow to occur at ultra-low level concentrations (i.e. ng L<sup>-1</sup> levels).

In the 1 µg L<sup>-1</sup> E2 and Mixture groups, *VEPA*, *VEPB*, *VEPG*, *ERA1* and *ERA2* remained significantly increased vs CON-2 but all were lower compared to the d7 elevations, so the gene transcript inductions were recovering toward baseline levels (Figure 7-10). *VTG* was not significantly elevated at d14 in the Mixture group, but it was in the E2 group. *ERB2* was still reduced, but less so than *ERB2* at d7, showing it was also returning to baseline (Figure 7-10). At d14, the ERs appear to be differentially responsive to the estrogen hormones since *ERB1* was elevated in both E1 and EE2 groups but *ERB1* was not ever altered in the E2 and Mixture groups (Figure 7-10). As well, at d14, *ERB2* was significantly increased in the E1 and EE2 groups, but it was decreased in the E2 and Mixture groups (Figure 7-10). Further study is needed to characterize the response of the four hepatic ER isoforms with various concentrations of estrogen hormones, since this may provide information that relates downstream signalling pathways to the roles ERs have in physiological functions (Nagler et al., 2007).

## **Summary and Conclusions**

### ***Estrogenicity of the Effluents***

The two tertiary-treated STP effluents contained few estrogen hormones, with only 2 ng L<sup>-1</sup> E1 seen in STP#2 5% effluent. STP#2 5% effluent and 2 ng L<sup>-1</sup> E1 had several effects on Chinook in common, including increased plasma cortisol concentrations, decreased gill NKA activity, and increased hepatic *VEPG* expression.

Although few indicators of estrogenicity existed, it appeared that the estrogenic content of STP#2 5% was related to E1 presence.

### **0.5 ng L<sup>-1</sup> EE2**

A water quality guideline exists for 0.5 ng L<sup>-1</sup> EE2 (30 d mean) in freshwater aquatic systems in BC, Canada (BC MOE, 2010). This concentration was evaluated in this study and there were few effects on Chinook after 7 d of exposure, but those seen included elevated plasma cortisol concentrations, indicating general stress, and increased *VEPA* transcript in both males and females. After 7 d of recovery (at d14), effects were found on many gene transcripts: *VEPB*, *VEPG*, *ERA1*, *ERB1*, *ERB2*, which indicated that this low concentration of EE2 takes time to alter parameters. It is not apparent that 7 d of exposure to this concentration seriously impairs Chinook, but indications are that the longer fish are exposed, the more alterations there are that could occur. Possibly, a new water quality guideline could be developed for E1, since E1 is present in STP effluents from BC (Furtula et al., 2012), it has close potency to E2 (Thorpe et al., 2003), and it is gaining in recognition as an important estrogen hormone in surface waters worldwide (Dammann et al., 2011).

### **Recovery**

There is substantial value in including a recovery phase after xenoestrogen exposures to study the return to baseline levels of altered parameters. Without this phase, the gene expression alterations related to vitellogenesis activities would have

been missed in the E1 and EE2 concentrations, which are environmentally-relevant levels of these estrogen hormones (Kolpin et al., 2002; Jeffries et al., 2010; Williams et al., 2012). Thus, it turns out that not all parameters are altered during the treatment phase and it takes time for actions to become evident. For the higher concentrations of estrogen hormones (E2 and the Mixture), nearly all gene expression alterations did not return to baseline levels given 7 d to recover. Nor did VTG protein inductions, which was not surprising given this has been reported elsewhere regarding VTG protein clearance (Hemmer et al., 2002; Osachoff et al., 2013). Alterations of GSI in male Chinook were not evident until the d14 timepoint, which was important to characterize since it is a physiologically relevant endpoint that relates to reproductive success in salmonids (Jobling et al., 1996; Schultz et al., 2003). In summary, most, but not all, xenoestrogen-altered parameters can return to baseline levels after a depuration period and parameters not found altered during the treatment phase can become significantly different during the recovery phase.

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## Tables

**Table 7-1** Concentrations (ng/L) of sterol estrogen hormones or  $\beta$ -sitosterol in Control well water, sewage treatment plant (STP) #1 5% and 100% effluents, and STP#2 5% and 100% effluents. Results are mean  $\pm$  SEM of duplicate composite water samples taken at day 0.

Sterol Compound	Abbreviation	Type	Function	Concentration (ng/L)				
				Control	STP#1 5%	STP#1 100%	STP#2 5%	STP#2 100%
Estrone	E1	Natural	oxidized form of E2	<i>nd</i>	<i>nd</i>	<i>nd</i>	2 $\pm$ 0.2	28 $\pm$ 1
17 $\beta$ -Estradiol	E2	Natural	main vertebrate estrogen hormone	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Estriol	E3	Natural	pregnancy hormone	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
17 $\alpha$ -Ethinylestradiol	EE2	Synthetic	contraceptive or hormone replacement therapy	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
$\beta$ -Sitosterol	$\beta$ -SIT	Natural	phytosterol	6 $\pm$ 3	188 $\pm$ 10	3,211 $\pm$ 61	74 $\pm$ 9	629 $\pm$ 24

*nd* = not detected

**Table 7-2 Hematological parameters in Rainbow trout exposed to sewage treatment plant (STP) effluents or estrogen hormones. Results are presented as mean  $\pm$  SEM (n=14). WBC = white blood cells, RBC = red blood cells. MCV = mean corpuscular volume (Hematocrit / RBC). MCH = mean corpuscular hemoglobin (hemoglobin / RBC). MCHC = mean corpuscular hemoglobin concentration (hemoglobin / hematocrit). CON-1 = negative control for STP effluents. CON-2 = negative control for estrogen hormones. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$**

Timepoint	Treatment	WBCs (cells/ml)	Hematocrit (Hct)	RBCs (cells/ml)	Hemoglobin (g/dL)	MCV ( $\times 10^{-15}$ L/cell)	MCH (pg/cell)	MCHC (g/dL)
d7	CON-1	$1.59 \times 10^7 \pm 2.80 \times 10^6$	$40.1\% \pm 0.8\%$	$3.93 \times 10^9 \pm 8.19 \times 10^8$	$6.06 \pm 0.21$	$161 \pm 27$	$23.4 \pm 3.5$	$14.8 \pm 0.4$
	STP#1 1%	$9.55 \times 10^6 \pm 1.75 \times 10^6$	$39.0\% \pm 1.0\%$	$4.21 \times 10^9 \pm 8.19 \times 10^8$	$5.81 \pm 0.18$	$157 \pm 31$	$23.5 \pm 4.7$	$15.0 \pm 0.4$
	STP#1 5%	$1.09 \times 10^7 \pm 2.15 \times 10^6$	$38.3\% \pm 0.9\%$	$4.28 \times 10^9 \pm 8.22 \times 10^8$	$5.93 \pm 0.21$	$135 \pm 23$	$20.3 \pm 3.0$	$15.4 \pm 0.4$
	STP#2 1%	$8.91 \times 10^6 \pm 1.04 \times 10^6$	$40.0\% \pm 0.9\%$	$3.49 \times 10^9 \pm 5.27 \times 10^8$	$6.24 \pm 0.28$	$156 \pm 23$	$24.4 \pm 3.8$	$15.6 \pm 0.6$
	STP#2 5%	$8.88 \times 10^6 \pm 1.33 \times 10^6$	$41.9\% \pm 0.7\%$	$3.34 \times 10^9 \pm 5.77 \times 10^8$	$6.43 \pm 0.30$	$164 \pm 22$	$25.1 \pm 3.5$	$15.3 \pm 0.5$
	CON-2	$3.16 \times 10^7 \pm 2.28 \times 10^6$	$37.4\% \pm 0.7\%$	$1.61 \times 10^9 \pm 8.37 \times 10^7$	$6.86 \pm 0.32$	$240 \pm 11$	$41.1 \pm 1.8$	$17.3 \pm 0.6$
	2 ng/L E1	$3.59 \times 10^7 \pm 4.91 \times 10^6$	$39.3\% \pm 0.9\%$	$1.36 \times 10^9 \pm 1.10 \times 10^8$	$6.17 \pm 0.15$	$315 \pm 28$	$55.1 \pm 5.5$	$17.5 \pm 0.7$
	0.5 ng/L EE2	$2.53 \times 10^7 \pm 3.41 \times 10^6$	$37.1\% \pm 0.8\%$	$1.37 \times 10^9 \pm 1.03 \times 10^8$	$6.47 \pm 0.27$	$292 \pm 23$	$48.6 \pm 3.9$	$16.7 \pm 0.4$
	1 $\mu$ g/L E2	<b><math>1.77 \times 10^7 \pm 1.40 \times 10^6</math>***</b>	$38.7\% \pm 0.6\%$	<b><math>1.36 \times 10^9 \pm 4.17 \times 10^7</math> *</b>	$6.58 \pm 0.22$	$289 \pm 10$	$49.0 \pm 2.1$	$16.9 \pm 0.4$
	Mixture	$2.12 \times 10^7 \pm 2.18 \times 10^6$	$38.8\% \pm 0.9\%$	$1.40 \times 10^9 \pm 1.00 \times 10^8$	$6.39 \pm 0.22$	$291 \pm 18$	$47.3 \pm 2.3$	$16.5 \pm 0.4$



Timepoint	Treatment	WBCs (cells/ml)	Hematocrit (Hct)	RBCs (cells/ml)	Hemoglobin (g/dL)	MCV (x 10 <sup>-15</sup> L/cell)	MCH (pg/cell)	MCHC (g/dL)
d14	CON-1	3.98 x 10 <sup>7</sup> ± 4.51 x 10 <sup>6</sup>	39.3% ± 0.5%	1.64 x 10 <sup>9</sup> ± 7.46 x 10 <sup>7</sup>	6.54 ± 0.26	247 ± 12	41.2 ± 2.6	16.6 ± 0.6
	STP#1 1%	3.92 x 10 <sup>7</sup> ± 2.61 x 10 <sup>6</sup>	37.7% ± 1.2%	1.51 x 10 <sup>9</sup> ± 3.70 x 10 <sup>7</sup>	6.65 ± 0.21	251 ± 9	44.3 ± 1.4	17.7 ± 0.3
	STP#1 5%	4.03 x 10 <sup>7</sup> ± 2.87 x 10 <sup>6</sup>	39.9% ± 0.9%	1.51 x 10 <sup>9</sup> ± 7.72 x 10 <sup>7</sup>	6.91 ± 0.20	273 ± 14	47.1 ± 2.2	17.3 ± 0.4
	STP#2 1%	4.11 x 10 <sup>7</sup> ± 2.86 x 10 <sup>6</sup>	39.7% ± 1.0%	1.59 x 10 <sup>9</sup> ± 8.69 x 10 <sup>7</sup>	6.69 ± 0.27	263 ± 19	44.1 ± 2.7	16.9 ± 0.5
	STP#2 5%	4.72 x 10 <sup>7</sup> ± 3.41 x 10 <sup>6</sup>	39.7% ± 0.9%	1.43 x 10 <sup>9</sup> ± 5.03 x 10 <sup>7</sup>	6.12 ± 0.37	282 ± 10	43.8 ± 3.1	15.4 ± 0.7
	CON-2	3.05 x 10 <sup>7</sup> ± 2.45 x 10 <sup>6</sup>	36.1% ± 0.8%	1.48 x 10 <sup>9</sup> ± 9.94 x 10 <sup>7</sup>	5.83 ± 0.27	258 ± 17	41.3 ± 2.9	16.1 ± 0.6
	2 ng/L E1	<b>4.20 x 10<sup>7</sup> ± 1.95 x 10<sup>6</sup> **</b>	<b>38.8% ± 1.0% *</b>	1.57 x 10 <sup>9</sup> ± 6.95 x 10 <sup>7</sup>	6.29 ± 0.23	252 ± 9	40.8 ± 1.7	16.2 ± 0.3
	0.5 ng/L EE2	<b>3.95 x 10<sup>7</sup> ± 2.37 x 10<sup>6</sup> *</b>	37.1% ± 0.6%	1.57 x 10 <sup>9</sup> ± 6.29 x 10 <sup>7</sup>	6.14 ± 0.17	241 ± 9	39.8 ± 1.6	16.6 ± 0.4
	1 µg/L E2	<b>4.33 x 10<sup>7</sup> ± 2.43 x 10<sup>6</sup> ***</b>	37.3% ± 1.0%	1.42 x 10 <sup>9</sup> ± 4.47 x 10 <sup>7</sup>	6.26 ± 0.20	272 ± 10	45.5 ± 1.7	16.8 ± 0.5
	Mixture	3.72 x 10 <sup>7</sup> ± 2.07 x 10 <sup>6</sup>	<b>39.4% ± 0.7% *</b>	1.46 x 10 <sup>9</sup> ± 7.26 x 10 <sup>7</sup>	6.84 ± 0.23	275 ± 19	47.4 ± 3.1	17.3 ± 0.5

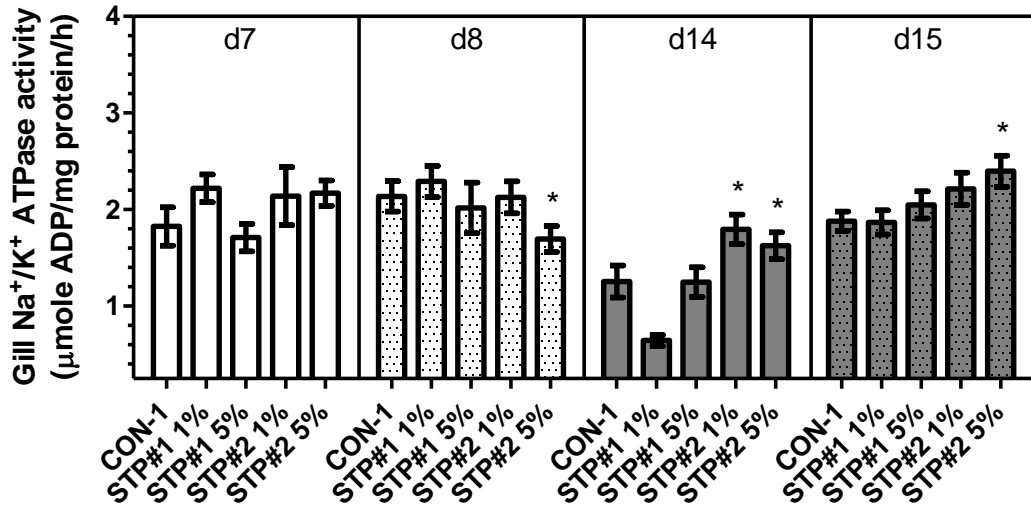
## Figures

	<b>TREATMENT</b>	
DAY: -----	7	----- 8
	Exposure to: <u>Set 1: Sewage Effluents</u> CON-1 STP#1 1% and 5% STP#2 1% and 5% <u>Set 2: Estrogen Hormones</u> CON-2 2 ng/L E1 0.5 ng/L EE2 1 µg/L E2 Mixture	24 h Salt Water Challenge (SWC)
	<b>DEPURATION</b>	
DAY: -----	14	----- 15
	Recovery Period: All treatments exposed to well water	24 h Salt Water Challenge (SWC)

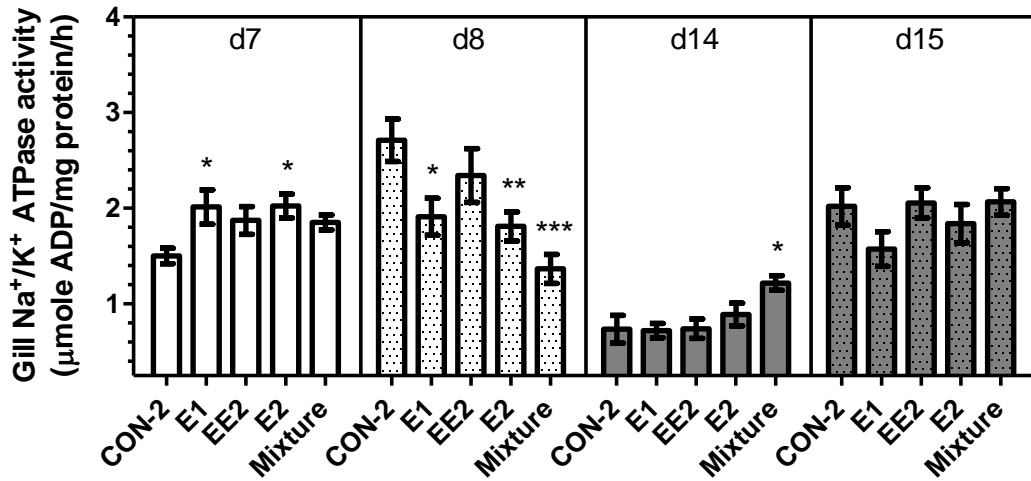
CON – Control  
 STP – Sewage Treatment Plant  
 E1 – Estrone  
 EE2 – 17 $\alpha$ -ethinylestradiol  
 E2 – 17 $\beta$ -estradiol  
 Mixture – E1 + EE2 + E2

**Figure 7-1** Study exposure design with two sets of treatments (sewage effluents or estrogen hormones) and 2 phases evaluated (treatment and recovery), plus a 24 h saltwater challenge (SWC) after each phase. Fish tissues were sampled at d7, d8, d14 and d15.

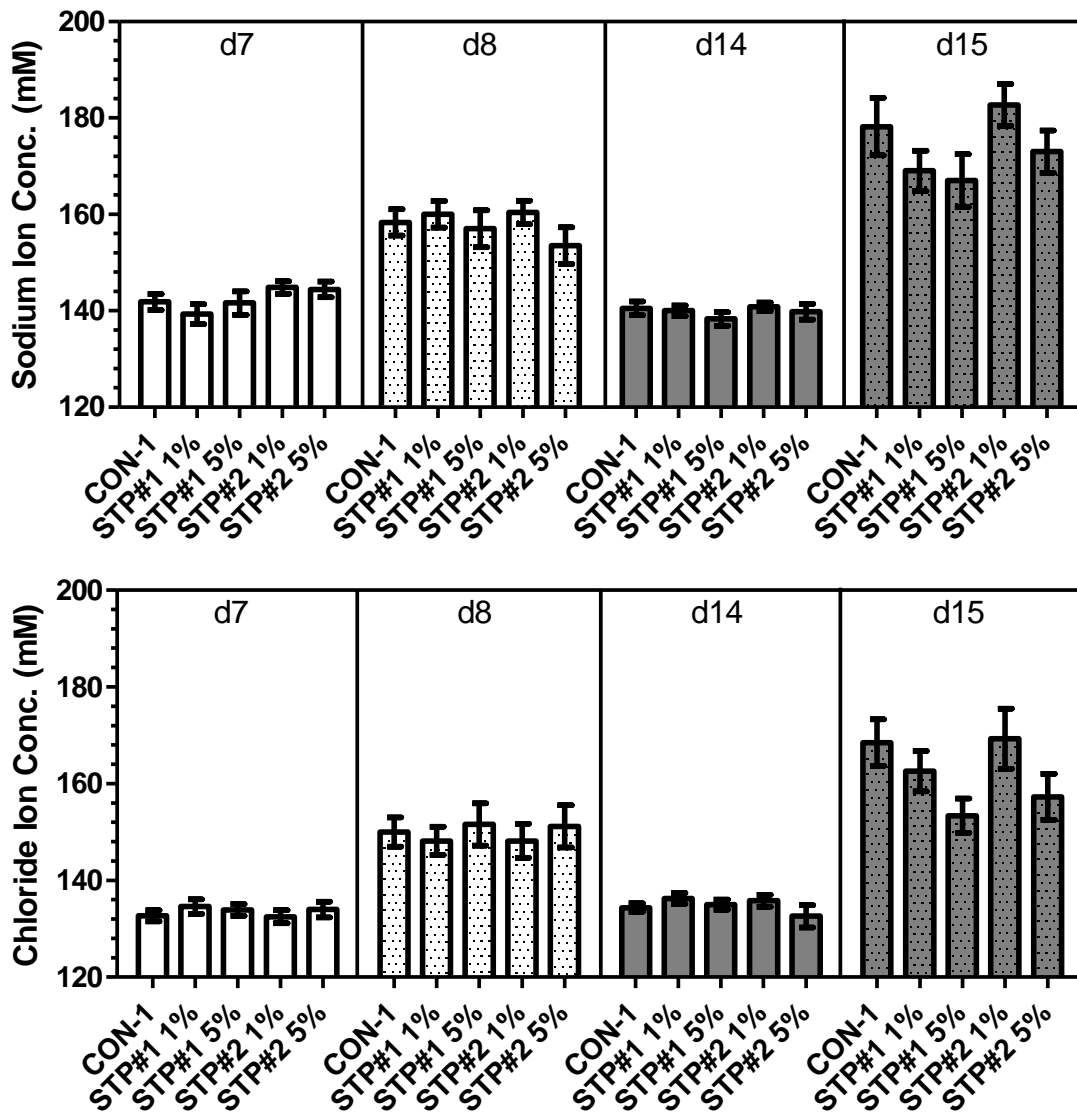
A.



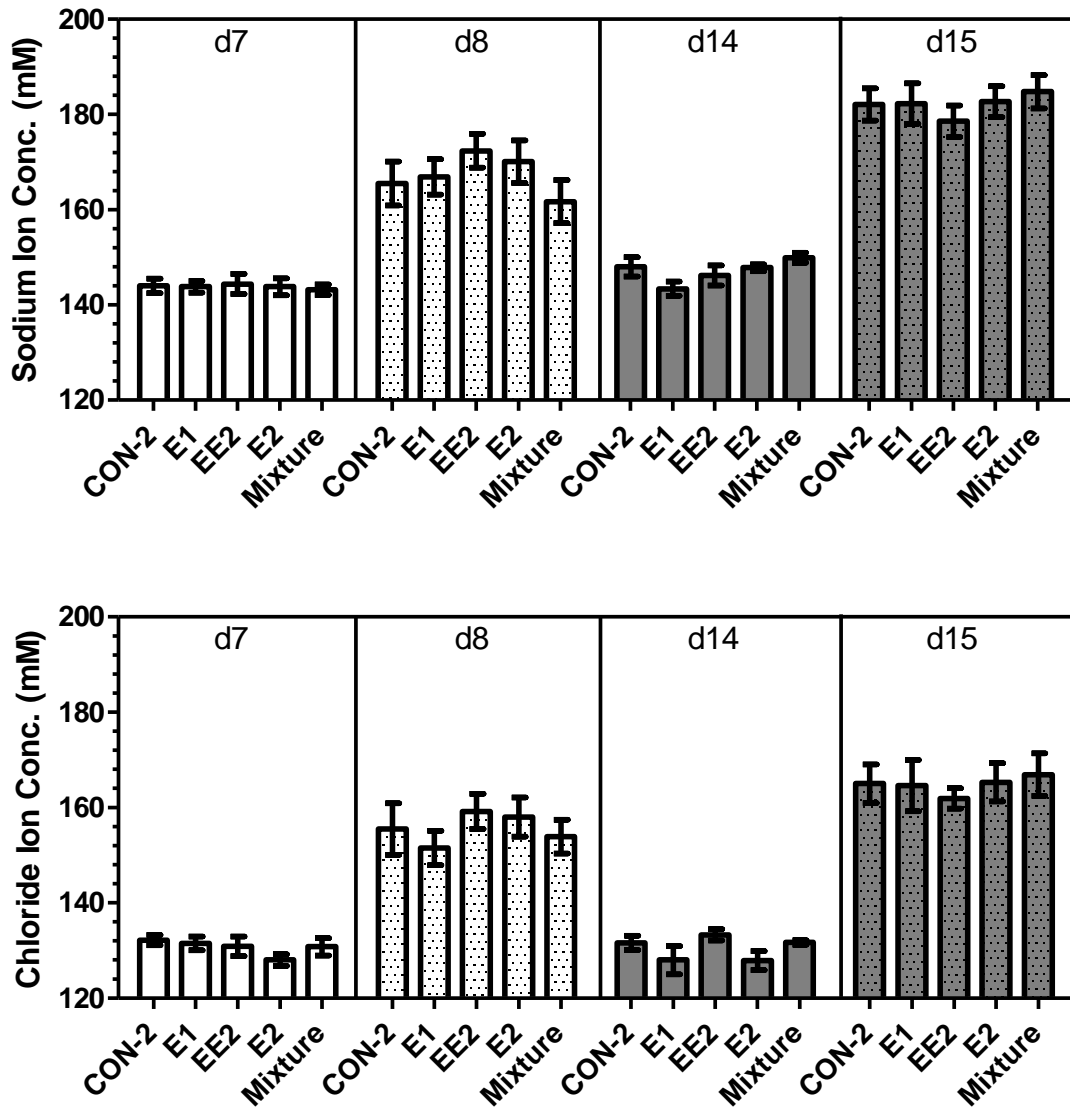
B.



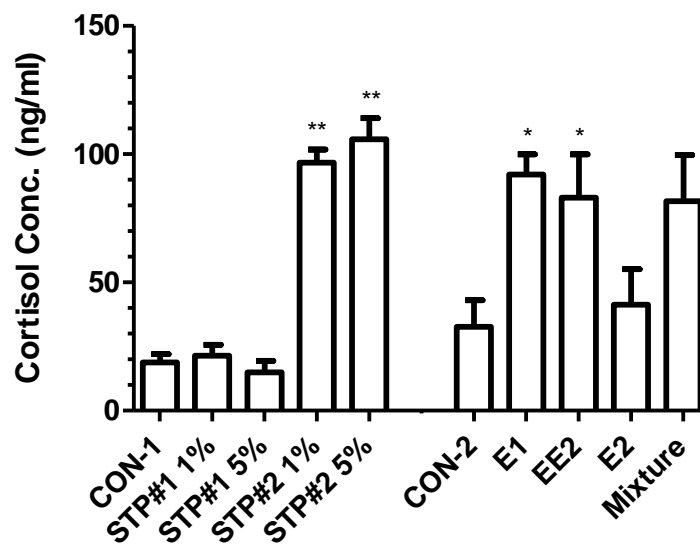
**Figure 7-2** Gill Na<sup>+</sup> / K<sup>+</sup> ATPase (NKA) activity in Chinook exposed for 7 d (d7) followed by a saltwater challenge (d8) or followed by 7 d of recovery (d14) and a second saltwater challenge (d15). A. Sewage treatment plant (STP) effluent treatments and negative control (CON-1). B. Estrogen hormones and negative control (CON-2). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



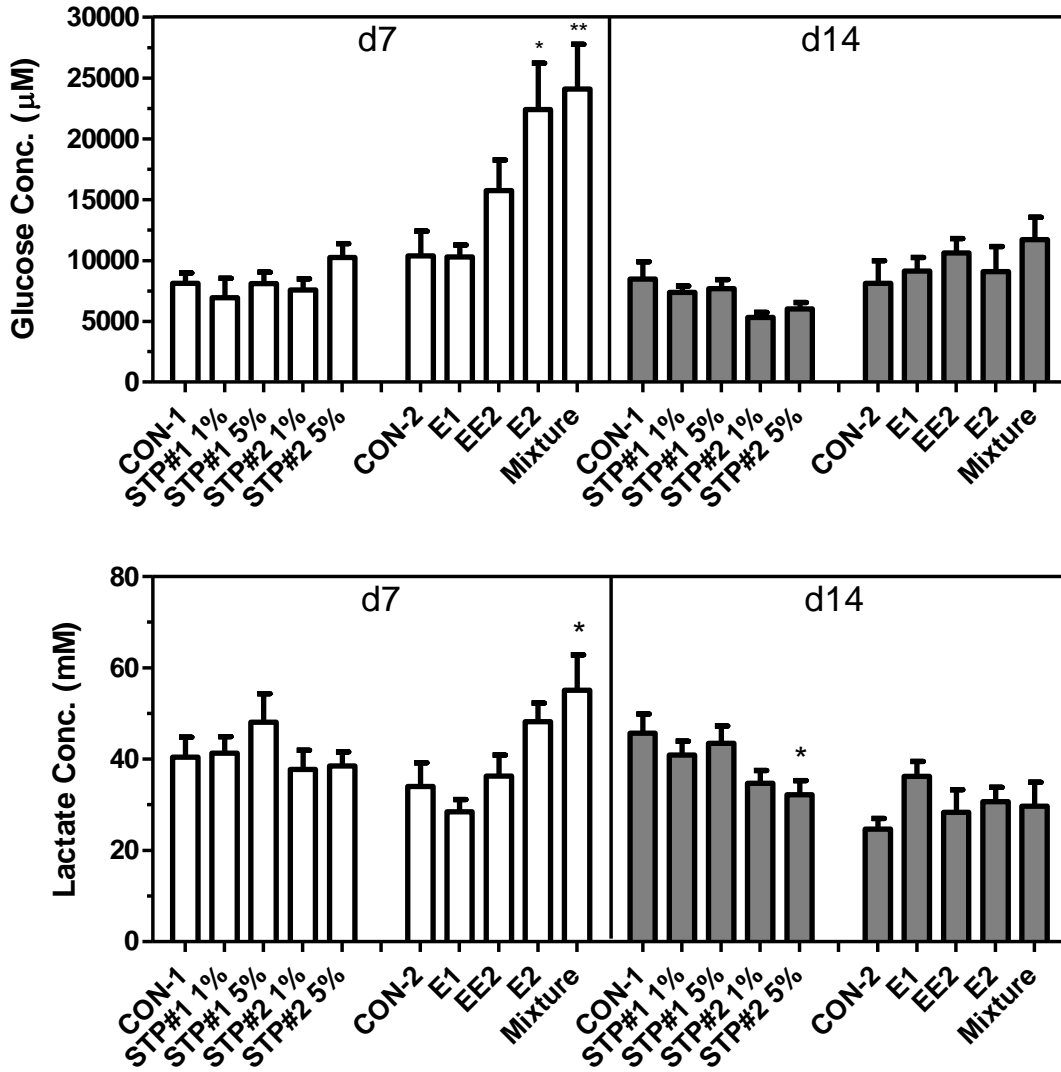
**Figure 7-3** Plasma ions in sewage treatment plant (STP) effluents in Chinook exposed for 7 d (d7) followed by a saltwater challenge (d8) or followed by 7 d of recovery (d14) and a second saltwater challenge (d15).



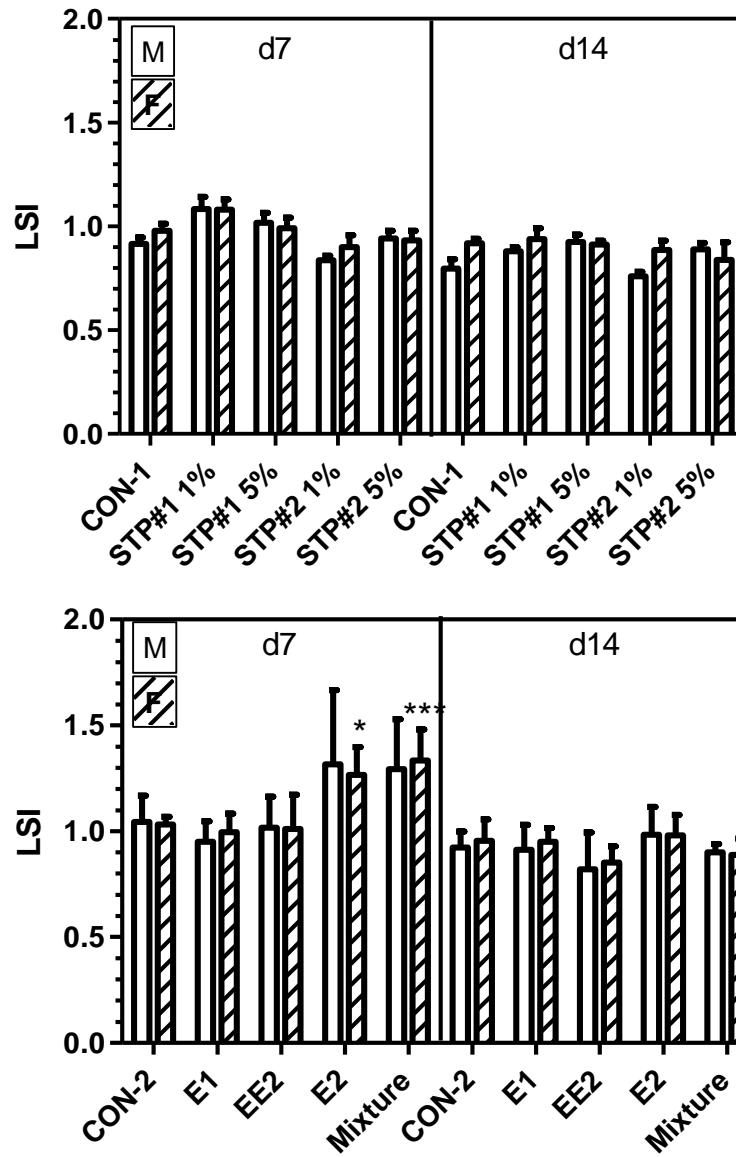
**Figure 7-4** Plasma ions in estrogen hormone treatments in Chinook exposed for 7 d (d7) followed by a saltwater challenge (d8) or followed by 7 d of recovery (d14) and a second saltwater challenge (d15).



**Figure 7-5** Cortisol plasma concentrations after treatment for 7 days (d7) in chinook exposed to two sets of treatments: (1) negative control 1 (CON1) and sewage effluents (STP #1 1% and 5%, STP#2 1% and 5%), and (2) negative control 2 (CON2) and pure estrogenic sterol compounds (2 ng/L estrone (E1), 0.5 ng/L 17 $\alpha$ -ethinylestradiol (EE2), 1  $\mu$ g/L 17 $\beta$ -estradiol (E2) and a mixture of those three compounds (Mixture)). \*  $p < 0.05$ , \*\*  $p < 0.01$

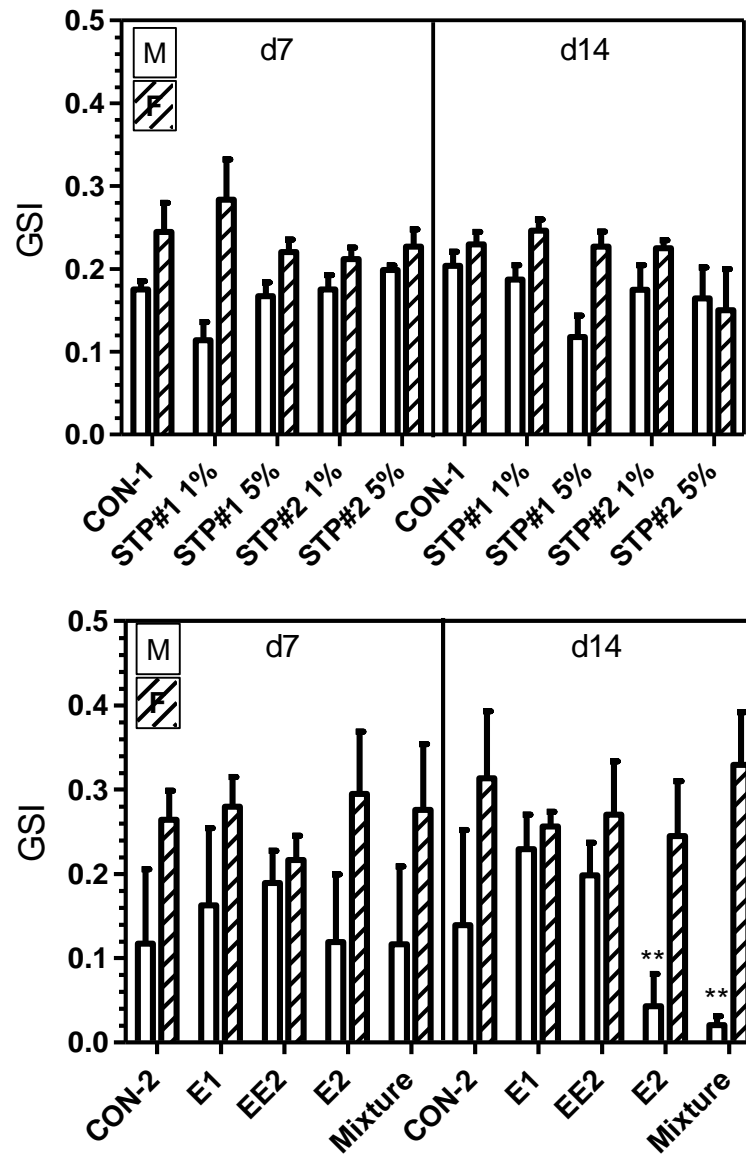


**Figure 7-6** Plasma glucose and lactate concentrations after 7 d of exposure (d7) followed by 7 d of recovery (d14) in chinook exposed to two sets of treatments: (1) negative control 1 (CON1) and sewage effluents (STP #1 1% and 5%, STP#2 1% and 5%), and (2) negative control 2 (CON2) and pure estrogenic sterol compounds (2 ng/L estrone (E1), 0.5 ng/L 17 $\alpha$ -ethinylestradiol (EE2), 1  $\mu$ g/L 17 $\beta$ -estradiol (E2) and a mixture of those three compounds (Mixture)). \*  $p < 0.05$ , \*\*  $p < 0.01$

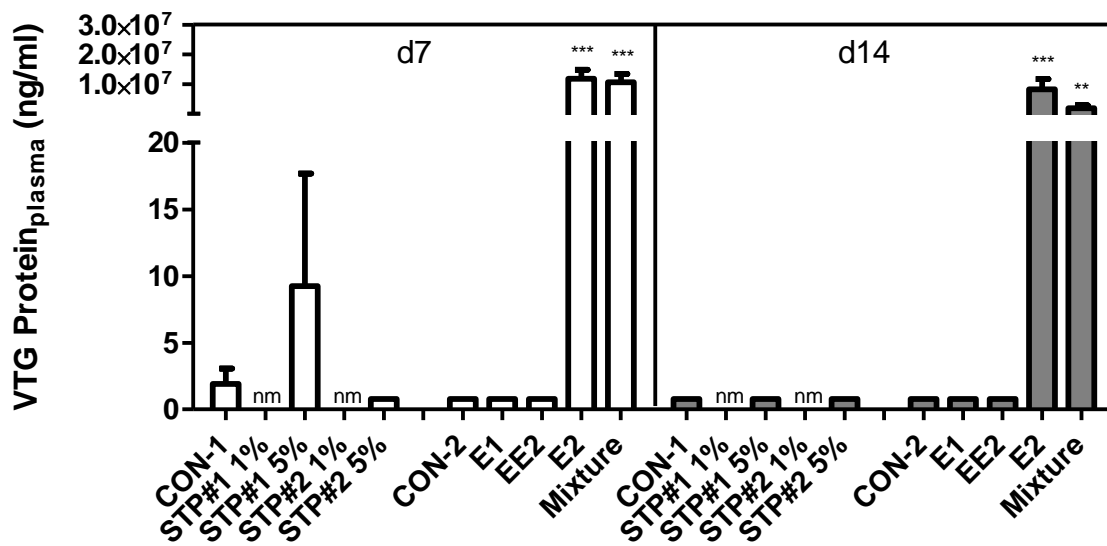


**Figure 7-7** Liver somatic index (LSI) for males (open bars) and females (hatched bars) in each treatment after 7 d of exposure (d7) followed by 7 d of recovery (d14). Treatments compared to their respective control for the same sex and timepoint. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

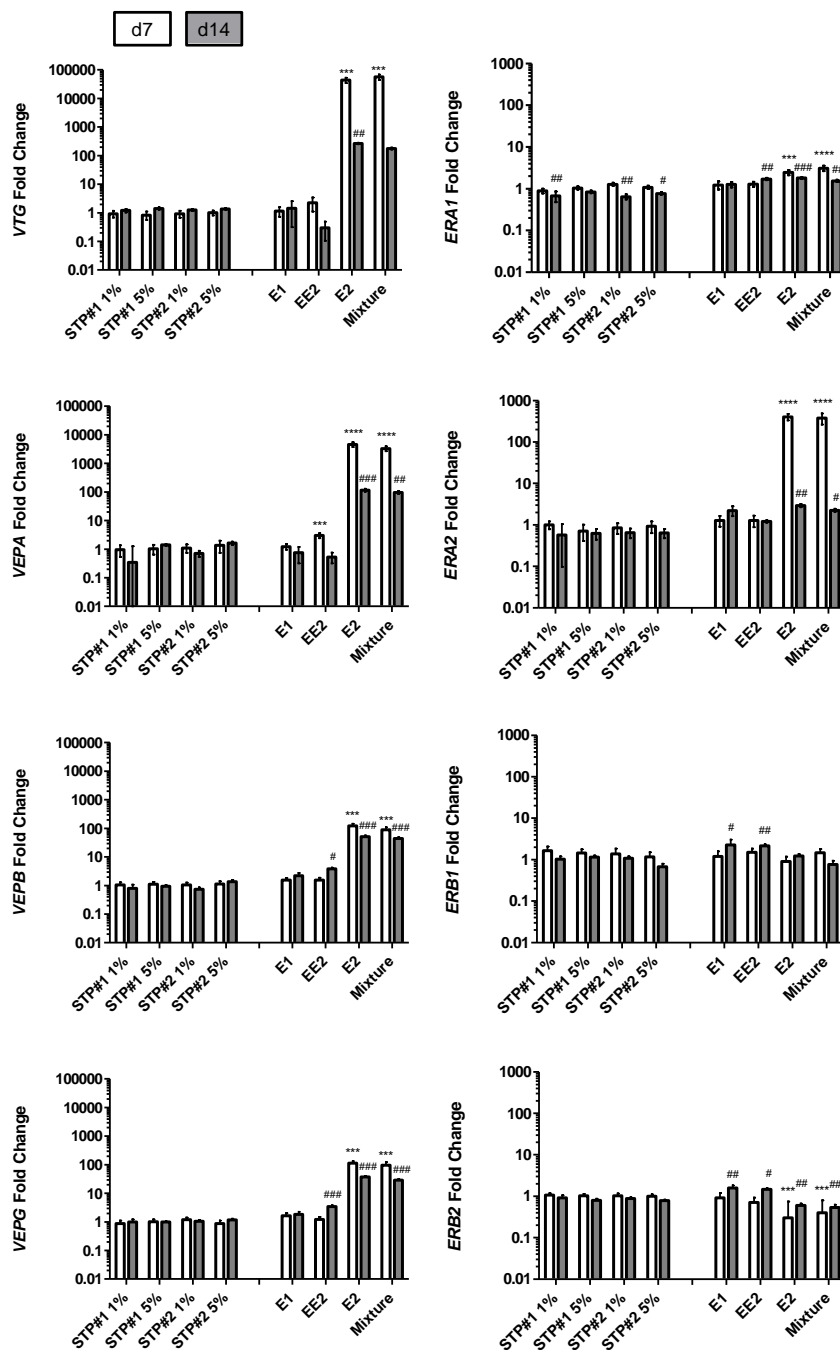




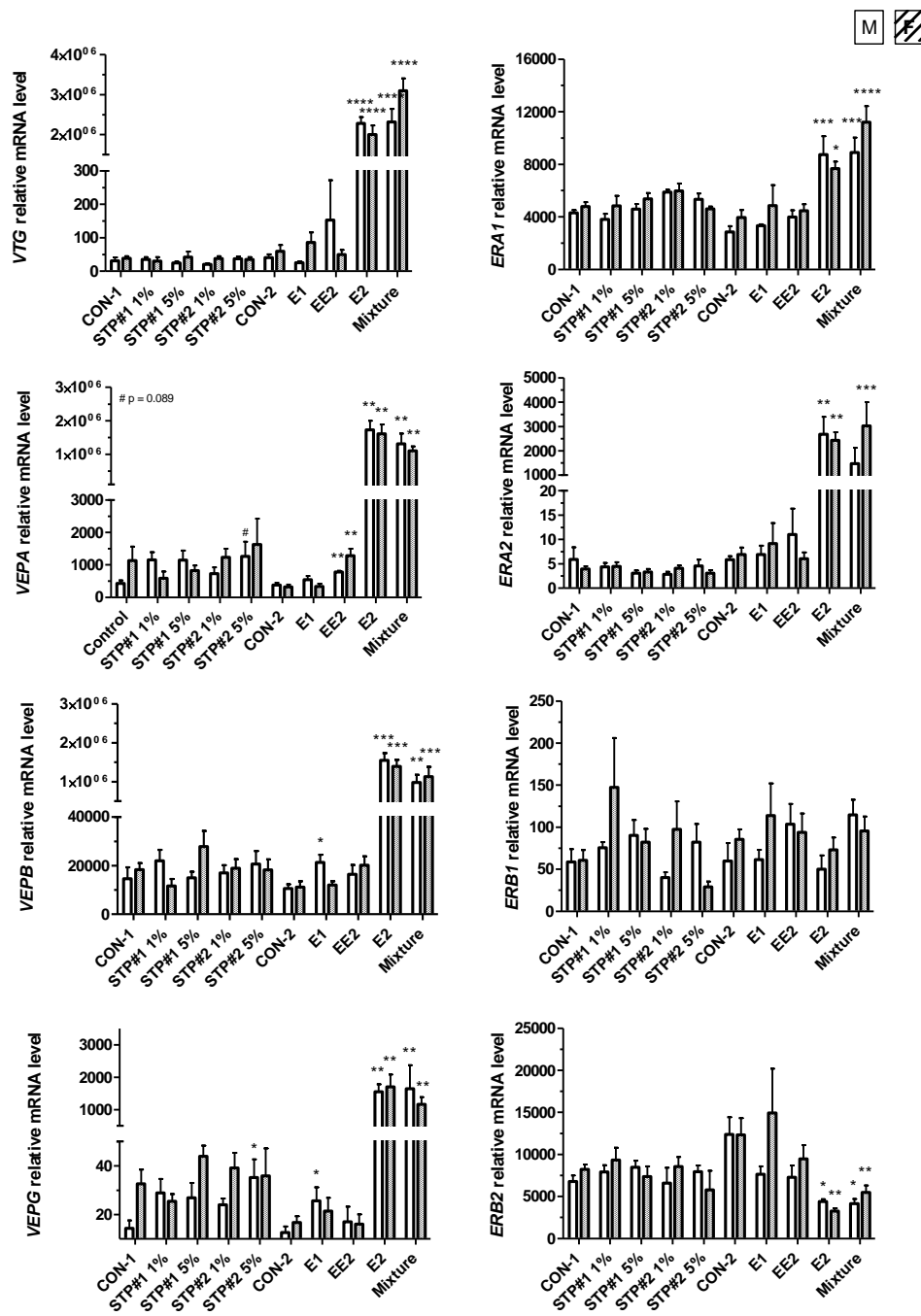
**Figure 7-8** Gonadal somatic index (GSI) for males (open bars) and females (hatched bars) in each treatment after 7 d of exposure (d7) followed by 7 d of recovery (d14). Treatments compared to their respective control for the same sex and timepoint. \*  $p < 0.05$ , \*\*  $p < 0.01$



**Figure 7-9** Plasma VTG Protein concentrations in each treatment after 7 d of exposure (d7) followed by 7 d of recovery (d14). nm = not measured; \*  $p < 0.05$ , \*\*  $p < 0.05$ , \*\*\*  $p < 0.001$



**Figure 7-10** Fold change (vs controls) in hepatic vitellogenesis-related gene transcripts in each treatment after 7 d of exposure (d7) followed by 7 d of recovery (d14). ERA1 = estrogen receptor  $\alpha$ 1, ERA2 = estrogen receptor  $\alpha$ 2, ERB1 = estrogen receptor  $\beta$ 1, ERB2 = estrogen receptor  $\beta$ 2, VTG = vitellogenin, VEPA = vitelline envelope protein  $\alpha$ , VEPB = vitelline envelope protein  $\beta$ , VEPG = vitelline envelope protein  $\gamma$ . \*  $p < 0.05$ , \*\*  $p < 0.05$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$



**Figure 7-11** Fold change (vs controls) in vitellogenesis-related gene transcripts in each treatment after 7 d of exposure (d7) for males (open bars) and females (hatched bars). ERA1 = estrogen receptor  $\alpha$ 1, ERA2 = estrogen receptor  $\alpha$ 2, ERB1 = estrogen receptor  $\beta$ 1, ERB2 = estrogen receptor  $\beta$ 2, VTG = vitellogenin, VEPA = vitelline envelope protein  $\alpha$ , VEPB = vitelline envelope protein  $\beta$ , VEPG = vitelline envelope protein  $\gamma$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$

## Supplementary Information

**Table S7-1 Concentrations ( $\mu\text{g/L}$ ) of sterol compounds in Control well water, sewage treatment plant (STP) #1 5% and 100% effluents, and STP#2 5% and 100% effluents. Results are mean  $\pm$  SEM of duplicate composite water samples taken at day 0. MDL = method detection limit.**

Sterol Compound	MDL	Control	Concentration ( $\mu\text{g/L}$ )			
			STP#1 5%	STP#1 100%	STP#2 5%	STP#2 100%
Mestranol	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Norethindrone	0.2	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Equol	0.2	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Estrone	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	0.002 $\pm$ 0.0002	0.028 $\pm$ 0.001
Equilin	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Norgestrel	0.2	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
17 $\alpha$ -Ethinylestradiol	0.02	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
17 $\alpha$ -Estradiol	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
17 $\beta$ -Estradiol	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Coprostanone	0.005	<i>nd</i>	0.014 $\pm$ 0.003	0.373 $\pm$ 0.039	0.041 $\pm$ 0.007	0.421 $\pm$ 0.018
Estriol	0.02	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Cholestanone	0.005	<i>nd</i>	0.004 $\pm$ 0.001	0.082 $\pm$ 0.002	0.006 $\pm$ 0.001	0.056 $\pm$ 0.001
Coprostanol	0.005	<i>nd</i>	0.029 $\pm$ 0.007	0.856 $\pm$ 0.022	0.125 $\pm$ 0.014	1.317 $\pm$ 0.055
Epicoprostanol	0.005	<i>nd</i>	0.003 $\pm$ 0.001	0.062 $\pm$ 0.001	0.012 $\pm$ 0.001	0.104 $\pm$ 0.005
Epicolestanol	0.005	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
Cholesterol	0.005	0.026 $\pm$ 0.016	0.210 $\pm$ 0.011	3.438 $\pm$ 0.058	0.180 $\pm$ 0.014	1.602 $\pm$ 0.036
Dihydrocholesterol (cholestanol)	0.005	0.001 $\pm$ 0.000	0.029 $\pm$ 0.008	0.695 $\pm$ 0.006	0.029 $\pm$ 0.005	0.227 $\pm$ 0.009
Desmosterol	0.005	<i>nd</i>	0.017 $\pm$ 0.004	0.285 $\pm$ 0.022	0.024 $\pm$ 0.006	0.128 $\pm$ 0.016
Campesterol	0.005	0.001 $\pm$ 0.000	0.054 $\pm$ 0.015	1.198 $\pm$ 0.009	0.033 $\pm$ 0.006	0.244 $\pm$ 0.007
24-Ethylcoprostanol	0.005	<i>nd</i>	0.017 $\pm$ 0.004	0.428 $\pm$ 0.009	0.054 $\pm$ 0.010	0.499 $\pm$ 0.021
Stigmasterol	0.005	0.006 $\pm$ 0.002	0.197 $\pm$ 0.003	3.374 $\pm$ 0.006	0.051 $\pm$ 0.009	0.342 $\pm$ 0.007
$\beta$ -Sitosterol	0.005	0.006 $\pm$ 0.003	0.188 $\pm$ 0.010	3.211 $\pm$ 0.061	0.074 $\pm$ 0.009	0.629 $\pm$ 0.024
Stigmastanol	0.005	0.004 $\pm$ 0.001	0.010 $\pm$ 0.003	0.197 $\pm$ 0.026	0.013 $\pm$ 0.002	0.124 $\pm$ 0.014

*nd = not detected*

**Chapter 8.**  
**Final Discussion**

## Summary of Key Research Findings

### *Xenoestrogen Impacts*

Immune function and swimming performance were two areas identified as non-reproductive impacts in salmonids that occurred as a result of treatment with xenoestrogens, but the mechanisms of how these effects occurred have not been identified (Madsen et al., 2004; Wenger et al., 2011; Shelley et al., 2012). The studies presented here detail the impacts of xenoestrogens on these functions and have identified potential mechanisms for these effects.

Several experiments described in this thesis on immune system effects show that blood leukocyte counts decreased upon exposure to 17 $\beta$ -estradiol (E2) and 17 $\alpha$ -ethinylestradiol (EE2) (Chapters 3 and 7) and that T cell proliferation was reduced with E2 exposure (Chapter 2), both of which could impair immune system function resulting in lower pathogen resistance. Estrogen receptor (ER) transcripts were responsive to E2 in purified leukocytes (Chapter 2), which demonstrated that the ability exists for leukocytes to directly respond to E2. This was an important discovery, contributing to the knowledge of how immune function repression can be caused by xenoestrogens resulting in increased disease susceptibility (Wenger et al., 2011, Shelley et al., 2012).

Swimming performance was decreased by E2 and EE2 exposure, possibly due to reduced oxygen carrying capacity in the blood or an altered ability to maintain osmoregulatory balance (Chapter 3). Since xenoestrogen exposure has been demonstrated to impact migration performance (Madsen et al., 2004; Bangsgaard et al., 2006), and is linked to poor smolt returns after environmental applications of pesticide

formulations (Fairchild et al., 1999), the reductions seen in burst swimming speeds here provide a mechanism by which these impacts might have occurred. Burst swimming is an ability needed for many behaviours, including prey capture, predator avoidance, and swimming over turbulent waters. Therefore, xenoestrogen-induced reductions in burst swimming speed likely impair fish fitness, resulting in reduced survival.

### ***Time Course of Vitellogenesis-related Events***

Estrogen hormone exposure caused increases in liver somatic index (LSI), plasma vitellogenin (VTG) protein, and hepatic gene transcript levels, including *ERs*, *VTG* and vitelline envelope proteins (*VEPs*) (Chapters 4, 5 and 7). The time frame for these increases were characterized in detail for E2 (Chapter 4), estrone (E1) (Chapter 5), and E1, EE2 and E2 (Chapter 7) exposure, as was the time frame for the return of these altered parameters during a non-exposure period (Chapter 5) or at the end of the depuration phase (Chapters 4 and 7). New insights were gained such as: *VEPs* are gene expression biomarkers for E1-treated fish that quickly return to baseline levels, the timeframe for biomarker sampling to evaluate gene expression is not necessarily the same as that for VTG protein, and that there are differences between salmonid species since Rainbow trout had gene transcript alterations return to baseline levels (Chapters 4 and 5) while Chinook did not (Chapter 7). Evaluating the time course of changes in vitellogenesis-related responses to xenoestrogen exposure provided insight into using various vitellogenesis-related parameters as biomarkers, which is important given the widespread reports of the presence of xenoestrogens in fish habitats.



## ***Sewage Treatment Plant Effluents***

Two experiments made significant advances in knowledge regarding STP effluents and their effects (Chapters 6 and 7). First, when typical percent xenoestrogen removal levels were achieved by conventional activated sludge (CAS) treatment, the remaining bioactive components had sufficient activity to cause adverse effects in fish (Chapter 6). This knowledge can be used by STP managers and engineers because it suggests that advanced treatment processes are needed to remove nearly all (> 99%) of xenoestrogens to reduce the adverse effects caused by effluents. Second, in two tertiary-treated STP effluents, the only estrogen hormone found was E1. It appeared that E1 was the sole contributor to the STP effluent estrogenicity, although few indicators of estrogenicity were seen in Chinook (Chapter 7). These findings suggest that a water quality guideline should be developed for E1 to protect aquatic life in British Columbia (BC).

## **Recommendations for Future Studies**

### ***Impacts of Reduced Burst Swimming Speed***

The identification of reduced burst swimming speed was important and can be further investigated with lower concentrations of estrogen hormones to determine the thresholds of adverse effects and with other classes of xenoestrogens to determine if adverse effects exist for compounds other than estrogen hormones. The impact of reduced burst swimming speeds on predator avoidance and prey capture can be evaluated using predator-prey tests, so that impairments can be quantified in terms of

risk of predation, or decreased growth and the potential for starvation. Additionally, there are swim performance endpoints that can be evaluated that represent other types of swimming, such as critical swimming speed ( $U_{crit}$ ), which is a measurement of sustained swimming ability (Kieffer, 2000). Further explorations of the diversion of metabolic resources by xenoestrogens need to be performed, one area of study could be evaluating intra-muscular metabolic substrates and products in fish subjected to swim tests, because potentially there are ramifications on physiological systems that were not studied in this research yet could relate to reductions in fish fitness.

### ***Linking Molecular or Biochemical Results to Physiological Outcomes***

Making linkages between genomics and physiological results is a long-term challenge in toxicology, yet it is highly important to achieve, so that relevance can be given to gene expression changes and what they represent to the organism in terms of adverse effects (vs merely an alteration in transcript level). It was difficult to achieve linkages between molecular and physiological approaches in this research in the intended areas of reproductive and immunological effects. It was not possible to link the alterations in vitellogenesis-related responses (molecular, biochemical and tissue levels) to reproductive endpoints (tissue, organism or population levels) due to the use of Oncorhynchid species, which are difficult to maintain post-exposure because they have a long maturation time (between 1 – 3 years) and substantial housing requirements due to their size. To be able to do this in the future, a fish species that is small in size, has a relatively short life cycle, and has a well-characterized vitellogenesis model (i.e. including knowledge of both the transcripts and proteins for *ERs*, *VTGs* and other egg

proteins) would be advantageous (e.g. fathead minnow or zebrafish). If this is accomplished, then the sensitive, predictive power of gene expression alterations could be utilized to identify harmful actions prior to impairment. The attempt to link immune transcript alterations to changes in immune functions was not successful due to the lack of significant effects on immune transcripts even though changes in immune functional endpoints were seen (Chapter 2). Before this can be achieved, immune transcripts of Oncorhynchids need to be identified and annotated (e.g. the toll-like receptors and complement factors) because much of the current annotation relates to mammalian immune systems and indications exist that salmonid immune systems are somewhat different (Iwanowicz & Ottinger, 2009).

Further research with Oncorhynchids should attempt comprehensive studies with multi-level approaches to link together parameters from the molecular, biochemical, tissue, and organism or physiological levels to adverse outcomes. This is important information to obtain regarding wild salmonid fitness, if feral fish are exposed to xenoestrogens. It will require a relatively large, inter-disciplinary research group with a long-term (i.e. decade) research plan and large-scale fish housing facilities. In regards to this, the implications of parameters that do not recover post-xenoestrogen exposure need to be explored. Excess VTG protein is known to cause toxic effects in salmonids, but little work has been performed on this topic and few studies have used waterborne exposures. Thus, there is an opportunity to identify the xenoestrogen concentrations and exposure durations that relate to adverse effects of VTG protein in males and juveniles. If VTG protein inductions were related to the impairment of salmonid health, which may include reproductive outcomes, direct tissue damage or the diversion of metabolic resources from critical life functions, then this could link a biochemical

parameter to physiological endpoints. Then, the level of VTG protein in wild fish, which is already used as a biomarker of exposure, could possibly be used to provide context to the degree of potential harm.

### ***Monitoring for Xenoestrogens***

Very little information exists for the presence of xenoestrogens in the water bodies of BC, Canada. This research showed that E1 can be present in BC STP effluents (Furtula et al., 2012; Table 1-2 and Chapter 7); thus, environmental monitoring for estrogen hormones needs to be conducted downstream of STPs. Alternatively, final STP effluents could be evaluated to determine xenoestrogen content and then predictions of environmental concentrations could be made based on known receiving water dilution values. Pacific salmon are incredibly important economically, socially and culturally, and it should be a priority to identify if they are being exposed to xenoestrogens or not. Since STP effluents discharge chronically to the environment and estrogen hormones can bioaccumulate in salmonid food sources (such as algae (Lai et al. 2002), biofilm (Writer et al., 2011) and fish (Scott et al., 2005; Maunder et al., 2007)), there should be a higher level of local concern regarding estrogen hormones, or other xenoestrogens, and their potential impacts on Pacific salmonids. If estrogen hormones are found in surface waters, there are possible implications for juvenile salmonid health (including reduced immune defense and burst swimming ability, or repercussions from vitellogenesis-related inductions) and further study is needed to determine impacts on other salmonid life stages.

## Conclusions

Overall, these studies have demonstrated that environmentally relevant concentrations of xenoestrogens affect several physiological systems in salmonids. These chemicals can impair immune function and burst swimming ability, both of which have implications for wild fish survival. In juvenile salmonids, vitellogenesis-related transcripts (*VEPs*, *ERs* and *VTG*) can be biomarkers of estrogen hormone exposure. However, clear establishment of adverse reproductive outcomes in male or juvenile salmonids will be needed in combination with these results of altered vitellogenesis-related transcripts to make future use of the sensitive and predictive power of gene expression alterations. It appears that salmonids can mostly recover from xenoestrogen exposure, but that the elimination of VTG protein takes more than two weeks. The relevance of this to juvenile fish health is unknown. Estrone is the estrogen hormone found in BC STP effluents, suggesting that a water quality guideline is needed for this compound. The concentrations of xenoestrogens that remain in STP effluents may appear low, yet effluents can still cause effects on juvenile salmonid fish (in particular relating to initiation of vitellogenesis) due to the presence of potent and bioactive estrogen hormones and the summation of xenoestrogen components. The presence of xenoestrogens in the aquatic environment needs to be monitored in BC and, if found, there are implications for juvenile salmonid health.

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## **Appendices**

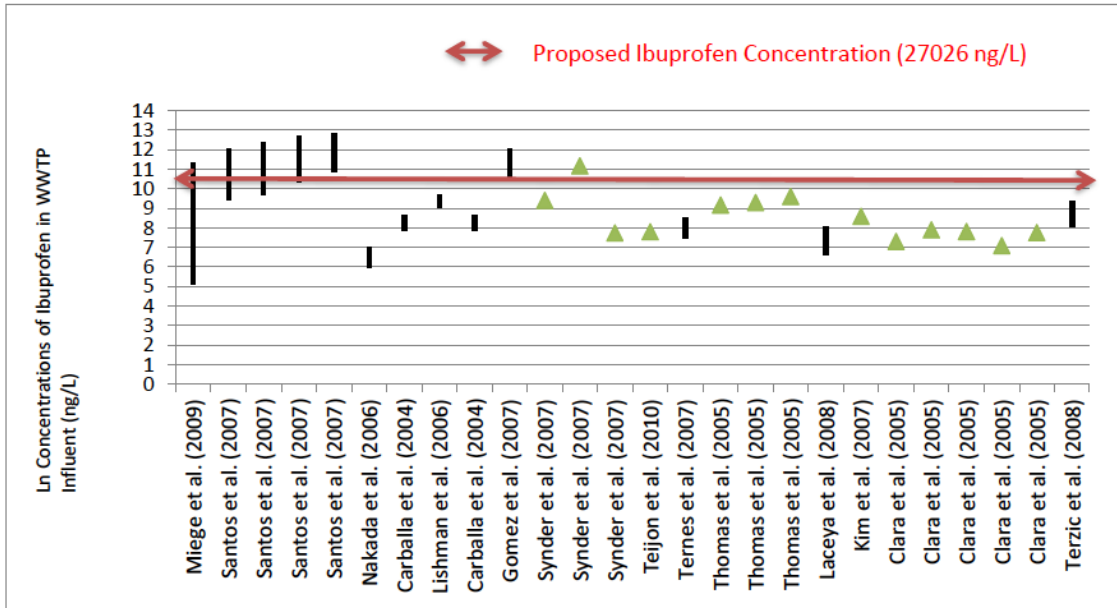


## **Appendix 1.**

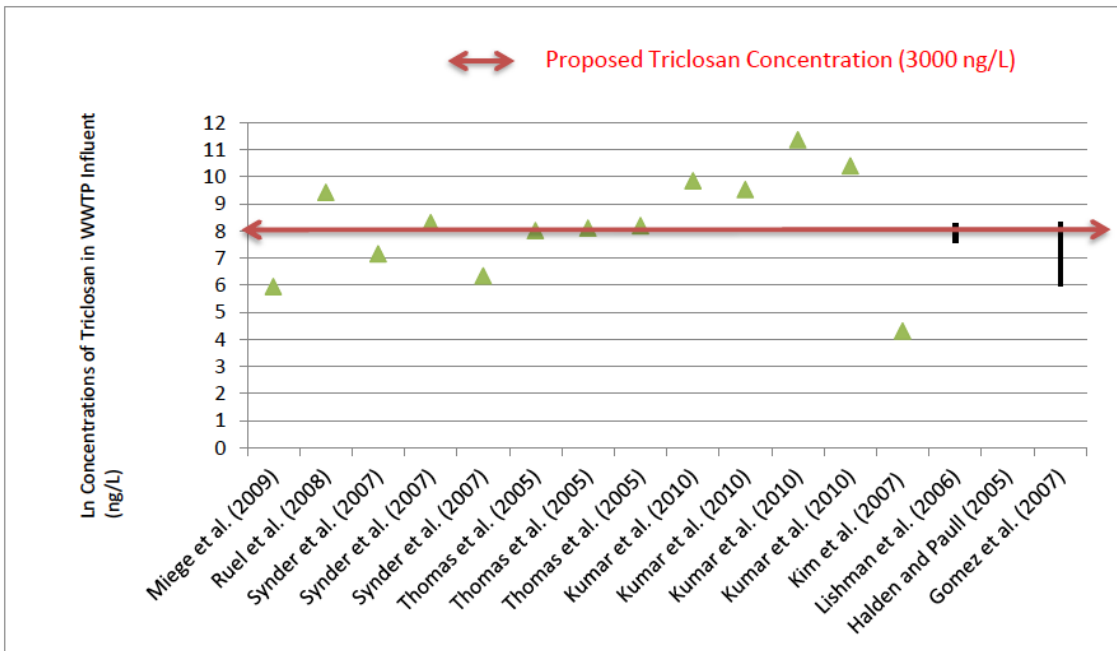
### **Literature values for derivation of target influent concentrations, supporting information for Chapter 6**

Figures prepared by Mehrnoush Mohammadali, PhD candidate, Dept. of Civil Engineering, University of British Columbia, Vancouver, BC

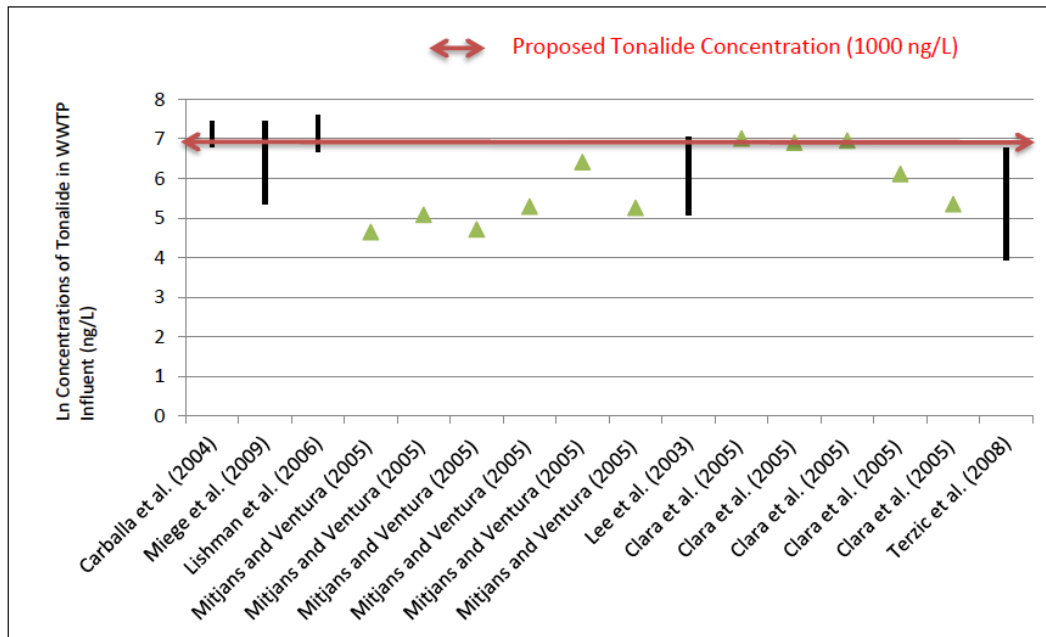
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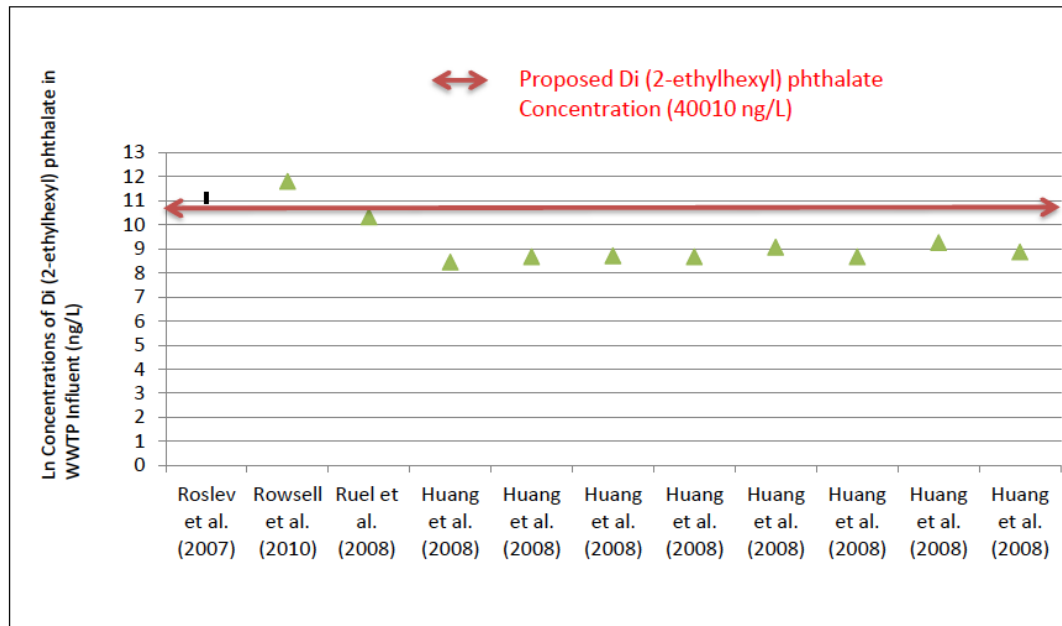
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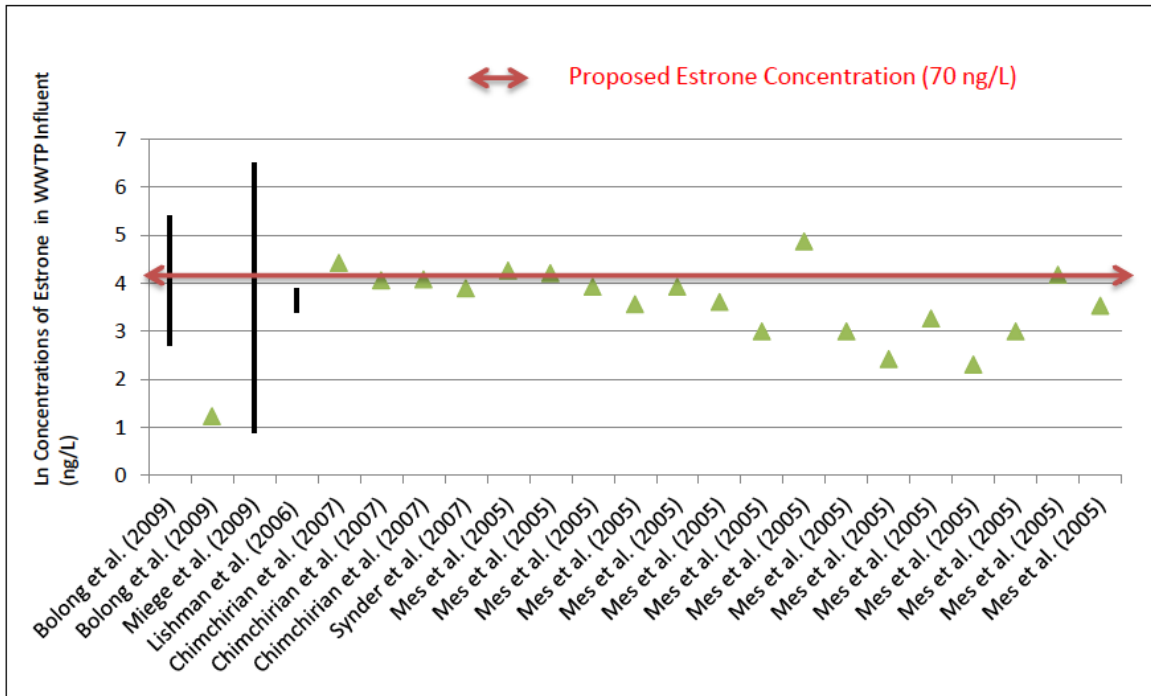
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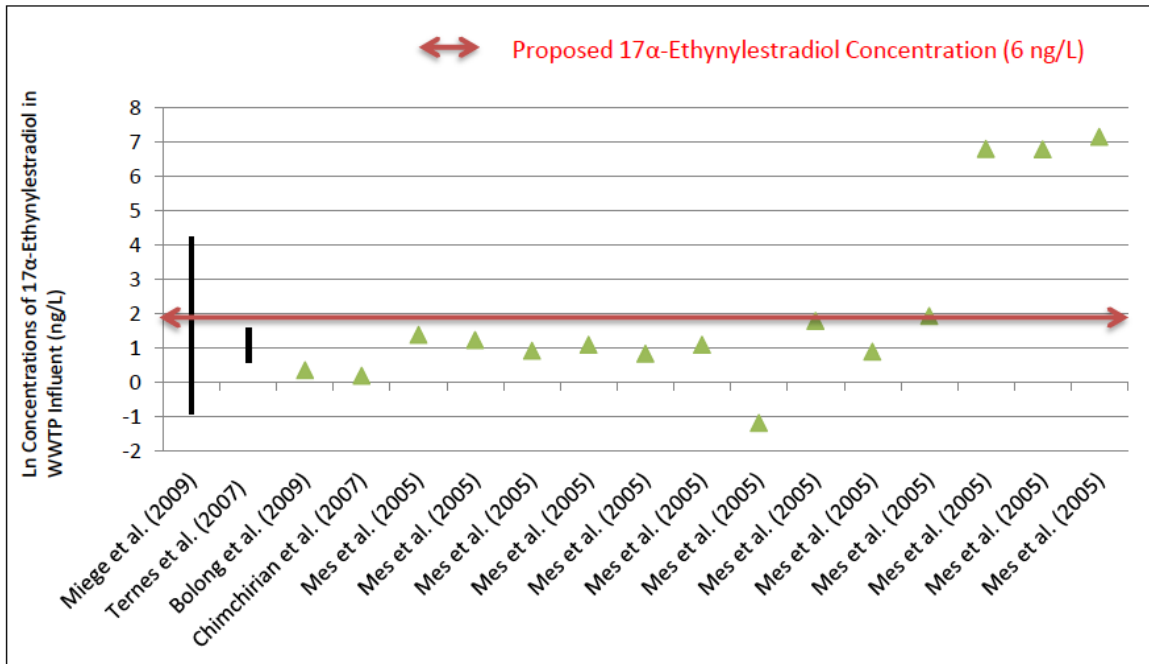
D. DEHP.



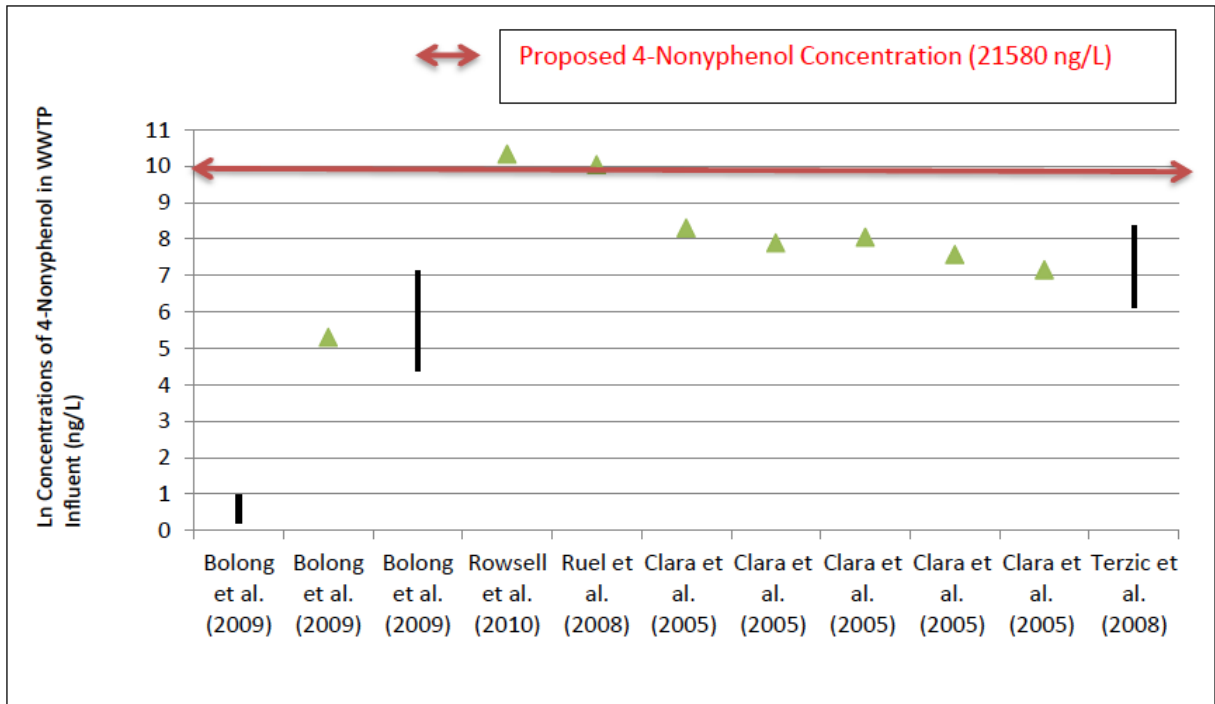
E. Estrone.



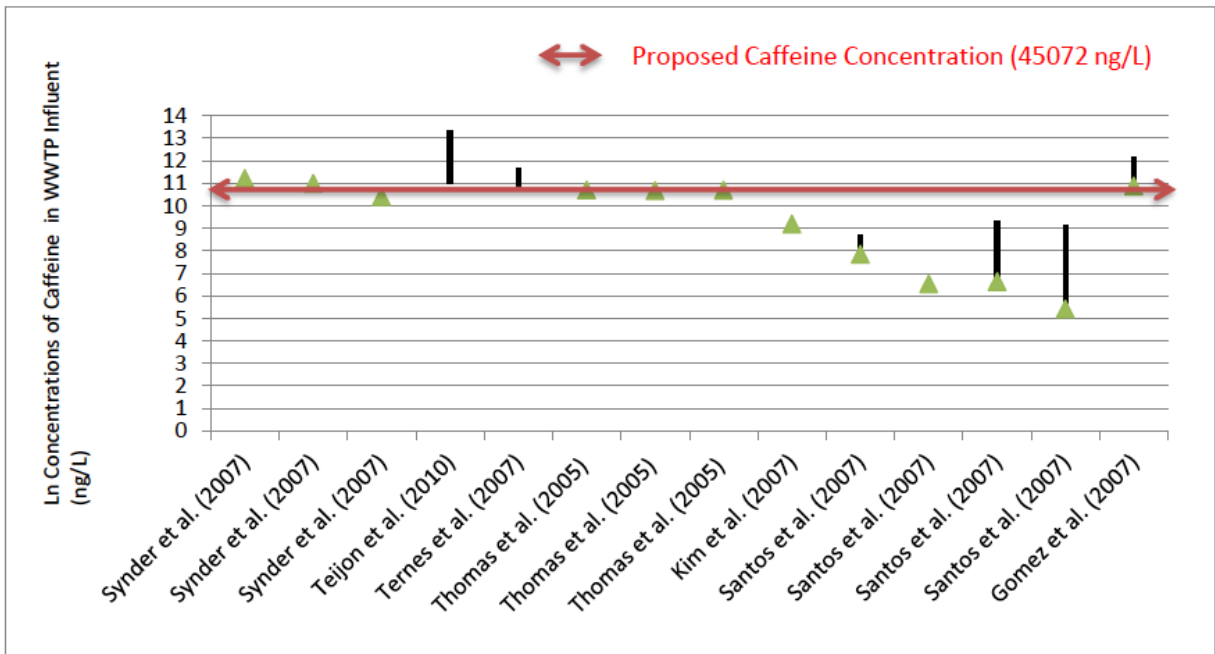
F. 17 $\alpha$ -ethynylestradiol.



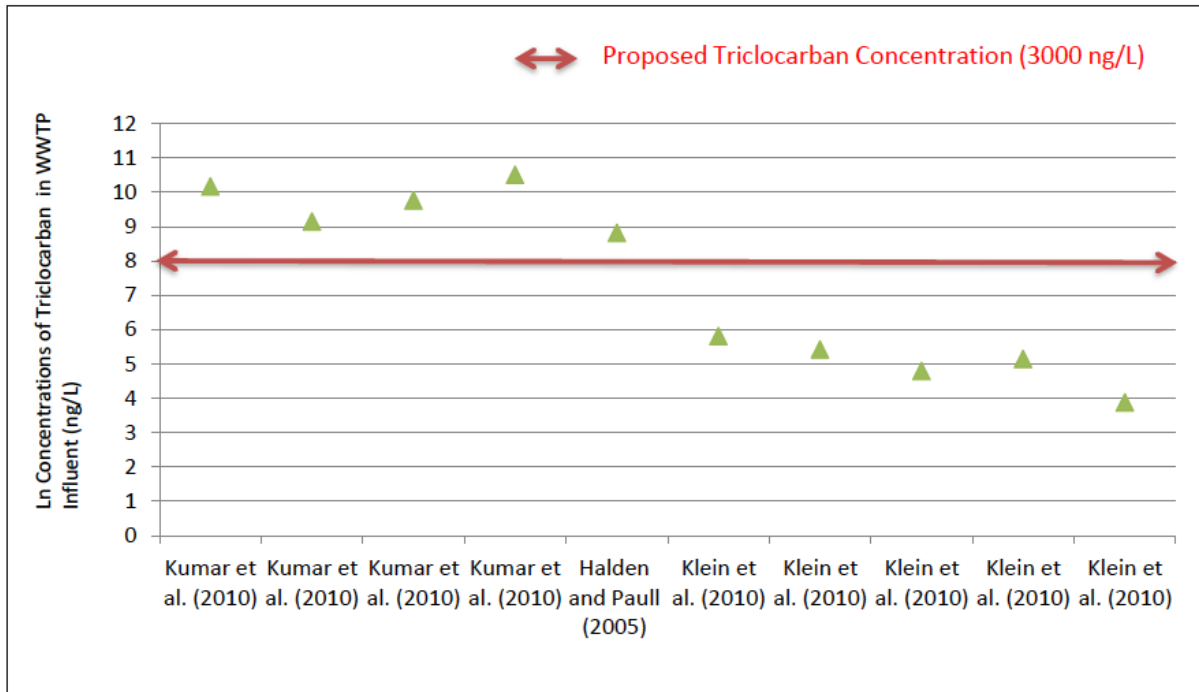
### G. Nonylphenol



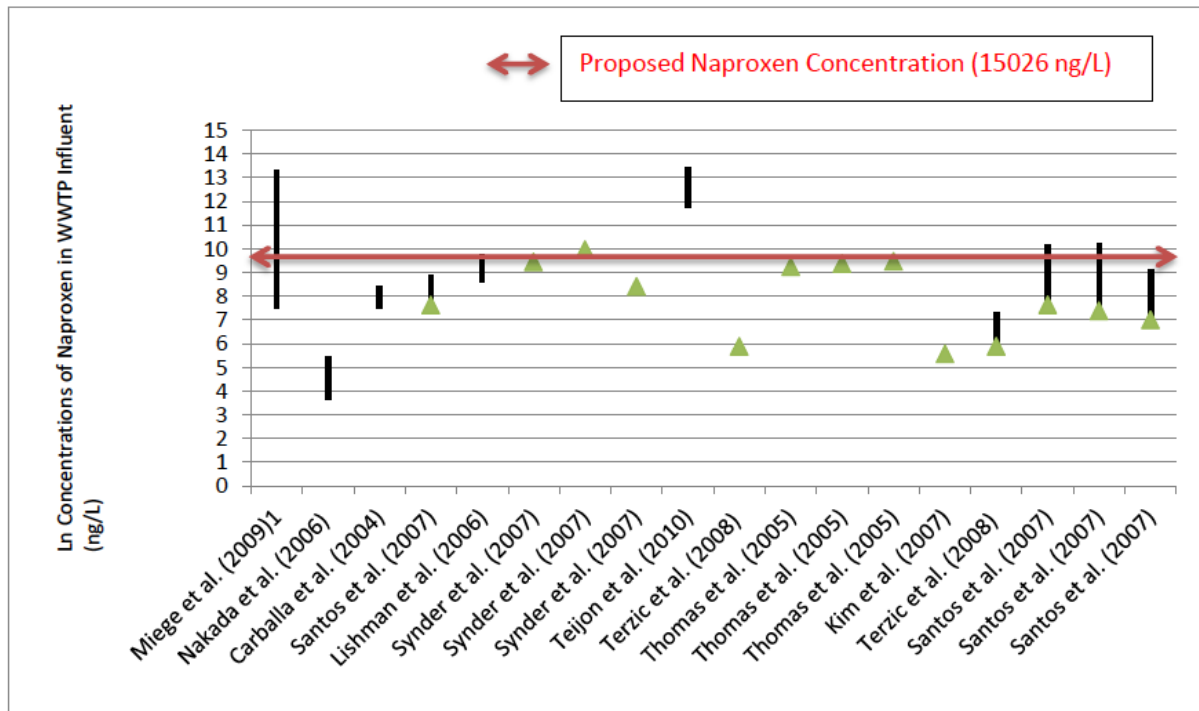
### H. Caffeine.



I. Triclocarban



J. Naproxen.



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