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Impacts of Calgary's Municipal Wastewater Treatment Plant Effluents on Reproduction of Fish in the Bow River

by

Vaidehi Patel

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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Abstract

The purpose of this study was to investigate the reproductive impairment in fish exposed to treated municipal effluents in the Bow River. Adult fathead minnows were caged at five different sites along the Bow River for 26 days at upstream and downstream from the City of Calgary municipal wastewater treatment plants (WWTPs).

The results provide evidence that exposure to treated municipal WWTP effluents discharged into the Bow River adversely affect reproduction in both male and female fish. The evidence is based on changes in gonadosomatic index, spermatogenesis by flow cytometry, gonadal morphology, caspase activity as a measure of apoptosis, measurement of transcripts for enzymes involved in the control of steroidogenesis, and gonadal steroid levels.

The results provide evidence that treated WWWP effluents discharged in the Bow River from Bonnybrook and Fish Creek WWTPs contains contaminants of emerging concern with hormone-like activity and highlights a need to improve wastewater treatment process.

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I would like to express my heartfelt gratitude to Dr. Hamid R. Habibi for his supervision, guidance, support and all the opportunities he has given me to advance my knowledge and learn new skills. Despite the mistakes I have made, Dr. Habibi has been very calm and taught me to overcome those hurdles positively. His suggestions and teachings have strengthened my understanding of the field and thus develop ideas for research. Dr. Hamid Habibi has taught me to be a good researcher and teacher thus shaping me professionally.

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Dedication

To my loving parents

For their support and encouragement

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List of Symbols, Abbreviations, and Nomenclature

Symbol	Definition
HPG Axis	Hypothalamus - Pituitary - Gonadal Axis
EDC	Endocrine Disrupting Chemicals
WWTPs	Wastewater Treatment Plants
Esr	Estrogen Receptors
AR	Androgen Receptors
E2	Estradiol
11 - KT	11 - Ketotestosterone
RT - qPCR	Real-time quantitative Polymerase chain reaction
cDNA	Complementary Deoxyribonucleic Acid
mRNA	Messenger Deoxyribonucleic Acid
ANOVA	Analysis of Variance
GSI	Gonadosomatic Index
CYP11a	Cytochrome P450 family 11
CYP19a	Gonadal Aromatase
CECs	Contaminants of emerging concern
VTG	Vitellogenin
T	Testosterone
HSD	Hydroxysteroid Dehydrogenase
NSD	No significant difference
GnRH	Gonadotrophin releasing hormone
FSH	Follicle stimulating hormone

LH	Luteinizing hormone
GnRH-R	Gonadotrophin releasing hormone receptor
FSHR	Follicle stimulating hormone receptor
LHR	Luteinizing hormone receptor
E	Efficiency

Chapter 1 – Introduction

In an attempt to provide proper health and sanitation to the ever-increasing global population with the limited supply of water resources, the idea of waste disposal system in many countries across the globe was established (Habibi, 2014). For the very first time, the idea of wastewater treatment was implemented in the 1850s to deal with the cholera outbreak. The wastewater treatment technique then was focused on chlorine treatment to purify the water, which paved the way for water disinfection. It was in the early 1970s that the purification techniques shifted the focus from treatment of water to remove the disease-causing microorganisms that cause waterborne illness, to anthropogenic water pollution such as pesticide residues and industrial sludge and organic chemicals (Ashton and Ubido, 1991). Owing to rapid scientific evolution, other physiochemical methods such as rapid sand filtration, coagulation, and sedimentation were implemented for the first time in the early 1890s. Until the 1990s, the primary concern for the WWTPs was to remove the presence of non-polar hazardous substances (persistent organic pollutants-POPs). Later, the WWTP technologies were upgraded to remove various dangerous compounds such as macro-sized contaminants, particulate matter, and a percentage of endocrine disrupting chemicals (EDCs) from wastewater (Petrovic, 2002). Today, the top-notch conventional water treatment plants which use advanced physicochemical techniques are designed to manage better and regulate the biological oxygen demand (BOD), chemical oxygen demand (COD) and to eliminate suspended solids from the industrial and domestic wastewater. These WWTPs also stresses on the reduction of specific nutrients as well as metals, chemicals (organic and inorganic) and toxic substances (Petrovic, 2002). Although wastewater treatment techniques have been upgraded radically over the years in most of the provinces across the globe, there are still concerns about degradation of ecosystem health in receiving waters. Despite all the upgradation of advanced scientific techniques such as wastewater treatment technologies such as UV treatment, activated carbon, ozonation (O3), and

ultrafiltration, however, trace amounts of contaminants make their way into the natural environment via treated effluents released from WWTP (Jefferies et al., 2008a, b; Mailler et al., 2015; Melcer and Klecka, 2011; Toro-Velez et al., 2016). Further research is required to study the fate of these toxicants along with their byproducts into the receiving waters and biosolids (Haarstad et al., 2012).

The contaminants of emerging concerns (CECs), today, are found just about everywhere and in everything including but not limited to: cosmetics, shampoos and other personal care products, electronics, pesticides, herbicides, fertilizers from agricultural sources, pharmaceuticals, natural and synthetic hormones, industrial chemicals and of course as additives or contaminants in the processed foods we eat every day. These contaminants enter the WWTPs through several pathways such as, but not limited to disposal and use of consumer products like soap, shampoo, disinfectant washes, and toothpaste which contain biologically active compounds, farm runoff, toxic spills, and excretion via the urine and feces of those consuming pharmaceuticals. The pharmaceuticals exclusively designed for the human body consumption, only metabolizes a percentage of each drug taken, expelling the rest into wastewater, which releases these contaminants into the sewer system are then transported to a wastewater treatment plant (WWTP). Therefore, the final effluent contains a certain percentage of mixture of nutrients and contaminants, including pharmaceuticals and personal care products (PPCPs), metals, and polycyclic aromatic hydrocarbons and their byproducts, which varies in composition depending on the waste sources.

1.1. Conventional Municipal Wastewater Treatment Plants

Wastewater is a combination of domestic, industrial, commercial, agricultural activities, and surface runoff or stormwater which is made up of grit, debris, suspended solids, disease-causing pathogens, decaying organic wastes, nutrients and over more than 200 identified chemicals.

Wastewater treatment is a complex series of processes that involves several steps to remove solids, nutrients, and contaminants (Metcalf & Eddy et al., 2003). The conventional WWTP applies the following steps to treat the wastewater. The sequence of processes is as follows:

The wastewater which enters the WWTP facility, passes through to large filter screens that facilitate the removal of coarse debris and larger solid particles such as plastic bags, toilet paper, sticks large solids, trash, garbage and then passed into grit removers. The wastewater then flows into grit tanks, which allows the solid particulate debris such as sand, gravel, etc., to settle down at the bottom, where it is collected and transported to the landfills. The wastewater coming out of the grit tanks is known as Primary Influent.

- **Primary Clarifier**

The primary influent then makes its way into the large open-air tanks known as primary clarifiers. Within the clarifiers, the particulate matter settles to the bottom becoming sludge, which takes approximately four hours. The bottom sludge and top waste which includes scum and grease are collected and pumped to the anaerobic digesters. The remaining wastewater from the primary clarifiers or primary effluent moves into the secondary liquid treatment process (Metcalf & Eddy, 1972).

- **Bio Reactor**

Within the bioreactors, the primary effluent is treated with a mixture of micro-organisms in the presence of air, phosphorus and nitrogen. Thus, providing an appropriate environment for the microorganisms and the organic matter to react with each other. The retention time in the bioreactors is approximately seven hours. The mixed liquid, the combination of activated sludge and primary effluent, then moves to the secondary clarifiers for further processing (Metcalf & Eddy, 1972).

- **Secondary Clarifier**

Secondary clarifiers are aerobic tanks which facilitates the settling of influents to form activated sludge for about seven hours. The activated sludge is pumped to the bioreactors and aeration tanks for further processing while the remaining sludge requires thickening. While the secondary effluents flow to the disinfection process (Metcalf & Eddy, 1972).

- **Disinfection (Ultraviolet Rays)**

Secondary clarifier effluent flows into the disinfection tanks, which is then subjected to high-intensity ultraviolet radiation (UV light). The UV light interferes with the microbial community genetics and subsequently preventing the ability to reproduce and to avoid disease. After disinfection, the effluent flows directly into the Bow River. The final effluent is considered transparent, high in oxygen, low in organic matter, ammonia, phosphorus, and microorganisms (Metcalf & Eddy, 1972).

Despite the advances in the wastewater treatment plants, these treatment plants focus on removal of BOD, nutrients, solid waste and certain microorganisms, while certain contaminants are partially or seldom treated. Sometimes incoming volumes of wastewater are too much for the treatment plant to handle, leading to the sewer bypassing, thus the raw or partially treated sewage enters the environment. This phenomenon happens more frequently with combined systems (i.e., stormwater) during or just after rainstorms (City of Calgary., 2009). The pharmaceuticals which are exclusively designed for the human body consumption, only metabolizes a percentage of each drug taken, while expelling the rest into wastewater, which releases these contaminants into the sewer system are then transported to a wastewater treatment plant (WWTP). Therefore, the

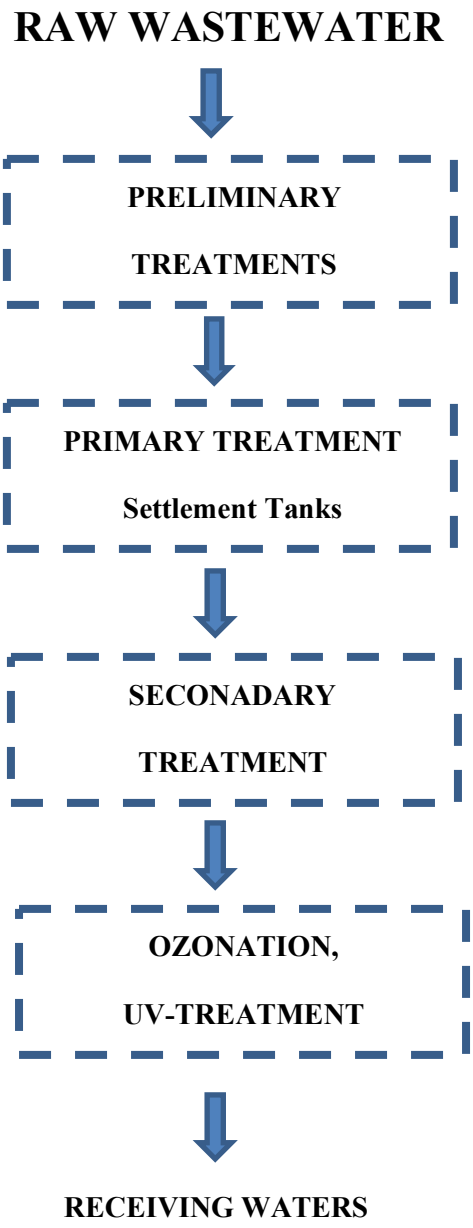

Figure 1.1 Schematic diagram of the process involved in a conventional wastewater treatment plant (WWTP).

final effluent contains a certain percentage of mixture of nutrients and contaminants, including pharmaceuticals and personal care products (PPCPs), metals, and polycyclic aromatic hydrocarbons and their byproducts, which varies in composition depending on the waste sources.

1.2. Treated Effluents and its Mode of Action

The treated effluents represent the largest point source discharges of pollutants to Canadian surface waters and often contain a number of emerging contaminants. The emerging contaminants are detected in the environment, but lack sufficient information to determine potential environmental and public health risk (Drewes and Shore, 2001). Many of these emerging contaminants act as endocrine disruptors as they may mimic or block the activity of endogenous steroid hormones by binding to their receptors. These contaminants present in the treated effluents possess the capacity to alter the biosynthesis, secretion, transport, binding action, and metabolism of endogenous hormones. In addition, they have been demonstrated to interfere with synthesis and metabolism of hormone receptors. There is substantial evidence demonstrating the presence of the CECs - contaminants of emerging concern in the treated effluents negatively impacts the growth, development, maturation, and reproduction in the aquatic organisms (Lee et al., 2003). The emerging contaminants also demonstrate low acute toxicity but cause significant health effects at very low levels of exposure.

The endocrine system consists of endocrine tissues and glands such as hypothalamus, pituitary, thyroid, gonads and liver. Each of the aforementioned organs are well integrated system which produces a unique suite of hormones that help regulate the crucial processes for growth, metabolism, reproduction, and behaviour (Trudeau, 1997). The endocrine system is highly

receptive to the contaminants of emerging concern which is present in the treated WWTP effleunts (Mills and Chichester, 2005). The mode of action by which the contaiminants target the endocrine system varies greatly. Some toxicants appear to target the binding sites via agonists and antagonist's mechanisms and thus alter the activity of the cell. They are also capable to change the natural pattern of hormone synthesis and metabolism, resulting in improper balance or quantity of circulating hormones (Habibi, 2014; Hontela and Habibi, 2014; Jeffries et al., 2008, 2010). Additionally, the same chemicals have the potential to bind to several different receptors in different tissues, subsequently affecting several hormone pathways (Diamanti-Kandarakis et al., 2009; Jordan et al., 2012; Kinch et al., 2015, 2016).

These contaminants present in the treated effleunts are biologically active at low concentrations (parts per trillion - ng/L). These CECs are also known as "pseudo-persistent" because they are discharged into the aquatic environment on a continual basis and are short lived. (Jordan et al., 2012; Kinch et al., 2015, 2016; Sumpter and Jobling, 2013). These compounds tend to bioaccumulate within animals and biomagnify along the food web, causing a wide variety of adverse health effects on wildlife and humans (Beyer and Biziuk, 2009). Bioaccumulation is usually associated with environmental exposure where biota is exposed through multiple pathways such as ambient water, food, sediment, etc. The contaminants of emerging concern that are present in treated effluents, released into the aquatic ecosystem, where they may absorb to sediments and persist for relatively long periods. Researchers hypothesized that EDCs could be hydrophobic substances with affinity to bind to particles and accumulate on bottom sediments.

The mechanisms by which the treated effluents exert their impacts involve divergent pathways including (but not limited to) estrogenic, ant androgenic, thyroid, peroxisome proliferator-activated receptor γ, retinoid, and actions through other nuclear receptors; steroidogenic enzymes;

neurotransmitter receptors and systems; and many different pathways that are highly conserved in wildlife and humans (Diamanti-Kandarakis et al., 2009; Forner-Piquer et al., 2017; Santangeli et al., 2017).

The occurrence and fate of the contaminants and their byproducts in the WWTP effluents and the environment is extremely complicated. Researchers have showed that the biodegraded contaminants have disrupting properties with similar or even more adverse effects relative to the parent compound. For instance, during the WWTP treatment NP biogrades to form nonylphenol ethoxylates. Although almost 95% NP can be removed from the effluent, while there have been reports of increased concentrations of nonylphenol ethoxylates discharged into the receiving rivers (Barber et al., 2015).

Interestingly, laboratory studies and regulatory decisions are mostly based on single exposure chemicals. Although these contaminants are present in low concentrations, there is clear evidence that when compounds are present in mixtures, they can elicit dose additive, synergistic, or antagonistic effects. The synergistic effect is particularly important when these compounds present in the mixture act through the same pathway and the concentration of each compound is below its respective threshold effect, but cumulative exposure to these chemicals elicits adverse effects (Kortenkamp, 2007; Laetz et al., 2009; Rider et al., 2008).

Synergistic effects of these contaminants of emerging concern in a mixture, which are mostly reported in the case of CECs with similar modes of action, have been well documented using various endpoints. For instance, the male fathead minnows exposed to environmentally relevant concentrations of two anti-androgens, the herbicide linuron, and DEHP in synergism resulted in

decreased levels of testosterone and E2 by mixture exposure, which was not observed in the single exposure treatments (Crago and Klaper, 2012).

The additive effects of CECs in a mixture also have been reported in the case of compounds that induce the same effects through various mechanisms (Kortenkamp, 2007). For example, a mixture of seven anti-androgens that are responsible for disrupting the androgen signaling pathway via various mechanisms were shown to cause reproductive disruption in rats in a cumulative and dose-additive manner (Rider et al., 2008). CECs with different properties (e.g. anti-estrogenic or androgenic) are theoretically capable of neutralizing estrogenic effect of estrogenic compounds (Santos et al., 2006; Sun et al., 2009; Witorsch, 2002). Several co-exposure studies have been conducted to clarify the combined effects of CECs with different modes of action. Anti-estrogens may be able to neutralize estrogenicity of some estrogenic compounds in a mixture to a degree at the level of biomarkers. However, the adverse effects of estrogenic compounds on reproductive function were narrowly improved, and in some cases, became even more adverse (Sun et al., 2009).

1.3. Treated effluents and HPG Axis

Reproduction in teleost's and vertebrates is under the tight control of the HPG axis (Habibi et al., 2012; Habibi and Andreu-Vieyra, 2007; Kim et al., 2012). Upon stimulation from environmental and internal cues, the gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, which binds to its receptor (GnRH-R) in the pituitary and triggers the production and release two gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. These hormones regulate gametogenesis and steroidogenesis through interaction with their respective receptors, luteinizing hormone receptor (LHR), and follicle-stimulating hormone receptor (FSHR), within the gonads (Habibi and Andreu-Vieyra,

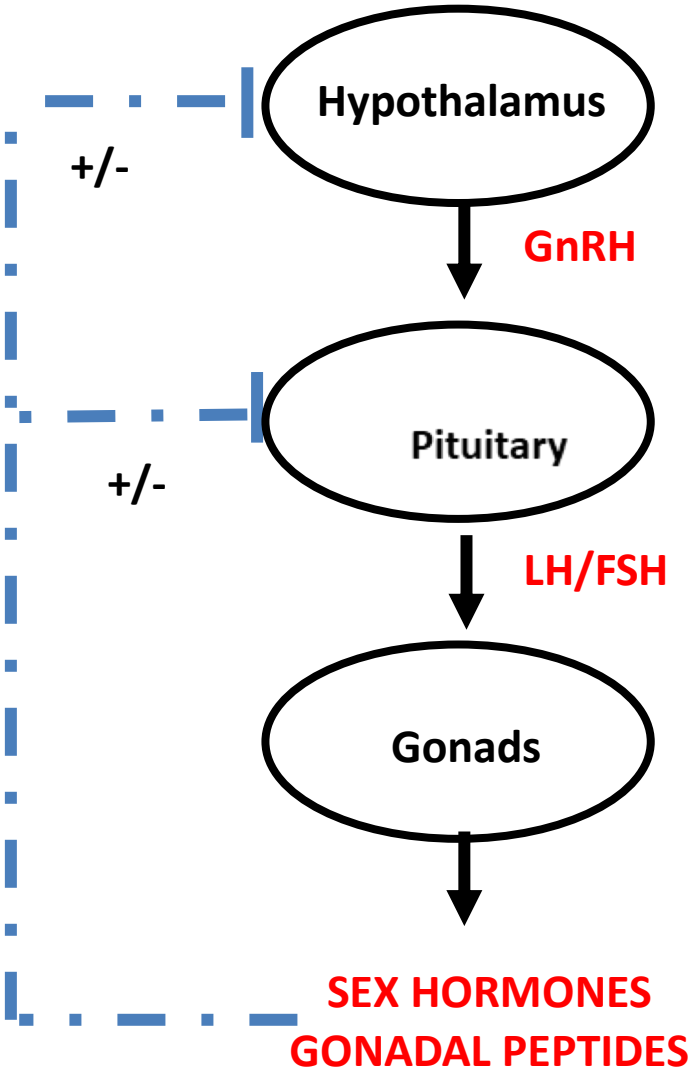

Figure 1.2 Overview of the teleost hypothalamic pituitary gonadal axis. The linkages between the components of the axis show how homeostasis is regulated. Solid arrows indicate synthesis; dashed arrows indicate positive and negative feedback mechanisms.

2007; Moussavi M, et al., 2012, 2013; Rempel and Schlenk, 2008). In teleosts, gonadotropins stimulate the synthesis of gonadal sex steroids such as 17β estradiol (E2) and testosterone (T) (Guzman et al., 2014; Kobayashi et al., 2008; Kwok et al., 2005; Weltzien et al., 2003). The sex steroids are responsible for the stimulation of vitellogenesis, gonadal development, oocyte maturation, spermatogenesis, activin and inhibin synthesis/release, as well as gonadal steroidogenesis (Kobayashi et al., 2008; Kwok et al., 2005; Weltzien et al., 2003). Any disruptions in the hypothalamus-pituitary-gonadal axis that cause hormone level changes could potentially lead to reproductive impairment.

The following is a brief synopsis of treated effluents toxicity reported to date using a wide range of model organisms from aquatic invertebrates to rodents.

1.3.1. Aquatic Invertebrates

Various studies reported induction of Vn (Vitellins) like proteins in male and juvenile females and reproductive impairment in aquatic invertebrates. There was a significant induction of vitellins (Vn), which acts as energy reserves for embryo development in oviparous organisms, reported in soft shell clams (Mya arenaria) collected at the sites closer to local sewer outfall zone (Gagné et al., 2002). Vn-like protein levels increased in both the hemolymph and the gonad of the female and male mussels when exposed to municipal effluent. In another study, mussels were caged along the St. Lawrence River which resulted in higher Vg-like protein levels in hemolymph at the downstream site when compared to the upstream site where females showed higher Vg-like protein levels than males (Blaise et al., 2003). A notable effect of municipal effluents on mussel sex ratio was also observed at the polluted sites along the St. Lawrence River, indicating that the exposure to a municipal effluent induced feminization in freshwater mussels (Blaise et al., 2003).

1.3.2. Amphibians

An increased occurrence of male testicular oocytes was observed following exposure to 50%, and 100% treated effluents when compared to the control treatment in the Northern leopard frog, Rana pipiens (Sowers et al., 2009). Female-biased sex ratios have been also seen in previous studies following exposure to estrogenic compounds, suggesting that amphibian species are susceptible to sex reversal resulting in genotypic males becoming phenotypic females (Hogan et al., 2008; Pettersson and Berg, 2007). Exposure to treated effluents in Xenopus laevis and Northern leopard frog, Rana pipiens resulted in delayed metamorphosis by targeting the HPT axis (Hogan et al., 2008). An increased incidence of male frogs containing testicular oocytes in response to larval exposure to estrogenic compounds present in treated wastewater has been observed in prior studies (Hogan et al., 2008). Larval exposure to final municipal wastewater treatment effluent delayed metamorphosis and altered male gonadal development in R. pipiens juveniles (Sowers et al., 2009).

1.3.3. Mammals

Wistar rats exposed to treated effluents in the northern region of India exhibited a decreased level of gonadotrophic hormones and increased testosterone level, histopathological changes were observed in kidneys and liver of the exposed male rats. Also, a significant difference in the abundance patterns of the vital steroidogenic enzymes was noted in adrenal and testis: CYP11a, CYP17, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase was observed (V. Kumar et al., 2008). Behavioral changes such as increased aggression and cannibalism in albino rats exposed to treated effluents (Ogwu et al., 2014). Following the exposure of lactating female rats to treated effluents, the lactating male pups upon reaching adulthood, displayed delayed spermatogenesis and decreased sperm counts and motility (Aravindakshan et al., 2004b).

1.3.4. Fish

Aquatic ecosystems are especially susceptible to contaminant loadings as they receive continuous inputs through discharge and runoff. Fish play an integral role in the aquatic ecosystem food web, and any effects that change the population structure of fish may also alter community and food web dynamics. Fish carrying high loads of CECs in their body tissue thus, suffer impaired health and can deliver high concentrations of CECs to their consumers. Many species of fish, in particular those species near the top of the food chain, are generally regarded as integrators of environmental conditions and may reflect, therefore, the health of aquatic ecosystems and therefore, they are excellent indicators of the relative health of aquatic ecosystems and their surrounding watersheds. Today, many of the teleosts are extensively used to study field based toxicological studies. Teleost fish are the most varied and abundant group of vertebrates on Earth (Annett et al., 2014; Golshan et al., 2014, 2016; Kinch et al., 2015, 2016). They are essential both commercially and scientifically.

While the impacts of treated effluents have been examined in a variety of model organisms, the effects have been explored most frequently in fish species and are summarized in (Table 1.1).

1.4. Evidence for endocrine disruption in wild fish

Scientific evidence has demonstrated that a large number of environmental contaminants can modulate or interfere with the actions of steroid hormones and impair reproductive function in adults, while also leading to irreversible malfunctions when exposed during susceptible developmental periods (Huang et al., 2015). The adverse effects of treated effluents on fish have been documented since the early 1980s when a group of sports fishermen noticed estrogenic impacts that expressed elevated levels of VTG in fish caged in receiving waters of sewage effluents in the UK (Purdom et al., 1994; Tyler and Jobling 2008). Subsequent investigations have

demonstrated that exposure to environmental contaminants can result in hormonal imbalance and feminization in fish. For example, high incidence of intersex characteristics (co-occurrence of ovarian and testicular tissue in gonads) has been observed in wild populations of roach (Rutilus rutilus), flounder (Platichthys flesus) and gudgeon (Gobio gobio) (Bjerregaard et al., 2006; Jobling et al., 1998). Further, sewage effluent has been suggested to be the causal factor for elevated levels of VTG and inhibition of gonadal growth observed in male bream (Abramis brama) (Vethaak et al., 2005) and depressed T levels in male carp (Cyprinus carpio) (Lavado et al., 2004; Petrovic et al., 2002). Environmental androgens and anti-androgens have until recently been overlooked, most likely because of the lack of a sensitive system for the detection of androgenic activity (Katsiadaki et al., 2006). The most obvious case of androgenic disruption in the aquatic environment is the masculinization of fish living downstream of pulp mill effluent discharges (Howell et al., 1980). Moreover, the exposure to treated effluents may also affect reproductive behavior. Another study conducted by Jefferies et al. (2008a, 2008b) observed endocrine disruption occurring along these rivers at a basin-wide scale. Vitellogenin, an estrogen mediated process was induced in male Longnose Dace (Rhinichthys cataractae) at every site along the Oldman and Bow Rivers indicating widespread exposure to the treated effluents. These findings are in conjunction with the detection of many compounds with endocrine activity in sewage effluent and their receiving waters throughout Alberta (Sosiak and Hebben 2005, Jeffries et al. 2010, Evans et al. 2012).

1.5. Model Organism

For this study, I used Fathead Minnows (Pimephales promelas) as they are native to the Bow River. Fathead minnows, freshwater species, belongs to ecologically important Cyprinidae family, widely distributed across North America and Europe. Fathead minnows are small bodied fish, adult male fathead minnow is usually larger than the female (3–5 g versus 2–3 g, respectively).

The reproductively active males exhibit several secondary sex characteristics such as dark banding, dorsal pad, and nuptial tubercles not generally seen in females, thus making it an excellent model to study reproductive toxicology. Fathead minnows, make an excellent model organism for toxicological studies as they have shorter lifespans (achieves reproductive maturity within 4–5 months of hatch and, depending on water temperature and photoperiod). They can spawn continually for several months (Brungs, 1971a). It spawns multiple times per breeding season, every 3 to 4 days (Watanabe et al. 2007; Jensen et al. 2001) for females while males can breed continuously. The main spawning season of the fathead minnow is from mid-May to early August. Due to its relatively well-define reproductive/developmental cycle, fathead minnows have been extensively used to study ecotoxicological studies. The other reason which makes fathead minnows a good model for toxicology studies is due the fact that the genome of fathead minnow is well established, and many molecular methods have been developed using its genetic information. From a survival standpoint, fathead minnows, make an excellent model organism for toxicological studies as they are tolerant of a wide range of essential water quality parameters including pH, alkalinity/hardness, turbidity, and temperature. Fathead minnows are suitable study model in various physiology, endocrinology and neurobiology research (Harries et al., 2000; Jensen et al., 2001), and have been used successfully in toxicology and endocrine disruption studies (Garcia – Reyero et al., 2009; Filby et al., 2007).

The most frequently used adult fish species in treated effluents toxicity studies has been the fathead minnow and effects have ranged from adversely affecting fecundity (reproductive rate), altering levels of sex steroid synthesis (11-ketotestosterone, testosterone, and 17β-estradiol), reduction in secondary sexual characteristics, and altering the transcript abundances of genes and enzymes involved in gonadotropins, estrogen-responsiveness, metabolism of endogenous and exogenous

substrates, oxidative stress, apoptosis, and immune function (Bjerregaard et al., 2006; Jobling et al., 1998; van Aerle et al., 2001).

1.6. Previous studies in Southern Alberta

The efficiency of the effluent treatment techniques at sewage treatment plants is of critical importance for preventing the contamination of the aquatic environment by contaminants. Removal efficiencies of pollutants have shown a considerable variability depending on treatment technologies in the WWTPs and the varying nature and the composition of the incoming wastewater. Previous studies conducted by Metcalfe et al., 2003 demonstrated the presence of different types of pharmaceuticals and personal care products in the Bow River. Efficiency removal studies conducted by Metcalfe et al., 2003 reported the presence of ibuprofen, carbamazepine, naproxen, diclofenac, and bezafibrate in Calgary's treated WWTP effluents. Also, low levels of antibiotics (<0.6 μg/L) were detected in Calgary's WWTP effluents; the results indicate that these compounds are not eliminated from wastewater during treatment (Chen et al., 2015) in the Bonnybrook WWTP and Fish Creek WWTP. The compounds detected in the Bow River and Oldman River included synthetic estrogens (birth control pill compounds and hormone therapy drugs) downstream of municipal wastewater effluents and natural hormones downstream of municipal wastewater effluents and in agricultural areas. Greater concentrations of cholesterol and derivatives, pharmaceuticals (diclofenac, gemfibrozil, ibuprofen and indomethacin), Bisphenol A, DEHP, β-Sitosterol, phytosterols, and fecal sterols were measured at the most downstream sites along Bow River (Sosiak and Hebben 2005, Jeffries et al., 2008, 2010, Evans et al. 2012). In addition, other pharmaceuticals such as erythromycin, tetracyclines, fluoroquinolones, antidepressants, an anti-inflammatory like ibuprofen, naproxen, carbamazepine are also detected in the WWTP effluents in waters at other Canadian locations (Miao et al., 2004,

Metcalfe and Eddy, 2003; Lishman et al., 2006). Also, natural hormones such as 17β-estradiol and estrone (Servos et al., 2001), and industrial compounds such as bisphenol-A (Lee et al., 2003) and nonylphenol (Servos et al., 2001) can be detected in the aquatic ecosystem. Individual chemicals may exert adverse impact in a particular manner. However, it is important to understand the effect of these contaminants as mixtures. The synergistic effect of these pollutants may significantly have different potency or exert different effects by unpredictably interacting with each other and target tissues (Jordan et al., 2012).

1.7. Rationale, Hypothesis, and General Objective

The vast majority of studies on fish toxicity have been carried out with particular contaminants present in the treated effluents. Although there is evidence suggesting the presence of endocrine disruptors in the Bow River, our knowledge on the harmful effects of contaminants in the treated effluents discharged into the Bow River from Bonnybrook WWTP and Fish Creek WWTP is limited. Previous studies in our lab have observed a severe endocrine disruption and feminization at multiple sites in the Oldman River (AB, Canada) among the longnose dace (Rhinichthys cataractae) (Evans et al., 2012; Jeffries et al., 2008; Jeffries et al., 2010). A female-biased sex population with elevated levels of VTG mRNA abundance in male fish, and increased incidents of intersex formation (development of the ovarian tissue in the testis or vice versa) were observed (Evans et al., 2012; Jeffries et al., 2008; Jeffries et al., 2010). Female-biased sex ratios, skewed up to 90%, were observed in the Oldman River; however, sex ratios in the Bow River were not significantly female-biased (Henderson et al., 2014, M. Sc thesis, University of Calgary, 2014). While studies such as those of Jefferies et al., 2008a, 2008b are essential in understanding basin-wide patterns of endocrine disruption, they do not focus on the molecular mechanisms involved in impacts of treated effluents on reproductive impairment.

This information led me to make my main hypothesis that, EDCs present in Alberta Rivers are responsible for abnormalities such as female-biased sex ratio, intersexualization, and the increase in VTG expression of fish in the Oldman River. Thus, the primary objective of the study was to better understand the mechanism involved in the reproductive impairment caused by treated WWTP effluents released into the Bow River. In the present study, I measured gonadosomatic index, spermatogenesis by flow cytometry, gonadal morphology, caspase activity as a measure of apoptosis, measurement of transcripts for genes involved in the control of steroidogenesis, and gonadal steroid levels to investigate disruption of reproduction in fathead minnows caged for 26 days in five different sites (2 upstream site and 3 downstream sites) along the Bow River.

Hypothesis - 1 -

I hypothesize that the treated WWTP effluents released into the Bow River alter the abundance of VTG and sex steroid receptors in the male fathead minnows.

Hypothesis - 2 -

I hypothesize that the treated WWTP effluents adversely impact the gonadal development in the fathead minnows by targeting the process of gonadal apoptosis.

Hypothesis - 3 -

I hypothesize that the treated effluents adversely impact the process of gonadal steroidogenesis by targeting the expression of steroidogenic enzymes in the fathead minnows exposed to the treated WWTP effluents along the Bow River.

The physiological response to the treated effluents to assess the endocrine disruption focusing mainly on reproductive endpoints using previously develop biomarkers (Table 1.2).

Table 1.1. Adult teleost species effects following exposure to treated effluents

Fish Species	Exposure to treated effluents	Reported Effects	Citations
Rainbow Darter *(Etheostoma caeruleum*)	Grand River - Waterloo, Canada	Induction of VTG and Intersex in exposed males, altered sexual differentiation and development in both the sexes	P.A. Bahamonde et al., 2014
White Suckers (Catostomus commersoni)	City of Boulder, USA	Gonadal intersex, altered sex ratios, reduced gonad size, disrupted ovarian and testicular histopathology, and vitellogenin induction in exposed male	Vajda et al., 2008
Male fathead minnows (*Pimephales promelas*)	48h, USA	Altered gene expression in gonads and liver involved in reproduction, fish sexual behavior, more aggressive behavior in caged male fishes exposed to effluents	Garcia-Reyero et al., 2009, 2011
Male fathead minnows (*Pimephales promelas*)	UK waters	Alteration in gene expression in gonads and liver involved in growth, development and reproduction	Filby et al., 2007
Smallmouth bass, sucker	Pennsylvania - USA	Induction of VTG in males, presence of testicular oocytes in smallmouth bass	Blazer et al., 2014

Table 1.2. Biomarkers measured in the current study to assess the effects of treated effluents on endocrine disruption mainly focussing on the reproductive endpoint on the caged fathead minnows along the Bow River, Calgary, Alberta

Biomarkers	**Indicator**
Caspase Activity	Gonadal apoptosis - To measure the gonadal degeneration
Histology	Gonadal morphology - To detect the presence of intersex condition in the gonads, if any
Flow Cytometry	Spermatogenesis - To study the process of spermatogenesis, and androgen disruption
Vitellogenesis	Estrogenic disruption - biomarker of exposure to estrogenic compounds
Sex steroid receptors	To study the alteration in hormone signalling
Steroidogenesis	To investigate the mechanistic basis of disruption in the process of steroidogenesis
Hormone Measurement	To measure the circulating levels of sex steroid as a reproductive endpoint to evaluate the impact of an effluent on fish

Chapter 2 – Methodology

2.1. Study System

The City of Calgary is one of the fastest growing populations within the province of Alberta (BRBC, 2010). The City of Calgary covers an area of approximately 825 km^2. The Bow River flows roughly about 645 km from the continental divide of the Rocky Mountains southeast to its confluence with the Oldman River. The City of Calgary has a population roughly over 1.12 million residents, producing more than 200 megaliters of raw wastewater per day (BRBC, 2015). The stormwater and municipal wastewater outfalls contributes to the contaminant load in the Bow River. Stormwater includes the urban runoff which discharges into the Bow River or may send stormwater to retention ponds relying heavily on sedimentation to remove pollutants (City of Calgary, 2011).

2.1.1. Bonnybrook WWTP

The Bonnybrook WWTP is one of the oldest WWTP, located in the center of the City of Calgary. Bonnybrook WWTP has a capacity of 500,000 m3/day and serves roughly 880,000 citizens and some industries, or approximately 85% of Calgary's wastewater. This treatment plant is the top-rated facility in Canada (Bathory et al., 2005), and has several steps in its treatment process: primary treatment to remove and digest organic material; secondary (biological) treatment for the removal of nutrients; and tertiary (UV disinfectant) treatment to process the wastewater prior to its release into the Bow River (Bathory et al., 2005).

2.1.2. Fish Creek WWTP

Fish Creek WWTP has a capacity of 500,000 m3/day and serves 150,000 citizens or 15% of Calgary’s wastewater. The Fish Creek WWTP is located downstream of the Bonnybrook facility and upstream to Pine Creek WWTP. The Fish Creek WWTP is also a tertiary treatment plant with chemical phosphorus removal and UV disinfection. In contrast to the Bonnybrook WWTP, the

Fish Creek WWTP does not employ BNR. Furthermore, the hydraulic retention time is also shorter in Fish Creek versus Bonnybrook WWTP (Chen et al., 2006).

2.1.3. Pine Creek WWTP

The Pine Creek WWTP is the newest of the three WWTPs in the City of Calgary commissioned in October 2008. The facility can treat up to 100 megaliters of wastewater per day (ML/d) to serve a population of 250,000, and the capacity could further be expandable to 700ML/d in future. The Pine Creek WWTP employs biological removal technologies as well as ultraviolet light disinfection (Lazaro – Cote, 2017, M.Sc Thesis, University of Calgary). Variances in treatment capacity and treatment type result in differences in contaminant profiles of each facility's final effluent (Chen et al., 2006).

2.2. Sampling Sites

For this study, five different sites were chosen, each location is different from each other, along the Bow River within the city limits of Calgary. Two major tertiary MWWTPs that are the focus of this study release effluent into the Bow River in this reach. The sampling sites for this study are as follows

- The first site was Bearspaw (BEAR -51°06'01.8"N 114°16'27.1"W) which is upstream to all the contamination sites and acts as the reference site.
- The second site was Cushing Bridge (CUSH -51°02'16.1"N 114°00'42.1"W), upstream to WWTP facilities but downstream to the untreated stormwater outfalls.
- The third site was 15 m downstream of the Bonnybrook WWTP outfall (Glenmore reservoir GLEN -51°00'32.6"N 114°01'08.5"W) which utilizes biological secondary treatment.
- The fourth site was 250 m downstream of the Fish Creek WWTP outfall (Highway 22X-H22X -50°54'27.1"N 114°00'39.3"W), utilizes chemical secondary treatment.

- The fifth site was (Uphigh-UPHI -50°49'09.0"N 113°47'32.3"W) which is 40 km downstream the Fish Creek WWTP and approximately 18 km downstream the Pine Creek WWTP, which means the impact of effluents could get diluted as we move farther away from the WWTPs. The figure 2.1 represents the five different caging studies along the Bow River.

2.3. Fish Acclimatization

The study was performed on adult fathead minnow, Pimephales promelas, age 4 – 5 months, with the approval of the institutional ethical committee. They were housed in in a recirculating system in 10 L tanks (Pentair Aquatic Habitats, Apopka FL) with 14 h light: 10 h dark schedule and the other parameters are as follows 750 µS conductivity, and pH 7.6. Fish were fed twice daily with a well-balanced fish fed consisting of goldfish flakes food in the morning (Pentair Aquatic Habitats) and bloodworms in the afternoon. The animals were acclimatized in the lab condition for two weeks prior to the exposure in the Bow River. Water temperature was gradually decreased to 16°C in a gradient manner during the course of two weeks. All experimental protocols were approved by the Animal Care and Use Committee at the University of Calgary and were in accordance with guidelines established by the Canadian Council for Animal Care.

2.3.1. Fish Sampling

The caging experiment was carried out in the Bow River, upstream and downstream of the two-municipal wastewater treatment plant, in Calgary, Alberta, Canada in September to October 2016. Five sites were selected: two upstream sites, and three downstream sites that were chosen. At each site, approximately 35 adult fathead minnows of unknown gender were caged in galvanized steel fathead minnow traps with 16.5-inches in overall length and 7.5-inches in diameter and mesh sizes approximately 8mm with the openings at each end closed with aquarium grade sealant to cover

any sharp edges. A PVC pipe (150mm long, 100mm diameter section) was fitted in the cage to provide shelter for the fish. The cages were held in the horizontally position with the aid of two steel reinforcing bars. Fish were not fed for the duration of the experiment. During the exposure period, we visited the sites every other day to check the cages and water chemistry, including temperature, dissolved oxygen, conductivity and pH (Table 2.1). One site was visited at one day and the all the fish were sampled at similar times (9.30 am to 11 am) of day. At each site, all fish were removed simultaneously from the tank and immediately anesthetized in MS 222 (0.1g/L tricaine methane sulphonate). Fork length, total body weight and sex were recorded, and fish were sacrificed by decapitation. Later, in the lab the weight of the gonads was recorded, the gonadosomatic index was calculated. Liver (VTG and sex steroid receptor mRNA abundance) and one gonad were dissected, placed in Eppendorf tubes and stored at -80C until analysis. The other gonad was transported to the lab in L-15 medium for histology, caspase activity and flow cytometry.

2.4. Caspase 3 Assay

Gonadal apoptosis was measured using caspase 3 enzyme activity in the gonads of the fishes exposed along the Bow River. Gonadal caspase 3 enzyme activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes, Invitrogen). The tissues from all the sites were brought to the lab in the L-15 medium. These gonadal tissues were homogenized, and the supernatant was collected as per manufacturer's instructions to be stored in -20°C for further analysis. Briefly, 50 µl of the sample supernatant and 50 µl of the 2× working substrate (5 µM Z-DEVD-R110) was added to an individual well of 96 well plate and further incubated for 2 hours. In this kit, the rhodamine 110-derived substrate (Z-DEVD-R110) used in this assay is a non-fluorescent bisamide compound that, upon enzymatic cleavage of caspase enzymes, is converted

in a two-step process to the fluorescent monoamide and then to the even more fluorescent R110 product. Fluorescent product of the substrate Z-DEVD-rhodamine 110 generated by caspase-3-like proteases was measured at 485 nm excitation and 538 nm emission using a Spectramax spectrofluorometer (Molecular Devices, Union City, CA). Caspase-3 activity was expressed as arbitrary units of fluorescence with respect to body weight (caspase activity/mg).

2.5. Histological Analysis

Gonad morphology and sex determinations of exposed fish and reference fish were performed by histological examination. A section of ovary or testis of the fathead minnow was immersed in 500µl of paraformaldehyde - glutaraldehyde solution for 24h at 4°C, dehydrated in graded ethanol solutions. The gonads were stored in 70% ethanol until the samples could be processed. The fixed gonadal tissues were oriented longitudinally and embedded in methacrylate historesin. The methacrylate blocks were sectioned at 4µm in a step-wise fashion, and tissue sections were placed on microscope slides, stained with toluidine blue stain and cover-slipped with Slide Mounting Media Cytoseal 60 (Richard Allan Scientific, Thermo Scientific). Males were classified into either immature or mature. The criterion for mature gonads of males was the presence of spermatozoa. Gonads with simultaneous presence of oocytes and testicular tissue were classified as intersex. Five males from each site were selected to evaluate the testiscular development. From each of these fish, five random images at 40× magnification were collected from each fish and analyzed using scoring the cell population. Recorded cell types were spermatogonia, spermatid, and spermatozoa. The relative proportions of each cell type within a fish were further calculated using flow cytometry. A cross section of an ovary from 5 female fish from each site was processed. Using a microtome, six to twelve thin sections (5–8 µm thickness) were produced, placed on glass slides and stained with toluidine blue before coversliping. Images of entire cross sections of each

ovary were prepared by stitching image fields at 2.5× magnification. All cell types containing a visible nucleus within these cross sections were scored and reported. Recorded cell types were primary, cortical alveolar and vitellogenic oocytes.

2.6. Flow Cytometry

Flow Cytometry analysis was performed to sort testicular cells into haploid and diploid populations. Adult fathead minnow's testes were minced in 1 ml of 1X phosphate buffered saline (PBS) supplemented with 5 mM ethylenediaminetetraacetic acid (EDTA). The minced testes were incubated for 15 min at 4°C with gentle agitation. Samples were treated with 800 µL of 0.05% trypsin-EDTA and dissociated for 10 min at room temperature. After neutralizing the trypsin-EDTA by Calf Bovine Serum (Thermo Fisher Scientific, Gibco®, Canada), the cell suspensions were centrifuged at 3500 rpm for 15 min and samples were filtered through a 40-mm nylon mesh. The obtained single cells were fixed in 70% ethanol until further analysis. Later the cells were treated with 20 mg/ml RNase and stained with propidium iodide (50 mg/ml). FACS analysis was performed using the FACSCalibur machine (BD Biosciences) and digital data was analyzed using BD CellQuest Pro software (BD Biosciences. San Jose, USA). The fluorescence signals of 10000 cells were measured at 565-595 nm. Blank (PBS supplemented with PI) and unstained cells were used as controls.

2.7. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the liver and gonads using TRIZOL reagent (Invitrogen, CA, USA). Purified RNA was quantified by use of a NanoDrop ND-2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), by measuring the absorbance at two wavelengths - 260 nm and 230 nm. RNA was reconstituted in din-diethylpyrocarbonate (DEPC) water and was then stored at −80C before cDNA analysis. RNA was then treated with DNAse (Invitrogen) according to the

manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions. The cDNA samples were stored at −20°C until further qRT-PCR.

2.7.1. SYBR Green Real-Time qPCR

β-actin was used as the housekeeping control for gonadal samples, and 18s RNA was used as an internal control for liver samples. Primers for qRT-PCR were designed from the known fathead minnows sequence. All forward and reverse primers along with their annealing temperature for liver, and gonadal tissue is listed in Table. 2.1, respectively. Genes were selected based upon their role in sex differentiation, sex steroid signaling, and reproduction. (liver: vitellogenin (VTG), estrogen receptor (esr1), estrogen receptor 2a (esr2a), estrogen receptor 2b (esr2b) and androgen receptor (ar). Gonad: estrogen receptor 1 (esr1), estrogen receptor 2a (esr2a), estrogen receptor 2b (esr2b), androgen receptor (ar), follicle stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), Steroidogenic acute regulatory protein (StAR), polysaccharide side chain (cyp11a), 3β hydroxysteroid dehydrogenase (3β hsd), cytochrome P450 17 (cyp17), 17β hydroxysteroid dehydrogenase (17β hsd), 11β hydroxysteroid dehydrogenase (11β hsd) and cytochrome P450 19a (cyp19a). Optimal annealing temperatures for primers were between 52°and 60°C. PCR was first performed to confirm the specificity of primers. PCR thermocycling parameters were as follows; initial activation at 95°C for 2 min, followed by 40 cycles of 10 s at 95°C, 30 s of the annealing temperature (52°–60°C), and 10 s at 95°C. PCR products underwent a final elongation at 72°C for 10 min. The efficiencies of the reactions were determined by performing the QPCR on serial dilutions of cDNA. To ensure that the primers amplified a single product, a melt curve for every qPCR plate was performed. All primers used were shown to amplify only one size of the template, melting at only one temperature. Amplification efficiency

is the rate at which a PCR amplicon is generated, commonly measured as a percentage value. The value assigned to the efficiency of a PCR reaction is a measure of the overall performance of a real-time PCR assay. The efficiencies for all genes were between 90-105%, which is considered in the optimal range for qPCR analysis.

PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA), according to the manufacturer's instructions. Briefly, each well contained a PCR reaction mixture of 10 µL of SYBR Green Supermix (Bio-Rad, USA), 0.3 µL of sense/antisense gene-specific primers and 0.75 µL of cDNA diluted in Ultrapure water (Gibco, USA). Relative abundance of mRNA levels was assayed using the CFX96TM Real-time PCR System (Bio-Rad), starting with an initial activation of 95°C for 2 mins, then 39 cycles of 10s at 95°C and 30 s at the annealing temperature. Following this, samples were subjected to incremental increases in temperature (0.5°C) from 65°C to 95°C every 5 s to generate a melt curve. Each experimental sample was run in triplicate to ensure consistency Changes in abundances of transcripts of target genes were quantified according to the pfaffl method (Pfaffl et al., 2001).

2.8. Sex Steroid Measurement

For the extraction of the steroids, the fishes were partially thawed, weighed and sexed before homogenizing it. The whole-body homogenates were stored in -20°C for further analysis. Whole body homogenates were digested with 50mM of Tris - protease inhibitor at room temperature. The samples were later reconstituted in ultrapure water, vortexed for 45s and the steroids were extracted using Diethyl ether. The organic phase was collected, allowed to evaporate, and re-dissolved in 0.1% BSA in PBS. The desiccated samples were stored at −20°C until use. The levels of E2 and T in whole-body homogenates were measured using a competitive ELISA from Cayman

Chemicals, in accordance with the manufacturer's instructions and in the protocol previously described by Liu et al., 2009.

2.9. Statistical Analysis

Statistical tests were performed using R studio™ (v. 11.0). All analyses were run separately for males and females. Data are reported as a mean ± standard error mean. A p-value ≤ 0.05 is regarded as significantly different. For qPCR studies, the fold-changes are calculated using median values. For caspase activity assay and flow cytometry analysis, the relative change in caspase activity and testicular cell population, respectively is expressed as percent change concerning the body weight. A p-value ≤ 0.05 was regarded as significantly different. If data were normally distributed, differences in mRNA levels and relative expression between sites were analyzed using ANOVA followed by Tukey's pair-wise comparisons to identify which pairs of sites differed. In case of non-parametric data, Kruskal-Wallis tests were performed.

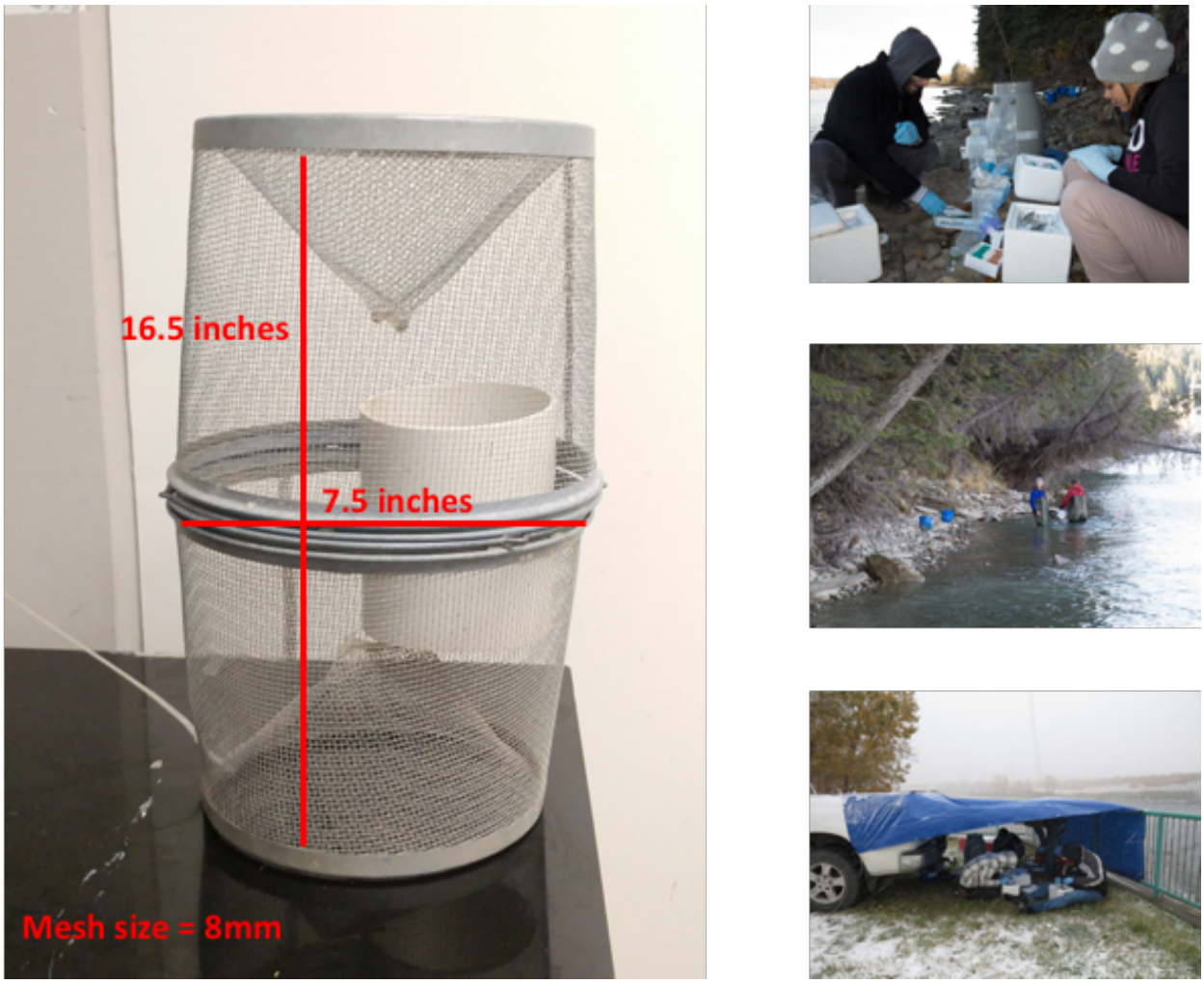

Figure 2.1 Cage used to cage the fathead minnows at the different sites along the Bow River (left side). Fathead minnows were caged for 26 days at five sites along the Bow River and sampled at the riverside (right side).

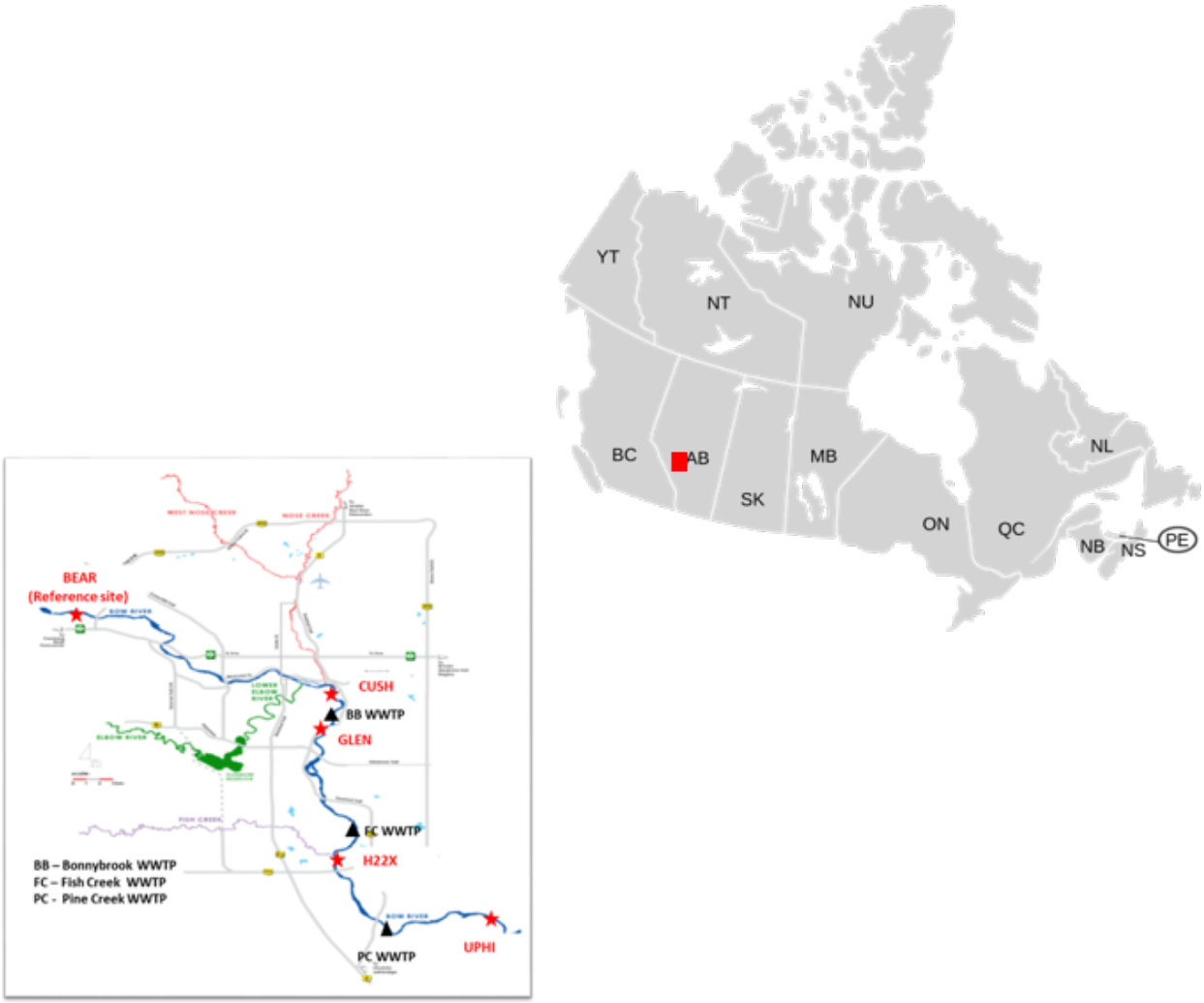

Figure 2.2 Stars represent the sampling sites along the Bow River. Sites (GLEN - downstream to Bonnybrook WWTP, H22X - downstream, to Fish Creek WWTP and UPHI) are the polluted sites along the Bow River, and Site BEAR and CUSH is upstream (BEAR acts as the Reference Site) of the Bow River.

Table 2.1. Water quality parameters at each caging site along the Bow River, Calgary, Alberta from September 12 to September 30, 2016. Values represent mean ± SEM. (Lazaro-Côté et al., 2017, MSc Thesis, University of Calgary)

Site	Temperature (°C)	Dissolved Oxygen (mg/L)	Conductivity (µs/cm)	pH	Turbidity (NTU)
BEAR	12.0 ± 0.3[a]	9.63 ± 0.51[b]	298.3 ± 1.5[a]	8.25 ± 0.03[b]	4.28 ± 1.37
CUSH	12.0 ± 0.5[a]	10.45 ± 0.14[b]	325.8 ± 2.1[b]	8.18 ± 0.08[b]	3.27 ± 0.36
GLEN	18.4 ± 0.2[b]	6.06 ± 0.25[a]	928.5 ± 3.6[e]	7.23 ± 0.08[b]	4.32 ± 0.53
H22X	12.5 ± 0.4[a]	11.04 ± 0.56[b]	376.0 ± 2.8[c]	8.19 ± 0.09[a]	5.06 ± 0.60
UPHI	12.3 ± 0.3[a]	9.91 ± 0.53[b]	397.4 ± 2.3[d]	8.00 ± 0.08[b]	4.33 ± 1.14

Table 2.2. Nucleotide Sequence and Efficiencies of Primer Pairs Used in qPCR

Gene	Primer Sequence (5' – 3')	AT	E (%)
VTG	F- GAAGTGCGCATGGTGGCTTGTA R- AGCTGCCATATCAGGAGCAGTGA	55	102
Esr1	F- CGTGGCTCTGAGCGAATATC R- CACCTCACACAGACCAACAC	55	95
Esr2a	F- GGCTGAGATTTTCGACATGCTT R- AAATTCCTCCAGCTTCAGTTTTAG	55	92.7
Esr2b	F- GCCACCTCCAGATTCAG R- CACGACTCTCCACACCTTCAG	58	99.7
AR	F- TACCCTAACGTGCCCTGTGTGA R- CCGCATCAAACCTGCCATCTGT	56	98
FSH-R	F- CCCCATCGTTCTGGACATCT R- GAAGCTTAAGGGTCCACAGCAT	55	103
LH-R	F- CGTCCGATCAGGTCGTTCA R- TGCACGCTCCCAACGAA	53	105
StAR	F- TGTCCGCTGTGCCAAAC R- GCTCTTACAAATCCTTTCTTCTC	52	97.3
CYP11a	F- CGACACCCGGACTTGCA R- CACGTCTCCTTTAGAGGTGATACG	53	96
3β HSD	F- ATGAGATGCCCTACCCAAAGAC R- CCCTTTACCTTTGTGCCATTG	56	91
CYP17	F- AAAGGAAGATGAATGGATTTG	55	96

	R- GCAGTTGTTGAAAGAAGATG		
17β HSD	F- ATCCAGAGTGTGCTGCCTTT R- AGGGAAATAGCCGTTGGTCT	55	95
11β HSD	F- GCATCGGCGAGCAGTTG R- CTCCTCGCCGTGATAACGA	58	94
CYP19a	F- GCTGCACAAGAAGCACAAG R- CGTGGCTCTGAGCGAATATC	60.3	86
Caspase 3	F- GTAACGGGACACACGAGGAT R- GTGATCCTGCCGAGACACTT	55	99.5
18s RNA	F- ATACCGCAGCTAGGAATAATGG R- CGGTCCAAGAATTTCACCTCTA	58.4	105
β Actin	F- AGCTGTGTCCGTATCTTTGGGCTT R- GAGCAGTTGAGCAAGGCA TTCA	57	96.8

Chapter 3 - Adverse Impact of Treated Effluents on Gonadal Structure and Development in the Fathead Minnows exposed downstream to WWTPs

3. Introduction

3.1. Gonadal Development in Fathead Minnows

The mode of reproduction in teleost's can be gonochoristic, hermaphroditic (sequential and even simultaneous) or parthenogenetic (unisexual) (Strüssmann and Nakamura, 2002). Most of the teleosts are gonochoristic, meaning the undifferentiated gonad develops either in testis or ovary. There are two types of gonochoristic teleosts: differentiated and undifferentiated. In the differentiated gonochorists, ovarian and testicular differentiation proceed directly from undifferentiated gonads (Strüssmann and Nakamura, 2002). In the undifferentiated gonochorists, all individuals initially develop ovarian-like structures which in presumptive males later transform into testes (e.g., zebrafish and other similar cyprinids) (Strüssmann and Nakamura, 2002; Takahashi, 1977).

The mode of reproduction in fathead minnows is gonochoristic, meaning the undifferentiated gonad develops either in testis or ovary. Gonadal development in fathead minnows begins between 10 - 20 days post-fertilization. Fathead minnows are differentiated gonochorists, meaning the primordial germ cells differentiated into either oocyte, which later leads to the development of ovaries, or spermatogonia, which develops into testes. The primordial germ cells from undifferentiated gonad, leads to the development of ovaries, or spermatogonia, which develops into testes. The undifferentiated gonad (primordial gonad) contain primordial germ cells and somatic cells. Primordial germ cells (PGCs) can become oogonia or spermatogonia in the ovary and testis, respectively (Strüssmann and Nakamura, 2002).

3.2. Testicular Development and Spermatogenesis

The testes of the adult male fathead minnows are compartmentalized in to two sections: the interstitial and the tubular chamber. The interstitial compartment typically contains only somatic

cells, Leydig cells, blood vessels, connective tissue, immunological cells such as macrophages and mast cells, lymphatic vessels and carries neural cells. The Leydig cells, located in the interstitial compartment, especially near the blood vessels and produce testosterone. The seminiferous epithelium is filled with fluid, which consists of two cell types – germ cells and epithelial cells. Germinal cells are surrounded by the sertoli cells. Germ cells include all the stages of differentiation of the spermatogenic cells (Strüssmann and Nakamura, 2002). Spermatogenesis is a complicated process in which spermatogonial stem cells undergo differentiation and mitotic proliferation to form primary spermatocytes and, following two meiotic divisions, secondary spermatocytes, and round spermatids. These spermatids subsequently enter spermiogenesis to give mature spermatids (Nagahama, 1994). During the spermatogenesis cycle, various signals promote the sperm production and cell proliferation during sexual maturation and reproductive seasons in order to regulate the process of spermatogenesis and cellular apoptosis during a breeding cycle (Schulz et al., 2010).

During the spermatogenesis cycle, a spermatogonial stem cell undergoes a cell division to give rise two daughter cells, through the process of differentiation into spermatogonia. Spermatogonial stem cells differentiates into spermatogonia type A (it lacks heterochromatin), Spermatogonia type A further differentiates into spermatogonia type B (has high amounts of heterochromatin in their nuclei). Spermatogonia B undergoes mitosis, converting itself into primary spermatocytes, which undergo the first meiotic division. During the first meiotic division, the primary spermatocytes enter metaphase I, anaphase I, and telophase I, which causes recombination of DNA takes place, which is now termed secondary spermatocytes. Secondary spermatocytes are seen less frequently than primary spermatocytes as they divide rapidly, after a short interphase between the two divisions of meiosis.

The haploid spermatids are cells with diameters of 2–4 μm, are spherical and have a spherical nucleus. They do not divide anymore but undergo a morphological transformation, becoming spermatozoa through the process called spermiogenesis. Within this process, several distinctive morphological changes occur (Miura & Miura, 2004). The morphological changes include the formation of a sperm head containing DNA in a condensed nucleus, construction of a mid-piece containing mitochondria, establishment of a flagellum for propulsion and transformation of the Golgi-apparatus into an acrosome (at least in teleost fish). At the end of the spermiogenesis, during the spermiation, the cysts open and the spermatozoa move to the efferent ducts.

The gonadotrophins and sex steroids play a crucial role in the process of spermatogenesis. For instance, FSH released from the pituitary, directly stimulates the steroli cells to release various growth factors, which are directed in a paracrine manner towards the spermatogonia proliferation. Another role of Sertoli cell products is to reduce apoptosis. Next, to regulating these specific spermatogenic processes, the Sertoli cell products also allow spermatogenesis, in general, to take place (Rhoades & Bell, 2009).

In the other hand, LH is directly involved in stimulation of the Leydig cells to produce 11-ketotestosterone (11-KT) and testosterone (T) in fathead minnows. 11-KT and T have an accelerating effect on the Sertoli cells, which enables these cells to nurture the maturing spermatogonia need. In this way, LH also has an indirect impact on Sertoli cells involved in the proliferation of spermatogonia and decrease of apoptosis of the developing germ cells (Rhoades & Bell, 2009).

In male fathead minnows, testosterone assumes the lead role in both morphological development and reproductive function, while estrogen is essential for the fertility. Estrogens have shown to inhibit the spermatogonial proliferation, induced apoptosis of undifferentiated spermatogonia,

accelerated late events in spermatogenesis and induced post-spawning processes such as infiltration of acidophilic granulocytes (Chaves-Pozo et al., 2007). Androgens strongly influence testicular gene abundance and involved in supporting either the whole process of spermatogenesis, or at least some steps such as spermatogonial multiplication and spermatocyte formation (Billard et al., 1982; Fostier et al., 1983; Nagahama, 1994; R. W. Holdcraft and R. E. Braun., 2004).

Apart from the sex steroids produced by the gonads, certain gonadal peptides such as activin and inhibin are also produced in the gonads. Both activin and inhibin, produced in response to FSH, are involved in the regulation of spermatogenesis. Activin promotes spermatogenesis: it stimulates the proliferation and differentiation of spermatogonia. While inhibin improves steroidogenesis in the Leydig cells via an increased abundance of enzymes and the Lh-receptor (Rocha, Arukwe & Kapoor, 2008).

3.3. Oocyte Development and Oogenesis

A mature ovary consists of an ovarian cavity, the germinal or surface epithelium, and the stromal compartment. Follicles are present in the stromal compartment where oocytes grow, and steroid hormones are produced. Oocytes in all teleosts undergo the same basic pattern of growth: oogenesis, primary oocyte growth, cortical alveolus stage, vitellogenesis, maturation, and ovulation.

Ovarian differentiation commences with the transformation of primordial germ cells to oogonia. Ovarian differentiation begins with the conversion of primordial germ cells to oogonia. Ovarian differentiation of fathead minnow occurs between 10 and 25 days post-hatch (dph). At 25dph, the germ cells are converted to primary oocytes vis a series of mitotic divisions. Towards the end of 40 dph, the majority of the germ cells enter the primary oocytes stage and contain only a few oogonia. From 60 to 90 dph, by the oocyte development, there is an increase in the size of the

nucleus (germinal vesicle) and the cytoplasm is uniformly scattered within the oocyte. This stage is known as the perinucleolar stage. Towards the end of this stage, the oocytes enter the vitellogenesis stage. During this phase, there is an enormous growth of the oocyte, accounting for as much as 95% of the final egg size (Tyler et al., 1991). At vitellogenic stage, the smaller size yolk proteins, made up of vitellogenin is synthesized in the liver. Vitellogenin is a glycophospholipoprotein whose molecular weight is 250 to 600 KDa, At this stage, the oocyte also accumulates RNA (known as maternal RNA). During the process of vitellogenesis, the oocytes are subjected to increase in size and volume, thereby the GSI in the fathead minnows increases. By this time the oocyte remains in meiotic arrest, at the end of prophase and in the diplotene stage. Upon sexual maturation, the increasing levels of LH released by the hypothalamus, causes resumption of meiosis in oocytes. This is characterized by the peripheral migration of nucleus (germinal vesicle) and germinal vesicle breakdown (GVBD), the formation of a monolayer of cortical alveoli and yolk platelet dissolution undergoes hydration. The first meiotic division results in two cells differing in size, the small cell with first polar body degenerates, and the large secondary oocyte is formed, and finally, ovulation takes place at the end of the maturation process. The secondary oocyte then moves into the ovarian lumen or abdominal cavity. The second meiosis results in the formation of haploid female gamete, ovum and secondary polar bodies which also degenerate (Miura et al., 2002).

Oogenesis in fathead minnows is regulated by various hormones such as gonadotropin, thyroxine, triiodothyronine, growth hormone, insulin and insulin-like growth factors (IGFs). 17β-estradiol (E2), acts directly on oogonial proliferation, while 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) promotes oogonial proliferation and initiates the first meiotic division, leading to the development of the first stage in primary growth (Miura et al., 2002). Gonadotropins - FSH, and LH play a

crucial role in early oogenesis. The gonadotropin has been suggested to function as anti-apoptotic agents during oogenesis (Baron et al., 2005). Early vitellogenesis is characterized by further increases in plasma FSH and E2 and increased the abundance of ovarian FSH receptor (Breton et al., 1998; Kwok et al.,2005; Kobayashi et al., 2008, 2009; Swanson, 1991; Tyler et al., 1998). LH plays a vital role in the steroidogenic pathway from the production of predominantly E2 during vitellogenesis, to the production of maturation-inducing steroids (MIS) (Nagahama and Yamashita, 2008). FSH and Activin regulate the granulosa proliferation, and Activin also plays an essential role in modulation of the sensitivity of granulosa cells to FSH (Alviggi et al., 2009). Inhibins stimulate the production of androgens in thecal cells and are essential for regulating the LH-dependent stages of follicle development (Alviggi et al., 2009). Termination of vitellogenesis and resumption of meiosis is characterized by an acute increase in plasma LH and ovarian LH receptor. During fertilization, the haploid ovum nucleus fuses with the haploid nucleus of the spermatozoon and forms the diploid egg.

3.4. Gonadal Apoptosis

Apoptosis is one form of programmed cell death (PCD), in which the cell has the ability to activate an inherent suicide mechanism that ultimately eliminates the cell in an ordered fashion. In addition to the proliferation of somatic and germ cells in the ovary and the testis during normal gonadal development, degeneration of gonadal cells plays an essential physiological role and results in the depletion of a majority of germ cells in both sexes. Gonadal apoptosis is the cellular death mechanism, very crucial for gonadal development and differentiation in fish. Massive cell death characterizes the healthy development of both female and male gonads. It is the dynamic balance between cellular proliferation and apoptosis in the gonads that maintain homeostasis and allows for spermatogenesis/oogenesis to occur (Nagahama, 1994). During normal testicular development

50–75% of the germ cells from the potential germ cell pool undergo apoptosis at different stages of spermatogenesis. In contrast, the testis somatic cells (Leydig and Sertoli cells) do not show any signs of degeneration. In the testis, GnRH and sex steroids have the direct impact on apoptosis. In the testes, FSH/LH and sex steroids (testosterone) have shown to regulate and promote cell division and proliferation of spermatogonia in the gonad (Andreu-Vieyra et al., 2007). Gonadal GnRH has a direct role in testicular germ cell apoptosis. Low circulating levels of sex steroids fail to provide protection against degeneration of spermatogonial cells at specific stages of spermatogenesis (H.Billig et al., 1996). Testosterone and the synergistic action of FSH with oestradiol support germ cell survival (Shaha et al., 2010). Androgens can act as survival factors in the testis, and treatment with androgens prevents apoptotic DNA fragmentation of testis tubules and spermatogenic cells. These hormones also act as a positive or negative feedback on both the adenohypophysis and the hypothalamus.

3.4.1. Ovarian Follicular Atresia

Ovarian atresia is a common phenomenon in vertebrate ovaries under both natural and experimental conditions during which a number of ovarian follicles recruited into the vitellogenesis pool fail to complete maturation and ovulation (Saidapur, 1978).The process of ovarian atresia is highly regulated and is essential for maintaining healthy ovarian hemostasis at the end of each reproductive cycle (Krysko et al., 2008).Atresia is a common phenomenon and can be induced by various factors such as stress, inadequate hormone levels, endocrine disruptors, high temperature, starvation (Miranda et al., 1999). The decrease in the levels of circulating gonadotropins due to some alterations in HPG axis or blockade in the ovulatory surge of LH and FSH lead to massive induction of gonadal apoptosis (H.Billig et al., 1996). GnRH is one of the critical hormones which play a crucial role in the induction of gonadal apoptosis. Previous studies

have shown that GnRH and its receptors on fish ovary may have been involved in the induction of ovarian apoptosis during gonadal development due to regression inappropriate ovulatory LH surge in goldfish (Habibi and Andreu-Vieyra, 2007). In the ovary, sex steroids are necessary for the ovarian regulation of follicular atresia. Estrogens which is known as "female hormone" has a positive impact on follicular atresia whereas androgens and treatment with testosterone have shown to have stimulated the process of ovarian atresia.

In general, any alteration in the production of sex steroids or modification in hormonal regulation involved in the HPG axis due to the interference of endocrine disruptors which leads to the change in the production of the sex steroids leads to induction of gonadal apoptosis. Therefore, it can be concluded that sex steroids play an essential role in the regulation of gonadal apoptotic cell death

3.5. Results

3.5.1. Water Quality Parameters

In this study BEAR acts a reference site. The difference between the reference and control site is subtle and often subjected to debate. A 'control site' is often considered identical to the treatment site, with the exception being the treatment action. A reference site is defined as pristine state, with conditions untouched or minimal alteration by human activities. During the course of this study, the effluent flow from Bonnybrook, Fish Creek, and Pine Creek WWTPs made up approximately 8.3 %, 0.6 %, and 1.9 % of the total flow of the river, respectively (Lazaro – Cote et al., M.Sc. Thesis, University of Calgary, 2017). There was no significant difference in the temperatures at were measured during the course of exposure between reference site (BEAR -12.0 ± 0.3) and exposed site (CUSH- 12.0 ± 0.5, H22X - 12.5 ± 0.4 and UPHI - 12.3 ± 0.3) except for the temperature at GLEN (18.4 ± 0.2). Similarly, the pH in all sites (BEAR, CUSH, H22X and UPHI) was on average 8 pH SU. There was significant decrease in pH at GLEN (7.17 ± 0.06). There was

significant decrease in dissolved oxygen at GLEN - 6.06 ± 0.25 when compared to the reference site (Table 2.1). There was no significant difference in the dissolved oxygen between reference site BEAR and exposed site CUSH, H22X and UPHI. The specific conductivity at CUSH (325.8 ± 2.1), GLEN (928.5 ± 3.6), H22X (376.0 ± 2.8) and UPHI (397.4 ± 2.3) was significantly higher than the reference site BEAR (298.3 ± 1.5) (Table 2.1).

3.5.2. Gonadosomatic Index

There was significant decreased gonadosomatic index (GSI) in males and females sampled along the downstream of WWTPs. GSI was significantly lower at the site – GLEN (4.2 ± 0.33) in male fathead minnows when compared to the GSI of fish from the reference site BEAR (9.8 ± 0.65) (Figure 3.1A; $p<0.001$). I observed a considerable decrease in the GSI of the male fathead minnows exposed at CUSH and H22X (5.9 ± 1.06 and 8.8 ± 1.5, respectively) (Figure 3.1A; $p\leq0.05$) when compared to the male fathead minnows caged at BEAR (Figure 3.1A). The relative gonad size of the female fish exposed at CUSH, GLEN and H22X (13.8 ± 1.9, 14.5 ± 1.3, 15.9 ± 1.7) was smaller than fish exposed to the reference site (24 ± 1.5) (Figure 3.1B; $p<0.036 – 0.045$). The gonadosomatic index of the male (13.30 ± 3.5) (Figure 3.1A) and female fish (22 ± 1.2) (Figure 3.1B) exposed at UPHI was not significantly different from the GSI of the male and female fathead minnows exposed at the upstream reference site BEAR. The same trend was noticed in the female fathead minnows exposed along the Bow River for the period of 26 days.

3.5.3. Caspase 3 Activity

Compared to the reference site, significantly higher caspase 3 activity was observed at CUSH and at GLEN and H22X, downstream to Bonnybrook WWTP and Fish Creek WWTP, respectively. In male fish, there were 20%, 40%, and 25% increase in the caspase activity at CUSH, GLEN and H22X (Figure 3.2A; $p<0.01 - 0.05$), respectively. In the female fathead minnows, there were also

significant increases (35%, 45%, and 30%) in caspase 3 activity at CUSH, GLEN and H22X, respectively (Figure 3.2A; $p < 0.03 - 0.05$) when compared to the fathead minnows exposed at BEAR. There was no significant difference in the caspase 3 activity in the fathead minnows of both the sexes caged at UPHI (far-field site) when compared to the reference site BEAR.

3.5.4. Caspase 3 mRNA abundance

This result was supported by the increase in caspase 3 mRNA levels occurring in conjunction with the same locations in both sexes. Abundances of transcripts of caspase 3 were more significant in testes from male caged at CUSH, GLEN, and H22X by 1.7 –fold ($p<0.05$), 7 – fold ($p<0.001$) and 1.7-fold ($p<0.05$), respectively, compared to abundances in testes from male fish exposed to upstream reference site BEAR (Figure 3.2B). Abundances of transcripts of caspase 3 were more significant in ovaries from female caged at CUSH, GLEN, and H22X by 3 -, 7 -, and 3 - fold, respectively, compared to the reference site BEAR (Figure 3.2B; $p<0.025 - 0.05$).

3.5.5. Testicular Tissue

In male fathead minnows exposed to treated effluents along the Bow River for 26 days, there were no cases of intersex in any of the sites CUSH, GLEN, H22X, and UPHI. No histopathological changes other than testicular fibrosis, and testicular disorganization were detected in the male fathead minnows exposed to GLEN and H22X. In the male fishes exposed at BEAR, showed the typical organization of the testicular compartments with clear cell type proportion of spermatogonia and spermatozoa. This is similar to testicular organization of fathead minnows reported in the literature. In the male fathead minnows exposed to GLEN and H22X, downstream to Bonnybrook WWTP and Fish Creek WWTP, shows disorganization of the testicular compartments with increased fibrotic enlargement of interstitial tissue. Histological assessment of the testicular tissue demonstrated alterations in testicular structure including: thickening of the

interstitial tissue, increased apoptosis of germ cells, and altered spermatogenesis as evidenced by decreased proportion of spermatogonia and relative increase in spermatids in germinal epithelium. Some of the testicular lobules contained relatively lesser spermatogonia population. Decreased spermatogonia population, along with degeneration of the testicular structures revealed suppression of spermatogenesis in the male fishes exposed to treated effluents along the Bow River.

3.5.6. Ovarian Tissue

In the female fishes exposed along the Bow River, the ovarian tissues were examined based on the five follicular stages of maturation present in the ovarian tissue. The five stages of ovarian development include - Primary growth stage, Cortical alveolus stage, Vitellogenic stage, Mature stage and Atretic (degenerated) follicles. Histological sections of the fishes exposed along the Bow River shows the presence of oocytes at the primary perinucleolar (PV), cortical alveolar (CA), early vitellogenic (EV) stages and late vitellogenic (LV) stages in all individuals exposed at BEAR, CUSH, GLEN, and H22X. The proportions of oocytes at the different development stages varied among sites. At the sites, downstream to Bonnybrook WWTP and Fish Creek WWTP - GLEN and H22X, the ratio of atretic oocytes proportionally increased when compared to the reference site BEAR (Figure 3.4.).

3.5.7. Flow Cytometry

The testicular population was quantified using propidium iodide flow cytometry. The flow cytometry showed prominent 1C peak including spermatozoa and spermatids, 2 C peak including spermatogonia and somatic cells at the G1 phase and secondary spermatocytes, and 4 C peak including primary spermatocytes and somatic cells at the G2 and M phases. Flow cytometric analyses disclosed that haploid and diploid populations were significantly affected. Exposure to

the treated WWTP effluents, downstream to Bonnybrook WWTP and Fish Creek WWTP, I observed a significantly decreased diploid cell population (spermatogonia) at GLEN ($p<0.01$) and H22X ($p<0.025$) with a resultant increase in the haploid population (spermatozoa and spermatids) in the male testicular tissue exposed at GLEN ($p<0.01$) and H22X ($p<0.025$) (Figure 3.5).

3.6. Discussion

In this study, we exposed adult fathead minnows (*Pimephales promelas*, FHM) for 26 days to treated WWTP effluents along the Bow River, AB and observed abnormal gonadal development and structure. Physiological and molecular responses associated with reproduction were found to be altered at each site. The results demonstrate site-specific gonadal abnormalities in the fishes as the treated effluents discharged from the WWTPs are unique since the concentration of contaminants continuously change depending on human use and environmental factors including storms, season and human activity.

In the present study, the presence of various contaminants (including personal care products, pharmaceuticals, natural and synthetic hormones) were detected in the treated effluents discharged from the Bonnybrook WWTP and Fish Creek WWTP (Evans et al., 2012; Jeffries et al., 2008; Jeffries et al., 2010). The presence of certain contaminants was till detectable 18 km from the most downstream WWTP in Calgary (Lazaro – Cote, 2017, M.Sc. Thesis, University of Calgary). The gonadosomatic index of the fishes exposed at each site was calculated the end of the exposure period (Figure 2). Gonadosomatic index is an indicator of the reproductive status of the fish and chemical exposure, and it is related to reproductive success. At GLEN, 15m downstream to Bonnybrook WWTP, there was significant decrease in the GSI in fathead minnows of both the sexes caged at that particular site. I observed 50% reduction in GSI in both male and female fathead minnows at GLEN when compared to the GSI of the fishes caged at BEAR. Both male and female

fathead minnows exposed at H22X, downstream to Fish Creek WWTP had relatively smaller gonads when compared to the gonads of the fathead minnows exposed at the reference site BEAR. Interestingly, at CUSH, a site upstream from Calgary's WWTPs showed reduction of GSI in fathead minnows of both the sexes comparable to GSI of fathead minnows at H22X. The reduction in GSI at the contaminated sites CUSH, GLEN and H22X may be due to the contaminants present in the treated effluents discharged, resulting in inhibition of testicular and ovarian differentiation and development, thus contributing to lower GSI. Interesting, the GSI of the fathead minnows exposed at UPHI, showed no significant changes in both the sexes. This may be due to diluting effect impact of treated effluents. Alterations of GSI value have been reported in several fish species exposed to the treated WWTP effluents in Rainbow darter and Brooke stickleback (Bahamonde et al., 2016), Rainbow and Greenside darter (Tetreault et al., 2011), Roach (Jobling et al., 2002) and Bream (Hecker et al., 2008). In order to understand the process of gonadal degeneration, we studied gonadal apoptosis using an assay for caspase 3; an essential cysteine protease activated during apoptosis. The results demonstrate elevated caspase 3 enzyme activity in both male and female fathead minnow gonads downstream to Bonnybrook WWTP at GLEN. This is consistent with reduced GSI and suppressed spermatogenesis parameters studied using flow cytometry. Additionally, water temperature was the highest immediately downstream from Bonnybrook WWTP (GLEN), which may have led to influence the induction of caspase activity in the gonads of the caged fish. Also, there is a correlation between the smaller elevation of caspase activity at CUSH and H22X and reduced GSI at that respective sites. Although CUSH is an upstream to the WWTPs, it is also located downstream from over a hundred stormwater outfalls, and a creek that receives urban and rural runoff. Stormwater drains collect urban runoff containing polycyclic aromatic hydrocarbons, nutrients, sediments, and metals (City of Calgary, 2011).

The observed caspase activity results are supported by the increases in caspase 3 mRNA levels at the same locations in both male and female fathead minnows. From this result, we may conclude that specific contaminants present in the treated effluents discharged from the WWTP and stormwater runoffs in case of CUSH, induce oxidative stress, leading to apoptosis. Another reason for induction of gonadal caspase 3 activity may involve effluents induced alterations in follicular and testicular survival factors (gonadotropins, E2, T, 11-KT) which results in induction of apoptosis. Reduced circulating levels of E2 and T in the fathead minnows exposed to GLEN and H22X, downstream to WWTP. Recent studies have shown that exposure to municipal effluents can trigger gonadal degeneration and germ cell loss in fish of both sexes, but the mechanism behind this pathology is still not completely explored (Garcia-Reyero et al., 2011). Increased gonadal apoptosis causes degeneration of gonadal cells, that plays an essential physiological role and results in the depletion of most germ cells in both sexes. These results are consistent with a previous report demonstrating a significant increase in caspase 3 mRNA transcript abundance in the female fathead minnows exposed to treated effluents (Cavallin et al., 2016). A similar induction of the caspase 3 mRNA transcript abundance was observed in the gonads of the fathead minnows exposed to treated effluents (Garcia- Reyero et al., 2011). Interesting there was no significant induction of caspase activity in the gonads to the caged fathead minnows at UPHI, a far field site, suggesting that the effects on caspase activity may be mitigated several kilometers downstream from WWTPs due to dilution with similar water temperature as the reference site BEAR. The results from my study do not provide the kind of cells which are more susceptible to the impacts of effluents. But from the hormone analysis studies, this study may suggest that effluents induce depletion of germ cells in both the sexes.

Histopathology, another important biomarker for analyzing the morphological changes of tissues. Histological analysis of the male fathead minnows exposed to treated effluents at CUSH, GLEN, and H22X showed no signs of the intersex condition. The testis in the males exposed to the upstream reference site BEAR appeared to have better organized testicular compartmentalization with more spermatogonia, spermatozoa and spermatid population. The histologic analysis of testes from the exposed males at GLEN and H22X found a decrease in early stage germ cells (spermatogonia and spermatocytes), an increase in apoptotic germ cells and a generalized thickening of the interstitium. This massive increase in the interstitial tissue and increase apoptotic germ cells has led to a disorganization of the testicular compartment in male fishes. The mechanism underlying testicular apoptosis produced by the contaminants present in the Bow River are still unclear. Interestingly I also observed a considerable disorganization of the testicular tissue in the fathead minnows caged at CUSH. The sampling site CUSH is downstream of over 100 stormwater outfalls, and a creek that receives rural and urban runoff, all of which may contribute nutrients and contaminants to the Bow River. These results are consistent with the field studies conducted by Bouma and Nagler, 2001; B. de Montgolfier et al., 2008 showing that there was suppression of spermatogenesis in fish exposed to treated effluents. In another study showed, delayed spermatogenesis, widespread fibrosis, and tubule malformations were determined in the testis of fathead minnow (Pimephales promelas) exposed to EE2 (Kidd et al., 2007). Studies conducted by (Aravindakshan et al.,2004; Jobling et al., 2002), have demonstrated that treated effluents can alter the process of spermatogenesis in fish. These morphological effects indicate an abnormal testicular development and spermatogenesis of the male fathead minnows, which could influence the testicular steroidogenic capacity of the male fish.

This study also demonstrates a significant alteration in the histology of female gonadal development in the adult female fathead minnow exposed to treated WWTP effluents at GLEN and H22X downstream to Bonnybrook WWTP and Fish Creek WWTP, respectively. In the ovaries exposed to GLEN and H22X, there was proportional increase in atretic oocytes. Atretic ovarian follicles are identified by disorganization of the nucleus and cytoplasm of the oocyte and folding of the oocyte wall. Apart from the atresia, thickening of the somatic cells as well as regressed oogenesis which showed a decrease in the number of vitellogenic oocytes and increase in perinucleolar oocytes in the ovarian tissue was observed. The other contributing factor to induce ovarian apoptosis in the ovaries at GLEN, could the increase in temperature at that particular site. Although these studies suggest that there are potential adverse impacts of treated effluents on females, we found these hard to separate from the effects of other stressors in the wild. The changes observed in females in this study tended to occur both upstream and downstream of the WWTPs, suggesting that any impacts present may have been due to cumulative urban effects rather than just treated effluents. The results in this study is similar to the studies conducted in Fathead minnow (Pimephales promelas) exposed to EE2 has also exhibited a high incidence of ovarian atresia when compared to reference fish (Kidd et al., 2007). Similarly, gonadosomatic index was found to reduce, with regressed oocyte development and increased atretic oocytes in female fathead minnows exposed to CUSH, GLEN and H22X. Reduction in gonadal development in treated effluents exposed fish has been reported in Roach (Jobling et al., 1998), Flounder (Allen et al., 1999), Bream (Hecker et al., 2008), White Sucker (Vajda et al., 2008), and Rainbow and Greenside Darters (Tetreault et al., 2011).

Flow Cytometry

Spermatogenesis is a continuous and highly organized process in which diploid spermatogonial stem cells develop through multiple phases into haploid spermatozoa (Schulz et al., 2010). Spermatogenesis is a complicated process in which spermatogonial stem cells undergo reduction of the DNA content ranging from 4C in spermatocytes at G1 phase to 1C spermatozoa. The primary objective of this investigation was to quantitatively measure the changes in the haploid and diploid population, thus determine if there was any alteration in the testicular population in the male fishes exposed along the Bow River to treated effluents at all sites. Quantitative changes of the relationships between can be selectively investigated by flow cytometry. This technique was used to sort and count the number of cells in different phases of cell cycle during the process of spermatogenesis. The cells from the exposed testicular tissue were dissociated, fixed and stained with propidium iodide (PI). The results demonstrate both inhibitory and stimulatory effects of treated effluents downstream to Bonnybrook WWTP at GLEN and Fish Creek WWTP at H22X on testicular cell proliferation and differentiation in adult fathead minnows exposed for 26 days. Flow cytometry results reveal that there was a significant decrease in the diploid spermatogonia population and increase in the haploid spermatids population, suggesting inhibition of testicular cell proliferation, thereby suppressing spermatogenesis in male fishes exposed to WWTP effluent at GLEN and H22X. The cycle of spermatogenesis is under the influence of gonadotropins. Throughout this period, the testes increase in size which can be measured by an increase in the gonadosomatic index (GSI; gonad weight/body weight x 100) and is usually maximal in the breeding season (Dziewulska and Domagala, 2003). Testicular growth (GSI) and spermatogenesis in male fathead minnows were arrested following exposure to treated effluents for 26 days compared to control fish. Spermatogenesis acts a vital biomarker to study androgen disruption as the process of the spermatogenesis is mainly under the influence of androgens. The flow cytometry

results along with a histological examination of the gonadal tissue clearly demonstrate disruption in testicular structure and function in the male fathead minnows exposed to treated WWTP effluents downstream to Bonnybrook WWTP and Fish Creek WWTP. Additionally, water temperature was the highest immediately downstream from Bonnybrook WWTP (GLEN), which may have led to the influence the reduction of diploid population (Spermatogonia population). A comparison of morphology and flow cytometric analysis yielded characteristic quantitative distribution patterns of the different germ cell and somatic cell populations in regular and disturbed spermatogenesis observed at BEAR (reference site) and GLEN. Although the results from the flow cytometry analysis pointed out that the effluents from the treatment plants had inhibitory impact on spermatogenesis but the results require further analysis due to the small size which is the main limitation of this analysis.

In conclusion, the reproductive and physiological status shows clear signs of disruption in fathead minnows influenced by contaminants present downstream to the Bonnybrook WWTP and Fish Creek WWTP along the Bow River in the City of Calgary after 26 days exposure. The biomarker data revealed a remarkable disruption in the gametogenesis activity as evidence the increase caspase activity and abnormal gonadal development. Physiological responses measured at each site demonstrated different variations of the response depending upon the differences in the chemical composition of treated effluents and the concentrations reported in surface waters can vary considerably due to differences in sampling season, wastewater treatment type, and influent source.

Gonadosomatic Index

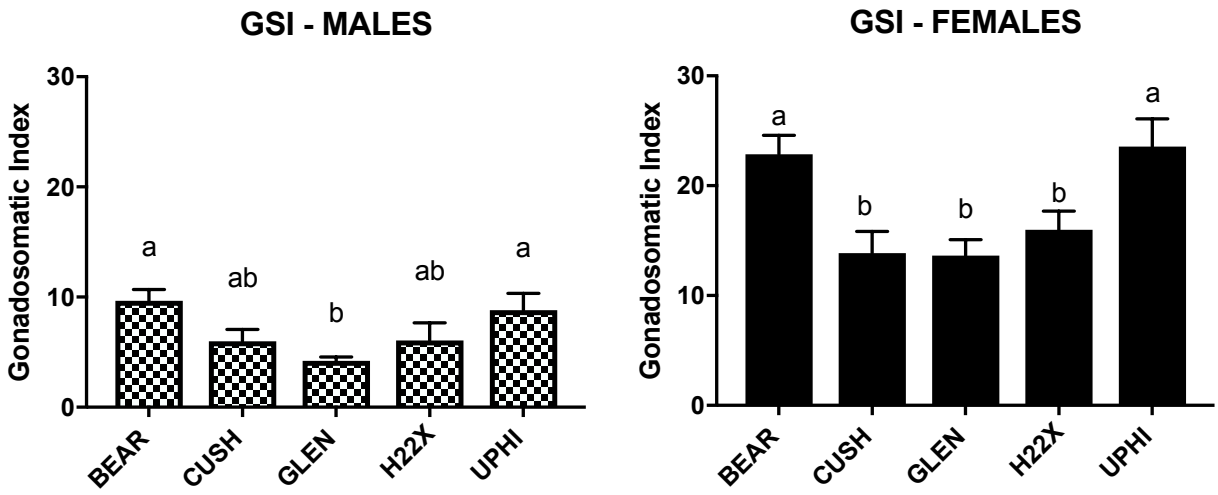

Figure 3.1 Mean gonadosomatic index (GSI) of male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; (n = 8-14). BEAR is the reference site.

Caspase 3 Activity

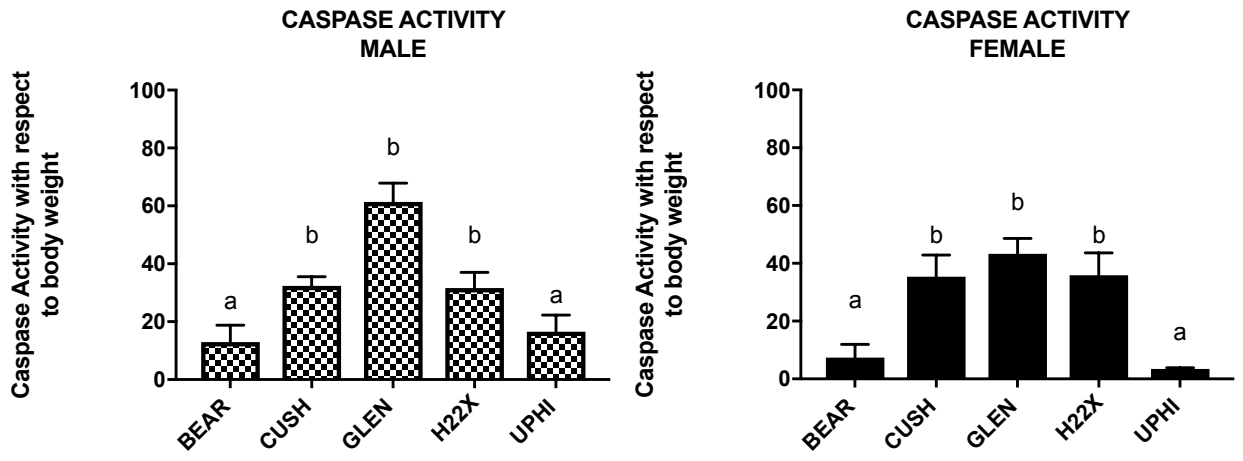

Figure 3.2A Mean caspase 3 activity in male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM;(n=2-8). BEAR is the refernce site.

Caspase 3 mRNA abundance

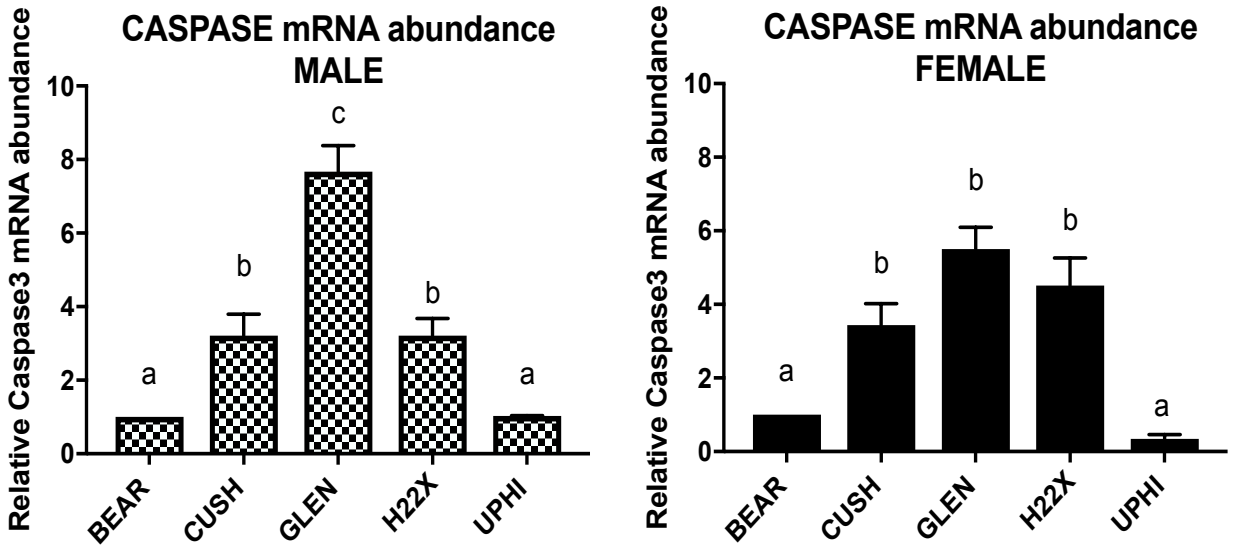

Figure 3.2B Mean caspase 3 mRNA abundance in male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

Testicular Tissue

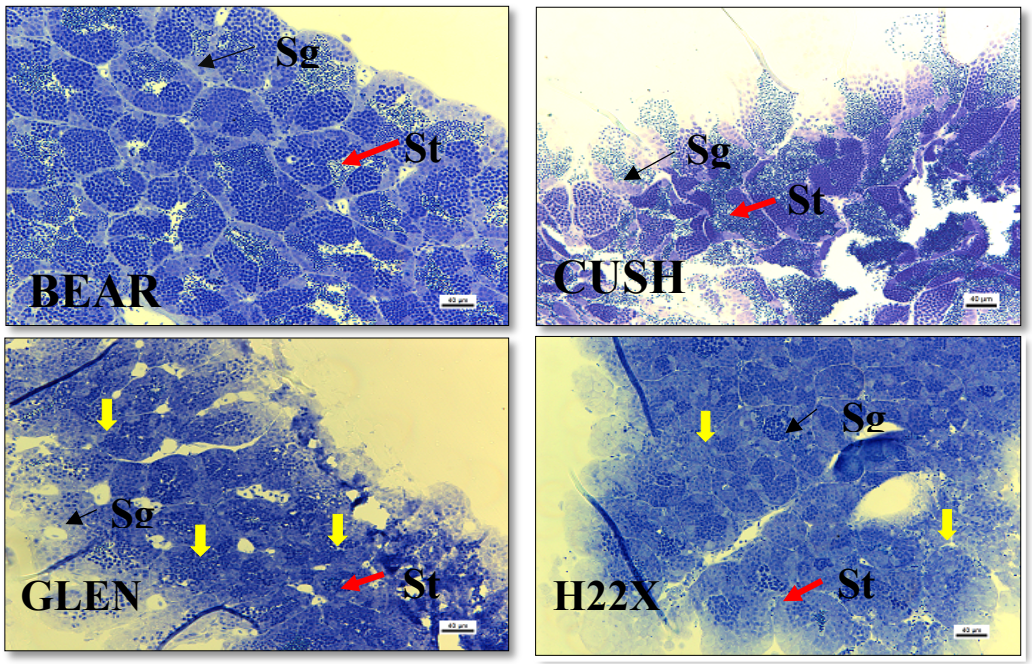

Figure 3.3 Histological sections of fathead minnow testicular fragments exposed to treated effluents for 26 days. (A) BEAR - reference site shows the testicular organization with seminiferous tubules and interstitial tissues. Seminiferous lobes containing germ cells: spermatogonia (Sg), spermatocytes (black arrow), spermatids (St) (red arrow). Yellow arrows represent a notable increase in the number of dead germ cells was observed with fibrotic enlargement of interstitial tissue (Bars = 40 µm; n =5).

Ovarian Tissue

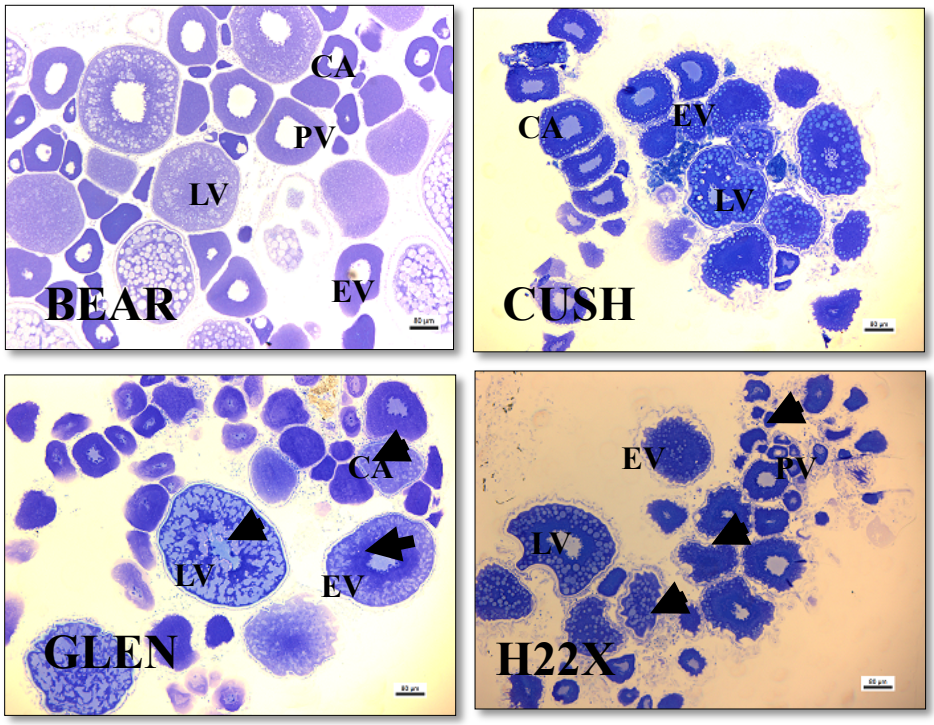

Figure 3.4 Histological sections of fathead minnow ovaries exposed to treated effluents for 26 days.; BEAR - reference site shows the clear organization with black arrows indicate the atretic follicles. The five stages of ovarian development include - Cortical alveolus stage (CA), perinuclear stage (PV), vitellogenic stage – early (EV) and late (LV), mature stage and aretic (degenerated) follicles. Ovarian atretic follicles are identified by disorganization of the nucleus and cytoplasm of the oocyte and folding of the oocyte wall (Bars = 20 µm; n =5).

Flow Cytometry

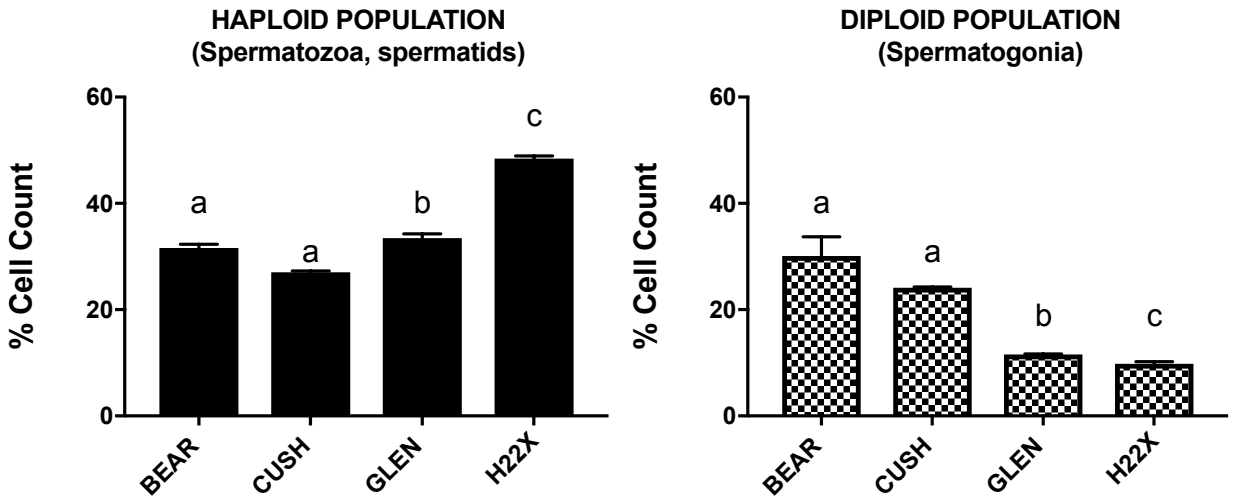

Figure 3.5 Quantification of the testicular population of spermatozoa/ spermatogonia in the testicular tissue of fathead minnow exposed to treated effluents along the Bow River for 26 days. Different letters indicate statistical significance. Bars represent SEM; n =2,3.

Chapter 4 - Adverse Impacts of treated WWTP effluents on endocrine parameters in Fathead Minnows exposed downstream WWTPs

4.1. Introduction

A wide range of biologically active contaminants with hormone-like activity are regularly measured in the treated WWTP effluents, receiving rivers, and surface waters in Canada (Kolpin et al. 2002; Sosiak and Hebben 2005). These contaminants are biologically active at environmentally relevant low concentrations (ng/L) but high enough to exert adverse health effects on aquatic organisms (Diamanti-Kandaraki et al., 2009). The phenomenon of endocrine disruption in fish due to treated effluents ranges from minor changes to significant permanent changes in the reproductive function. Endocrine disruption in many parts of the world was shown to be associated with the feminization of male fish, characterized by increased plasma vitellogenin level, and in extreme cases, leading to sex reversal or the presence of intersex phenotype (Diamanti-Kandaraki et al., 2009).

Disruption in endocrine profile (vitellogenesis, steroidogenesis and circulating levels of sex steroids) leading to reproductive impairment may be due by targeting the HPGL axis, crucial for reproduction and sexual development. The complex interaction between the hypothalamus, pituitary, gonads, and liver are often targeted by the contaminants of emerging concern present in the treated effluents. Both the gonads and the liver are downstream organs in the HPGL axis, which plays a crucial role in gonadal development, induction of vitellogenesis, production, and biotransformation of sex steroids, can be affected by xenobiotics present in the municipal effluent.

In this chapter, I hypothesize that the treated effluents cause reproductive impairment in the fathead minnows caged along the Bow River by altering the endocrine profile, thus disrupting the process of gonadal steroidogenesis and vitellogenesis.

4.2. Gonadal Steroidogenesis

Upon sexual maturation, the gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. In gonads, gonadotropins stimulate and regulate the processes of gonadal development as well as the production of steroid hormones: 17β-estradiol in females, testosterone (T) and predominantly 11- ketotestosterone (11KT) in males. Production of the sex steroids is catalyzed using series of reactions primarily involving several cytochrome P450s and hydroxysteroid dehydrogenases (Arukwe et al., 2008; Leusch and MacLatchy, 2003; Skolness et al., 2013). Cholesterol acts as the precursor molecule to produce steroids in the body. The transport of cholesterol from the outer mitochondrial membrane to inner mitochondrial membrane under the influence of Steroidogenic Acute Regulatory Protein (StAR). After that, several steroidogenic enzymes come into play. StAR is expressed in steroidogenic tissues including the adrenal cortex, ovarian theca, granulosa, testicular Leydig and Sertoli cells, as well as the brain. StAR is extremely sensitive to endocrine disruptors present in the treated municipal effluents (Kortner et al., 2009). Depending on the availability of the cholesterol molecule, the transfer of cholesterol from outer to inner mitochondria membrane occurs rapidly within seconds to few minutes (Ramsby et al., 1997). Thus, it is evident that activity of StAR is a rate-limiting step in steroidogenesis due to its role in transporting cholesterol which is the precursor molecule within the mitochondria.

Once cholesterol molecule reaches the inner mitochondrial membrane, P450scc enzyme (cyp11a1) is responsible for the conversion of cholesterol into pregnenolone. CYP11a is present in all steroidogenic cells. The abundance of CYP11a determines the net steroidogenic capacity of the

cell, hence it is another rate-limiting step involved in gonadal steroidogenesis (Cheshenko et al., 2008; Miller et al., 1999).

3β hydroxysteroid dehydrogenase (3β HSD) converts pregnenolone to progesterone. 3β HSD are membrane-bound enzymes present in steroidogenic tissues such as adrenal cortex, gonads, and placenta. 3β hydroxysteroid dehydrogenase (3β HSD) activity is essential to produce androgen during the process of gonadal steroidogenesis (Ramsby et al., 1997).

Progesterone is then mainly converted into androstenedione mediated by bifunctional enzyme CYP hydroxylase/lyase (CYP17). 17α Hydroxylase (CYP17) is a membrane-bound enzyme present in adrenal gland and gonadal tissue and essential for the synthesis of glucocorticoids and sex steroids (Miller et al., 1999; Ramsby et al., 1997).

Androstenedione, a weak androgen, is mainly converted into testosterone by the 17β-HSD (Cheshenko et al., 2008). 17β HSD is also a membrane-bound enzyme exclusively present in the gonadal tissue and catalyze later steps in gonadal steroidogenesis (Payne and Hales, 2004).

The essential androgens produced in teleosts are testosterone (T), 11-ketotestosterone (11-KT) and dihydroxy testosterone (DHT) (Borg, 1994). In testes, 11-ketotestosterone, the prominent androgen, is formed from testosterone by 11β hydroxysteroid dehydrogenase (11β hsd) in the Leydig cells present in the testes (Zhang et al., 2010). The 11-KT is a direct stimulator of spermatogenesis. T, on the other hand, impacts spermatogenesis through its feedback to the hypothalamus and pituitary (Borg, 1994).

In the ovaries, the androgen is converted to estrogen in the presence of the aromatase, CYP19a isoform which is present in the gonads. CYP19 is located in the endoplasmic reticulum in the

gonads and brain cells. Two CYP19 genes are present in most teleosts, CYP19a and CYP19b, primarily expressed in the ovary and brain, respectively. CYP19 aromatase enzymes mediate the conversion of androgens to estrogens. Any modulation in the aromatase CYP19 abundance and function can alter the rate of estrogen production, thereby resulting in the disruption of the circulating levels of estrogen in the body (Cheshenko et al., 2008).

In fishes, an essential estrogen is 17β-estradiol (E2), just like in most vertebrates. Estrogens are known to inhibit androgen synthesis (de Waal, 2009). 11-KT and T are the most critical androgens in fathead minnows. The difference between T and 11-KT is that 11-KT is non-aromatizable.

The steroidogenic pathway has feedback loops that tightly regulate the circulating hormone concentrations and their homeostasis. Sex steroids influence regulation of GnRH and GtH release, receptor abundance, and hormone concentrations in the hypothalamus and pituitary (Meng et al., 2010). The feedback loops can act at the hypothalamus level by either inhibiting GnRH secretion in times of increased sex steroid concentrations, or decreasing inhibition of GnRH secretion if circulating sex steroid hormone concentrations are low. Feedback loops also regulate the flow of FSH and LH from the pituitary, which in turn control the synthesis of sex steroid hormones. Because of the intricate interactions of the HPG-axis, a inhibition of GnRH secretion if circulating sex steroid hormone concentrations are low. Feedback loops also regulate the flow of FSH and LH from the pituitary, which in turn control the synthesis of sex steroid hormones. Because of the intricate interactions of the HPG-axis, a change in one component of the axis can potentially result in changes in the other components.

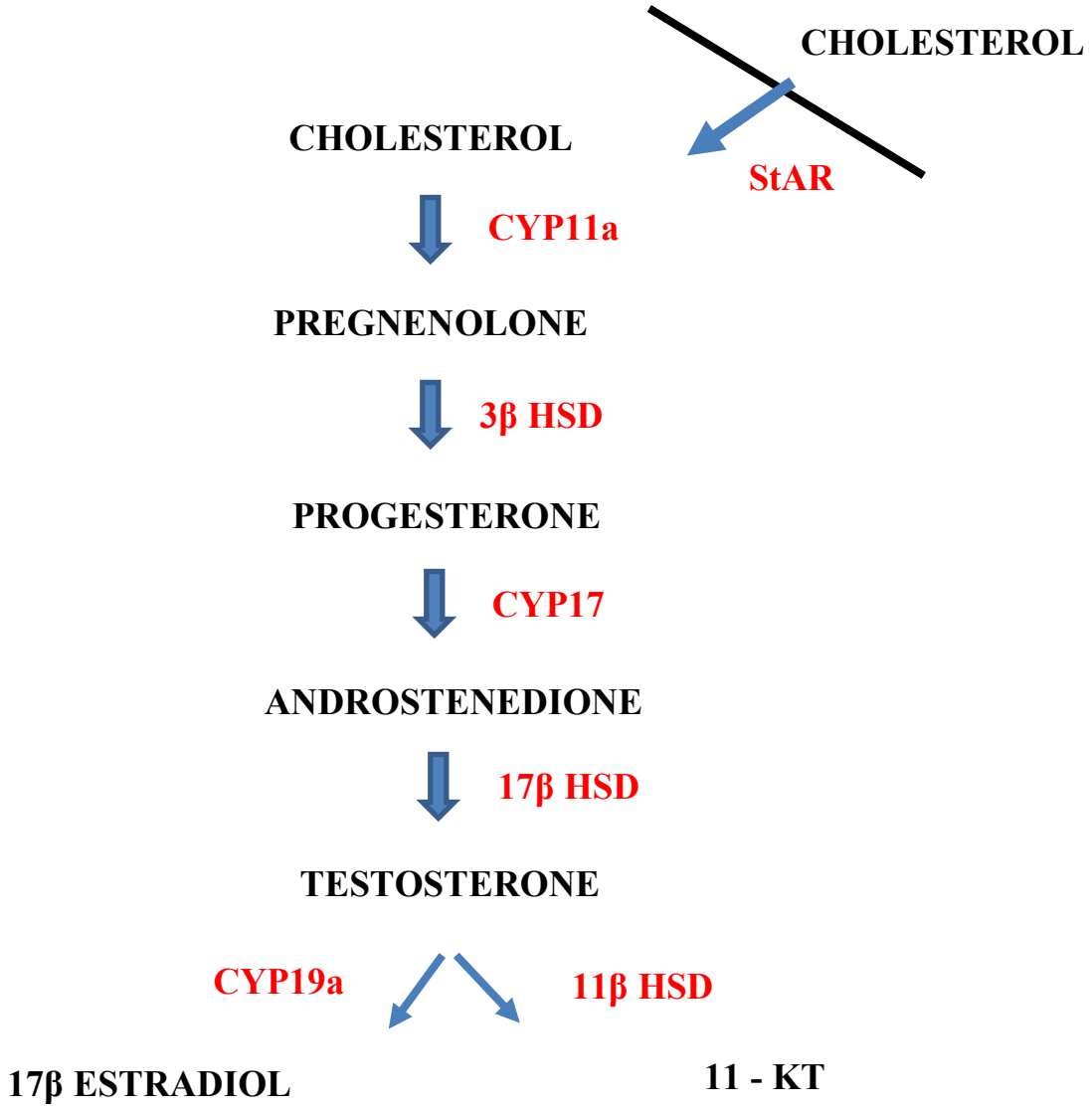

Figure 4.1. Schematic representative of gonadal steroidogenesis pathway and showing enzymes involved in the biosynthesis of androgens and estrogens.

These changes can be natural cycling of steroidogenesis and reproduction, disease-related, or artificially induced through environmental exposures.

4.3. Impact of EDCs on Steroidogenesis

Numerous studies have demonstrated that sewage effluent and waters receiving the discharge from WWTPs have detectable levels of endocrine disruptors, including natural and synthetic estrogens, androgens and other biologically active compounds (Kolpin et al., 2010; Zhang et al., 2010). Disruption of enzymes involved in the steroidogenic pathway at any levels could result in the alteration in the production of sex steroid hormones E2, T, and 11-KT. Various studies have reported that the exposure to contaminants or EDCs disrupts the process of steroidogenesis by reversible or irreversible catalytic inhibition of steroidogenic enzymes, which leads to either increase or reduction of the enzymes involved in steroidogenesis, ultimately resulting in variations in the synthesis of sex steroids (E2 and T). 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 a reproductive failure (Filby et al., 2005; Garcia Reyero et al., 2009, 2011). Recent studies have reported modulations of sex steroid levels and impairment of gonadal development in wild fish collected from contaminated sites (Hecker et al. 2011; Lavado et al., 2004; Noaksson et al., 2001,2003) suggesting that wild fish populations are indeed exposed to substances that can perturb steroidogenesis and potentially the sexual development of fish.

4.4. Vitellogenesis

A significant function of the liver during ovarian development in female teleost is the process of vitellogenesis (Kime, 1998). The process of vitellogenesis consists of five events: synthesis of vitellogenin in the liver, delivery of vitellogenin to oocyte through the bloodstream, uptake of vitellogenin by the oocyte and last two events include utilization of vitellogenin for the production and storage of egg yolk proteins (Reading et al., 2011). Upon sexual maturation, the 17β estradiol produced by the ovaries enters the liver through blood circulation (Peter and Yu., 1997). In the liver, 17β estradiol binds to the various isoforms of hepatic estrogen receptors, stimulates the production of yolk protein vitellogenin. Although multiple isoforms are present in liver, estrogen receptor (esr1) in particular is involved in the process of vitellogenesis (Filby et al., 2005; Nelson and Habibi., 2010). Vitellogenin synthesized in the female liver reaches the oocyte through blood circulation. In the oocyte, vitellogenin gains access through intercellular channels and absorbed by receptor-mediated endocytosis beginning at the previtellogenic (late) and early–vitellogenic stages. Vitellogenins are directly involved in the transport of vital biomolecules (lipids, sugars, minerals) into the egg–yolk for the development of the organism, and the derived yolk–proteins also play a role in maturation (hydration). The yolk–proteins act as a growth substrate for developing organisms through the embryo stage (Nicholas et al., 1999).

Although there are estrogen receptors present in the liver of the male fish, the process of vitellogenesis is undetectable in the male fish as they do not produce high concentrations of estrogen to trigger the process of vitellogenesis in males. (Matozzo et al., 2008; Tyler et al., 1996). Hence it can be concluded that vitellogenesis is an estrogen-mediated process which is normally restricted to females (Tyler et al., 1996). Receptors present in male fish are fully functional and

capable of binding to estradiol and, hence, VTG synthesis may be induced. In juvenile fish and mature males, exposure to exogenous estrogens or compounds with estrogen-like activity can induce synthesis of this protein; therefore, VTG abundance is a useful biomarker of estrogenic activity (Purdom et al., 1994). Most xenoestrogens act by a similar mechanism but have less affinity for the ER, thus, less potency when compared to 17β- estradiol (Nimrod and Benson, 1996).

The induction of vitellogenesis in the male fish act as a common biomarker of exposure to environmental estrogens or estrogenic activity of municipal effluents. Evidence of feminization in male fish is one classic example of the abnormal reproductive function, which occurs in the presence of estrogenic compounds or androgen-signaling inhibitors. Exposure to exogenous estrogens can be triggered to cause abnormal induction of vitellogenesis in male and juvenile fish (Matozzo et al. 2008; Tyler et al. 1996) and in extreme cases, exposure to endogenous estrogen can even develop intersex testis, a condition wherein the oocytes are mixed with sperm (Kidd et al., 2007). Genotypic males exposed to exogenous estrogens during an early developmental stage can influence change into phenotypic females (Parrott & Blunt, 2005) thus, skew the sex ratio and influence population dynamics (Ankley et al., 2008; Jeffries et al., 2008, 2010; Kidd et al., 2007). Other effects include a decrease in spawning activity, spawning success and male secondary sex characteristics (Parrott & Blunt, 2005). Several studies have reported that correlations between increased VTG and decreased egg production, effects on ovarian structure, inhibition and disruption of testicular growth and development, respectively (Panter et al., 1998). The investigation into the abundance of the ER has been of interest regarding exposure to estrogenic EDCs (Skillman et al., 2006).

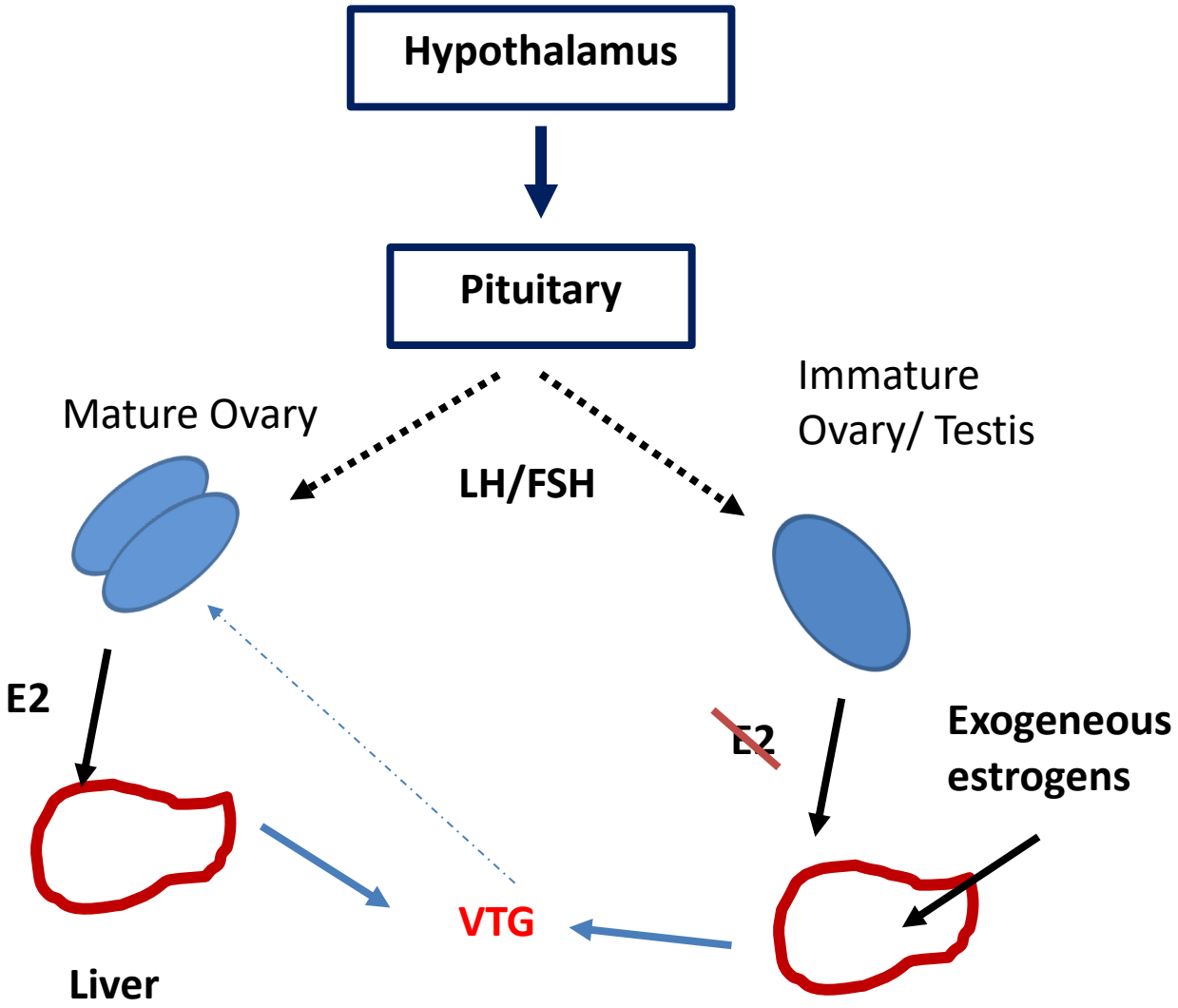

Figure 4.2. Overview of the vitellogenin production in teleost. Vitellogenesis is the process by which maturing oocytes accumulate yolk. Vitellogenin (VTG) is normally synthesized in the liver and is regulated by 17β-estradiol (E2). Male fish possess VTG gene, but expression is triggered as inappropriate response to exogenous estrogen, leads to production of VTG in male fish.

Laboratory and field experiments have revealed that contaminants present in the treated WWTP effluents can exert their actions through altering nuclear receptor signaling such as ERs, ARs, progesterone receptors, TRs, and retinoid receptors. CECs are also capable of interacting with nonsteroid receptors and interfering with steroid biosynthesis and/or metabolism at every level of HPG axis. This results in various disorders in reproductive functions as outlined below (Schug et al., 2011). Due to similarities in structural confirmation, contaminants can interact with receptor agonists and exert agonist or antagonist activities. They can also alter the receptors synthesis and metabolism and therefore influence the natural response to endogenous hormones. Hence it is crucial to also study the impact of the treated effluents on the sex steroid receptors in order to understand the endocrine disruption caused by the fathead minnows caged along the Bow River.

In this study, I measured transcript levels for genes involved in regulation of gonadal steroidogenesis and vitellogenesis on fathead minnow. Interaction of the gonadotropins - luteinizing hormone and follicle stimulating hormone with its respective membrane-bound receptors - luteinizing hormone receptor and follicle stimulating hormone receptors are also important factors in the regulation of gonadal steroidogenesis and steroid-dependent feedback mechanisms (Kumar and Trant, 2001; Schulz et al., 2001; Yaron et al., 2003). An additional component of the HPG axis is androgen receptor and different types of estrogen receptors (ER1, ER2α, and ER2β). The abundance of these receptors can influence androgen or estrogen responsiveness.

4.5. Results

Expression of transcripts involved in Vitellogenesis and receptor signaling in the liver and the gonads

4.5.1. Vitellogenesis

Females upstream the WWTPs produced significantly higher vitellogenin mRNA abundance than the male fathead minnows exposed downstream to the WWTPs at all sites. There was a significantly lower abundance of VTG mRNA in the female fathead minnows caged at GLEN H22X, and UPHI by 0.4 – fold ($p<0.05$), 0.3 - fold ($p<0.05$), and 0.7 - fold ($p<0.01$), in comparison to BEAR (reference site), respectively (Figure 4.3). There was no significant difference in the VTG mRNA abundance in male fishes in a different site, although appear to be insignificant variations in the male liver vitellogenin mRNA abundance from site to site with no consistent pattern (Figure 4.3).

Sex steroid receptors in Liver

4.5.2. Estrogen Receptor (Esr1)

Hepatic estrogen receptor (esr1) mRNA levels were generally higher in females than in males. Abundances of transcripts of estrogen receptors (esr1) were not significantly different in the liver of male fish caged at the sites CUSH, GLEN, H22X, and UPHI compared to the reference site BEAR (Figure 4.4A). In female fish, the relative abundance of the hepatic estrogen receptors (esr1) decreased at CUSH, GLEN, and H22X by 0.4 –fold ($p<0.05$), 1.2-fold ($p<0.01$), and 0.3- fold ($p<0.05$), respectively. There was no significant difference in the female fishes exposed at UPHI in comparison to the upstream reference site BEAR (Figure 4.4A).

4.5.3. Estrogen Receptors (Esr2a)

Hepatic estrogen receptor (esr2a) mRNA levels were generally higher in females than in males. Abundances of transcripts of estrogen receptors 2a (esr2a) were not significantly different in the liver of the male fish exposed to treated effluents at the sites when compared to the abundance in gonads from male exposed to upstream reference site BEAR (Figure 4.4B). Abundances of estrogen receptors (er2a) were significantly lower in the liver of the female fathead minnows exposed at the sites CUSH, GLEN, H22X and UPHI by 0.2-fold ($p<0.05$), 0.3-fold ($p<0.05$), 0.5-fold ($p<0.05$), and 0.6-fold ($p<0.034$) respectively, when compared to the liver of the female fish exposed to upstream reference site BEAR (Figure 4.4B; $p<0.05$)

4.5.4. Estrogen Receptors (Esr2b)

Hepatic estrogen receptor (esr2b) mRNA levels were generally higher in females than in males. Abundances of transcripts of estrogen receptors (esr2b) were significantly lower in the liver of the male fish exposed to treated effluents at the sites UPHI 0.4 - fold by compared to the abundance of esr2b in testis from male fish exposed to upstream reference site BEAR (Figure 4.5A; $p<0.05$). In female fish, the relative abundance of the hepatic estrogen receptors (esr2b) was lower at CUSH and UPHI by 0.4 -fold ($p<0.05$) and 0.5-fold ($p<0.05$) respectively, whereas there was no significant difference in the transcript abundance of the estrogen receptors (esr2b) in the liver of the female fathead minnows at GLEN and H22X when compared to the reference site BEAR.

4.5.5. Androgen Receptors (AR)

Abundances of androgen receptors (ar) was significantly lower in the liver of the male fish exposed at CUSH, GLEN, H22X, and UPHI by 0.5-fold ($p<0.05$), 0.4-fold ($p<0.05$), 0.7- fold ($p<0.01$), and 0.5-fold ($p<0.05$) respectively, when compared to the upstream reference site BEAR (Figure 4.5B). The abundance of hepatic ar in females was significantly higher at the sites (GLEN and

H22X) by 0.5- fold ($p<0.05$) at both the sites and lesser abundance of ar transcripts at CUSH and UPHI by 0.7 - fold ($p<0.04$), and 0.8-fold ($p<0.037$) in comparison to reference site. (Figure 4.5B). I observed site – and sex – specific differences in the relative expression of androgen receptors in the fathead minnows exposed along the Bow River.

Sex Steroid Receptors in Gonads

4.5.6. Estrogen Receptors (Esr1)

Gonadal expression of estrogen receptor (esr1) mRNA levels were generally higher in females than in males. Relative abundance of esr1 in males was not significantly different at the sites CUSH, GLEN and H22X, the abundance of esr1 at UPHI was lesser by 0.4- fold ($p<0.05$), when compared to the reference site BEAR (Figure 4.6A). Abundances of esr1 were more significant in the female fish at CUSH, GLEN by 1.5- fold ($p<0.01$), and 2.5-fold ($p<0.01$), respectively, compared to upstream reference site BEAR (Figure 4.6A). There were no significant differences in esr1 abundances in H22X in female fathead minnows (Figure 4.6A).

4.5.7. Estrogen Receptors (Esr2a)

Relative abundance of esr2a in males has increased the sites GLEN and H22X by 1.6-fold ($p<0.027$) and 0.8-fold ($p<0.045$), and the abundance of esr2a at CUSH and UPHI was not significantly different when compared to the reference site BEAR (Figure 4.6B). Abundances of esr2a were greater in female fish exposed at CUSH, GLEN, and H22X by 0.3-fold ($p<0.05$), 1.1-fold ($p<0.008$) and 1.2- fold ($p<0.008$) respectively, the abundance of esr2a was no significant difference at UPHI when compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.6B).

4.5.8. Estrogen Receptors (Esr2b)

Gonadal expression estrogen receptor (esr2b) mRNA levels were generally higher in males than in females. Relative abundance of esr2b in males was lower at the sites GLEN by 1.6-fold ($p<0.05$), and the abundance of esr2b at other sites showed no significant difference when compared to the reference site BEAR (Figure 4.7A). Abundances of esr2b were greater in female fish exposed at CUSH, and H22X by 1.1- fold ($p<0.01$), and 2.4 - fold ($p<0.01$) respectively. There was a 0.7-fold ($p<0.05$) reduction in the abundance of esr2b at UPHI, no significant difference of er2b abundance was seen at GLEN when compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.7A).

4.5.9. Androgen Receptors (AR)

Abundances of transcripts of androgen receptors (ar) were not statistically different in the gonads from male fish exposed to treated effluents at CUSH, GLEN, H22X when compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.7B). There was significant decrease in ar abundances in testis exposed at UPHI by 0.7 – fold ($p<0.05$). There was significant decrease in ar abundances in testis exposed at UPHI by 0.7 – fold ($p<0.05$). Abundances of ar were lesser in female fish exposed at CUSH, GLEN, H22X, and UPHI by 0.7- fold ($p<0.05$), 0.7- fold ($p<0.05$), 0.9 –fold ($p<0.046$), and 0.9 –fold ($p<0.046$), respectively, compared to abundances in female fish exposed to upstream reference site BEAR (Figure 4.7B).

Gonadal Steroidogenesis

4.5.10. StAR

Abundances of transcripts of StAR was greater in gonads from male exposed to GLEN and H22X by 1.1-fold and 1-fold (Figure 4.8A; $p<0.001$) respectively, whereas there was significant decrease in the abundance of StAR by 0.6-fold at CUSH (Figure 4.8A; $p<0.05$) when compared to relative

abundance of StAR in the gonads of the male fish exposed to upstream reference site BEAR. There was no significant difference in the abundance of StAR in the female fathead minnows caged along the Bow River (Figure 4.8A).

4.5.11. CYP11a

The relative abundance of cyp11a mRNA levels were generally higher in females than in males. Abundances of transcripts of CYP11a was significantly higher in gonads from female fathead minnows exposed at CUSH, GLEN, H22X, and UPHI by 1-fold ($p<0.05$), 1.8 –fold ($p<0.035$), 1.7 -fold ($p<0.037$) and 0.9 –fold ($p<0.05$) respectively respectively when to compared to abundances in gonads from female fish exposed to upstream reference site BEAR (Figure 4.8B). Abundances of transcripts of CYP11a was not significantly different in gonads from male exposed to CUSH, GLEN, H22X when compared to its relative abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.8B).

4.5.12. 3β HSD

Abundances of transcripts of 3β hsd was lesser in gonads from male fish caged at CUSH, GLEN, H22X, and UPHI by by 0.7- fold, 0.7 -fold, 0.6 - fold and 0.8 -fold, respectively, compared to male fish exposed to upstream reference site BEAR (Figure 4.9A; $p < 0.05$). Abundances of transcripts 3β hsd in gonads from female fish exposed to CUSH, GLEN, H22X and UPHI (0.9 and 0.72 - fold, 0.6 - fold, 0.4 - fold, respectively) were less than abundances in gonads from male fish exposed to BEAR (Figure 4.9A; $p < 0.03$-0.05).

4.5.13. CYP17

Abundances of transcripts of CYP17 was lesser in gonads from male fish exposed at CUSH, GLEN, H22X, and UPHI by 0.2- fold, 0.2-fold, 0.3- fold and 0.4 -fold, respectively, compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.9B; $p < 0.01$). Abundances of transcripts of CYP17 was lesser in gonads from female fish exposed at CUSH, GLEN, and H22X by 0.5- fold, 0.8 -fold and 0.4-fold respectively. There was no significant difference in the abundance of CYP17 in female fishes exposed at UPHI when compared to abundances in gonads from female fish exposed to upstream reference site BEAR (Figure 4.9B; $p < 0.01$-0.03).

4.5.14. 17β HSD

Abundances of transcripts of 17β hsd were lesser in gonads from male fish exposed at CUSH, GLEN, and H22X by 0.7- fold ($p<0.01$), 0.6 - fold ($p<0.01$), and 0.2 - fold ($p<0.05$) respectively, compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.9C). In contrast, there were no significant differences of 17β hsd transcripts at UPHI in comparison to the reference site. Abundances of transcripts of 17β hsd was lesser in gonads from female fish at CUSH, GLEN, H22X, and UPHI by 0.78-fold ($p<0.03$), 0.8 –fold ($p<0.03$), 0.6 - fold ($p<0.045$) and 0.9 –fold ($p<0.026$) respectively, compared to abundances in gonads from female fish exposed to upstream reference site BEAR (Figure 4.9C).

4.5.15. 11β HSD

Abundances of transcripts of 11β hsd was lesser in gonads from male fish exposed at CUSH, GLEN, H22X and UPHI by 0.5- fold ($p<0.05$), 0.6 –fold ($p<0.05$), 0.7 – fold ($p<0.024$) and 1 –

fold ($p<0.012$), respectively, compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.10A; $p < 0.05$). Abundances of transcripts of 11β hsd were lesser in gonads from female fish exposed at CUSH, H22X and UPHI by 0.9- fold ($p<0.01$), 0.5 –fold ($p<0.05$), and 0.6 –fold ($p<0.05$), respectively. In contrast to this, there was no significant difference in the abundance of 11β hsd at GLEN compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.10A).

4.5.16. CYP19a

The relative abundance of cyp19a mRNA levels were generally higher in females than in males. Abundances of transcripts of gonadal aromatase (CYP19a) was lesser in gonads from male fish exposed at CUSH, GLEN, H22X, and UPHI by 1- fold ($p<0.01$), 0.7 –fold ($p<0.035$), 1 – fold ($p<0.01$), and 1 –fold ($p<0.01$), respectively, compared to abundances in gonads from male fish exposed to upstream reference site BEAR (Figure 4.10B). Abundances of transcripts CYP19a in gonads from female fish exposed to GLEN, H22X, and UPHI (0.5 - fold, 0.6 - fold, 0.4 - fold, respectively) were less than abundances in gonads from male fish exposed to BEAR (Figure 4.10B; $p < 0.01$).

Gonadotropin Receptors

4.5.17 Follicle stimulating hormone receptor (FSHR)

The relative abundance of follicle stimulating hormone receptor (FSHR) mRNA levels were generally higher in females than in males. Abundances of transcripts of FSHR were statistically no different in gonads from male fish exposed to the sites CUSH and UPHI, compared to abundances in gonads from male fish exposed to the upstream reference site (BEAR). Conversely,

there were lesser abundances of FSHR mRNA abundance in the male fishes exposed to GLEN and H22X (Figure 4.9A) site approximately by 0.75-fold ($p<0.01$), and 0.3-fold ($p<0.05$), respectively, in comparison to the reference site BEAR. In female fathead minnows exposed along the Bow River, the abundance of transcripts of FSHR was greater in ovaries exposed to H22X ($p<0.01$), and UPHI ($p<0.01$), while there was no significant difference in the abundances of FSHR exposed at CUSH compared to the abundance of transcripts of FSHR in ovaries exposed to upstream reference site BEAR (Figure 4.9A). I observed a site – and sex – specific differences in the fathead minnows caged along the Bow River.

4.5.18. Luteinizing hormone receptor (LHR)

The relative abundance of luteinizing hormone receptor (LHR) mRNA levels were generally higher in females than in males. Abundances of transcripts of LHR were greater in gonads from male fish exposed downstream to WWTPs at the sites CUSH, GLEN, H22X and UPHI (Figure 4.11B; $p < 0.05$) by 1-fold ($p<0.01$), 0.8- fold ($p<0.034$), 2.7-fold ($p<0.002$), and 2.4-fold ($p<0.004$) respectively compared to abundances in gonads from male fish exposed to the upstream reference site BEAR. In ovaries from female fish exposed to GLEN and H22X (Figure 4.11B) abundances of transcripts of LHR were higher by 1- fold ($p<0.01$), and 0.8 –fold ($p<0.023$) compared to abundances in ovaries from female fish exposed to reference site BEAR. There was a significant decrease in the abundance of the LHR mRNA transcripts exposed to the CUSH and UPHI when compared to the reference site BEAR. I observed a site – and sex – specific differences in the fathead minnows caged along the Bow River.

4.6. Sex Steroid Levels

4.6.1. Testosterone Levels

The levels of testosterone in male and female fishes exposed to CUSH, H22X and UPHI were not significantly different to those at the upstream reference site - BEAR whereas there was a significant decrease in mean testosterone levels at GLEN (Figure 4.12A; $p < 0.05$ in both the sexes).

4.6.2. 17β Estradiol Levels

In male fishes exposed downstream to WWTPs at GLEN and H22X (Figure 4.12B; $p < 0.05$), there was a significant decrease in the estradiol when compared to upstream reference site BEAR. In female fishes exposed to GLEN and H22X showed a substantial reduction in estradiol levels when compared to the reference site BEAR.

4.7. Discussion

Vitellogenesis is an excellent biomarker to detect the presence of estrogenic contaminants in the treated effluents released into the Bow River. Vitellogenesis is an estrogen-mediated process, predominantly seen in female fish, as the male fish has a lesser amount of endogenous estrogen. In certain circumstances, exposure to exogenous estrogens, the male fish also induces the synthesis of vitellogenins in the liver. Induction of vitellogenin synthesis in male fish is mainly due to the presence of exogenous estrogen. In this study, quantitative real-time PCR (qRT-PCR) was used to study transcript abundances. It represents a rapid and reliable method for the detection and quantification of mRNA transcription levels of a selected gene of interest. The sensitivity of qRT-PCR allows to work with a minimal amount of starting material, still achieving an accurate

quantification of poorly transcribed mRNAs. Quantitative RT- PCR has an added advantage over the array technique as the name suggests it is a quantitative technique. qPCR is restricted in the number of genes that can be screened at one time. In the current study, I observed no significant induction of hepatic VTG mRNA abundance in male liver exposed to treated effluents along the Bow River (Figure 4.3). Contributing factors include lack of compounds with estrogen-like activity, or more likely the resulting mixture of contaminants in the WWTP effluents affect vitellogenin expression in a different way. One possibility is that WWTP effluents may contain a large number of antiestrogens which is used commonly for the treatment of estrogen-dependent cancers. This is, in fact, consistent with the observed reduction in the vitellogenin transcript levels in the female in the downstream sites in the Bow River. In this context, the presence of compounds with estrogenic activity has been documented in the effluents released from the Bonnybrook WWTP and Fish Creek WWTP (Jeffries et al., 2010; Sosiak and Hebben 2005). Another contributing factor could be the presence of compounds with androgen - like activity that counteracts the endogenous estrogens and the environmental contaminants with estrogen-like activity. The vitellogenin results are consistent with previous findings in southern Alberta (Jeffries et al., 2008b) and a study conducted on Longnose Dace in the Bow River (Henderson et al., 2014, M.Sc Thesis, University of Calgary). Similar results were observed in wild roach (Jobling et al., 1998), fathead minnows (Nicholas et al.,1999) and Brook trout (Montogoflier et al., 2007). The presence of aromatase inhibitors in the treated effluents could also be responsible for the significant downregulation of CYP19a (key regulator in the conversion of testosterone to 17β estradiol) which can reduce plasma levels of estradiol by inhibiting the conversion of testosterone to estradiol. This can lead to a reduction in VTG (Ankley et al., 2009) as observed in the present study. In previous studies conducted in North American and European waters, show no consistent

pattern in changes in the VTG levels in wild-type female fishes exposed to treated effluents. Previous studies have reported an increase in plasma VTG (Thorpe et al., 2003), no change in VTG (Jobling et al., 2002; Thorpe et al., 2003), or a decrease in VTG levels (Jobling et al., 1998; Nichols et al., 1999) in female fish exposed to treated effluents. The different trends in VTG mRNA abundance among individuals may be due to the varying complexity of the chemical compositions of the effluent. The response of VTG mRNA abundance is dependent on sex, location, and stages of gonadal maturation in fishes exposed to treated effluents.

In this is study we measured the mRNA abundance of esr1, esr2a, and esr2b in the liver as biomarkers for the estrogenic characteristic of treated effluents released into the Bow River. In the liver of both the sexes, higher relative mRNA abundance of esr1 and esr2b is observed (Filby et al., 2005). Some studies have reported induction of esr1, an ER subtype, in male fish following exposure to environmental concentrations of EE2 (Filby et al., 2007; Nelson et al., 2007; Lange et al., 2008). In this study, the male fish exposed to effluents along the Bow River, showed no significant differences in esr1 mRNA abundance, whereas there was significant reduction of esr1 abundance in the female fathead minnows caged at CUSH, GLEN, and H22X coincident with observed VTG abundance observed in both sexes along the Bow River. Often environment contaminants with demonstrated estrogen-like activity are not purely estrogenic and could interact with other receptor types as an agonist or antagonist. Contributing factors could possisbly be a potential reduction in estrogen responsiveness and steroid signaling in females, or, the blocking of esr1 mRNA abundance in females by estrogen antagonists. Gender differences in esr1 have been noted in previous studies that showed small levels of esr1 mRNA abundance in male zebrafish (Meng et al., 2010), which is consistent with the present results. There was no significant

difference in esr2b mRNA abundance in male fishes exposed to treated effluents along the Bow River. These results are consistent with the study by Benninghoff and Williams, 2008 who reported no change in esrb in the liver of juvenile male rainbow trout exposed to estrogenic compounds (i.e., 17β-estradiol(E2), tamoxifen (TAM), diethylstilbestrol (DES), and others). Similar results were also reported by Esterhuyse et al., 2010 in the liver of adult male tilapia (*O. mossambicus*) exposed to 60 ng/L E2 for 12 h. In female fishes, there was a significant increase in esr2b mRNA abundance at GLEN and H22X. There was considerable downregulation of esr2b mRNA abundance at CUSH and UPHI in female fish.

There was a significant decrease in ar mRNA abundance in the liver of the male fishes exposed to treated effluents along the Bow River. Interestingly, the androgen receptor in fathead minnows appears to be more abundant in females than males. The level of ar mRNA was significantly higher in females from GLEN and H22X sites compared to males at the upstream reference area. The mRNA abundance patterns of the estrogen and androgen receptors in females and males are consistent with what has been reported in other species. For example, Maruska and Fernald, 2010 stated that both esr1 and ar mRNA abundance is higher in female liver compared to males in African cichlids (*Astatotilapia burtoni*) and that there is no difference in the overall mRNA abundance of esrb between the sexes.

Impact of treated effluents on Sex Steroid Receptors in Gonads

Three nuclear estrogen receptor subtypes, ER1, ER2a, and ER2b, mediate the physiological effects of estrogen by regulating the transcription of target genes possessing the estrogen response element (ERE) in their promoter regions. Synthesis of ERs, in turn, is regulated by estrogen itself (Filby et

al., 2007; Nelson et al., 2007; Nelson and Habibi, 2010). The role of ER subtypes in the gonads of fish is not well understood. Previous studies have demonstrated that there is higher abundance of esr2a and esr2b, when compared to esr1 receptor. Interestingly, esr2b in the testis is significantly involved in normal physiological of testicular tissue and spermatogenesis whereas esr2a in the ovaries plays a dominant role in ovarian physiology and development (Filby et al., 2005). There were greater abundances of the estrogen receptors (esr1, esr2a and esr2b) in the male fathead minnows caged along CUSH, GLEN and H22X (except for esr2b in ovaries at GLEN). Alterations in the endogenous hormone receptors and estrogen responsiveness can other possible mode of action for adverse effects of these contaminants. Unlike the observed effect of E2, however, exposure to the contaminants tested did not increase the ER subtypes, suggesting that their adverse effect is not purely estrogenic. In male fathead minnows, there was significant increase in the abundances of estrogen receptors in esr2a in the male fathead minnows caged at GLEN and H22X. This induction in esr2a demonstrates that the contaminants present in the Bow River have the potential to change the sensitivity of these tissues to estrogen. In my study, I observed a significant decrease in esr2b abundance in the male fathead minnows caged downstream to Bonnybrook WWTP at GLEN, which is consistent with the suppressed spermatogenesis observed in males at that particular site (Chapter 3).

In the present study, the contaminants did not significantly alter the abundances of androgen receptors in the testes but decreased AR abundances in the females, although the function of this gene in female fish is not certain. This is consistent with a previous study in in African cichlids (*Astatotilapia burtoni*) exposed to treated municipal effluents.

Gonadotrophins (LH and FSH) released from the pituitary plays a crucial role only in gametogenesis, but also is essential to regulate the process of steroidogenesis. StAR is considered as the key regulator of the steroidogenesis pathway, as it is involved in the transportation of cholesterol from outer mitochondria membrane to inner mitochondria membrane to initiate sex steroid biosynthesis (Stocco, 2000). CYP11a encodes the P450scc enzyme involved in the conversion of cholesterol to pregnenolone. P450scc/cyp11a1 play an essential role during early steroidogenesis (Arukwe, 2008). 3β hsd is responsible for the conversion of pregnenolone to progesterone. The CYP17 enzyme catalyzes the conversion of progesterone to androstenedione, also the key enzyme in androgen biosynthesis. 17β HSD enzyme catalyzes the final step of sex steroid biosynthesis, which involved in the conversion of androstenedione to testosterone which controls estrogen and androgen concentrations. 11β HSD is involved in the biosynthesis of 11-ketotestosterone (11-KT), the potent androgen. It is involved in the conversion of testosterone to 11- ketotestosterone.

Effects on Males

In the male fathead minows exposed at GLEN, 15m downstream to Bonnybrook WWTP and H22X, 250 m downstream to Fish Creek WWTP, there was significant decrease in the abundances in FSHR, suggesting it might be in response to lower circulating levels of FSH released from the pituitary. This could be one of the contributing factors to suppressed spermatogenesis in the testes (Filby et al., 2007), whereas there were greater abundances of LHR, suggesting the higher circulating levels of LH from the pituitary.

There was significant increase in the abundances of StAR in males at both the sites. Relating my finding to other studies shows both similarities and differences. In a study conducted by Filby et al., 2005 the fathead minnows exposed to effluents present in UK waters, showed a significant increase in mRNA abundance of StAR in males. Another study conducted by Garcia - Rereyo et al., 2009 also showed no inhibitory impact of treated effluents on the mRNA abundance of StAR. There was no significant difference in CYP11a mRNA abundance in male fathead minnows. This is in accordance with the study conducted by P.A. Bahamonde et al., 2014 where there was no significant change in mRNA abundance of CYP11a in the testis of rainbow darter collected from the Grand River, Canada. There was significant decrease in the abundances of key enzymes involved in androgen biosynthesis (3b HSD, CYP17 and 17b HSD) in the male fathead minnows exposed at GLEN and H22X. Inhibition of this enzyme results in changes in the production of sex steroid in the gonads, as well as changes in plasma concentrations of sex steroids in both male and female fathead minnow (Filby et al., 2007). These results indicate the genes involved in androgen production (3β hsd, cyp17, 17β hsd) are the crucial targets of the treated effluents discharged from WWTP into the Bow River. There was a significant decrease of the key enzymes could be due to the presence of specific contaminants which counteract the standard mechanism which is involved in the androgen biosynthesis.

At CUSH, an upstream site, there was no significant difference in the FSHR, whereas there was significant increase in LHR abundances. FSH plays a regulatory role in early follicular development and spermatogenesis, while LH mainly regulates steroidogenesis, oocyte maturation, and ovulation/spermiation. Interestingly, there was significant downregulation in the abundances of StAR, whereas there were no significant differences in CYP11a abundances. In case of the

abundances of the key enzymes (3b HSD, CYP17 and 17b HSD) in androgen biosynthesis, also had significantly decreased levels of abundances of 3b HSD, CYP17 and 17b HSD than the upstream reference site BEAR, with levels comparable to fish caged at H22X. The sampling site CUSH is downstream of over 100 stormwater outfalls, and the Calgary Zoo, and a creek that which may contribute nutrients and contaminants to the Bow River (Lazaro – Cote et al., 2017, MSc Thesis, University of Calgary).

At UPHI, abundances of StAR, CYP11a, 3b HSD and CYP17 significantly lower than the reference site BEAR in fish sampled 18 km from Pine Creek WWTP (UPHI), suggesting that effects on steroidogenesis may be not have mitigated several kilometers downstream from WWTPs due to dilution. A similar trend was observed in the females exposed at UPHI.

11β HSD is involved in the biosynthesis of 11-ketotestosterone (11-KT), the potent androgen. It is involved in the conversion of testosterone to 11- ketotestosterone. The mRNA abundance of the 11β HSD was significantly decreased at CUSH, GLEN, H22X, and UPHI in the fathead minnows both the sexes. A similar trend was observed in the mRNA abundance of 11β HSD was found in the fathead minnows exposed to municipal effluents (Filby et al., 2007).

Effects on Females

In the current study, the mRNA abundance of StAR in the ovaries was not influenced by the contaminants present in effluents at all sites. Relating my finding to other studies shows both similarities and differences. In a study conducted by Filby et al., 2005 the fathead minnows exposed to effluents present in UK waters, showed a significant increase in mRNA abundance of StAR in both sexes. Another study conducted by Garcia - Rereyo et al., 2009 also showed no

inhibitory impact of treated effluents on the mRNA abundance of StAR. There was a considerable increase of CYP11a mRNA abundance in female fathead minnows exposed to CUSH, GLEN, H22X and UPHI. The results of the current study were in contrast to another study conducted in rainbow darter obtained from the Grand River, Canada showed the significant decrease of CYP11a mRNA abundance in female rainbow darter (P.A. Bahamonde et al., 2014). From the results from my study, we may conclude that the treated effluents from the WWTPs do not inhibit the initial steps of steroidogenesis. There was significant decrease in the abundances of key enzymes involved in androgen biosynthesis (3b HSD, CYP17 and 17b HSD) in the male fathead minnows exposed at CUSH, GLEN, H22X and UPHI.

In female fathead minnows, I observed a significant increase of FSHR at GLEN and H22X, suggesting increased circulating levels of FSH in the female fishes at those sites. Thus, it can be concluded that there was is sex-specific and site-specific response in FHSR abundance. I observed significant upregulation of LHR mRNA abundance in fathead minnows caged at GLEN and H22X in both the sexes, suggesting the increased circulating levels of the LH in fathead minnows exposed at those sites. Exposure to treated effluents had a stimulatory impact on the LHR mRNA abundance in the fathead minnows.

Aromatase (cyp19) is the key enzyme which catalyzes the irreversible transformation of androgen into estrogen and plays a vital role in maintaining a physiological balance between sex and steroid hormones (Hinfray et al., 2008). In the ovary, androgens synthesized in the thecal cells are converted into estrogens by CYP19 in the granulosa cells located nearby. The highest mRNA abundance of aromatase cyp19a is reported to occur in stage III oocytes (vitellogenic oocytes), consistent with the function of estrogen in fish ovarian development (Goto-Kazeto et al., 2004).

Aromatase is also found in the testis, however, at much lower mRNA abundance levels. Abundances of transcripts CYP19a in gonads from both the sexes exposed to GLEN, H22X, and UPHI when compared to the fishes exposed at reference site BEAR. The similar downward tendency was seen in studies conducted by Garcia- Reyero et al., 2011, Filby et al., 2007, Garcia-Reyero et al., 2009. The presence of aromatase inhibitors in the treated effluents could also be responsible for the significant downregulation of CYP19a (key regulator in the conversion of testosterone to 17β estradiol) can reduce plasma levels of estradiol by inhibiting the conversion of testosterone to estradiol, which reflects reduction in VTG (Ankley et al., 2002). Interestingly, there was no significant difference in the abundance of CYP19a in the females caged at CUSH. This is consistent with the VTG abundance in the female fathead minnows.

Sex Steroid Levels

The measurement of circulating sex steroids in the plasma of fish has been used as a reproductive endpoint to evaluate the impact of an effluent on fish (McMaster et al., 1995; Van Der Kraak et al., 1992). This information provides greater insight into the reproductive status of the fish under study and potential mechanisms of action of compounds of concern. Gonadotropins stimulate the production of sex steroid and gonadal peptides in the gonads. Sex steroids can exert feedback regulation on the production and release of gonadotropins which will, in turn, affect the sex steroid production (Schulz and Miura, 2002).

Circulating steroid levels are generally used to determine steroid levels of an individual fish. However whole-body sex steroid levels have also been used in many studies to determine steroid levels in smaller size fish i.e., fish that are too small to provide an adequate plasma sample. One

of the limitation of using whole body homogenate is that the presence of body fluids which dilutes the circulating steroid in the body, thus causes binding interference within the assay due to excess body fluids. The hormone concentration is correlated to the body size in both the sexes. I observed a significant decrease in the circulating T levels in the male fathead minnows exposed at GLEN - downstream to Bonnybrook WWTP (Figure 4.12A), this could be due to a significant decrease of the critical enzymes involved in testosterone biosynthesis (Chapter 4). Although we did not determine which individual or mixture of contaminants is responsible for depressing levels at testosterone in male fathead minnows caged at GLEN, we understand that there is the presence of chemicals like β sitosterol accountable for the decrease in the testosterone levels by targeting the enzymes involved in androgen biosynthesis. Interestingly I observed no significant changes in the T levels in the male fathead minnows exposed at CUSH, H22X, and UPHI (Figure 4.12A). Although there was a significant decrease in the mRNA abundance of enzymes involved in testosterone biosynthesis, we were unable to notice any reduction in the testosterone levels and could be that the changes in testosterone synthesis was too small to be translated into the plasma changes at the contaminated sites (Sharpe et al., 2004; Bjorkblom et al., 2008).

A significant depression in 17β estradiol was also observed in GLEN, and H22X in female fathead minnows when compared to the female fathead minnows caged at the reference site (BEAR). Previous studies conducted in Bow River suggested the presence of estrogen-like chemicals in the treated effluents released from the Bonnybrook WWTP and Fish Creek WWTP (Jefferies et al., 2009). These xeno-estrogen present in treated effluents intervenes with the negative feedback mechanism involved in the gonadotropin production, which results in depressed levels of 17β estradiol in the fathead minnows exposed to treated effluents. The results I observed in my studies

were consistent with another study conducted in wild roach present in the UK waters, also showed significantly downregulated levels of 17β estradiol. A significant depletion of E2 in the in the sites downstream the WWTPs could lead to due to low availability of androgen serving as substrates for estradiol production, consistent with the suppressed mRNA abundance of CYP19a, a key enzyme involved in the production of 17β estradiol. In female fathead minnows, 17β estradiol levels in individuals collected at the far-field site UPHI returned to comparable levels as those fish obtained at the reference site (BEAR), suggesting that the impact of xeno-estrogens may get diluted as we go farther from the WWTPs.

In conclusion, the results of this study demonstrate the mRNA transcript profiles of a set of genes along the HPG axis in male and female fathead minnows exposed to the treated effluents along the Bow River for 26 days show that the responses are complex and site- and gender-specific. Overall, clear patterns of adverse impacts on the hepatic function in the fathead minnows exposed downstream of WWTPs in the Bow River suggest that reproductive impairment is likely caused by contaminants derived from sewage wastewater effluents. The results of this chapter, suggests that the treated effluents cause alteration in the critical enzymes involved in androgen production, thus, in turn, causes impairment of sex steroid production in the fathead minnows caged along the Bow River. This study also provides a strong foundation for understanding the mechanistic model of action involved in the alteration of the reproductive biomarkers due to treated effluents present in the Bow River. The majority of studies to date have focused on the estrogenic properties of wastewater effluent and their feminizing effects (Filby et al., 2007; Tyler and Jobling, 2008) and the effects of non-estrogenic features are still relatively unclear. And while numerous organic contaminants with endocrine disrupting activity have been detected in Calgary's wastewater

effluent, the complete characterization of the contaminant composition is unknown. Additional water chemistry analyses are needed to further understand the variation in the presence and concentration of contaminants. The present findings provide strong support for the hypothesis that environmental contaminants, discharged from the WWTPs, are causing endocrine disruption of fish populations in the Bow River.

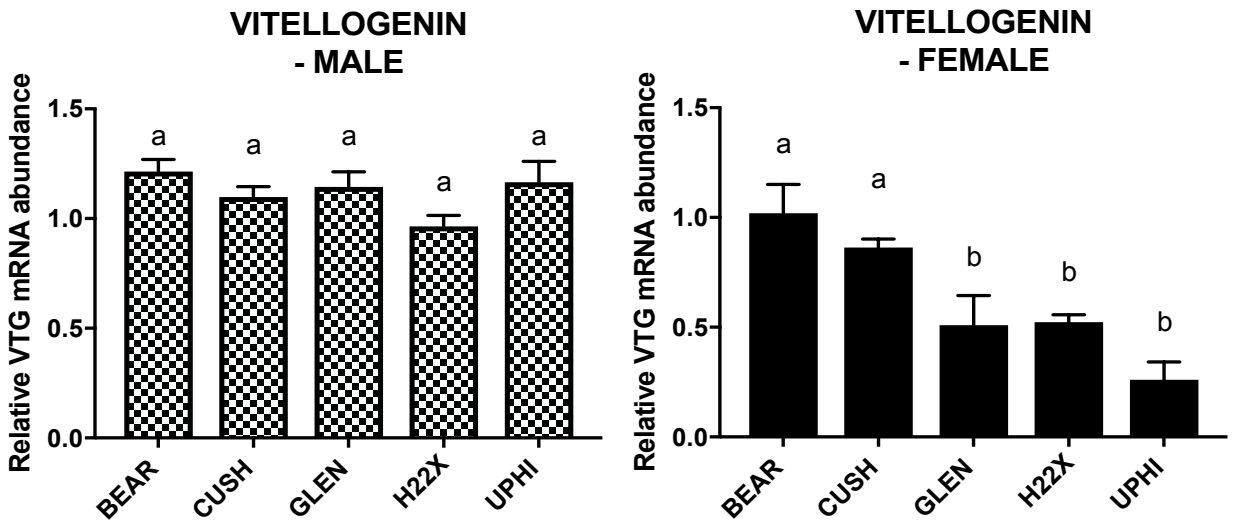

Figure 4.3 Relative vitellogenin mRNA abundance in the liver of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

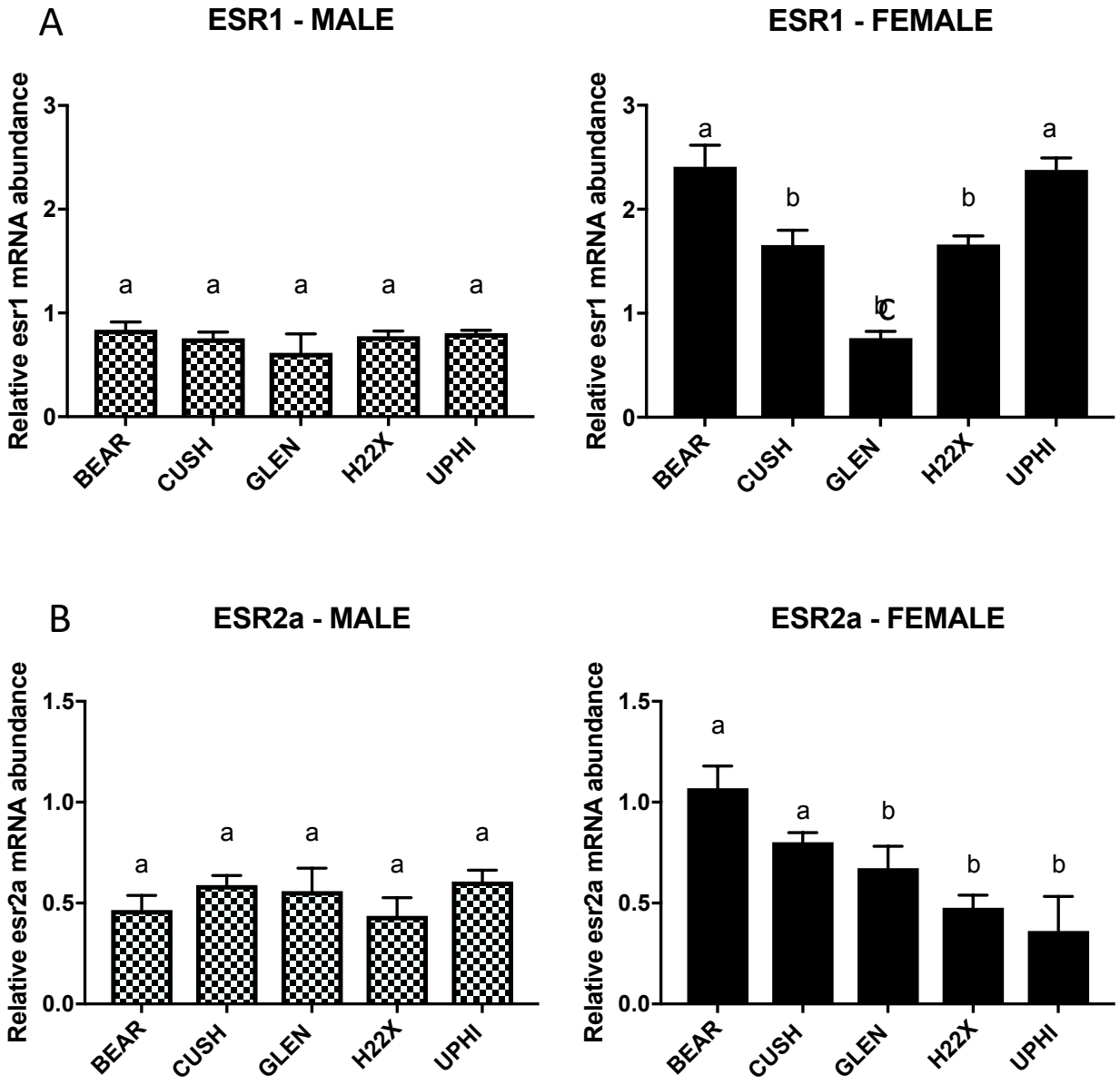

Figure 4.4 Relative estrogen receptor (esr1) (A) and estrogen receptors (esr2a) (B) mRNA abundance in the liver of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

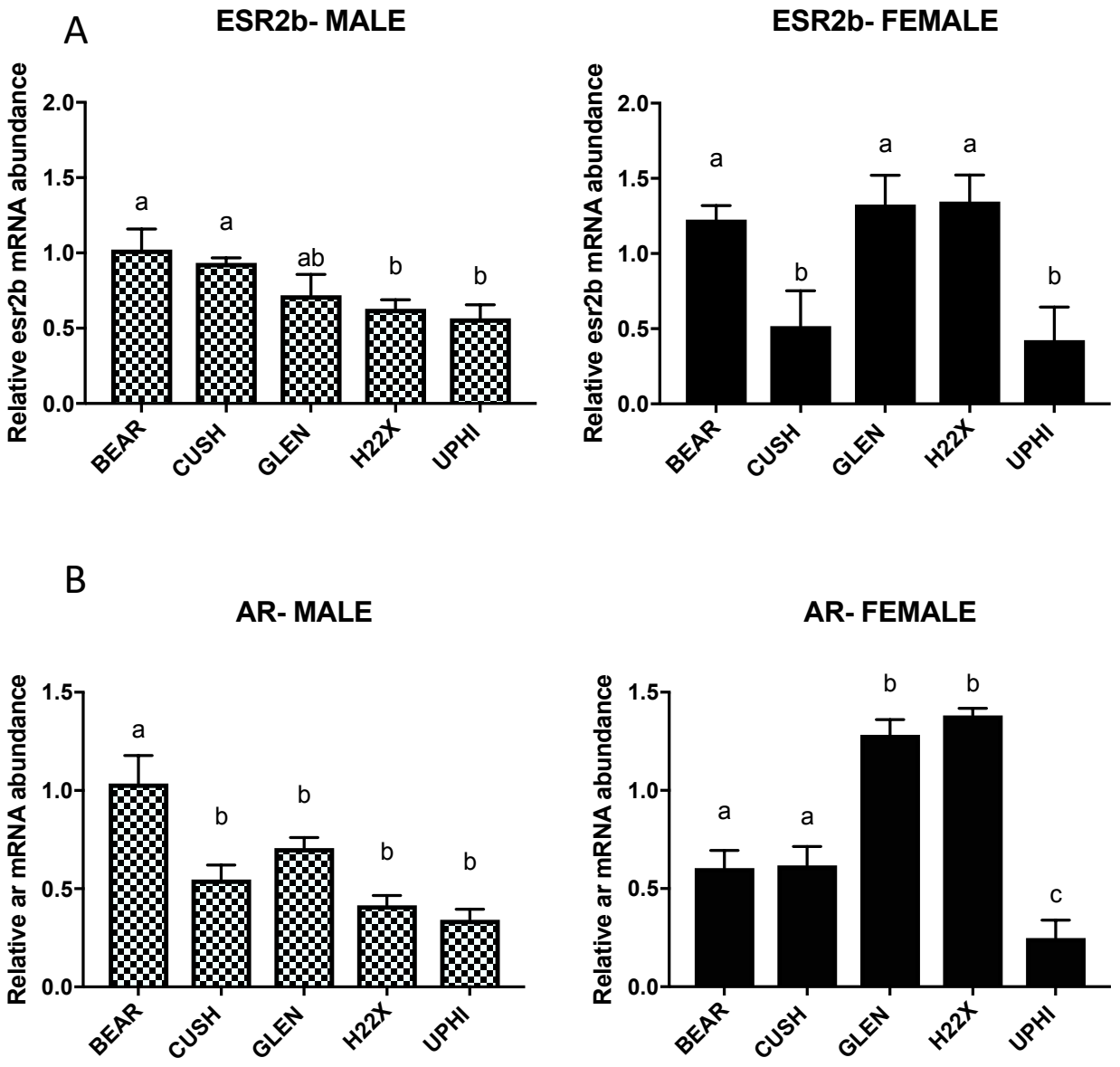

Figure 4.5 Relative estrogen receptor (esr2b) (A) and androgen receptors (AR) (B) mRNA abundance in the liver of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

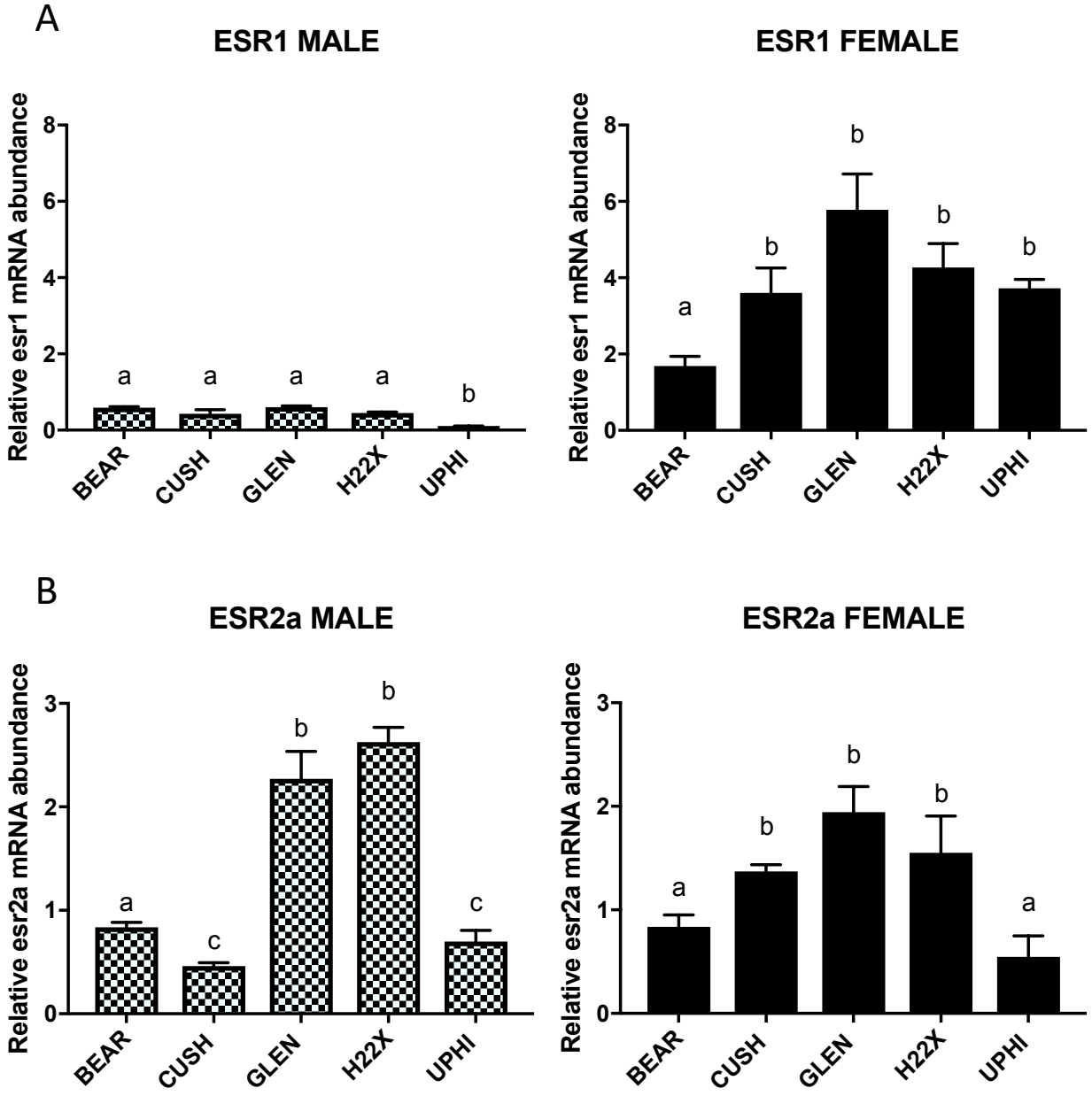

Figure 4.6 Relative estrogen receptor (esr2b) (A) and androgen receptors (AR) (B) mRNA abundance in the gonads of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

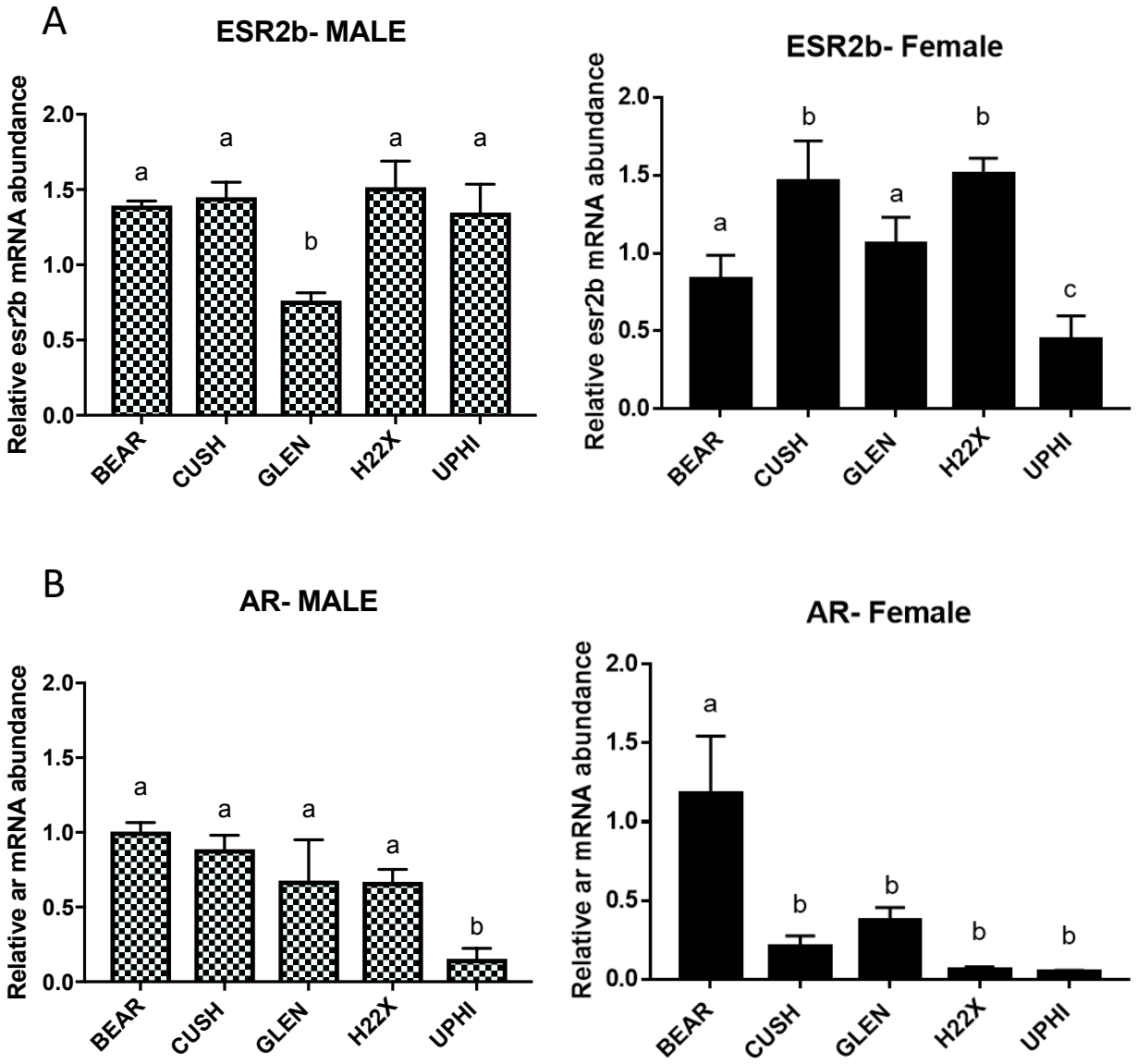

Figure 4.7 Relative estrogen receptor (esr2b) (A) and androgen receptors (AR) (B) mRNA abundance in the gonads of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

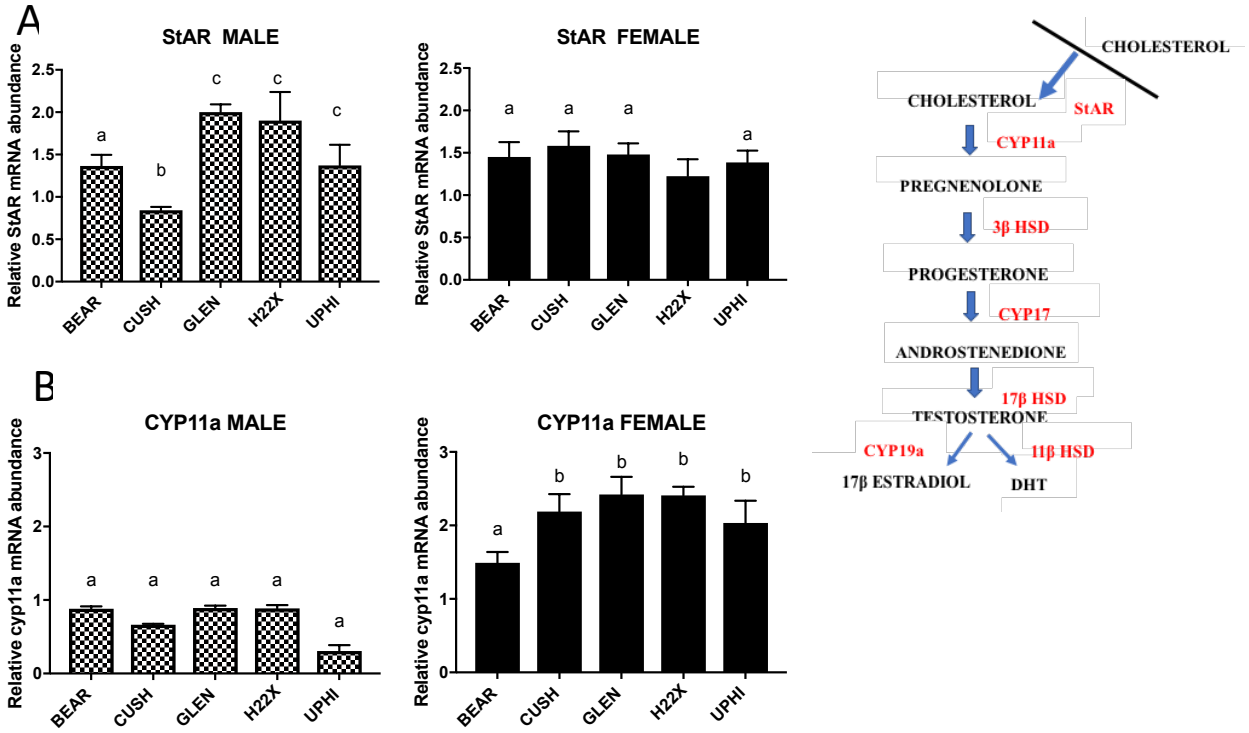

Figure 4.8 Relative Steroidogenic acute regulatory protein (StAR) (A) and CYP11a (B) abundance in the gonads of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

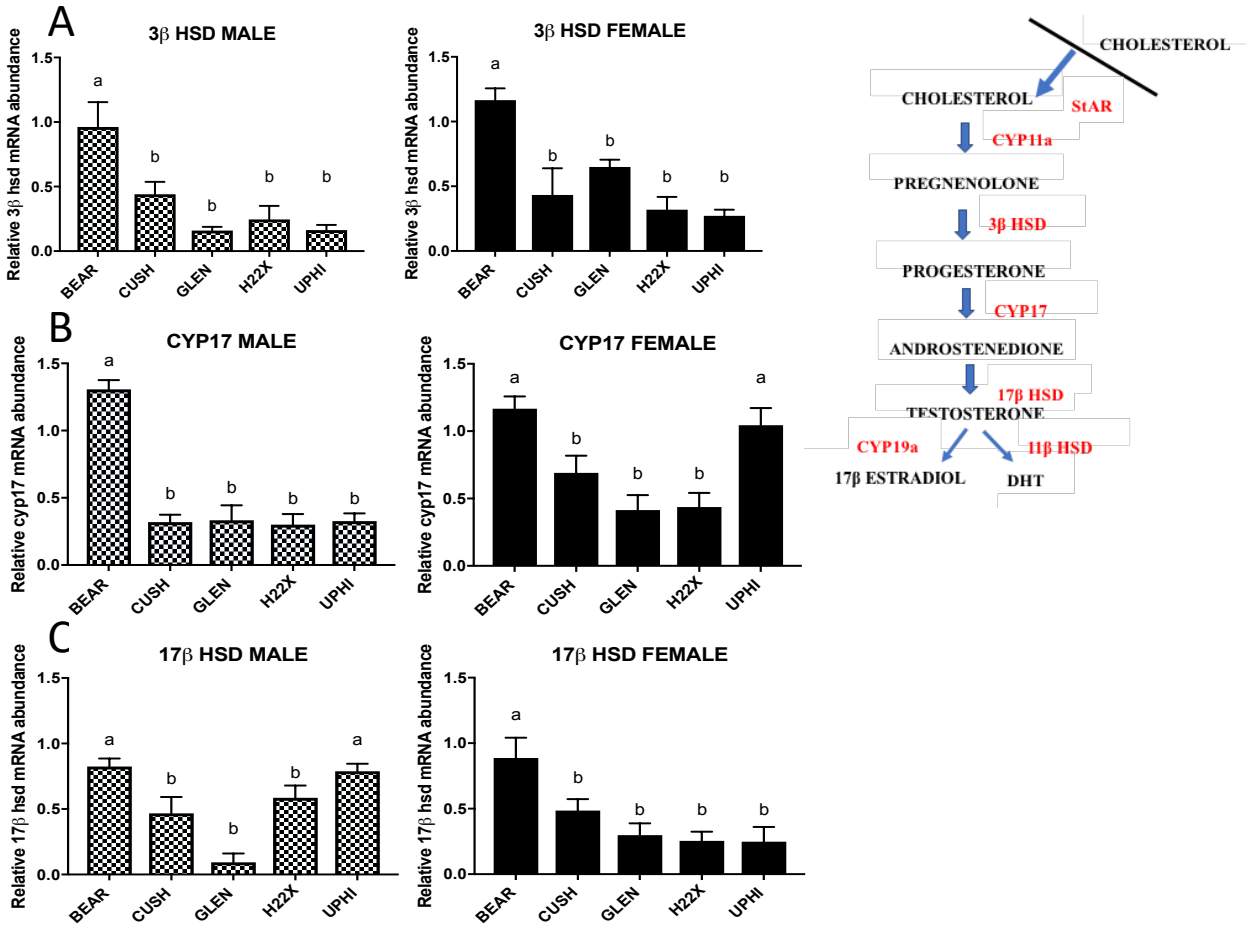

Figure 4.9 Relative 3β HSD (A), CYP17 (B) and 17β HSD (C) abundance in the gonads of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

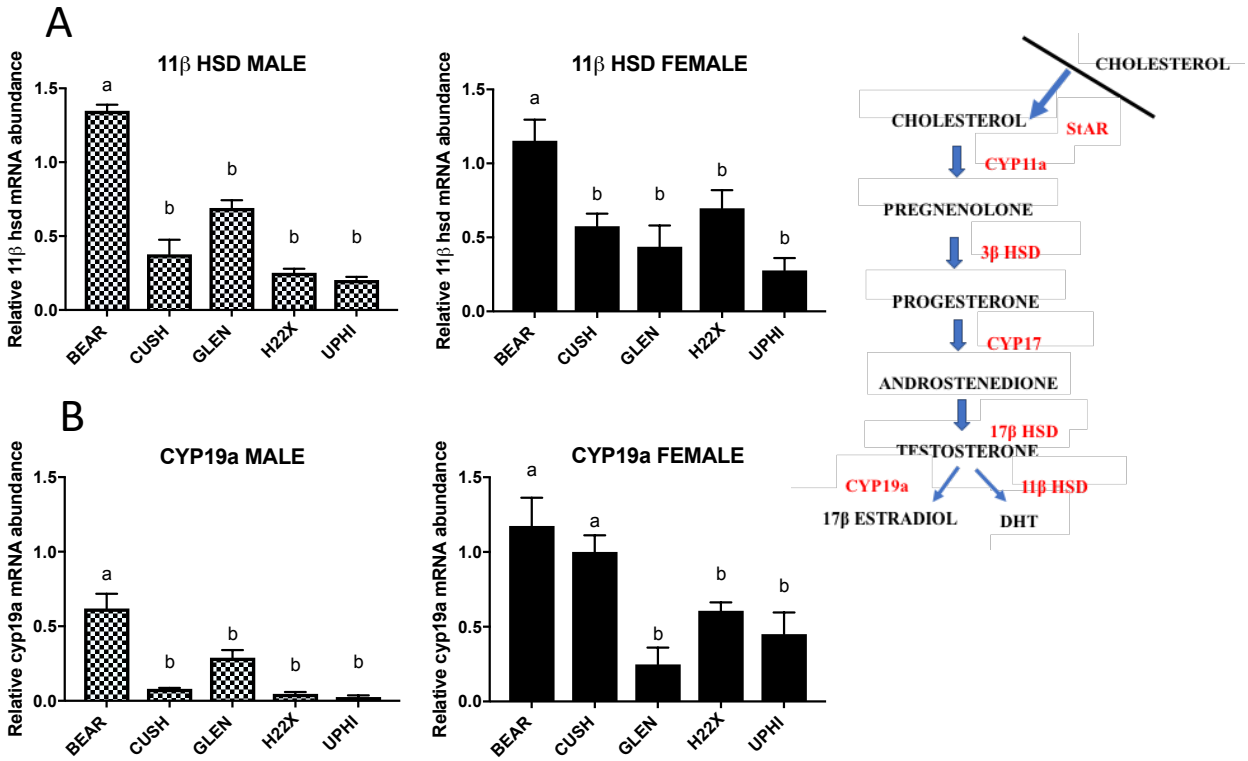

Figure 4.10 Relative 11β HSD (A) and gonadal aromatase CYP19a (B) abundance in the gonads of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

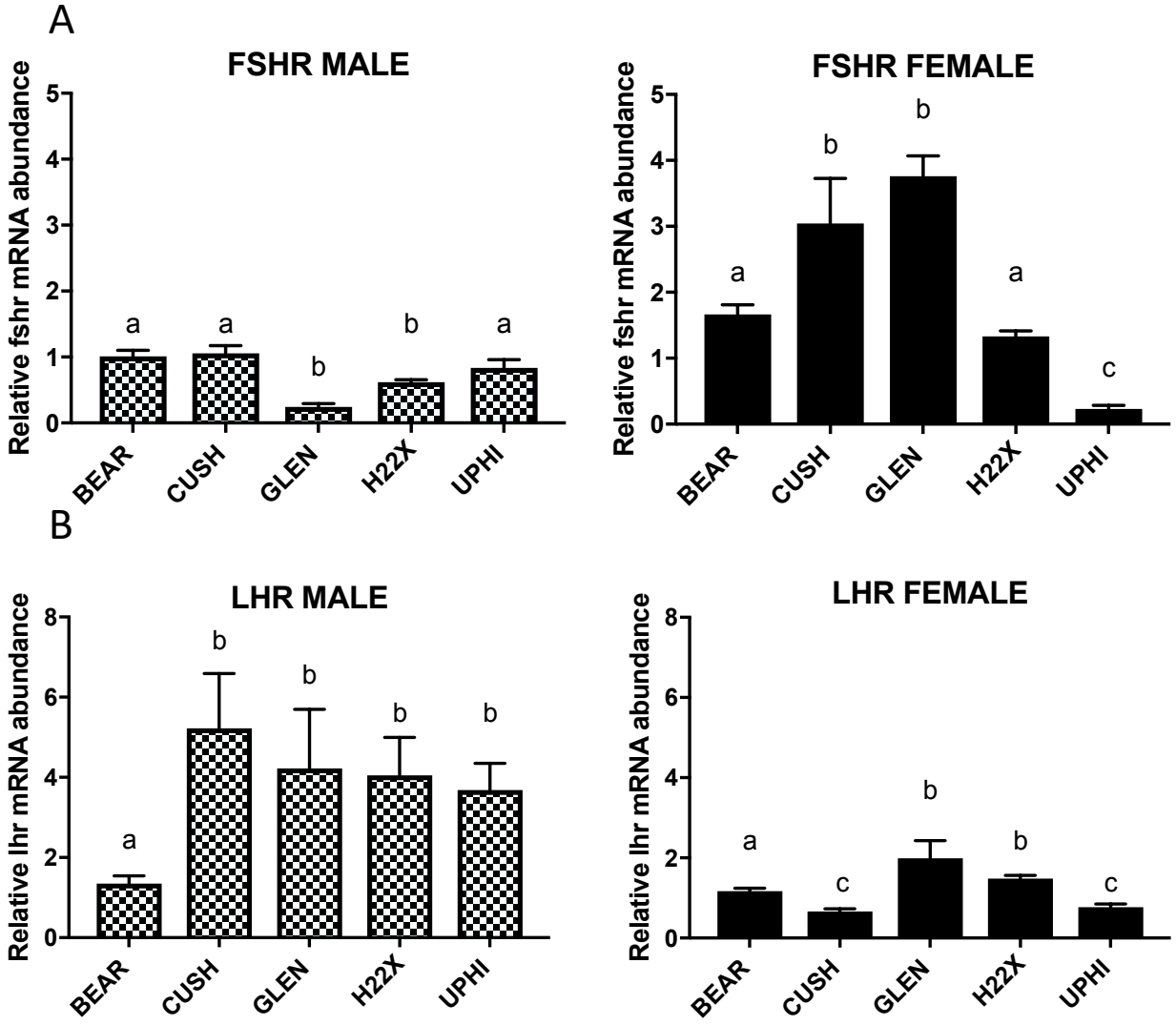

Figure 4.11 Relative follicle stimulating hormone receptor (FSHR) (A) and luteinizing hormone receptor (LHR) (B) abundance in the gonads of the male (A) and female (B) fathead minnows exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. Different letters indicate statistical significance. Bars represent SEM; n=4 and expressed as fold-increase in relative mRNA abundance from the reference site BEAR.

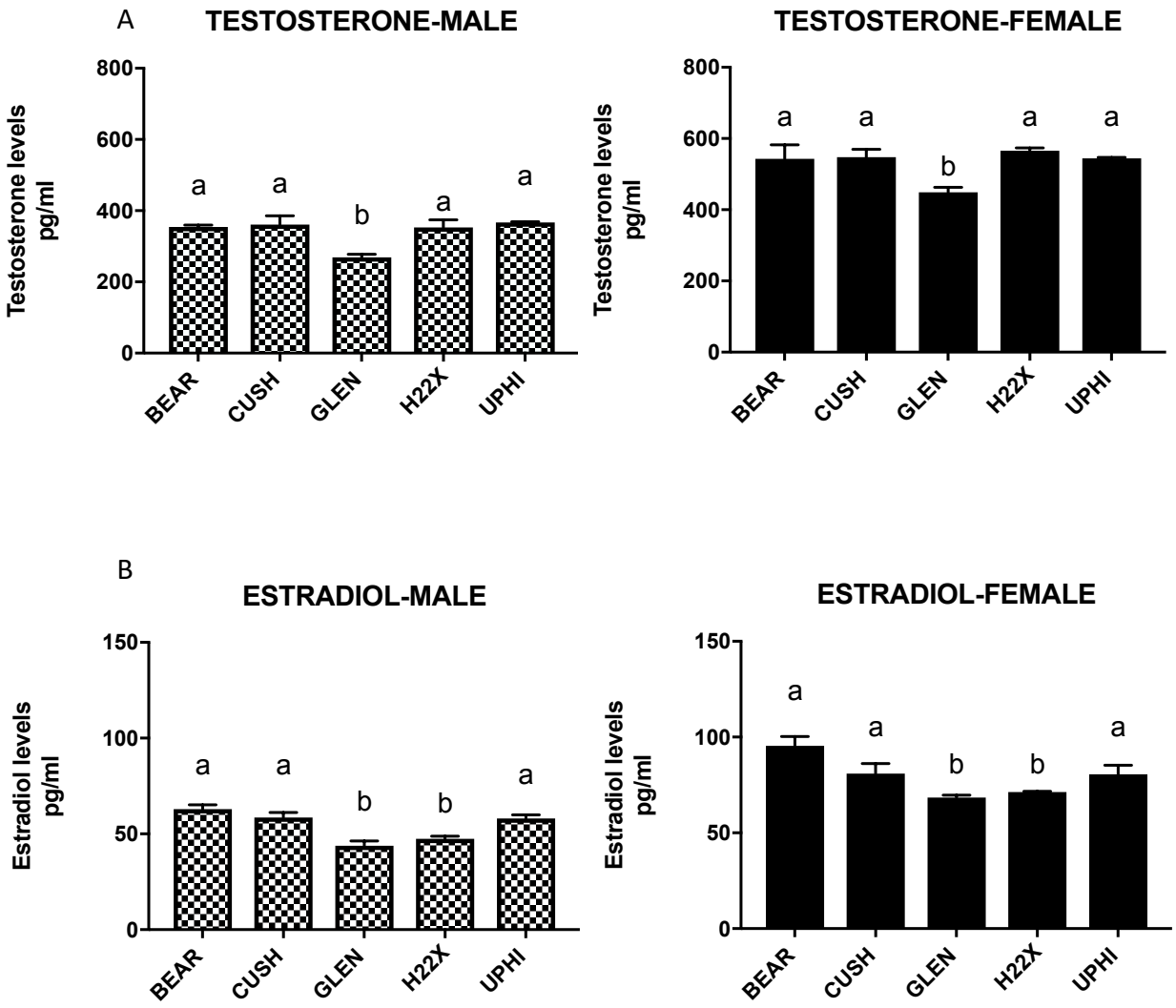

Figure 4.12 Mean testosterone (T) levels (A) and 17β estradiol (E2) levels (B) (pg/g fish ± S.E.M.) in adult male and female fathead minnow exposed at upstream (BEAR, CUSH) and downstream exposed (GLEN, H22X, UPHI) sites along the Bow River. BEAR is the reference site. Different letters indicate statistical significance. Bars represent SEM; n = 3.

Chapter 5 - General Discussion and Conclusion

5.1. General discussion and Conclusion

The research presented in this thesis documents the adverse impacts of treated effluents on fathead minnows exposed along the Bow River. Despite the presence of low levels of environmental contaminants in Alberta rivers, the occurrence of endocrine disruption has been documented in these sites (Evans et al., 2012; Jeffries et al., 2008; Jeffries et al., 2010). The primary objective was accomplished through a site-specific field study in the vicinity of treated effluents discharges looking at the health and reproductive status of fathead minnows caged along Bow River, AB, Canada for the duration of 26 days. However, the effects of treated effluents documented in previous studies varies depending upon various factors such as whether the exposure occurred in the field or in the lab, suggesting that additional environmental variables in the field may be contributing to the disruption in the stress performance of fish.

For my research, three objectives were studied to examine the adverse effects of treated WWTP effluents discharged into the Bow River on the endocrine function, mainly focusing on the reproductive endpoint in fathead minnows, caged along the five different sites. In chapter 3, I describe the adversity of the treated effluents on the gonadal development and morphology, and in chapter 4, the impact of processed effluents on the endocrine profile which includes gonadal steroidogenesis and vitellogenesis in liver was studied using RT-qPCR technique.

For the first objective, I hypothesized that the treated effluents adversely impact the gonadal development in fathead minnows. In the exposed fathead minnows at CUSH (an upstream site) and GLEN, H22X (downstream to WWTP) demonstrated a significant reduction in the gonadosomatic index (GSI), indicative of suppressed gonadal development. Histopathological examination and flow cytometric analysis of the testicular tissue exposed at GLEN and H22X (downstream to WWTP) revealed increased haploid population (spermatids/spermatozoa) and

decreased diploid population (spermatogonia), suggesting that there is suppression in the process of spermatogenesis in the exposed male fathead minnows. Histological examination of the ovarian tissue shows the proportional increase in atretic germ cells, adversely impacting the production of 17β estradiol in exposed female fathead minnows (Chapter 4). The fathead minnows exposed at GLEN and H22X (downstream to Bonnybrook WWTP and Fish Creek WWTP) showed elevated caspase 3 activity in both the sexes, which indicates increased gonadal apoptosis in gonads. Increased gonadal apoptosis suggests depletion of the majority of the germ cells, which possibly impact the production of sex steroids in the fathead minnows exposed at those site in both the sexes. There was considerable induction in caspase 3 activity and mRNA abundance at CUSH (an upstream site).

Various caging studies conducted worldwide demonstrated a high degree of endocrine disruption in the aquatic organisms exposed to estrogenic treated effluents or at sites downstream to WWTPs. Induction of vitellogenin synthesis (an estrogen-mediated process) is the common indicator for the presence of compounds with estrogen-like activity in the treated municipal effluents. There was no significant induction of VTG mRNA level observed in the male fathead minnows caged at GLEN and H22X along the Bow River. The results reveal complex nature of contaminants in the WWTP effluents that can mask estrogen-like activity. This view is supported by the decreased abundance of esr1 and esr2b transcripts in the male fathead minnows at the same sites. In the female fathead minnows caged at GLEN and H22X downstream to Bonnybrook, WWTP and Fish Creek WWTP demonstrated suppressed VTG mRNA abundances which signifies disruption of estrogen-induced response in female fathead minnows caged along the Bow River.

The next objective of the study was to study the impact of contaminants on steroidogenesis. Steroidogenic acute regulatory (StAR) and P450-mediated cholesterol side-chain cleavage

(P450scc), are important enzymes that take part in early gonadal steroidogenesis (Kortner et al., 2009). Exposure to treated effluents did not impact either enzyme, thus treated effluents released into Bow River does not influence the early gonadal steroidogenesis in fathead minnows of both the sexes. In both the sexes sampled at CUSH, GLEN and H22X there was significant reduction of the critical enzymes involved in androgen production (3β HSD, CYP17, 17β HSD), suggesting that lesser androgen substrates may be available for conversion to 17β estradiol in females and 11-ketotestosterone in males. This is consistent with the observed suppression of testosterone levels in male fathead minnows caged at GLEN, and 17β estradiol in female fathead minnows caged at GLEN and H22X. No significant changes were observed in the mean testosterone levels in the fishes exposed to CUSH and H22X, and this could be because the effects on testosterone synthesis may be too small to be translated into the plasma titers, but large enough to have physiological consequences since steroids also exert paracrine effects in the gonads that are not always translate to big changes in circulatory hormone level. Also, there was significant decrease in the FSHR transcript abundances at GLEN and H22X, in male fathead minnows, in agreement with suppressed spermatogenesis in the testes of the male fathead minnows exposed downstream to the Bonnybrook WWTP at GLEN (Chapter 3; Table 5.1 & Table 5.2). These results, are consistent with contaminants analysis carried out previously in the Bow River. The findings provide evidence that contaminants present in WWTP effluent disrupt the normal reproductive process of fathead minnows exposed along the Bow River for 26 days.

Figure 5.1. illustrates that upon sexual maturation, the hypothalamus produces gonadotropin releasing hormone (GnRH), which triggers the pituitary to produce and release two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. These hormones regulate gametogenesis and steroidogenesis in both male and females by binding to their

respective receptors, luteinizing hormone receptor (LHR), and follicle-stimulating hormone receptor (FSHR). LH and FSH then stimulate the synthesis of 17beta-estradiol (E2) and testosterone (T), which lead to vitellogenesis and early gonadal development. This figure 5.1. also shows the mechanisms through which the treated effluents discharged from the Bonny brook WWTP and Fish Creek WWTP disrupts the endocrine profile of the fathead minnows caged along the Bow River for the period of 26 days.

This study highlights several impacts of emerging contaminant on the reproductive system of fishes. The reproductive impairments caused in the caged fathead minnows suggests the presence of the multiple endocrine disruptors in the Bow River. In conclusions, this study presents information on the mechanisms by which contaminants disrupt reproduction in fish. My study provides a better comprehension pre-dominant nature of the disruption based upon the existing treatment used in the WWTPs within the City of Calgary. My study provides a better understanding to employ better bio-monitoring techniques based upon the type of disruption caused in response to the treated effluents.

My studies help to increase the knowledge about the impacts on organisms downstream from WWTP and promote a better understanding among the end-users to characterize exposure risks of emerging contaminants relative to other constituents in municipal wastewater. This research highlights the need to bring about potential changes in the in practice (for municipalities) about monitoring of downstream waters from wastewater treatment plants (WWTP). In a longer run, the data from my studies will help to formulate better bio remediation process. My project is part of a larger team research to investigate how novel treatment technologies achieve improved removal of contaminants, and how can we critically evaluate the adverse impact of diverse compounds present in treated WWTP effluents. My project is part of a larger team research to investigate how

novel treatment technologies achieve improved removal of contaminants, and how can we critically evaluate the adverse impact of diverse compounds present in treated WWTP effluents. Therefore, the significance is far reaching with global implications.

Table 5.1. Summary of the results. ↑ represents the significant up-regulation relative to the reference site BEAR, and ↓ shows the significant down-regulation compared to the reference site in male fathead minnows caged along the Bow River. NSD = no significant difference.

GENES	CUSH	GLEN	H22X	UPHI
StAR	↓	↑	↑	↑
CYP11a	↓	NSD	NSD	↓
3β HSD	↓	↓	↓	↓
CYP17	↓	↓	↓	↓
17β HSD	↓	↓	↓	NSD
CYP19a	↓	↓	↓	↓
11β HSD	↓	↓	↓	↓

Table 5.2. Summary of the results. ↑ represents the significant up-regulation relative to the reference site BEAR, and ↓ shows the significant down-regulation compared to the reference site in female fathead minnows caged along the Bow River. NSD = no significant difference.

GENES	CUSH	GLEN	H22X	UPHI
StAR	NSD	NSD	NSD	NSD
CYP11a	↑	↑	↑	↑
3β HSD	↓	↓	↓	↓
CYP17	↓	↓	↓	NSD
17β HSD	↓	↓	↓	NSD
CYP19a	↓	↓	↓	↓
11β HSD	↓	↓	↓	↓

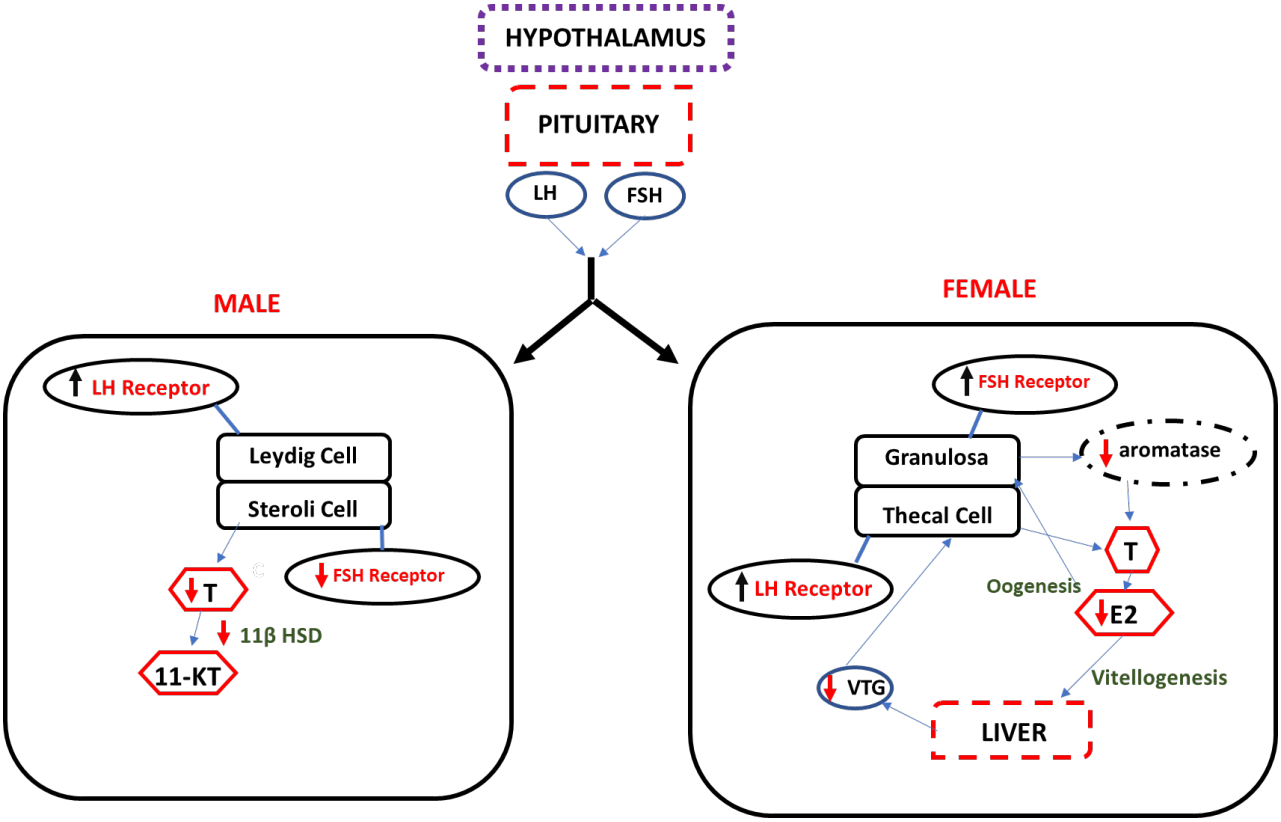

Figure 5.1 Fathead minnows hypothalamic–pituitary–gonadal (HPG) axis model and physiological response profile in male and female fathead minnows caged at GLEN and H22X downstream to Bonnybrook WWTP and Fish Creek WWTP. Abbreviations: GnRH - Gonadotropin-releasing hormone, LH - luteinizing hormone, FSH - follicle-stimulating hormone, E2 - 17β -estradiol, T - testosterone, 11 - KT – Ketotestosterone.

Chapter 6 – References

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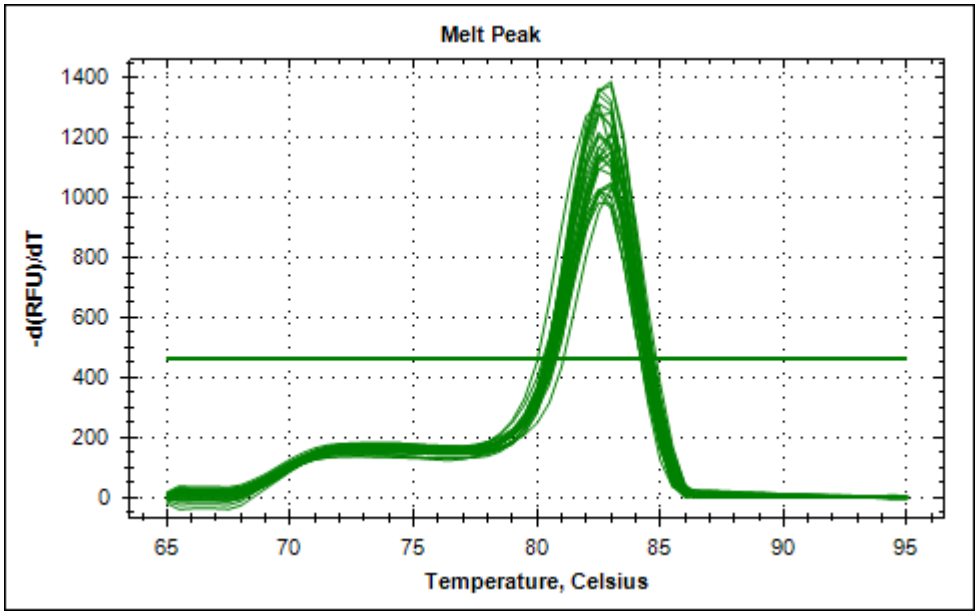
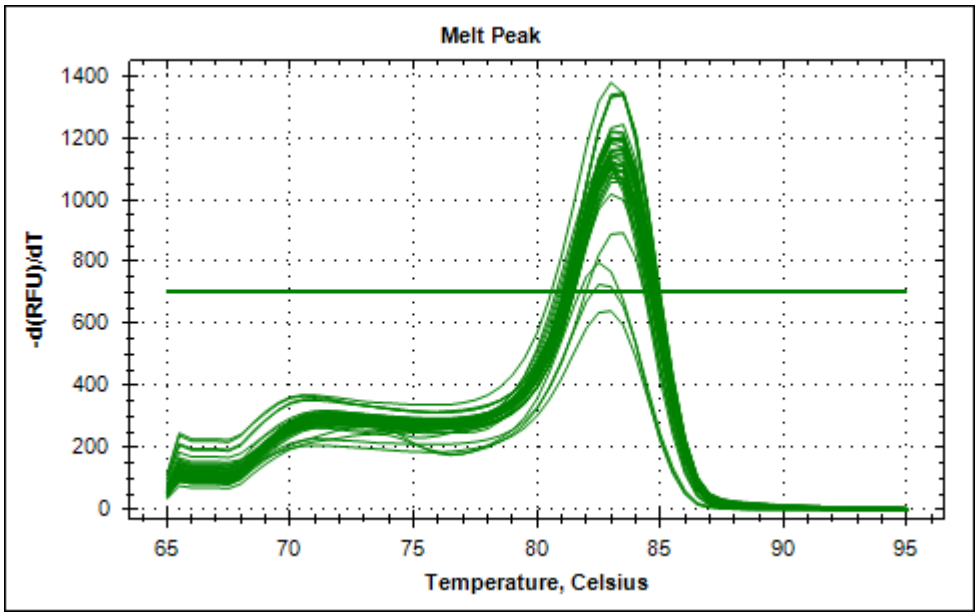
Chapter 7 – Appendix

QPCR Curves

Actin



18s RNA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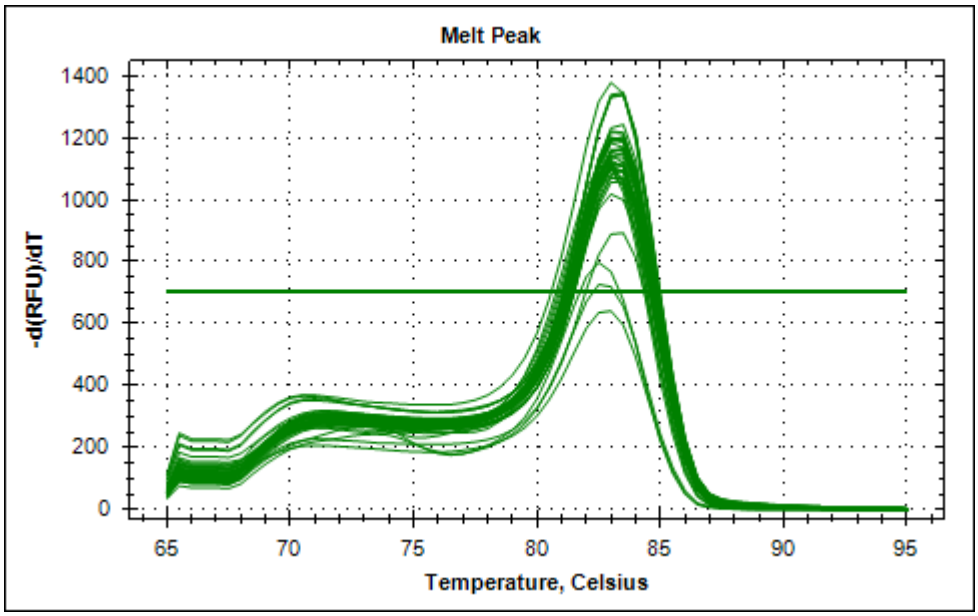
95
Temperature, Celsius

VTG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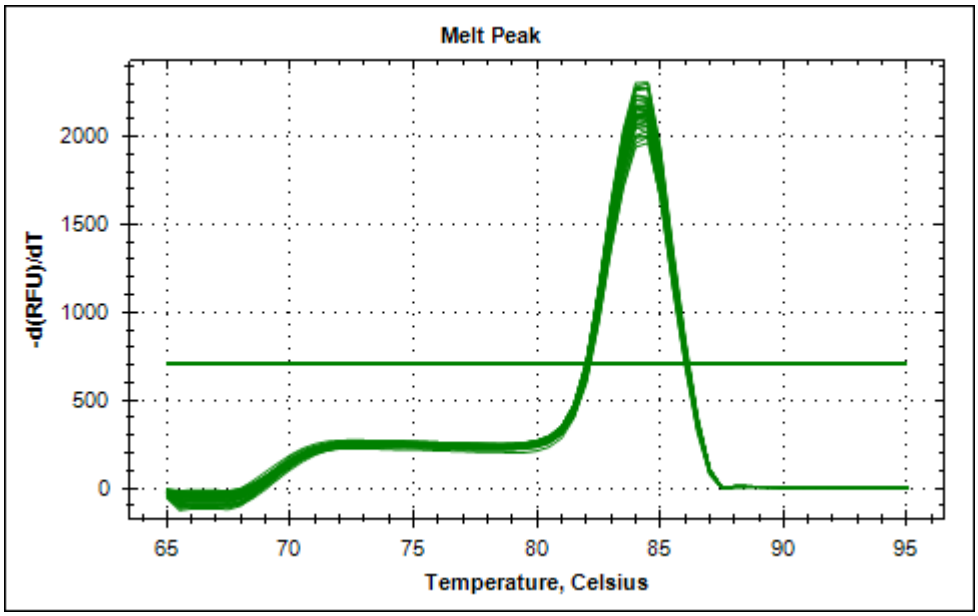
Temperature, Celsius

ESR1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Temperature, Celsius

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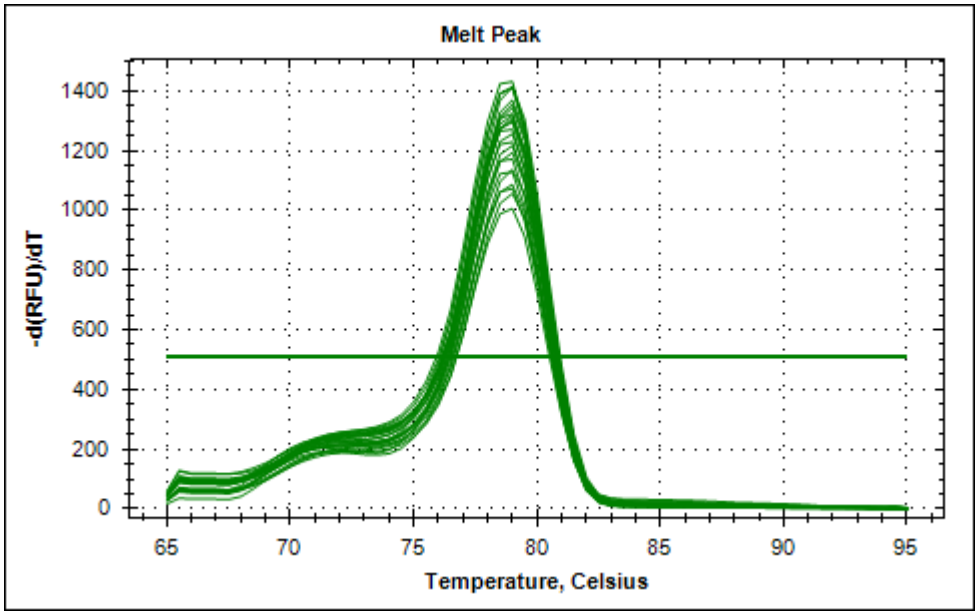
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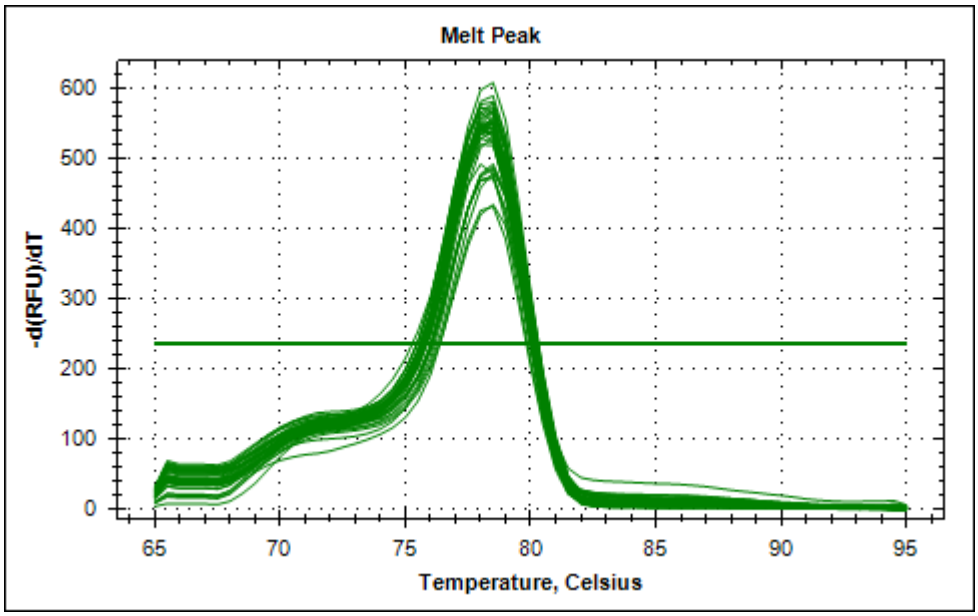
Temperature, Celsius

ESR2b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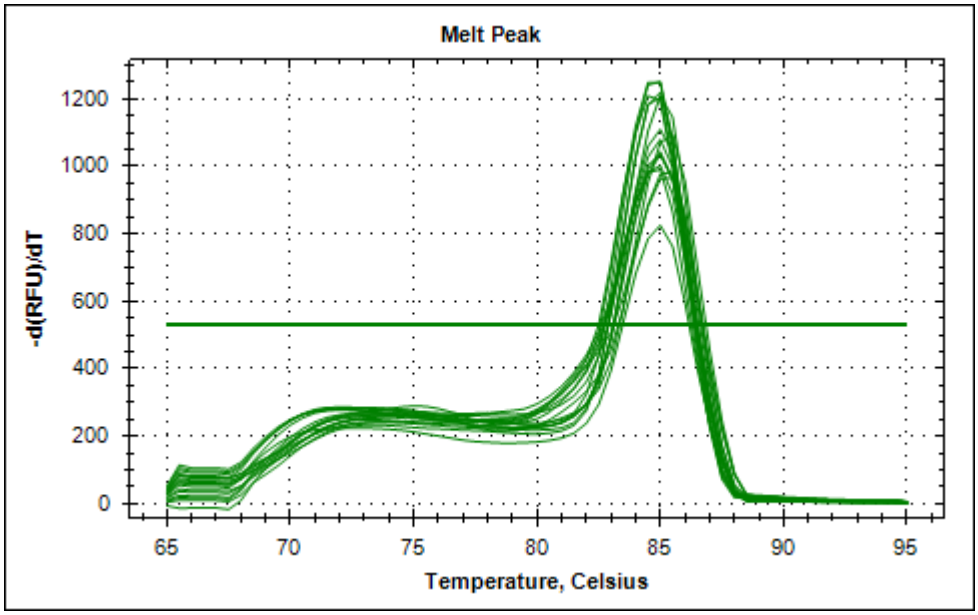
Temperature, Celsius

AR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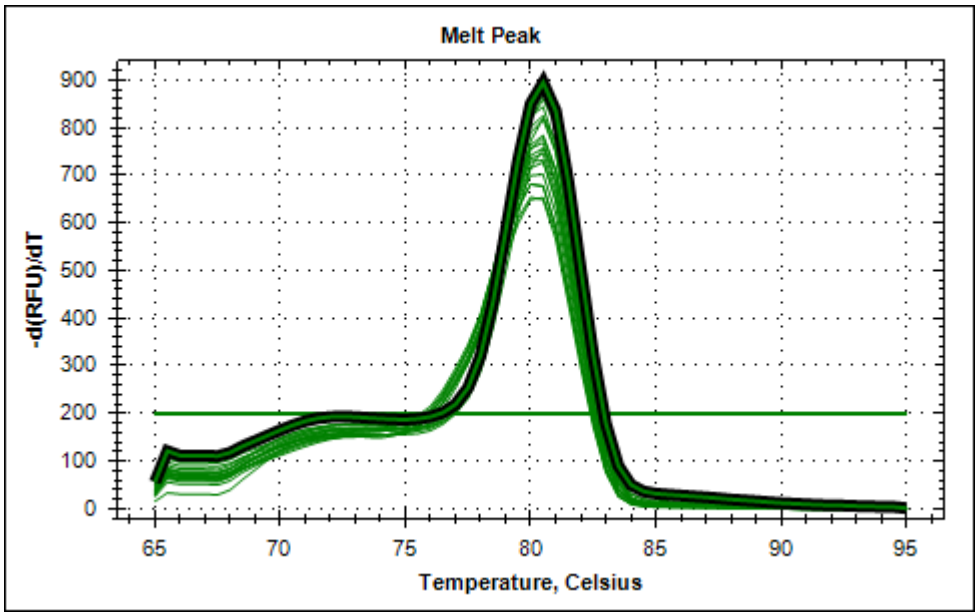
Temperature, Celsius

FSHR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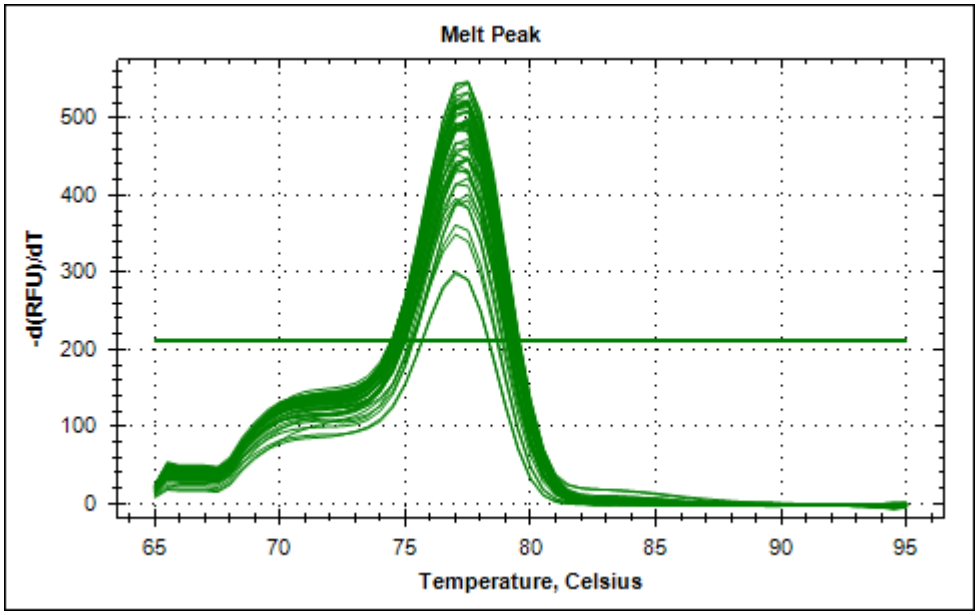
Temperature, Celsius

LHR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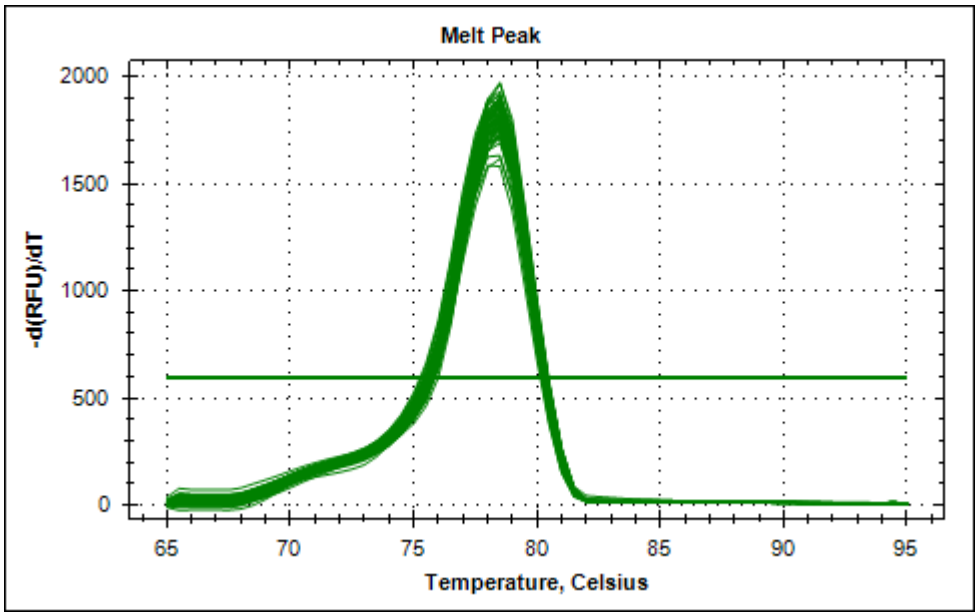
Temperature, Celsius

STAR



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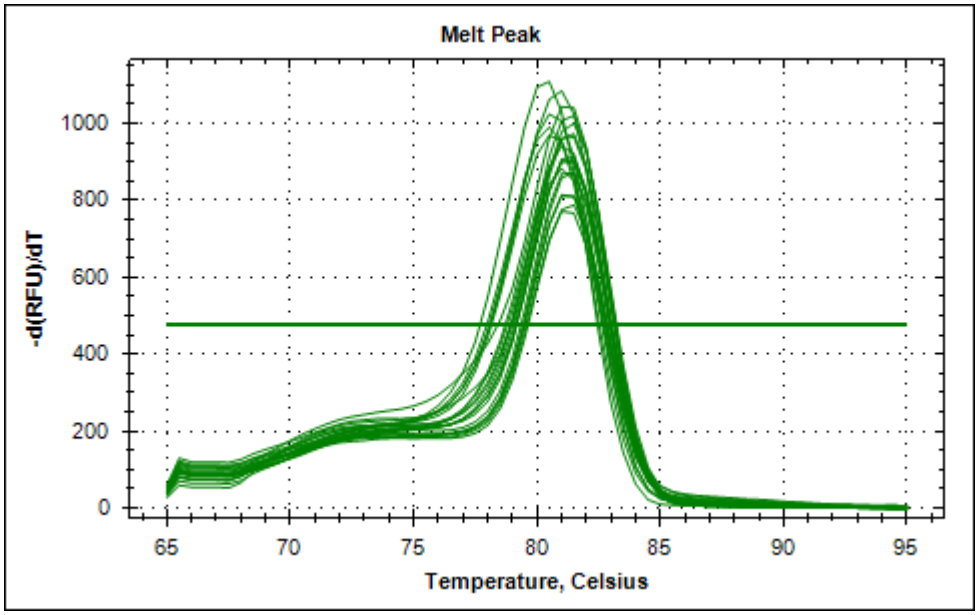

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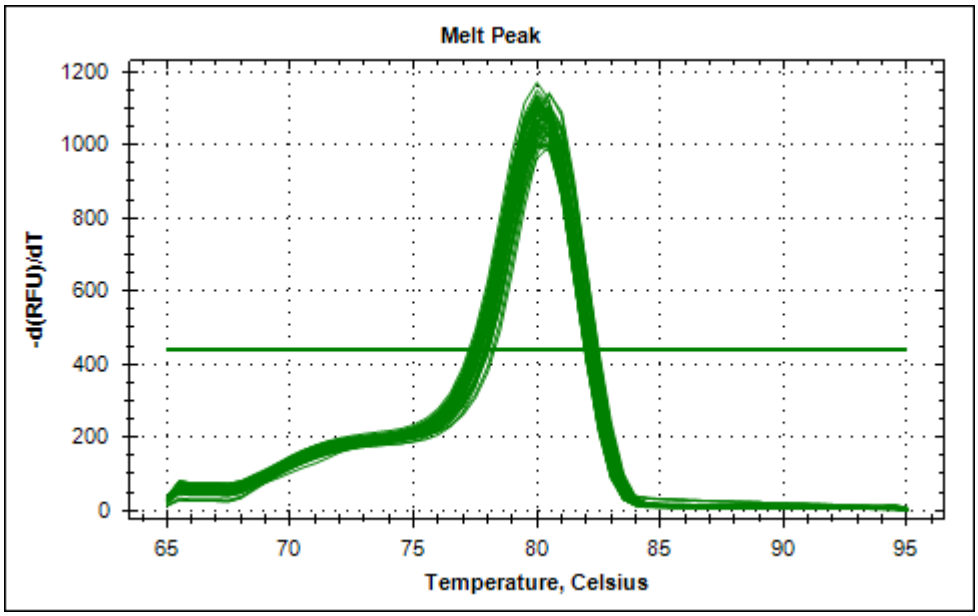
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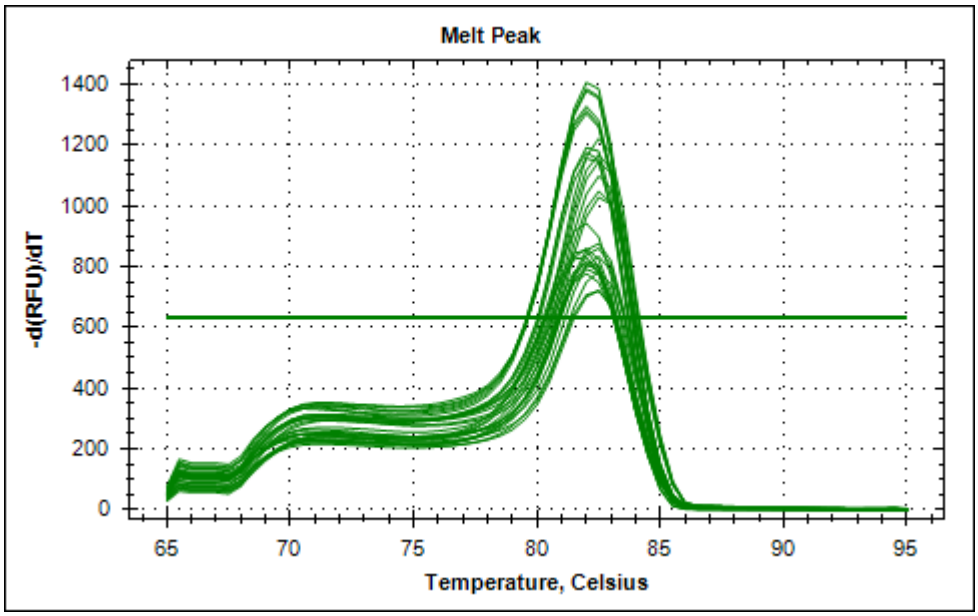
Temperature, Celsius

CYP17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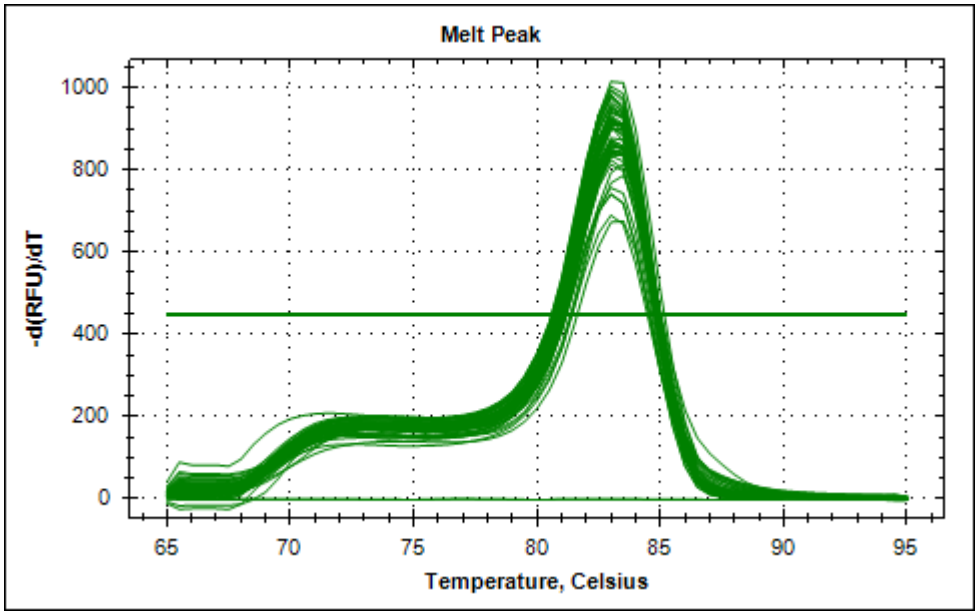
Temperature, Celsius

17B HSD



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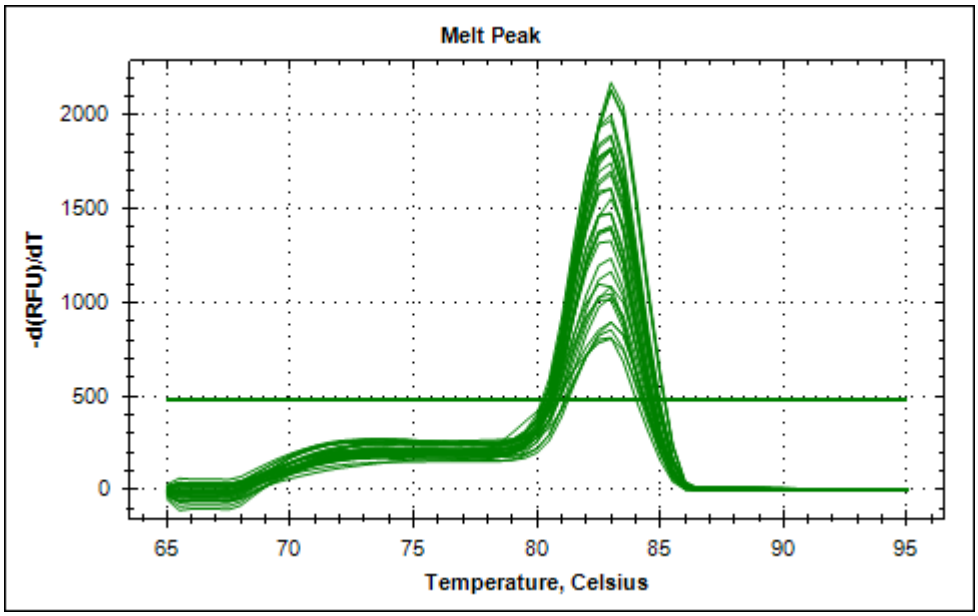
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Temperature, Celsius

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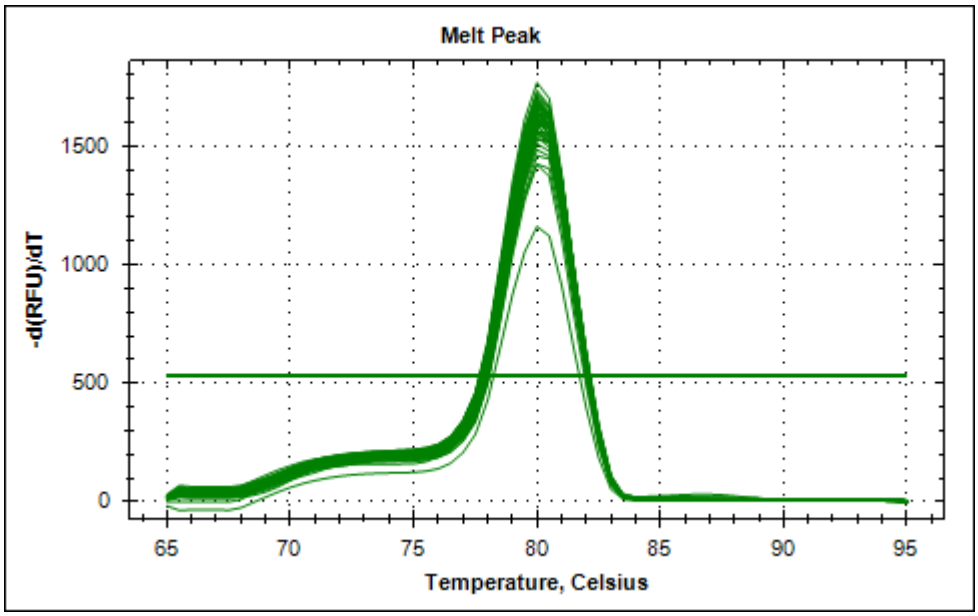
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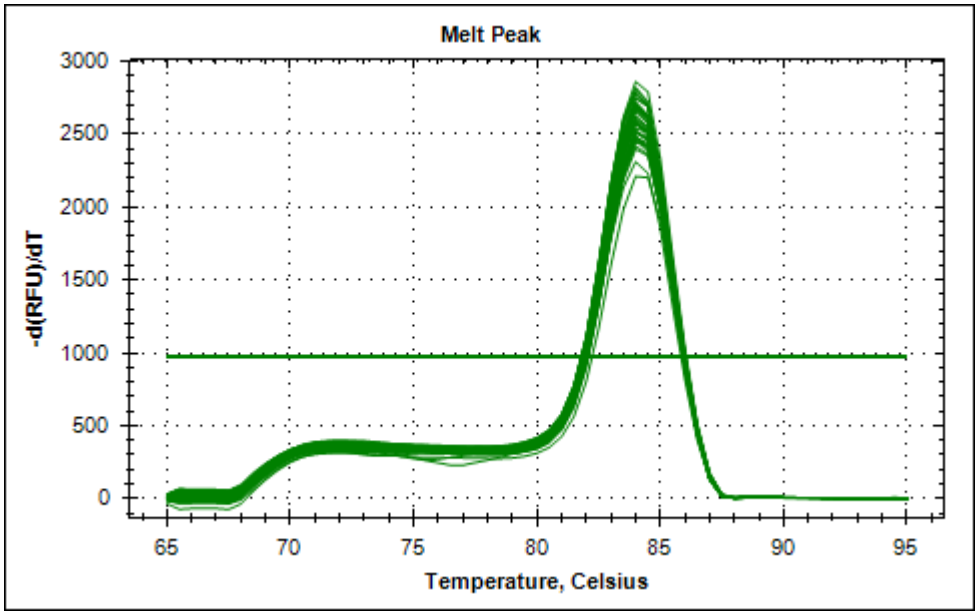
Temperature, Celsius

ESR1



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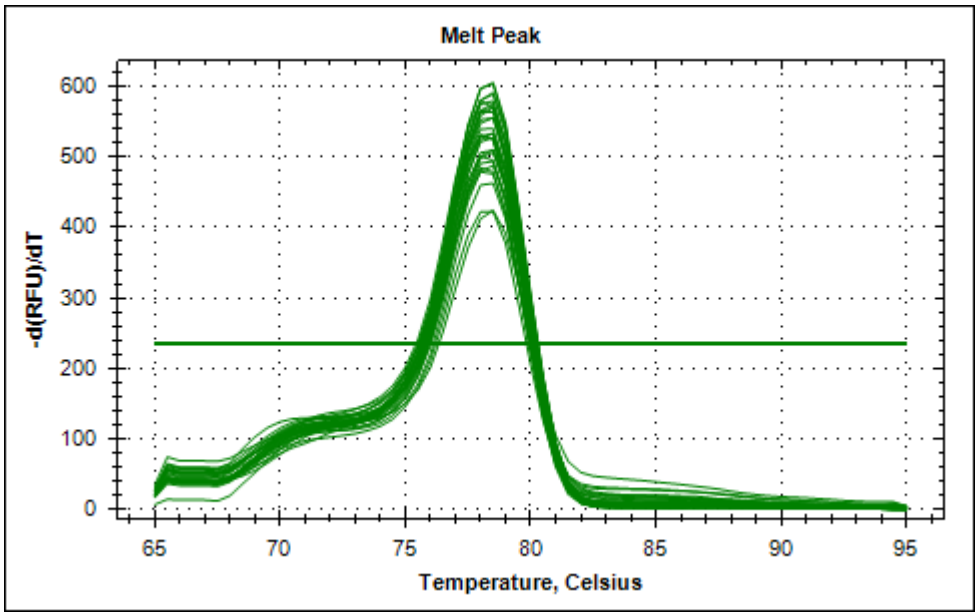

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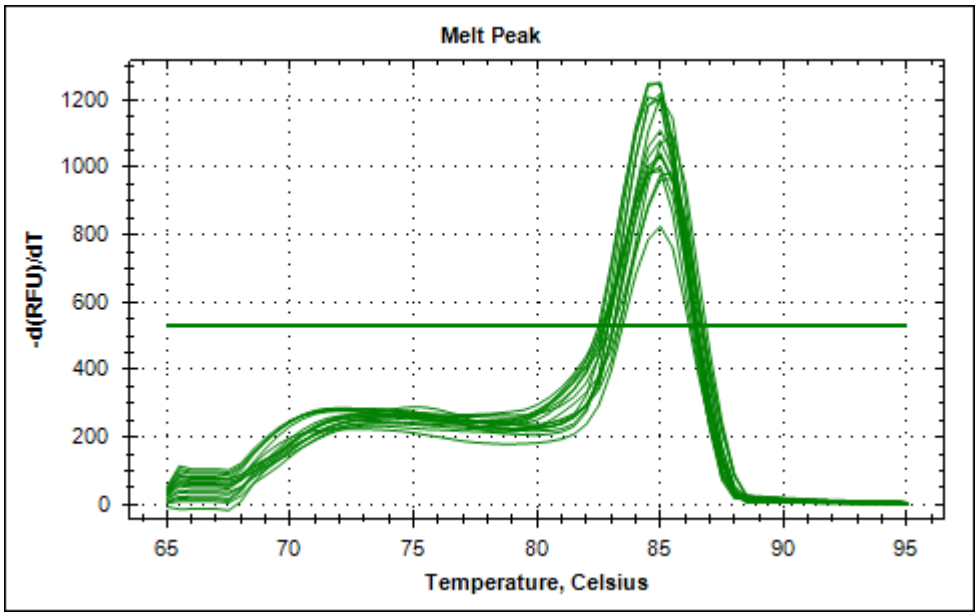
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Temperature, Celsius

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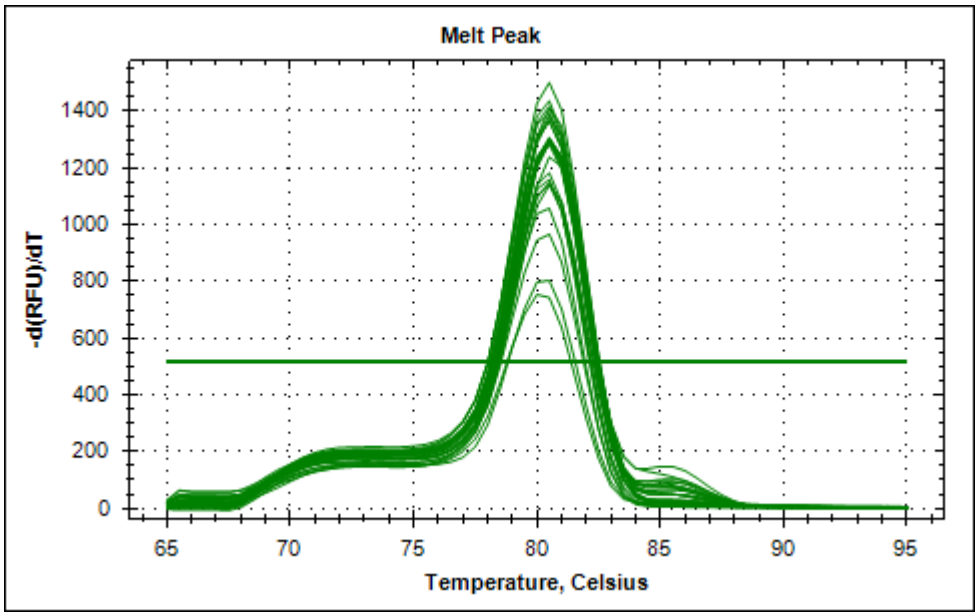
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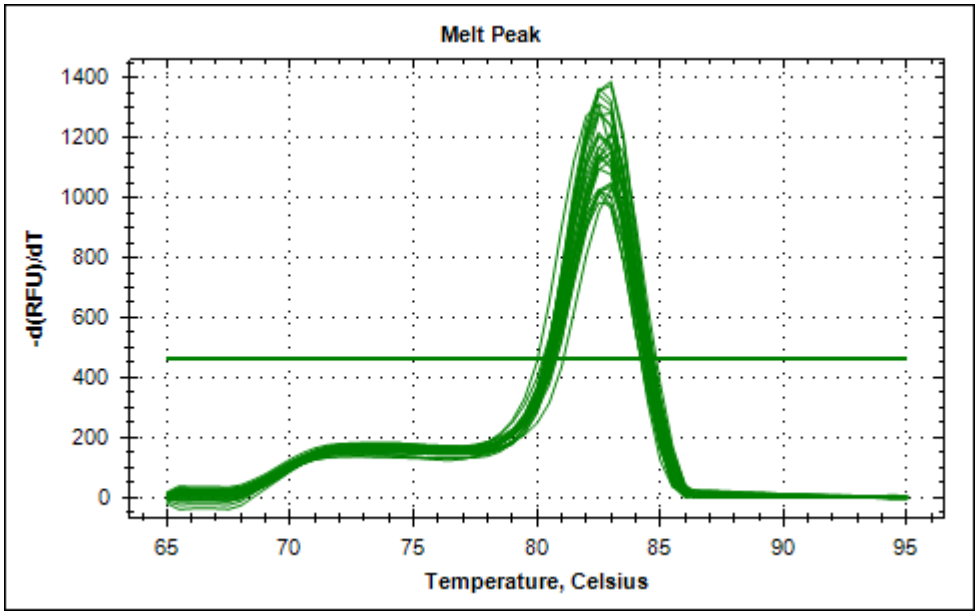
Temperature, Celsius

Caspase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Temperature, Celsius