EFFECTS OF EMERGING CONTAMINANTS ON NATIVE CANADIAN FISH SPECIES

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Masters of Science

In the Toxicology Graduate Program

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

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ABSTRACT

The presence of emerging contaminants (ECs) detected in municipal wastewater effluents (MWWE) is increasing in much of the developed and developing world. For this reason, effects of these compounds to aquatic wildlife in receiving water bodies is becoming a growing concern globally. However, toxicity of many ECs, including 17α-ethynylestradiol (EE2), fluoxetine (FLX), and hexabromocyclododecane (HBCD), have limited or no data regarding their toxicity to aquatic organisms, particularly with native North American species of interest. Additionally, while testing of the effects of ECs to native species is warranted, challenges exist when conducting whole animal studies with wild fish species. Therefore, alternative assays such as *in vitro* assays or short-term molecular mechanistic studies are increasingly being implemented, including the tissue explant assay and the whole transcriptome experiment described in this thesis. Specifically, the primary objectives of this study were to 1) characterize and further expand our knowledge on the toxicity of selected ECs for which little toxicological knowledge regarding fish exist; and 2) to establish an *in vitro* approach that enables assessing the responsiveness of four selected fishes of relevance to North American fresh waters to three priority ECs. To address the information gap regarding toxicity of selected ECs, high-throughput, next generation sequencing technologies were utilized to identify key molecular pathways that were altered in liver of rainbow trout (*Oncorhynchus mykiss*) after a 96-hr waterborne exposure to the EC, fluoxetine (FLX). Pathway analysis yielded changes in a total of 144 different pathways, many of which were shared with previous studies. Altered pathways were predominantly involved in oxidative stress (downregulated) as well as metabolic function and other biological processes (upregulated). *In vitro* tissue explant assays with gonads and livers

from lake trout (*Salvelinus namaycush*) and northern pike (*Esox lucius*), as well as livers from rainbow trout and white sturgeon (*Acipenser transmontanus*), exposed to serial concentrations of EE2, FLX or HBCD were then conducted and transcript abundances of reproductive and antioxidant genes were measured to characterize species-specific changes in gene expression to these compounds. Antioxidant genes to be measured with exposure to FLX were chosen based on pathway analysis in Chapter 2. Results from the *in vitro* tissue explant experiments with rainbow trout exposed to fluoxetine confirmed and validated the findings from the first study; however, gene expression was highly variable, and other *in vitro* endpoints should be explored. Rainbow trout were consistently one of the more sensitive species in our study, and they appear to represent an appropriate model organism for many scenarios; however, in a few cases other species were more sensitive (with exposure to HBCD, for example), caution should be taken when extrapolating across species. Results of the tissue explant assays successfully characterized gene expression in native fish species to these ECs of concern and, if validated, could represent a useful tool for toxicity screening of chemicals in the future.

ACKNOWLEDGEMENTS

I would first like to thank my M.Sc. supervisor, Dr. Markus Hecker, for his continuous support, guidance, and the endless opportunities he provided me throughout my tenure at the Toxicology Centre, both as a research technician and as a graduate student. Without the experiences that I have obtained through my time in Markus's laboratory, I would not be the scientist, or person, I am today. I would also like to thank my M.Sc. committee members, Dr. Natacha Hogan and Dr. Paul Jones, for their time and support over the last few years, as well as my external examiner Dr. Lucy Lee.

Also, I would like to thank Jon Doering and Shawn Beitel for hiring me as an undergraduate research assistant in 2013 and starting my career as a Toxicologist. Specifically, Jon was an inspiration and mentor for me throughout my time at the Toxicology Centre, and this thesis would not have been possible without the guidance he has provided over the years. Special thanks also to James Alcaraz and Dayna Schultz for their assistance with experiments and their invaluable friendship.

I would like to acknowledge the funding sources for this project, which was supported through the Canada Research Chair program and Department of Fisheries and Oceans grant to Markus Hecker.

Lastly, I would like to express my sincerest gratitude to my family and friends, especially my mother and step-father, Sheila and Keith Gregoire, and my supportive boyfriend, Stefan Gonari. I want to dedicate this thesis to my late father, Neil Eisner, from whom I attribute my inspiration and drive.

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LIST OF ABBREVIATIONS

°C – degree Celsius

α – alpha

β – beta

γ – gamma

µg – microgram

µg/L – microgram per litre

µL – microliter

ANOVA – analysis of variance

ATRF – Aquatic Toxicology Research Facility

BC – British Columbia

BCV – biological variation

BFR – brominated flame retardant

bp – base pair

CA – California

CAT – catalase

cDNA – complimentary deoxyribonucleic acid

CIA – coinertia analysis

CPM – count per million

CYP1A – cytochrome P450, family 1, subfamily A

d – day or days

DLC – dioxin-like compound

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

E2 – 17β-estradiol

EC – emerging contaminant

EDC – endocrine disrupting chemical

EE2 – 17α-ethynylestradiol

ER – estrogen receptor

ERα – estrogen receptor alpha

ERβ – estrogen receptor beta

ERE – estrogen response element

FBS – fetal bovine serum

FLX – fluoxetine

g – gram

GABA – gamma-aminobutyric acid

GO – gene ontology

GPX – glutathione peroxidase

GST – glutathione S-transferase

GVL – Genomics Virtual Lab

h or hrs – hour or hours

HBCD – hexabromocyclododecane

IL – Illinois

KEGG – Kyoto Encyclopedia of Genes and Genomes

kg – kilogram

L – litre

LDH – lactate dehydrogenase

LOEC – lowest observed effect concentration

LT – lake trout

mm^3 – cubic millimetre

mg – milligram

mg/L – milligram per litre

MI – Michigan

min – minute

mL – millilitre

MOA – mechanism of action

mRNA – messenger ribonucleic acid

MS-222 – tricaine methanesulfonate

mV – millivolt

MWWE – municipal wastewater effluent

n – sample size

NCBI – National Center for Biotechnology Information

ng/L – nanogram per litre

NGS – next generation sequencing

NP – northern pike

ON – Ontario

PA – Pennsylvania

POP – persistent organic pollutant

PPCPs – pharmaceutical and personal care products

qPCR – quantitative polymerase chain reaction

ReR – relative response

RIN – RNA integrity number

ROS – reactive oxygen species

RT – rainbow trout

RNA – ribonucleic acid

SEM – standard error of the mean

SK – Saskatchewan

SSRI – selective serotonin reuptake inhibitor

T3 – triiodothyronine

T4 – thyroxine

THR – thyroid hormone receptor

US-EPA – United States Environmental Protection Agency

VTG – vitellogenin

WA – Washington

WS – white sturgeon

CHAPTER 1

1 GENERAL INTRODUCTION

PREFACE

This thesis is assembled in the manuscript-style. Therefore, there is some repetition between the introduction, materials and methods, and discussion sections in each chapter. Chapter 1 is a general introduction and literature review of emerging contaminants and their known effects on native North American fishes. Chapters 2 and 3 are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 4 is a general discussion and relates both Chapters 2 and 3 to each other, and to other *in vivo* and *in vitro* findings in the literature.

Author contributions:

Bryanna Eisner (University of Saskatchewan) reviewed the literature and wrote the chapter.

Dr. Markus Hecker (University of Saskatchewan) provided guidance as well as commented on and edited the chapter.

1.1 Emerging contaminants in municipal wastewater effluent

The widespread use and ubiquity of emerging contaminants (ECs), such as flame retardants and pharmaceuticals and personal care products (PPCPs), has raised concerns regarding their possible risks to the environment (Boxall et al., 2012; Ela et al., 2011). Many of these chemicals occur at high concentrations in municipal wastewater effluents (MWWEs) and are frequently detected in aquatic environments, especially downstream of wastewater treatment plants. PPCPs and other chemicals that are found in MWWEs have been identified by Environment Canada as one of the leading sources of pollution to date (Environment Canada, 2001). Therefore, there are concerns regarding their potential to cause harm to aquatic ecosystems (Boxall et al., 2012; Ela et al., 2011). Current wastewater treatment technologies are often insufficient in removing select ECs, and regions downstream of communities with minimal or no wastewater treatment, such as northern or rural communities, are at particular risk of increased exposure to these chemicals. As many of these chemicals are found in waterways, safety of fishes downstream from discharge points are the primary concern with regards to adverse ecological effects. Much of the toxicological concern pertaining to pharmaceuticals stems from the fact that they are often designed to elicit biological effects at low concentrations. For this reason, nontarget organisms may be adversely affected by these compounds due to differences in fish and mammal physiology and biochemistry (Corcoran et al., 2010). Furthermore, a number of ECs, including flame retardants and pharmaceuticals, may act as endocrine disrupting compounds (EDCs), targeting growth, development, and reproduction in non-target organisms such as fish. ECs may therefore impact fish populations exposed to MWWEs downstream of treatment facilities. However, adverse effects of many ECs to fish are not well characterized to date and further

information is needed to better protect aquatic environments. Native species to Canada are of particular interest due to the very limited, or even absent, information available on their susceptibility or sensitivity to these chemicals. Many freshwater fishes are significantly important to local ecosystems and protection of these fishes is essential.

1.2 Emerging contaminants of interest

Many ECs are found at increasing concentrations in MWWEs due to anthropogenic activities (Boxall et al., 2012; Ela et al., 2011), and from which they are then released into the aquatic environments with potential to cause adverse effects downstream. Several of these ECs are relatively new and have very little toxicological information available. Hence, there is increasing need for further testing to better understand the fate and effects of these chemicals to organisms that are at risk of exposure. Fluoxetine (FLX) and hexabromocyclododecane (HBCD) are two such chemicals for which little toxicity data is available. Although more data on 17α-ethynylestradiol (EE2) is available compared to FLX and HBCD, there are knowledge gaps concerning its effects to native North American species which need to be addressed.

1.2.1 17α-ethynylestradiol

One EC of particular concern in MWWEs is 17-α-ethynylestradiol (EE2). EE2 is a potent estrogen receptor agonist modeled after the natural endogenous hormone, 17β-estradiol (E2) (Aris et al., 2014). It is one of the most ubiquitously used compounds in oral contraceptives due to its high binding affinity to the estrogen receptor (ER; Aris et al., 2014). As such, EE2 has been

detected in aquatic environments around the world at concentrations as high as 42 ng/L, particularly in areas with higher population density (Chen et al., 2006; Ternes et al., 1999). Estrogens such as EE2 can competitively bind to either membrane bound ERs or the more widely studied nuclear ERs. Membrane ERs, although less well known, can also elicit an estrogenic response when activated by an estrogenic compound. For example, zebrafish oocytes showed a delay in maturation when membrane bound ERs were activated (Pang and Thomas, 2010). Nuclear ERs are more commonly studied and remain inactive in the nucleus, bound to heat shock proteins until an estrogenic compound interacts with it (Boelsterli, 2007; Figure 1.1). After an estrogenic compound, such as EE2, binds to the nuclear ER, the ER then homodimerizes and interacts with the estrogen response element (ERE; Macgregor and Jordan, 1998). Once bound to the ERE in the nucleus, EE2 is then able to trigger transcription of genes involved in estrogenic response pathways (Macgregor and Jordan, 1998). This PPCP acts as an EDC through inducing an estrogenic response, thus disrupting normal endocrine functions, such as reproduction, development, behaviour, and homeostasis (Nasuhoglu et al., 2012).

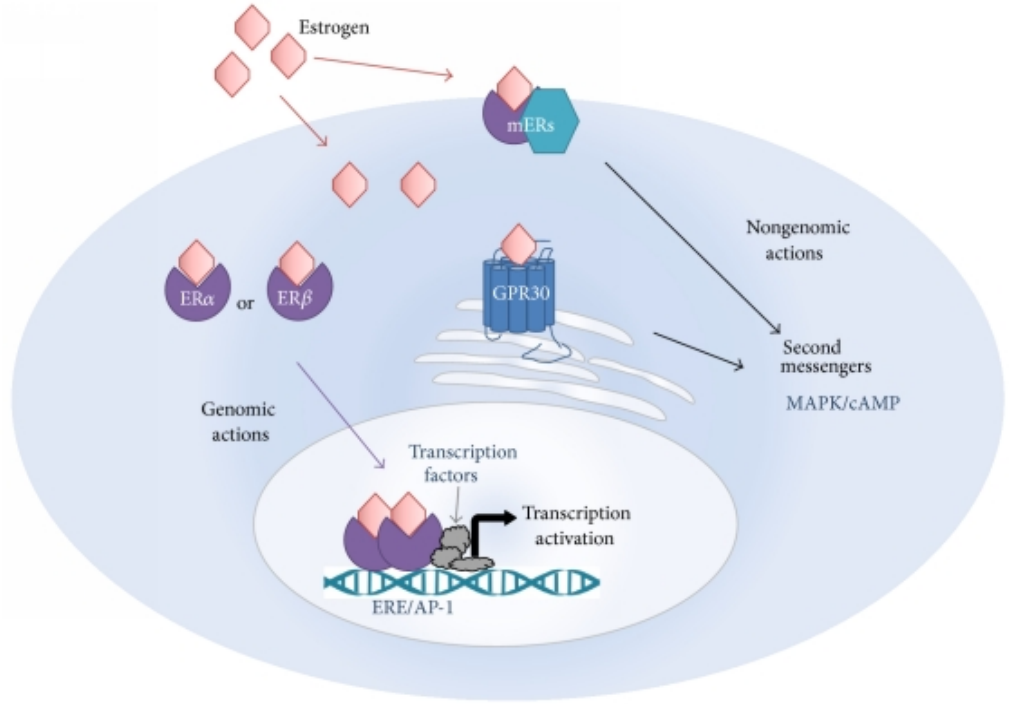

Figure 1.1 Mechanism of action (MOA) for 17β-estradiol (E2), as well as other estrogen receptor (ER) agonists such as 17α-ethynylestradiol (EE2). Estrogen can interact with either membrane bound or nuclear ERs. In the estrogenic response involved with activation of a nuclear ER, the estrogen diffuses across the plasma and binds to the ER which then diffuses across the nuclear membrane and interacts with the estrogen response element (ERE), forming homodimers. Activation of transcription of genes related to the estrogenic response pathways is initiated and a response in the cell of the organism is then triggered (Gogos et al., 2015).

EE2 is a lipophilic compound with a considerably lower solubility than E2 and is moderately persistent in surface waters, with a half-life between 1.5 and 17 days, and in soils, with a half-life between 3 and 7.7 days (Colucci and Topp, 2001; Jurgens et al., 2002; Zuo et al., 2006). Due to its relatively high lipophilicity and persistence, EE2 may bioconcentrate in fishes, therefore making it an increased concern to aquatic environments as it is primarily released into aquatic systems through MWWEs (Larsson et al., 1999).

Due to its high prevalence in municipal raw sewage, removal of EE2 during wastewater treatment is necessary (Corcoran et al., 2010). However, only approximately 70 to 80% of total steroidal estrogens are typically removed during conventional treatment, allowing 20 to 30% to escape into the aqueous phase to the effluent and into the environment (Auriol et al., 2006; Johnson et al., 2007). At environmentally relevant concentrations, EE2 has shown to induce (de)feminization, (de)masculinization, or intersex in aquatic organisms, and is particularly potent to fish (Chikae et al., 2003; Orn et al., 2003, 2006; Seki et al., 2002; Tyler et al., 1999). Reproductive failure has been observed in zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), and roach (*Rutilus rutilus*) at life time exposure concentrations between 4-6 ng EE2 L^{-1} or with a seven-day exposure at 10 ng EE2 L^{-1} (Kidd et al., 2007; Lange et al., 2009; Lister et al., 2009; Nash et al., 2004).

1.2.2 Fluoxetine

Fluoxetine (FLX) is a pharmaceutical drug primarily used to treat depression, but is also administered for personality and eating disorders, as well as compulsive behaviours (Brooks et al., 2003). As a selective serotonin reuptake inhibitor (SSRI), FLX improves mood levels and

decreases hostility and appetite in humans by competitively binding to serotonin receptors, thereby blocking reuptake of the neurotransmitter serotonin in the brain (Gaworecki and Klaine, 2008; Figure 1.2). With reuptake of serotonin blocked by FLX, extracellular serotonin levels are increased (Gaworecki and Klaine, 2008).

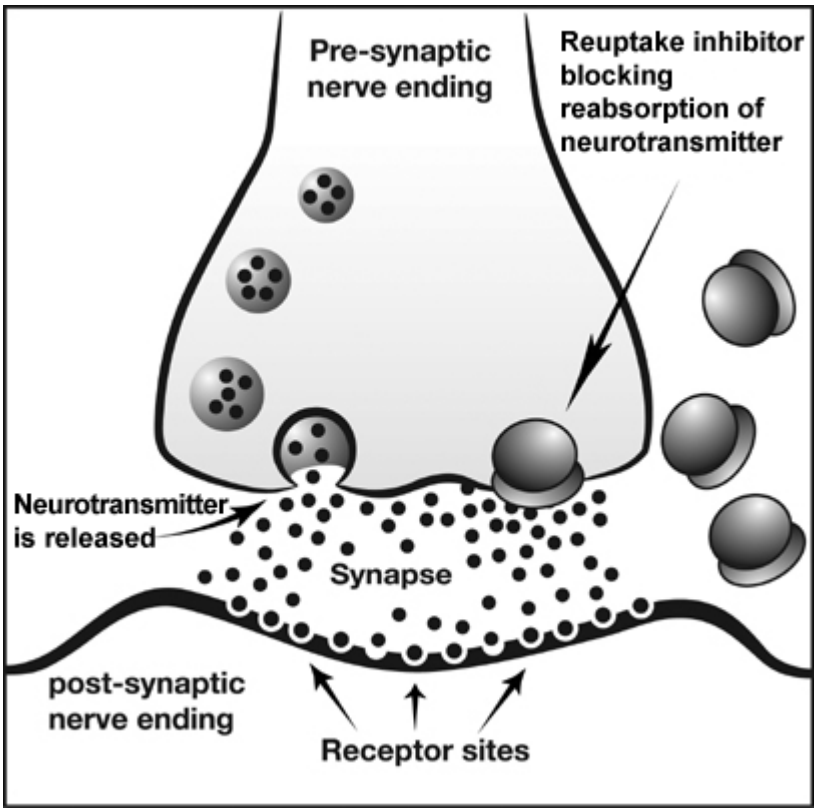

Figure 1.2. Diagram depicting the mechanism of action (MOA) of selective serotonin reuptake inhibitors (SSRIs) at the synapse. SSRIs, such as fluoxetine (FLX) competitively bind to the presynaptic membrane, actively blocking the reuptake of the neurotransmitter, serotonin. Thus, serotonin concentrations are increased in the synapse and remain bound to the postsynaptic membrane (Lattimore et al., 2005).

The PPCP FLX is biotransformed to its active metabolite, norfluoxetine, by cytochrome P450 enzymes and is excreted in the urine with less than 10% of the initial compound present (Brooks et al., 2003). Drugs such as FLX require continuous administration in order for desired therapeutic effects to be observed and, for this reason, aquatic systems are not exposed acutely, but rather chronically and continuously, to low doses of FLX (Gaworecki and Klaine, 2008; Kreke and Dietrich, 2008). Furthermore, FLX is a polar, non-volatile drug that adsorbs partially to activated sludge during treatment with percent removal of FLX usually between 60-88% (Bedner and MacCrehan, 2006; Brooks et al., 2003; Jones-Lepp et al., 2001). Along with EE2, SSRIs such as FLX are among the most commonly measured drugs in wastewater effluents and are predominantly detected in the water column, but have also been shown to moderately adsorb to sediments (Corcoran et al., 2010). Like many antidepressants, FLX is a weak base and its uptake and accumulation is largely dependent on the pH of water (Metcalfe et al., 2009). At low pH, FLX is predominantly in its ionic form, while at higher pH (freshwater generally around pH of 7), FLX is in its neutral form and more readily accumulates in fish tissue (primarily in the brain and liver) as it can easily pass through the phospholipid membrane of cells (Brooks et al., 2005; Metcalfe et al., 2009). Once in the brain, FLX has an estimated half-life of nine days, after which it is excreted via the urine or in breast milk at a dose below the level of toxic concern to fishes (Kristensen et al., 1999; Wong et al., 2013).

Due to its wide and prolonged use and its incomplete elimination during wastewater treatment processes, FLX is continuously released into the environment through wastewater effluents with concentrations as high as 540 ng FLX L^{-1} detected in receiving aquatic systems (Bedner and MacCrehan, 2006; Brooks et al., 2003; Weston et al., 2001). Although FLX has been used since 1986, very little is known about its fate in the environment or its toxicity to

aquatic life (Brooks et al., 2003; Myers, 2007). Much like in humans, serotonin functions as an important neurotransmitter in fish and is involved in various functions such as endocrine parameters, reproduction, as well as behaviours such as aggression and appetite (Caamano-Tubio et al., 2007; Corcoran et al., 2010; Overli et al., 1998). In fathead minnow, decreased feeding rate or ability to catch prey and decreased growth were observed with exposure to concentrations of 51.0-170 µg FLX L^{-1} and 51.0-53.0 µg FLX L^{-1}, respectively (Gaworecki and Klaine, 2008; Stanley et al., 2007). Additionally, Japanese medaka (*Oryzias latipes*) exhibited developmental abnormalities as well as increased estradiol levels with exposure to environmentally relevant concentrations of 0.10-5 µg FLX L^{-1} and 0.10-0.50 µg FLX L^{-1}, respectively (Brooks et al., 2003). Fish in general are less proficient at metabolizing FLX, as well as other PPCPs, than mammals, due to their lack of the cytochrome $P_{450}2C$ enzyme family in the liver (Gagné et al., 2006). This group of enzymes are essential for drug metabolism and their absence may hinder the elimination of PPCPs, particularly FLX (Figure 1.3; Gagné et al., 2006). While there has been some work done on behavioural or select apical effects of a few model fish species, little to nothing is known about the effects of FLX on the molecular level to fishes native to North America.

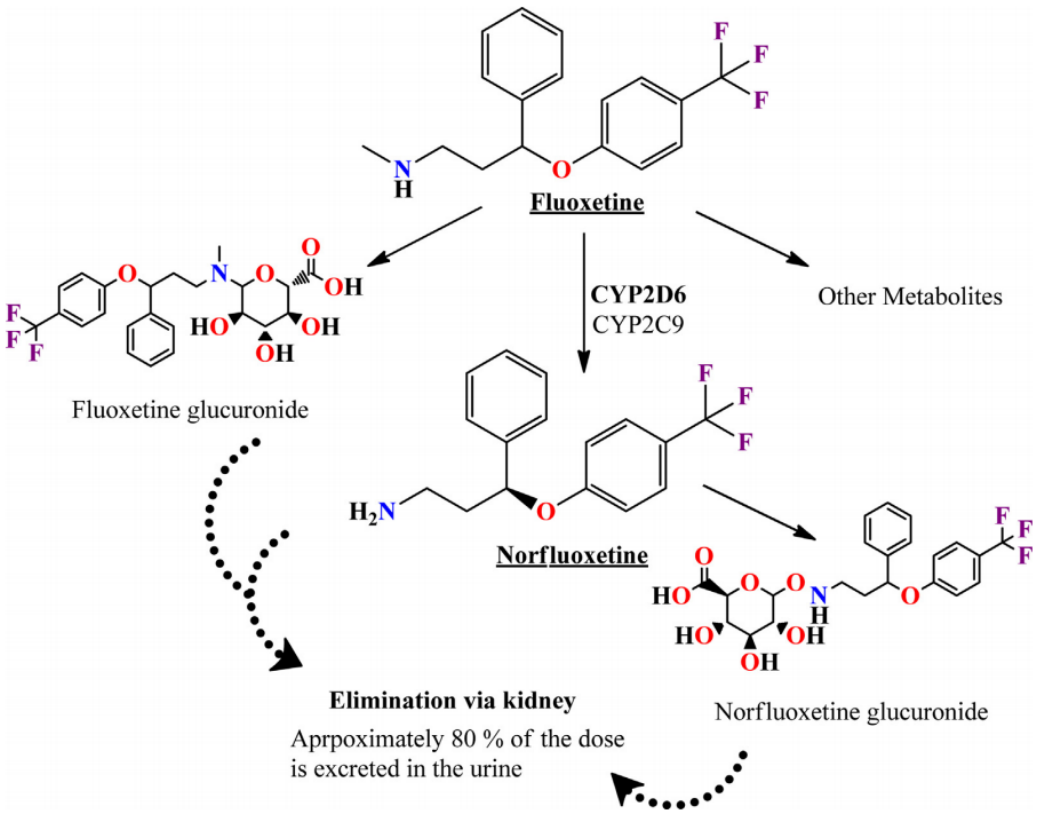

Figure 1.3. Schematic depiction of the metabolic pathway for fluoxetine (FLX). The parent compound, FLX, is first broken down into FLX glucuronide, norfluoxetine, and other metabolites through the action of several cytochrome P450 enzymes, namely CYP2D6 and CYP2C9. Norfluoxetine is further broken down into norfluoxetine glucuronide and is eliminated, along with FLX glucuronide, via the kidneys (Alves et al., 2015).

1.2.3 Hexabromocyclododecane

Hexabromocyclododecane (HBCD) is a persistent organic pollutant (POP) that has been widely used in electrical equipment, textiles, and other building materials, and in recent years has been the third most used brominated flame retardant (BFR) worldwide (Berntssen et al., 2011; Meng et al., 2011). As such, HBCD is detected in soils, sediments, and air at increasing concentrations (Birnbaum and Staskal, 2004; Hu et al., 2009). With a log K_{OW} value of 5.8, HBCD demonstrates high bioaccumulative properties and has been detected at concentrations as high as 10 000 µg/kg wet weight in aquatic organisms (Law et al., 2006; Hu et al., 2009). Due to its hydrophobic nature, HBCD preferentially binds to solid particles, rather than remaining in the water column, and has been detected in sewage sludge, soils and sediments at concentrations as high as 1700 ng HBCD g^{-1} dry weight downstream of HBCD production plants (Covaci et al., 2006; Sellstrom et al., 1998). As a POP, HBCD possesses qualities characteristic of this class of chemicals, such as persistence in the environment, bioaccumulation and possible biomagnification in lipid-rich tissues, long-range transport, as well as potential toxicity to aquatic and terrestrial organisms (Covaci et al., 2006). Even at sampling sites without a known source of HBCD contamination, low concentrations of less than 10 ng HBCD g^{-1} dry weight were detected (Covaci et al., 2006). At these particular sites, it is hypothesized that long range transport is the source of HBCD contamination, and therefore, multiple non-target species of interest may be affected without our knowledge (Covaci et al., 2006). Due to its common use in consumer products, HBCD has been detected in dust collected from homes (Stapleton et al., 2008), as well as in municipal wastewater sludge (La Guardia et al., 2010). Contaminants such as HBCD may be released into the environment via various point sources, including emission or leaching from

BFR manufacturing plants, and non-point sources, such as improper disposal of BFR-containing consumer products (Covaci et al., 2011). Although HBCD is not commonly detected in water samples, it may still cause harm to aquatic organisms through consumption of contaminated feed from the sediment or soil (Hu et al., 2009). Concentrations up to 0.3 ng HBCD g^{-1} dry weight (dw) have been detected in sediments (Klosterhaus et al., 2012) and up to 60 ng L^{-1} in water (Robson et al., 2013). With dietary exposure to HBCD, certain fish species, such as rainbow trout (*Oncorhynchus mykiss*), have exhibited altered metabolism and thyroid disruption (5 ng HBCD g^{-1} bodyweight; Berntssen et al., 2011; Palace et al., 2008; 2010). Furthermore, zebrafish exposed to concentrations of 0.1 mg/L have shown toxicity to the brain and liver (Deng et al., 2009; Feng et al., 2013a). Due to these factors, as well as its abundance in the environment, HBCD has become an emerging contaminant of concern (Covaci et al., 2006).

Due to its persistence in and potential toxicity to wildlife including fish, HBCD is slowly being phased out of use or banned in many parts of the world and replaced with other flame retardants; yet, because of its wide presence in anthropogenic materials, particularly in insulation, HBCD will continue to leach into the environment for years to come (GEO, 2007). However, in Europe and North America for example, the general trend suggests that HBCD concentrations in the environment are on the decline (Ismail et al., 2009; Rudel et al., 2011). The α-isomer of HBCD is present in aquatic organisms in greater quantities than the β- or γ-isomers, especially in the muscle; however, all HBCD isomers have been detected in liver, muscle and brain tissue in fish (Berntssen et al., 2011; Haukas et al., 2009; Remberger et al., 2004). Additionally, the α-isomer has the highest biomagnification factor (BMF) among the three isomers, with a value of 9.2 (Law et al., 2006). With the complicated breakdown of HBCD, more data is needed on the fate and effects of specific isomers as well as temporal trends of

biodegradation of each isomer and the conditions that influence it (Covaci et al., 2006). Furthermore, HBCD is an additive BFR and little data is available on its effects in mixtures (Cruz et al., 2015). Although HBCD was added to the Stockholm Convention's list of POPs in 2013 and meets criteria to trigger section 64 of the Canadian Environmental Protection Act (CEPA) of 1999, a large gap in data, particularly relative to risk assessment, exists and further research on the toxicity of this ubiquitous chemical is warranted (Cruz et al., 2015; UNEP, 2013).

1.3 Regulatory relevance of ECs and implications to species of interest

Many ECs are frequently found in surface water systems, and for this reason, effects on aquatic organisms are a main concern with regard to exposure to these chemicals. However, adverse effects of ECs of concern are not widely characterized in many aquatic organisms, particularly in fish species native to northern Canadian freshwater ecosystems. This is of great concern to ecological risk assessors as it has been shown that large differences in sensitivities exist among freshwater fishes (Doering et al., 2013; Elonen et al., 1998). Current environmental risk assessment strategies for the determination of chemical toxicity in aquatic organisms typically rely on standard model species that may not be representative of the diversity of fishes, particularly those native to Canada. Extrapolation of data from model species to native species of interest requires application of uncertainty factors, which may considerably overestimate the risk of that chemical, in some cases by multiple orders of magnitude (CEC., 1996; Chapman et al., 1998; Forbes and Calow, 2002). Additionally, sensitivity to chemicals has been assumed to be similar among phylogenetically related organisms; however, studies have shown that this is not

always true (Allard et al., 2010; Sanderson and Solomon, 2009; Vardy et al., 2013). Therefore, extrapolation of data from model species to native species in the same class or family may significantly underestimate the sensitivity of that species to the chemical of concern (Allard et al., 2010; Sanderson and Solomon, 2009; Vardy et al., 2013). For these reasons, it is necessary to also test native fishes rather than conclude from existing data for model species.

Examples of species of interest in Canadian freshwater systems include lake trout (*Salvelinus namaycush*), rainbow trout, northern pike (*Esox lucius*), and white sturgeon (*Acipenser transmontanus*). These species are abundant in North America and hold cultural, recreational, and commercial significance to Canadians (Scott & Crossman, 1998). With the exception of white sturgeon, these species are also top predators and aid as key indicators of ecosystem health as they tend to accumulate contaminants (Scott & Crossman, 1998). Rainbow trout, in addition to being a well-known sport fish like lake trout and northern pike, are a widely used model laboratory species in toxicity testing due to their relative sensitivity to contaminants, including metals and EDCs (Hansen et al., 2002; Jobling et al., 1996; Scott & Crossman, 1998). White sturgeon, while no longer an important species commercially due to its endangered status in most parts of Canada, are very important from a cultural and conservation perspective. White sturgeon are also long-lived and may therefore be exposed to greater concentrations of contaminants in their lifetime, thereby putting them at greater risk than shorter-lived species (Doering et al., 2012; Scott & Crossman, 1998). Furthermore, very few studies have been conducted with white sturgeon, as with lake trout and northern pike, as they are difficult to culture and maintain under laboratory conditions and few toxicity tests are tailored to these species. With the exception of rainbow trout, knowledge of the sensitivities and susceptibilities

of these species to ECs is limited and, therefore, assessment of effects of ECs to these species is warranted.

1.4 Alternative testing methods

Traditionally, effects of contaminants of concern are assessed using whole animal *in vivo* toxicity assays with standard laboratory animals (Krewski et al., 2007). However, numerous challenges exist when conducting *in vivo* assays, particularly with non-model species. For example, it is often very difficult to culture and maintain wild fish species under laboratory conditions, and there are ethical concerns when working with whole fish, especially endangered species such as white sturgeon. Additionally, *in vivo* toxicity assays are expensive as well as time- and space-consuming (Villeneuve and Garcia-Reyero, 2011). For these reasons, alternative, high-throughput *in vitro* testing methods are increasingly being used to test toxicity of contaminants in place of traditional *in vivo* methods. *In vitro* toxicity assays may include primary cell cultures, stable cell lines, and as described in this study, tissue explants. Of these *in vitro* approaches, tissue explant assays are particularly of interest with regard to the conduct of species-specific toxicity assays as they require fewer animals relative to *in vivo* toxicity assays, while maintaining some species-specific tissue functions, such as paracrine interactions among different cell types, which is not always the case for cell-line based assays (Gray et al., 1997; Tan and Schirmer, 2017). Furthermore, with primary *in vitro* assays, it is possible to test multiple contaminants for each individual for a wider range of concentrations than *in vivo* assays. Additionally, *in vitro* assays allow for the identification of the mechanism of action (MOA) of a compound, thereby making it possible to determine species-specific effects to that compound.

1.5 Objectives

To date, very little is known about the sensitivities and susceptibilities of native North American fish species to emerging contaminants of concern. Therefore, approaches to address the lack in data are warranted. The overall objective of this study was to establish and validate an alternative *in vitro* tissue explant assay to characterize the sensitivity of four species of commercial, recreational or Aboriginal relevance in Canada, namely lake trout, rainbow trout, northern pike and white sturgeon, to three priority ECs, namely 17α-ethynylestradiol (EE2), fluoxetine (FLX) and α-hexabromocyclododecane (HBCD). Specific objectives, hypotheses, and experiments included:

1) **Transcriptomic response of rainbow trout to fluoxetine, *in vivo* (Chapter 2).**

The pharmaceutical drug FLX is widely used by humans to treat depression as well as eating and personality disorders (Brooks et al., 2010). Removal of FLX during wastewater treatment is incomplete, and therefore, it is frequently detected in aquatic systems (Corcoran et al., 2010). Due to its ubiquity in the environment and partial elimination from wastewater, there is increasing concern regarding its risk to aquatic life. Although its mechanism(s) (MOA) of action is well understood in humans, its direct effect on aquatic life, as well as its fate in the environment, is uncertain (Brooks et al., 2003; Myers et al., 2007).

This portion of the study aimed to identify the biological pathways that are altered with exposure to FLX, *in vivo*, using whole transcriptome analysis. With whole transcriptome analysis, we are able to more accurately define the MOA of a chemical and use that information in screening for chemicals through alternative testing in the future. This will reduce animal use in

future studies as *in vitro* endpoints can then be assessed once more knowledge on a chemicals MOA becomes known. The PPCP FLX was chosen for this study rather than EE2 or HBCD for several reasons. Firstly, the MOA of EE2 has been well characterized in fishes while information regarding FLX is limited. Furthermore, while information regarding the MOA of HBCD is also limited, it is present in the environment at lower concentrations than FLX with lesser effects reported in fish. Additionally, as FLX is specifically designed to affect neuroendocrine systems at low levels, there is a pressing need to further characterize its MOA to non-target species. Therefore, the transcriptome in rainbow trout livers were assessed and analyzed and the specific objectives and null hypotheses were:

1) Characterize molecular toxicity pathways for FLX in rainbow trout using a whole transcriptome sequence-by-synthesis approach.

   H_0: There is no statistically significant alteration in any genes in livers from rainbow trout following exposure to FLX, *in vivo*.

2) Inform on *in vitro* molecular endpoints to be assessed in the tissue explant assays established in Chapter 3.

   H_0: There are no endpoints identified that could be assessed *in vitro*.

2) In vitro comparison of responses among four Canadian fish species to emerging contaminants of concern (Chapter 3).

Use of ECs, such as flame retardants and select pharmaceuticals, has become widespread and many of these chemicals are being released into the environment through MWWE at increasing concentrations (Boxall et al., 2012; Ela et al., 2011). Although many of these chemicals have been implicated in disrupting endocrine function in aquatic organisms, effects of

several ECs are still not fully understood in native fish species (Boxall et al., 2012; Ela et al., 2011). Therefore, an *in vitro* tissue explant assay was conducted to investigate and compare the responses of four Canadian fish species, specifically lake trout, rainbow trout, northern pike, and white sturgeon, to serial concentrations of EE2, FLX, and HBCD. Transcript abundances of target genes were analyzed in liver and gonad tissues for each species and each chemical tested. The specific objectives and null hypotheses were:

1) To characterize the transcript abundances of selected target genes in lake trout, rainbow trout, northern pike or white sturgeon with exposure to EE2, FLX and HBCD in liver tissue.

   H_0: There is no statistically significant and concentration-dependent change in the abundance of target gene transcripts in liver slices of lake trout, rainbow trout, northern pike or white sturgeon exposed to EE2, FLX, or HBCD compared to controls.

2) To characterize transcript abundances of selected target genes in lake trout, rainbow trout, northern pike or white sturgeon with exposure to EE2, FLX and HBCD in gonad tissue.

   H_0: There is no statistically significant and concentration-dependent change in the abundance of target gene transcripts in gonad slices of lake trout, rainbow trout, northern pike or white sturgeon exposed to EE2, FLX, or HBCD compared to the controls.

3) Comparison of responses at the gene expression level after exposure to EE2, FLX and HBCD among lake trout, rainbow trout, northern pike and white sturgeon.

H_0: There is no statistically significant difference in the abundance of target gene transcripts in liver or gonad slices among lake trout, rainbow trout, northern pike, or white sturgeon exposed to EE2, FLX, or HBCD.

4) Comparison of changes in gene expression patterns after exposure to EE2, FLX and HBCD in lake trout, rainbow trout, northern pike and white sturgeon *in vitro* to available *in vivo* data in the literature to determine if observed *in vitro* responses are predictive of *in vivo* sensitivity.

   H_0: *In vitro* responses from this study will not differ from *in vivo* responses previously reported in the literature.

CHAPTER 2

2 TRANSCRIPTOMIC RESPONSE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) TO FLUOXETINE EXPOSURE *IN VIVO*

PREFACE

As little data on the effects of the pharmaceutical drug fluoxetine (FLX) on fishes are available, this Chapter aimed to further characterize the molecular mechanism of action of FLX. I therefore investigated the effects of FLX on the whole transcriptome of rainbow trout (*Oncorhynchus mykiss*) livers following a 96-hr *in vivo* exposure.

Author contributions:

Bryanna Eisner (University of Saskatchewan) designed and managed the experiment, generated and analyzed all data, and drafted the manuscript.

James Alper Alcaraz (University of Saskatchewan) helped with the design of the experiment and assisted with experiment set-up and bioinformatics analyses.

Dr. Markus Hecker (University of Saskatchewan) provided guidance and inspiration for the conception and design of the experiment, offered comments and edits to the manuscript, and provided research funding.

2.1 Abstract

Prevalence of pharmaceuticals and personal care products (PPCPs) detected in municipal wastewater effluents (MWWE) is increasing in much of the developed and developing world. For this reason, effects of these compounds to aquatic wildlife in receiving water bodies, particularly to native fish populations, is becoming a growing concern globally. One such PPCP ubiquitously found in MWWEs is the pharmaceutical drug fluoxetine (FLX), which is administered to treat depression as well as other behavioural disorders in humans. However, the specific effects FLX has on non-target organisms such as fish including its molecular mechanism of toxic action (MOA) are not fully understood to date. To address this information gap, high-throughput, next generation sequencing technologies were utilized to identify key molecular pathways that were altered in livers of rainbow trout (*Oncorhynchus mykiss*) after a 96-hr waterborne exposure to 125 µg/L FLX. A total of 547 and 945 contigs were significantly upregulated and downregulated after exposure to FLX, respectively. However, only 236 (43.1%) and 642 (67.9%) contigs that were up-regulated or down-regulated could be annotated with a gene name, respectively. Pathway analysis yielded changes in a total of 144 different pathways. Altered pathways were predominantly involved in oxidative stress (downregulated) as well as metabolic function and other biological processes (upregulated), which is consistent with previous data. Future work will compare effects at the transcriptome level with whole proteome responses as well as apical outcomes in order to characterize toxicity pathways of FLX in fish that enable predicting outcomes of regulatory relevance.

2.2 Introduction

The potential impacts municipal wastewater effluents (MWWE) have on aquatic systems are becoming a major concern due to the ubiquitous presence of emerging contaminants (ECs) at increasing frequencies in these effluents (Boxall et al., 2012; Ela et al., 2011). Particularly, pharmaceuticals and personal care products (PPCPs) are often detected in MWWEs due to the insufficient removal of these substances during wastewater treatment (Boxall et al., 2012; Ela et al., 2011). Since common wastewater treatment technologies are not designed to, and are inefficient in, eliminating select ECs, regions downstream of communities, especially those with minimal or no wastewater treatment such as northern or rural communities in Canada, are at particular risk of increased exposure to these chemicals. Additionally, many pharmaceuticals are designed to elicit biological effects at low concentrations, and for this reason, non-target organisms may be at risk to be adversely affected by these compounds even at low levels. However, effects of many PPCPs on aquatic organisms are not well characterized to date, and as such, more information is needed to better understand the possible implications these chemicals pose to wildlife, particularly to native freshwater fishes in North America.

One PPCP that is predominant in MWWEs is the selective serotonin reuptake inhibitor (SSRI), fluoxetine (FLX) (Bedner and MacCrehan, 2006; Brooks et al., 2003; Jones-Lepp et al., 2001; Weston et al., 2001). Drugs such as FLX are among the most commonly measured drugs in wastewater effluents and are frequently detected in the water column of wastewater (Corcoran et al., 2010). Furthermore, FLX is a polar, non-volatile drug that adsorbs partially to activated sludge during treatment with percent removal usually between 60-88% (Bedner and MacCrehan, 2006; Brooks et al., 2003; Jones-Lepp et al., 2001). Due to its wide and prolonged use and its

incomplete elimination during the wastewater treatment process, FLX is continuously released into the environment through wastewater effluents with concentrations as high as 540 ng FLX L^{-1} detected in receiving aquatic systems (Bedner and MacCrehan, 2006; Brooks et al., 2003; Weston et al., 2001). In humans, FLX is largely administered to treat depression, compulsive behaviours, as well as personality and eating disorders (Brooks et al., 2003). However, very little is known about its fate in the environment and its toxicity to aquatic life (Brooks et al., 2003; Myers, 2007). In fathead minnows (*Pimephales promelas*), decreased feeding rate or ability to catch prey and decreased growth was observed with exposure to concentrations of 51.0-170 µg FLX L^{-1} and 51.0-53.0 µg FLX L^{-1}, respectively (Gaworecki and Klaine, 2008; Stanley et al., 2007). Additionally, Japanese medaka (*Oryzias latipes*) exposed to 0.10-5 µg FLX L^{-1} and 0.10-0.50 µg FLX L^{-1} exhibited developmental abnormalities as well as increased estradiol levels, respectively (Brooks et al., 2003). As in humans, serotonin is an essential neurotransmitter in fish and disruption of serotonin levels may affect multiple systems, including the endocrine, reproductive, and behavioral systems (Caamano-Tubio et al., 2007; Corcoran et al., 2010; Overli et al., 1998). Since FLX exposure may affect various physiological systems, its mechanism(s) of action (MOA) are complicated and still not well understood in fishes. Therefore, information to identify the cascade of key biological events leading to an adverse outcome is needed to characterize the MOA of FLX in native fishes.

To explore the key initiating events leading to an adverse outcome of the exposure to FLX in fishes, high-throughput molecular tools were utilized in this study to identify acute response patterns of FLX on the rainbow trout (*Oncorhynchus mykiss*) liver transcriptome. RNA-seq (whole transcriptome shotgun sequencing) is a relatively new approach, which utilizes next-generation sequencing to characterize whole transcriptome profiles (Wang et al., 2009). With

this technology, the complete suite of genes expressed by an organism at the time of sampling can be assessed. Rainbow trout were chosen as the test species in this study due to their cultural, recreational, and commercial significance to North Americans (Scott and Crossman, 1998). Additionally, due to their sensitivity, rainbow trout are a widely used model laboratory species used in toxicity testing and are often used in risk assessment for determination of chemical guidelines (Abnet et al., 1999; Bak et al., 2013; Doering et al., 2014, 2015; Scott and Crossman, 1998). To date, effects of FLX exposure on rainbow trout have not been characterized. This study aimed to characterize molecular toxicity pathways induced *in vivo* by short-term exposure to FLX in rainbow trout using RNAseq analysis and confirm effects through quantitative real-time polymerase chain reaction (qRT-PCR).

2.3 Materials and methods

2.3.1 *In vivo* exposure

Juvenile, sexually differentiated rainbow trout, approximately five months of age, were randomly selected from an in-house stock reared from eggs acquired from the Troutlodge Hatchery (tetraploid; WA, United States). A total of 20 fish were assigned to 4 tanks (n = 5), with two replicate treatment groups, in an exposure chamber at approximately 12°C. After an acclimation period of 24 h, each pair of replicate tanks was dosed with either an ethanol (EtOH; ≥99%; final concentration of 0.01%) control or 125 µg/L FLX hydrochloride (FLX; purity ≥99%; VWR International, Radnor, PA, United States of America; product of Alfa Aesar, Ward Hill, MA, United States of America). 125 µg/L FLX was chosen as the test concentration as it

was observed to cause malformations in rainbow trout after sub-chronic exposure in a parallel *in vivo* study (unpublished data). A 50% water change was conducted every 24 h and water quality (pH, dissolved oxygen, and temperature) was measured daily for a duration of 96 h. Fish were not fed throughout the exposure. At termination of the experiment, fish were euthanized using buffered MS-222 and livers were immediately extracted, flash frozen in liquid N_2, and then stored at -80°C until analysis. The present study was approved by the Animal Research Ethics Board at the University of Saskatchewan and followed the Canadian Council on Animal Care guidelines for humane animal use (CCAC, 2005).

2.3.2 Transcriptomics

The RNeasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON, Canada) was used according to the manufacturer's protocol to extract total RNA from livers from each individual (approximately 30-50 mg tissue per individual) for each dose. Purified RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples were stored at –80 °C until analyzed. Quality of RNA was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States) for RNA Integrity Number (RIN). Only samples with a RIN ≥ 8 were used for sequencing. Equal amounts of RNA were pooled from each individual to form one RNA-Seq library for each replicate (n = 5) in each treatment. RNA samples were sent to McGill University's Genome Quebec Innovation Centre for Next Generation Sequencing. The TruSeq RNA Sample kit (*Illumina*, San Diego, CA, United States) was used according to the protocol provided by the manufacturer to prepare one library per

sample and libraries were run as 125 base-pair (bp) paired-end reads on a Hi-Seq 4000 instrument (Illumina Inc, San Diego, CA, United States).

Public databases for the genome or transcriptome for rainbow trout were only approximately 40% matched to our sequences; therefore, as described in previous studies, *de novo* assembly of all reads generated for rainbow trout livers in this study was utilized to construct a complete reference transcriptome (NCBI; Berthelot et al., 2014; Doering et al., 2014). The University of Queensland's Galaxy Genomics Virtual Lab (GVL) next generation sequencing (NGS) tools were used to filter and trim raw sequencing reads (Afgan et al., 2015). Quality of raw reads was evaluated using FASTQ (http://usegalaxy.org), and to ensure high sequence qualities, a Phred quality score of ≥30 was used to trim the terminal 5' and 3' nucleotides from all sequences prior to transcriptome assembly. These cleaned, paired reads were then merged to combine any overlapping reads into a single read. CLC genomics workbench v.5.0 (CLC Bio, Aarhus, Denmark) was then used to assemble contigs (continuous, overlapping sequences assembled from individual sequencing reads) *de novo* from merged reads and unmerged paired-end reads for the reference transcriptome using default parameters. The minimum contig length was set to 200 bp. The assembly process generated 35,348 unique contigs with a mean contig size of 936 bp. BlastX searches in Blast2GO v.2.5.0 software (Conesa et al., 2005) with a minimum *E*-value of $<10^{-6}$ was used to annotate the contigs comprising the reference transcriptome. Contigs were annotated against the non-redundant protein database for zebrafish in NCBI.

Merged and unmerged paired-end reads for each treatment were aligned to the reference transcriptome and the total number of effective counts were 30,508,388 for EtOH-1 treatment group, 33,553,262 for EtOH-2 treatment group, 29,295,151 for FLX-1 treatment group, and

33,127,834 for FLX-2 treatment group. The 'edgeR' package (Robinson et al., 2010) in R software v.3.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was utilized to normalize mapped reads after reads were filtered based on count per million (CPM) of >5. CPM in the treatment relative to CPM in the control was used to calculate fold-changes for each contig and a biological variation (BCV) value was set to 0.2. The cutoff *p*-value was set to 0.05 and was used to identify contigs in the treatment that were significantly differentially expressed from control contigs.

2.3.3 qPCR confirmation

Results of transcriptome analysis were confirmed using quantitative real-time polymerase chain reaction (qRT-PCR). The same pool of RNA that was extracted from livers from individuals for transcriptomics was used for qRT-PCR validation. The QuantiNova Reverse Transcription Kit (Qiagen) was used according to the manufacturer's protocol to synthesize first-strand cDNA from 1 µg of total RNA. RNA from livers were pooled by replicate to assess variance for a subset of four genes (Doering et al., 2012, 2014; Eisner et al., 2016). Samples were then stored at –20 °C until analyzed. Gene expression of target genes was measured using qRT-PCR conducted in 96-well plates using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). 2x concentrated QuantiNova SYBR Green master mix, an optimized concentration of cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water were combined in a 25 µL reaction mixture and prepared for each primer combination for each cDNA from each pooled sample for liver. Each PCR reaction was performed in duplicate with 10 µL reaction volumes per well. Before the first PCR cycle, the

reaction mixture was denatured at 95 °C for 2 min. The PCR thermal cycle was then performed and consisted of denaturing at 95 °C for 5 s and primer annealing and template amplification at 60 °C for 10 s for 40 PCR cycles in total. Six genes were targeted for confirmation of transcriptome analyses. These included two genes that were significantly downregulated in the rainbow trout whole transcriptome (stx41a and bcap2a) and two that were significantly upregulated (cyp2ad2 and agmo; Table 2.1). Transcript abundance of target genes was quantified by normalizing to the reference genes, β-actin and EF-1, as described previously (Simon, 2003). Primer3 software (Rozen and Skaletsky, 2000) was used to design qRT-PCR primers for genes of interest from sequences generated in the rainbow trout transcriptome described here (Table 2.1).

Table 2.1. Sequences, efficiencies, annealing temperatures, and amplicon size for primers used in quantitative real-time polymerase chain reaction (qRT-PCR) for transcriptome validation.

Target Gene[1]	Molecular Function[2]	Primer Sequence (5'-3')	Efficiency (%)	Annealing Temp (°C)	Amplicon Size
Stx51	Intracellular protein transport	F: GACACATTCCAACACCATCG R: TGTAGATGGGAGGAGGATGC	122	60	159
Bcap29	Intracellular protein transport	F: GAAAATGATAGCGTGCGTGA R: GGGAAGCCTTGAAAAAGTC	111	60	221
Cyp2ad2	Monooxygenase	F: GGCGGGACTGTCCTATCACT R: ACTGGGATCCAGAAGACCCC	114	60	246
Agmo	Oxidoreductase	F: CATCGCATAGTTTGGCAGGC R: TGTCTCTGTTCCGTTTGGCA	116	60	199
EF-1	Protein biosynthesis	F: AGAACCATTGAGAAGTTCGAGAAG R: GCACCCAGGCATACTTGAAAG	116	60	70
β-actin	ATP and nucleotide binding	F: TCCTCGGTATGGAGTCTTGC R: AGCACTGTGTTGGCGTACAG	108	60	100

[1] Stx51 – syntaxin-5; Bcap29 – B-cell receptor-associated 29; cyp2ad2 – cytochrome family subfamily polypeptide 2; Agmo – alkylglycerol monooxygenase; EF-1 – elongation factor 1; β-actin – beta-actin.

[2] Gene molecular functions were retrieved from UniProt website (https://www.uniprot.org/).

2.3.4 Data analysis

Associations between FLX replicates and between ethanol control replicates were assessed by use of coinertia analysis (CIA) to ensure duplicity of results (Figure 2.1; Dray et al., 2003). Accession numbers for each transcript were converted to protein accession numbers from zebrafish using the bioDBnet database to database (db2db) tool (http://biodbnet.abcc.ncifcrf.gov/db/db2db.php). The Cytoscape platform (v3.6.0) was then utilized for pathway analysis for transcripts that were successfully annotated with a gene name and significantly differentially expressed from the controls. Ontologies based on zebrafish in Kyoto Encyclopedia of Genes and Genome (KEGG) and the GO Consortium Biological Processes were used for pathway analysis and were visualized by use of ClueGo through Cytoscape. KEGG and GO pathways enable the mapping of high level functions in a biological system (e.g., cell, tissue, whole organism). In addition to the standard biogeochemical processes, these pathways comprise all molecular pathways, including: “metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases, and drug development” (GenomeNet Database Resources). A one-tailed t-test was used to analyze qRT-PCR data for confirmation of transcriptome analyses ($p \leq 0.05$).

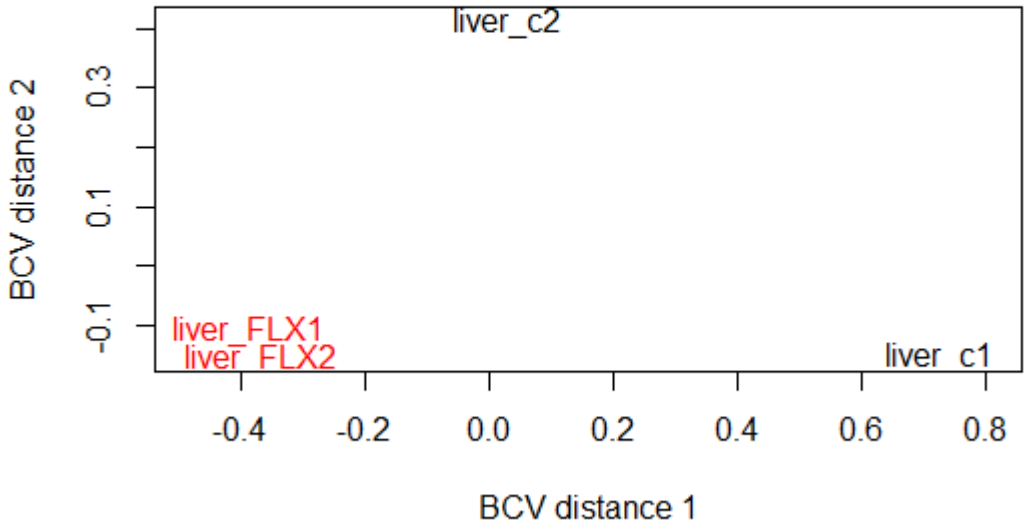

Figure 2.1. Association between replicates by use of coinertia analysis (CIA) of FLX (liver_FLX1 and liver_FLX2) and ethanol control (liver_c1 and liver_c2) responses in rainbow trout liver. Dispersion coefficient was 0.003118 and the Biological Coefficient of Variation (BCV) was 0.1766.

2.4 Results and discussion

2.4.1 Alterations in transcriptome pathway

The present study analyzed the effects of FLX on the global transcriptome in rainbow trout livers. Transcript abundances of 1492 contigs were significantly altered in rainbow trout livers, compared to controls ($p < 0.05$). Among the altered transcripts, 547 and 945 contigs were significantly upregulated and downregulated in livers, respectively. However, only 236 (43.1%) and 642 (67.9%) contigs that were upregulated and downregulated could be annotated with a gene name, respectively. A lack of sequences in the public database for species phylogenetically related to rainbow trout likely accounted for the low annotation frequency observed in our results, particularly with upregulated genes (Hou et al., 2011). Genes involved in a variety of pathways were affected by exposure to FLX and included cellular biosynthetic processes, protein transport, oxidoreductase and monooxygenase activity, and RNA processing, among others (Figure 2.2). Two genes observed to be upregulated and two genes observed to be downregulated in the rainbow trout transcriptome were confirmed with qRT-PCR and the fold change of transcript abundance was comparable between the two methods; however, fold-change of each gene was generally greater with qRT-PCR than with transcriptome analyses (Table 2.2). A list of the most highly differentially expressed genes significantly in the liver transcriptome are shown in Table 2.3. In total, 144 pathways were altered at the gene expression level, and these processes were grouped into four major functional categories by their GO or KEGG pathway. Over half (57%) of differentially regulated pathways were involved in general biological processes while the remaining pathways were specifically involved in cellular components (23%), molecular function (14%), and genetic information processing (6%). Of the total

pathways involved in biological processes, 27% were specific to metabolic processes. Metabolism encompasses a vast system of interconnected reactions, which are dependent on one another and disruption of these reactions is very sensitive (Berg et al., 2002). Metabolism dysfunction with FLX exposure may lead to many complications for an organism, increasing their vulnerability to development of disease (Heindel et al., 2017). For example, with the disruption or loss of insulin signaling in the liver, resistance to insulin has been observed in mice, which in turn causes failure to suppress hepatic glucose production (Michael et al., 2000). The insulin signaling pathway was also disrupted in rainbow trout livers in the present study, indicating that there is potential for unregulated hepatic glucose production in fishes as well. Without the ability to suppress glucose production, glucose homeostasis, which is an integral aspect in sustaining liver function, is compromised and liver disease may ensue (Michael et al., 2000). In addition to metabolic effects, genes related to responses in other organisms, or an external biotic stimulus, were also significantly downregulated (heat shock protein family A, member 5 (hspa5); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a (nfkbiaa); deoxynucleoside triphosphate triphosphohydrolase (SAMHD1); serin incorporator 5 (serinc5); TNF receptor superfamily member 9 (tnfrsf9); among others). This a notable finding as liver is not normally considered to be directly involved with mediating behavioural responses. To our knowledge, this phenomenon has not been observed by other studies. Therefore, this demonstrates that common pathways can be detected among tissues, even in those with no clear involvement with the biological manifestation of a response. Alteration of such genes may reduce an organism's ability to react to predators in their environment, thus decreasing their chance of survival.

As rainbow trout used in this study were too young to visually differentiate between males and females (i.e., gonads were not yet sufficiently developed), both sexes fish were potentially exposed. However, due to their early life stage, sex was not likely to play a confounding factor in gene expression as gender specific gene regulation process associated with sexual maturation and reproductive cycling were unlikely to have taken place. Conversely, with adult fish, studies have eluded that effects of fluoxetine at the gene expression level can be sex-specific and, therefore, studies with adult fish should differentiate between males and females (Mennigen et al., 2011; Weinberger and Klaper, 2014). Additionally, sex-specific differences with exposure to fluoxetine have been widely observed in adult (?) mice (Hodes et al., 2010; Jones and Lucki, 2005). For example, male mice chronically exposed to fluoxetine (injected daily with 10 mg/kg for 21 d) remained unaffected, while the survival rate in females decreased (Hodes et al., 2010).

Although changes in the transcriptome give us insight into specific molecular pathways that were differentially regulated in the liver, it is difficult to directly link these changes to adverse apical effects in fishes, and further transcriptome analysis, perhaps with rainbow trout brain, should be conducted to better understand the behavioral MOA of FLX. Additionally, transcriptome analyses need to be paired with biochemical, histological, behavioural, and other apical markers in order to confidently establish FLX's MOA.

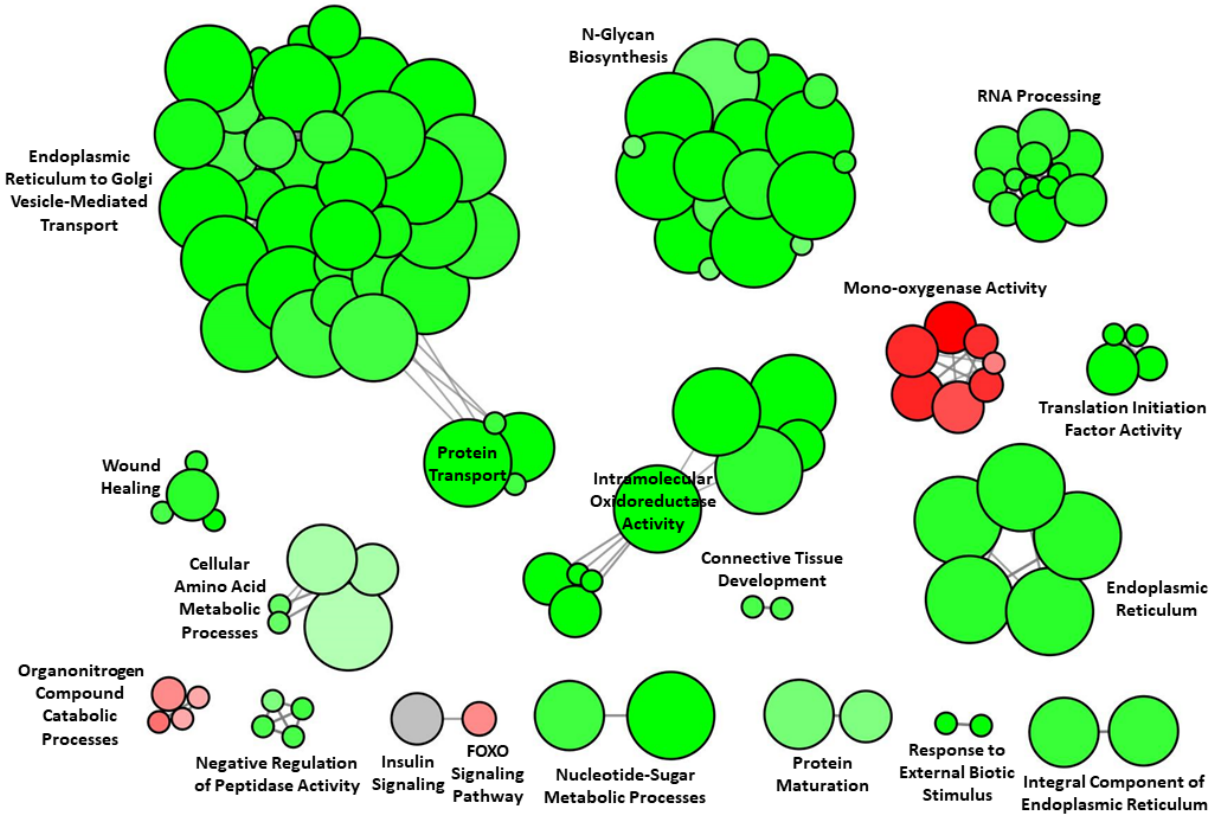

Figure 2.2. ClueGO clustering results of shared physiological processes in rainbow trout liver tissue altered by FLX exposure at the transcriptome level. Red clusters depict a greater proportion of upregulated processes, while green clusters depict a greater proportion of downregulated processes. Relative abundance of upregulated vs. downregulated processes are depicted by degree of red or green in each cluster, while gray clusters contain 50% upregulated processes and 50% downregulated processes. The relative number of processes in each cluster is represented by the size of cluster. Gray interconnecting lines show connections between pathways indicating that these processes share transcript(s).

Table 2.2. Comparison of fold-changes for abundance of transcripts by use of transcriptome sequencing and by use of quantitative real-time polymerase chain reaction (qRT-PCR) in livers of rainbow trout following a 96-hr waterborne exposure to 125 µg/L FLX.

Gene	Transcriptomics	qRT-PCR
Stx51a	0.459	0.371
Bcap2a	0.271	0.141
Cyp2ad2	2.335	2.403
Agmo	1.810	2.793

Table 2.3. Top ten differentially expressed genes ($p < 0.05$) that were upregulated or downregulated in the rainbow trout liver transcriptome. Genes that were not annotated with a gene name are not listed.

Upregulated Genes		Downregulated Genes	
Gene	Fold Change	Gene	Fold Change
DNA damage-inducable transcript 4 isoform X1	6.2	12-(S)-hydroxy-5,8,10,14-eicosatetraenoic acid receptor	0.03
Purine nucleoside phosphorylase 5a	4.7	CD59 glyco-like	0.06
Solute carrier family member 9a	4.3	Leukocyte cell-derived chemotaxin-2	0.08
Kinesin KIF26A isoform X1	3.9	Microfibril-associated glycol 4	0.08
Crumbs homolog 2	3.6	Myeloid 1-like	0.09
Heme oxygenase 1	3.4	Early growth response 1	0.09
Sodium voltage-type beta b precursor	3.2	Carbonic anhydrase 7	0.09
Cdc42 effector 3 isoform X1	3.1	Tubulin beta-4B chain-like	0.10
CD27 antigen-like isoform X2	3.0	S-acyl fatty acid synthase medium chain isoform X1	0.10
1,25-dihydroxyvitamin D(3) 24-mitochondrial	3.0	Tumor necrosis factor receptor superfamily member 21-like	0.12

2.4.2 Comparison of response with previous studies

The generation of global transcriptomes is increasingly being used to identify specific MOAs and toxicity pathways of emerging chemicals to fishes. To date, the changes in the global transcriptome with exposure to FLX have been elucidated in adult goldfish (*Carassius auratus*) brain (Mennigen et al., 2008), larval zebrafish (whole body; Park et al., 2012), adult zebrafish brain (Wong et al., 2013), juvenile rainbow trout liver (this study), as well as in Asian clams (*Corbicula fluminea*; Chen et al., 2013). Among these studies there is overlap in pathways that were altered (Table 2.4). Specifically, metabolic processes were also affected in goldfish, zebrafish, and clams. Furthermore, the oxidative response was also dysregulated in zebrafish brain (Wong et al., 2013). Since these pathways have been observed to be altered across brain and liver tissues, as well as the whole body in the case of the Asian clam and larval zebrafish, it is hypothesized that molecular pathways affected by FLX exposure are conserved among several tissue types. Moreover, similar pathways were observed to be altered across several species and taxonomic groups (goldfish, zebrafish, rainbow trout, and Asian clam), further revealing the comparability of FLX exposure among the diversity of fishes. With similar results observed among species at the transcriptome level, it is possible that the key events leading to an adverse outcome with FLX exposure are related and common among different species. It is likely that FLX exposure may activate oxidative pathways, triggering an antioxidant response in fishes, and subsequently enhancing the expression of genes encoding for antioxidant enzymes on the molecular level. The increased expression of antioxidant enzymes produces elevated reactive oxygen species (ROS) that are otherwise maintained in a steady-state (Stoliar and Lushchak, 2012). ROS are always present in cells in small concentrations as they are involved in cell

signaling, however, adverse effects, including DNA, protein, and lipid damage, can arise when concentrations exceed those which a cell can combat (Stoliar and Lushchak, 2012). Damage to DNA, proteins, and lipids may be key events that lead to more severe adverse outcomes, such as carcinogenesis and neurodegenerative diseases that have previously been observed in humans in response to oxidative stress (Toyokuni, 1999). While these diseases may manifest differently in fish (reduced feeding or ability to catch prey, anxiety), they have the potential to severely affect fish survival. Furthermore, as the brain is a lipid-rich tissue that consumes high amounts of oxygen, it is at increased risk of oxidative damage (Salim, 2014).

Table 2.4. Shared gene ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of significantly differentially expressed genes between the current study liver and four other studies ($p < 0.05$) are marked with an "X". GO terms were acquired by use of the global transcriptome generated in each study.

GO or KEGG Pathway (this study)	Asian clam, whole body[1] (Chen et al. 2013)	Goldfish, brain[2] (Mennigen et al. 2008)	Zebrafish (larval), whole body[3] (Park et al., 2012)	Zebrafish, brain[3] (Wong et al. 2013)
Oxidoreductase activity	-	-	-	X
Cellular biosynthetic processes	-	X	X	X
Cellular catabolic processes	-	X	X	-
Lipid metabolism	X	-	X	X
Coenzyme metabolism	X	-	-	-
Response to wounding	-	X	-	-

[1]*Corbicula fluminea*
[2]*Carassius auratus*
[3]*Danio rerio*

In addition to alterations in metabolic processes, Chen et al. (2013) confirmed that FLX exposure led to changes in genes that are influenced by other environmental stressors, such as food deprivation or thermal stress, which may affect the manifestation of FLX toxicity. These genes include antioxidant, heat-shock protein, cytochrome P450 and gamma-aminobutyric acid (GABA) receptor genes, which were also observed to be altered in our study. Alteration of these genes with exposure to FLX may prove to be useful biomarkers for exposure to environmental stressors (Chen et al., 2013).

A previous study by Mennigen et al. (2008) with goldfish also saw an increase in genes involved with reproductive functions in the brain; however, no change in abundance of such genes was observed in our study with rainbow trout livers or previous studies with larval (Park et al., 2012) or adult (Wong et al., 2013) zebrafish brain. One possible cause for the difference in reproductive pathway alterations could be the method by which fish were exposed to FLX. The goldfish were intraperitoneally injected with a total of 5 µg FLX g^{-1} twice a week for 17 d (Mennigen et al., 2008) whereas a waterborne exposure was used for the larval (25 and 250 µg FLX L^{-1} for 96 h; Park et al., 2012) and adult (100 µg FLX L^{-1} for 14 d; Wong et al., 2013) zebrafish. Our study was also a waterborne exposure where we examined the changes in the liver and brain transcriptome. Previous studies have shown that uptake rates after intraperitoneal injection are up to three times faster than uptake rates for waterborne exposures, which may influence gene expression along reproductive pathways (Levine et al., 1994). Furthermore, the duration of the exposures among previously mentioned studies varied significantly. For example, the present study was conducted over 96 hr, while studies by Mennigen et al. (2008) and Wong et al. (2013) conducted the exposure over 17 d and 14 d, respectively. Over a longer period of time, it is likely that secondary effects may begin to manifest in the global transcriptome, thus

initiating alterations in pathways that might not have otherwise been affected, such as reproduction. This is supported by the absence of changes in genes or pathways involved with reproduction in our short-term study.

In addition to the method and duration of exposure, life-stage of the test organism would also likely influence the effects of FLX among organisms (Park et al., 2012). For example, the global transcriptome for larval and adult zebrafish share very few similarities in differentially expressed genes, which coincides with previous studies with mammals where FLX is known to have life-stage dependent effects (Bouet et al., 2012; Homberg et al., 2011; Olivier et al., 2011; Park et al., 2012; Wong et al., 2013). Therefore, effects observed on the global transcriptome in juvenile rainbow trout in this study may not be observed in larval rainbow trout, and vice versa. While reproductive effects have been reported in goldfish, a study by Foran et al. (2004) did not observe any effects on egg production, spawning, rate of fertilization, or fertilization success in adult Japanese medaka. Furthermore, serotonin involvement with regards to reproduction has been shown to be species-specific and variations in its function have been observed. In Japanese medaka, for example, serotonin has been shown to aid in the maturation of oocytes (Iwamatsu et al., 1993); however, in mummichog (*Fundulus heteroclitus*), maturation was inhibited by serotonin (Cerda et al., 1998).

2.5 Conclusions

The present study was conducted to further our understanding of the molecular toxicity pathways triggered by exposure to the common pharmaceutical drug, FLX, in rainbow trout as it is a prevalent drug in MWWEs and is becoming a growing concern worldwide. Overall, metabolic pathways were significantly altered in the rainbow trout liver whole transcriptome. While many alterations in genes and pathways observed here were also observed in other species, some were unique to rainbow trout, thus stressing the importance for further analyses of the whole transcriptome in native North American fish species. Additionally, comparison of altered pathways with previous studies underlines the complexities of how FLX toxicity manifests within different tissues, species, and life stages. With our results, we can begin to comprehend what key changes are occurring on the molecular level; however, analysis of higher level organizational changes including whole proteome, physiological and behavioral alterations with exposure to FLX exposure must also be assessed in order to verify whether, and which, changes across the whole transcriptome would translate to impairments of organism health.

CHAPTER 3

3 *IN VITRO* COMPARISON OF RESPONSES AMONG FOUR CANADIAN FISH SPECIES TO EMERGING CONTAMINANTS OF CONCERN

PREFACE

This Chapter builds on the results of Chapter 2 where I investigated the effects of fluoxetine (FLX) on the whole transcriptome in rainbow trout liver. Specifically, information obtained in Chapter 2 with rainbow trout was used to inform on endpoints to measure in the present study. This chapter further explores the effects of FLX on rainbow trout (*Oncorhynchus mykiss*), as well as two additional compounds (17α-ethynylestradiol (EE2) and hexabromocyclododecane (HBCD)), and three additional species of commercial, recreational, and cultural interest. These species include lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*) and white sturgeon (*Acipenser transmontanus*). Additionally, due to the challenges associated with maintaining non-model fish species, such as the aforementioned species, under laboratory conditions, as well as the high cost and ethical concerns with using whole animals, an *in vitro* approach was used rather than an *in vivo* approach. This chapter will be submitted to Environmental Chemistry and Toxicology.

Author contributions:

Bryanna Eisner (University of Saskatchewan) designed and managed the experiment, generated and analyzed all data, and drafted the manuscript.

Shawn Beitel (University of Saskatchewan) helped with the design of the experiment and assisted with experiment set-up.

Dr. Markus Hecker (University of Saskatchewan) provided guidance and inspiration for the conception and design of the experiment, offered comments and edits for the manuscript, and provided research funding.

3.1 Abstract

There is an increasing number of emerging contaminants (ECs), such as 17α-ethynylestradiol (EE2), fluoxetine (FLX), and hexabromocyclododecane (HBCD), detected in municipal wastewater effluents (MWWEs), for which limited or no data regarding their toxicity to aquatic organisms is available. While data for some of these compounds are available for model laboratory species, including rainbow trout (*Oncorhynchus mykiss*), little is known of the effects of these ECs to other species of cultural, recreational and commercial importance to Canadians including lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*) and white sturgeon (*Acipenser transmontanus*). As such, there is a need for approaches that allow assessment of potential effects to these species. At the same time, there are increasing concerns with regard to live animal testing, particularly in the context of endangered or long-lived species such as white sturgeon. Therefore, alternative testing methods are needed that enable characterizing the potential toxicological effects to these species while reducing the need for live animal testing. The aim of the current study was to establish and validate an alternative *in vitro* tissue explant assay to characterize the sensitivity of four species of cultural, recreational and commercial importance in North American freshwater environments. Specifically, *in vitro* tissue explant assays with gonads and livers from lake trout and northern pike, as well as livers from rainbow trout and white sturgeon exposed to serial concentrations of EE2, FLX or HBCD were conducted and transcript abundances of genes involved with reproductive and antioxidant processes were measured to characterize species-specific changes in gene expression to these compounds. Rainbow trout had the greatest vitellogenin induction among species when exposed to EE2, followed by white sturgeon, northern pike, then lake trout. With exposure to FLX, gene

expression was highly variable. However, rainbow trout and white sturgeon had an equal magnitude of effect while lake trout and northern pike had a lesser magnitude of effect. With exposure to HBCD, induction of antioxidant genes, including catalase, glutathione peroxidase, and glutathione-*S*-transferase was observed. Rainbow trout was again the most responsive species with regard to gene expression, followed by northern pike, lake trout, and white sturgeon. As rainbow trout were consistently one of the more sensitive species in our study, they appear to represent an appropriate model organism for many scenarios; however, in a few cases other species were more sensitive (white sturgeon with exposure to HBCD, for example) and caution should be taken when extrapolating across species.

3.2 Introduction

Widespread use and ubiquity of emerging contaminants (ECs), such as pharmaceuticals and personal care products (PPCPs) and flame retardants, has raised concerns regarding their possible risks to humans and wildlife (Boxall et al., 2012; Ela et al., 2011). One of the primary sources of ECs to surface waters are municipal wastewater effluents (MWWEs) because current wastewater treatment technology is often insufficient in removing select ECs. The discharge of PPCPs into aquatic systems via MWWEs has been identified by Environment and Climate Change Canada as one of the leading sources of pollution of Canadian surface waters to date (Environment Canada, 2001). Accordingly, many of these chemicals are frequently detected downstream of wastewater treatment plants (Boxall et al., 2012; Ela et al., 2011). In particular, regions downstream of communities with minimal or no wastewater treatment, such as many northern or rural communities, are at increased risk of exposure to these chemicals.

Much of the toxicological concerns pertaining to ECs, particularly pharmaceuticals, stems from the fact that they are often designed to elicit biological effects at low concentrations (Corcoran et al., 2010). For this reason, exposure of non-target organisms to these compounds can result in unwanted and adverse effects. For example, several ECs have been shown to act as endocrine disrupting compounds (EDCs) that can impair growth, development, and reproduction of vertebrates, including fish (Beitel et al., 2015; Cruz et al., 2015; Mennigen et al., 2010a). While significant information is available on select chemicals such as 17-α-ethynylestradiol (EE2), many other ECs commonly found in aquatic environments, such as fluoxetine (FLX) and hexabromocyclododecane (HBCD), are not well characterized to date and further information is needed to better understand their potential impacts on aquatic organisms of interest, including fish.

The PPCP EE2 is a potent estrogen agonist modeled after the natural endogenous hormone, 17β-estradiol (E2), and has been found in MWWE with concentrations as high as 42 ng/L (Chen et al., 2006; Ternes et al., 1999). Due to its high binding affinity to the estrogen receptor, EE2 is one of the most ubiquitously used compounds in oral contraceptives, and at environmentally relevant concentrations, has shown to cause (de)feminization, (de)masculinization, or intersex in fish (Aris et al., 2014; Chikae et al., 2003; Örn et al., 2003, 2006; Seki et al., 2002; Tyler et al., 1999). The selective serotonin reuptake inhibitor (SSRI), FLX, is primarily used to treat depression in humans, and has also been found in MWWEs at concentrations as high as 540 ng FLX L^{-1} (Bedner and MacCrehan, 2006; Brooks et al., 2003; Jones-Lepp et al., 2001; Weston et al., 2001). Much like in humans, serotonin functions as an important neurotransmitter in fish and is involved in various functions including endocrine parameters, reproduction, and behaviours such as aggression and appetite (Caamano-Tubio et al., 2007; Corcoran et al., 2010; Overli et al.,

1998). The persistent organic pollutant (POP), HBCD, has been widely used in electrical equipment, textiles, and other building materials (Berntssen et al., 2011; Meng et al., 2011). As a POP, HBCD possesses characteristics of this class of chemicals, such as persistency in the environment, bioaccumulation and possible biomagnification in lipid-rich tissues, long-range transport and toxicity to aquatic and terrestrial organisms (Covaci et al., 2006). Since it is widely used in consumer products, HBCD has been detected in dust collected from homes (Stapleton et al., 2008), as well as in municipal wastewater sludge (La Guardia et al., 2010). Due to the limited information regarding the effects of these chemicals to North American fishes, especially FLX and HBCD, they are becoming an increasing concern to the environment (Gaworecki and Klaine, 2008; Kreke and Dietrich, 2008). Furthermore, the specific MOA of FLX or HBCD in fish remains unclear, necessitating additional studies that focus on characterization of the mechanism of action (MOA) of these chemicals, and how it may be linked to the sensitivities and susceptibilities of fish species of interest.

Current environmental risk assessment strategies for the determination of chemical toxicity in aquatic organisms typically rely on standard model species that are unlikely to be representative of the diversity of fishes, and which has been shown in several cases (Abnet et al., 1999; Bak et al., 2013; Doering et al., 2014, 2015; Rigaud et al., 2014). Extrapolation of data from model species to native species of interest requires application of uncertainty factors, which may considerably overestimate or underestimate the toxicological risk of a given chemical or chemical mixture, in some cases by multiple orders of magnitude (CEC, 1996; Chapman et al., 1998; Forbes and Calow, 2002). Fish species native to Canada, namely lake trout (*Salvelinus namaycush*), rainbow trout, northern pike (*Esox lucius*), and white sturgeon (*Acipenser transmontanus*), are of particular interest for North American ecosystems due to the very limited,

or even absent, information available on their susceptibility or sensitivity to the aforementioned ECs. These freshwater fishes all hold cultural, recreational, and commercial significance to North Americans and furthermore represent indicators of ecosystem health due to their position in the food chain. In addition, white sturgeon are endangered in all of Canada and parts of the United States, and thus, represent a particular protection goal. With the exception of rainbow trout, knowledge of the sensitivities and susceptibilities of these species is limited, and therefore, assessment of effects of ECs to these species is warranted.

Traditionally, effects of contaminants of concern are assessed using whole animal *in vivo* toxicity assays with standard laboratory animals (Krewski et al., 2007). However, numerous challenges exist when conducting *in vivo* assays, particularly with non-model species. For example, it is often very difficult to culture and maintain wild fish species under laboratory conditions, and there are ethical concerns when working with whole organisms, especially endangered species such as white sturgeon (Beitel et al. 2015). Additionally, *in vivo* toxicity assays are expensive as well as time- and space-consuming (Villeneuve and Garcia-Reyero, 2011). For these reasons, alternative, high-throughput *in vitro* testing methods are increasingly being proposed to test toxicity of contaminants in place of traditional *in vivo* methods. *In vitro* toxicity assays may include primary cell cultures, stable cell lines and, as described in this study, tissue explants. Among these tests, tissue explant assays were shown to have greater, although slightly more variable, specificity of response than primary cells or stable cell lines (Gray et al., 1997). While there is an increasing diversity of species- and tissue-specific cell lines and approaches being developed to address this information gap, these systems are still limited and require further development for routine and standard use. Specifically, explants are advantageous over cell-line based assays in that they maintain some species-specific tissue functions, such as

paracrine interactions among different cell types (Gray et al., 1997). Like other *in vitro* assays, tissue explant assays still require significantly fewer animals relative to *in vivo* toxicity assays. Additionally, *in vitro* assays allow for the identification of the MOA of a compound, thereby making it possible to determine the specific properties that drive species-specific sensitivity to that chemical. Molecular endpoints, specifically changes in gene expression, are tools that were previously and successfully utilized in determining species sensitivities to different compounds of concern, and previous studies have shown that use of these endpoints can be very useful in predicting species sensitivities *in vitro* (Beitel et al., 2015; Eisner et al., 2016).

The overall goal of this study was to characterize the changes in transcript abundance of four native fish species with exposure to three ECs of concern, namely EE2, FLX and HBCD, using an alternative *in vitro* tissue explant assay. Specifically, transcript abundance of genes related to reproductive functions and oxidative stress were measured in liver and gonad tissue explants exposed to each chemical. This study aided in further understanding the differences of sensitivities among species of interest and allows us to better understand effects on non-model, native fish species.

3.3 Materials and methods

3.3.1 Fish

Adult lake trout were collected from Lac la Plonge (SK, Canada) using gill nets. Adult northern pike were collected by use of gill nets and angling from Lake Diefenbaker (SK, Canada). Juvenile rainbow trout were randomly selected from an in-house stock reared from eggs acquired from the Troutlodge Hatchery (tetraploid; WA, United States). Juvenile white

sturgeon were randomly selected from an in-house stock reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Rainbow trout and white sturgeon were reared at the Aquatic Toxicology Research Facility at the Toxicology Centre at the University of Saskatchewan (Saskatoon, SK, Canada). All studies were approved by the Animal Research Ethics Board at the University of Saskatchewan and followed the Canadian Council on Animal Care guidelines for humane animal use. Experiments with white sturgeon were conducted under Species At Risk Act (SARA) permit XRSF 20 2013. Liver (hepatic; HSI) and gonad (GSI) somatic indices for each species were calculated using the followinh equations (Equations 1 and 2):

$$\text{HSI} = \frac{\text{Liver Weight}}{\text{Organism Weight}} \quad (1)$$

$$\text{GSI} = \frac{\text{Gonad Weight}}{\text{Organism Weight}} \quad (2)$$

3.3.2 Chemicals

Stock solutions of EE2 (purity ≥98%; Sigma-Aldrich, Oakville, ON, Canada) and FLX hydrochloride (purity ≥99%; VWR International, Radnor, PA, United States of America; product of Alfa Aesar, Ward Hill, MA, United States of America) were prepared in ≥99% pure ethanol. The stock solution of 1,2,5,6,9,10- hexabromocyclododecane (HBCD; purity ≥95%; Sigma-Aldrich) was prepared in dimethyl sulfoxide (DMSO).

3.3.3 Exposure protocol

Liver and gonad tissue explant assays were conducted following methods outlined previously (Beitel et al., 2014; Doering et al., 2015; Eisner et al., 2016). All individuals were euthanized by blunt-force trauma and livers were excised from each species to be subsequently treated with EE2 (n = 4, 3, 3, and 6 for lake trout, northern pike, rainbow trout and white sturgeon, respectively), FLX (n = 8, 6, 6, and 6 for lake trout, northern pike, rainbow trout and white sturgeon, respectively), and HBCD (n = 7, 12, 6, and 6 for lake trout, northern pike, rainbow trout and white sturgeon, respectively). Gonad tissues were excised from lake trout and northern pike only and exposed to the same concentrations of EE2, FLX, and HBCD. Tissues were immediately rinsed in 1X phosphate-buffered saline (Sigma Aldrich, Oakville, ON, Canada) and then sliced into 1 mm^3 pieces. Tissue slices were rinsed multiple times with supplemented Leibovitz L-15 media (13.8 g of L-15 powder / L medium, 420 mg $NaHCO_3$ / L, 10% fetal bovine serum, 1% antibiotic-antimicotic solution (100 units penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B per mL), pH 7.6). Two pieces of liver or gonad tissue

were placed into each well of a 24-well culture plate containing 1 mL of supplemented L-15 media. Tissue explants were exposed to serial concentrations of EE2, FLX, and HBCD with concentrations ranging from 3 to 3000 ng/L, 0.32 to 5000 μg/L, or 0.2 to 30 mg/L, respectively. Tissue explants were incubated at 15 °C on a platform rocker for 24 hrs and were then placed into microcentrifuge tubes and immediately frozen at –80 °C. Cell viability of tissue explants was assessed by quantifying lactate dehydrogenase (LDH) in the media at 24 hrs in accordance with the protocol provided by the manufacturer (Cayman Chemical, Ann Arbor, MI, United States). Explants for liver and gonad tissues were considered viable across all experiments and treatment groups because lactate dehydrogenase in media of exposed explants did not differ more than 20% in media of control explants exposed for 24 h (Beitel et al., 2014).

3.3.4 Quantitative real-time PCR

RNA was extracted from liver and gonad explants from each individual for each dose using the RNeasy Lipid Tissue Mini Kit (Qiagen, Toronto, ON, Canada) according to methods described by the manufacturer. Concentrations of RNA were quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States) and samples were stored at –80 °C until analyzed. The QuantiTect Reverse Transcription Kit (Qiagen) was used according to the manufacturer's protocol to synthesize first-strand cDNA from 1 μg of total RNA. Samples were then stored at –20 °C until analyzed.

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to determine abundance of target gene transcripts as described previously (Doering et al., 2012; 2015a; Eisner et al., 2016). Glutathione peroxidase (GPX), glutathione S-transferase (GST) and catalase (CAT)

genes were chosen to measure antioxidant responses in liver and gonad tissues of each species exposed to FLX. Anti-oxidant responses were observed to be significantly altered in the whole transcriptome of rainbow trout liver tissues in a previous study and were therefore selected as an endpoint for this study (Chapter 2). These genes (GPX, GST, and CAT) were also analyzed in each tissue exposed to HBCD. In addition, genes associated with reproductive functions, including vitellogenin (VTG), estrogen receptor α (ERα) and estrogen receptor β (ERβ), were measured in each species exposed to EE2. Expression of target genes in liver explants was measured using qRT-PCR conducted in 96-well plates using the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). 2x concentrated Quantifast SYBR Green master mix, an optimized concentration of cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water were combined in a 50 µL reaction mixture and prepared for each primer combination for each cDNA from each individual for each dose. Each PCR reaction was performed in duplicate with 20 µL reaction volumes per well. Before the first PCR cycle, the reaction mixture was denatured at 95 °C for 10 min. The PCR thermal cycle was then performed and consisted of denaturing at 95 °C for 10 s and primer annealing and template amplification at 60 °C for 1 min for 40 PCR cycles in total. Transcript abundance of target genes was quantified by normalizing to expression of reference genes β-actin and ribosomal protein L9 (RPL9), according to methods described previously (Simon, 2003). Briefly, expression of target genes were normalized separately to each reference gene, and data presented was calculated as the geometric mean of these two expression values. Primers for genes associated with oxidative stress for white sturgeon were published previously (Zee et al., 2016). Oxidative stress primers for rainbow trout and northern pike were designed by use of Primer3 software from partial mRNA sequences for each species found in the NCBaI database (Rozen and Skaletsky, 2000).

Primers for VTG, ERα, ERβ and β-actin for white sturgeon and northern pike were published previously (Beitel et al., 2015; Eisner et al., 2016). VTG, ERα, ERβ and RPL9 primers for rainbow trout were also designed by use of Primer3 software from partial mRNA sequences found in the NCBI database (Rozen and Skaletsky, 2000). The β-actin primers for rainbow trout were published previously (Eisner et al., 2016). All lake trout primers were designed based off rainbow trout primers from this study. All primers for each species were validated through sequencing the purified PCR products (Qiagen; Toronto, ON, Canada) by the University of Calgary's University Core DNA Services (Calgary, AB, Canada).

3.3.5 Statistical Analysis

Concentrations of each chemical that elicited a statistically significant increase in expression of target genes ($p \leq 0.05$) relative to the ethanol or DMSO control treatment was determined by use of a one-way ANOVA followed by a Dunnett's post-hoc test using SPSS version 20.0 (SPSS, Chicago, IL, United States). Data was assessed for normality and homogeneity of variance using a Kolmogrov-Smirnov test and Levene's test, respectively. To ensure that homogeneity of variance was not violated, a logarithmic transformation was used when necessary. A Kruskal-Wallis test, followed by a Mann-Whitney *U* test, was used to analyze nonparametric data ($p \leq 0.05$).

Relative responses (ReRs) were calculated for gene expression in liver tissues by using the formula (Equation 3):

$$\text{ReR} = \frac{\text{LOEC Rainbow Trout}}{\text{LOEC Species xx}} \quad (3)$$

Where: LOEC is the lowest observable effect concentration for rainbow trout, lake trout, northern pike, or white sturgeon.

3.4 Results

3.3.6 Maturation and life-stage of fish

Northern pike and lake trout used in this study were mature adults (> 1 year of age) in the pre-spawning stage of development (i.e., gonads were fully developed; Table 3.1). White sturgeon and rainbow trout were juveniles and were not yet at their reproductive stage as gonads were not sufficiently developed (Table 3.1).

Table 3.1. Sample sizes, lengths, hepatic somatic indices, and gonadosomatic indices of test organisms used for *in vitro* tissue explant assays. Data are presented as the mean ± standard error of the mean (SEM). 'N/A' – data not available. HSI and GSI were calculated by Equation 1 and 2, respectively.

Species	n	Length (cm)	Hepatic Somatic Index	Gonadosomatic Index
White Sturgeon	6	39.1 ± 0.5	2.81 ± 0.13	N/A
Rainbow Trout	8	29.0 ± 0.6	1.34 ± 0.05	N/A
Northern Pike	9*	50.7 ± 2.8	0.89 ± 0.26	1.15 ± 0.26
Lake Trout	7	57.7 ± 1.4	1.50 ± 0.12	5.18 ± 0.64

* Sample size for northern pike length, liver somatic index, and gonadosomatic index was 9, 6, and 6 individuals, respectively. Liver and gonad weights were not taken for three northern pike and are therefore not included in the average.

3.3.7 Gene expression in tissues exposed to EE2

Transcripts of VTG, ERα, and ERβ were measured in liver tissue explants from lake trout, northern pike, rainbow trout and white sturgeon exposed to EE2 (Figure 3.1). However, ERβ could not be quantified in white sturgeon because no primer with appropriate efficiency could be designed. Exposure to EE2 yielded no change in transcript abundance of VTG in lake trout (Figure 3.1A), while VTG significantly increased at the highest dose of 3000 ng/L by 3.6-, 182-, and 36-fold compared to controls in northern pike, rainbow trout, and white sturgeon, respectively (Figure 3.1A). The ERα gene was significantly upregulated in northern pike, downregulated in rainbow trout, and did not change in either lake trout or white sturgeon livers (Figure 3.1B). Furthermore, no change in transcript abundance of ERβ was observed in lake trout or northern pike livers (Figure 3.1C). In rainbow trout, however, ERβ was significantly upregulated at 30 ng/L (Figure 3.1C). Rainbow trout were the most responsive species compared to lake trout, northern pike, and white sturgeon by 1000-, 333-, and 10-fold, respectively (Table 3.2).

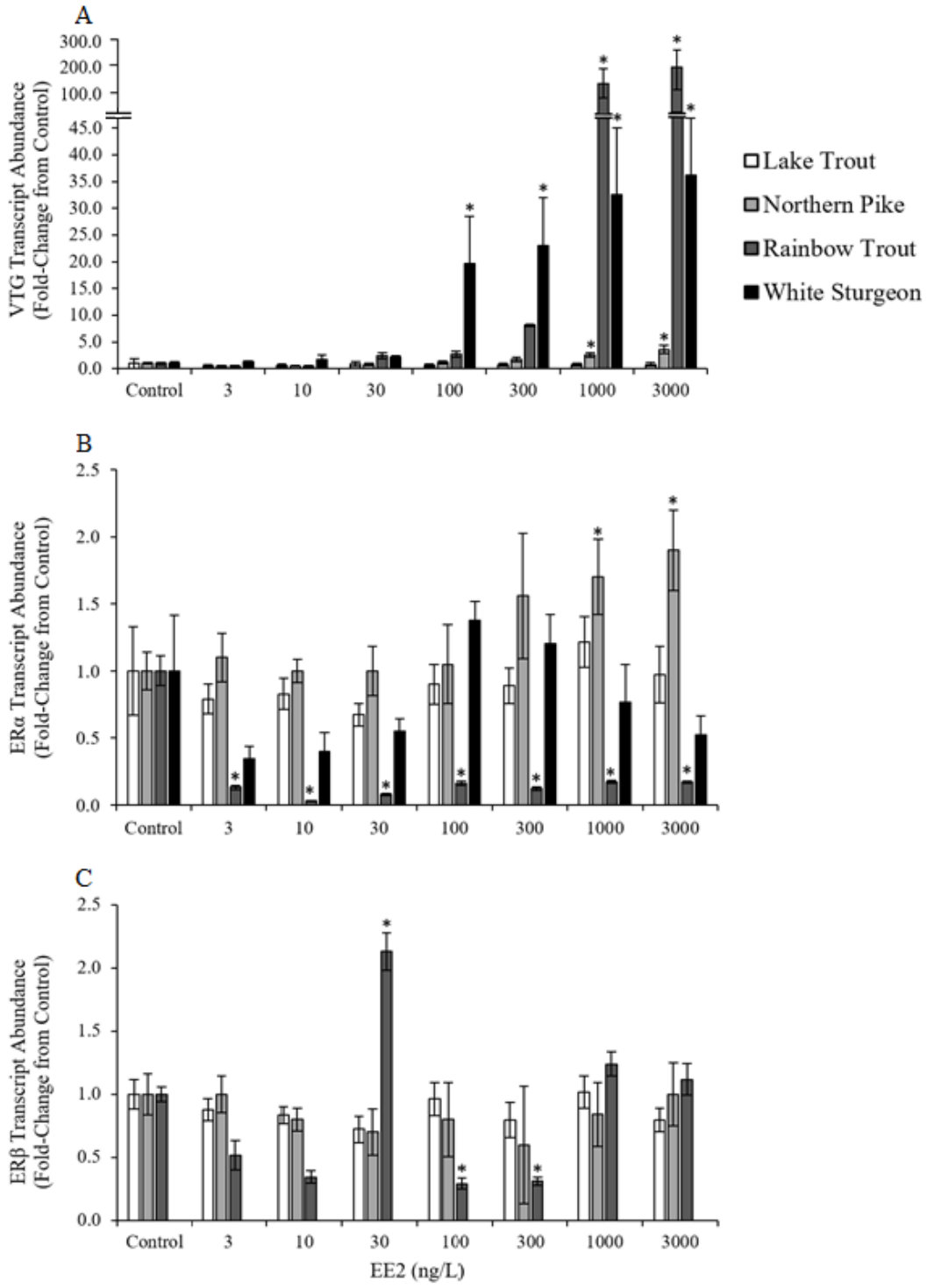

Figure 3.1. Transcript abundances of VTG (A), ERα (B), and ERβ (C) in liver explants of male lake trout (n = 4), northern pike (n = 3), rainbow trout (n = 3), and white sturgeon (n = 6) following exposure to a solvent control (DMSO) or EE2. Data are presented as the mean ± standard error of the mean (SEM). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

Table 3.2. Relative responses (ReRs) of liver explants of rainbow trout, lake trout, northern pike, and white sturgeon to EE2, FLX, and HBCD based on the lowest observed effect concentrations (LOEC). Values that could not be calculated are indicated with '-'. The ReRs were calculated by use of Equation 3.

	EE2	**FLX**	**HBCD**
Rainbow Trout	1.0	1.0	1.0
Lake Trout	0.001	-	0.01
Northern Pike	0.003	-	1.0
White Sturgeon	0.1	1.0	0.006667

Abundances of ERα and ERβ transcripts were also measured in lake trout and northern pike gonad tissues exposed to EE2 (Figure 3.2). In both lake trout and northern pike, ERα was significantly upregulated at the highest dose, 3000 ng/L, by 11- and 33-fold from the controls, respectively. At 30 ng/L, ERβ was significantly upregulated in lake trout gonad tissues; however, transcript abundance decreased back to the control level at 1000 and 3000 ng/L (Figure 3.2). In northern pike, ERβ was significantly downregulated by approximately 10-fold compared to the controls at the highest dose of 3000 ng/L (Figure 3.2).

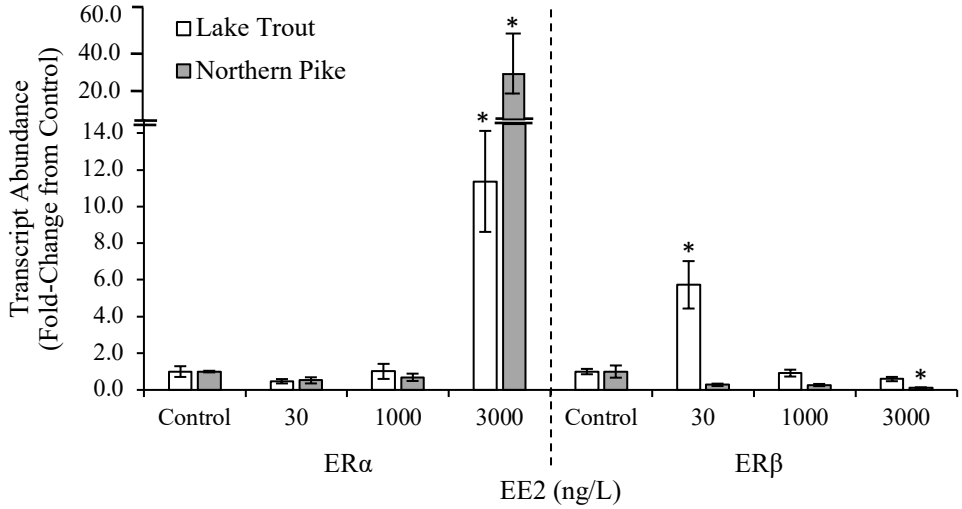

Figure 3.2. Transcript abundances of ERα and ERβ in gonad explants of male lake trout (n = 4) and northern pike (n = 3) following exposure to a solvent control (DMSO) or EE2. Data are presented as the mean ± standard error of the mean (SEM). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

3.4.1 Gene expression in tissues exposed to FLX

With exposure to FLX, transcripts of genes related to oxidative stress, including CAT, GPX, and GST, were measured in liver explants of lake trout, northern pike, rainbow trout, and white sturgeon (Figure 3.3). The reproductive marker, VTG, was also measured in each species but no significant effect was observed at any concentration in any species tested (Figure 3.4). No changes in transcript abundance of CAT and GPX were observed in lake trout, northern pike, or white sturgeon (Figures 3.3A, 3.3B). However, in rainbow trout, CAT and GPX were significantly upregulated by 4.7- and 2.2-fold, respectively, where the greatest increase in transcript abundance was observed at 200 and 1000 µg/L for each CAT and GPX (Figures 3.3A, 3.3B). GST was significantly upregulated in northern pike, rainbow trout, and white sturgeon while no change in transcript abundance of GST was observed in lake trout (Figure 3.3C). The concentration at which the greatest increase in transcript abundance of GST was observed varied in each species. GST was induced the greatest in northern pike at 5000 µg/L (13-fold), in white sturgeon at 0.32 µg/L (12-fold), and in rainbow trout at 1000 µg/L (3.5-fold; Figure 3.3C). Rainbow trout and white sturgeon were the most responsive species with exposure to FLX followed by northern pike while no change in transcript abundance was observed at any concentration for any of the genes measured for lake trout (Table 3.2).

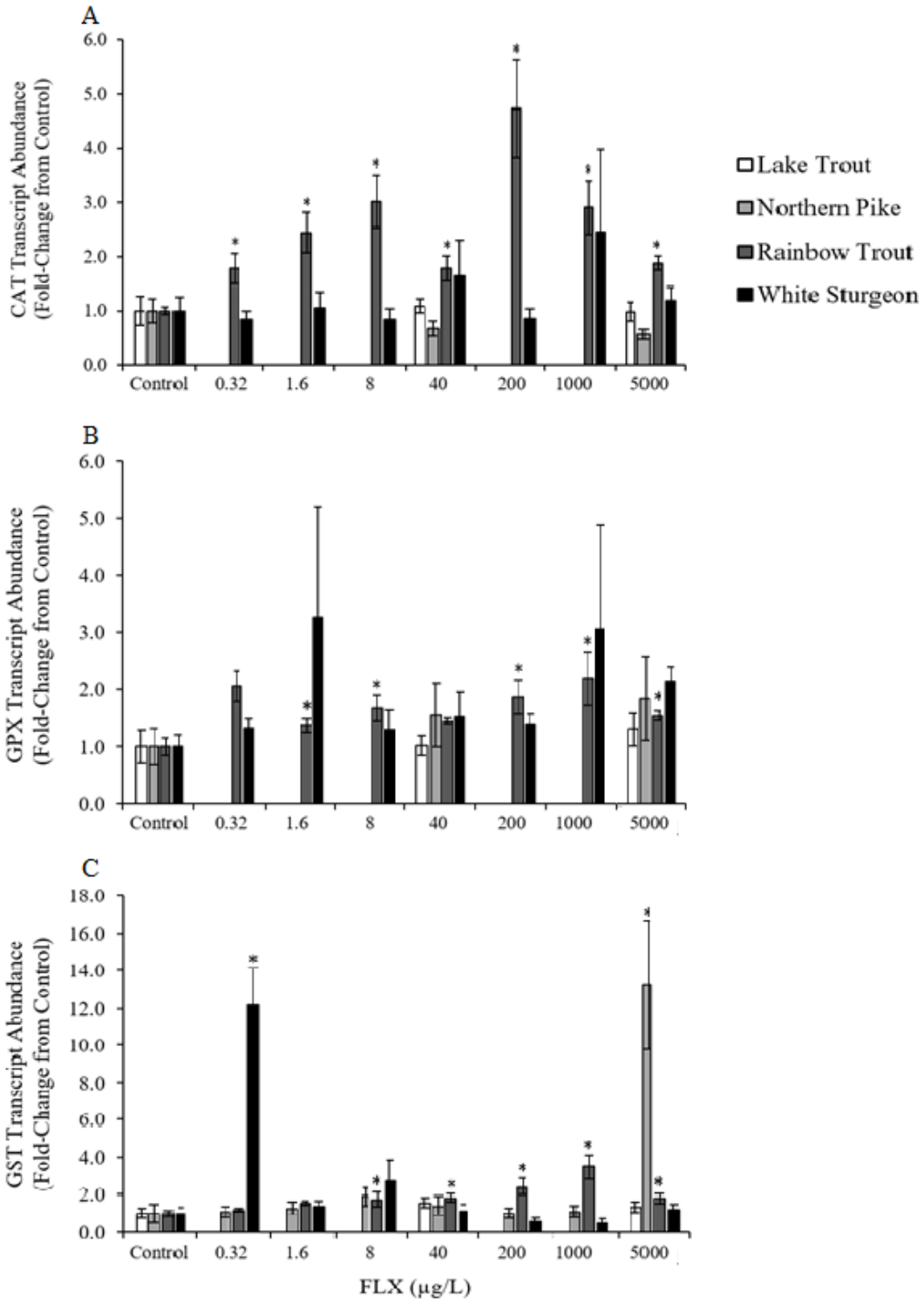

Figure 3.3. Transcript abundances of CAT (A), GPX (B), and GST (C) in liver explants of lake trout (n = 8), northern pike (n = 6), rainbow trout (n = 6), and white sturgeon (n = 6) following exposure to a solvent control (ethanol) or FLX. Data are presented as the mean ± standard error of the mean (SEM). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

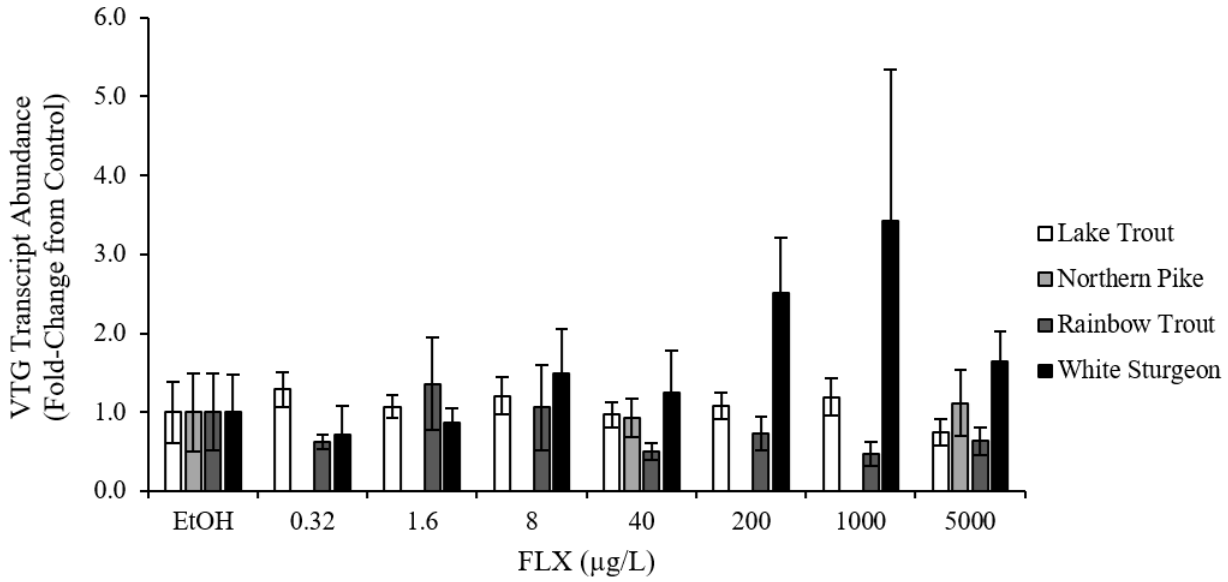

Figure 3.4. Transcript abundance of VTG in liver explants from lake trout, northern pike, rainbow trout, and white sturgeon following exposure to FLX. Data presented as the mean ± standard error of the mean (SEM) based on lake trout (n = 8 individuals), northern pike (n = 6 individuals), rainbow trout (n = 6 individuals), and white sturgeon (n = 6 individuals). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

Transcript abundances of CAT, GPX, and GST with exposure to FLX exposure were also measured in lake trout and northern pike gonad tissues (Figure 3.5). Transcript abundance of antioxidant genes, particularly CAT, were highly variable. A significant increase of CAT was only observed at 200 µg/L FLX in lake trout gonad tissues (Figure 3.5). In lake trout gonad tissues, GPX was significantly downregulated at every dose, while it was significantly upregulated at 200, 1000, and 5000 µg/L in northern pike (Figure 3.5). Additionally, GST, similar to GPX, saw an overall trend of downregulation in lake trout gonad tissues and an overall trend of upregulation in northern pike.

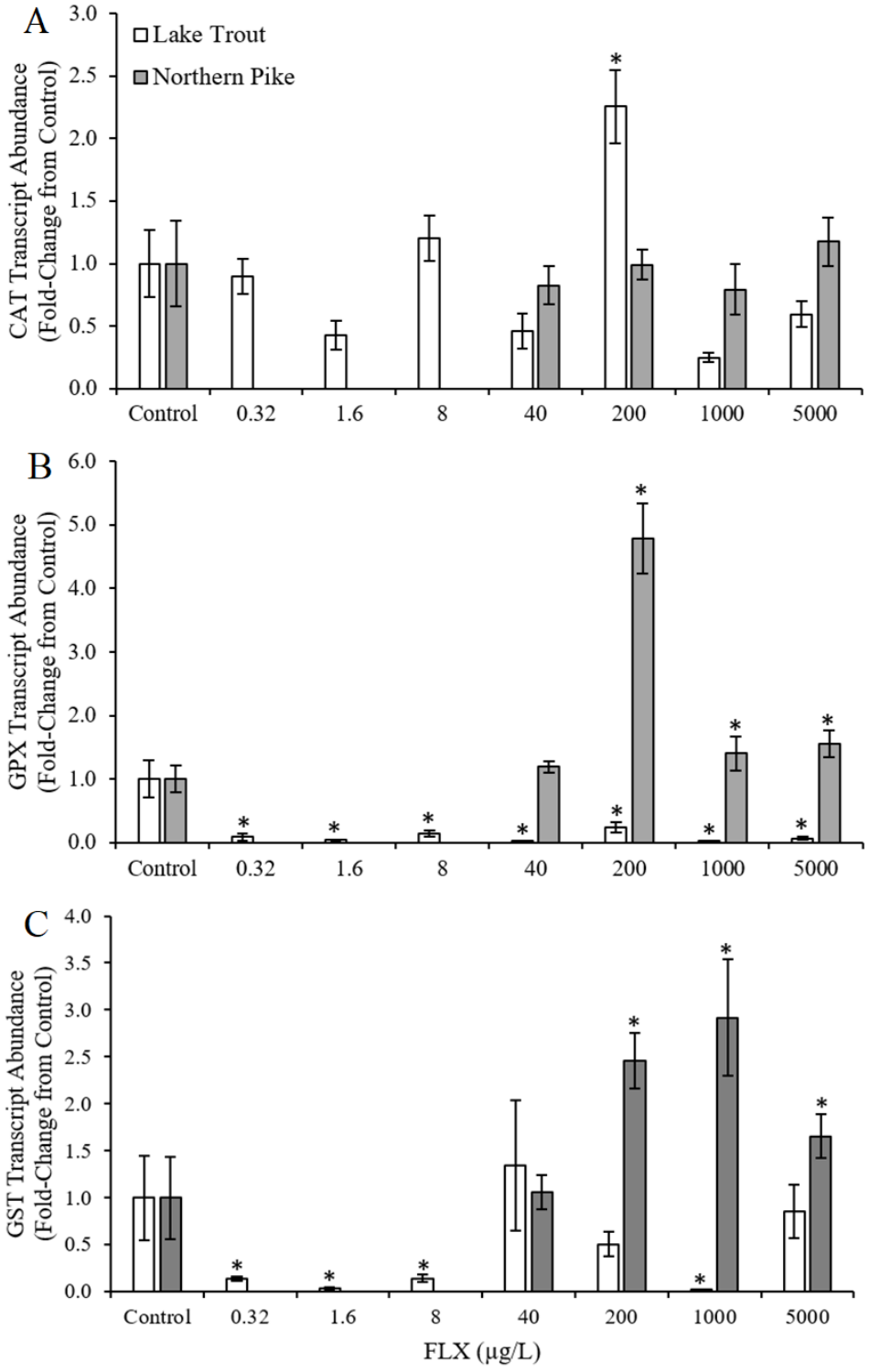

Figure 3.5. Transcript abundances of CAT (A), GPX (B), and GST (C) in gonad explants of lake trout (n = 8) and northern pike (n = 6) following exposure to a solvent control (ethanol) or FLX. Data are presented as the mean ± standard error of the mean (SEM). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

3.3.8 Gene expression in tissues exposed to HBCD

As with exposure to FLX, transcripts of oxidative stress genes were also measured with exposure to HBCD in liver explants of lake trout, northern pike, rainbow trout, and white sturgeon (Figure 3.6). Both GPX and GST were significantly induced in livers of all species while CAT was only induced in lake trout, northern pike, and rainbow trout (Figure 3.6). While CAT and GPX were significantly induced in lake trout, northern pike, and rainbow trout, a significant decrease in transcripts was observed at the highest dose of 30 mg/L (Figures 3.6A, 3.6B). For example, CAT was induced at 20 mg/L by 3.4- and 1.9-fold in lake trout and northern pike, respectively, and decreased by approximately 15- and 2.7-fold at 30 mg/L, respectively. A significant change in transcript abundance of CAT was not induced in white sturgeon livers with exposure to HBCD (Figure 3.6A). Rainbow trout was again the most responsive species to HBCD, followed by northern pike, lake trout, and white sturgeon (Table 3.2). Transcript abundances of antioxidant genes in gonad tissues of lake trout and northern pike exposed to HBCD were, again, highly variable (Figure 3.7). In northern pike, GPX was the only gene significantly altered and was downregulated at 0.2 mg/L only (Figure 3.7). Conversely, in lake trout, all three genes were significantly downregulated at the highest dose of 30 mg/L, which is consistent with observed effects in lake trout liver tissues (Figure 3.7).

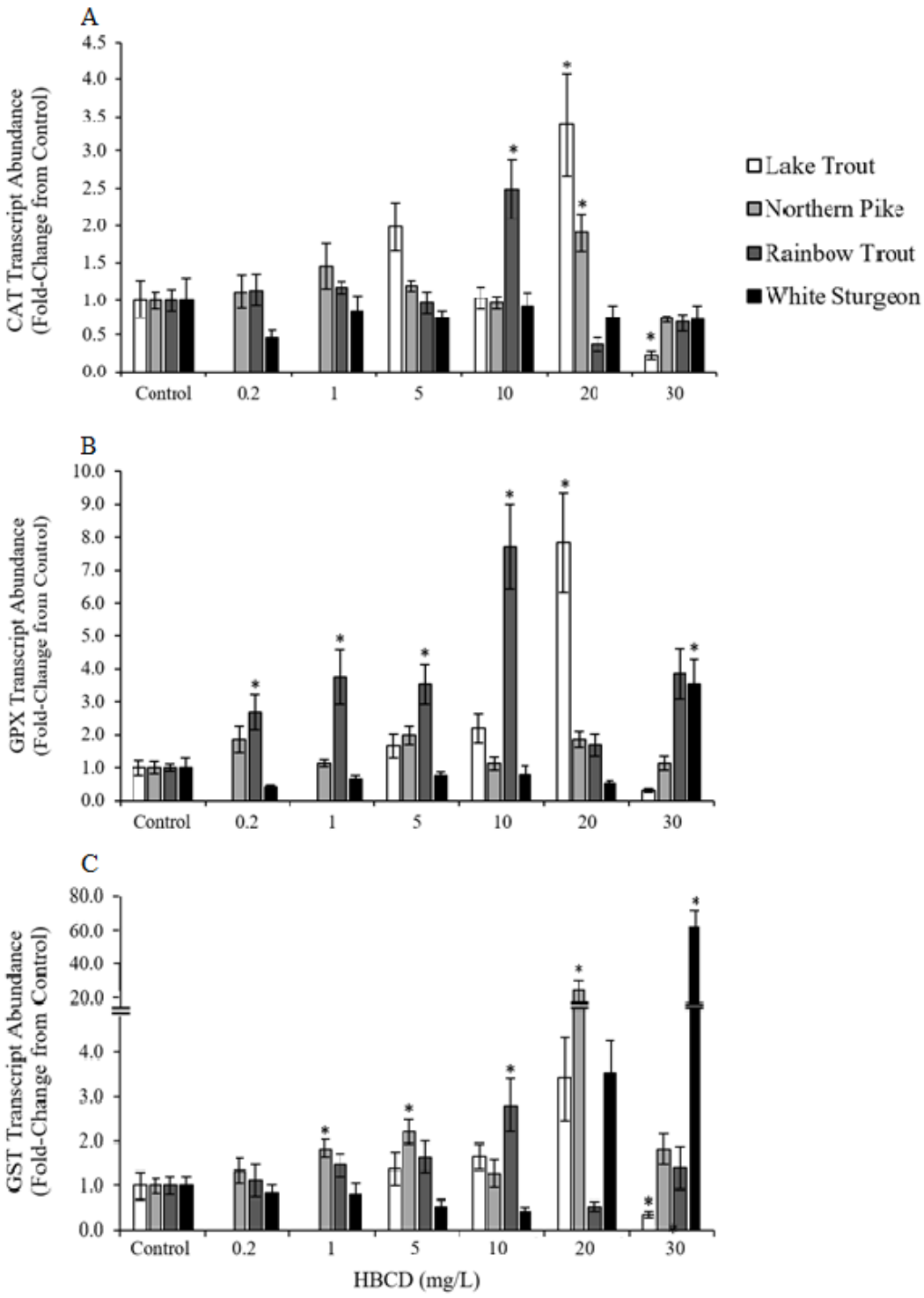

Figure 3.6. Transcript abundances of CAT (A), GPX (B), and GST (C) in liver explants of lake trout (n = 7), northern pike (n = 12), rainbow trout (n = 6), and white sturgeon (n = 6) following exposure to a solvent control (DMSO) or HBCD. Data are presented as the mean ± standard error of the mean (SEM). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

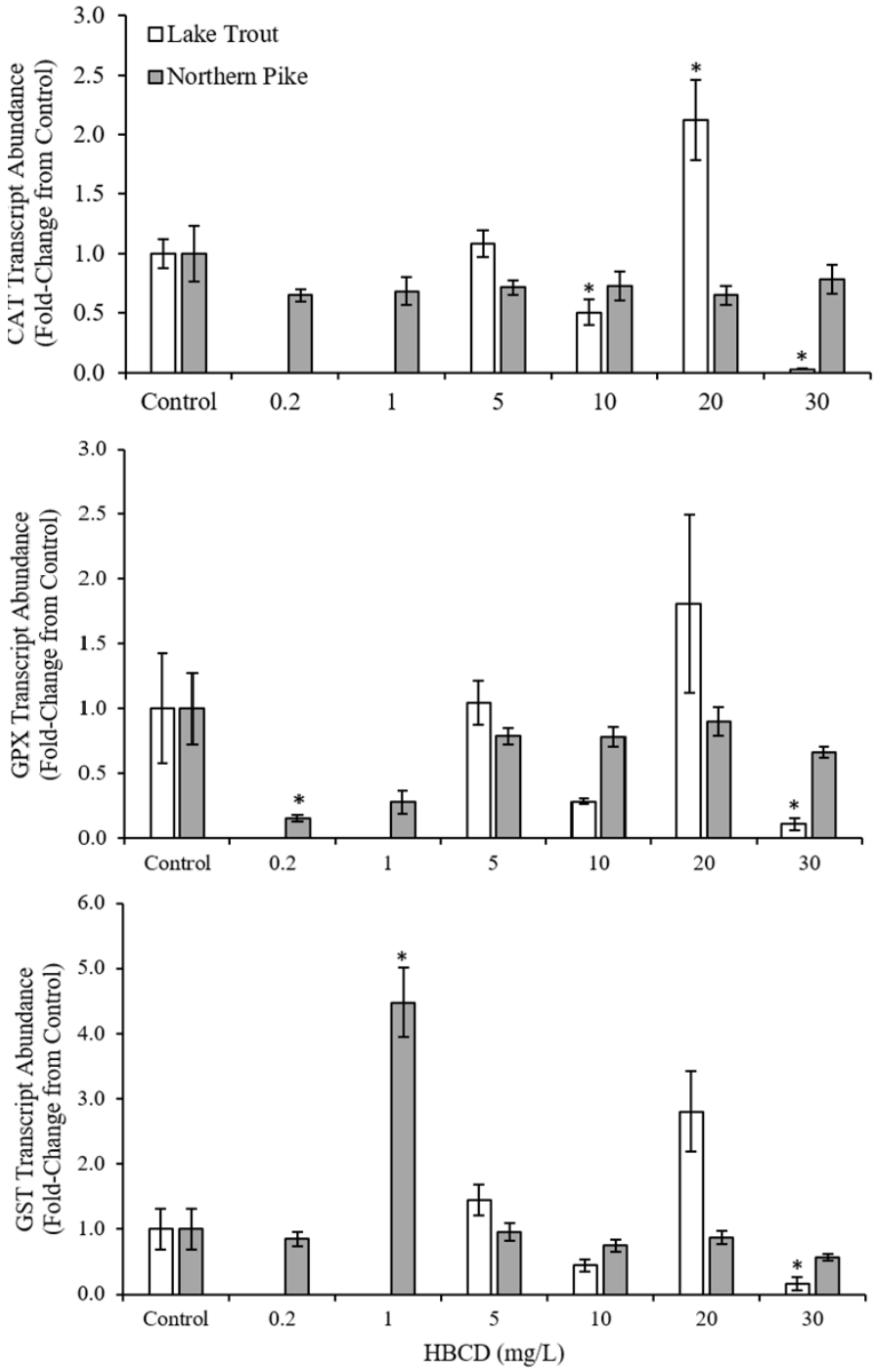

Figure 3.7. Transcript abundances of CAT (A), GPX (B), and GST (C) in gonad explants of lake trout (n = 7) and northern pike (n = 12) following exposure to a solvent control (DMSO) or HBCD. Data are presented as the mean ± standard error of the mean (SEM). An asterisk (*) represents a significant change from solvent controls (ANOVA with Dunnett's post-hoc test; $p \leq 0.05$).

3.5 Discussion

Prevalence of pharmaceuticals and flame retardants in water systems is on the rise due to their constant release into the environment, primarily via wastewater effluent (Boxall et al., 2012; Ela et al., 2011; Ternes et al., 1999). The present study successfully utilized an *in vitro* tissue explant assay to assess species-specific effectss among four North American fish species to the emerging contaminants EE2, FLX, and HBCD. Significant differences in ReRs among fishes were observed *in vitro*, which were comparable to ReRs derived for EE2 from previous *in vitro* studies (Beitel et al., 2015).

3.4.1 Relative responses of fishes to EE2, FLX, and HBCD

A number of differences in *in vitro* ReRs for EE2, FLX, and HBCD were observed among species, and where data were available these were compared to *in vitro* and *in vivo* findings from the literature. However, it should be noted that FBS was used as a supplement in the exposure media, which may potentially bind up lipophilic chemicals, such as those studied here as serums such as FBS may contain lipids and hormones. Additionally, lipophilic chemicals have the potential to bind to the plastic well in which the exposure was conducted, thereby reducing the concentration that tissue explants were exposed to. Therefore, future studies should evaluate the concentration of chemical that was taken up by the tissues to determine the exact dose given. However, many previous *in vitro* studies also used FBS (or other serums, such as bovine serum albumin) in culture medium and, therefore, data would be comparable across these

studies (Beitel et al., 2015; Bjorkblom et al., 2007; Eide et al., 2014). As seen in previous liver tissue explant studies with EE2, large differences in ReRs were also observed in our study (Table 3.2; Table 3.3; Beitel et al., 2015). Induction of the expression of the gene or protein of the egg-yolk precursor protein, VTG, is one of the most sensitive and widely used biomarkers of estrogen exposure as it is expressed in both male and female fish and is under direct control of the estrogen receptor pathway (Beitel et al., 2015; Matozzo et al., 2008). Although present in both males and females, VTG is only actively expressed in the livers of mature female fishes. However, VTG can be induced in males and juvenile females with exposure to environmental estrogens (Harries et al., 1997; Larsson et al., 1999; Nichols et al., 1999; Thorpe et al., 2001). Therefore, VTG serves as a useful indicator of exposure to estrogenic EDCs. In this study, VTG was the most responsive gene after exposure of liver tissue explants to EE2, followed by ERα and ERβ (Figure 3.1). LOECs for VTG induction in this study were comparable to those reported for northern pike but differed by 10-fold for white sturgeon when compared to another study by Beitel et al. (2015; Table 3.3). White sturgeon were more sensitive in our exposure compared to the previous study, which may be due to a potential difference in age between fishes tested in each study (> 4 years in our study compared to 4 years in another study; Table 3.3; Beitel et al., 2015). Additionally, because juvenile white sturgeon were used, we were unable to determine the sex of each fish and, therefore, both males and females were likely exposed. Responses of white sturgeon in our study were also among the most sensitive species compared to other *in vitro* liver explant studies with other fish species (Table 3.3). The VTG LOEC values for rainbow trout and northern pike were also comparable in our study and were approximately 3- and 10-fold greater than walleye (*Sander vitreus*) and white sturgeon LOECs, respectively), suggesting that they would also rank among the more sensitive species *in vivo* (Table 3.3; Beitel et al. 2015). *In vivo*,

however, the LOEC for VTG induction in juvenile rainbow trout is 200-fold lower than the *in vitro* LOEC obtained in this study (Van den Belt et al., 2003). The VTG LOEC values for white sucker (*Catostomus commersoni*) and Atlantic cod (*Gadus morhua*) from a previous *in vitro* study were 30- and 300-fold greater than that of white sturgeon determined in our study, respectively, while lake trout did not exhibit any change in VTG transcript abundance (Table 3.3; Beitel et al., 2015; Eide et al., 2014). An *in vivo* study in which lake trout were exposed to waterborne EE2 also found lake trout to be more tolerant to EE2 (LOEC: 400 ng/L; Figure 3.1; Werner et al., 2003), and which would be in accordance with our *in vitro* findings. Furthermore, lake trout in a lake treated with environmentally relevant concentrations of EE2 over a 3-year period did not display any reproductive abnormalities or population changes while pearl dace (*Margariscus margarita*) in the same lake saw decreased reproductive fitness and a reduction in their overall populations (Palace et al., 2006; Werner et al., 2006). These studies further suggest that lake trout are relatively tolerant to EE2; however, additional testing of higher concentrations would be needed to confirm this conclusion.

Table 3.3. Lowest observable effect concentrations (LOEC) of VTG mRNA induction from male fishes exposed to EE2 in *in vitro* liver explants.

Species	**LOEC (ng/L)**	**Exposure Duration (h)**	**Reference**
White sturgeon (*Acipenser transmontanus*)	100	24	This study
	1000	24	Beitel et al. (2015)
Walleye (*Sander vitreus*)	300	24	Beitel et al. (2015)
Rainbow trout (*Oncorhynchus mykiss*)	1000	24	This study
Northern Pike (*Esox lucius*)	1000	24	This study
	1000	24	Beitel et al. (2015)
White sucker (*Catostomus commersoni*)	3000	24	Beitel et al. (2015)
Atlantic cod (*Gadus morhua*)	30000	24, 48	Eide et al. (2014)
Lake trout (*Salvelinus namaycush*)	NA	24	This study

When compared to the study by Beitel et al. (2015), northern pike LOEC values for ERα in liver tissues were comparable. The LOEC value for rainbow trout ERα was approximately 333-fold lower than that of northern pike, while no statistically significant change in gene expression was observed in white sturgeon and lake trout from this study or white sucker and white sturgeon from a previous study (Figure 3.1B; Beitel et al., 2015). In contrast, a significant induction in ERα was observed in lake trout and northern pike gonad tissues. In northern pike gonad tissues, ERβ was significantly reduced, which has also been observed in livers of other teleost species, including zebrafish, Atlantic salmon (*Salmo salar*), and Japanese medaka exposed *in vivo* to other estrogenic compounds (Menuet et al., 2004; Mortensen and Arukwe, 2007; Yost et al., 2014). In livers, a statistically significant increase in ERβ transcript abundance was only observed in rainbow trout at 30 ng/L in our study and no effect was observed in northern pike, white sturgeon, and white sucker liver explants from a previous study (Figure 3.1C; Beitel et al., 2015). While *in vitro* assays do not take into account all the species-specific distribution, metabolism, and excretion of a compound (ADME properties), absorption rates in liver and gonad tissues are relevant and may differ among species. For example, EE2 has a relatively high lipophilicity (octanol-water partition coefficient, $K_{OW} = 4.15$), and therefore, it is more readily taken up into fatty tissues (Laurenson et al., 2014). Since white sturgeon livers, particularly in our study, are more fatty than other species, EE2 may be absorbed at a faster rate, thereby lowering the concentration at which an effect is observed compared to other species. Additionally, abundance and specificity of receptors as well as basal expression of some genes may differ among species, thus potentially altering species sensitivities (Beitel et al., 2015).

Previous studies with FLX have largely focused on whole animal behavioural endpoints due to its behavioural effects in humans and other mammals (Barry, 2013; Kreke and Dietrich,

2008; Wong et al., 2013). However, our study examined molecular parameters, *in vitro*, associated with FLX exposure in order to gain a better understanding of FLX's MOA in fishes, which has not been well characterized to date. As an SSRI, FLX has the potential to disrupt physiological pathways in which serotonin is involved; however, previous studies with fish also suggested that FLX has the potential to alter reproductive pathways (Corcoran et al., 2010; Gaworecki and Klaine, 2008; Mennigen et al., 2010a, 2010b, 2011). Therefore, VTG was measured in our study as a known biomarker of reproductive disruption (Figure 3.4). However, no change in VTG transcript abundance was observed in lake trout, northern pike, rainbow trout, or white sturgeon (Figure 3.4). Although white sturgeon exhibited a dose-dependent increasing trend in VTG transcript abundance, no statistical significance was found (Figure 3.4). A waterborne exposure to 0.54 µg/L FLX for 14 d also elicited a dose-dependent increase of VTG mRNA transcript abundance, as well as ERα mRNA transcript abundance, in male goldfish (*Carassius auratus*); however, a significant effect was only obtained when goldfish were exposed to FLX in combination with 5 ng/L EE2 (Silva de Assis et al., 2013). This suggests that FLX alone is not a potent enough EDC to exhibit significant estrogenic effects but can be harmful when exposed in mixtures, as is often the case in MWWEs. Therefore, a significant effect might have been attained in our study if our species were exposed to FLX in conjunction with EE2, as was observed in male goldfish (Silva de Assis et al., 2013). Results of our study demonstrate that lake trout, northern pike, and rainbow trout may be more resistant to reproductive disruption by FLX while white sturgeon may be comparably more susceptible; however, further analysis of additional reproductive biomarkers is needed to be certain.

The SSRI, FLX, has also been shown to influence oxidative metabolism in fishes due to its relatively high oxidative potential (1100 mV) compared to other PPCPs (Gagné et al., 2006).

With the disruption of oxidative metabolism, FLX may cause oxidative damage in tissues, particularly in livers, of some fish (Gagné et al., 2006). Furthermore, a study in which juvenile rainbow trout were exposed to FLX *in vivo* found oxidative metabolism to be one of the main pathways affected across the whole liver transcriptome and included dysregulation of antioxidant genes (Chapter 2). Transcriptome-level effects on oxidative response was also observed in goldfish (*Carassius auratus*; Mennigen et al., 2008) and zebrafish (*Danio rerio*; Park et al., 2012; Wong et al., 2013). In the present study, the transcript abundances of the antioxidant genes commonly employed in defense of oxidative stress, CAT, GPX, and GST, were measured in fishes exposed to FLX and significant differences were observed (Figures 3.3, 3.5; Zee et al., 2016). Each gene was upregulated in a dose-dependent manner in rainbow trout livers only, peaking at 200, 1000 and 1000 µg/L for CAT, GPX, and GST, respectively, and downregulated at the next highest dose(s), indicating that the system may have been overwhelmed or other pathways were initiated to combat oxidative damage (Figure 3.3; Zee et al., 2016). Expression of GST was relatively lower and more variable among the different species tested for both liver and gonad tissues, which may be explained by a number of factors. As mentioned earlier, other genes or antioxidant response pathways may compensate for the low expression of GST (Zee et al., 2016). Furthermore, GST has many isoforms expressed in multiple tissues and differences in gender have also shown to influence expression of GST isoforms in mice (Knight et al., 2007). Although there was not a clear pattern in GST expression between males and females in our study, differences in GST isoform expression in different tissues among individuals may account for the variability observed. However, abundance of GST in different tissues cannot be confirmed as only liver tissue was exposed in this study. Many free radical scavengers, including molecules the genes tested in this study encode for, act in conjunction with each other, which

may explain the high expression of CAT and low expression of GPX and GST in rainbow trout (Figure 3.3; Zee et al., 2016). CAT has also been shown to have the largest role in fighting oxidative stress among the three genes measured, which may explain its relatively higher expression in our study compared to GPX and GST (Mates, 2000). Oxidative stress endpoints in fishes, especially *in vivo*, have not been well-studied in the past, making comparison with our *in vitro* study difficult. A study with marine molluscs, however, assessed levels of antioxidant enzymes, including CAT and GST, after a two-week exposure to 75 ng FLX L^{-1} and increased enzyme activities were observed, which is consistent with our results. Most studies with FLX have reported metabolic effects, such as decreased food intake and energy metabolism as well as behavioural effects, including reduced aggression, swim speed and schooling behaviour, at environmentally relevant concentrations (Airhart et al., 2007; Barry, 2013; Dzieweczynski and Hebert, 2012; Kohlert et al., 2012; Mennigen et al., 2010b; Pittman and Ichikawa, 2013; Silva de Assis et al., 2013; Wong et al., 2013). Additionally, in fish, fluoxetine has shown to affect males and females differently. For example, fathead minnow (*Pimephales promelas*) males showed greater displays of aggression and defense against other male fish, while behaviours of female fish were unaffected (Weinberger and Klaper, 2014). These studies prove that FLX exposure can exhibit sex-specific effects. Furthermore, FLX may affect multiple targets in peripheral organs as well as the neuroendocrine brain, making characterization of effects incomplete and comparison among species difficult (Mennigen et al., 2011).

Most toxicological studies currently available on HBCD focused on the degradation and elimination of specific HBCD isomers as well as their distribution profiles in organisms, rather than the direct toxicity of HBCD to an organism (Abdallah et al., 2014; Esslinger et al., 2011; Huhtala et al., 2006; Sanders et al., 2013; Szabo et al., 2010; 2011; Zegers et al., 2005).

Furthermore, very few *in vitro* studies have been conducted with HBCD. However, in most studies conducted to date, whether *in vitro* or *in vivo*, oxidative stress appears to be the main driver of toxicity in organisms exposed to HBCD. In our study, significant and concentration-dependent increases in transcript abundance of antioxidant genes were observed in all species at most test concentrations, indicating that HBCD disrupted oxidative metabolism. Additionally, at the highest dose, a significant decrease in transcript abundance was observed with lake trout, northern pike, and rainbow trout liver tissues and in lake trout gonad tissues (Figures 3.6 and 3.7). Similar to the effects observed in fishes exposed to FLX, this decrease in transcript abundance may be due to an overwhelmed system at these great concentrations or induction of other pathways to compensate for a decrease in gene activity (Zee et al., 2016). In goldfish and Wistar rats, hepatic oxidative stress was detected after exposure to HBCD, with a significant increase in GST transcripts observed especially in female rats (Canton et al., 2008; Feng et al., 2013a). Feng et al. (2013a) hypothesized that oxidative stress, particularly in liver tissue of organisms, may be due to slow metabolism and elimination of HBCD, as well as its high potential to bioaccumulate. Additionally, Canton et al. (2008) saw sex-specific concentrations of HBCD in livers and expression of phase II biotransformation genes, such as GST. Female livers had the highest HBCD concentrations and GST transcript abundance, suggesting that female rats eliminated HBCD at a slower rate than male rats and, therefore, were at risk of greater toxicity. While there are no *in vitro* studies conducted to date that measured these specific genes following HBCD exposure, a study with HepG2 and human SH-S75Y cells exposed to HBCD saw elevated reactive oxygen species (ROS) production and cell apoptosis as well as a significant reduction of cell viability at concentrations ranging between 0.7 μM to 60 μM (Al-Mousa and Michelangeli 2012; 2014; Jing et al., 2014). An antioxidant defense is initiated in

response to tissue damage, particularly impairment of cell membrane permeability, which is caused by reactive oxygen molecules, and therefore, antioxidant genes are extremely important in combating effects brought about by ROS (Zee et al., 2016). Since we saw alteration of these antioxidant genes, it is evident that oxidative stress is an important MOA of HBCD toxicity to fishes, particularly *in vitro*, and further studies are needed to confirm these findings, *in vivo*. Developmental toxicity, particularly malformations of the heart, in zebrafish and marine medaka (*Oryzias melastigma*) fry after exposure to environmentally relevant concentrations of HBCD has also been observed, and oxidative stress, including generation of ROS to trigger apoptosis, was suggested as the main cause for these impacts (Deng et al., 2009; Du et al., 2012; Hong et al., 2014). Moreover, upregulation of CAT transcript abundance in the gills of marine clams (*Venerupis philippinarum*) following HBCD exposure was also found (Zhang et al., 2013). Alteration of antioxidant genes in our *in vitro* study, along with the antioxidant defenses observed in organisms in the aforementioned studies, is further evidence for oxidative stress as the main driver of HBCD toxicity to wildlife and should be assessed in greater detail in fishes native to North America.

Although oxidative stress is the most commonly found effect with exposure to HBCD, disruption of thyroid function has also been described due to HBCD's high affinity for the thyroid hormone receptor (THR; Palace et al., 2008; 2010; Ven et al., 2006; Yamada-Okabe et al., 2005). However, we were unable to successfully design primers for genes related to thyroid disruption and, therefore, effects on the thyroid axis are not reported here.

3.4.2 Implications for ecological risk assessment

Current environmental risk assessment of ECs found in MWWE is made difficult due to the limited toxicological information available on these chemicals to native species of interest. The present study aimed to fill in some of the existing knowledge gaps with regard to toxicity of EE2, FLX, and HBCD to North American fishes to aid in prioritizing ECs and species for toxicity testing. Customarily, regulatory guidelines for fishes are developed using standard laboratory animals, including rainbow trout (Krewski et al., 2007). Our study assessed effects of the above chemicals on lake trout, northern pike, and white sturgeon, as well as the model species rainbow trout, in order to compare sensitivities of all species to rainbow trout (Table 3.2). With exposure to EE2, the greatest increase in transcript abundance was elicited in rainbow trout. Transcripts of target genes in northern pike and white sturgeon were comparable to those in rainbow trout, indicating that current regulatory guidelines that are based on rainbow trout, including some Canadian Water Quality Guidelines, are protective of these species (Table 3.2; CCME, 2007; 2013). Interestingly, lake trout liver tissues did not respond to EE2, suggesting that this species is relatively insensitive to exposure with this compound. Therefore, we would also expect that current rainbow-trout based guidelines would protect this species; however, additional tests including higher biological level endpoints are needed to confidently make this conclusion. Transcripts of target genes following FLX exposure *in vitro* were much more variable in our study compared to those following EE2 and HBCD. Overall, rainbow trout was again the most responsive species, with upregulation observed in all three antioxidant genes which, again, may implicate that regulations set by rainbow trout may be protective of lake trout, northern pike, and white sturgeon (CCME, 2007). Nevertheless, results were highly variable and additional endpoints need to be measured to establish a clear MOA in fishes. Furthermore, FLX may affect multiple pathways and its effects may also be influenced by external environmental

stressors (Kreke and Dietrich, 2008; Heugens et al., 2002). Therefore, establishing a MOA that is representative across all fish species is crucial to develop regulations for the diversity of fishes. Antioxidant genes measured with exposure to HBCD were altered the greatest, again, in rainbow trout. Yet, it is still unclear whether alteration of antioxidant genes is a true indicator of sensitivity in fish species due to their compensatory nature and additional information is needed to better calibrate the antioxidant responses observed in our study at the gene expression level with endpoints that verify that actual damage has occurred, both *in vitro* and *in vivo* (e.g., lipid peroxidation, presence of ROS). In general, rainbow trout was the most responsive species in our study, which is beneficial since it is already one of the main model species used in risk assessment in cold freshwater systems. However, some studies with dioxin-like chemicals showed that this is not always the case and species sensitivities are chemical-specific (Eisner et al., 2016). Therefore, further studies are needed to confirm our findings.

3.6 Conclusion

The current study successfully utilized an *in vitro* liver explant assay to establish species-specific differences in gene expression in four Canadian fish species to assess the impact of the emerging contaminants EE2, FLX, and HBCD on the expression of selected target genes. However, measurements of gene expression are of a functional nature and do not necessarily indicate toxicity, which would require the parallel assessment of apical outcomes, and further research is needed. Our results demonstrated differential gene expression among species to these contaminants, which draws attention to deficiencies with current risk assessment approaches that only use model laboratory species for determination of guidelines. Furthermore, this study

highlights the need for characterization of more reliable endpoints. For example, oxidative stress has proven, both here and in previous studies, to be a difficult endpoint to classify due to the unclear sensitivity of antioxidant genes (Palace et al., 1998). More research is needed to better describe the toxicities of these chemicals alone, and in mixtures, as well as to determine if *in vitro* responses are truly indicative of *in vivo* effects. Our future work includes comparing the *in vitro* responses measured in this study with responses in a parallel in *vivo* study conducted with the same chemicals and species.

CHAPTER 4

4 GENERAL DISCUSSION

Increased use of pharmaceuticals and personal care products (PPCPs) as well as flame retardants in consumer products has raised concerns regarding their risk to the environment and their potential toxicity to aquatic life (Boxall et al., 2012; Ela et al., 2011). Pollution of the aquatic environment with these emerging contaminants (ECs) is becoming increasingly widespread, particularly in developed areas where they are often detected in municipal wastewater effluents (MWWEs) and downstream water bodies. While toxicity of some of these ECs to fish has been studied using small-bodied neo-tropical model species, few studies have been conducted with native North American species of interest. This focus on a few test species is of great concern considering the large diversity of fishes (> 28,000 species worldwide), which display significant differences in physiological characteristics and adaptations. For this reason, extrapolation of data from model species to native North American fishes is often not indicative of their true sensitivity to these chemicals. Therefore, a large information gap currently exists, complicating ecological risk assessment of ECs for non-model species (CEC., 1996; Chapman et al., 1998; Forbes and Calow, 2002). While testing of the effects of ECs on native species is warranted, challenges exist when conducting whole animal studies with wild fish species, including difficulties with rearing fishes in a laboratory setting, ethical issues, as well as the large expense and space that *in vivo* assays use. Therefore, alternative assays such as *in vitro* assays or short-term molecular mechanistic studies are increasingly being implemented in toxicity testing, including the tissue explant assay and the whole transcriptome experiments described in this thesis. Specifically, the primary objectives of this study were to 1) characterize and further expand our knowledge on the toxicity of selected ECs for which little toxicological knowledge exist regarding fish; and 2) to establish an *in vitro* approach that enables assessing the

responsiveness of four selected fishes of relevance to North American fresh waters to three priority ECs.

Two PPCPs, 17α-ethinylestradiol (EE2) and fluoxetine (FLX), and the flame retardant, hexabromocyclododecane (HBCD), are frequently found in aquatic systems and may pose a risk to freshwater organisms inhabiting these areas. While ample information regarding the specific mechanism of action (MOA) through which EE2 causes adverse effects in fish exists, less is known about the MOA of FLX and HBCD in fishes. Therefore, the first study of this thesis aimed to characterize whole transcriptome responses in a model species (rainbow trout; *Oncorhynchus mykiss*) through identification of key molecular pathways that are altered with exposure to waterborne FLX. Based on the outcomes from this study and additional mechanistic toxicity information available through the literature, the second study of this thesis then aimed to establish and validate alternative *in vitro* tissue (liver and gonad) explant assays to characterize the effects of FLX, as well as HBCD and EE2, to three species native to Canada and determine how they rank in sensitivity compared to a model fish, rainbow trout, used in regulatory toxicity testing (rainbow trout; CCME, 2007; 2013). It should be noted that conducting a second whole transcriptome study with HBCD was outside the scope of my work given that this is part of a Masters of Science thesis and, therefore, limited time and resources were available. Thus, conducting an *in vivo* study with HBCD and assessing changes in the whole transcriptome is still necessary.

The results from the *in vitro* tissue explant experiments confirmed and validated the findings from the first study, in which transcript abundances of antioxidant genes were significantly altered with exposure to FLX. Therefore, with further sequencing analysis of

additional fishes, a more comprehensive characterization of the MOA of FLX in native North American fish species can begin to be understood. The results from the second study also demonstrated that *in vitro* tissue explants assays are a valuable approach to investigate dysregulation of key genes involved in the toxic manifestation of ECs in lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*), white sturgeon (*Acipenser transmontanus*), and rainbow trout. Each of EE2, FLX, and HBCD elicited a change in gene expression in each species tested in both liver and gonad explants that, in most cases, were indicative of physiological alterations previously reported in the literature where data was available. Furthermore, rainbow trout were consistently one of the more sensitive species in our study, and therefore, it is evident that this species appears to be an appropriate model organism for assessment of toxicity of select ECs to fishes.

4.1 Determining the predictability of *in vitro* data to *in vivo* data

As mentioned earlier in this thesis, numerous challenges exist when conducting *in vivo* studies with non-model, or wild fish species. Furthermore, significant economic and ethical concerns exist when using live animals. Hence, there is a push for the implementation of *in vitro* testing as a preliminary screen prior to conducting whole body toxicity tests, which often require thousands of individual organisms and upwards of tens of thousands of dollars. This thesis therefore aimed to evaluate the predictability of *in vitro* tissue explant assays to *in vivo* results, as well as compare transcriptomic responses in rainbow trout *in vivo* changes in gene expression observed *in vitro*. While a large number of whole organism toxicity tests with EE2 have been conducted in fishes, few have been conducted with FLX and HBCD. Furthermore, even fewer

studies, *in vitro* or *in vivo*, with EE2, FLX, and HBCD have been conducted with non-model fish species native to North America. Therefore, there is an obvious data gap in the literature regarding the scalability of *in vitro* results to whole organism effects in these species.

In order to develop *in vitro* assays that are capable of predicting toxicity *in vivo*, one must first understand the specific mechanisms by which chemicals of concern affect biology of the species in question. Therefore, assessment of the transcriptomic changes in rainbow trout conducted in the first study of this thesis aimed to further characterize effects of FLX to the model species, rainbow trout. Characterization of the pathways altered *in vivo* can help inform targeted *in vitro* assays and endpoints to be analyzed as a first pass screening tool in the future. Whole transcriptome sequencing of rainbow trout liver revealed significant dysregulation of individual genes and pathways after exposure to FLX. Specifically, metabolic function pathways were significantly affected across the rainbow trout transcriptome. When compared to previous *in vivo* studies in which zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) were exposed to FLX, all species shared several altered pathways, including lipid metabolism and cellular biosynthetic processes (Mennigen et al., 2008; Park et al., 2012; Wong et al., 2013). In addition to these pathways, genes related to responses to other organisms, or external biotic stimuli, were also significantly downregulated in rainbow trout livers, which is significant as livers are not typically considered to be directly involved in mediating behavioural responses.

Furthermore, pathways involved in regulating oxidative stress were also altered, which is in accordance with previous observations in zebrafish brain (Wong et al., 2013). As alterations of oxidative stress pathways were a common effect among fishes exposed to FLX, antioxidant genes dysregulated in the *in vivo* assay were used to inform the second study of this thesis in which *in vitro* tissue explants were used to characterize the effects of this drug across four fishes

native to Canadian freshwater systems (Chapter 3). However, future studies should also probe some of the less well-described toxicity pathways identified in this study for FLX in rainbow trout.

In vitro tissue explant assays confirmed significant dysregulation of genes involved with antioxidant responses across the four test species. Therefore, rainbow trout exposure to FLX *in vitro* was shown to be relatively predictive of responses *in vivo*, and the responses observed in rainbow trout liver explants were indicative of what would occur in the whole tissue. However, it is uncertain whether effects measured at the level of gene expression are truly translating to physiological alterations (i.e., oxidative stress). Therefore, different endpoints that are truly indicative of oxidative stress *in vitro*, including quantification of lipid hydroperoxides, should be measured to better predict what would occur *in vivo*.

Based on the outcomes from the whole transcriptome analysis and reports in the literature, production of ROS was identified as potentially critical mechanism of toxicity for FLX in fishes as oxidative stress pathways were significantly altered in rainbow trout livers. While ROS are present in small amounts in cells, excess ROS production can lead to negative effects, including DNA, protein, and lipid damage (Stoliar and Lushchak, 2012). Damage to these molecules may be the key events that lead to more severe adverse outcomes, such as carcinogenesis or neurodegenerative diseases (Toyokuni, 1999). While information gaps regarding effects on the remaining North American fishes still exists, this study represents a step forward in the identification of the key events triggering a toxic effect of FLX in fishes. Further *in vivo* studies with additional species, as well as with exposure to HBCD, will help identify species-specific differences that may explain the differences in sensitivities observed *in vitro*.

Since three of the four fish species tested in this study have not been well studied to date, I was unable to compare responses between *in vitro* and *in vivo* studies for all species. However, rainbow trout are a well-studied species and have been assessed for their sensitivity to EE2 both *in vivo* and *in vitro*. While the lowest observable effect concentration (LOEC) for rainbow trout exposed to EE2 *in vitro* (1000 ng/L) was approximately 200-fold higher than the *in vivo* LOEC, rainbow trout were still among the more sensitive species with both tests (Chapter 3; Van den Belt et al., 2003; Werner et al., 2003). Therefore, although *in vitro* assays were not predictive of the exact concentrations at which an effect would be observed *in vivo*, *in vitro* assays may be predictive of the relative species sensitivity distribution with exposure to EE2. Conversely, lake trout exposed to EE2, both *in vitro* and *in vivo* were relatively more tolerant compared to other species (Chapter 3; Van den Belt et al., 2003; Werner et al., 2003.) Therefore, it is apparent that the *in vitro* test was predictive of *in vivo* sensitivity to EE2 as lake trout were more tolerant in both assays. The generally greater concentrations required to elicit an effect *in vitro* compared to *in vivo* may be due to the toxicokinetic differences between *in vitro* and *in vivo* assays, complicating the comparability of responses between the two studies and highlighting the need for toxicokinetic models for non-model species. In this case, the mode of uptake differs between the tissue explant assays and whole animal studies. For example, lipophilic chemicals such as EE2 have a higher likelihood for accumulation of the chemical at greater concentration occurring at the target tissues *in vivo* and, therefore, adverse effects are more likely to occur at lower concentrations *in vivo* than *in vitro*.

4.2 Differences in species sensitivity

Currently, environmental risk assessment of ECs found in surface waters primarily uses model laboratory species to assess effects on fishes. Therefore, there is very limited data regarding effects of ECs to native fish species of interest. This thesis aimed to bridge the gap in toxicological knowledge that exists for non-model fish species to improve our understanding of how fish species native to North American waters, lake trout, northern pike, and white sturgeon, rank compared to a model organism commonly used in aquatic toxicology, rainbow trout.

Results of Chapter 2 successfully illustrated that many pathways affected by exposure to FLX were conserved among species. The species tested in this study, rainbow trout, shared many dysregulated pathways with other species, including goldfish and zebrafish (Mennigen et al., 2008; Park et al., 2012; Wong et al., 2013), indicating that the effects of FLX exposure at the transcriptome level are relatively conserved among evolutionarily related species. The similarities in affected pathways observed among these species are an important outcome in informing cross-species assessments to chemicals such as FLX. Results from studies such as this can therefore aid in identifying specific key molecular key events that manifest as adverse effects in fishes, which can then, in some cases, be extrapolated across species. Furthermore, characterizing toxicity with these methods (high-throughput 'omics tools) is becoming increasingly prominent in ecological risk assessment and more studies assessing toxicity of chemicals in this way are warranted (Perkins et al., 2013).

Results of Chapter 3 demonstrated that sensitivity to a compound can vary greatly by species. While the magnitude of differences in sensitivities varied, rainbow trout were consistently the most responsive with exposure to ECs tested in this study. In particular, the greatest differences in sensitivities among species tested in this study were observed with exposure to EE2 (Table 3.2). Rainbow trout were 1000-fold more sensitive to induction of the

estrogenic biomarker, vitellogenin (VTG), than the most tolerant species in this study, lake trout. It is therefore evident that the processes that drive sensitivity or responsiveness of a species differ between the sensitive rainbow trout and more tolerant species such as northern pike. One explanation may be that EE2 could bind to the estrogen receptor (ER) with higher affinity and greater specificity in rainbow trout than other species. Alternatively, more tolerant species may eliminate and metabolise EE2 at a faster rate than rainbow trout, thus reducing the time EE2 is available to bind to the ER. The latter hypothesis may also be applicable to the differences observed with FLX and HBCD exposure among the four species tested in this study (Yost et al., 2014). However, to date there is a lack of baseline metabolic activities in the four species tested, and thus, this hypothesis remains speculative.

Chapter 3 also assessed the effects of EE2, FLX, and HBCD on northern pike and lake trout gonad tissue explants. As these two species were collected from the wild, we attempted to predict the most sensitive gonadal stage based on spawning cycles. Previous studies have shown that the maturation stage of gonad tissues is essential for the production of hormones and rates of gonad tissue development are different between species as well as between sexes (Beitel et al., 2015). However, it is difficult to be sure that the most sensitive stage for each species was in fact captured without further histological analyses, as their spawning cycles are different. Therefore, the differences observed in gene expression between northern pike and lake trout gonad explant may be attributed to differences in the stage of the spawning cycle that fishes were collected at. For example, lake trout spawn in the fall, while northern pike spawn in the spring. Therefore, it is difficult to capture the exact same stage of the cycle for both species when they cannot be collected concurrently. While northern pike liver tissue explants were most tolerant to EE2, lake trout gonad tissue explants were less responsive than those of northern pike (Figure 3.2).

Additionally, as hypothesized with liver explants earlier, it is also likely that EE2 binds with higher affinity to the ER in northern pike gonadal tissues than in lake trout. While transcript abundance increased in a dose-dependent manner in gonad explants with exposure to EE2, responses to FLX and HBCD was highly variable. As described earlier in this thesis, variability in response to FLX or HBCD is likely due to the compensatory nature of antioxidant genes in which certain genes may be induced to counterbalance the reduction of another antioxidant gene (Chapter 3). Therefore, there are uncertainties regarding whether measurement of antioxidant genes through quantitative real-time polymerase chain reaction (qRT-PCR) is truly representative of physiological changes occurring and this endpoint must be evaluated further.

4.3 Future research

Next generation RNA sequencing of the whole transcriptome proved a useful tool in identifying key molecular pathways affected by FLX in rainbow trout liver. With these tools, we can begin to inform toxicity patterns for ECs in fish, and therefore, better understand the MOA of these priority ECs. With further testing of additional ECs and fish species, it will then possible to identify important biomarkers for EC exposure that can be used to screen with *in vitro* methods and reduce the need for whole animal studies. While this thesis provides important preliminary results with regards to both the MOA of FLX *in vivo* and the species sensitivity distribution with exposure to EE2, FLX, and HBCD *in vitro*, there are a number of areas that can be expanded to better characterize effects of these compounds to native fishes of interest.

In the *in vivo* rainbow trout exposure to FLX (Chapter 2), I successfully identified key pathways affected by FLX in rainbow trout liver. Given the success of determining toxicological pathways and mechanisms for FLX, and the conservation of these pathways among fish species, these results show great promise for use in native species as well. However, analysis of pathways affected in the brain with exposure to FLX should also be assessed as it would shed light on additional pathways that may not be expressed in liver. Additionally, conservation of pathways among species may aid in informing cross-species assessments in ecological risk assessment in the future. A previous study conducted by Doering et al. (2016) had already successfully sequenced the whole transcriptome of liver of the native fish species, white sturgeon, following exposure to dioxins and dioxin-like chemicals (DLCs), further proving the usefulness of RNAseq technologies across model and wild fish species. For example, while fewer genes were altered across the whole transcriptome compared to other species, many genes affected by DLCs in white sturgeon were also shared among other species, including zebrafish, killifish (*Fundulus heteroclitus*), and roach (*Rutilus rutilus*; Brinkmann et al., 2016; Li et al., 2013; Whitehead et al., 2010), demonstrating the importance of toxicity pathway analyses for cross-species extrapolation. However, changes at the transcriptomic level in native fish species of interest has not been previously conducted with exposure to FLX *in vivo* and it is hypothesized that this analysis would reveal the specific mechanisms that could drive sensitivities of lake trout, northern pike, and white sturgeon to FLX. Specifically, it would allow a comparison among species to be conducted, and therefore, develop a more refined species sensitivity distribution for North American fish species. Furthermore, it may aid in developing a mechanistic model that may explain the specific differences that drive species sensitivities to ECs among diverse fishes. Additionally, while many pathways on the transcriptomic level were shared among other fishes

reported in the literature (including zebrafish, Asian clam, and goldfish; Chen et al., 2013; Mennigen et al., 2008; Park et al., 2012; Wong et al., 2013), many were also unique to rainbow trout, further stressing the importance of analyzing effects in multiple fish species, and not relying solely on the effects on rainbow trout for development of regulatory guidelines.

In the gonad explant assay, gonads from northern pike and lake trout were taken during the time leading up to spawning. This period of time or maturation stage is presumably the most sensitive stage for these tissues as fish are expending significant amounts of energy to build up and mature their gonads (Beitel, 2014). However, since histological analyses on gonad tissues was not conducted, we cannot be sure that the most sensitive stage was in fact captured in this study and future studies could acquire histological samples at the time of tissue collection to verify the maturation stage of the fish.

In the tissue explant assays, future research could test additional concentrations of some of the ECs to describe more complete dose-response patterns of rainbow trout, lake trout, northern pike and white sturgeon. Specifically, further tests with additional EE2 concentrations could be conducted to determine an *in vitro* LOEC for all species, which would allow for comparison to *in vivo* studies. Alternative markers of oxidative stress with exposure to FLX and HBCD, including generation of ROS, should also be measured to better predict effects of these chemicals *in vitro*, and to reduce the variability observed with expression of antioxidant genes. Furthermore, additional *in vitro* endpoints should be assessed, to better inform on alterations observed in other pathways. For example, analysis of the whole transcriptome revealed alterations in a number of different pathways with exposure to FLX, and therefore, endpoints that reflect these pathways could be measured, giving a more robust understanding of FLX toxicity, *in vitro*. Toxicokinetic models with these species would also shed light on the physiological species differences that

exist, as well as determine rates of adsorption, distribution, metabolism, and excretion to further refine extrapolation from *in vitro* to *in vivo* responses.

4.4 Conclusions

A primary aim of this thesis was to establish molecular toxicity response profiles for a key EC, FLX, with little associated toxicity information in native fishes. Results of the 96-hr *in vivo* exposure of rainbow trout to FLX identified alteration of biological pathways at the transcriptome level that were similar to those observed in other fishes previously. The results of the pathway analysis in rainbow trout liver identified oxidative damage as a primary process affected by FLX exposure, and was therefore used to inform which genes to assess *in vitro*. The second study successfully established and validated an alternative *in vitro* tissue explant assay to characterize the sensitivity of four species of commercial, recreational, and cultural relevance in Canada (lake trout, rainbow trout, northern pike, and white sturgeon) to three priority ECs (EE2, FLX, and HBCD). In the tissue explant assays, transcript abundances of target genes were measured in liver tissues exposed to serial concentrations of EE2, FLX, and HBCD and significant and specific-specific responses were obtained. Liver explant assays revealed that rainbow trout were consistently the most sensitive species tested, particularly with exposure to EE2, whereas the sensitivities of the remaining species varied. Transcript abundance of VTG and ERα was similar to that observed in other fishes as well as other studies with the same species. However, FLX exposure was variable, limiting the ability to deduce clear species-response patterns. Thus, further research is needed to confirm sensitivities of these species *in vitro*. Transcript abundances of target genes were also successfully measured in lake trout and northern

pike gonad tissue explants after which variable changes in transcript abundances were observed. With exposure of gonadal tissue to EE2, northern pike was the most responsive species while lake trout were generally the most responsive to FLX and HBCD. As with liver explants, additional endpoints must be assessed to determine the true type of toxicity of FLX to gonad explants as measurement of antioxidant genes was highly variable. Overall, the *in vitro* tissue explant assays reported in this thesis represent an important preliminary analysis of the toxicity of these ECs to native fishes of interest, which may assist in guiding or prioritizing further testing of ECs for protection of aquatic life. However, significant remaining uncertainty with regards to the applicability of comparing effects across species exists, and therefore, further research is needed to better standardize testing and account for environmental variation when conducting studies with wild fish.

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