ASSESSMENT OF THE SENSITIVITY OF NORTH AMERICAN FISH SPECIES TO ENDOCRINE DISRUPTING CHEMICALS *IN VITRO*

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ABSTRACT

There is concern regarding exposure of aquatic organisms to chemicals that interfere with the endocrine system. Disruption of the endocrine system can lead to impacts on sexual development, altered hormone levels, intersex, and ultimately reproductive failure. While effects of endocrine disrupting chemicals (EDCs) on standard laboratory species have been subject of intense study, to this day there is a large gap in knowledge and a high degree of uncertainty regarding the sensitivity of wild fish species to these compounds. One of the main concerns with current toxicity testing approaches is that they require the use of a large number of live animals, particularly when working with native species. Therefore, the aim of this study was to develop *in vitro* tissue explant assays that would enable the assessment of the sensitivity of different wild fish species native to North America to the exposure with EDCs. Specifically, two *in vitro* assays were developed: 1) A liver explant assay to assess effects of EDCs that can interact with the estrogen receptor (environmental estrogens), and 2) a gonadal explant assay to assess effects of EDCs on sex-steroid production. The test species selected were northern pike (*Esox lucius*), walleye (*Sander vitreus*), and white sucker (*Catostomus commersoni*) that were sampled from Lake Diefenbaker, Saskatchewan, Canada, and white sturgeon (*Acipenser transmontanus*) that were randomly selected from an in house stock reared from eggs. Liver tissue was excised from male fishes and exposed for 24 h to a synthetic estrogen, 17α- ethinylestradiol (EE2). Transcript abundance of vitellogenin (VTG), estrogen receptor (ER) α and β in liver tissue were quantified using qPCR. Gonad tissue from both male and female were excised and exposed for 24 h to a model inducer (forskolin) and inhibitor (prochloraz) of steroidogenesis. 11-ketotestosterone (11-KT) and estradiol (E2) were quantified in media by use of ELISA. Exposure to EE2 resulted in a concentration dependent increase in VTG in all species, and an increase in ERα in northern pike.

Walleye males showed the greatest sensitivity to EE2. Gonad tissues exposed to forskolin showed a concentration dependent increase in 11-KT and E2. Exposure to prochloraz resulted in a decrease of 11-KTand E2. Male and female white sucker showed greatest sensitivity to forskolin, while male and female walleye showed greatest sensitivity to prochloraz. The seasonal time point during which gonad explants were excised and exposed had an impact on the potency and magnitude of response, resulting in a seasonal effect on sensitivity. Also, gonad explants from these species were found to have greater sensitivity than responses previously reported for *in vitro* explants of other fish species such as the fathead minnow (*Pimephales promelas*), and stable cell lines currently used as screening applications to detect chemicals that might disrupt the endocrine system. Therefore, current approaches that use stable cell lines or tissue explants from standardized small bodied laboratory species might not be protective of some wild fish species. These tissue explants represent a promising approach to help understand species sensitivity to EDCs, and if appropriately validated, could be a powerful tool for chemical screening.

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LIST OF ABBREVIATIONS

°C – degree Celsius

µg – microgram

µg/L – microgram per litre

µL – microlitre

µM - micromolar

11-KT – 11-ketotestosterone

ANOVA – analysis of variance

AR – androgen receptor

AR α – androgen receptor alpha

AR β – androgen receptor beta

ATRF – Aquatic Toxicology Research Facility

cDNA – complimentary deoxyribonucleic acid

cm – centimetre

CYP 17 – cytochrome P450, family 17

CYP 19 – cytochrome P450, family 19

DDE – dichlorodiphenyldichloroethylene

DDT – dichlorodiphenyltrichloroethane

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

E2 – 17-β estradiol

EC_{50} –concentration of chemicals causing 50% effect

EDC – endocrine disrupting chemical

EDSP – Endocrine Disruptor Screening Program

EE2 – 17α ethinylestradiol

ELISA – enzyme linked imunosorbent assay

ER – estrogen receptor

ER β1 – estrogen receptor beta 1

ER β2 – estrogen receptor beta 2

ERE – estrogen response element

ERα – estrogen receptor alpha

FBS – fetal bovine serum

FSDT – fish sexual development test

FSH – follicle stimulating hormone

g – gram

GnRH – gonadotropin releasing hormone

GSI – gonadosomatic index

h – hour

H295R – human adenocarcinoma cell line

HPG – hypothalamic pituitary gonadal

HSI – hepatosomatic index

kg – kilogram

L – litre

LH – luteinizing hormone

LOEC – lowest observed effect concentration

mm^3 – cubic millimetre

mg – milligram

mg/L – milligram per litre

min – minute

mL – millilitre

mRNA – messenger ribonucleic acid

n = sample size

ng/L – nanogram per litre

ng/L – nanograms per litre

ng/mL-g – nanogram per litre per gram

nM – nanomolar

NSERC – Natural Sciences and Engineering Research Council of Canada

qPCR – quantitative polymerase chain reaction

RNA – ribonucleic acid

rpm - revolutions per minute

S.E.M – standard error of the mean

SBP – steroid binding protein

T – testosterone

US-EPA – United States Environmental Protection Agency

VTG – vitellogenin

WPS – weeks post spawn

PREFACE

Chapter 1 of this thesis is a general introduction and Chapters 2 and 3 are organized as manuscripts for publication in scientific journals. Thus, there is some repetition between the introduction and the materials and methods sections in each chapter. Chapter 2 has been published in *Aquatic Toxicology* (2014) Volume 152, Pages 273-283.

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Endocrine disruption in the aquatic environment

There is significant concern regarding chemicals in the environment that have the potential to disrupt normal endocrine functions and associated physiological processes in wildlife and humans (Tyler et al., 1996; Sumpter 1998; Hutchinson et al., 2005; Jobling et al., 2006). Numerous regulatory bodies including the United States Environmental Protection Agency (US-EPA) have recognized the importance of identifying chemicals that can affect the endocrine systems of fish and other wildlife. To address these needs, screening programs such as the US-EPA Endocrine Disruptor Screening Program (EDSP) were developed and implemented (Fenner-Crisp et al., 2000). These programs aim to identify chemicals with specific endocrine disrupting properties such as interaction with the estrogen receptor (ER), androgen receptor (AR), and steroidogenic pathways, which might ultimately cause an adverse effect on reproduction. However, there is a lack of harmonized approaches and programs to assess environmental endocrine disruption, especially in aquatic systems.

Fishes are predominantly at risk of being exposed to endocrine disrupting chemicals (EDCs) as many of these compounds are directly released into the aquatic environment through industrial and communal effluents, along with agricultural runoff, resulting in either an intermittent or continuous exposure. Exposure to EDCs has been linked to a wide variety of developmental and reproductive effects in multiple fish species throughout the world (Vos et al.,

2000; Jobling and Tyler, 2003; Palace et al., 2009; Scholz et al., 2013). Unfortunately, limited data is available regarding endocrine disruption in wild fishes, particularly species native to North America. The sensitivity to the exposure with EDCs has only been studied in a small proportion of wild freshwater fish species, with the majority of data having been derived from few selected cyprinids and salmonids (Jobling and Tyler, 2003). The majority of data used to date in support of environmental risk assessment of EDCs in aquatic systems relies on standard, small bodied, laboratory species such as the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*). Unfortunately, it is not known whether these laboratory species are actually predictive of species that are indicators for the health of ecosystems, and it is unknown if these standard laboratory fish species can be used as a model for the most sensitive fish in an ecosystem. This leaves a large gap in knowledge and a high degree of uncertainty regarding the sensitivity of fishes native to northern ecosystems such as pike (Esoxidae), perch (Percidae), suckers (Catostomidae), sturgeon (Acipenseride) and others to EDCs. Therefore, to enable more objective risk assessments, it is critical to identify the sensitivity of species native to the environments of concern.

1.2 Types of endocrine disruption

Currently, the main focus in context with EDCs has been on exogenous chemicals causing endocrine disrupting effects by agonistically or antagonistically binding to sex steroid receptors, mainly the ER and AR. However, there are a number of other equally relevant non-receptor mediated processes that can significantly disrupt endocrine function. These can include disruption of enzymes involved with synthesis, as well as transformation, transportation, metabolism, and elimination of steroid hormones (Hecker et al., 2002; Villeneuve et al., 2007, 2009; Hecker and Giesy, 2008; Yeung et al., 2011).

1.2.1 Receptor mediated effects of endocrine disruption

Steroid hormones exert their action through binding to specific receptors, and when bound by an agonist, a receptor-mediated response is initiated. Steroid hormone receptors form a large family of receptors, which contain a ligand-binding domain and a DNA-binding domain (Boelsterli, 2007). The two most well-researched receptors that have been investigated in context with receptor mediated effects of endocrine disruption are the ER and AR.

1.2.1.1 Estrogen receptor

The ER agonistically binds (xeno)estrogens and induces processes that are associated with female reproductive functions such as expression of primary female sexual characteristics and ovarian sexual maturation processes (Mills and Chichester, 2005). There are two main types of ERs, membrane bound ERs and nuclear ERs. Although much less is known regarding membrane bound ERs compared to nuclear ERs, it has been established that interaction with membrane bound ERs can result in rapid responses that can lead to cytoplasmic alterations, activation of signaling cascades and regulation of gene transcription (Marino et al., 2006). In fact, in a study by Pang and Thomas, (2010), it was suggested that activation of membrane bound ERs by estrogens were responsible for a delay in spontaneous maturation of zebrafish oocytes. Since the majority of research related to ER mediated effects of endocrine disruption in fishes focuses on the nuclear ER and associated trancriptional response, the background information presented in this section will focus on the nuclear ER.

The nuclear ER is inactive within the nucleus where it is blocked from binding to DNA by heat shock proteins until it comes in contact with a ligand (Boelsterli, 2007; Fig. 1.1). When a (xeno)estrogen binds to the nuclear ER, there is a conformational change of the receptor,

resulting in its activation and dissociation of heat shock proteins. The activation can lead to the formation of a homodimer, followed by binding of a receptor-ligand complex to its DNA binding domain, promoting synthesis of mRNA, and ultimately leading to the translation of a specific protein, enzyme or receptor (Bolsterli, 2007; Fig. 1.1). Teleost fishes express at least three distinct types of ERs, ERα, ERβ1 and ERβ2 (Lange et al, 2012; Yost et al., 2014). Any chemical that resembles the structure of 17-β estradiol (E2) can bind to each of these receptors. Along with the subtype of ERs differing in tissue distribution, ligand binding affinity and pattern of transcript regulation upon exposure to a ligand, they have been shown to differ in their role in the estrogenic response (Yost et al., 2014).

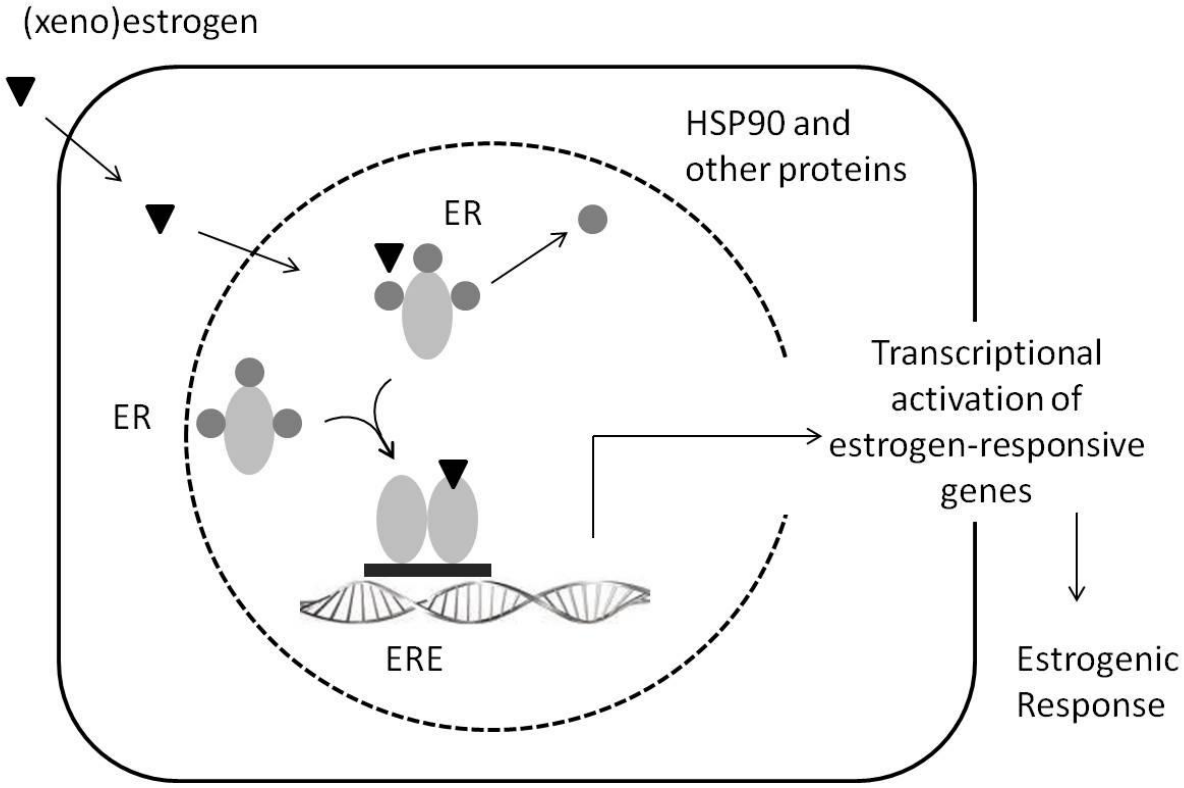

Fig. 1.1. Mechanism of (xeno)estrogen-induced activation of the estrogen receptor (ER). The compounds diffuse across biomembranes into the nucleus, where it binds to the ER. The ER forms homodimers and binds to estrogen-response elements (ERE) on DNA, leading to transcriptional activation of estrogen-responsive genes, and an estrogenic response (Boelsterli, 2007).

The role of the different ER subtypes regarding the regulation of vitellogenin (VTG) in response to estrogen exposure is not yet fully understood (Yost et al., 2014). VTG is an egg-yolk precursor protein synthesized in the liver of female fishes that is under strict control of estrogens. Originally, it was thought that ERα was the primary subtype responsible for the regulation of VTG, as numerous studies illustrated an induction of VTG along with the induction of ERα transcript abundance, and no change in ERβ (Boyce-Derricott et al., 2009; Yost et al., 2014). Recently, there has been increased research into the role of ERβ in the estrogenic response of exposed male fishes (Nelson and Habibi, 2010; Yost et al. 2014). Even with the abundance of ERβ not changing upon exposure of hepatocytes of goldfish (*Carassius auratus*) to E2, ERβ is thought to be responsible for induction of VTG and ERα, with ERβ1 having been hypothesized to be responsible for the maintenance of basal concentrations of ERα (Nelson and Habibi, 2010). In addition, a study conducted by Griffin et al. (2013) concluded ERβ2 was responsible for induction of ERα and VTG when exposed to estrogens, while the role of ERβ1 is unknown. It appears that the role of the ER subtypes in response to environmental estrogen exposure is species specific and additional research is required to identify if there is a common underlying role of the ER subtypes among teleosts.

The ER is known to bind numerous compounds that resemble E2, which can lead to unintended physiological responses. These compounds, also known as environmental estrogens, enter the aquatic environment primarily through municipal wastewater treatment plant effluents, making exposure of fish a particular concern. One environmental estrogen of great concern is 17α-Ethinylestradiol (EE2). EE2 is a synthetic estrogen and the active ingredient of most contraceptive pills. Canadian wastewater treatment plant effluents have been found to have average concentrations of EE2 in the low ng/L range, with maximum concentrations as high as

42 ng/L (0.14 nM) (Ternes et al., 1999). EE2 has a 10- to 50-fold greater potency than some natural estrogens and has the ability to bioconcentrate up to 332-fold in the body of a fish relative to concentrations in the surrounding water (Lai et al., 2002). These attributes make environmentally relevant concentrations of EE2 a potential threat to populations of fishes. Exposure to EE2 has been shown to lead to feminization of male fish including the induction of VTG, reduced male secondary characteristics, intersex, reduced fertilization success, and altered sex ratios (Lange et al., 2001; Parrott and Blunt, 2005; Kidd et al., 2007). Furthermore, a whole lake study conducted in the Experimental Lakes Area in Ontario, Canada, demonstrated that treatment with an environmentally relevant concentration of 5 ng EE2/L resulted in the collapse in populations of resident fathead minnow (Kidd et al., 2007; Palace et al., 2009).

VTG is one specific gene that is inducible upon binding of an environmental estrogen to the ER. The VTG gene resides in the male genome as well. However, very little, if any, VTG is produced in male fishes under natural conditions, as circulating estrogen levels are too low to trigger significant expression of the VTG gene (Sumpter and Jobling, 1995). It is, however, inducible upon exposure to low concentrations of estrogens, making it one of the most utilized and sensitive biomarkers of exposure to these compounds.

1.2.1.2 Androgen receptor

The AR binds androgens and compounds with structural similarities to natural androgens to promote a receptor mediated response. In fish, the endogenous ligand testosterone (T), and more importantly 11-ketotestosterone (11-KT), are responsible for testes development, and the expression of male secondary sexual characteristics. This receptor, like the ER, is a ligand-activated nuclear receptor. The AR is primarily located in the cytosol in an inactive state. Once a ligand associates with the ligand binding domain on the AR, the complex translocates into the

nucleus, is phosphorylated and dimerises, followed by binding to a specific androgen response element on the DNA (Boelsterli, 2007). The AR is regulated at the transcriptional level and protein level, mainly by androgens. However, other hormones and growth factors are involved in this process as well (Boelsterli, 2007). There are many xenobiotics that cause their endocrine disruptive effects by interacting with the AR receptor. One of the most famous examples is a metabolite of the pesticide DDT, DDE. DDE has been shown to interfere with sexual differentiation and maturation of alligators in the contaminated Lake Apopka, Florida, USA. It was demonstrated that DDE antagonistically interacts with the AR, making the complex unstable and unable to translocate into the nucleus to bind with the androgen response element. This leads to an inhibition of AR-mediated gene activation and associated downstream effects (Guillette et al., 1994). It therefore must be acknowledged that interaction of a sex steroid receptor does not just lead to an enhanced response. Interaction with the receptor of its associated ligand or response elements can also lead to a lesser or inhibited response, having alternate downstream effects.

1.2.2 Non-receptor mediated effects of endocrine disruption

There are multiple mechanisms within an organism which aid in regulating the concentrations of circulating sex steroid hormones responsible for normal growth, development and reproduction. A few of these processes involved in maintaining this homeostasis are synthesis, metabolism, transportation, and elimination. Synthesis of sex steroid hormones, also known as steroidogenesis, is required to increase the total concentration of sex steroid hormones within the body. Sex steroids are transported throughout the body by transporter proteins, namely sex steroid binding protein (SBPs), and to a lesser extent by albumin (Burton and Westphal, 1972). When bound to a binding protein, the steroid is inactive and thus cannot

interact with its target receptor. When functioning properly, SBPs protect against hormone metabolism and excretion, along with buffer against sudden changes in active concentrations of hormones (Burton and Westphal, 1972). Metabolism and excretion of steroid hormones are responsible for maintaining optimal concentrations, along with eliminating hormones when a lesser total concentration is required. It is important to note that all of these processes work together to maintain homeostasis of the concentrations of steroid hormones, and disruption of any of these processes could lead to negative effects.

There is a misconception that exogenous chemicals cause their reproductive endocrine disrupting effects by solely acting on sex steroid receptors, mainly the ER and AR. In fact, many additional non-receptor mediated processes can alter endocrine function, including disruption of processes involved in synthesis, metabolism, transportation, and elimination of steroid hormones (Baker 2001; Hecker and Giesy, 2008). Specifically related to sex steroid hormone metabolism, compounds such polychlorinated biphenyls and polyhalogenated aromatic hydrocarbons have been shown to inhibit estrogen sulfonotransferase, an enzyme responsible for metabolism of estrogens (Song 2006; Diamanti-Kandarakis et al., 2009). Disruption of these metabolizing enzymes could lead to elevated concentrations of sex steroid hormones. Xenobiotics have been shown to interact with SBPs, resulting in the release of bound steroid hormones and elevated concentrations of circulating steroid hormones (Danzo et al., 1997). Since disruption of sex steroid synthesis is a focus of this thesis, a more thorough description of this process is given below.

1.2.2.1 Disruption of steroidogenesis

Steroidogenesis is a critical process in context with sexual development, growth and reproduction. Sex steroid hormones need to be at proper concentrations at specific times during development and maturation, with disruption being detrimental for normal development and reproductive success. Sex steroid hormone homeostasis is regulated through the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1.2). Signals from the brain, resulting from external seasonal or local cues, are translated by the hypothalamic-pituitary system into changes in hormone secretion. Secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus causes release of gonadotropins from the pituitary gland into the blood stream. Specifically, luteinizing hormone (LH) and follicle stimulating hormone (FSH), two peptide hormones, are released from the pituitary in response to GnRH signalling, and act on the gonad to stimulate secretion of sex steroid hormones (Kime, 1998). These in turn initiate changes in secondary sexual characteristics (if present), behaviour and courtship patterns, and regulate development of the gametes, their maturation and eventually spawning (Kime, 1998).

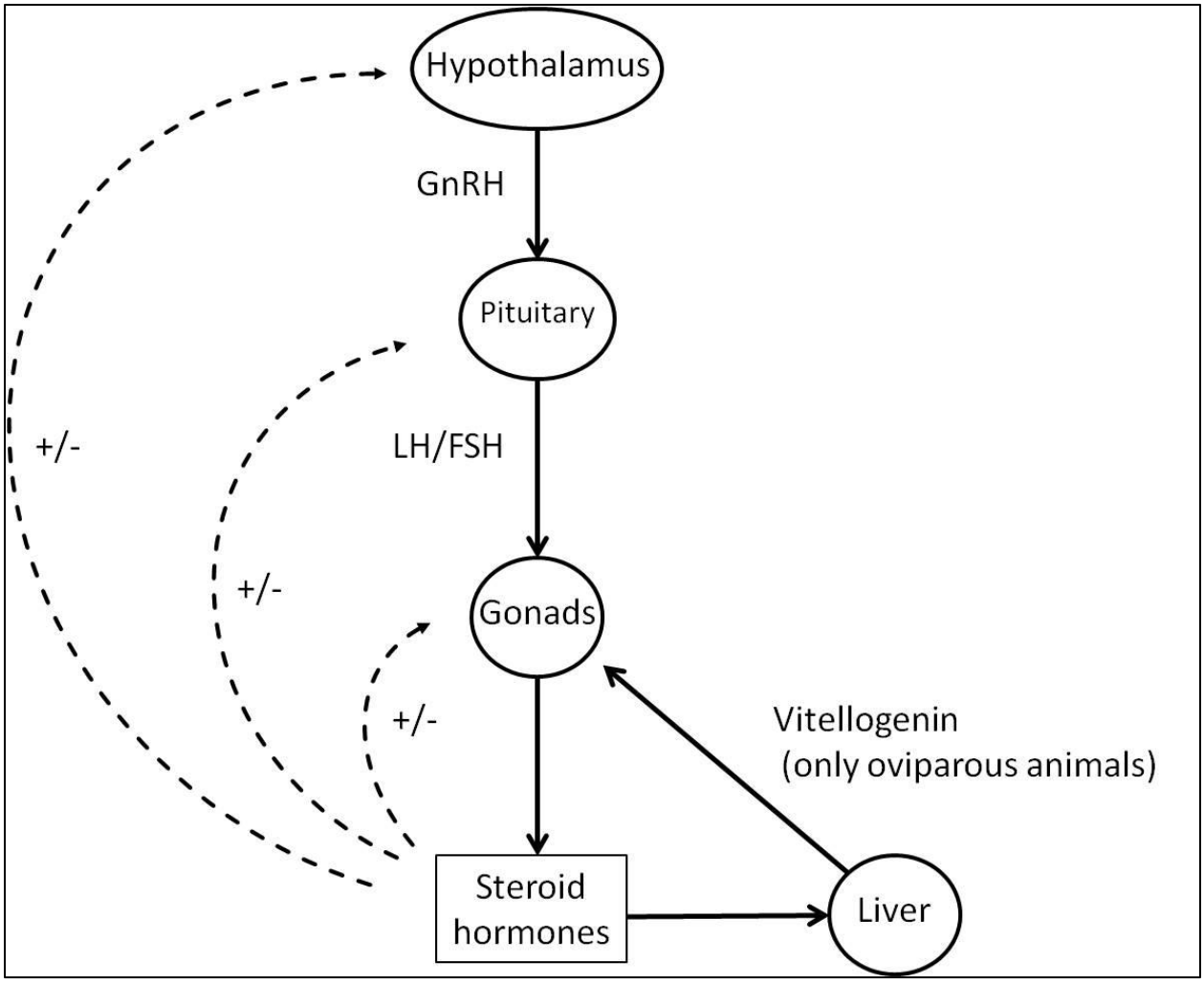

Fig. 1.2. Overview of the teleost hypothalamic pituitary gonadal axis. The linkages between the components of the axis show how homeostasis is regulated. Solid arrows indicate synthesis, dashed arrows indicate positive and negative feedback mechanisms.

Steroidogenesis is one part of the HPG axis. It regulates synthesis of circulating steroid hormones responsible for reproduction and development. These sex steroids are produced in the gonads from cholesterol in a process involving many intermediates and enzymatic reactions (Fig. 1.3) (Leusch and MacLatchy, 2003; Arukwe, 2008; Hogan et al., 2010). The steroidogenic pathway has feedback loops that tightly regulate the circulating hormone concentrations and their homeostasis. These feedback loops can act at the hypothalamus level by either inhibiting GnRH

secretion in times of increased sex steroid concentrations, or decreasing inhibition on GnRH secretion if circulating sex steroid hormone concentrations are low. Feedback loops also regulate the secretion of FSH and LH from the pituitary, which in turn regulate the synthesis of sex steroid hormones. The complexity of this pathway encompasses numerous potential targets for disruption. Disruption of certain enzymes involved in the steroidogenic pathway can result in alteration of the production of the primary sex steroid hormones E2, T, and 11-KT. Proper homeostasis of these sex steroid hormones is critical for successful growth, development, and reproduction in fishes (Noris, 1997). Therefore, alterations in hormone levels due to effects on steroidogenesis can lead to disruption of gonadal maturation, abnormal gonad morphology, impaired sexual development, and ultimately reproductive failure (Kime, 1998; Cooper and Kavlock, 2001; Hecker et al., 2002; Nadzialek et al., 2011). Numerous compounds, including fadrozole, ketoconazole, prochloraz, forskolin, and vinclozolin (Gray et al., 1997; Powlin et al., 1998; Hecker et al., 2006; Villeneuve et al., 2007), are known to alter steroid hormone synthesis through induction or inhibition of specific or multiple enzymes in the steroidogenic pathway. Forskolin and prochloraz were chosen as chemicals of interest for this study due to their previous use as model and reference substances in context with the assessment of effects of contaminants on steroidogenesis (Hecker et al. 2011), and therefore, are discussed in further detail below.

Forskolin is a compound produced from the Indian coleus plant *Coleus forskohlii.* Although this is a natural compound, it is not an environmentally relevant contaminant, as it is metabolized quickly *in vivo.* It is, however, used in numerous *in vitro* assays as it is a well known general inducer of steroidogenesis that acts via the activation of cAMP pathways (Hecker et al, 2006), and has been shown to induce the production of steroid hormones with the same processes and pattern as luteinizing hormone (LH) (Hedin and Rosberg, 1983). Considering that

forskolin induces steroidogenesis in the same manner as LH does through the HPG axis, it provides a useful tool as a positive control substance for gonad explant tests that are isolated from the HPG axis.

Prochloraz is a fungicide that is used as a model compound in studies focusing on different mechanisms of action, due to the effects it has on the vertebrate HPG axis (Gray et al., 2006; Ankley et al., 2009). Prochloraz is known to inhibit multiple enzymes in the steroidogenic pathway, including aromatase (CYP19) and cytochrome P450 c17α-hydroxylase/17,20-lyase (CYP17), resulting in decreased E2 and T production, respectively (Ankley et al., 2009; Nielsen et al., 2012). In addition to a decrease in concentrations of circulating sex steroid hormones, subsequent effects can manifest. For instance, exposure of prepubertal trout to prochloraz resulted in spermatogenesis being significantly inhibited (Skolness et al., 2011). In addition, female fathead minnows exposed to prochloraz exhibited decreased gonadal E2 production, decreased plasma concentrations of VTG, and reduced fecundity (Ankley et al., 2005, 2009).

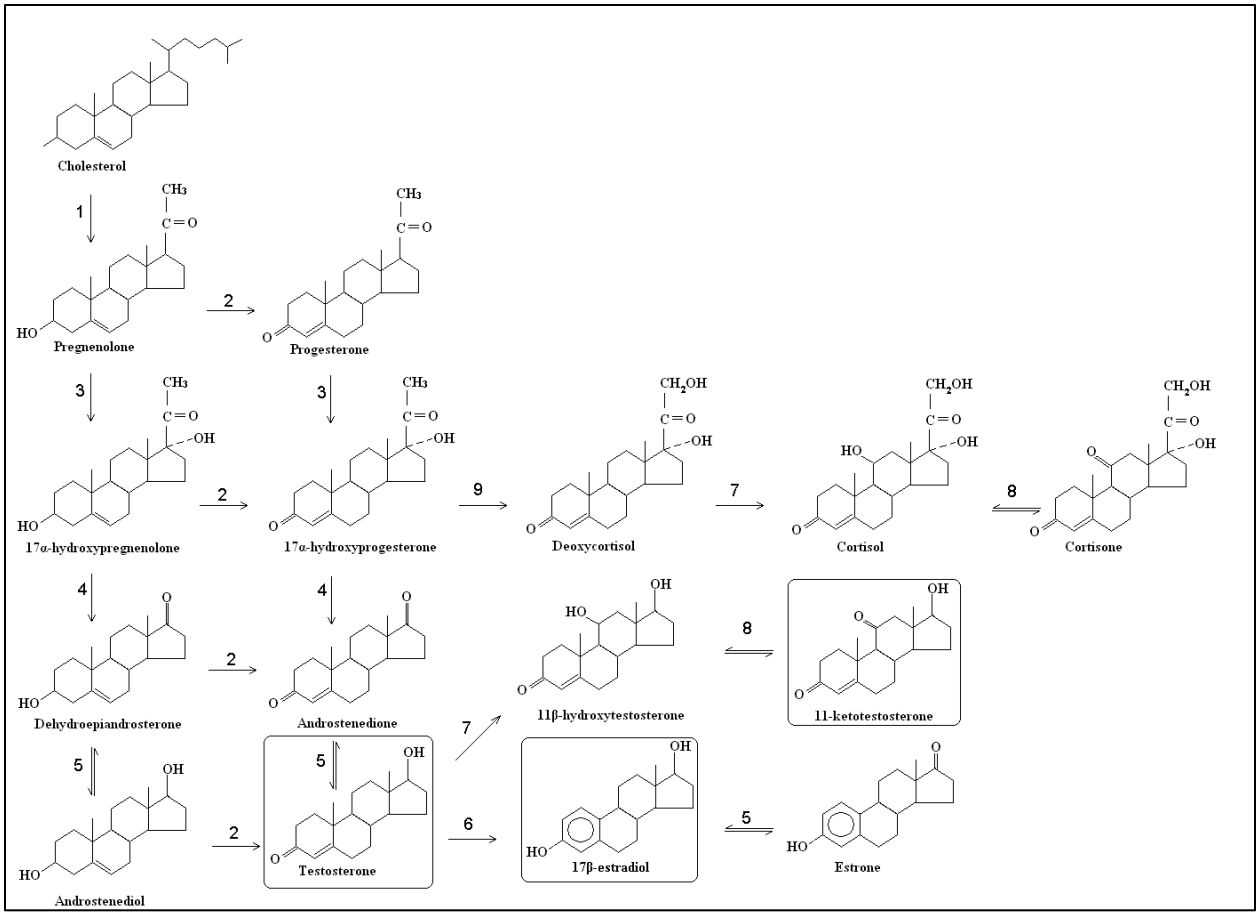

Fig. 1.3. Steroidogenic pathway in teleost fish, depicting the formation of estrogens and androgens and the enzymes involved. 1) cholesterol side chain cleavage enzyme (P450scc); 2) 3β-hydroxysteroid dehydrogenase (3β-HSD); 3) 17α-hydroxylase; 4) $C_{17,20}$-lyase; 5) 17β-hydroxysteroid dehydrogenase (17β-HSD); 6) P450 aromatase (CYP19A); 7) 11β-hydroxylase (CYP11B); 8) 11β-hydroxysteroid dehydrogenase (11β-HSD); 9) 21-hydroxlase (P450c21). Boxes indicate major circulating hormones.

1.3 Fish species of interest

Northern pike (*Esox lucius*), walleye (*Sander vitreus*), white sucker (*Catostomus commersoni*), and white sturgeon (*Acipenser transmontanus*) are ecologically, economically and culturally relevant species present in northern ecosystems, and are at risk of exposure to EDCs. These species, are found throughout North America, are from four different taxonomic orders (Esoxidae, Percidae, Catostomidae, Acipenseridae), and comprise two trophic levels (predator, bottom feeder). Northern pike and walleye are predatory fish positioned high in the food chain. While they fill an important ecological niche, they can be at a greater risk of accumulation of contaminants. Along with their ecological relevance, pike and walleye are fished for sport, adding economic value to these species. White sucker and white sturgeon are bottom feeders, which, through close contact with the sediment, can have an increased risk of exposure to contaminants that accumulate in sediments and/or sediment dwelling organisms. White sturgeon are classified as endangered within Canada, which renders them of great interest in context with ecological risk assessment. White sturgeon can live to be over 100 years old, reaching sexual maturity later in life compared to most fish species. This prolonged time prior to reaching sexual maturity allows more time for individuals to become exposed to toxicants that could impact sexual differentiation and cause other potential complications in their reproduction (LeBreton et al, 2004). Therefore, these are excellent test species for the assessment of the sensitivity of North American fish species to EDCs.

1.4 In vivo versus in vitro

Current approaches involved in environmental risk assessment primarily assess the risk of a chemical based on live animal testing. These approaches are primarily used because

exposure of live animals accounts for toxicokinetic processes such as adsorption, distribution, metabolism and excretion (ADME), and effects observed in individuals can be translated or extrapolated to populations (Gray et al., 1997). However, there are many challenges with assessing the effects of contaminants on native species of interest. These challenges include difficulties in maintaining wild fish species under laboratory conditions, ethical concerns when working with endangered species (which can be of particular interest with regard to their sensitivity to environmental contaminants), and high investments in time, labour and cost. *In vivo* studies often require large animal numbers to fulfil statistical requirements, which can be difficult to obtain for some wild species, especially if one is dealing with species that are endangered or threatened in their environment.

In vitro approaches use cells, tissues, or organ systems outside their natural environment (within an organism) as a surrogate of the targeted system within an organism. *In vitro* tests have a number of advantages over *in vivo* approaches because they often have greater specificity in their response, are amendable to higher throughput, and have a lesser cost (Gray et al., 1997). They also address current animal welfare concerns associated with toxicity testing approaches that use large numbers of live animals. In addition, multiple compounds, concentrations and species can be tested with ease using an *in vitro* approach. However, while *in vitro* approaches are promising, it should be noted that there are still a number of uncertainties regarding the use of *in vitro* tests as a replacement of *in vivo* assays. *In vitro* systems often represent simplified systems that lack the complexity of an organism with its feedback loops, cross-talk between different biological pathways, realistic representation of ADME, etc. Some of these issues can be circumvented through use of tissue cultures that typically maintain some of the organismal properties such as paracrine interactions and tissue specific metabolic process. In fact, gonad

tissue explants have been found to be reasonably predictive of *in vivo* effects in the fathead minnow (Villeneuve et al., 2007). *In vitro* and *in vivo* approaches can use similar endpoints when identifying an exposure or potential indicator of an adverse effect to estrogenic EDCs, including transcript abundances, or concentrations of proteins or hormones, among others. The ability to compare the sensitivity of some endpoints between *in vitro* and *in vivo* approaches, especially when using an indicator of an adverse effect, makes *in vitro* approaches increasingly attractive and relevant to chemical risk assessment. Therefore, the overall purpose of this study was to establish and validate tissue explant based *in vitro* assays to aid in the assessment of the sensitivity of native fishes to EDCs.

1.5 Objectives

Since little is known regarding the sensitivity of fishes native to North America to the exposure with EDCs, there is a need for efficient and objective methods to elucidate differences in sensitivity among such species and compare this information to standard laboratory model species. Therefore, the overall objective of the research in this M.Sc. thesis was to develop *in vitro* approaches that enable the assessment of species-specific sensitivity of native fish species to selected EDCs. The specific research objectives, hypotheses and the experimental approaches employed are outlined below:

1. **Assessment of the sensitivity of three North American fish species to disruptors of steroidogenesis using *in vitro* tissue explants (Chapter 2).**

   Disruption of sex steroid hormone synthesis can lead to disruption of gonadal maturation, abnormal gonad morphology, impaired sexual development, and ultimately reproductive failure (Kime, 1998; Cooper and Kavlock, 2001; Hecker et al., 2002; Nadzialek et al., 2011). It is uncertain whether small bodied laboratory fish species

currently used in risk assessments are protective of fish species native to northern ecosystems, as there is little known about the sensitivity of wild fish species to disruptors of steroidogenesis. In addition, there are ethical concerns along with great investments of time, labour and cost with *in vivo* studies. Therefore, the main objective of this portion of the study was to establish an *in vitro* gonadal explant test that enabled assessment of effects on sex-steroid production in northern pike, walleye and white sucker. The specific objectives and associated null hypotheses were:

1) To determine whether the gonad explant assay responded in a manner previously reported for various other *in vitro* steroidogenesis test systems exposed to forskolin and prochloraz by inducing and inhibiting, respectively, sex steroid hormone production (E2 and 11-KT).

   H_0: Exposure of gonad explants to forskolin or prochloraz does not change sex steroid hormone production (E2 and 11-KT) compared to controls.

2) To determine whether seasonality of reproductive function represented a critical factor in the magnitude of response and sensitivity of the *in vitro* assay.

   H_0: Seasonality of reproductive function does not change the magnitude of response or sensitivity of the *in vitro* assay.

3) To determine whether there was a species specific sensitivity of gonad explants of northern pike, walleye and white sucker to the exposure with disruptors of steroidogenesis.

H_0: There is no difference in sensitivity among northern pike, walleye and white sucker gonad explants to the exposure with disruptors of steroidogenesis.

2. **Comparison of the sensitivity of four native Canadian fish species to 17-α Ethinylestradiol, using an *in vitro* liver explant assay (Chapter 3).**

Exposure to environmental estrogens and other endocrine-active chemicals have been shown to impact reproduction of freshwater fish species. One estrogenic endocrine disrupting chemical of particular concern is the synthetic estrogen EE2. Little is known about the potential adverse effects of estrogenic EDCs to freshwater fish species native to North America or their sensitivity to these effects. Therefore, an investigation of the sensitivity of four native Canadian species, namely northern pike, walleye, white sucker, and white sturgeon to EE2 using an *in vitro* liver explant approach was conducted. Liver explants were exposed to increasing concentrations of EE2, with abundance of transcripts of VTG, ERα and ERβ being the endpoints. The objectives and associated null hypotheses of this study were:

1) To determine the transcript abundances of VTG, ERα and ERβ in liver explants of northern pike, walleye, white sucker and white sturgeon upon exposure to EE2.

H_0: There is no change in transcript abundance of VTG, ERα and ERβ of liver explants of northern pike, walleye, white sucker, or white sturgeon upon exposure to EE2.

2) To determine whether there was a difference in species specific sensitivity of liver explants to the exposure with the potent environmental estrogen EE2.

H_0: There is no difference in sensitivity among northern pike, walleye, white sucker, and white sturgeon liver explants exposed to EE2.

CHAPTER 2

2 ASSESSMENT OF THE SENSITIVITY OF THREE NORTH AMERICAN FISH SPECIES TO DISRUPTORS OF STEROIDOGENESIS USING *IN VITRO* TISSUE EXPLANTS[1]

[1]This chapter has been published in Aquatic Toxicology (2014) Volume 152, Pages 273-283, under joint authorship with Jon A. Doering (University of Saskatchewan), Sarah E. Patterson (University of Saskatchewan) and Markus Hecker (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis. A brief description of the methods development for this chapter is illustrated in the Appendix.

2.1 Abstract

There is concern regarding exposure of aquatic organisms to chemicals that interfere with the endocrine system. One critical mechanism of endocrine disruption is impairment of steroidogenesis that can lead to altered hormone levels, altered or delayed sexual development, and ultimately reproductive failure. With the current large gap in knowledge and a high degree of uncertainty regarding the sensitivity of fishes native to northern ecosystems to endocrine disrupting chemicals (EDCs), the aim of this study was to develop an *in vitro* gonadal explant assay enabling the assessment of endocrine disrupting chemicals on sex-steroid production in wild fish species native to North America. Northern pike (*Esox lucius*), walleye (*Sander vitreus*), and white sucker (*Catostomus commersoni*) were sampled from a reference location in Lake Diefenbaker, Saskatchewan, Canada, at spawn and multiple post-spawn time-points. Gonads were excised and immediately exposed for 24 h to a model inducer (forskolin) or inhibitor (prochloraz) of steroidogenesis in L-15 supplemented media. Furthermore, seasonal profiles of plasma 11-ketotestosterone (11-KT) and estradiol (E2) concentrations were characterized. Enzyme-linked immunosorbent assays were used to quantify hormone concentrations in plasma and media. The seasonal profile of plasma hormones was significantly correlated with basal *in vitro* hormone production. Gonad tissue exposed to forskolin showed a concentration-dependent increase in E2 and a general increase in 11-KT. Gonad tissue exposed to prochloraz resulted in a decrease of concentrations of 11-KT and E2. These results illustrated that gonadal tissue is undergoing steroidogenesis in an *in vitro* setting that is comparable to *in vivo* hormone profiles, and is responsive to chemical exposure in a concentration-dependent manner. The seasonal time point during which gonad explants were excised and exposed had an impact on the potency and magnitude of response, resulting in a seasonal effect on sensitivity.

Male and female white sucker showed greatest sensitivity to forskolin, while male and female walleye showed greatest sensitivity to prochloraz. Also, gonad explants from these species were found to have greater sensitivity than responses previously reported for *in vitro* explants of other fish species such as the fathead minnow (*Pimephales promelas*), and stable cell lines currently used as screening applications to detect chemicals that might disrupt the endocrine system. Therefore, current approaches that use stable cell lines or tissue explants from standardized small bodied laboratory species might not be protective of some wild fish species. Future research is required that investigates whether this *in vitro* gonadal explant assay is predictive of *in vivo* effects in wild species of fishes.

2.2 Introduction

Numerous chemicals in the aquatic environment are known to interact with the endocrine system of aquatic vertebrates (Kavlock et al., 1996; McMaster et al., 2005; Tyler et al., 2005; Jobling et al., 2006; Gerbron et al., 2010). Exposure to these endocrine disrupting chemicals (EDCs) has been linked to a wide variety of developmental and reproductive effects in multiple fish species throughout the world (Vos et al., 2000; Jobling and Tyler, 2003; Palace et al., 2009; Scholz et al., 2013). Numerous regulatory bodies including the United States Environmental Protection Agency (US-EPA) have recognized the importance of identifying chemicals that can affect the endocrine systems of fish and other wildlife. To address these needs, screening programs such as the US-EPA Endocrine Disruptor Screening Program (EDSP) were developed and implemented (Fenner-Crisp et al., 2000). These programs aim to identify chemicals with specific endocrine disrupting properties such as interaction with the estrogen receptor (ER), androgen receptor (AR), and steroidogenic pathways, which might ultimately cause an adverse effect on reproduction.

To enable more objective risk assessments of EDCs, it is critical to identify the sensitivity of species native to the environments of concern. However, the majority of data used to date in support of environmental risk assessments of EDCs in aquatic systems relies on standard, small bodied, laboratory species such as the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) that often are not representative of the environments of concern. The sensitivity to the exposure with EDCs has only been studied in a small proportion of wild freshwater fish species, with the majority of the data having been derived from few selected cyprinids and salmonids (Jobling and Tyler, 2003). This leaves a large gap in knowledge and a high degree of uncertainty regarding the sensitivity to EDCs of

fishes native to northern ecosystems such as pike (Esoxidae), perch (Percidae), suckers (Catostomidae), and others.

To date, most focus in context with EDCs has been on exogenous chemicals causing endocrine disrupting effects by agonistically or antagonistically binding to sex steroid receptors, mainly the estrogen and androgen receptor (Snyder et al., 2001; Goksoyr, 2006; Hecker and Giesy, 2008). However, there are a number of other equally relevant non-receptor mediated processes that are known to significantly disrupt endocrine functions. These can include disruption of enzymes involved in synthesis, as well as transformation, transportation, and elimination of steroid hormones (Hecker et al., 2002; Villeneuve et al., 2007, 2009; Hecker and Giesy, 2008; Yeung et al., 2011).

The steroidogenic pathway involves the production of a number of different steroid hormones, including sex steroids, glucocorticoids and mineralocorticoids. During production of sex steroid hormones, the precursor compound cholesterol is being converted into active hormones through a series of enzymatic reactions involving multiple cytochrome P450 enzymes and hydroxysteroid dehydrogenases (Parker and Schimmer, 1995; Leusch and MacLatchy, 2003; Arukwe, 2008; Hogan et al., 2010; Skolness et al., 2013). This process is tightly regulated through the hypothalamus-pituitary-gonadal (HPG) axis with positive and negative feedback loops (Ankley et al., 2009; Yeung et al., 2011). The complexity of this pathway encompasses numerous potential targets for disruption. Disruption of certain steroidogenic enzymes can result in alteration of the production of the primary sex steroid hormones 17-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT). Proper homeostasis of sex steroid hormones is critical for successful growth, development, and reproduction in fishes (Noris, 1997). Therefore, alterations in hormone levels due to disruption of steroidogenesis can lead to disruption of gonadal

maturation, abnormal gonad morphology, impaired sexual development, and ultimately reproductive failure (Kime, 1998; Cooper and Kavlock., 2001; Hecker et al., 2002; Nadzialek et al., 2011). There are numerous compounds known to alter steroid hormone synthesis through induction or inhibition of specific or multiple enzymes in the steroidogenic pathway, including fadrozole, ketoconazole, prochloraz, forskolin, and vinclozolin (Gray et al., 1997; Powlin et al., 1998; Hecker et al., 2006; Villeneuve et al., 2007).

There are multiple challenges in assessing the effects of contaminants to native species of interest. These challenges include difficulties in maintaining wild fish species under laboratory conditions, ethical concerns when working with endangered species (which can be of particular interest with regard to their sensitivity to environmental contaminants), and high investments in time, labour and cost involved with *in vivo* assays. Therefore, *in vitro* assays are increasingly used as tools to investigate the toxicity of chemicals because they often have greater sensitivity to low concentrations, specificity of response, high throughput, and have a lesser cost than *in vivo* assays (Gray et al., 1997). Additionally, *in vitro* assays require fewer numbers of animals compared to *in vivo* assays, which is of growing interest in toxicity testing. One method for *in vitro* testing involves the use of tissue explants. Testes and ovarian tissues maintain some of their natural functions, including steroidogenesis, outside their natural environment (e.g. the body of the fish), as all the necessary machinery required for the cell- or tissue-specific function is present (Gray et al., 1997; Powlin et al., 1998). It is hypothesized that species-specific tissue function is preserved within these tissues, and therefore, a test system using gonad explants could be used to identify sensitivity to EDCs which disrupt steroid synthesis. It should be acknowledged, however, that though numerous advantages exist for *in vitro* assays, there are remaining uncertainties regarding their use as surrogates for *in vivo* assays. For example,

adsorption, distribution, metabolism and excretion of the test chemical are often not, or only partially, accounted for by *in vitro* assays. This can lead to false positive or false negative results (Gray et al., 1997).

Northern pike (*Esox lucius*), walleye (*Sander vitreus*), and white sucker (*Catostomus commersoni*) are ecologically and economically relevant species in northern ecosystems that are at risk of exposure to EDCs. These three species are found throughout North America, are from three different orders (Esoxidae, Percidae, Catostomidae), and comprise two trophic levels (predator, bottom feeder). Northern pike and walleye are predatory fish positioned high in the food chain. While they fill an important ecological niche, they can be at a greater risk of accumulation of contaminants. Along with their ecological relevance, pike and walleye are fished for sport, adding economic value to these species. White sucker are bottom feeders, which through close contact with the sediment can have an increased risk of exposure to contaminants that accumulate in sediment and/or sediment dwelling organisms. Little is known about the sensitivity of northern pike, walleye or white sucker to exposure to EDCs. Therefore, the aim of this study was to develop an *in vitro* assay to enable the assessment of species-specific sensitivity of these three wild species of fish to disruptors of steroidogenesis. Specifically, gonads were excised and exposed to forskolin or prochloraz, model inducers and inhibitors of steroidogenesis (Hecker et al., 2011), respectively. Sex–steroid production (E2, 11-KT) was used as the endpoint to identify differences in species sensitivity by use of gonad explants. The ultimate goal of this research is to generate information that will allow more objective future risk assessments of EDCs to wild fish species native to northern ecosystems.

2.3 Materials and methods

2.3.1 Chemicals

Forskolin from *Coleus forskohlii*, (CAS 66575-29-9; purity: ≥ 98%), and prochloraz (CAS 67747-09-5; grade: analytical standard), were purchased from Sigma Aldrich (Oakville, ON, Canada). Serial dilutions of forskolin and prochloraz were prepared in dimethyl sulfoxide (DMSO).

2.3.2 Field sampling and tissue collection

Sexually mature northern pike (*E. lucius*), walleye (*S. vitreus*), and white sucker (*C. commersoni*) ranging from 0.5 to 6.1 kg, 0.6 to 4.7 kg, 0.6 to 1.6 kg in mass, respectively, were sampled using gill nets from a reference location in Lake Diefenbaker, Saskatchewan, Canada. The reference location had water quality indices that ranged from good to excellent as monitored by the Water Security Agency (Water Security Agency 2012). Sample collection occurred between May 2012 and September 2013 during spawn, 8-10 weeks post spawn (WPS), 16-18 WPS, and 24 WPS. During certain sampling events it was not possible to collect the desired numbers of male and female fishes (see Appendix, Fig. C2.S1). Mass (± 0.1 kg) and fork length (±0.5 cm) were measured and recorded for each individual. Immediately after catching, blood was sampled from the caudal vein of each fish using a sterile, heparinised 18-gauge needle and syringe. Blood was stored in microcentrifuge tubes on ice. Gonad tissue was excised, weighed (±1.0 g) and recorded in order to calculate gonadosomatic indices (GSI) (GSI = gonad weight/body weight x 100). Gonad tissues were sliced into smaller pieces and immediately transported to the Toxicology Centre, University of Saskatchewan in ice cold supplemented

Leibovitz L-15 media (13.8 g of L-15 powder per litre medium, 420 mg $NaHCO_3$/L, 1% antibiotic-antimicotic solution [100 units penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B per mL], pH 7.6) (Sigma Aldrich). The time between sampling of fish and initiation of the exposure did not exceed 5 h. Plasma was separated by centrifugation (6000 rpm for 5 min) and frozen at -80 °C for subsequent hormone analysis.

2.3.3 Exposure protocol

Gonad tissue was sliced into 1mm^3 pieces and rinsed several times with supplemented L-15 media. Multiple pieces of gonad, approximately 100 mg total, were placed into each well of a 24-well culture plate containing 999µL of supplemented L-15 media with 1 mg cholesterol/L. Test chemicals and the solvent control were added to the sample wells to a final concentration of 0.1% DMSO, 0.3, 1.0, 3.0, 10.0 µM forskolin, or 0.03, 0.1, 0.3, 1.0 µM prochloraz for the 2012 season, and 0.1% DMSO, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 µM forskolin, or 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 µM prochloraz for the 2013 season. Each concentration was dosed in quadruplicate (2012 season) or triplicate (2013 season) for each individual. Samples were incubated at 15 °C for 24 h on a platform rocker. Upon termination of the exposure, tissue was removed from each well and placed into pre-weighted microcentrifuge tubes, weighed and frozen at -80 °C for subsequent analysis as part of a parallel study that will be reported on elsewhere. Media was placed into microcentrifuge tubes and frozen at -80 °C for subsequent analysis of steroid hormones.

2.3.4 Hormone extraction and quantification

Steroid hormones were quantified using enzyme-linked immunosorbant assay (ELISA) purchased from Cayman Chemical (Ann Arbor, MI, USA). Steroid hormones were extracted

from the media and plasma using a liquid-liquid extraction method in accordance with Chang et al. (2009) with minor modifications. Briefly, 700 µL of sample and 700 µL nanopure water were combined and extracted twice with 2 mL of a 1:1 Hexane:Ethyl Acetate mixture by vortexing the sample mixture for 1 min, followed by centrifugation at 2000 rpm for 3 min. The supernatant was collected and evaporated to dryness under a stream of nitrogen and brought up into a buffer provided by the manufacturer (Cayman Chemical) for quantification of steroid hormones. 11-KT and E2 were quantified in accordance with protocols provided by the manufacturer (Cayman Chemical). Intra- and inter-assay variation did not exceed 20%. Hormone concentrations were expressed as ng per mL plasma (ng/mL; plasma) and ng per mL medium per g tissue (ng/mL-g; gonadal explant).

2.3.5 Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA) and data were expressed as mean $\pm$ standard error of the mean (S.E.M). Data was analyzed by one-sample Kolmogorov-Smirnov test for normality, and was analyzed by Levene's test for homogeneity of variance. Parametric data was analyzed by analysis of variance (ANOVA), followed by a 2-tailed Dunnett's test or Tukey's test. Non-parametric data was analyzed by Kruskal Wallis test followed by Mann Whitney-U test with Bonferroni adjustment to correct for ties. A probability of $p \leq 0.05$ was considered statistically significant. The concentrations of 50% maximal effect (EC_{50}) were calculated using Graph Pad Prism version 6.0 (GraphPad, La Jolla, CA, USA) by fitting data to a four-parameter logistic model.

2.4 Results

2.4.1 Seasonal profiles of GSI and plasma sex steroid hormones

GSI changed with season, generally being greatest during spawning season (Fig. 2.1). GSI decreased to its minimum after spawning (8-10 weeks post spawn [WPS]), followed by an increase at later sampling times (Fig. 2.1). No data was generated for female northern pike during the spawning season as no fish could be collected during this time point. The increase in GSI of females post spawn was slower than in males for all species, with female walleye and white sucker reaching approximately 5 % and 42 % of the spawning GSI, respectively, during the final sampling time point (Fig. 2.1A). Male northern pike, walleye and white sucker had reached a GSI similar to the GSI observed during spawning by 16-18 WPS (Fig. 2.1B).

Plasma E2 and 11-KT concentrations were detectable in all species at all sampling times, and showed distinct seasonal profiles in all fish with exception of male northern pike and male walleye (Fig. 2.2). Plasma E2 concentrations in female northern pike, walleye and white sucker were lowest at time of spawn and 8-10 WPS, (1.4, 1.1 and 0.4 ng/mL, respectively), followed by an increase reaching maximum concentrations at 16-18 WPS (2.5, 7.2 and 4.9 ng/mL, respectively) (Fig. 2.2A). Male walleye and white sucker plasma 11-KT concentrations had a similar seasonal trend, with absolute plasma 11-KT being greatest at spawn (8.5 and 56.9 ng/mL, respectively) and least during 8-10 WPS (0.1 and 0.4 ng/mL, respectively) (Fig. 2.2C).

Basal E2 production by female northern pike gonad explants showed no statistically significant difference between the 8-10 and 16-18 WPS time points with an average of 10.3 ng/mL-g (Fig. 2.2B). Female walleye and white sucker gonad explants had the least basal E2 production at spawn (0.5 and 2.3 ng/mL-g, respectively), and increased through 16-18 WPS to

maximum concentrations (8.3 and 9.7 ng/mL-g, respectively) (Fig. 2.2B). Basal 11-KT production by gonadal explants of male northern pike, walleye and white sucker showed similar trends among species, with a general decrease in production from their maximal production at spawn (65.3, 79.5 and 171 ng/mL-g, respectively) until reaching the least production at 16-18 WPS (10.6, 4.0 and 56.7 ng/mLg, respectively), followed by an increase in production after 16-18 WPS (Fig. 2.2D).

There was a statistically significant correlation between seasonal plasma E2 concentrations and basal *in vitro* E2 production in females (ρ = 0.498, n = 37, p = 0.002). A statistically significant correlation was also found between GSI and plasma E2 in females (ρ = 0.401, n = 55, p = 0.002). However, there was no correlation between GSI and basal *in vitro* E2 production in females (ρ = 0.095, n = 27, p = 0.637). In males, there was a significant correlation between seasonal plasma 11-KT concentrations and basal *in vitro* 11-KT production (ρ = 0.497, n = 35, p = 0.002). Furthermore, a statistically significant correlation occurred between GSI and basal *in vitro* 11-KT production in males (ρ = 0.466, n = 25, p = 0.019) but not plasma 11-KT production (ρ = 0.305, n = 29, p = 0.107).

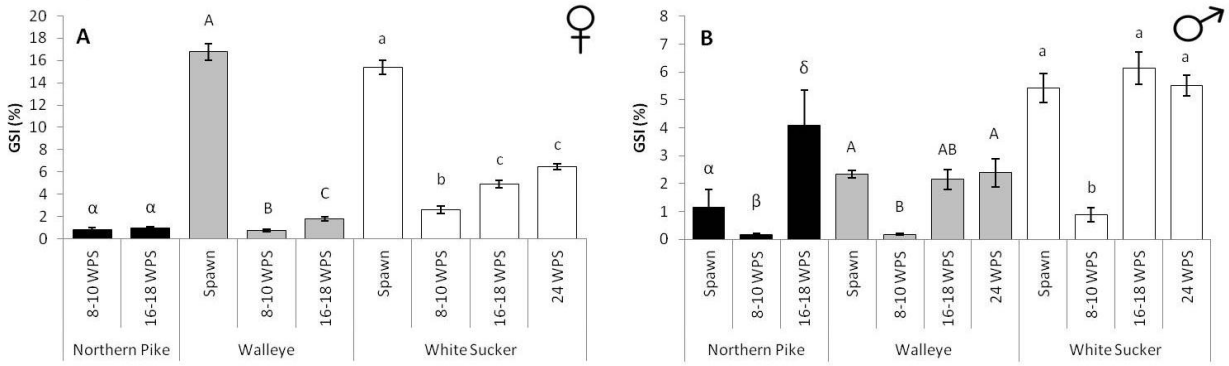

Fig. 2.1.(A) Gonadosomatic indices (GSI) of female northern pike (n = 5,3 individuals), walleye (n = 3, 21, 4 individuals) and white sucker (n = 3, 25, 8, 7 individuals) collected during spawn, 10-16, 16-18, and 24 weeks post spawn (WPS). (B) GSI of male northern pike (n = 3, 12, 3 individuals), walleye (n = 2, 3, 4, 6 individuals), and white sucker (n = 2, 5, 4, 3 individuals) collected during the spawn, 8-10, 16-18, and 24 WPS. Data represented as mean $\pm$ S.E.M. Different letters indicate significant difference (Tukey's test; $p \leq 0.05$).

Female 11-KT plasma and basal *in vitro* hormone production (Fig. 2.3) and male E2 plasma and basal *in vitro* hormone production (see Appendix, Fig. C2.S1), were also quantified. Plasma E2 was approximately 10- to 30-fold greater in females than in males, whereas plasma 11-KT was approximately 35- to 100-fold greater in males than in females. Plasma 11-KT in female walleye and white sucker had significant seasonal changes where concentrations were greatest at spawn, decreasing to their minimum at 16-18 WPS for walleye and 8-10 WPS for white sucker, followed by an increase for white sucker at later sampling times (Fig. 2.3). There were no significant seasonal changes in plasma 11-KT concentrations and basal *in vitro* 11-KT production for female northern pike (Fig. 2.3). However, a statistically significant correlation occurred between seasonal plasma 11-KT concentrations and basal *in vitro* 11-KT production in females ($\rho = 0.516$, $n = 32$, $p = 0.002$). A statistically significant correlation was found between GSI and plasma 11-KT in females ($\rho = 0.764$, $n = 54$, $p < 0.001$), and between GSI and basal *in vitro* 11-KT production in females ($\rho = 0.491$, $n = 22$, $p = 0.02$).

There were significant seasonal changes in plasma E2 concentrations in male northern pike; however, no significant seasonal changes occurred in walleye or white sucker. There was a seasonal trend for all species, where concentrations decreased from spawn to their lowest point at 8-10 WPS, increased to their maximum concentration at 16-18 WPS, followed by another decrease (see Appendix, Fig. C2.S1). There were significant seasonal changes in basal *in vitro* E2 production in male walleye, but not in northern pike or white sucker (see Appendix, Fig. S2.C1). There was no significant correlation found among seasonal plasma E2 concentrations, basal *in vitro* E2 production, and GSI in males.

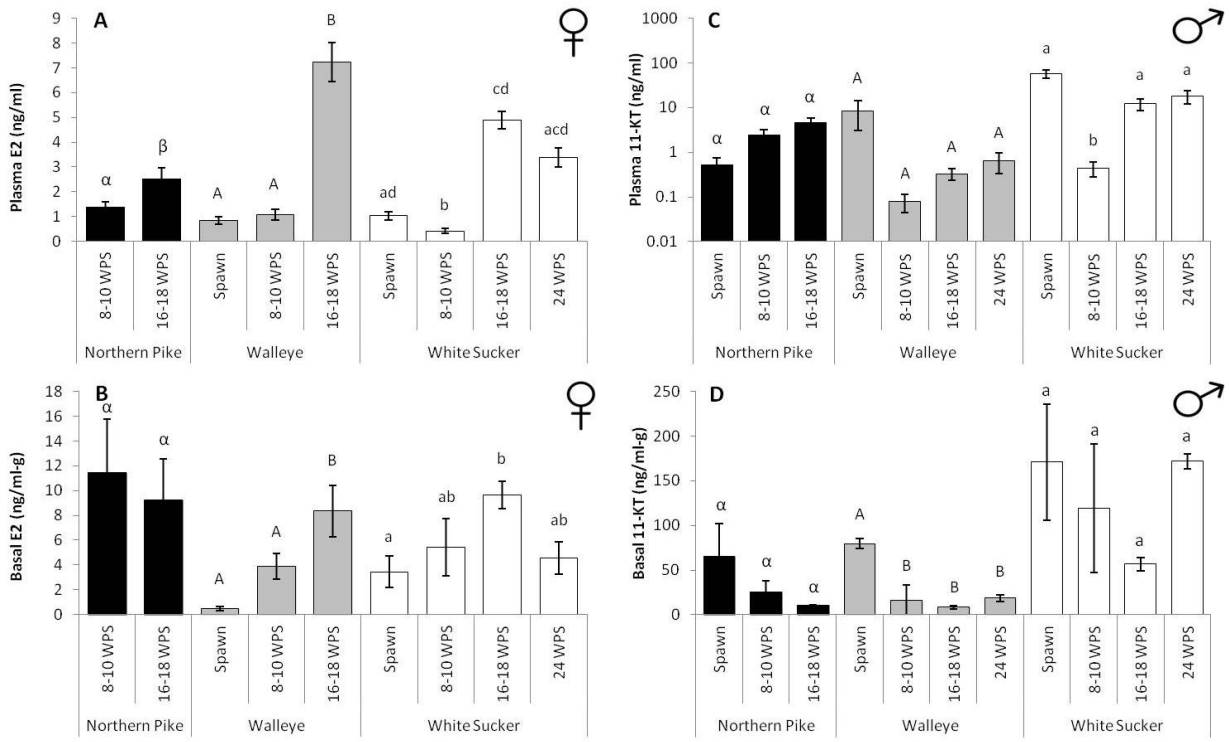

Fig. 2.2. (A) Plasma E2 concentrations of female northern pike (n = 5, 3 individuals), walleye (n = 3, 18, 4 individuals), and white sucker (n = 5, 17, 8, 7 individuals) collected at spawn, 8-10 weeks post spawn (WPS), 16-18 WPS, and 24 WPS. (B) Basal *in vitro* E2 production by gonadal explants of female northern pike (n = 3, 3 individuals), walleye (n = 3, 7, 3 individuals) and white sucker (n = 6, 2, 8, 3 individuals) at spawn, 8-10, 16-18 and 24 WPS time points. (C) Plasma 11-KT concentrations of male northern pike (n = 3, 11, 3 individuals), walleye (n = 4, 3, 4, 5 individuals), and white sucker (n =2, 5, 4, 3 individuals) collected at spawn, 8-10 WPS, 16-18 WPS, and 24 WPS. (D) Basal *in vitro* 11-KT production by gonadal explants of male northern pike (n = 3, 4, 3 individuals), walleye (n = 4, 2, 4, 5 individuals) and white sucker (n = 3, 3, 4, 2 individuals) at spawn, 8-10, 16-18 and 24 WPS time points. Plasma data represented as mean $\pm$ S.E.M in ng/ml plasma. Basal *in vitro* hormone production data represented mean $\pm$ S.E.M in ng/ml-g. Different letters indicate significant difference (Tukey's test; $p \leq 0.05$).

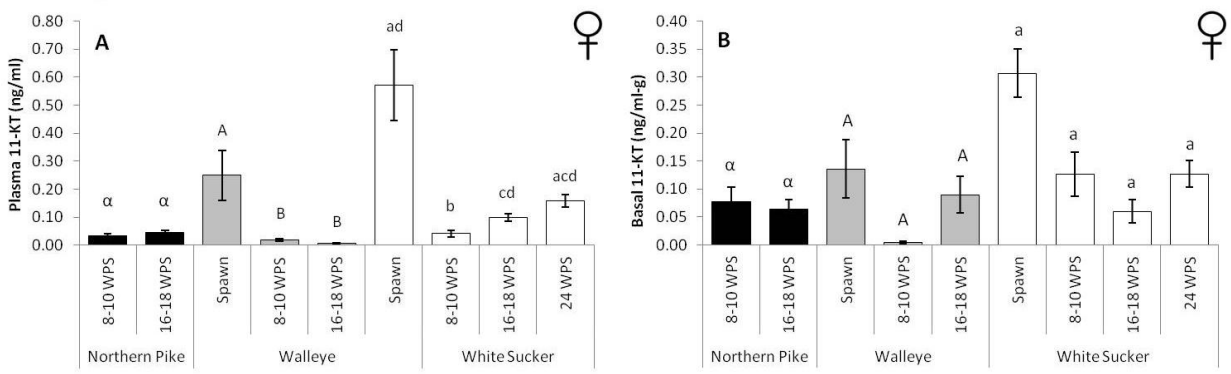

Fig. 2.3. (A) Plasma 11-KT concentrations of female northern pike (n = 5, 3 individuals), walleye (n = 3, 18, 4 individuals), and white sucker (n = 5, 16, 8, 7 individuals) collected at spawn, 8-10 weeks post spawn (WPS), 16-18 WPS, and 24 WPS. (B) Basal *in vitro* 11-KT production by gonadal explants of female northern pike (n = 3, 3 individuals), walleye (n = 4, 2, 4 individuals) and white sucker (n = 4, 2, 4, 3 individuals) at spawn, 8-10, 16-18 and 24 WPS time points. Plasma data represented as mean ± S.E.M in ng/ml plasma. Basal *in vitro* hormone production data represented mean ± S.E.M in ng/ml-g. Different letters indicate significant difference (Tukey's test; $p \leq 0.05$).

2.4.2 Effects of forskolin and prochloraz on in vitro steroid hormone production

Exposure to forskolin and prochloraz resulted in significant and chemical-specific effects on sex steroid hormone production by gonadal explants (Fig. 2.4 & 2.5). Exposure of female walleye and white sucker gonad tissues to forskolin resulted in a concentration dependent increase in E2 (Fig. 2.4A-C). Sampling and exposing female tissues to forskolin at different states of maturation had a significant effect on the capacity of gonad tissue to produce hormones in all species (Fig. 2.4A-C). The lowest observable effect concentrations (LOEC) of female gonad explants exposed to forskolin were 3.0, 0.1, and 0.03 µM forskolin for northern pike, walleye and white sucker, respectively (Fig. 2.4A-C). All LOECs occurred during the 16-18 WPS time point. The lowest EC_{50} values for induction of E2 production in female gonad explants were at 16-18 WPS for walleye (0.115 µM) and white sucker (0.136 µM) (Table 2.1). EC_{50} values for northern pike could not be calculated because of the absence of concentration dependent response.

Exposure of female gonad tissue to prochloraz resulted in a decrease in E2 during all sampling times except 24 WPS for white sucker (Fig. 2.4D-F). Maturation state had a significant effect on the capacity of gonad tissue to produce hormones for all species, except northern pike (Fig. 2.4D-F). The LOECs of female gonad explants exposed to prochloraz were 0.1, 0.03 and 0.1 µM prochloraz for northern pike, walleye and white sucker, respectively, at 16-18, 8-10 and 8-10 WPS time points. The lowest EC_{50} values for inhibition of E2 production occurred during 8-10 WPS in female northern pike (0.029 µM), walleye (0.017 µM) and white sucker (<0.1 µM) (Table 2.1).

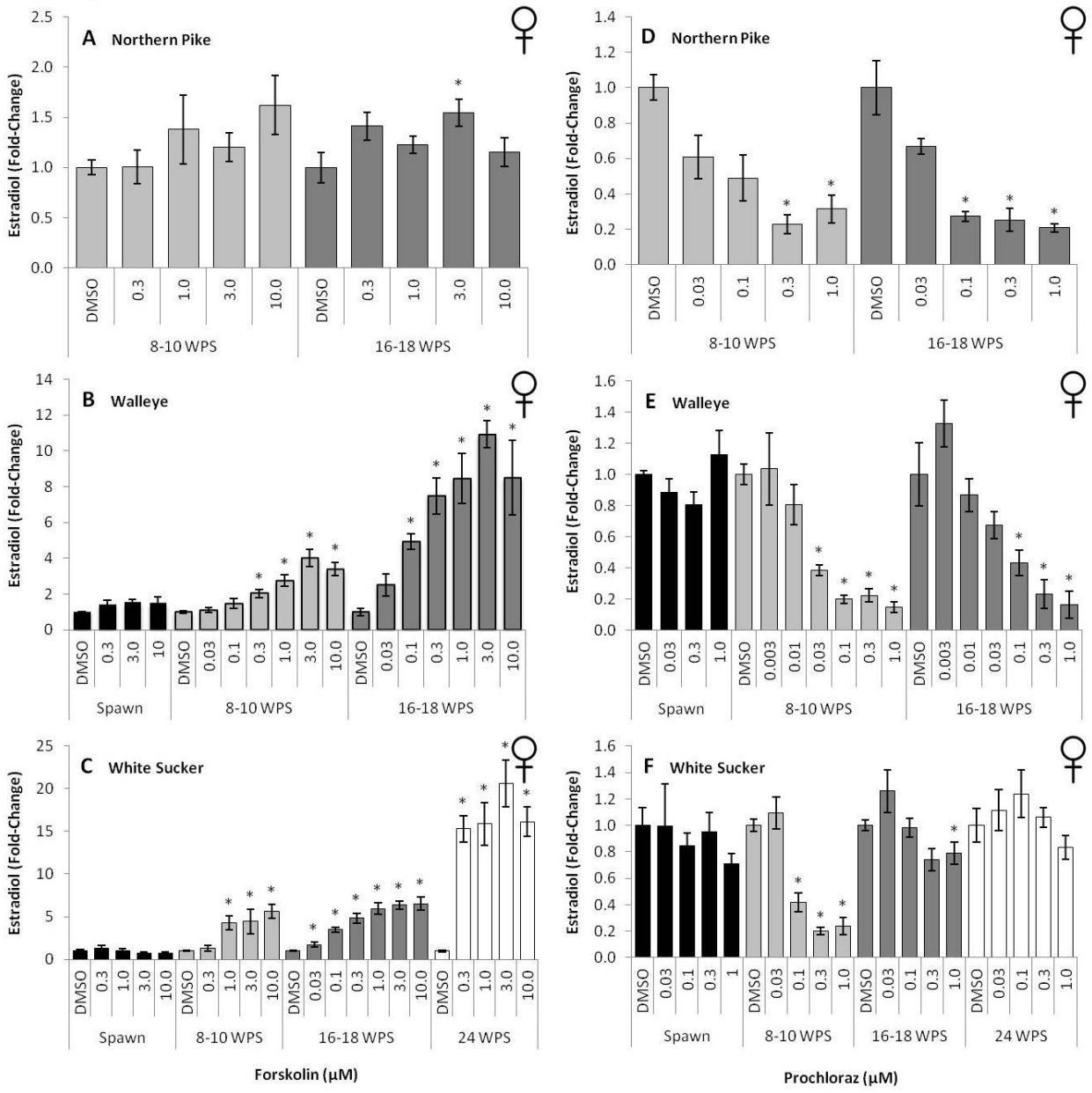

Fig. 2.4. Comparison of fold-change in hormone production of females at spawn, 8-10 weeks post spawn (WPS), 16-18 WPS, and 24 WPS compared to the average response in the solvent controls exposed to forskolin and prochloraz. (A) Northern pike (n = 8, 8 wells) (B) walleye (n = 6-8, 6-18, 6 wells) and (C) white sucker (n = 8, 8, 6-18, 8 wells) exposed to forskolin. (D) Northern pike (n = 8, 8 wells) (E) walleye (n = 6-8, 6-18, 6 wells) and (F) white sucker (n = 8, 8, 18, 8 wells) exposed to prochloraz. Data were normalized for tissue mass following exposure for 24 hours. The range of doses is a result of the year in which fish were collected and dosed. Results are reported as the mean ± S.E.M. * statistically different from solvent controls (Dunnett's test; $p \leq 0.05$).

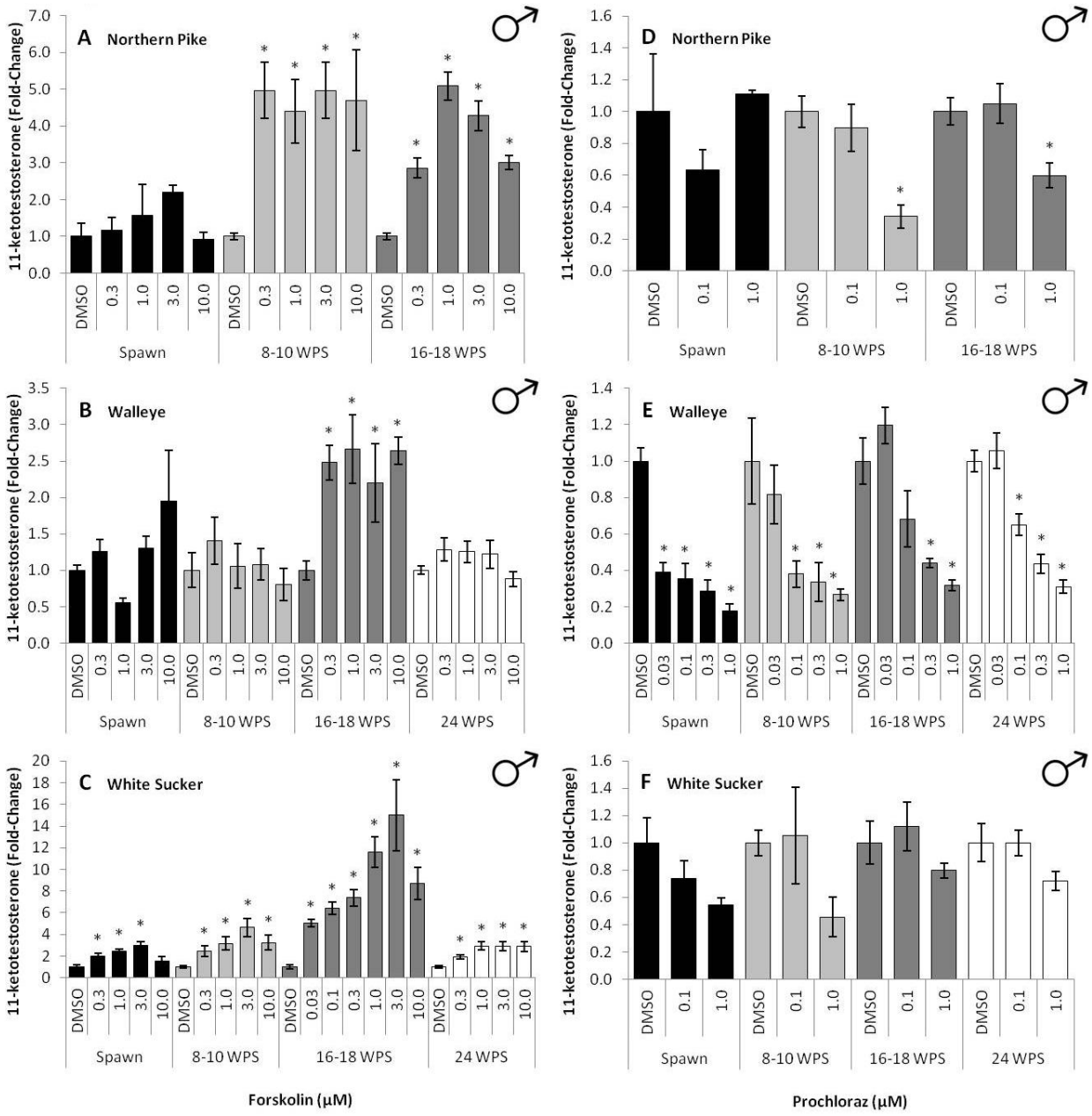

Fig. 2.5. Comparison of fold-change in hormone production of males at spawn, 8-10 weeks post spawn (WPS), 16-18 WPS, and 24 WPS compared to the average response in the solvent controls exposed to forskolin and prochloraz. (A) Northern pike (n = 4, 7, 8 wells) (B) walleye (n = 8, 6, 6, 8 wells) and (C) white sucker (n = 8, 8, 6-8, 8 wells) exposed to forskolin. (D) Northern pike (n = 4, 7, 8 wells) (E) walleye (n = 8, 6, 6, 8 wells) and (F) white sucker (n = 8, 8, 6-8, 8 wells) exposed to prochloraz. Data were normalized for tissue mass following exposure for 24 h. The range of doses is a result of the year in which fish were collected and dosed. Results are reported as the mean ± S.E.M. * statistically different from solvent controls (Dunnett's test; $p \leq 0.05$).

Table 2.1. Concentrations of 50 % maximal effect (EC_{50}) and lowest observable effect concentration (LOEC) values of hormone production from male and female gonad explants exposed to forskolin and prochloraz. All concentrations are presented in μM. LOEC or EC_{50} values which were unable to be calculated are represented with '-'. '*' indicates EC_{50} values were approximated based on three doses.

Species	Chemical		Spawn		8-10 WPS		16-18 WPS		24 WPS	
			Female	Male	Female	Male	Female	Male	Female	Male
Northern Pike	Forskolin	EC50	-	-	-	<0.3	-	0.298*	-	-
		LOEC	-	-	-	0.3	3.0	0.3	-	-
	Prochloraz	EC50	-	-	0.029	0.282*	0.034	0.745*	-	-
		LOEC	-	-	0.3	1.0	0.1	1.0	-	-
Walleye	Forskolin	EC50	-	4.518	0.486	-	0.115	<0.3	-	-
		LOEC	-	-	0.3	-	0.1	0.3	-	-
	Prochloraz	EC50	-	0.010	0.017	0.044	0.033	0.097	-	0.096
		LOEC	-	0.03	0.03	0.1	0.1	1.0	-	0.1
White Sucker	Forskolin	EC50	-	0.339	0.638	0.313	0.136	0.100	<0.3	0.320
		LOEC	-	0.3	1.0	0.3	0.03	0.03	0.3	0.3
	Prochloraz	EC50	-	0.088*	0.03<0.1	1.088*	-	-	-	0.298*
		LOEC	-	-	0.1	-	1.0	-	-	-

Exposure of male gonad tissue to forskolin resulted in a general increase in 11-KT production for all species (Fig. 2.5A-C). Maturation state had a significant effect on the capacity of the gonad tissue to produce hormones for all species (Fig. 2.5A-C). The LOECs of male gonad explants exposed to forskolin at 8-10 and 16-18 WPS, 16-18 WPS, and 16-18 WPS for northern pike, walleye and white sucker were 0.3 µM, 0.3 µM and 0.03 µM, respectively (Fig. 2.5A-C). The lowest EC_{50} values for induction of 11-KT production occurred at 16-18 WPS for northern pike (0.298 µM), walleye (<0.300 µM) and white sucker (0.100 µM) (Table 2.1).

Exposure of male gonad tissue to prochloraz resulted in a decrease of 11-KT production in all species (Fig. 2.5D-F). Maturation state had a significant effect on the capacity of the gonad tissue to produce hormones for all species except northern pike (Fig. 2.5A-C). Maturation stage did not have as great of an effect on the capacity of gonad tissue to produce hormones with prochloraz exposure compared to forskolin exposure (Fig. 2.5). The LOEC of male gonad explants exposed to prochloraz was 1.0 µM at 8-10 and 16-18 WPS for northern pike, and 0.03 µM at spawn, for walleye (Fig. 2.5D-E). No LOEC could be calculated for white sucker males. The lowest EC_{50} values for inhibition of 11-KT production in northern pike, walleye and white sucker occurred at 8-10 WPS (0.282 µM), spawn (0.010 µM) and spawn (0.088 µM), respectively (Table 2.1).

2.5 Discussion

This study illustrated that *in vitro* gonad explant assays represent a useful tool to evaluate disruption of steroidogenesis in wild fish species including northern pike, walleye and white sucker. The explant assay responded in a manner expected by inducing or inhibiting sex steroid hormone production (E2 and 11-KT) in a concentration dependent manner when exposed to

forskolin or prochloraz, respectively (Hecker et al., 2006). Furthermore, this study demonstrated that gonadal maturation stage represents an important factor with regard to the capacity of gonad tissue to produce hormones, as well as its responsiveness and sensitivity to exposure to disruptors of steroidogenesis. It should be noted that in a few cases the data are based on a limited sample size due to constraints naturally associated with the type of field work conducted here.

2.5.1 Capacity of hormone production

Fishes were sampled and exposed throughout their reproductive and gonadal maturation season to identify if reproductive cycling influenced the capacity of the gonad tissue to produce hormones *in vitro*, along with their sensitivity to exposure to a model inducer and inhibitor of steroidogenesis. GSI and plasma hormone concentrations were used to illustrate reproductive cycling throughout the season, along with characterization of *in vivo* profiles of basal hormone production at the various sampling time points. Northern pike, walleye and white sucker spawn during spring, and like most spring-spawning freshwater fish, males undergo gonadal growth throughout the summer, with females continuing through autumn and winter (Medford and Mackay, 1977; Malison et al., 1994). GSI changed with season, generally being greatest during spawning season. GSI decreased to its minimum after spawning, followed by an increase at later sampling times. Although the current study did not sample fish beyond 24 WPS, studies within the literature illustrate a gonad maturation profile of spring-spawning freshwater fish (Medford and Mackay, 1977; Malison et al., 1994) similar to what was observed in this study (Fig. 2.1).

This study revealed that maturation stage was important for the capacity of the exposed gonad tissue to produce hormones. Gonad tissue develops at different rates in different species,

and since maturation stage was found to be important with regards to capacity to produce hormones, the use of histology in identifying maturation stage would be desirable in future studies as it would give more detailed insights into the specific maturation stage at which capacity of hormone production is the greatest for a particular species. To the best of the author's knowledge, previous gonad explant assays, including common laboratory models such as the fathead minnow, have not considered reproductive seasonality as a factor in the assessment of effects of EDCs on steroidogenesis. Thus, data obtained by these assays can be variable among experiments and might not be indicative of the most sensitive maturation stage. If a specific maturation stage proves to have greatest sensitivity for multiple species, additional research would need to be done to identify whether the maturation stage currently used with fathead minnow gonad explant assays in risk assessments is representative of the most sensitive maturation stage.

Increasing sex-steroid hormone concentrations have been associated with the onset and regulation of gonad maturation, along with increase in GSI (Lee and Yang, 2002; Schulz et al., 2010). The increase in plasma E2 observed in female northern pike, walleye and white sucker within this study is similar to previously observed increases in E2 associated with the onset of vitellogenesis in most teleosts (Malison et al., 1994). The gradual increase in plasma E2 observed in northern pike is similar to that of rainbow trout (*Oncorhynchus mykiss*) and striped mullet (*Mugil cephalus*) (Whitehead et al., 1978; Tamaru et al., 1991), whereas the rapid increase of E2 in walleye and white sucker is similar to the increase observed in another study involving walleye (Malison et al., 1994). Since a full seasonal profile of plasma hormones was not attained in this study, it should be noted that plasma E2 concentrations in most spring-spawning teleost species reach a maximum around the beginning of winter, and gradually

decrease through the course of spawning as final maturation of gonadal tissue continues, mediated by progestins (Fitzpatrick et al., 1986; King et al., 1994; Malison et al., 1994). This decrease in E2 production at later stages of gonad maturation could result in a decreased capacity to produce hormones in the *in vitro* model.

The pattern of plasma 11-KT decreasing from spawn through 8-10 WPS in walleye and white sucker males (Fig. 2.2) corresponds to the decreasing pattern of 11-KT observed previously in pre-spawn, spawn and spent rainbow trout and white sucker (Scott et al., 1984; Baynes and Scott, 1985). A correlation between increasing 11-KT and maturation state has been shown in this study and in other fishes, including coho salmon (*Oncorhynchus kisutch*) (Fitzpatrick et al., 1986). However, some studies reported that T was better correlated with increasing GSI since 11-KT had a lesser increase compared to T relative to GSI, and prior to spawn, there was a major increase in 11-KT where GSI maintained stable (Baynes and Scott, 1985; Fitzpatrick et al., 1986; Malison et al., 1994). Although T has been found to increase prior to 11-KT, the concentrations of 11-KT were greater than T throughout the entire reproductive season (Baynes and Scott, 1985; Fitzpatrick et al., 1986; Malison et al., 1994). The pattern of plasma 11-KT reaching the greatest concentration during spawn in female walleye and white sucker, followed by a decrease post-spawn (Fig. 2.3), corresponds to the pattern of plasma T observed in pre-spawning, spawning and spent female white sucker and coho salmon (Scott et al., 1984; Fitzpatrick et al., 1986). It has been suggested that the peak of T prior to 11-KT might serve to stimulate secondary sexual behaviours, or serve as a precursor to other steroids including 11-KT and E2 (Malison et al., 1994).

In most cases the least responsive time point after exposure to forskolin was during the spawning season (no such characterization could be conducted for female northern pike). An

increase in hormone production capacity was identified with an increase in GSI, peaking at 16-18 WPS for males, and the final exposure time point for females. It is hypothesized that when steroid hormones and GSI have reached their maximum level (16-18 WPS for males) a negative feedback mechanism was initiated to reduce the ability of gonadotropins to further induce hormone production and subsequent gonad growth and development. Therefore, during gonad development, the ability to induce steroidogenesis to aid in development increases with gonadal maturation, resulting in the observed increase in capacity of hormone production with GSI when stimulated. At 24 WPS, male walleye and white sucker gonad explants had a decreased capacity similar to the spawning time point (Fig. 2.3), aiding in the hypothesis that a negative feedback mechanism was initiated to pause gonad development. Unfortunately, exposure time points beyond 16-18 WPS for northern pike and walleye, and 24 WPS for white sucker were not tested as no fish could be collected during these time points. Therefore, for females, it is uncertain if capacity of gonad tissues to produce hormones in the presence of forskolin would have continued to increase or decrease in a similar manner as in males, once maximum E2 concentrations were attained. *In vitro* gonad explant studies conducted with female spotted seatrout (*Cynoscion nebulosus*) and rainbow trout during mid-vitellogenesis had approximately 7- and 10-fold increases in E2 production, respectively, when exposed to 10 µM forskolin (Singh and Thomas, 1993; Leatherland et al., 2005). Post-vitellogenic common carp (*Cyprinus carpio*) gonad explants were exposed to 10 µM forskolin, resulting in approximately 6.5-fold increase in E2 production (Paul et al., 2010). Another gonad explant study involving female white sucker during spawn resulted in a maximum induction of T production of approximately 2-fold when exposed to 1.0 µM forskolin (Van der Kraak et al., 1992). This low capacity to induce hormone production is in accordance with the conclusions that during spawning the capacity for sex

steroid production is the least compared to other maturation stages in fishes (Fig. 2.4). An *in vitro* gonad explant study conducted with male coho salmon resulted in a maximum induction of 11-KT of 8-fold (Planas et al., 1993). The coho salmon in the study by Planas et al., (1993) were sampled when testes were at the same maturation stage as male coho salmon exhibiting maximum 11-KT levels (Fitzpatrick et al., 1986). This is in accordance with the hypothesis that during times of gonad maturation there is the potential for greatest induction of hormone production in explant assays. It is uncertain if maturation stage is of similar importance in other species of fishes with regard to capacity of hormone production, as previously published *in vitro* gonad explant studies were only conducted at a single maturation time point.

Effects of the exposure of gonad explants to prochloraz were in accordance with results obtained by previous *in vitro* studies (Hecker et al., 2006), with a concentration dependent decrease in E2 and 11-KT production by gonadal explants of northern pike, walleye and white sucker (Fig. 2.4 & 2.5). Maturation stage was found to significantly impact the responsiveness of gonads to the exposure with prochloraz, as was also seen with forskolin. Unlike forskolin, the maximum response resulting from exposure to prochloraz was observed during multiple sample time points with most species, and a similar maximum response of approximately a 4-fold inhibition compared to control was found with most species. This maximum response is similar to that previously seen in fathead minnow and brown trout (*Salmo trutta*) gonad explants (Villeneuve et al., 2007; Marca Pereira et al., 2011).

2.5.2 Species sensitivity

Assessment of sensitivity of fishes for the purpose of this study was based on LOEC and EC_{50} values (Table 2.1). The use of these two measurements for comparing sensitivity among

species and time points of exposure is somewhat limited, as the range of exposure concentrations differed in some cases. This is especially true for scenarios where the LOEC was the lowest dose tested. EC_{50} values could only be calculated when a concentration dependent response was attained. Unfortunately, there were cases where the maximum response was observed at the lowest exposure concentration leading to the inability to calculate an EC_{50} value. In these cases, a range was stated. Since the time point of exposure was found to have an effect on sensitivity, the most sensitive time point for each species was used to compare among species.

This study found male and female white sucker gonad explants to be the most sensitive to the exposure with forskolin, with LOECs of 0.03 µM and EC_{50} values of 0.102 and 0.136 µM, respectively. Species of greatest to least sensitivity for female gonad explants exposed to forskolin were white sucker (this study), common carp (Paul et al., 2010), walleye (this study), northern pike (this study), and rainbow trout (Leatherland et al., 2005) with LOECs of 0.03, 0.1, 0.1, 1.0, 3.0, and 10.0 µM, respectively. Species of greatest to least sensitivity for male gonad explants exposed to forskolin were white sucker (this study), coho salmon (Planas et al., 1993), northern pike and walleye (both this study) with LOECs of 0.03, 0.1, 0.3 and 0.3 µM, respectively. It should be noted the lowest dose studied for carp and rainbow trout showed significant results, and therefore, they could have greater sensitivity to forskolin than what was illustrated within the study. An alternative *in vitro* steroidogenesis assay using an immortal human adenocarcinoma cell line (H295R) was previously conducted with forskolin (Hecker et al., 2007). Within that study, mean EC_{50} values for T and E2 production were 0.71 and 0.56 µM forskolin, respectively (Hecker et al., 2007). These results suggest white sucker and walleye male and female, along with northern pike male gonad explants have a greater sensitivity to forskolin than H295R cells, which are currently used as a screening application for identifying

endocrine active compounds. Therefore, the H295R test would underestimate *in vitro* toxicity of forskolin to these species.

Walleye gonad explants were found to be the most sensitive when exposed to prochloraz, resulting in LOEC values of 0.03 and 0.03 µM, and EC_{50} values of 0.017 and 0.010 µM for females and males, respectively. Other studies involving fish gonadal explants exposed to prochloraz reported lesser sensitivities for gonad explants of fathead minnows and brown trout. Female fathead minnow gonad explants exposed to prochloraz had an LOEC of 3.0 µM (Villeneuve et al., 2007), and female and male brown trout gonad explants exposed to prochloraz had an LOEC of 0.66 µM (250 ng/L) (Marca Pereira et al., 2011). Of the species that have been tested, walleye was found to have greatest sensitivity to prochloraz, followed by northern pike, white sucker, brown trout, and fathead minnow, having approximately 3-, 3-, 20- and 100-fold greater LOEC values, respectively. These data clearly indicate that current fish models such as the fathead minnow that is commonly used in risk assessments of endocrine active chemicals might not be protective of wild fish species of concern such as northern pike, walleye and white sucker. However, it should be noted that there was only one time point used for the studies involving fathead minnows and brown trout, and juvenile brown trout gonad tissue was used. Since it was found that maturation stage plays an important role in the sensitivity of the gonad explants, the time point tested might not have represented the greatest sensitivity. In addition, the brown trout study consisted of a limited range of concentrations, of which the lowest concentration showed significant effects. Therefore, brown trout might have greater sensitivity to prochloraz than what was illustrated within the study, making sensitivity less comparable to the fathead minnow.

When compared to the H295R steroidogenesis assay (Hecker et al., 2007), it could be shown that female walleye gonad explants had greater sensitivity in detecting steroidogenesis inhibition after exposure to prochloraz, whereas male walleye had equal sensitivity based on the EC_{50s}, but were less sensitive based on the LOEC. The H295R test would underestimate *in vitro* toxicity of prochloraz to some of these species.

2.5.3 In vitro versus *in vivo*

It is often difficult to compare *in vitro* and *in vivo* studies because the organ system being tested is isolated and tested independent of other tissues that can interact under *in vivo* conditions. However, unlike some immortal cell-based *in vitro* assays, which deal with only one cell type, gonad explants include multiple cell types that comprise all the enzymes required to produce sex steroid hormones. Furthermore, gonad explants comprise paracrine properties and a greater level of organization than some other *in vitro* assays, and have been found to be reasonably predictive of *in vivo* effects in fathead minnows (Villeneuve et al., 2007). Although no *in vivo* testing was conducted as it was beyond the scope of the presented study, the correlation between basal hormone production and plasma hormone concentrations provides evidence that the gonad tissue was undergoing steroidogenesis in an *in vitro* setting that is reflective of reproductive seasonality, indicating a connection between responses *in vitro* and *in vivo*. *In vivo* endpoints of fathead minnows exposed to prochloraz, including alterations in hormone production and decreased fecundity, had LOECs of approximately10-fold less than alterations in hormone production by *in vitro* gonad explants (Ankley et al., 2005). The fish sexual development test (FSDT) has also been conducted with fathead minnow and zebrafish exposed to prochloraz. Effects of prochloraz included alterations in sexual differentiation, plasma vitellogenin concentrations and delayed maturation (Kinnberg et al., 2007; Thorpe et al.,

2011; Baumann et al., 2013). The FSDT resulted in LOECs approximately 10-fold less than alterations in hormone production by *in vitro* gonad explants for fathead minnow, similar to other observed *in vivo* endpoints. Zebrafish were found to have greater sensitivity to prochloraz in the FSDT than fathead minnow, with a LOEC of 0.042 μM (Kinnberg et al., 2007), which is of similar sensitivity as alterations in hormone production by walleye *in vitro* gonad explants as determined in this study. Since fathead minnows were found to be the least sensitive species with regard to endocrine disrupting effects following *in vitro* gonad explants exposed to prochloraz, *in vivo* testing could reveal even greater sensitivity of wild fish species including northern pike, walleye and white sucker, assuming that the *in vitro* responses in these species would also be less sensitive than *in vivo* effects. Although *in vitro* gonad explants were shown to be reasonably predictive of *in vivo* effects, some *in vivo* endpoints have been seen to be of greater sensitivity, and thus, more research is needed to identify how predictive this *in vitro* gonadal explant assay is for *in vivo* effects in wild fish species.

2.6 Conclusion

This study successfully established an *in vitro* gonadal explant assay for wild fish species found in northern ecosystems to assess the effects of EDCs on sex steroid production. The gonad explant assays responded in a manner previously reported for various other *in vitro* systems exposed to forskolin and prochloraz by inducing and inhibiting, respectively, sex steroid hormone production (E2 and 11-KT). Seasonality of reproductive function represented a critical factor that needs to be considered when using an *in vitro* gonad explant assay to enable objective assessment of responses of wild fish species to disruptors of steroidogenesis. Gonad explants of male and female white sucker, and male and female walleye were found to have the greatest

sensitivity to forskolin and prochloraz, respectively. Gonad explants from these species were found to have greater sensitivity than the H295R assay which is currently used as a screening application to detect potential EDCs. It is likely the H295R assay is not protective of fish of all species or of both sexes, and thus, the H295R test could underestimate *in vitro* toxicity for some species. It is recommended to increase the sample size in future studies to have greater statistical power to aid in the statistical confirmation of observed sensitivities and correlations. Finally, additional research into identifying how predictive this *in vitro* gonadal explant assay is for *in vivo* effects in wild fish species should be the focus of future research.

Acknowledgements

The study reported was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. The authors would like to thank S. Pryce, E. Higley, H. Prodahl and all other staff and students who provided laboratory and/or field assistance throughout this study. Research was supported through the Canada Research Chair program and an NSERC Discovery Grant to M. Hecker.

CHAPTER 3

3 COMPARISON OF THE SENSITIVITY OF FOUR NATIVE FISH SPECIES TO 17-α ETHINYLESTRADIOL, USING AN *IN VITRO* LIVER EXPLANT ASSAY[2]

[2] This chapter will be submitted to Environmental Science and Pollution Research under joint authorship with Jon A. Doering (University of Saskatchewan), Bryanna K. Eisner (University of Saskatchewan) and Markus Hecker (University of Saskatchewan). The tables, figures and references cited in this article have been re-formatted here to the thesis style. References cited in this chapter are listed in the reference section of this thesis. A brief description of the methods development for this chapter is illustrated in the Appendix.

3.1 Abstract

Exposure to environmental estrogens and other endocrine disrupting chemicals (EDCs) has been shown to impact reproduction of freshwater fish species. One compound of particular concern in this context is the synthetic estrogen 17α-Ethinylestradiol (EE2). While estrogenic EDCs have been extensively researched in standard laboratory fish models, little is known about the sensitivity of freshwater fish species native to North America to these compounds. With numerous economical and ethical challenges in assessing the effects of contaminants on native species *in vivo, in vitro* assays are increasingly being used for their high throughput, specificity, and lesser cost compared to *in vivo* assays. The aim of this study was to investigate the sensitivity of four native Canadian species, namely northern pike (*Esox lucius*), walleye (*Sander vitreus*), white sucker (*Catostomus commersoni*), and white sturgeon (*Acipenser transmontanus)* to EE2 using an *in vitro* liver explant approach. Transcript abundances of vitellogenin (VTG) as well as the estrogen receptors (ER) α and β were used as the measuring endpoints as they are known biomarkers previously used to assess exposure to environmental estrogens. The assay responded in a manner previously reported in other *in vitro* liver explant and hepatocyte assays, as well as *in vivo* assays. Specifically, transcript abundance of VTG was up-regulated in a concentration dependent manner in each species. Liver explants of male walleye were found to have the greatest sensitivity to EE2, with a LOEC of 300 ng/L (1.0 nM) for VTG transcript abundance, with juvenile white sturgeon having the greatest magnitude of VTG transcript induction in exposed tissue (15-fold relative to control). Exposure of liver explants to EE2 resulted in no alteration in transcript abundance of ERβ, whereas induction of ERα was observed in northern pike only. Based on *in vitro* expression of VTG, northern pike, walleye, white sucker, and white sturgeon were among the species with greatest sensitivity to estrogenic EDCs

of the species studied to date with sensitivities similar to those previously reported for rainbow trout and roach *in vitro* and *in vivo.* Although tissue slices have been shown to be a relatively realistic *in vitro* model with comparable response profiles to those previously observed in *in vivo* studies, more research is needed to identify how predictive this *in vitro* liver explant assay is to *in vivo* effects in wild fish species.

3.2 Introduction

Numerous natural and synthetic chemicals present in the aquatic environment have been shown to interact with the endocrine system of humans and wildlife (Tyler et al., 1996; Sumpter 1998; Hutchinson et al., 2005; Jobling et al., 2006). Among these endocrine disrupting chemicals (EDCs), environmental estrogens have received particular attention over the past decades (Scholz et al., 2004; Flick et al., 2013). Environmental estrogens are chemicals that agonistically bind to the estrogen receptor (ER), and can disrupt male sexual development and reproductive functions by inducing processes associated with female reproductive functions. The primary sources for estrogenic EDCs to the environment are municipal wastewater treatment plant effluents, rendering exposure of aquatic organisms including fish a particular concern. In fact, it has been shown by numerous authors that exposure of fish to estrogenic EDCs can result in disruption of normal gonad development, feminization of males, and a decrease in reproductive success (Sumpter 1998; Jobling and Tyler., 2003; Tyler et al., 2005).

One estrogenic EDC of particular environmental concern is 17α-Ethinylestradiol (EE2). EE2 is a synthetic estrogen and the active ingredient of most contraceptive pills. Canadian waste water treatment plant effluents have been found to have average concentrations of EE2 in the low ng/L range, with maximum concentrations as high as 42 ng/L (0.14 nM) (Ternes et al., 1999). EE2 has a 10- to 50-fold greater potency than some natural estrogens and has the ability to bioconcentrate up to 332-fold in the body of a fish relative to concentrations in the surrounding water (Lai et al., 2002). These attributes make environmentally relevant concentrations of EE2 a potential threat to populations of fishes. Exposure to EE2 has been shown to lead to feminization of male fish including the induction of vitellogenin (VTG), reduced male secondary characteristics, intersex, reduced fertilization success, and altered sex

ratios (Lange et al., 2001; Parrott and Blunt, 2005; Kidd et al., 2007). Furthermore, a whole lake study conducted in the Experimental Lakes Area in Ontario, Canada, demonstrated that treatment with an environmentally relevant concentration of 5 ng EE2/L resulted in the collapse in populations of resident fathead minnow (*Pimephales promelas*) (Kidd et al., 2007; Palace et al., 2009).

There is a host of information on the effects of EE2 and other estrogenic EDCs on standard laboratory fish species such as the fathead minnow, Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*). However, little is known regarding the sensitivity of the large number of freshwater fish species native to water bodies in North America, Europe, or other parts of the world that could be at risk to exposure with estrogenic EDCs. With risk assessments for fresh water fish species currently being based on extrapolation from responses to EDCs of standard laboratory fish species, it is uncertain whether the majority of wild fish species are adequately protected (Jobling and Tyler, 2003). Therefore, there is a need to assess the sensitivities of species native to the environments in question to enable more objective risk assessments of estrogenic EDCs.

There are several challenges in assessing the effects of contaminants to native species of concern. These challenges include difficulties in maintaining wild fish species under laboratory conditions, ethical concerns when working for example with endangered species, and the high investments in time, labour and cost associated with *in vivo* testing. In an effort to address some of these concerns, *in vitro* approaches are increasingly used as tools to assess toxicity of chemicals. *In vitro* tests have a number of advantages over *in vivo* approaches because they often have greater specificity in their response, have higher throughput, and have a lesser cost (Gray et al., 1997). They also address current animal welfare concerns associated with toxicity

testing approaches that use large numbers of live animals. *In vitro* tests can be based on the use of stable cell lines or primary cell cultures and tissue explants. While primary tissue explants can show some greater variability in their responses relative to immortalized and/or stably transfected cell lines, they have the advantage that they can maintain some of their natural functions outside their natural environment (e.g. the body of the fish) because all necessary machinery required for cell- or tissue-specific functioning is present (Gray et al., 1997; Powlin et al., 1998). An earlier study by our group demonstrated that in a gonadal explant assay, species-specific tissue functions were well preserved, which enabled distinguishing sensitivities to disruptors of steroidogenesis among three native fish species (Beitel et al., 2014). It is hypothesized that similar species-specific properties occur for other tissues, suggesting that a test system using liver explants could be used to identify differences in sensitivity to estrogenic EDCs among different native fish species.

One of the most utilized and sensitive biomarker of exposure to environmental estrogens is the induction of VTG in male fish. VTG is an egg-yolk precursor protein synthesized in the liver of female fishes that is under strict control of estrogens. The VTG gene resides in the male genome as well. However, very little, if any, VTG is produced in male fishes under natural conditions, as circulating estrogen levels are too low to trigger significant expression of the VTG gene (Sumpter and Jobling, 1995). It is, however, inducible upon exposure to low concentrations of estrogens, making it a powerful biomarker of exposure to these chemicals. Studies have revealed correlations between increased VTG and decreased egg production, effects on ovarian structure, and inhibition and disruption of testicular growth and development, respectively (Panter et al., 1998; Van den Belt et al., 2004). However, uncertainty remains regarding the capacity of up-regulation of VTG *in vitro* to predict endocrine-mediated responses *in vivo*

(Segner et al., 2002). Induction of transcript and protein abundance of VTG have been endpoints of interest for exposure studies with both *in vivo* and *in vitro* systems (Van den Belt et al., 2004; Gerbron et al., 2010; Nagler et al., 2010; Woods and Kumar., 2011). In fact, transcript abundance of VTG has been shown to be as effective in detecting exposure to estrogens as the abundance of VTG protein (Thomas-Jones et al., 2003; Hutchinson et al., 2006; Flick et al., 2014), making transcript abundance an ideal endpoint for short term *in vitro* assays, as up-regulation can be observed within a few hours of exposure compared to a few days that are required when quantifying protein abundance (Flouriot et al., 1996; Hutchinson et al., 2006). In addition to VTG being the most commonly used biomarker of exposure to estrogenic EDCs, it has also been used to quantify the potency of various environmental estrogens (Thomas-Jones et al., 2003; Woods and Kumar, 2011). Investigation into the expression of the ER has been of interest regarding exposure to estrogenic EDCs (Skillman et al., 2006; Humble et al., 2014). Along with using the ER as a biomarker of estrogen exposure, there has been recent interest in the role of the ER subtypes regulating VTG and other estrogenic responses (Humble et al., 2014; Yost et al., 2014).

The main objective of this study was to develop an *in vitro* approach to enable the assessment of species-specific sensitivity of four native species of fish to estrogenic EDCs using the model compound EE2. The species used in this study were northern pike (*Esox lucius*), walleye (*Sander vitreus*), white sucker (*Catostomus commersoni*), and white sturgeon (*Acipenser transmontanus*). These species were selected based on their presence in northern ecosystems, risk of exposure to estrogenic EDCs, economic, cultural and ecological relevance, along with the limited knowledge about the sensitivity of these fishes to EDCs. Specifically, liver explants from northern pike, walleye, white sucker, and white sturgeon were excised and exposed to the

synthetic estrogen, EE2, with transcript abundance of VTG, ER alpha (ERα) and ER beta (ERβ) used as endpoints to identify potential differences in sensitivity among liver explants of these species.

3.3 Materials and methods

3.3.1 Chemicals

Leibovitz L-15media, and antibiotic antimicotic solution were purchased from Sigma Aldrich (Oakville, ON, Canada). Fetal Bovine Serum (FBS) was purchased from Life Technologies (Burlington, ON, Canada). 17-α Ethinylestradiol was purchased from Sigma Aldrich and serial dilutions were prepared in dimethyl sulfoxide (DMSO) from a stock concentration.

3.3.2 Field sampling and tissue collection

Sexually mature male northern pike (*E. lucius*), walleye (*S. vitreus*), and white sucker (*C. commersoni*) ranging from 0.8-1.2 kg, 0.8-2.4 kg, and 0.8-1.2 kg, respectively, were sampled with gill nets (Lakefish Net and Twine Ltd., Edmonton, AB, Canada) from a reference location in Lake Diefenbaker, SK, Canada. Sample collection occurred between July 2013 and September 2013. Juvenile, non-sexually differentiated white sturgeon (*A. transmontanus*) ranging from 2.9-4.7 kg were randomly selected from an in-house stock reared from eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Mass (± 0.1 kg) and fork length (±0.5 cm) were measured and recorded for each individual. Livers were excised and weighed (±1.0 g) to quantify hepatosomatic indices (HSIs). Liver tissue was sliced into pieces and placed in ice cold supplemented Leibovitz L-15 media (13.8 g of L-15 powder per litre

medium, 420 mg $NaHCO_3$/L, 10% FBS, 1% antibiotic-antimicotic solution [100 units penicillin, 0.1 mg streptomycin and 0.25 ug amphotericin B per mL], pH 7.6). With exception of white sturgeon that were raised in house, tissues from wild-caught species were extracted and transferred to medium in the field and then immediately transported on ice to the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada). The time between sampling of fish and initiation of the experiment did not exceed 5 h.

3.3.3 Exposure protocol

Liver tissue was sliced into $1mm^3$ sections and rinsed several times with supplemented L-15 media. Two to three pieces of liver were added to each well of a 24-well plate containing L-15 medium. EE2 was added to the sample wells to a final concentration of 0, 3, 10, 30, 100, 300, 1000, 3000 ng/L in 0.1% DMSO. Each concentration was dosed for each of northern pike (n = 4), walleye (n = 5), white sucker (n = 4) and white sturgeon (n = 7), respectively. Samples were incubated at 15 °C for 24 h on a platform rocker. Upon termination of the exposure, tissue was removed from each well and placed into microcentrifuge tubes and stored frozen at -80 °C for subsequent analysis of transcript abundance.

3.3.4 Transcriptome assembly and primer design

Little information about gene sequences, including VTG and ER, was available for the four fish species investigated in this study. Since the development of degenerate primers for these species was unsuccessful, a *de novo* approach to sequencing was utilized similar to that described by Wiseman et al. (2013) and Tompsett et al. (2013). Briefly, a complementary DNA (cDNA) library was created for each species by use of a TruSeq RNA Sample Prep Kit (*Illumina,* San Diego, CA, USA), according to the protocol provided by the manufacturer. Each

cDNA library began with 4 μg of pooled mRNA from liver tissue of northern pike, walleye or white sucker, where tissues from 2, 3, and 2 individuals of each species, respectively, were pooled. The cDNA library for northern pike, walleye and white sucker was sequenced using an Illumina MiSeq sequencer (*Illumina*, San Diego, CA, USA) at the Toxicology Centre, University of Saskatchewan. Transcriptomes were *de novo* assembled using CLC Genomics Workbench 5.0 (CLC Bio, Aarhus, Denmark). The transcriptome used to acquire receptor primers for white sturgeon was generated by paired-end sequencing by use of the *Illumina* HiSeq 2000 platform (*Illumina*) according to methods described in Doering et al. (2014). Contigs of each of the four species were identified using Blast2Go 2.5.0 software (Conesa et al., 2005), and primers were designed for the desired genes and receptors using the obtained sequences (see Appendix, Table C3.S1; Fig. C2S1-C2S11). Primers for β-actin of white sturgeon have been published previously (Doering et al, 2012). Primers for VTG of white sturgeon were based off of primers of VTG for Chinese sturgeon (*Acipenser sinensis*) (Zhang et al., 2005). Primers for β-actin of northern pike were based off of primers of β-actin for rainbow trout (Wiseman et al, 2011). All primers were synthesized by Invitrogen (Burlington, ON, Canada) and validated using efficiency curves to ensure their suitability for real-time polymerase chain reaction (qPCR).

3.3.5 Quantitative real-time PCR

Approximately 30 mg of tissue was used to extract total RNA by use of the RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON, Canada), according to the protocol provided by the manufacturer. Concentrations of RNA were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE, USA). Samples of RNA were stored at −80 ˚C until first-strand cDNA was synthesised by use of the QuantiTect Reverse

Transcription Kit (Qiagen) with 1 µg of total RNA according to the protocol recommended by the manufacturer. Samples of cDNA were stored at −20 °C until analyzed.

Transcript abundances of VTG, ERα and ERβ were quantified by qPCR. qPCR was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 50 µL master mix consisting of 25 µL of 2x QuantiFast SYBR Green Master Mix (Qiagen), 2.5 µL of gene-specific primers at a concentration of 10 pmol, 2.5 µL of cDNA, and 20 µL of molecular grade water was prepared for each sample of cDNA and primer combination. All reactions were performed in duplicate with 20 µL per well. The thermal cycle profile was the same as detailed in Doering et al. (2014). Abundances of transcripts were quantified by normalizing to β-actin according to the method of Simon (2003). Nucleotide sequences of primers and efficiency of each qPCR assay (determined by construction of standard curves of serially diluted cDNA) are presented in Appendix, Table C3.S1. Walleye ERα and ERβ primers that resulted in a single product could not be successfully designed.

3.4 Statistical analysis

All statistical analyses were performed with SPSS version 20.0 (SPSS, Chicago, IL, USA) and data were expressed as mean ± standard error of the mean (S.E.M). Data were analyzed by one-sample Kolmogorov-Smirnov test for normality, and by Levene's test for homogeneity of variance. Parametric data was analyzed by analysis of variance (ANOVA), followed by a 2-tailed Dunnett's test. Non-parametric data were analyzed by Kruskal Wallis test followed by Mann Whitney-U test with Bonferroni adjustment to correct for ties. A probability of $P \leq 0.05$ was considered statistically significant.

3.5 Results

3.5.1 Assay performance and validation

Hepatocyte isolation was attempted for all species, however, was unsuccessful for white sucker and white sturgeon. Since exposure of liver explants has been shown to have similar sensitivity and response to estrogenic EDCs as primary hepatocytes (Gerbron et al., 2010), liver explants were chosen to maintain the same exposure procedure among all species tested to compare relative sensitivities. Concentration related induction of transcript abundance of VTG in EE2 exposed liver explants indicated the assay was functional in its ability to respond to exposure to EE2 (Fig. 3.1). β-actin did not change upon exposure of EE2, confirming it was a valid housekeeping gene for these species.

3.5.2 Basal expression of VTG and ERs

Transcripts of VTG, ERα and ERβ were amplified from liver explants exposed to the solvent control (Fig. 3.2). Abundance of transcripts of VTG was 96-, 192- and 235-fold greater in walleye males relative to northern pike, white sucker and white sturgeon, respectively (Fig. 3.2). There were no significant differences in basal transcript abundances of ERα among the species tested (Fig. 3.2). Abundance of transcripts of basal ERβ was 17- and 21-fold greater in white sucker and white sturgeon relative to northern pike, respectively (Fig. 3.2).

3.5.3 Effects of EE2 on expression of VTG and ERs

Exposure of liver explants to EE2 resulted in a significant and concentration-dependent up-regulation of transcript abundance of VTG in all species tested (Fig. 3.1). The lowest observable effect concentrations (LOECs) of liver explants exposed to EE2 within this study

were 300 ng/L (1.0 nM), 1000ng/L (3.4 nM), 1000 ng/L (3.4 nM), and 3000 ng/L (10 nM) for walleye, northern pike, white sturgeon, and white sucker, respectively (Fig. 3.1; Table 3.1). The maximum up-regulation of transcript abundance of VTG compared to controls was approximately 3.5-, 2.75-, 4.0-, and 15- fold for northern pike, walleye, white sucker, and white sturgeon, respectively.

Exposure to EE2 resulted in a statistically significant induction of transcript abundance of ERα in male northern pike liver explants, while no change was observed in liver explants of male white sucker or juvenile white sturgeon (Fig. 3.3). The LOEC of male northern pike liver explants exposed to EE2 was 1000 ng/L (3.4 nM) (Fig. 3.3). Exposure of EE2 resulted in no statistical difference in transcript abundance of ERβ compared to control in liver explants of male northern pike, male white sucker, or juvenile white sturgeon (Fig. 4). Walleye ERα and ERβ primers could not be successfully designed.

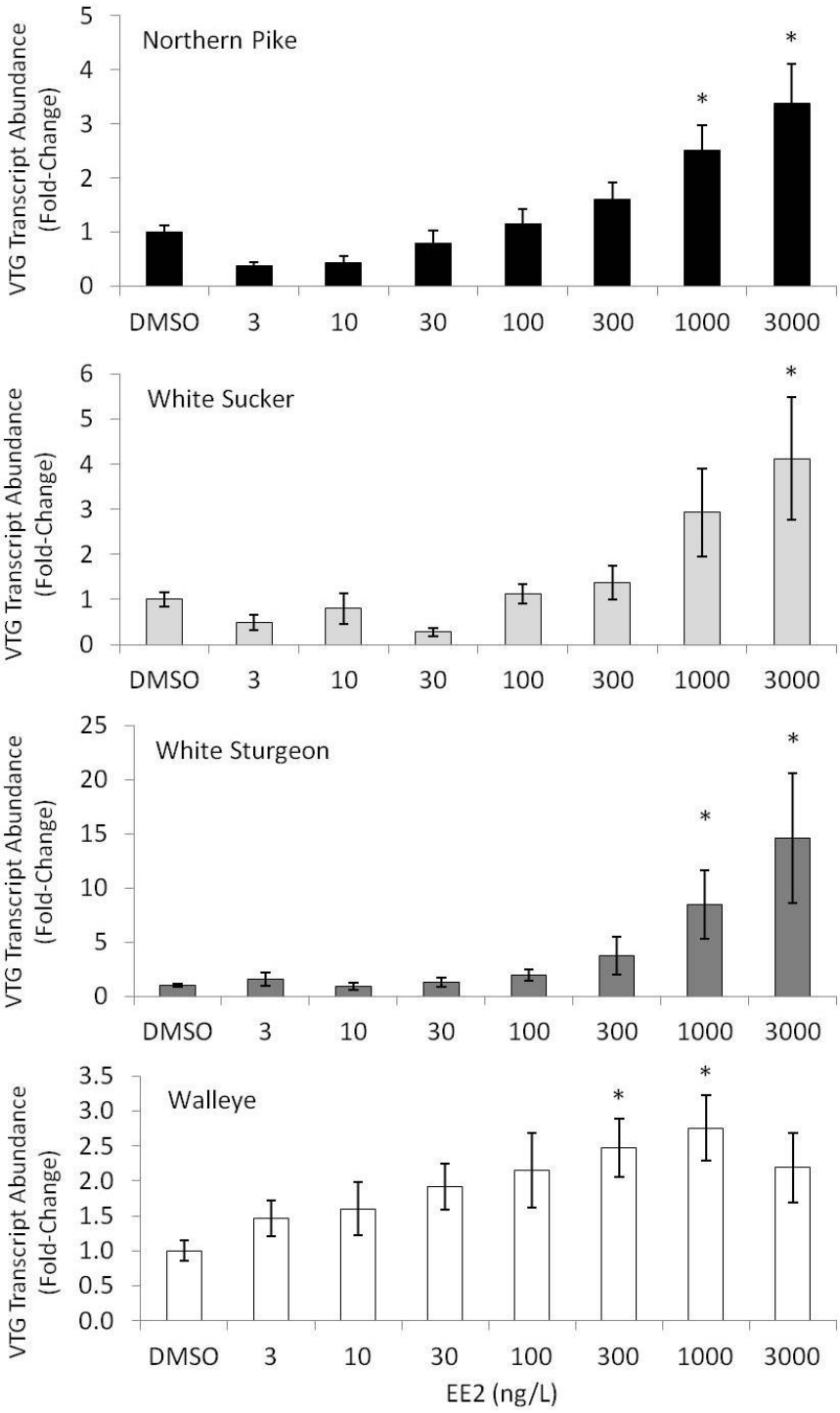

Fig. 3.1. Abundances of transcripts of VTG in liver explants of northern pike (n = 4 individuals), walleye (n = 5 individuals), white sucker (n = 4 individuals), and white sturgeon (n =7 individuals), following exposure to DMSO or serial concentrations of EE2. Data are reported as the mean $\pm$ S.E.M. * statistically different ($p \leq 0.05$) from solvent controls.

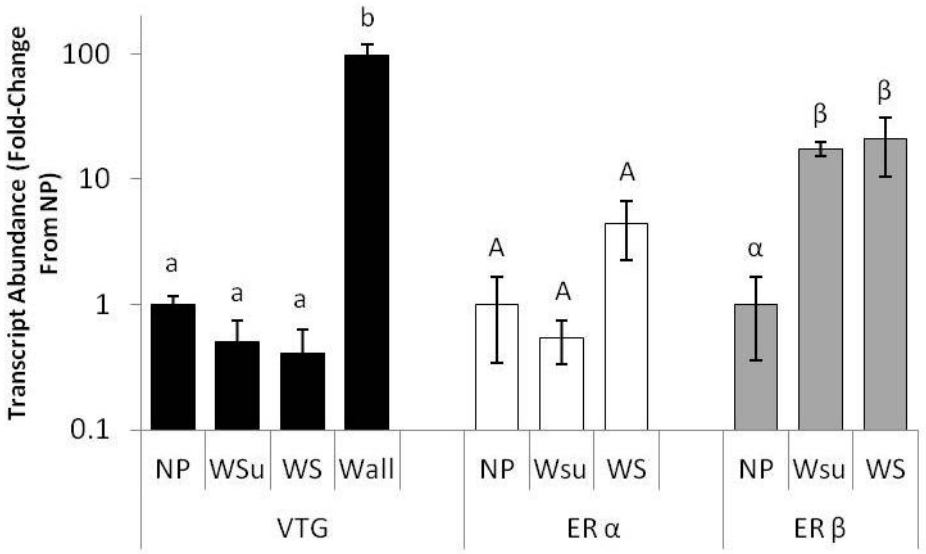

Fig. 3.2. Basal expression of VTG, ERα and ERβ in liver explants of northern pike (*n* = 4 individuals), white sucker (*n* = 4 individuals), white sturgeon (*n* = 4-7 individuals), and walleye (*n* = 5 individuals) following exposure to DMSO. Data are reported as the mean ± S.E.M. Different letters indicate significant difference among species within VTG, ERα, or ERβ. Walleye data is not included for ERs as design of ERα and ERβ primers was unsuccessful.

Table 3.1. Lowest observable effect concentration (LOEC) of VTG induction from male fishes exposed to estrogenic EDCs. All concentrations are presented as nM, with the exposure compound. '*' indicates LOEC values that were the lowest concentration tested.

In vitro tissue explants

Species	LOEC (compound)	Endpoint	Reference
Rainbow Trout (*Oncorhynchus mykiss*)	0.1 (E2) 1.0 (E2)	protein mRNA	Shilling and Williams, 2000 Schmieder et al., 2004
Roach (*Rutilus rutilus*)	1.0 (E2)	protein	Gerbron et al., 2010
Walleye (*Sander vitreus*)	**1.0 (EE2)**	**mRNA**	**This Study**
White Sturgeon (*Acipenser transmontanus*)	**3.4 (EE2)**	**mRNA**	**This Study**
Northern Pike (*Esox lucius*)	**3.4 (EE2)**	**mRNA**	**This Study**
White Sucker (*Catostomus commersoni*)	**10 (EE2)**	**mRNA**	**This Study**
Atlantic Cod (*Gadus morhua*)	100 (EE2)	mRNA	Eide et al., 2014a
Three-Spined Stickleback (*Gasterosteus aculeatus*)	10,000 (E2)	protein	Bjorkblom et al., 2007

In vitro hepatocytes

Species	LOEC (compound)		Reference
Atlantic Salmon (*Salmo salar*)	0.001 (E2) * 1000 (EE2)	protein mRNA	Tollefsen et al., 2003 Braathen et al., 2009
Rainbow Trout (*Oncorhynchus mykiss*)	0.01 (E2) 0.1 (E2) 1.0 (E2) 1.0 (E2) 10 (EE2) * 10 (E2)	protein protein mRNA mRNA mRNA protein	Jobling and Sumpter, 1993 Tollefsen et al., 2008 Flouriot et al., 1996 Islinger et al., 1999 Finne et al., 2007 Marlatt et al., 2006
Carp (*Cyprinus carpio*)	2.0 (E2) 10 (E2) * 100 (EE2)	protein protein protein	Smeets et al., 1999 Bickley et al., 2009 Rankouhi et al., 2004
Goldfish (*Carassius auratus*)	10 (E2) *	mRNA	Nelson and Habibi, 2010
Brown Trout (*Salmo trutta*)	50 (E2)	protein	Christianson-Heiska and Isomaa, 2008
Zebrafish (*Danio rerio*)	100 (EE2) *	mRNA	Eide et al., 2014b
Tilapia (*Oreochromis niloticus*)	100 (E2) * 180 (E2)	protein protein	Riley et al., 2004 Liu et al., 2007
Three-Spined Stickleback (*Gasterosteus aculeatus*)	200 (EE2)	protein	Bjorkblom et al., 2007
Bream (*Abramis brama*)	1000 (EE2)	protein	Rankouhi et al., 2004

In vivo

Species	LOEC (compound)		Reference
Fathead Minnow (*Pimephales promelas*)	0.002 (EE2)	protein	Flick et al., 2014
	0.010 (EE2)	mRNA	Lange et al., 2012
Rainbow Trout (*Oncorhynchus mykiss*)	0.005 (EE2) *	mRNA	Lange et al., 2012
	0.017 (EE2)	mRNA	Veldhoen et al., 2013
Roach (*Rutilus rutilus*)	0.010 (EE2)	mRNA	Lange et al., 2012
Atlantic Salmon	0.017 (EE2)	protein	Mortensen and Arukwe, 2007
	0.169 (EE2)	mRNA	Mortensen and Arukwe, 2007
Zebrafish (*Danio rerio*)	0.031 (EE2)	mRNA	Lange et al., 2012
Medaka (*Oryzias latipes*)	0.031 (EE2)	mRNA	Lange et al., 2012
	0.169 (EE2)	mRNA	Zhang et al., 2008
	0.337 (E2)	mRNA	Yamaguchi et al., 2005
Three-Spined Stickleback (*Gasterosteus aculeatus*)	0.032 (EE2)	mRNA	Lange et al., 2012
Sand Goby (*Pomatoschistus minutus*)	0.037 (EE2)	mRNA	Humble et al., 2014
Killifish (*Fundulus heteroclitus*)	0.236 (EE2) *	mRNA	Hogan et al., 2010

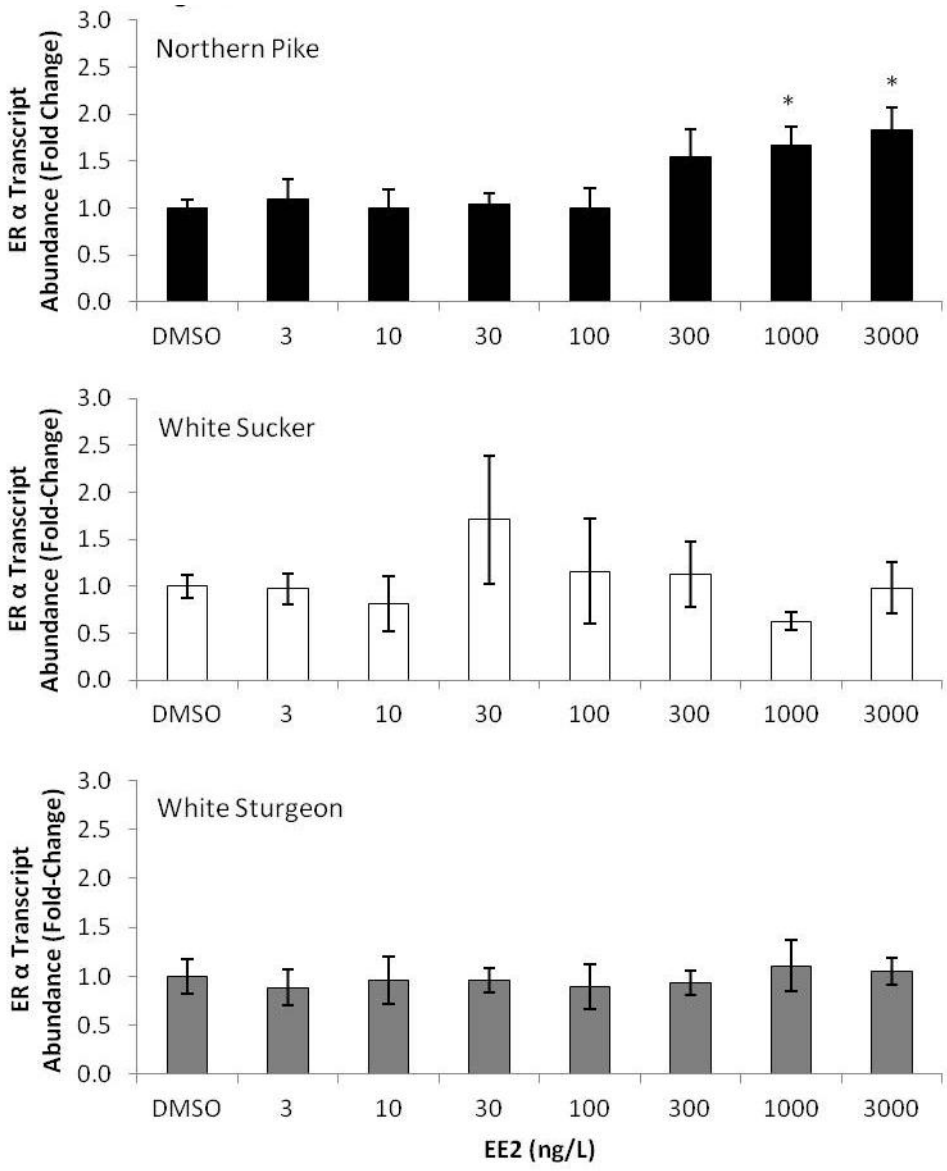

Fig. 3.3. Abundances of transcripts of ERα in liver explants of northern pike (n = 4 individuals), white sucker (n = 4 individuals), and white sturgeon (n = 4 individuals), following exposure to DMSO or EE2. Data are reported as the mean ± S.E.M. * statistically different ($p \leq 0.05$) from solvent controls. Walleye data is not included as design of ERα primers was unsuccessful.

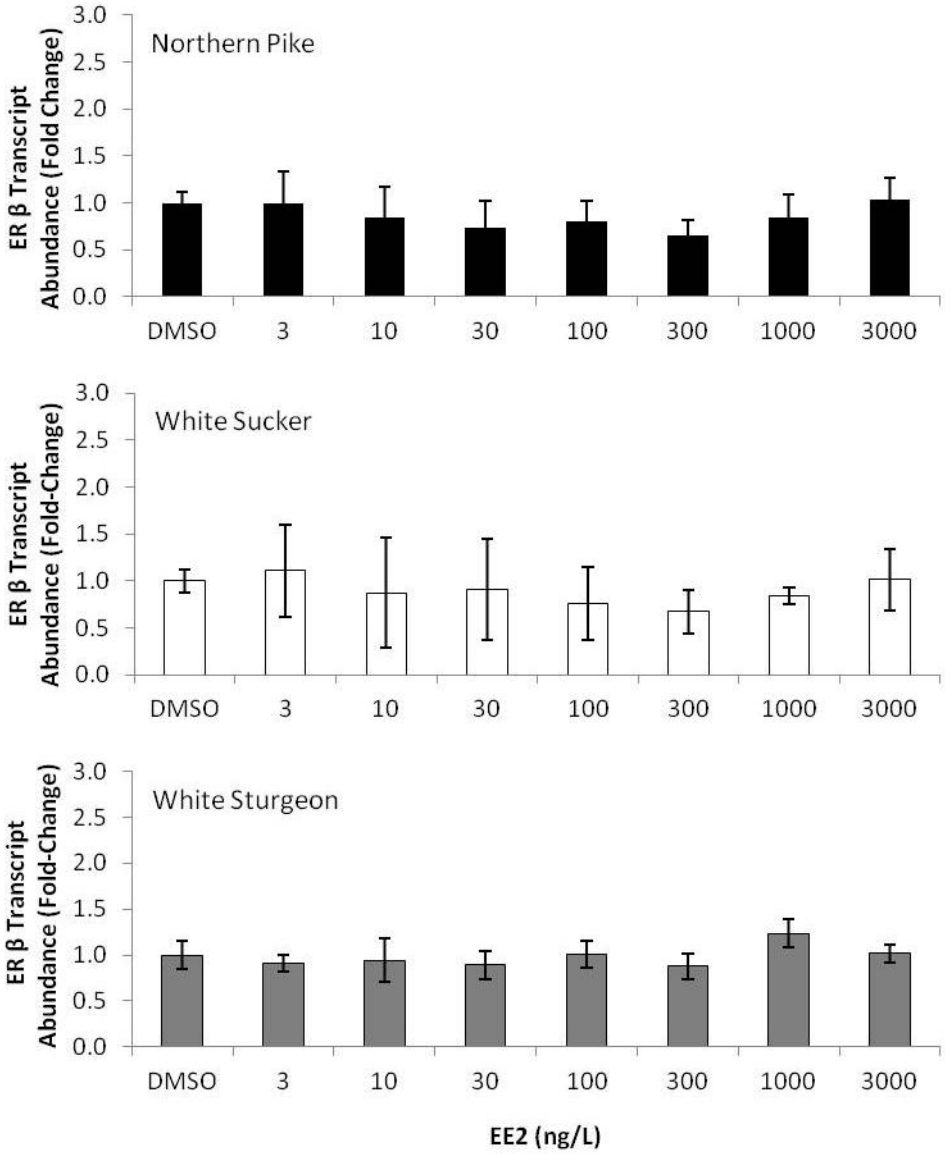

Fig. 3.4. Abundances of transcripts of ERβ in liver explants of northern pike (*n* = 4 individuals), white sucker (*n* = 4 individuals), and white sturgeon (*n* = 4 individuals), following exposure to DMSO or EE2. Data are reported as the mean ± S.E.M. * statistically different ($p \leq 0.05$) from solvent controls. Walleye data is not included as design of ERβ primers was unsuccessful.

3.6 Discussion

This study successfully established an *in vitro* assay enabling comparison of relative responses to the exposure with estrogens among different fish species native to North America, with the potential to predict species sensitivity *in vivo*. The assay responded in a manner previously reported in other *in vitro* liver explant and hepatocyte assays, as well as *in vivo* assays through up-regulation of transcript abundance of VTG in a concentration dependent manner when exposed to EE2 (Braathen et al., 2009; Veldhoen et al., 2013; Eide et al., 2014a, 2014b; Humble et al., 2014). While VTG was a sensitive and clear marker for exposure to estrogenic EDCs, ERα and ERβ were not good indicators for EE2 exposure in these species.

3.6.1 Transcript abundance of VTG and ERs in the presence of EE2

Liver explants of four North American fish species were exposed to increasing concentrations of EE2 to identify whether changes in transcript abundance profiles could be used to identify their sensitivity to the exposure with estrogenic EDCs. Effects observed were in accordance with previous reports of significant induction of transcript abundance of VTG upon exposure to estrogenic EDCs (Schmieder et al., 2004; Braathen et al., 2009; Nelson and Habibi, 2010; Veldhoen et al., 2013; Eide et al., 2014a, 2014b). When comparing LOECs obtained for walleye liver explants to those of other studies, responses obtained within this study were comparable to results obtained with liver explants of rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) exposed to E2 (17-β estradiol), which were among the most sensitive species tested using liver explants (Table 3.1). Northern pike, white sturgeon and white sucker liver explants had LOEC values between 3- to 100-times greater than rainbow trout, roach and

walleye, but they showed a greater sensitivity to estrogenic EDCs than Atlantic cod (*Gadus morhua*) and three-spined stickleback (*Gasterosteus aculeatus*)(Table 3.1).

The up-regulation of northern pike liver ERα transcript upon exposure to EE2 was consistent with other teleost species exposed to estrogenic EDCs *in vitro* (Flouriot et al., 1996; Grans et al., 2010; Nelson and Habibi, 2010) and *in vivo* (Menuet et al., 2004; Skillman et al., 2006; Zhang et al., 2008; Boyce-Derricott et al., 2009; Hogan et al., 2010; Humble et al., 2014). In contrast, transcript abundance of liver ERα in Atlantic salmon (*Salmo salar*) exposed to estrogenic EDCs decreased (not statistically) in comparison to control when exposed *in vitro* (Braathen et al., 2009) and statistically decreased in comparison to control when exposed *in vivo* (Mortensen and Arukwe, 2007). However, the reasons for these differences in responses are unclear. The LOEC of male northern pike liver explants exposed to EE2 was 1000 ng/L (3.4 nM) (Fig. 3.3). Species from greatest to least sensitivity for *in vitro* exposures to estrogenic EDCs were rainbow trout (Flouriot et al., 1996), northern pike (this study) and goldfish (*Carassius auratus*) (Nelson and Habibi, 2010), with LOECs of 1.0 nM E2, 3.4 nM EE2 and 100 nM E2, respectively. It should be noted that the lowest dose studied for goldfish showed significant results. Therefore, this species is likely to have a greater sensitivity to E2 than what was reported. The lack of response of ERα of white sucker and white sturgeon might be due to concentrations tested not being high enough to induce a response, as previous studies identifying an induction of ERα in teleosts upon exposure to estrogenic EDCs have used concentrations beyond 100-fold greater than the maximum concentrations used in this study. Future research involving an ERα antagonist in association with an estrogen exposure could give insight into the role of ERα in the estrogenic response.

Exposure of EE2 resulted in no statistical difference in transcript abundance of ERβ compared to control in liver explants of male northern pike, male white sucker, or juvenile white sturgeon (Fig. 3.4). These results are consistent with results obtained for numerous other species exposed to estrogenic EDCs *in vitro* (Grans et al., 2010; Nelson and Habibi, 2010) and *in vivo* (Sabo-Attwood et al., 2007; Zhang et al., 2008; Boyce-Derricotte et al., 2009). In contrast, transcript abundance of ERβ in the liver of some species, including Atlantic salmon, zebrafish and Japanese medaka has been observed to significantly decrease relative to control when exposed to estrogenic EDCs *in vivo* (Mortensen and Arukwe, 2007; Menuet et al., 2004; Yost et al., 2014). However, there is some discrepancy with Atlantic salmon as in one study the *in vitro* exposure of hepatocytes to EE2 resulted in an increase in transcript abundance of ERβ relative to control (Braathen et al., 2009). It should also be noted that the LOEC of EE2 to cause a significant increase in the transcript abundance of ERβ in Atlantic salmon hepatocytes was 100-fold greater than the maximum concentration used in this study. Recently, there has been increased attention into the role of ERβ in the estrogenic response of exposed male fishes (Nelson and Habibi, 2010; Yost et al., 2014). Even with the abundance of ERβ having not changed upon exposure of E2 to hepatocytes of goldfish, ERβ was believed to be responsible for induction of VTG and ERα, with ERβ1 believed to be responsible for the maintenance of basal concentrations of ERα (Nelson and Habibi, 2010). In addition, a study conducted by Griffin et al. (2013) concluded ERβ2 was responsible for induction of ERα and VTG when exposed to estrogens, while the role of ERβ1 is unknown. Therefore, the lack of response of ERβ in northern pike, white sucker and white sturgeon liver explants exposed to EE2 does not provide evidence that ERβ is not involved in the estrogenic response in these species. More research

with these species would be needed to identify the specific role of ERβ in the estrogenic response of these species.

3.6.2 Differences of species sensitivity

The *in vitro* sensitivities of the four species tested in this study rank among the fish species with the greatest sensitivity to exposure to environmental estrogens when compared to other *in vitro* studies (Table 3.1). Numerous reasons have been hypothesized to help explain differences in sensitivity among species exposed to EE2, including differences in chemical uptake rates, distribution profiles, abundances of nuclear receptor co-activators, and differences in receptor specificity and abundance (Blewett et al., 2014; Yost et al., 2014). As indicated above, abundance of transcripts of VTG was significantly greater in walleye males relative to other species tested (Fig. 3.2). Walleye males had the greatest basal transcript abundance of VTG and the least magnitude of response (3-fold), and juvenile white sturgeon, which had the least basal transcript abundance of VTG had the greatest magnitude of response (15-fold) in the induction of VTG in response to EE2. Along with walleye males having the greatest basal transcript abundance of VTG, they were also found to have the greatest sensitivity to VTG. However, it is uncertain whether basal expression of VTG has implications in context with the sensitivity or the maximum induction potential of the tissue investigated. It has been suggested, particularly with Atlantic salmon, that the greater basal expression of liver ER might explain the high level of sensitivity to estrogenic EDCs (Tollefsen et al., 2003). Within the species tested, white sucker and white sturgeon had significantly greater basal expression of ERβ than northern pike (Fig. 3.2); however, no trend regarding the basal expression of the ER and sensitivity was observed. Since we were unable to design a primer with a single product for walleye ERα or

ERβ, the basal levels of the ER in the most sensitive species tested in this study cannot be compared among the other species tested.

3.6.3 Comparison of *in vitro* responses with *in vivo* studies

With increasing ethical and economic concerns regarding the use of whole bodied animals in toxicity studies, the use of *in vitro* approaches is becoming increasingly attractive and relevant in support of chemical risk assessment. *In vitro* and *in vivo* approaches can use similar endpoints when identifying an exposure or potential indicator of an effect to estrogenic EDCs, including transcript and protein abundance of VTG. Since the transcript abundance profiles of VTG, ERα and ERβ among the species tested within this study were similar to other species tested using both *in vitro* and *in vivo* approaches, it is suggested that responses obtained with *in vitro* assays are predictive of *in vivo* effects with regard to these endpoints.

Differences between *in vitro* and *in vivo* sensitivities could be explained by the lack of toxicokinetic processes, including chemical uptake rates, for *in vitro* assays compared to *in vivo* assays, which can have a significant influence on species specific sensitivity. When comparing the LOEC values for the induction of VTG between *in vivo* and *in vitro* approaches, *in vivo* approaches had a greater sensitivity, ranging from 10- to 320 000- fold, with the exception of Atlantic salmon, in which *in vitro* approaches were 6000-fold greater or 6000-fold less sensitive depending on the study conducted (Table 3.1). Despite the greater sensitivity of *in vivo* approaches, there were similarities among the greatest and least sensitive species when comparing between *in vitro* and *in vivo* studies (Table 3.1). Rainbow trout (LOEC: 0.01 to 10 nM; 0.005 to 0.017 nM) and roach (LOEC: 1.0 and 0.01 nM) are among the species with greatest sensitivity, with three-spined stickleback (LOEC: 200 to 10,000 nM and 0.032 nM) being among

the least sensitive species to the exposure with estrogens *in vitro* and *in vivo*, respectively (Table 3.1). With walleye (LOEC: 1.0 nM), white sturgeon (LOEC: 3.4 nM), northern pike (LOEC: 3.4 nM), and white sucker (LOEC: 10 nM) having a similar sensitivity to estrogens as rainbow trout and roach *in vitro*, it is hypothesized that their sensitivity *in vivo* would rank them among the more sensitive fish species as well, and therefore, could be at risk of exposure to estrogenic EDCs found in the environment if this greater sensitivity translates to the whole organism. Future research should include *in vivo* exposures with these species investigating VTG induction, along with other common markers of exposure to estrogen EDCs. In addition, this *in vitro* assay should be conducted with fathead minnow, which is a standard test organism for endocrine disruption testing. To date, no *in vitro* assays have been conducted with the fathead minnow, and since this species has the greatest sensitivity to estrogenic EDCs *in vivo* (Table 3.1), comparison to this species would allow anchoring the data obtained with other species. While more research is needed to further validate this tissue explant assay for its ability to predict *in vivo* responses in wild fish species, it represents a promising first step in developing an alternative model for the prediction of the potential sensitivity of wild fish species to estrogenic EDCs.

3.7 Conclusion

This study successfully established an *in vitro* liver explant assay for wild fish species found in northern ecosystems to assess the effects of an estrogenic EDC, EE2, on transcript abundance of VTG. The assay responded in a manner previously reported in other *in vitro* and *in vivo* assays by up-regulation of transcript abundance of VTG in a concentration dependent manner when exposed to EE2. Liver explants of male walleye were found to have the greatest sensitivity to EE2, with an LOEC of 300 ng/L (1.0 nM). The liver explants from the species

tested within this study were found to have similar sensitivity to rainbow trout and roach, which are among the most sensitive species tested *in vitro.* Although tissue slices have been shown to be a relatively realistic *in vitro* model, with comparable response profiles to those previously observed *in vivo,* more research is needed to identify how predictive this *in vitro* liver explant assay is to *in vivo* effects in wild fish species.

Acknowledgements

The study reported was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Thanks to the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan's Toxicology Centre for supplying space and equipment for white sturgeon culture and exposure. Special thanks to Ron Ek and the team at the Kootenay Trout Hatchery for supplying white sturgeon embryos. The authors would like to thank H. Prodahl and all other staff and students who provided laboratory and/or field assistance throughout this study. Research was supported through the Canada Research Chair program and an NSERC Discovery Grant to M. Hecker.

CHAPTER 4

4 GENERAL DISCUSSION

4.1 Introduction

There is increasing concern regarding the numerous compounds that enter the aquatic environment, and have the potential to affect the endocrine system of fish and other aquatic wildlife. Exposure to these EDCs has lead to a variety of developmental and reproductive effects in fish including, but not limited to: disruption of gonad maturation, normal gonad development, circulating hormone concentrations, feminization of males, impaired sexual development, and reproductive failure (Sumpter 1998; Kime 1998; Cooper and Kavlock, 2001; Hecker et al., 2002; Jobling and Tyler, 2004; Tyler et al., 2005; Nadzialek et al., 2011). Small-bodied laboratory species have been, and still are, primarily being used to gather information in support of ecological risk assessment of these EDCs, as there are economic and ethical challenges when studying wild fish species. Unfortunately, these laboratory species are often not representative of the native species present in environments of concern. In fact, there is a large gap in knowledge related the sensitivity of native fish species to EDCs, and due to this gap in knowledge, it is uncertain whether current risk assessment approaches are sufficiently protective of these native species of concern.

The main objective of the research conducted in this thesis was to develop *in vitro* approaches to enable the assessment of species-specific sensitivity of native fish species to EDCs. The first study was designed to investigate the ability of an *in vitro* gonad explant assay to assess the sensitivity of native fish species to EDCs whose mechanism of action is through the

disruption of steroidogenesis. The results of this study illustrated that *in vitro* gonad explant assays represent a useful tool to evaluate disruption of steroidogenesis in wild fish species including northern pike, walleye and white sucker. The explant assay responded in a manner expected by inducing or inhibiting sex steroid hormone production (E2 and 11-KT) in a concentration dependent manner when exposed to forskolin or prochloraz, respectively. Furthermore, this study demonstrated that gonadal maturation stage represents an important factor with regard to the capacity of gonad tissue to produce hormones, as well as its responsiveness and sensitivity to exposure to disruptors of steroidogenesis. The second study was designed to investigate the ability of an *in vitro* liver explant assay to assess the sensitivity of native fish species to estrogenic EDCs, with EE2 used as the model compound. From this study, it was concluded that liver explants of northern pike, walleye, white sucker, and white sturgeon responded to EE2 in a similar manner as other fish species tested *in vitro* and *in vivo,* through the up-regulation of VTG. It was also concluded that while VTG was a sensitive and clear marker for exposure to estrogenic EDCs, ERα and ERβ were not good indicators for EE2 exposure in these species. Within both studies, it was illustrated that the native fish species explants tested ranked among the species with the greatest sensitivity to the EDC of interest, when compared to *in vitro* studies within the literature. In fact, in some cases the native fish explant assays tested had greater sensitivity to EDCs than laboratory species or other *in vitro* screening methods that are currently used in national and international regulatory EDC screening programs.

4.2 Differences in species sensitivity

Current environmental risk assessments rely on the use of laboratory species to predict if there would be a risk associated with the exposure to an environmental contaminant to cause

adverse effects in wild fishes. It has been discussed within this thesis that in order to enable more objective ecological risk assessments there is a need to identify the sensitivity of native fish species to environmental contaminants, and to compare these with sensitivities of common laboratory model species and other standardized testing tools. Therefore, the main objective of this study was to investigate the sensitivity of fish species of concern in North American freshwater systems including northern pike, walleye, white sucker, and white sturgeon to different types of EDCs. Of the species tested, it was found that the species with the greatest sensitivity differed depending on the mechanism of action of the EDC. Female and male white sucker and walleye gonad explants were found to have the greatest sensitivity to forskolin and prochloraz, respectively, with walleye male liver explants having the greatest sensitivity to EE2. Several reasons have been hypothesized to help explain differences in sensitivities among species exposed to environmental estrogens, including differences in chemical uptake rates, distribution profiles, abundances of nuclear receptor coactivators, and differences in receptor specificity and abundance (Blewett et al., 2014; Yost et al., 2014). Similarly, differences in chemical uptake rates, distribution profiles, metabolism, and steroidogenic enzyme abundance could explain some differences in sensitivity among species exposed to steroidogenic disruptive EDCs.

One of the objectives of this thesis was to compare the sensitivity of these native fish species with previously tested fish species. Within both gonad and liver explant assays, it was determined that the *in vitro* sensitivities of the species tested were among the fish species with the greatest sensitivity to exposure to the corresponding EDC when compared to other *in vitro* studies. For the gonad explant assay, white sucker was found to have the greatest sensitivity to forskolin relative to all other fish species tested. When comparing species sensitivities to

prochloraz, walleye, northern pike and white sucker gonad explants had greater sensitivities than all other fish species tested previously and in this study. It should be noted that amphotericin B, which was present in the media as part of the antibiotic-antimicotic solution, has been previously shown to be a weak inhibitor of steroidogenesis in mammals (Poff et al., 1988). Therefore, its presence could have resulted in an underestimation of the sensitivity of the gonadal explant assay in these species, and which render these species possibly even more sensitive as reported here. Future research should identify if the sensitivity of the gonad explant assay could be increased without amphotericin B in the media. In the liver explant assay (Chapter 3), it was determined that all four species tested were among those with the greatest sensitivity to EE2 *in vitro* and *in vivo,* with walleye males having similar sensitivity to rainbow trout and roach.

When comparing the data generated by this study to the H295R steroidogenesis assay, which is currently used as a mandatory screening application to identify endocrine active compounds by the US-EPA (Hecker et al., 2007; U.S. EPA. 2011), white sucker, walleye male and female, along with northern pike male gonad explants had a greater sensitivity to the exposure with forskolin. Furthermore, gonad explants from walleye females had a greater sensitivity in detecting inhibition of steroidogenesis after exposure to prochloraz than the H295R assay, with walleye male explants having equal or less sensitivity to prochloraz depending on the endpoint used. Therefore, the H295R assay would likely underestimate *in vitro* toxicity of forskolin and prochloraz to some of the native species.

Taken together, current *in vitro* screening assays used for regulatory purposes might not be protective of these native fish species. It was thoroughly illustrated that gonad tissue explants from some of the species tested within this study had a greater sensitivity than the H295R Steroidogenesis Assay at detecting disruptors of steroidogenesis. However, the H295R assay is

only one assay used in risk assessment; laboratory fish species are also used. It was demonstrated that fathead minnow gonad explants were less sensitive to prochloraz than gonad explants of the native fish species tested in this study. Unfortunately, forskolin was not tested on fathead minnow gonad explants, or any other laboratory fish species commonly used in risk assessment. Therefore, more research is needed to identify how sensitive *in vitro* gonad explants of laboratory fish species are to inducers of steroidogenesis.

4.3 Relating in vitro to in vivo

In vitro approaches are becoming increasingly attractive and relevant in support of chemical risk assessment to address the numerous ethical and economic concerns regarding the use of live animals. However, additional concerns have been raised over how predictive *in vitro* tests are of *in vivo* effects, along with differences between the sensitivity of *in vitro* and *in vivo* tests. It is often difficult to compare *in vitro* and *in vivo* studies because the organ system being tested is isolated and tested independently of other tissues that interact under *in vivo* conditions. Although no *in vivo* testing was conducted as it was beyond the scope of the presented research, when consulting studies within the literature, there is evidence of a correlation between responses observed with *in vitro* and *in vivo* systems. Within the gonad explant study (Chapter 2), there was a correlation between basal hormone production and plasma hormone concentrations, which provides evidence that gonad tissues were undergoing steroidogenesis in an *in vitro* setting that is reflective of reproductive seasonality in whole fish. Within the liver explant study (Chapter 3), transcript abundance profiles of VTG, ERα and ERβ among the species tested within this study were similar to other species tested using both *in vitro* and *in vivo* approaches. Therefore, it is suggested that responses obtained with these *in vitro* assays can be

predictive of *in vivo* effects with regard to the specific endpoints tested in this study, namely alterations in hormone production and transcript profiles of VTG.

Similar endpoints can be used for *in vitro* and *in vivo* approaches when identifying an exposure or potential indicator of an adverse effect to EDCs, allowing comparison between the sensitivity of these different approaches. It was concluded that zebrafish (Kinnberg et al., 2007) and fathead minnow (Thorpe et al., 2011) exposed to prochloraz in the FSDT were of similar sensitivity, and approximately 10-fold less sensitive, respectively, than alterations in hormone production by walleye *in vitro* gonad explants. It was also concluded that *in vivo* approaches had 10- to 320 000-fold greater sensitivity of VTG induction upon exposure to environmental estrogens compared to *in vitro* approaches (Table 3.1). These differences in the sensitivity between *in vitro* and *in vivo* approaches could in part be explained by *in vitro* approaches lacking various toxicokinetic processes and mechanisms involved in the regulation of steroid hormone homeostasis, including feedback loops. Additional research involving rates of absorption and metabolism of potential EDCs in native fishes could give a better prediction of sensitivity *in vivo*, if combined with *in vitro* toxicity data.

Despite the greater sensitivity of *in vivo* approaches with regard to environmental estrogen exposure, there were similarities among the greatest and least sensitive species when comparing between *in vitro* and *in vivo* studies (Table 3.1). Since the native fish species tested within this study were among the species with the greatest sensitivity to environmental estrogens *in vitro* (Chapter 3), it is hypothesized their sensitivity *in vivo* would rank them among the more sensitive fish species. Taken together, the native fish species tested within these studies rank among the species with the greatest sensitivity to both environmental estrogen exposure and exposure to disruptors of steroidogenesis, *in vitro.* Thus, they could be at increased risk

concerning the exposure to these compounds in the environment if this greater sensitivity translates to the whole organism.

4.4 Future research

There are a number of areas within the studies conducted within this thesis that could be expanded to provide further insights into the sensitivity of native fish species to EDCs:

In the gonad explant assay (Chapter 2), it was identified that maturation stage influenced both the sensitivity and capacity of the gonad explants to produce hormones when exposed to disruptors of steroidogenesis. Concentrations of plasma hormones, basal hormone production and WPS were used to illustrate changes in maturation stage; however, the use of histology in identifying maturation stage would be desirable in future studies as it would give more detailed insights into the specific maturation stage at which capacity of hormone production is the greatest for a particular species. In a few cases, data are based on a limited sample size due to the constraints naturally associated with the type of field work conducted here. Future research could increase the sample size, especially for time points where fish were unable to be collected during this study. In addition, one could expand the range of doses tested to focus in on the LOEC and EC50 values.

Previous gonad explant assays, including common laboratory models such as the fathead minnow have not investigated maturation stage as a factor in the assessment of effects of EDCs on steroidogenesis. Therefore, data presented might not be representative of the maturation stage with greatest sensitivity. Additional research would need to identify if the maturation stage used with the fathead minnow gonad explant assay in current risk assessments is representative of the most sensitive maturation stage. Once the maturation stage with greatest sensitivity is identified

for the fathead minnow, direct comparisons of the *in vitro* sensitivity of other species, including the species tested within this thesis can be conducted. Finally, since this gonad explant assay responded in a manner predicted when tested with model compounds, future research could use other environmentally relevant EDCs to test the capacity and general applicability of this *in vitro* assay.

Within the liver explant assay (Chapter 3), the native fish species tested were among the species with greatest sensitivity to environmental estrogens *in vitro* (Table 3.1.). There were similarities among the species with the greatest sensitivity *in vitro* and *in vivo.* However, no *in vitro* data on exposure to environmental estrogens exists for the fathead minnow. In order to anchor this *in vitro* data to the literature, future research should include a study on the sensitivity of fathead minnow liver explants to EE2. This would be valuable information as the fathead minnow was found to be the most sensitive species to environmental estrogens *in vivo.*

With current uncertainties regarding the predictive nature of *in vitro* studies to *in vivo* effects, additional research is needed to investigate how effective these *in vitro* assays presented here are at predicting *in vivo* sensitivity. Future research could include *in vivo* exposures of EE2, looking at VTG induction, along with other common biomarkers of estrogenic EDC exposure in these native fishes. Additional research into the differences in absorption and metabolism among native species would give insight into how much these influence the differences in *in vitro* and *in vivo* sensitivity. Overall, these *in vitro* tissue explant assays represent a promising step towards the development of alternative models for the prediction of the potential sensitivity of wild fish species to EDCs.

4.5 Conclusion

This thesis successfully established two *in vitro* explant assays for wild fish species found in northern ecosystems, to assess the effects of various classes of EDCs. The gonad explant assays responded in a manner previously reported for various other *in vitro* systems exposed to forskolin and prochloraz by inducing and inhibiting, respectively, sex steroid hormone production (E2 and 11-KT). Seasonality of reproductive functions represented a critical factor that needs to be considered when using an *in vitro* gonad explant assay to enable objective assessment of responses of wild fish species to disruptors of steroidogenesis. Gonad explants of male and female white sucker, and male and female walleye were found to have the greatest sensitivity to forskolin and prochloraz, respectively. Gonad explants from these species were found to have greater sensitivity than the H295R Steroidogenesis Assay, which is currently used as a screening application to detect potential EDCs. It is likely the H295R Steroidogenesis Assay is not protective of fish of all species or of both sexes, and thus, it could underestimate toxicity for some species. The *in vitro* liver assay responded in a manner previously reported in other *in vitro* and *in vivo* assays by up-regulation of transcript abundance of VTG in a concentration dependent manner when exposed to EE2. Liver explants of male walleye were found to have the greatest sensitivity to EE2, with an LOEC of 300 ng/L (1.0 nM). The liver explants from the species tested within this study were found to have similar sensitivity to rainbow trout and roach, which are among the most sensitive species tested *in vitro* to date. Overall, these *in vitro* tissue explant assays represent advancement in the development of alternative models for the prediction of the potential sensitivity of wild fish species to EDCs. The gonadal explant assay could be used in place of the H295R Steroidogenesis Assay to detect potential disruptors of steroidogenesis as it was found to have a greater sensitivity along with

having greater environmental relevance. In addition, both *in vitro* assays could be used as a risk assessment tool to identify species with the greatest *in vitro* sensitivity to EDCs in an environment in question. This would allow not only the identification of native species with a potentially greater sensitivity *in vivo*, but also allow regulators to focus on these species and hopefully protect the greatest number of species, with less investment in time, cost and use of live animals.

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APPENDIX[3]

[3] Supplementary data are included in this chapter. The figure or table number is presented as Cx.Sy format, where 'Cx' indicates chapter number; 'Sy' indicates figure or table number.

Table C2.S1. Number of individuals collected and dosed for each sampling period.

Species	Sex	Spawn	8-10 WPS	16-18 WPS	24 WPS
Northern Pike	Female	0	2	2	0
	Male	1	2	2	0
Walleye	Female	2	5	2	0
	Male	2	2	2	2
White Sucker	Female	2	2	5	2
	Male	2	2	2	2

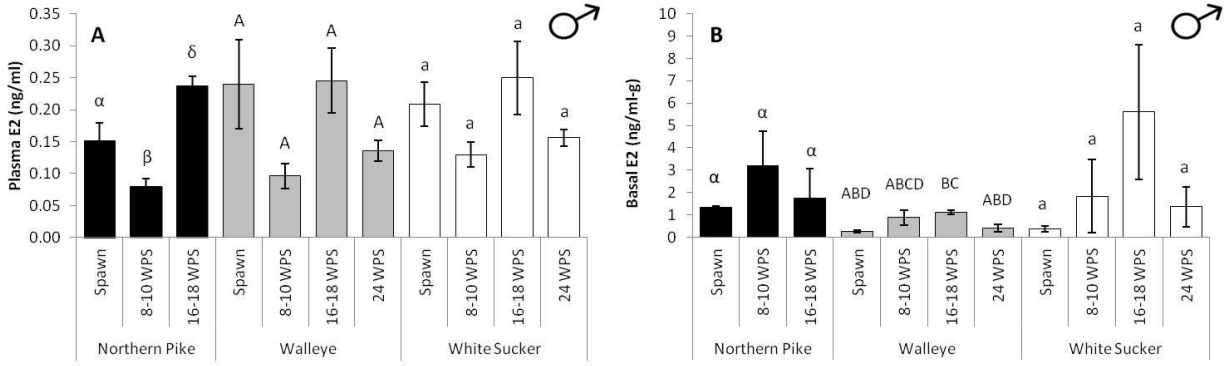

Fig. C2.S1. (A) Plasma E2 concentrations of male northern pike (*n* = 3, 11, 3 individuals), walleye (*n* = 3, 3, 4, 4 individuals), and white sucker (*n* = 2, 3, 4, 3 individuals) collected at spawn, 8-10 WPS, 16-18 WPS, and 24 WPS. (B) Basal *in vitro* 11-KT production by gonadal explants of male northern pike (*n* = 3, 4, 3 individuals), walleye (*n* = 3, 2, 4, 5 individuals) and white sucker (n = 3, 3, 4, 2 individuals) at spawn, 8-10, 16-18 and 24 WPS time points. Plasma data represented as mean ± S.E.M in ng/ml plasma. Basal *in vitro* hormone production data represented mean ± S.E.M in ng/ml-g. Different letters indicate significant difference within each species (Tukey's test; $p \leq 0.05$).

Table C3.S1. Sequence, annealing temperature, efficiency, and corresponding target gene Genbank accession number of northern pike (NP), walleye (WA), white sucker (WSU), and white sturgeon (WS) oligonucleotide primers used in quantitative real-time PCR.

Species	Target Gene	Accession #	Primer Sequence (5'-3')	Efficiency (%)	Annealing Temp (C)
NP	B-actin	AF157514	F: AGAGCTACGAGCTGCCTGAC	99	60
			R: GCAAGACTCCATACCGAGGA		
NP	VTG	NA	F: CGTGCAGTGAGAAAAGACCA	99	60
			R: GCCAGTTGTCATCTTCAGCA		
NP	ERα	NA	F: ATGTGGTAGATGAGGCGTC	100	60
			R: TGTTAAACTCCGGTGCCTTC		
NP	ERβ	NA	F: ACATCTGTCCCGCTACCAAC	103	60
			R: CATTCCACACTTGGTCATGC		
WA	B-actin	NA	F: GTGCCCATCTACGAGGGTTA	101	60
			R: CTCTCAGCTGTGGTGA		
WA	VTG	NA	F: TATTTGCCCCTGCAGAAGTC	99	60
			R: CCTTGAACTCCAGCCTCTTG		
WSU	B-actin	NA	F: GTGCCCATCTACGAGGGTTA	91	60
			R: TCTCAGCTGTGGTGGTGAAG		
WSU	VTG	NA	F: GGAAGTTGTCATGCTCGGAT	93	60
			R: TGCCCAGAACTTTTAGAGCC		
WSU	ERα	NA	F: TGTCTGATGTGGGAGAGCAG	93	60
			R: ACATGCTCTTGGCAACTGTG		
WSU	ERβ	NA	F: CAACTTTGCATGAGCAAGGA	94	60
			R: GTCCCACTCAGTCCGACAAT		
WS	B-actin	FJ205611	F: CCGAGCACAATGAAAATCAA	96	60
			R: ACATCTGCTGGAAGGTGGAC		
WS	VTG	AJ745099	F: GCACCAGCTCACTCCATTCAA	102	60
			R: CCTCCAAAACAAGCTTCTGCC		
WS	ERα	NA	F: GCGCCAGATAAAGACCGATCA	90	60
			R: ACTCACCAGTTTGGCTGACA		
WS	ERβ	NA	F: TACCGTCAGTGAGCAGCAAG	96	60
			R:CCGTAGGGTACAGGAGTCCA		

Fig. C3.S1. Contig for northern pike VTG.

AACCGCAATTTCACCAGGAATCACAAGGTCTCTCAAGCAATCTCCAAGACAAGTGT
CTCGAAAAGCAGGAGCAGTGGCTCTAGCTTCCAGCACATTTACAATGAGGCCAGAT
TCCTTGGCGAGACTCTTGCCCCTGAGGTGGTCATTCTGGTCCGTGCAGTGAGAAAAG
ACCAGAAGCAGGCTGGATACCAGGTGGCTGCTTACTTGGACAAAGCTACTTCCAGA
CTTCAGATCATTCTGGCTGCCATTGCTGAAGATGACAACTGGCAATTATGTGCTGAT
GGTGTTCTGCTCAGCAAACACAAAGTCAATGCCAAGATTGCTTGGGGTGCAGAGTG
CAAGGAATATAAGACCTTCATCACTGCAG

Fig. C3.S2. Contig for northern pike ERα.

CATATGTCGGATATGAGAGAGCAGGAGAAGGAGCTGTGCCTGCCGTCTGGACTGCT
GCTGCACCAAGGCTCCCGATTGGCCGATGTGGTAGATGAGGGCGTCAGTGATGTTGT
CCAGCATGTTCTGCACAGCCGGGCGGTTGTGTAGAGACTCCACTACGTTGGAACAGA
AGGAGAAGGCACCGGAGTTTAACAGGATGATGGCTTTGAGACACACAAACTCCTCC
GGCTTTAATCTGAGCGTGCGGAAACGAGAAACAGTGGCCAGGAGCATGTCAAAAAT
CTCACCGAAGCCCTCCACGCAATCCCCTTCACTCCGGTCCAGAATGAGGTCCTGGGC
GAAGATCAGTTTCCCTGGGCAATGGATGGACCTCCAAATGAGTCCGATCATCAGAA
CCTCAAGCCAGGAGCTCTCCAGCAATTGCACCTGGTCATGGAGGGACAGCTCCTGG
AATCCTGGTACTTTCTTGGCCCAGGCAATCATGTGTACAAGCTCCTTGTCGGCCATG
CTGGTAAGCAGAGTCATCACGGTGATCTCTGTGTAGGGCCGGGCCATCTTCTGGCGA
GAACACACGGCTGGAGGCTCTGCACCCTGCAGCTGGAACAACACCTGCTCAGGTGG
CATGGAAACCCTGGGCCTGACTCCTGGTCCTCCAGCACTGAGACTGCTGTTCCTGCT
ACTGTCCTGAGAGGGCGCTGTGCTTTGCTCCAGGACGCTGCTGTCAGCAGTAGGGCC
ATAACGCCGCTTATCCCTCCGGAGAACCCGCCCACCGCGGTCCTTACGCAAGCCTCC
TTTCATCATGCCAACTTCGTAACACTTTCTGAGACGGCATGCCTGGCAGCTCTTCCTG
CGGTTCCGGTCAATGGTACACTGGTTGGTCGCAGGGCACATATAGTCATTGTGACCT

Fig. C3.S3. Contig for northern pike ERβ.

AGGGCTGCAAGGCTTTCTTCAAAAGAAGTGTCCAAGGACACAATGACTACATCTGTC
CCGCTACCAACCAGTGCACTATTGACAAGAACCGTCGCAAGAGCTGCCAGGCCTGC
CGCCTCCGCAAATGCTATGAAGTTGGCATGACCAAGTGTGGAATGCGTCGCGA

Fig. C3.S4. Contig for walleye β-actin.

ATCTTCTACCTGTAACACATTCTCTTAAGTCGAAAAAAAACCAAACCTAAGTTGAGC
CATGGATGATGAAATTGCCGCACTGGTGGTTGACAACGGATCTGGTATGTGCAAAG
CCGGATTTGCTGGAGATGATGCTCCCCGTGCTGTCTTCCCATCAATCGTCGGTCGCC
CCAGACATCAGGGTGTGATGGTTGGTATGGGACAGAAGGACAGCTATGTTGGTGAT
GAAGCTCAGAGCAAGAGAGGTATCCTGACCCTGAAGTATCCCATCGAGCACGGTAT
TGTCACCAATTGGGATGATATGGAGAAGATCTGGCATCACACCTTCTACAATGAGCT
GCGTGTTGCACCTGAGGAGCACCCCGTCCTGCTCACAGAGGCCCCCCTGAACCCCAA
GGCCAACAGGGAAAAGATGACACAGATCATGTTCGAGACCTTCAACACCCCTGCCA
TGTACGTTGCCATCCAGGCTGTGCTGTCCCTGTATGCCTCTGGTCGTACCACTGGTAT
CGTGATGGACTCTGGTGATGGTGTCACCCACACTGTGCCCATCTACGAGGGTTACGC
ACTCCCCCATGCCATCCTTCGTCTGGACTTGGCTGGCCGTGACCTGACTGACTACCTC
ATGAAGATCCTGACCGAGAGAGGCTACAGCTTCACCACCACAGCTGAGAGGGAAAT
TGTCCGTGACATCAAGGAGAAGCTCTGCTACGTTGCCCTCGACTTTGAGCAGGAGAT
GGGCACTGCTGCTTCCTCCTCCTCCCTGGAGAAGAGCTATGAGCTGCCTGATGGACA
GGTCATCACCATTGGCAATGAGAGGTTCAGGTGCCCAGAGGCCCTGTTCCAGCCATC
ATTCCTGGGTATGGAGTCTTGCGGTATCCATGAGACAACCTTCAACTCCATCATGAA
GTGTGATGTGGACATCCGTAAGGATCTGTATGCCAACACCGTATTGTCTGGTGGCAC
CACCATGTACCCTGGCATTGCTGATAGGATGCAGAAGGAGATCACATCCCTGGCCCC
TAGCACAATGAAAATCAAGATCATTGCCCCACCTGAGCGTAAATACTCCGTCTGGAT
CGGAGGCTCCATCCTAGCTTCACTGTCCACCTTCCAGCAGATGTGGATTAGCAAGCA
AGAGTACGATGAGTCTGGGCCATCTATTGTCCACCGCAAATGCTTCTAAACGGACTG
TTACCACTTCACGCCGACTCAAACTGCGCAGAGAGGAAAAATTTCAAACGACAACA
TTGGCATGGCTTGTTATTTTTGGCGCTTGACTCAGGATCTAAAAACTGGAACGGTGA
AGGTGACGGCAATGTTTTTGGCAAATAAGCATCCCCGAAGTTCTACAATGCATCTGA
G

Fig. C3.S5. Contig for walleye VTG.

ACACGAACGGGACTGACATCGGGAACACCGCTCAGTTGTCTAGTTGGAACAAGGAC
TTAAACGGGAACTATTCTGGATACACATGTTCATACTCCGAGACGAAAACCCGCCAG
AGGGAGTACTTCCAGACCGGTCTCGACCAAATTTTCAGTCGTCGTTCCAAGAGGAGT
CACGACACTGTCTCTTATGGATGGACTACGTCGAGTACCTAAGAGTTTAGATACTCA
TGTCACCGTAGACCGGGCTCCTAAGAAAACGAGGTCGGTGATTTGAGTGGAGTCGT
GACCGAGGAGTTGAAGTCTAAGGGTAGTTCAAACTCATAAGATTACCACACCAACC
TTTCCATAAACGGGGACGTCTTCAGAGGGGATGANNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNAAGTCGAGTTGGATTTCTTCTGGGTNNNNNNNNNACTTGACGT
TCTCCGACCTCAAGTTCCTCACACATCCTGGGTGATATAGTAGTTACTTCTAAGTTTT
CGGTTGGTGTAATAACAGTGGTTTAGATTCCTAGACTCGGTGACGGTCCTCTCTTAG
TACTTCCTTCAGCCGAACCGTATGTGTCTCTTCACACAACTTACGTGGGTCTCCCAGT
TCCCAGACTAACTTCGCCGTTGAATGTTGATGTAGTNNNNNNNNCGACGGTTACCAC
ATGACTAGAGTCTCCGTTGTCAACTCCTTGACATAGTCAAGAGTGGGAAGTTACTCT
AGGTACCACNNNNNNNNNACCTTCGTTTTGTTTGGAACTGAATATAACTTTAACTCT
TCTGGGGGTAACGAGGGTAGGTTAGCCTAATAAACCGGGCACCTAGGGACGTCATA
CTCAAACGTAGACTTTAAGAAGTCTGAGGGTAAGATGAGGACTTCTAGTTACTACGT
GGTCGGGTCTAACACCTCCAGGATTTAGTGAACCAACTTTTGT

Fig. C3.S6. Contig for white sucker β-actin.

ATCTTCTACCTGTAACACATTCTCTTAAGTCGAAAAAAAACCAAACCTAAGTTGAGC
CATGGATGATGAAATTGCCGCACTGGTGGTTGACAACGGATCTGGTATGTGCAAAG
CCGGATTTGCTGGAGATGATGCTCCCCGTGCTGTCTTCCCATCAATCGTCGGTCGCC
CCAGACATCAGGGTGTGATGGTTGGTATGGGACAGAAGGACAGCTATGTTGGTGAT
GAAGCTCAGAGCAAGAGAGGTATCCTGACCCTGAAGTATCCCATCGAGCACGGTAT
TGTCACCAATTGGGATGATATGGAGAAGATCTGGCATCACACCTTCTACAATGAGCT
GCGTGTTGCACCTGAGGAGCACCCCGTCCTGCTCACAGAGGCCCCCCTGAACCCCAA
GGCCAACAGGGAAAAGATGACACAGATCATGTTCGAGACCTTCAACACCCCTGCCA
TGTACGTTGCCATCCAGGCTGTGCTGTCCCTGTATGCCTCTGGTCGTACCACTGGTAT
CGTGATGGACTCTGGTGATGGTGTCACCCACACTGTGCCCATCTACGAGGGTTACGC
ACTCCCCCATGCCATCCTTCGTCTGGACTTGGCTGGCCGTGACCTGACTGACTACCTC
ATGAAGATCCTGACCGAGAGAGGCTACAGCTTCACCACCACAGCTGAGAGGGAAAT
TGTCCGTGACATCAAGGAGAAGCTCTGCTACGTTGCCCTCGACTTTGAGCAGGAGAT
GGGCACTGCTGCTTCCTCCTCCTCCCTGGAGAAGAGCTATGAGCTGCCTGATGGACA
GGTCATCACCATTGGCAATGAGAGGTTCAGGTGCCCAGAGGCCCTGTTCCAGCCATC
ATTCCTGGGTATGGAGTCTTGCGGTATCCATGAGACAACCTTCAACTCCATCATGAA
GTGTGATGTGGACATCCGTAAGGATCTGTATGCCAACACCGTATTGTCTGGTGGCAC
CACCATGTACCCTGGCATTGCTGATAGGATGCAGAAGGAGATCACATCCCTGGCCCC
TAGCACAATGAAAATCAAGATCATTGCCCCACCTGAGCGTAAATACTCCGTCTGGAT
CGGAGGCTCCATCCTAGCTTCACTGTCCACCTTCCAGCAGATGTGGATTAGCAAGCA
AGAGTACGATGAGTCTGGGCCATCTATTGTCCACCGCAAATGCTTCTAAACGGACTG
TTACCACTTCACGCCGACTCAAACTGCGCAGAGAGGAAAAATTTCAAACGACAACA
TTGGCATGGCTTGTTATTTTTGGCGCTTGACT

Fig. C3.S7. Contig for white sucker VTG.

CACACCTGAATCAATTCAGGCTCTTGTAGTTGCTATGCNNNNNNNNNNNNNTGATTT
GGACACCATCAAGTTGACCGCTNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAATT
CCAGCTCTCCGGGAAGTTGTCATGCTCGGATATGGTTCCATGANNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNTCTCCTCAGGCCCCTCCATGAAATTGCTG
CAGANNNNNNNNNAAGAATGATATCTATGAAATCACTTTGGCTCTAAAAGTTCTG
GGCAAT

Fig. C3.S8. Contig for white sucker ERα.

TGGGATGGGCTGCATGACTCCGGGGCCTCTGGAGAGACTACTGCTGCTGGCTGTAGG
TGTAGAGGGTAAATTTTTCTCACTTGGTGCCCAGGGTCGCTGCACCTTGCCTGTGGA
GTGGAATCGTTGTGCATCCAGCATCTCCAGCAATAGATCGTACAGTGGTACTCGATT
CTTGCATTTCATTCTGTACAAGTGCTCCATTCCTTTGTTGCTCATGTGTCTGATGTGG
GAGAGCAGCAGCAGAAGTTGCGCCTGGCGTCGGGACTGCAGCTGCACTGAGGCACC
TGATTTACTGATGCAGTAAATGAGGACATCAGTGATGTTGTCCAGCATGCACTGCAC
CATAAAGCTGTCCGACTGGGGCGCCACGGGACTGGAGCAGAATGAAAATGCACCAG
AATTGAGAAGTATGATGGCTTTGAGACACACAAATTCCTCTGACTTGAGTTTAAGAC
TGTGGAATCGAGCCACAGTTGCCAAGAGCATGTCAAAAATCTCAGCCATCCCCTCA
ACGCATTCACCTTCATTCCTATCAAGAATAAGATCCTGA

Fig. C3.S9. Contig for white sucker ERβ.

CAAATTTTGTTTTGTTGAAGCAGGACTGAGAGTTGTATGTAAGCCCTGTTTGGTTCA
GGTTGTCGTTTGTCATTAAGTTCCATTCTGCAAAGTCCAAATCCAGTTCTGGATCCAG
GGAGGTTATTTCCCACAAAAGGGATATTGACAGTCTGTGCCCATGTCCTCTAGGTTC
AGCCTAGCATGCAACGTCTCTTCCACTCACTACTCGGGTTGTGAACTTTGGAGGATC
ACTGGGTCGCTCTCTTTCAGTGCAGGCTGCTGTCTGTATGTGTGGTGGGTTTCGGGG
ATCTGTTGAGTGTCTGGCCGGGTGAAGGTGTCTGTGTGAGCAGGAAGCATGTGGCTG
CTCTGAGATGTGTTCGCGTCCAGCATCTCCAGCAGAAGATCGTACAGAAGCACCAC
GTTTTTTCTCTTCATGCTCGACAGATGCTCAATACCTTTGTTGCTGAGGTGTCGTATA
TGTGACAGCAGCATCAGCAGGTGGGCGAGCCGAATGGATTGCTGCTGTGTGGACAG
CCCGGTTTTGGAAATGGCCCAAACCAGAGCATCAGTCACAGAGTCCAGCAGCCTCA
GAACCTTCCCACGACTCTCCACATCATCAGGGGTCTGTGGTGAGCTTGAACAGTTAT
TGGAGTTTAGAAGGATTACGGCTTTGAGACAGACATATTCCTCTCTTTGCAGCTTCA
GTTCTCTGAATCTGGAGGTGGTAGCCAGCAGCATGTCAAAGATCTCCATGATGCCTT
CAACACAGTTGCCCTCATCCCGGTTGAGTTTGAGGTCTGGTGAGAAGATGAGTTTCC
CGGGATGATCCACAGATCTCCACATTAATCCCAACATAAGAATATCCAGCCAGCAG
CATTCCAACAGATGCACCTGATCTGACAAACTCAGCTCCACAAAACCTGGTATCTTC
TTAGCCCAACTGATCATGAGCACCAACTCCTTGTCAGCGAGGTTGGTTAGGGACATC
ATCATGCTGGCCTCTGTATACGGCTTCTTTGCTGGATCTCGCAGGTAAATCTGAGGA
GGCTCCGCCTCCATTATACGGTTTACCAACTGCTCAGGGGAGAGGTTTAGGCCACAC
TGCTCAGCTTCGCCCCCTGAAGGGAAAAGGTGATGAGGGGGGTTGAGGGGAAATTC
TAGATGATGCTGGGAGTGACATTTGACCCCTATTGCTCCACCAGAGCTGTCTCTGAT
CTGGGGGGTACGCCGATGCCGAGCACCACGGTAACTGCAGCGTTCCCGCCTTACAC
CACACTTCATCATGCCCACTTCATAGCACTTGCGCAGTCGACAGGCCTGGCAGCTCT
TGCGTCTGCTCTTGTCGATAGTGCACTGGTTGGTGGCTGGACAAATGTAGTCATTGT
GTCCTTGAATGCTCCTTTTGAAGAAAGCCTTGCACCCCTCACATGACCAGACACCAT
AGTGATACCCAGAAGCGTAGTCATGACACACAGCACAGAACTGTTTGTCTCCTTTAC
TCACAATCCCTGGCAAAGGATTCAAGCCATCATCAACTTCCAGCTGCTGCCCTAACA
ACTTTGCATGAGCAAGGACAGAACTGCTCTGGTTAAGGCTGATTGTCTTAGCATCTT
CCCATCCGGTGTGTGCGTGAATTTCACTGTAATCCAGTGGATGCGGGCGGTGTAGAG
AGATTGCTGTGTGTGTGGTATGGGGAGGCCAGAAGATTGTCGGACTGAGTGGGACC
AGGCTTTCGGATACAGGAGGATGGGTGTACCCTAGCGCTGTGTAGGACATTGGGCT
GTAGAGTGTTAGGGCCCCGTGTGGTGGTGAGTAATCCTGACTGGCCTCCACATAGGG
AGATGGAATGCAGATGGTATGGTTGTAGAGCGGGGAGGAGTAGACAGAGGAGGAA
AGTTGAGGTGAGTCCCCTCGACTGGCCTTGCCAGAGTCCAGCTCCTGGTGCAGGGTA
GGGGTGGCAGACTCGGGGACTGGCCCAGGGGAGGAGCTCATTCTAGGGATGAGAAC
TCAAGCGAGGTCCTGGCTTAACACACTACAGCATCACAGGCCCAACTGATTGAGAG
GACGTTTGTGCAGTAGCAGGCAGCAGGTGATGTGGAGCTCGATTGTGTGTGTTTGAT
CGGCCGTGCACACGCTGCG

Fig. C3.S610. Contig for white sturgeon ERα.

TCCACCATACCCTCAACACAGCTTCCTTCATTCCTGTCAAAAGACAGATCTGGGGCA
AAAATTAACTTCCCAGGGAATTCCACAGAGCGCCAGATAAGACCGATCATTAGGAC
CTCTAACCAAGAGCATTCCAGCAGCTGTACCTGGTCATGGAGCGACAAATCCACAA
ACCCTGGTATCTTTTTTGCCCATGCTATCATGTGTACCAGTTCCTTGTCAGCCAAACT
GGTGAGTATATTCATCATGGAACTCTCAGTGTAGGGTCTATTTTGGTTCTGTTTTGAG
TATTGTGTTGGCGGTTCCGCTTCTAACAAATGGATGAGGACCTGATCAATCGACAGA
CCCAGTGCGCTGTTCTTCTTCCCATTGTTGATAGGTGTGGGCTCCAGGGTCAGAGTTA
GCCTTCTGTCATTGCCATTGACGTGGCTCTTCTCATCCTGCTCACTTGCTGTGCGCTT
CACTTTCAGCATGTGTCGCCCCCTACGGTCTTTACGCACTCCACCTTTCATCATCCCG
ACTTCATTGCACTTGCGCAGCCTGCATGCCTGGCAGCTCTTTCTCCGGTTCTTGTCGA
TGGTGCACTGGTTTGTAGCTGGGCACATGTAGTCATTGTGTCCTTGGATGCTTCTCTT
AAAGAAGGCCTTGCAGCCTTCACAGGACCAGACTCCATAATGGTAGCCGGAGGCGT
AGTCACTGCACACCGCACAGAACCTCATCTCCTTGCTCAATTTGGACGGGCCGCTGG
ATGGACTGCCTTTCTGCCCCATGTCGGAGAGCTGTTCCCTGACACCCTGATATCTGC
CTTCCATACTTGGCCTGAAAATAGGTTCAGGGGTGGCTTCCCT

Fig. C3.S11. Contig for white sturgeon ERβ.

ATGACAGCTTTGTCAAATAAAGAACCGCACTTGCTGCAGCTACAAGATGTTGGACCA
AGCAAAGTGTCAGGAATGACCTGCTCACCGGGAATCAGTTGCCCTGTTTCGTGCCGC
GGGGCGATGCCTGCATTGATGATGGAACGACATGCTGTTTGCATCCCTTCCCCGTAC
AGGGATAGCAACCACGATTACACAGCAGTGGCATTTTATAGTCCTTCGATGCGTGGG
TACAGTGGGCACAGTAATGGCAGCATTCCGGACAGCCCGACAGTAAGGCCGTGTTT
GAGCCCCTCTGCATTCTGGCCTCCACCAAGCCATATTTCATCATTAGCCCTTCAGTGC
CAGCAGATGCATACAGATCCTCCAAGGAGCCCTTGGAACGAGGAGAGATCAAGAAG
AGAGCAAATACCGTCAGTGAGCAGCAAGGAAAGCAACAGAATAAAGGAAAATTCA
GAAGATCCCACTGGACGCTGCTCAGGTTCAAAACAGGCCATGCACTACTGCGCGGT
GTGCAGTGACTTTGCCTCGGGTTATCACTACGGGGTCTGGTCATGTGAGGGGTGCAA
AGCGTTCTTTAAAAGGAGCATCCAAGGGCATAATGATTATATCTGCCCCGCTACAAA
CCAGTGCACCATAGACAAGAACAGAAGGAAAAGCTGCCAAGCCTGCCGACTAAGA
AAGTGCAATGAAGTTGGAATGATGAAATGCGGTACAAGAAGAGAGCGTTCTAATTA
TCGCATTGTACGACACAGGCGTCTTTCTCAAGGCCAAGGGCAGCCCAGTAGTAAAG
CCAGCAAAACCAGTGAAAGTGGCTTACTGCAGACAAGGAGGATTCACTTCAGTTCT
CTGAGCCCTGAAATGCTCATGTCTTCAGTAATAGAGGCTGAACCGCCTGAGATTTAT
TTGATGAGCTATCTCATGAAGCCATTCACTGAGGCCACCATGATGACATCATTAACC
ACCCTTGCAGACAAGGAACTCGTTTACATGGTCAGCTGGGCCAAAAAAATTCCAGG
GTTTGTGGAGCTCAGTGTGTATGACCAGGTATGCCTATTGGAGTGTTGCTGGTTAGA
GGTGCTGATGGTAGGGCTGATGTGGAGATCTATTAATCATCCAGGGAATCTCGTGTT
TGCATCTGACCTTATTTTAAACAGGGACGACGGCAACAGCGTGGAAGGATTACTGG
AGGTTTTCGACATGCTTTTGGCTCTAACTTCAAAGTTTCGAGAGCTGAATCTGCAGC

GAGAGGAGTATCTCTGCCTCAAGGTCATGGTCCTCCTCAACTCCACTATGTTCCCCG
GTCCCTCAGAGAAGCAAGAAAAAAGTGAAAGTAGAGATAATCTGCTTAAACTTCTG
GATGCAATCACTGATGCTTTAGCCTGGGTTATTTCGAAGAAAGGACTCTCTTTACAG
CAGCAGTCAGCACGCCTGGCTAACCTCCTGATGCTGCTCCCCCACATCAGACATGCA
AGTAACAAAGGTATTGAGCACCTCTACAGCATGAAGTGTAAAAATATAGTGCCTTTG
CGTGAATTGCTGCTGGAGATGCTGCACGCGCACACTCTACACTTCCCCAGAATGCCA
GCCATCACATCATCAGAATACAGCCCAAAGGAACAAACCAAGGAGCCTGTCACCTG
TTCAAAGCCAGAAGTATTTTGA

Methods development

Gonad explant assay

Sexually mature northern pike, walleye, white sucker and lake whitefish were sampled using gill nets from Lake Diefenbaker, Saskatchewan, Canada during September of 2011. Mass ($\pm$ 0.1 kg) and fork length ($\pm$ 0.5 cm) were measured and recorded for each individual. Gonad tissue was excised and weighted ($\pm$ 1.0 g) and recorded to calculate GSI. Gonad tissues were sliced into smaller pieces and immediately transported to the Toxicology Centre, University of Saskatchewan in ice cold supplemented Leibovit L-15 media (13.8g of L-15 powder per litre medium, 420 mg $NaHCO_3$/L, 1% antibiotic-antimicotic [100 units penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B per mL], pH 7.6) (Sigma Aldrich). The time between sampling of fish and initiation of the exposure did not exceed 5 h.

Gonad tissue was sliced into 1 mm^3 pieces and rinsed several times with supplemented L-15 media. Multiple pieces of gonad, approximately 100 mg total, were placed into each well of a 24-well culture plate containing 999 µL of supplemented L-15 media with 1 mg cholesterol/L. Test chemicals and the solvent control were added to the sample wells to a final concentration of 0.1% DMSO, 0.1, 1.0, 10.0 µM forskolin, or 0.1, 1.0, 10.0 µM prochloraz. Each concentration was dosed in quadruplicate. Samples were incubated at 15 °C for 12, 24 or 36 h. Upon termination of the exposure, tissue was removed from each well and placed into pre-weighted microcentrifuge tubes, weighed and frozen at -80 °C. Media was placed into microcentrifuge tubes and frozen at -80 °C for subsequent analysis of steroid hormones.

Steroid hormones were quantified using liquid chromatography tandem mass spectrometry (LC/MS/MS). Steroid hormones were extracted from the media and plasma using a liquid-liquid extraction method in accordance with Chang et al. (2009). Briefly, 500 µL of

sample media and 500 µL nanopure water were combined with an internal standard consisting of deuterated 17β-estradiol and testosterone. This mixture was extracted twice with 2 mL of a 1:1 Hexane:Ethyl Acetate mixture by vortexing the sample mixture for 1 min, followed by centrifugation at 2000 rpm for 3 min. The supernatant was collected and evaporated to dryness under a stream of nitrogen and brought up into 200 µL methanol. 100 µL of this sample was placed into a GC vial to quantify testosterone via a developed protocol for testosterone. The remaining 100 µL was dried under a stream of nitrogen, 100 µL of 1 mg/mL dansyl chloride (Sigma Aldrich) solution in acetone was added to the dried sample along with 100 µL of 0.1ml NaHCO3/Na2NO3 (PH 10.5) buffer. The solution was mixed for 1 min and incubated at 60 °C for 5 min. 1 mL of nanopure water was added, and the solution was extracted twice as described above. The extracted sample was evaporated to dryness under a stream of nitrogen and brought up into 100 µL acetone, transferred to a GC vial and quantified for 17β-estradiol.

Exposure of gonad explants to forskolin and prochloraz resulted in a concentration dependent increase and decrease, respectively for northern pike, walleye and white sucker (data not shown). Exposure of lake whitefish gonad explants showed no significant change in hormone production compared to control (data not shown). Among the different time points tested, the 24 h incubation time resulted in significant changes in hormone production, with less variation compared to the 36 h incubation. Therefore, 24 h was chosen as the incubation time for the future exposures. Due to the minimal response in lake whitefish, along with lake whitefish having a different reproductive cycle compared to the other three species tested (lake whitefish spawn in the fall), lake whitefish were removed from the study design and were not tested in future studies. Finally, due to the response of the tissues to the three concentrations of each

chemical, 0.3 to 10 μM forskolin and 0.03 to 1.0 μM was chosen as the range of concentrations to be tested in future studies.

Liver explant assay

Sexually mature northern pike, walleye, and white sucker were sampled using gill nets from Lake Diefenbaker, Saskatchewan, Canada during July of 2012. Mass (± 0.1 kg) and fork length (± 0.5 cm) were measured and recorded for each individual. Gonad tissue was excised and weighted (± 1.0 g) and recorded to calculate GSI. Gonad tissues were sliced into smaller pieces and immediately transported to the Toxicology Centre, University of Saskatchewan in ice cold supplemented Leibovit L-15 media (13.8g of L-15 powder per litre medium, 420 mg $NaHCO_3$/L, 1% antibiotic-antimicotic [100 units penicillin, 0.1 mg streptomycin and 0.25 μg amphotericin B per mL], pH 7.6) (Sigma Aldrich). The time between sampling of fish and initiation of the exposure did not exceed 5 h.

Liver tissue was sliced into $1mm^3$ sections and rinsed several times with supplemented L-15 media. Two to three pieces of liver were added to each well of a 24-well plate containing L-15 medium. EE2 was added to the sample wells to a final concentration of 0, 3, 10, 30, 100, 300, 1000, 3000 ng/L in 0.1% DMSO. Each concentration was dosed in triplicate for each of northern pike, walleye and white sucker. Samples were incubated at 15 °C for 24 or 48 h on a platform rocker. Upon termination of the exposure, tissue was removed from each well and placed into microcentrifuge tubes and stored frozen at -80 °C for subsequent analysis of transcript abundance. The same procedure was used to isolate total RNA, make cDNA and quantify transcript abundance VTG, ERα and ERβ of interest as described in section 3.35.

Exposure of liver explants to EE2 resulted in a concentration related increase in transcript abundance of VTG for the 24 and 48h incubation times (data not shown). The 48 h incubation time resulted in a greater increase in transcript abundance of VTG compared to the 24 h time point, however, there was greater variation among the replicates within the 48 h incubation time. Therefore, 24 h was chosen as the incubation time point for the future exposures. To keep the methods consistent among species tested and allow comparison among species, 24 h was chosen for the white sturgeon exposure as well.