COMPARING THE EFFECTS OF TWO MUNICIPAL WASTEWATER EFFLUENTS ON REPRODUCTIVE OUTPUT AND ENDOCRINE STATUS IN THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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By

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ABSTRACT

Municipal wastewater effluents (MWWEs) contain anthropogenic substances that can exhibit endocrine disrupting activity. These substances, which disrupt endogenous endocrine signalling, can impact normal fish reproduction and development and lead to effects that manifest at the individual and population level. In the Prairie provinces of Canada, increasing urban populations and industrial activities have resulted in greater water demand, and therefore, greater amounts of MWWEs released into the aquatic environment. Treatment plants often feature outdated technologies that inefficiently remove contaminants and when coupled with the increasing water demands, present a risk to receiving environments. The objective of this study was to utilize the 21-day fathead minnow reproductive assay to identify potential reproductive effects of MWWEs from Regina and Saskatoon. Fish were exposed for 21 days to 0 %, 10 % and 50 % of the effluents. Impacts on reproductive success (fecundity, fertility) as well as morphological, histopathological, and molecular/biochemical endpoints were assessed in male and female fish. Exposure to 10 % and 50 % Regina MWWE resulted in a significant decrease in fecundity compared to non-exposed minnows. There were significant differences in fecundity of minnows exposed to Saskatoon effluent, although the effect was not dose-dependent. Fertilization rate remained consistent regardless of treatment. Histological examination revealed a significant increase in proportion of spermatogonia and testicular degeneration in male fathead minnow testes in 50 % effluent concentrations compared to control minnows in both Regina and Saskatoon exposures. Increased oocyte atresia was observed in female minnows exposed to both Regina and Saskatoon 50 % treatment. No change in circulating sex steroid hormones (estradiol, 11-ketotestosterone) in male or female minnows was observed. Analysis of gene transcription revealed that exposure to Regina effluent induced ERα mRNA in female fathead minnows while exposure to Saskatoon effluent induced ERα in females and androgen receptor in males. No change in the expression of the vitellogenin was observed in any effluent-exposed group. The results from this study demonstrated that exposure to MWWE can impact the reproductive output and gonadal development of fathead minnows; however, due to the lack of change in key endocrine-related endpoints, it appears these effects are not due to estrogenic or androgenic compounds in the effluent. Additional work conducted by two parallel AIME studies supported this conclusion. Due to the complex nature of MWWE further work incorporating additional

endpoints (i.e. behaviour), exposure lengths, and multi-seasonal studies would provide valuable insight regarding the biological impacts of MWWE.

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LIST OF ABBREVIATIONS

11-KT	11-ketotestosterone
17βHSD	17β-hydroxysteroid dehydrogenase
18s	ribosomal rna 18s
3βHSD	3β-hydroxysteroid dehydrogenase
AIF	apopstosis inducing factor 3
AIME	aquatic impact assessment of municipal effluents
AR	androgen receptor
ARF	Aquatic Research Facility
ATRF	Aquatic Toxicology Research Facility
cDNA	complimentary deoxyribonucleic acid
CF	condition factor
cm	centimetre
CYP11A	cholesterol side-chain cleavage
CYP17	17α-hydoxylase
CYP19a	aromatase α
CYP19b	aromastase β
CYP1a	cytochrome p450 1a
CYP3a	cytochrome p450 3a
d	day
DEET	diethyltoluamide
E2	estradiol
EAC	endocrine active compounds
EDS	effluent-dominated stream
EE2	17α-ethinylestradiol
ELISA	enzyme-linked immunosorbant assay
ER	estrogen receptor
FSH	follicle stimulating hormone
g	gram
GSI	gonadal somatic index
GST	glutathione-s-transferase

h	hour
HPGL	hypothalamic-pituitary-gonadal-liver axis
HSI	hepato somatic index
HSP70	heat shock protein 70
LH	leutinizing hormone
mg/L	milligram per litre
mm	millimetre
mRNA	messenger ribonucleic acid
MWWE	municipal wastewater effluent
ng/L	nanogram per litre
N-NH3	ammonia nitrogen
N-NO2	nitrate nitrogen
PPCP	pharmaceutical and personal care products
StAR	steroidogenic acute regulatory protein
T	testosterone
ug/L	microgram per litre
US EPA	United States Environmental Protection Agency
VTG	vitellogenin
WWTP	wastewater treatment plant

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Therefore, there is some repetition between the material presented in each chapter. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 3 contains a general discussion and overall conclusion. Chapters 2 is organized as a manuscript for publication in a peer-reviewed scientific journal and a description of author contributions is provided following the preface for this chapter. References cited in each chapter are combined and listed in the References section of the thesis. Supporting information associated with the research chapter are presented in the Appendix section at the end of this thesis.

CHAPTER 1

1. LITERATURE REVIEW

1.1. Municipal Wastewater Effluent

Municipal wastewater treatment plants (WWTPs) are responsible for treating wastewater generated from the surrounding population by removing solids and compounds from the water before releasing the treated effluent into the environment. Municipal wastewater effluents (MWWEs) discharged from WWTPs contain a complex mixture of contaminants, both chemical and biological, including suspended solids, organic waste, nutrients such as nitrogen and phosphorus, bacteria, and both industrial and household chemicals (CCME, 2006). The occurrence and relative levels of these compounds depend on the levels of treatment. Release of MWWE can have significant environmental impacts on the quality of the receiving environment. Impacts of continuous discharges of MWWEs include the increase in toxic chemicals such as ammonia (Waiser et al., 2011a), eutrophication due to increased nutrient (i.e. phosphorus) inputs (McMaster et al., 2005; Yang et al., 2010), and lower dissolved oxygen levels (Tetrault et al., 2012). Recently, there has been concern about endocrine active compounds (EAC) contained within MWWE, with particular interest in pharmaceutical and personal care products (PPCPs). A wide range of PPCPs may be found in MWWEs including antibiotics, anti-inflammatories, analgesics, detergents, natural and synthetic estrogens (EE2), as well as their metabolites (Campbell et al., 2006; Waiser et al., 2011b). The prevalence of these chemicals in MWWE often arise from households where PPCPs are flushed down toilets or drains and consequently end up in the effluent (Campbell et al., 2006). Many of these compounds have been designed to interact with biological targets and elicit biological effects at low concentrations so there is concern regarding their impact on non-target aquatic organisms (Waiser et al., 2011b). In a whole lake exposure to low concentrations (ng/L range) of 17α-ethynylestradiol (EE2), the synthetic estrogen used in birth-control pills, researchers demonstrated near extinction of the lake's population of fathead minnow (*Pimephales promelas*) due to reproductive failure (loss of recruitment, intersex in males, altered oogenesis in females) (Kidd et al., 2007). This study is one of the few that have demonstrated decreased reproductive success and sustainability of wild fish populations following low level exposure to an endocrine-active compound. The low concentration effect levels of PPCP is especially disconcerting when coupled with evidence that WWTPs often cannot remove these contaminants efficiently (Thomas et al., 2005; Santos et al., 2007; Sui et al., 2010). As a result, effluents from treatment plants have been identified as the primary source of PPCP discharge into the aquatic environment (Daughton and Ruhoy, 2009).

There are also issues regarding the complexity of MWWE as they contain a mixture of domestic and industrial waste and are released into water systems that can be simultaneously influenced by chemicals from other sources. These include pesticides and fertilizers in agricultural run-off (Yang et al., 2010; Waiser et al., 2011a). Unfortunately, a chemical's toxicity is usually assessed in single exposure studies and does not adequately address the potential mixture toxicity associated with effluents such as MWWE.

The two largest urban centres in Saskatchewan, Saskatoon and Regina, each have a main municipal WWTP. Both cities have experienced rapid population growth in recent years and this has resulted in greater volumes of effluent released in to receiving bodies. This increase in effluent release coupled with extreme seasonal changes in water flows within streams and rivers in Saskatchewan can mean that some flows could be made up of primarily effluent at different times of the year. For example, Wascana Creek downstream of the WWTP for Regina can be up to 100% treated municipal wastewater during dry seasons (Waiser et al., 2011a). Due to the low flow and high effluent contamination of Wascana Creek during seasons of low water flow, it has been classified as an effluent dominated stream (EDS). These types of water systems present the greatest risk to aquatic organisms due to increased risk of exposure as a result of the decreased dilution (Brooks et al., 2006). Studies focusing on the effluent contamination in Wascana Creek have highlighted the environmental impact to local aquatic organisms (Tetrault et al., 2012; Waiser et al., 2011b). Conversely, the Saskatoon WWTP discharges its effluent into the South Saskatchewan River, which is a significantly larger water system. As a result of the increased volume of water, the effluent discharged into the river is more dilute than in Wascana Creek and presents a decreased risk to initiate adverse effects in the local aquatic organisms.

The WWTP located in Regina was built in 1956 and serves a population of ~ 230,000. It is a tertiary treatment facility, utilizing primary treatment (grit and sedimentation), a 5-cell biological-aerated lagoon system to remove solids, alum treatment to remove phosphorus, and UV disinfection (reduction of microorganisms) to treat its effluent before it is released into Wascana Creek (Zhao and Viraraghavan, 2004; Waiser et al., 2011a). As previously mentioned, during winter the water in the creek consists mostly of treated effluent until the creek meets the Qu'Appelle River approximately 60km downstream (Waiser et al., 2011a). The Saskatoon WWTP was built in 1971 and serves a population of ~260,000. This plant is considered a secondary treatment facility and utilizes a more advanced system compared to Regina, with

bioreactors that remove organic compounds such as excess carbon, phosphorus and nitrogen. Addition of these bioreactors to WWTPs has also demonstrated increased efficiency in removing PPCPs from wastewater influent (Barber, 2012). The effluent is also UV-disinfected before being released into the South Saskatchewan River. Differences in the technology employed can greatly affect the potential for PPCPs to be released and impact resident fish species. For example, a study conducted in Boulder, Colorado measured the biological responses of fathead minnow to wastewater treatment plant effluent before and after an upgrade to the facilities (Barber, 2012). When the facility switched from a biological filter to an activated sludge process, there was a significant decrease in levels of EACs such as EE2 and 4-nonyphenol as well as decrease in nitrogen (Barber, 2012). In order to accommodate an increasing population and to meet the requirements of current effluent water quality standards the Regina WWTP underwent a series of upgrades that took place between 2014 and 2016 (https://www.regina.ca/residents/ water-sewer/waste-water-treatment-plant). These upgrades increased the capacity of the facility (156M ML/day from 100 ML/d) and added a bacterial treatment process, similar to the facilities in Saskatoon (http://www.epcor.com/water/Pages/regina-project-page.aspx).

1.2. Hypothalamic-Pituitary-Gonadal Axis in Fish

The hypothalamic-pituitary-gonadal-liver axis (HPGL) is conserved among the vertebrate species and works via positive/negative feedback control that regulates the synthesis and secretion of hormones from the hypothalamus, pituitary gland, the gonads, and the liver (Arukwe and Goksøyr 2003; Ankley et al., 2004). The signalling pathway begins in the hypothalamus which when stimulated by external or internal cues, releases gonadotropin-releasing hormone (GnRH). This hormone interacts with the pituitary gland and signals the release of two gonadotropic hormones, GtH1 and GtH2 (Arukwe, 2001). These two hormones resemble follicle-stimulating hormone (FSH), which initiates spermatogenesis/oogenesis, and leutinizing hormone (LH), which initiates gamete maturation or sperm release (Ankley et al., 2004). These gonadotropins stimulate the secretion of endogenous steroid hormones, such as estrogens, testosterones and progestins, which are responsible for oocyte maturation and sperm development in the gonad (Ankley et al., 2004; Tubbs and Thomas, 2009). In female fathead minnow, the LH hormone signals the release of testosterone (T) from thecal cells (Ankley et al., 2004; Scholz and

Mayer, 2008). FSH then causes induction of aromatase, an enzyme that synthesizes estrogens from T (Ankey et al., 2004). These estrogens are critical in several reproductive processes such as signaling production and release of VTG from the liver as well as normal development of oocytes and secondary sex characteristics (Ankley et al., 2004). Estrogens are also responsible for a negative feedback loop that inhibits release of hormones from the hypothalamus and the pituitary gland (Scholz and Mayer, 2008).

Estrogens initiate their effects in males and females by interacting with estrogen receptors (Ankley et al., 2004). In males, the gonadotropic hormones interact with three cell types: germinal spermatogonia, Sertoli cells, and Leydig cells (Ankley et al., 2004; Scholz and Mayer, 2008). Stimulation of the germinal spermatogonia initiates their development into functional sperm. Sertoli cells respond to stimulation by FSH, initiating the sperm production as well as their maturation (Scholz and Mayer, 2008), while LH stimulate the production and release of T and 11-ketotestosterone (11-KT) from Leydig cells (Ankley et al., 2004). Like the estrogens in females, these androgens stimulate the development of secondary sex characteristics in males, as well as signaling negative feedback. It is important to note that in males, the liver is not naturally active in the HPG signaling pathway (Scholz and Mayer, 2008). However, hepatocytes in male fish are able to produce VTG when exposed to exogenous estrogens or estrogenic chemicals that bind to estrogen receptors (Sumpter and Jobling, 1995; Seki et al, 2005; Lange et al, 2012).

1.3. Endocrine Active Compounds

Endocrine active compounds (EACs) represent a diverse range of chemicals that are currently defined as chemicals that interact with endogenous endocrine signalling pathways and may result in effects leading to adverse outcomes for the individual and population (Mills and Clinton, 2005). The hypothalamic-pituitary-gonadal axis is one of these pathways that are responsible for a wide array of functions involving reproduction and is highly conserved among organisms (Arukwe and Goksøyr 2003; Mills and Clinton, 2005). There are many chemicals classified as EACs and there is a considerable research effort to understand their mechanism of action (MOA) as well any adverse effects (Hutchinson et al., 2006; Ankley et al., 2009; Scholz et al.,2013). EACs have been demonstrated to interact with the HPG of fish in several ways (reviewed in Arukwe, 2001). Firstly, they can resemble the structure of the natural hormone, and

are able to act directly with the targets, such as endogenous receptors and enzymes intended for the natural hormones. Secondly, they block sites that natural hormones interact with, effectively impairing the ability of endogenous hormones to elicit their effect and/or upregulate production of endogenous hormones. They can also increase or decrease the expression of estrogen or androgen receptors that bind the hormones, intensifying the effect of endogenous hormones (Goksøyr, 2006). The EACs may also impact hormones by altering normal hormone synthesis, metabolism, transport, transformation, and elimination (Arukwe, 2001; Goksøyr, 2006). All of these MOA have the potential to alter the normal endocrine function of the organism (Arukwe, 2001).

Several anthropogenic compounds studied to date have been reported to mimic endogenous estradiol and induce estrogenic effects in fish. These estrogenic compounds act by binding to the estrogens receptors (ER), such as ERα and ERβ (Scholz et Mayer, 2008). This has been demonstrated in studies where exposure of fathead minnow to ethynylestradiol (EE2) resulted increased VTG, reduction in egg production, and demasculinizing effects in males (Arukwe, 2001; Länge et al., 2001; Parrot and Blunt 2005; Schwindt et al., 2014). Such organism-level effects have been shown to precede a population collapse of fathead minnow in an EE2-contaminated lake system (Kidd et al., 2007). Some chemicals, including tamoxifen, show affinity to the ER and compete with endogenous estrogens for binding sites, but do not initiate the associated effects, and therefore effectively inhibit the natural function of estrogens (Arukwe, 2001; Williams et al, 2007). In addition, altering basal expression and function of aromatase (the enzyme responsible for production of estrogen) can result in downstream effects in fish, such as modulating estrogen production (Villeneuve et al., 2009), resulting in decreased fecundity and altered sexual behaviour (Cheshenko et al., 2008). A number of chemicals have the potential to inhibit aromatase. One study assessed the effects of fadrozole exposure (50μg/ L) to fathead minnows using a 21-day adult reproduction assay and reported an inhibition of aromatase in males and females leading to decreased circulating plasma estradiol in females as well as a decrease in mature oocytes (Ankley et al., 2002). In the same study, fadrozole exposure (50μg/ L) caused increased circulating plasma concentrations of both T and 11-KT in males, resulting in increased sperm production (Ankley et al., 2002).

Although the majority of research has focused on the occurrence of environmental estrogens, it is now apparent that contaminants can also possess androgenic and anti-androgenic

properties. Androgenic and anti-androgenic effects in fathead minnows are not as well characterised as estrogenic effects. The earliest studies of contaminant-induced androgenicity reported the masculinization of female mosquitofish (*Gambusia affinis*) downstream of pulp and paper mills where the females were observed to have developed a gonodopodium, a secondary sex characteristic specific to male fish (Howell 1980). Exposure to growth promoters 17α and 17β trenbolone for 21 days resulted in androgenic effects such as decreased fecundity, reduced sex steroids and VTG, and presence of male specific nuptial tubercles in female fathead minnows (Ankley et al., 2003; Jensen et al., 2006). Similar effects have also been observed in fathead minnows exposed to the synthetic testosterone, 17α-methyltestosterone (Pawloski et al., 2004). Anti-androgenic effects in fathead minnow have been difficult to assess due to a lack of androgen-regulated biochemical or molecular responses (Scholz et al. 2008). Male fathead minnows exposed to the anti-androgen flutamide had decreased expression of nuptial tubercles at 938.6 μg/ L and an induction of VTG in females in all treatment groups ranging to 95.3 μg/ L to 938.6 μg/ L (Panter et al., 2004). Similarly, male fathead minnows exposed to vinclozolin (255 and 450 μg/ L) had decreased tubercle score and size of dorsal pads following 21-day exposure (Martinovic et al., 2008). The same study also demonstrated an upregulation in androgen receptor expression in male gonad mRNA, which suggests a compensatory response to anti-androgenic effects (Martinovic et al., 2008).

1.4. Impacts of Wastewater Effluent on Fish Reproduction

The discharge of MWWEs into aquatic systems is a major source of EACs in the environment (Daughton and Ruhoy, 2009). As such, MWWEs have been associated with disruption of the normal reproductive function in various fish species, including fathead minnow. Induction of VTG expression (as protein or transcript levels) is consistently observed in males exposed to MWWEs (Barber et al., 2007; Martinovic et al., 2007; Vadja et al., 2008; Vadja et al., 2011). Since VTG expression is receptor-mediated by circulating estrogens, its measurement is used as an indicator of potential estrogen activity in effluent studies. Exposure to EACs measured in MWWE also has the potential to alter concentrations of sex steroid hormones in both male and female fish. In male fish, reduced circulating sex steroids (specifically 11-KT) has been reported following exposure to estrogenic chemicals, such as EE2, along with a lack of

aggression (Martinovic et al., 2007) and nest holding behaviour (Salierno and Kane, 2009; Garcia-Reyero et al., 2014). In the wild, aggression in males is needed to successfully reproduce, and it has been demonstrated that more timid males have a lowered capacity for successful reproduction (Martinovic et al., 2007). Effluents may also have the potential to demasculinize male fathead minnows; these alterations can manifest as reduction or complete lack of secondary sex characteristics such as nuptial tubercles, as well as decreased abundance of sperm in the testes (Vadja et al., 2011).

Fecundity is a key endpoint for many effluent studies and is also ecologically relevant as reduced fecundity in wild fish populations could result in population collapse (Sohoni et al., 2001; Jobling et al, 2003; Thorpe et al., 2009). Reduced fecundity in female fathead minnows has been observed following exposure to a number of estrogenic and androgenic chemicals (Ankley et al., 2003; Parrott et al., 2005; Jensen et al., 2006). Many of these chemicals (ethinylestradiol) have been detected in MWWEs and as such similar reductions in fathead minnow fecundity have been seen following exposures to MWWEs (Thorpe et al., 2009; Cavallin et al. 2016). However, it is important to note that while fecundity as an endpoint is generally considered population relevant, not necessarily endocrine specific and similar reductions in fecundity are observed following exposure to other contaminants found in MWWE, such as ammonia (Armstrong et al., 2012).

1.5. Fathead Minnow as a Test Species for EAC Research

Fathead minnows are members of the Cyprinadae family. They are found in freshwater systems, such as streams and lakes, throughout North America (Ankley and Villeneuve, 2006). They are found in abundance due to their tolerance for a wide range of water quality conditions including temperature, oxygen, pH, and turbidity (Ankley and Villeneuve, 2006). The species exhibits sexual dichotomy with mature males being generally larger (3-5 g) than the females (2-3 g; Jensen et al., 2001). In addition to the differences in size, males also exhibit a number of male-specific secondary sexual characteristics including banding, nuptial tubercles, and a fat pad (USEPA, 1987; Jensen et al., 2001). Female fathead minnows lay their eggs in nests, where the eggs are adhered to the nest surface. Males will then become highly territorial and guard the eggs (Ankley and Villeneuve, 2006; Martinovic et al., 2007). Females have the potential to lay

between 50-150 eggs every 3-4 days (Jensen et al., 2001). However, this number is variable and is highly dependent on several factors such as age, size and environmental conditions (Ankley et al., 2001). Under ideal laboratory conditions, fathead minnow eggs generally hatch within five days and can begin feeding immediately. These larvae will reach sexual maturity within 4-5 months (Ankley and Villeneuve, 2006).

Due to their well-understood reproductive and developmental cycle, as well as their tolerance for differing water qualities, fathead minnows can be cultured with relative ease in a laboratory setting. There are several established protocols that outline the ideal conditions required for successful spawning of fathead minnows (USEPA, 1987). This species has also been extensively used as a model species in research on the effects of EACs and guidelines have been established regarding the proper use (USEPA, 2009). Given their widespread distribution across North America, fathead minnows represent an environmentally relevant test species for effluent studies with relevance to Canada, unlike other standardized test species typically found in warmer climates. The USEPA has developed a number of standardized reproductive bioassays using fathead minnow to determine endocrine disrupting effects of a chosen contaminant. These tests have been developed to examine acute and chronic exposures, as well as exposures that highlight specific developmental stages. Fathead minnows exhibit a number of biological endpoints that are influenced by exposure to EAC such as measures of fertility/fecundity, as well as more specific biomarkers that involve interactions with the hypothalamic-pituitary-gonadal (HPG) axis. These biomarkers include plasma concentrations of sex steroids, VTG, gonadal histopathology, as well as alterations to secondary sex characteristics (Sumpter and Jobling 1995; Ankley et al., 2001; Jensen et al, 2001). Due to their frequent use as a model test species, there are also a number of target genes associated with HPGL functions (e.g. steroid receptors, steroidogenesis, VTG) that have been characterised and measured at the transcript level as indicators of exposure to EAC. Overall, endpoints ranging from individual to molecular level of biological organisation have been heavily used in regulatory programs to assess the potential reproductive effects of chemicals on fish, and specifically their effect on physiological processes controlled by estrogens and androgens (Ankley et al., 2004; USEPA, 2009).

1.6. Biomarkers of Endocrine Disruption in Fathead Minnow

1.6.1. Vitellogenin

One of the more prevalent biomarkers for determining exposure to EACs is the measurement of VTG. In oviparous vertebrates, the egg yolk protein VTG is important in the normal development of the growing oocytes (Sumpter and Jobling, 1995). High circulating levels of (E2) in the blood stimulates the production of VTG in the liver of females. Conversely, in immature and adult male fish with lower circulating levels of E2, the level of VTG is usually non-detectable or very low. However, exposure to an exogenous estrogen or estrogenic compound can induce VTG gene expresion and translation to VTG protein in male fathead minnows (Jensen et al., 2001; Shappell et al, 2010). There are a number of reasons why VTG is a valuable biomarker for endocrine disruption. Firstly, since VTG is dependent on E2, this means that VTG induction is specific for measurements involving estrogen agonists (Ankley et al., 2004). Secondly, males have naturally low levels of VTG, so induction is a sensitive measure of estrogen exposure (Ankley et al, 2004). Finally, since males do not naturally produce VTG, they do not possess the means to rapidly eliminate it from circulation, meaning it can be measured even after short-term exposures to estrogen-active contaminants (Ankley et al., 2004). Therefore, the level of VTG in male fish has become a principle endpoint to measure exposure to estrogenic contaminants in aquatic organisms (Sumpter and Jobling, 1995; Rotchell et al., 2003; Barber et al., 2012; Tetrault et al., 2012). Additionally, the relationship between reduced fecundity and VTG has been integrated into a population model. Miller et al. (2007) utilized fathead minnows in a 21-day exposure to five separate chemicals that inhibit VTG production to create a population model between fecundity and VTG concentration. They hypothesize that because VTG is essential in normal egg production, it could be used as a means of predicting reproductive success, and by using the population model, VTG could theoretically be used as a biomarker of individual and population level adverse effects (Miller et al., 2007).

VTG can be measured in a variety of ways including measuring protein levels in the blood using an enzyme linked immunosorbant assay (ELISA) as well as measurement of VTG mRNA by polymerase chain reaction (PCR) or quantitative PCR (Lattier et al., 2002; USEPA, 2009; Garcia-Reyero, 2011; He et al., 2012). Direct comparison of the two methods (protein vs. transcript) has shown that transcript levels change sooner following exposure and one can detect VTG mRNA at

picogram concentrations as compared to nanogram (Korte et al., 2000). In addition, transcript levels can be measured using liver samples while the ELISA utilizes blood plasma; thus, meaning when blood obtained from the fathead minnows is limited, the measurement of VTG transcripts in liver is a possible surrogate for circulating VTG (Korte et al., 2000).

1.6.2. Sex Steroids

Like all fish, fathead minnows produce a number of sex steroid hormones that control gonadal maturation, reproductive behaviour and expression of secondary sex characteristics. Steroidogenesis is the enzymatic conversion of cholesterol into various steroid hormones, including sex steroid hormones. In fish, the three main sex steroid hormones of interest are E2, T, and 11-ketotestosterone 11-KT, the latter which is a product of T biotransformation (Ankley et al., 2004). The concentrations of these hormones present in blood plasma are different between male and female fish, meaning they can be measured and used to indicate disturbances to the HPG axis (USEPA, 2009). Males have naturally higher concentrations of T and 11-KT, and low concentrations of E2 (Jensen et al., 2001; Ankley et al., 2004). Females will have measurable concentrations of both E2 and T but will have almost undetectable levels of 11-KT (Ankley et al., 2004). EACs have the potential to alter levels of these hormones at various points along the cascade, including disrupting steroidogenesis and causing feedback inhibition (Ankley et al., 2008). For example, feedback inhibition due to exposure to androgen agonists (e.g. 17β-trenbolone) resulted in decreased circulating concentrations of both E2 and T in fathead minnow (Ankley et al., 2003; Jensen et al., 2006). EACs can exert anti-androgen effects through inhibition of aromatase (CYP19A1; Villeneuve et al., 2009). This enzyme is involved in the final step that transforms the androgen precursor into estrogens. Inhibition of aromatase leads to a decrease in E2, as well as an increase in circulating androgens, due to the fact that they can no longer be synthesized into estrogens (Villeneuve et al., 2009). Another step in the sex steroid pathway is a rate-limiting step that involves the steroidogenic acute regulatory protein (StAR), which regulates the rate of cholesterol transport across the mitochondrial membrane (Socco, 2001). Exposure to the aromatase inhibitor fadrozole increased expression of StAR in fathead minnow ovaries, which was thought to be a compensatory mechanism and works to increase the overall rate of hormone synthesis (Villeneuve et al., 2009). Inhibition of 3β-hydroxysteroid dehydrogenase, which converts pregnenolone to progesterone, also represents a potential marker

for endocrine disruption as its inhibition in fathead minnows resulted in decreased VTG and alterations to testis size (Villeneuve et al., 2008). Following a 17β-estradiol exposure in rainbow trout (*Oncorhynchus mykiss)*, mRNA expression of genes involved in androgen production (CYP11A, CYP17, CYP11B) was decreased, indicating an inhibitory effect of estrogens on steroidogenesis in fish (Govoroun et al., 2001). In fathead minnow, exposure to 17β-estradiol resulted in similar inhibition of the same target genes (Filby et al., 2007). Conversely, exposure to the anti-androgen flutamide up-regulated expression of steroidogenic genes (Filby et al., 2007). The studies demonstrate the potential for using multiple steroidogenic genes for evaluating endocrine disruption. Evaluation of circulating sex steroid levels is achieved through collection of a blood sample and separation of plasma. ELISA is a common method for measurement of plasma hormone levels (Nichols et al., 1999; Beitel et al., 2014).

1.6.3. Secondary Sex Characteristics

Fathead minnows express a number of easily observed secondary sex characteristics that are sensitive to the presence of EACs, providing an effective method of evaluating apical effects. During breeding season, sexually mature males develop a dark banding pattern, develop a dorsal pad, and grow nuptial tubercles. The occurrence of these secondary sexual characteristics is androgen-dependent and highly responsive to endocrine active compounds. Of particular note is the dorsal pad and nuptial tubercles on the males, which are some of the most prominent visible features (Smith, 1974; Jensen et al., 2001). Previous studies have demonstrated that the growth of the dorsal pad, as well as the number and size of tubercles are reduced when the male fish are exposed to an estrogenic compound such as EE2 (Miles-Richardson et al., 1999; Pawloski et al., 2004). Reduced tubercle size has also been observed following exposure to effluents from a number of sources including MWTPs (Sowers et al., 2009; Vadja et al., 2011; Tetrault et al., 2012) which can be attributed to endocrine active compounds contained with the effluents. Exposure to androgenic compounds (17β-trenbolone) can also cause the development of tubercles in female fathead minnow, in addition to heightening their development in males (Ankley et al., 2003; Jensen et al., 2006). Banding and fin dot, which are characteristics in sexually mature males have also been observed in female fathead minnows when exposed to bleached kraft pulp mill effluent (Rickwood et al., 2006). The method for assessing these characteristics involves a scoring system, where size and occurrence are taken into account and

assigned a number. This number can either represent one feature, or can be combined to represent the overall condition of the specimen (USEPA, 2009).

1.6.4. Organosomatic Indices

In addition to the molecular and biochemical endpoints as indicators of exposure to EACs in fathead minnows, changes in organ size can also be used to make inferences on the health of the fish (Schmitt and Dethloff, 2000). These measurements are known as organosomatic indices. The two most common measurements used are the hepatosomatic index (HSI) and gonadosomatic index (GSI). These indices are obtained by comparing the weight of the individual organ to the overall weight of the fish. The result is standardized measurement that can be used to compare fish from the same species (Schmitt and Dethloff, 2000). In addition, the condition factor (K) is a general indicator of overall health and calculated by taking the weight of the fish divided by the length^3. Both the organosomatic indices and K can be increased or decreased by external stressors, such as change in temperature, or contaminant exposure (Schmitt and Dethloff, 2000). For this reason, they are considered reliable apical measurements when assessing fish health (Jensen et al., 2001).

GSI is the weight of the gonads divided by the total weight of the fish multiplied by 100. This measurement is used to assess the health of the gonads in fish. In fathead minnows, females will typically have a larger GSI (8-13%) than males (1-2%), as female ovaries are larger relative to the male testes (Jensen et al., 2001; US EPA, 2009). A number of studies have associated changes in the GSI of fathead minnow with exposure to EACs (Harries et al., 2000; Ankley et al., 2004; Sowers et al., 2009). Decreased GSI has been reported for both male and female fathead minnows following exposure to estrogenic compounds (Pawloski et al., 2004).

The hepato-somatic index (HSI) utilizes the same formula as GSI to assess the status of the liver. The liver is essential for nutrient and xenobiotic metabolism, and as such, changes in liver size are often the result of changes in metabolic status of a fish (Sumpter and Jobling, 1995). Estrogen receptors expressed on the liver act to initiate the production of VTG in females in response to circulating estrogen (Sumpter and Jobling, 1995), and as such, increased HSI is thought to be tied to increased VTG production (Li and Wang, 2005). Increases in HSI have been demonstrated in fathead minnows following 21-day exposure to pulp and paper mill effluent

(Rickwood et al., 2006; Rickwood and Dube, 2007), and in wild fish collected from MWWE-contaminated aquatic environments (Tetrault et al., 2012).

1.6.5. Histopathology

Effects of EAC exposure can also be observed through pathological changes at the tissue level in target organs. Histopathological examination of organs, particularly the gonads, in fathead minnow has been utilized in a number of studies to evaluate endocrine disrupting effects following chemical exposure (Miles-Richarson et al., 1999; Jensen et al., 2001; Ankley et al., 2003; Tetrault et al., 2012). The gonads are a major part of the HPG axis and histopathological changes are often the result of the integration of a large number of interactive physiological processes (van der Oost et al., 2003). Histology procedures and scoring criteria for fathead minnows are detailed in a USEPA guideline and generally include assessment of gonadal structure, gonadal sex ratios, and stage of developing oocytes/spermatogonia (USEPA, 2009). In males, parameters commonly assessed include the spermatocyte activity present as well as the number of germinal epithelium present (Ankley et al., 2001; Jensen et al., 2001; Rickwood et al., 2006, Tetrault et al., 2012). Following exposure to MWWEs, an increase in the immature gonadal cell spematogonia has been observed in male fathead minnow (Vadja et al., 2011; Tetralt et al., 2012). In certain cases, following prolonged exposure to estrogenic compounds, wild male fathead minnows have been observed with intersex tissues present in the testis (Kidd et al., 2007). The primary criteria for evaluating gonadal changes female fathead minnows in the fish short-term reproduction assay include oocyte atresia, perifollicular cell hyperplasia, egg yolk formation, and the proportion of different developmental stages of oocytes (primary, cortical alveolar, vitellogenic, atretic) (Miles-Richarson et al., 1999; Jensen et al., 2001; Ankley et al., 2001; Tetrault et al., 2012). Increased atretic follicles have been observed in fathead minnow females following exposure to EE2 and the anti-androgen flutamide (Miles-Richardson et al., 1999; Jensen et al., 2004). Reduced maturation in ovaries of fathead minnows has been observed following exposure to androgen agonists such as methyltestosterone and 17β-trenbolone (Ankley et al., 2001; Ankley et al., 2003).

1.6.6. Molecular and Transcriptomic Responses

Molecular responses following exposure to EACs are becoming increasingly studied as either a screening tool for exposure or to better understand mechanisms underlying reproductive impairment. Many studies focus on a small suite of targeted genes in tissues along the HPGL axis with some more recent studies evaluating changes across the transcriptome. Evaluating transcript level change in target genes offers the ability to detect potential disruption before other biomarkers (i.e. nuptial tubercles, histopathology) can be identified downstream. They also provide greater insight into the mechanism of action (e.g. receptor-mediated) by which a contaminant is exerting its effect. This is especially useful in regards to MWWE studies as the complexity of the mixture makes it difficult to elucidate how certain apical effects, such as fecundity, are occurring.

To identify whether specific pathways are disrupted along the HPG axis, several key target genes are often assessed together including receptors (e.g. estrogen receptor α/β) and enzymes involved in steroidogenesis (e.g. CYP19a). As mentioned previously, VTG is one of the most reliable endpoints in regards to estrogenic responses and mRNA measurement can be used in lieu of other methods, such as ELISA and RIA, for this endpoint. Following an embryo exposure to EE2 (2, 10, 50 ng/ L), VTG mRNA was induced in fathead minnows at all exposure concentrations (Johns et al, 2011). This induction in VTG is consistent to with studies where VTG protein was increased (Sumpter and Jobling, 1995; Rotchell et al., 2003; Barber et al., 2012; Tetrault et al., 2012). In addition, Johns et al. (2011) also reported that exposure to an antiestrogen ZM (1000 ng/L) caused a decreased expression of VTG mRNA, demonstrating the potential to use this endpoint to measure estrogenic and anti-estrogenic effects. Inhibition of VTG mRNA may be environmentally relevant as decreased concentration of VTG protein has been linked to decrease in reproductive success at the population level (Miller et al, 2007). Sex steroid (estrogen and androgen) and gonadotropin (FSH and LH) receptors can also provide insight in regards to estrogenic or androgenic potential of a contaminant. In male fathead minnow, both ER subtypes (ERα/β) will respond differently to E2 exposure, with an up-regulation seen in ERα mRNA and a down-regulation in ERβ mRNA (Filby et al., 2006).ERα has been considered the major ER involved in VTG synthesis (Nelson and Habibi, 2013), although studies conducted with E2 exposure using rainbow trout suggest that ERβ is likely the main mediator (Leanos-Castanada and Van Der Kraak, 2007). Androgen receptor (AR) transcript levels in fathead

minnows were induced with 21-day exposures to 17β-trenbolone (Ankley et al, 2003) and AR induction in fathead minnows has been used to determine androgenic potential of mixtures, such as pulp and paper mill effluents (Werner et al., 2010). Transcripts involved in steroidogenesis are important as alterations in expression can lead to changes in concentrations of sex steroids downstream. For example, a down-regulation of aromatase mRNA following fadrozole exposure resulted in increase of T and 11-KT in males, while a decrease in E2 was observed (Villeneuve et al., 2009). This makes aromatase a valuable biomarker, as its induction or inhibition leads directly to measurable downstream consequences. In terms of contaminant mixtures, such as MWWE, the effects caused by each compound may be masked or altered due to the presence of other chemicals in the mixture and their potential to agonistically or antagonistically interact with similar pathways. However, studies conducted with wastewater have utilized gene expression to identify patterns of estrogenic and androgenic effect of the overall effluent (Werner et al., 2010; Garcia-Reyero et al., 2011).

1.7. Project Rationale, Objectives and Hypotheses

The growing population in Saskatchewan has placed an increased demand on wastewater treatment facilities, as they are required to process a greater amount of wastewater. One of most important sources for endocrine active compounds are effluents from waste water treatment plants. Elimination of pharmaceuticals and personal care products as well as natural hormones and other contaminants is often incomplete and inefficient during conventional wastewater treatment. A number of these compounds are pharmaceuticals or personal care products, and as such, are designed to work at very low concentrations and are a significant risk to reproductive health and development of many fish species. This becomes problematic in some receiving environments (i.e. Wascana Creek), as there are times during the year when the water flow can be as high as 100% effluent (Waiser et al., 2011a).

The overall objective of this thesis research was to evaluate and compare the potential endocrine and reproductive impacts of effluents released from the Regina and Saskatoon WWTP on fish. The 21-day fathead minnow reproductive assay (USEPA, 2009) was used during which sexually mature male and female fish were exposed to various concentrations of the effluents in a laboratory flow-through system. Effects on fish reproduction were evaluated by measuring

including reproductive output, secondary sex characteristics, circulating steroids, gonadal histology, and targeted gene expression. This thesis research is part of a larger initiative focused on developing a screening and prioritization approach to characterize and assess potential effects of MWWEs to biota in aquatic ecosystems. This program consisted of (1) an effect-directed analysis (EDA) of municipal effluents with *in vitro* characterization of specific endocrine disruptive potentials MWWE as well as chemical analyses of candidate compounds (Bagatim et al., 2018); (2) a 21-day fathead minnow reproductive assay where fish are exposed to effluents (this thesis research); and (3) a wild fish study where fathead minnow are collected up- and down-stream of WWTPs and evaluated for differences in body condition and indicators of reproduction disruption (Hanson et al., 2018).

The specific objectives of this research were:

1) To evaluate and compare the reproductive performance of male and female fathead minnows during 21-day exposure to MWWEs from Regina and Saskatoon WWTP.
2) To characterise the physiological mechanisms by which MWWEs from Regina and Saskatoon WWTP induce reproductive effects in male and female fathead minnows
3) To evaluate the transcriptional changes in target genes to determine to identify potential mechanisms of action underlying physiological and apical effects of MWWE exposure in fathead minnows.
4) To compare responses measured in fish in this research to complimentary wild fish and *in vitro* AIME studies focused on South Saskatchewan MWWEs.

The main hypotheses of this research are:

1) Following 21-day exposure, fathead minnows exposed to Regina and Saskatoon MWWE treatments will have altered reproductive performance (i.e. fecundity and fertility) and secondary sex characteristics compared to minnows in the control exposure.
2) Following 21-day exposure, fathead minnow exposed to Regina and Saskatoon MWWE treatments will result in adverse alterations to physiological biomarkers, such as histopathology and sex steroid production, that will reveal the overall mechanisms by which the MWWEs induce their effects.
3) Following 21-day exposure, transcriptional changes in fathead minnows exposed to Regina and Saskatoon MWWE treatments will reveal by which mechanisms the MWWEs are acting and manifesting as adverse apical and physiological downstream effects.
4) Following a 21-day exposure, effects of MWWE will be consistent between the *in vitro*, wild-fish, and laboratory exposures conducted as part of the AIME project.

CHAPTER 2

2. ASSESSING BIOLOGICAL EFFECTS OF MUNICIPAL WASTEWATER EFFLUENT USING THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*) REPRODUCTIVE BIOASSAY

PREFACE

The objective of Chapter 2 was to evaluate and compare the potential endocrine and reproductive impacts of effluents released from the Regina and Saskatoon WWTP on fish. To meet this objective, the 21-day fathead minnow reproductive assay (USEPA, 2009) was used where sexually mature male and female fathead minnow were exposed to various concentrations of the effluents in a laboratory flow-through system.

Author contributions:

Kean Steeves (University of Saskatchewan) helped design and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript

Sara Hanson (University of Saskathewan) provided assistance throughout the project, including collection and transport of effluent and takedown of the exposures.

Tabata Bagatim (University of Saskathewan) provided assistance throughout the project, including collection and transport of effluent and takedown of the exposures.

Markus Hecker (University of Saskatchewan) provided scientific input and guidance on the design of the experiment, provided logistical support for effluent collection and transport, commented on and edited the chapter, and obtained funding for the research.

Alice Hontela (University of Lethbridge) provided scientific input and guidance on the design of the experiment, provided logistical support and resources for the exposures, co-supervised Kean Steeves, commented on and edited the chapter, and obtained funding for the research.

Natacha Hogan (University of Saskatchewan) provided scientific input and guidance on the design of the experiment, co-supervised Kean Steeves, commented on and edited the chapter, and obtained funding for the research.

2.1. Abstract

Municipal wastewater effluents (MWWEs) contain anthropogenic substances that can exhibit endocrine disrupting activity. In the Prairie provinces of Canada, increasing urban populations and industrial activities have resulted in greater water demand, and therefore, greater amounts of MWWEs released into the aquatic environment. Treatment plants often feature outdated technologies that inefficiently remove contaminants and when coupled with the increasing water demands, present a risk to receiving environments. The objective of this study was to utilize the 21-day fathead minnow reproductive assay to identify potential reproductive effects of MWWEs from Regina and Saskatoon. Fish were exposed for 21 days to 0 %, 10 % and 50 % of the effluents. Impacts on reproductive success (fecundity, fertility) as well as morphological, histopathological, and molecular/biochemical endpoints were assessed in male and female fish. Exposure to 10 % and 50 % Regina MWWE resulted in a significant decrease in fecundity compared to non-exposed minnows. There were significant differences in fecundity of minnows exposed to Saskatoon effluent, although the effect was not dose-dependent. Fertilization rate remained consistent regardless of treatment. Histological examination revealed a significant increase in proportion of spermatogonia and testicular degeneration in male fathead minnow testes in 50 % effluent concentrations compared to control minnows in both Regina and Saskatoon exposures. Increased oocyte atresia was observed in female minnows exposed to both Regina and Saskatoon 50 % treatment. No change in circulating sex steroid hormones (estradiol, 11-ketotestosterone) in male or female minnow was measured. Analysis of gene transcription revealed that exposure to Regina effluent induced ERα mRNA in female fathead minnows while exposure to Saskatoon effluent induced ERα in females and androgen receptor in males. No change in the expression of the vitellogenin was observed in any effluent-exposed group. The results from this study demonstrated that exposure to MWWE can impact the reproductive output and gonadal development of fathead minnow; however, due to the lack of change in key endocrine-related endpoints, it appears these effects are not due to estrogenic or androgenic compounds in the effluent.

2.2 Introduction

Municipal wastewater effluents (MWWE) are the remaining liquid portion of treated sewage wastes generated by wastewater treatment plants (WWTPs). In Canada, MWWE is the largest source of pollution (by volume) to the aquatic environment (Holeton et al., 2011) and level of treatment and disinfection varies among municipalities (Chambers et al., 1997; Holeton et al., 2011). Although treatment technologies have evolved and improved the quality of wastewater released, these systems are continually challenged by the increasing volume of municipal effluent requiring treatment from growing urban populations. Municipalities in Western Canada are currently experiencing some of the greatest population growth rates in the country with the cities of Saskatoon (12.5 %) and Regina (11.8 %) ranking third and fourth, respectively, in Canada (Statistics Canada, 2016). Each municipality has its own WWTPs to serve their growing populations. Until fall 2016, wastewater was treated at the Regina plant by phase separation in primary treatment (grit and sedimentation), followed by secondary treatment (biological-aerated lagoons), and finally tertiary treatment (chemical precipitation) prior to release into Wascana Creek (Zhao and Viraraghavan, 2004). Wascana Creek represents a low volume system that features periods of seasonal low flow (e.g. winter) that result in effluent comprising nearly 100 % of flowing water at certain times. The Saskatoon WWTP features more advanced treatment technologies and discharges MWWEs into the South Saskatchewan River, which is a larger volume system and dilutes the released effluent to a greater extent than Regina. With these differences in treatment technologies and environmental dilution, the effluent discharges from the Saskatoon and Regina WWTP are examples of a low risk and high risk exposure scenario, respectively.

Impacts of MWWEs on the receiving environment not only depend on the physical characteristics of the receiving water bodies but also on the amounts and type of contaminants released through the effluent. MWWEs are complex mixtures of biological and chemical compounds including nutrients, suspended solids, industrial and household chemicals, as well as chemicals of emerging concern. The latter consist of compounds such as human steroid hormones, and pharmaceuticals and personal care products (PPCPs) that enter municipal wastewater systems (Herberer, 2002; Richardson and Fulton, 2009), and currently, treatment facilities are ill-equipped to remove the diversity of these compounds. The release of processed

MWWEs provide a pathway for compounds, such as PPCPs, to enter aquatic environments, and measureable levels of emerging contaminants have been reported downstream of treatment plants in North America (Vadja et al., 2008; Waiser et al., 2011), Europe (Kasprzyk-Hordern et al., 2009), and Asia (Chen et al., 2016), highlighting the global scale of this problem.

The presence of emerging contaminants, in particular PPCPs and endocrine active compounds (EACs), is of significant concern given their potential for adverse effects on aquatic biota (Campbell et al., 2006). Many PPCPs and EACs exhibit high biological activity and while surface water concentrations are typically low, these compounds can disrupt endocrine signalling and affect the growth, development, and reproduction of aquatic organisms. Studies have reported impaired reproduction in wild fish exposed to MWWEs with one of the initial studies discovering that effluent discharge into UK rivers was resulting in an increased frequency of testicular oocytes (i.e. oocytes present in testes) in male roach (*Rutilus rutilus*) (Jobling et al., 1998). Subsequent studies identified the synthetic estrogen, ethynylestradiol (EE2), a key constituent of MWWE with high estrogenic potency, as a likely causative agent for the reproductive effects on wild fish (Rodgers-gray et al., 2000; Lange et al., 2012). Increased intersex can lead to decrease in fecundity, one of the most relevant endpoints for population health as it can result in populations collapse (Sohoni et al., 2001; Jobling et al, 2003; Thorpe et al., 2009). This was demonstrated through a study in which EE2 was added to a whole lake system and in subsequent years fish exhibited intersex and whole population collapse (Kidd et al., 2007; Kidd et al., 2014). Relevant to the treatment systems mentioned above, wild fathead minnow collected downstream of the Regina WWTP exhibited altered reproductive endpoints, including delayed spawning, altered gonadal development, and reduced expression of secondary sex characteristics in males (Tetreault et al., 2012).

Fathead minnows (FHM) are a small-bodied freshwater fish ubiquitous to aquatic systems in Canada. Fathead minnows are also a valuable model species for laboratory exposures due to their well-documented reproductive cycle and physiology. Male and female FHM exhibit several reproductive behaviours and characteristics that serve as endpoints in a standardised 21-day reproductive assay to evaluate the potential endocrine disrupting effects of compounds in fish (US EPA, 2009). The endpoints in the assay range from apical effects on reproductive output such as fecundity and fertility to changes in plasma hormone levels as an indicator of effects on steroidogenesis. Unique to FHM, secondary sex characteristics (i.e. nuptial tubercles and colour

banding on males) are also influenced by exposure to compounds with estrogenic (Vadja et al. 2011) or androgenic potential (Jensen et al., 2006). Gonad histopathology is also a valuable endpoint as changes (e.g. increased proportion of spermatogonia, oocyte atresia) are often the result of the integration of a large number of interactive physiological processes. Changes in male and female gonadal tissues have been observed in wild-caught FHM following exposure to wastewater effluents (Vadja et al., 2008; Tetreault et al., 2012).

In addition to the number of established apical endpoints, a number of molecular and biochemical endpoints exist that can be used to assess endocrine disruption in fathead minnows at a more mechanistic level. Plasma sex steroid hormone concentrations, such as 17β-estradiol (E2) and 11 ketotestosterone (11-KT), have been reported to change in FHM following exposures to wastewater effluents (Hewitt et al., 2008; Tetreault et al., 2012). Additionally, a number of target genes have been identified in fathead minnow that can be measured to assess processes along the hypothalamic-pituitary-gonadal-liver axis such as vitellogenin (VTG) production, hormone receptor signalling, and steroidogenesis. Measurement of VTG mRNA is particularly useful as its induction in males is correlated with exposure to estrogenic compounds, and inhibition following exposure to androgenic compounds (Miracle et al., 2006).

The objective of the present study was to utilize the 21-day fathead minnow reproductive assay to identify potential reproductive effects of MWWEs from the Regina and Saskatoon WWTPs. Fecundity and fertility was monitored over the duration of the exposure, with sampling at the end of the exposure to evaluate secondary sex characteristics, histopathological examination of the gonads, and measurement of circulating sex steroids, in order to identify potential underlying causes for effects on reproductive output. We also evaluated transcriptional changes in key target genes collected from multiple tissues in order to determine the potential mechanisms of action underlying the effects manifesting at the apical and physiological levels. The findings from this study were used as part of a larger project known as the Aquatic Impact Assessment of Municipal Effluents (AIME). The overall goal of AIME was to use a tiered approach to establish effective endpoints to create a toolbox for assessing the impacts of MWWEs on aquatic organisms.

2.3 Materials and Methods

2.3.1. Test species

Juvenile fathead minnow (*Pimephales promelas*) were purchased from Osage Catfisheries (Osage Beach, MO, United States) and housed in the Aquatic Research Facility (ARF), University of Lethbridge, Lethbridge, AB. Following arrival, fish were kept in a 421 L flow-through system for two weeks at a controlled temperature (25 ± 1°C) and photoperiod (16 h light and 8 h dark). During this time, fish were monitored daily for general health and any mortalities were noted. Temperature, dissolved oxygen concentration, and ammonia levels were monitored regularly. Minnows were fed bloodworms (Hikari Sales U.S.A. Inc., Hayward, CA, USA) twice daily. Experimental protocols were approved by the Animal Welfare Committee (University of Lethbridge) in accordance with the Canadian Council on Animal Care guidelines.

2.3.2. Effluent collection

Effluents were collected from the City of Regina and City of Saskatoon wastewater treatment plants in May and June 2014. Effluents were collected end-of-pipe and held in 40 L opaque carboys and stored in the dark. During each collection effort, ten carboys were collected from both the Regina and Saskatoon wastewater treatment plants and immediately transferred to University of Lethbridge. Upon arrival, containers were stored in the dark at 4°C until required for exposure. Carboys from each site were pooled in a larger container. Effluents used in the exposure were taken from the larger pooled effluent containers.

The effluents from both sites were analyzed for ammonia upon arrival and it was determined that levels were above acceptable limits outlined in US EPA assay guidelines for fish (<1 mg/L; US EPA, 2009). Therefore, a 96-hour LC50 assay was conducted using concentrations containing 50 %, 75 %, and 100 % effluent in order to establish a high dose concentration of effluent that would not result in mortality or otherwise overt toxicity. From the results of this study (see Appendix), 50 % effluent concentration was chosen as the high dose for the 21-day reproductive bioassay.

2.3.3. Pre-exposure and exposure initiation

Methods for exposure of fathead minnows to MWWE were followed according to the USEPA Endocrine Disruptor Screening Program Test Guidelines, OPPTS Number 890.1350:

Fish Short-Term Reproduction Assay (FSRA) (US EPA, 2009), and consisted of a pre-exposure and exposure period. The purpose of the pre-exposure, which took place over two weeks, was to acclimatize fish to exposure conditions (i.e. temperature, flow-through, light cycle), establish reproductive capacity of paired fish, and provide baseline data for fecundity and fertility. For both pre-exposure and exposure periods, fish were held in an experimental tower set-up with a flow-through system (Aquabiotech Inc, Coaticook, QC, Canada). Two separate towers were used for the exposures, one assigned to each effluent source (Regina and Saskatoon). The towers contained four individual shelves, each consisting of six 9 L tanks, and each shelf was connected to a separate header tank. At initiation of the pre-exposure period, four male and two female mature fathead minnows were assigned to each tank. Fish selected for evaluation during the pre-exposure had observable secondary sex characteristics and were similar in weight to minimize variability in egg production. Minnows were held at 25 ± 1°C and a 16:8 h light: dark photoperiod, and were fed bloodworms (Hikari Sales U.S.A. Inc., Hayward, CA, USA) twice daily.

The exposure regime and endpoints analysed for fathead minnow 21-day exposure to MWWE are shown in Figure 2.1. The exposure period was initiated after the two-week pre-exposure period and took place over 21 days, during which time male and female fathead minnows were exposed to the three concentrations of effluent: 0 % (control), 10 % effluent (low dose), and 50 % effluent (high dose). The effluent concentrations were fed into the towers using individual header tanks. The concentrations were mixed to the proper dilution using ARF facility water and then added to the header tanks. The effluent was renewed daily at a rate of 50 % tank volume per day. Fish were monitored daily for morbidity or mortality. Any dead fish were removed and preserved in formalin. Both ammonia and pH were recorded every three days during the pre-exposure and exposure periods. Un-ionized ammonia was measured using a Pocket Colorimeter II (Hach Company, Loveland, CO, USA) and pH was measured using a Pro10 pH Instrument (YSI Incorporated, Yellow Springs, OH, USA). Dissolved oxygen and temperature were monitored daily in the flow-through towers by facility systems (Aquabiotech Inc, Coaticook, QC, Canada).

To evaluate fecundity and fertilisation during the pre-exposure and exposure periods, spawning tiles were added to each tank. Tiles were checked twice daily for any spawning events - once in the morning one hour after lights in each rack were turned on and again in the afternoon,

as spawning events often occurred at these times in many of the tanks. If eggs were present, the spawning tiles were removed from the tank and eggs were collected into Petri dishes. After each collection, both the number of eggs spawned was counted and the fertilitization of the spawned eggs was assessed under a dissecting microscope and recorded. The average fecundity of both Regina and Saskatoon control treatments were used to account for differences between the two towers.

2.3.4 Tissue sampling and morphometrics

At the end of the 21-day exposure period, fish were anesthetised in Aquacalm (5-10 mg/L; Syndel Laboratories, Qualicum Beach, BC, Canada). The mass (to the nearest 0.01 g) and fork length (to the nearest 0.1 cm) of each fish was measured and the condition factor (CF) was calculated [CF = 100 × total wt (g)/fork length3 (cm)]. Blood was collected via heparinized needle from the caudal vein and the plasma was isolated by centrifugation (15,000 × g for 3 min). Secondary sex characteristics were assessed, which included recording the color banding of males, the presence of the dorsal fatpad, and the presence and size of nuptial tubercles. Tubercles were counted under a dissecting scope. A grading criteria was used to calculate the tubercle score of the fathead minnows, which represents both the amount and size of any nuptial tubercles present. The grading ranged from 0 to 3 and represented a range from non-existent to fully developed (see US EPA (2009) for further description of grading procedures). Both males and females were examined for presence or absence of tubercles. Fish were then euthanized by spinal severance, and three tissues (liver, gonads, and brain) were collected immediately. Gonads and livers were weighed in order to calculate the gonado-somatic index (GSI) [GSI = 100 × gonadal tissue wt (g)/total wt (g)] and liver somatic index (LSI) [LSI=100×liver tissue wt (g)/total wt (g)]. The gonads from males and females were divided into two equal portions. One portion was taken for histopathological analysis and fixed in Bouin's solution for 24 h, and then rinsed in water and stored in 70 % EtOH until histological processing. The remaining portion of the gonad, as well as liver and brain tissue, was flash frozen and stored at -80°C for gene expression analysis. All samples collected were transported to the University of Saskatchewan in a portable -80°C freezer (Stirling Ultracold, Athens, OH, USA) or at room temperature, as appropriate.

2.3.5. Histological analysis

Male and female fish exposed to the 0 % and 50 % effluent treatments from both Regina and Saskatoon MWWE were selected for histopathological analysis. Gonads from individual male and female fish were processed according to standard histological methods using an automatic tissue processor (Leica Microsystems Inc., Concord, ON, Canada) and embedded in paraffin wax. Testes and ovaries were sectioned at a thickness of 4μm and 7μm, respectively. Sections were mounted on glass slides and stained with hemotoxylin and eosin for further evaluation. A total of 10 slides, each containing five sections, were prepared for each specimen, each containing five sections. Sections were first collected halfway through the gonad, allowing for the largest section of tissue to be obtained. The following sections were taken from the remaining two-thirds of the tissue. Sections were mounted on glass slides and stained with hemotoxylin and eosin for further evaluation. Of the 10 slides prepared for each fish, three slides were chosen for histopathological analysis. Gonads were assessed microscopically to qualitatively evaluate relative germ cell numbers, stage of developmental maturity, and increased degenerative changes, according to US EPA guidelines (2009). For males, the primary criteria were: increased proportion of spermatogonia, presence of testicular oocytes, increased testicular degeneration, and interstitial cell hyperplasia/hypertrophy. For females, the primary criteria were: increased oocyte atresia, perifollicular cell hyperplasia/hypertrophy, decreased yolk formation, and change in gonadal staging. The histopathological criteria were scored using either a staging or severity index. Staging was used to compare relative maturational stage of ovaries and consisted of 7 criteria based on presence of specific stages of ovarian follicles: juvenile 0 = undeveloped, 1 = early development, 2 = mid-development, 3 = late development, 4 = late development/hydrated, 5 = post-ovulatory (see US EPA (2009) for detailed description of gonadal scoring and staging procedures). Severity scores were assigned based on percentage of affected tissue according to the following scale: grade 1 = minimal (<20% tissue affected), grade 2 = mild (30-50%) grade 3 = moderate (60-80 %), and grade 4 = severe (>80 %) (US EPA 2009). Any changes not amenable to grading instead were designated as “present.” Sections were examined using a Zeiss Axiostar Plus light microscope at 10x and 50x magnification and photographs were taken using an Axiocam MRC 5 MP camera (Carl Zeiss Canada Ltd., Toronto, ON, Canada) and recorded using Axiovion 4.8 Imaging software (Carl Zeiss Canada Ltd., Toronto, ON, Canada).

2.3.6. Quantification of plasma sex steroid hormone concentrations

Blood was collected from both male and female fish for measurement of plasma sex steroid hormones. Depending on collection volume, blood samples had to be pooled for analyses. A total of 7-10 or 5-8 replicate samples were processed per treatment for males and females, respectively. Circulating sex steroids were extracted from fathead minnow blood plasma sample using liquid-liquid extraction outlined in Chang et al. (2009), with minor modifications detailed in Beitel at al. (2014). Samples were extracted using a 1:1 hexane:ethylacetate mixture and by vortexing for 1 min, followed by centrifugation for 5 min at 2000 rpm. The sample supernatant was pipetted into vials and dried under a nitrogen stream. Buffer solution was then added to bring sample up to required volume (250 µL) for quantification. Both 11-ketotestosterone (11-KT) in males and 17-β estradiol (E2) in females were quantified with enzyme–linked immunosorbent assay (ELISA) kits provided by Cayman Chemicals (Ann Arbor, MI, USA) following the protocols outlined in the manufacturer's directions for each respective assay.

2.3.7. RNA extraction and qPCR of target genes

Total RNA was extracted from livers, gonad, and brain tissue samples using Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). A total of 8 biological samples for each tissue were RNA extracted per treatment (control, 10 %, 50 %). A Nanodrop 200 (Thermo Fisher Scientific, Toronto, ON, Canada) was used to determine the quantity of RNA present in each. First strand cDNA was synthesized from 1 ug of total RNA using QuantiTect Reverse Transcription Kit and diluted between 5-10 times to obtain working cDNA concentrations (Qiagen).

Target gene transcript levels were measured using quantitative polymerase chain reaction (qPCR) performed using a CFX96 real-time polymerase chain reaction system (Bio-Rad, Mississauga, ON, Canada) in 96-well PCR plates. Each reaction was run as a 20 µL reaction consisting of 1x Ssofast EvaGreen Supermix, 0.4 mM of both forward and reverse primer sample, and 2 µL input of diluted cDNA concentration. Before the first PCR cycle, the mix was denatured at 95°C for 10 min. The thermocycler was set at 15 sec at 95°C for denaturizing/annealing followed by an extension step for 1 min at 60°C for total of 40 cycles. Expression of various genes related to steroidogenesis and hormone signalling in the gonad and brain as well as hormone signalling and oxidative stress in the liver were evaluated (Table 2.1).

Each primer set was optimized for proper cDNA input and to obtain a single sequence specific peak amplification in the melt curve. Relative quantity of each target was determined using standard curves constructed using serially diluted cDNA template. Standard curves were used to assess efficiency, uniformity, and linear dynamic range of the qPCR assay and were run on every plate. All primer sets had reaction efficiencies between 90-110 %. Each biological replicate was run in technical triplicates and samples were normalized to the geometric mean of two reference genes (18S and rpL8). Dissociation curves were generated to ensure proper amplification of a single product. Assays also included appropriate negative controls: no template control (water only) and no reverse transcriptase control.

2.3.8. Statistical analysis

Fish exposed to MWWE from Regina and Saskatoon were analysed separately. Male and female fish were also analyzed separately. Normality of the data were tested using the Shapiro-Wilk test, while Levene's test was used to test for homogeneity of variance. Data that did not meet parametric assumptions were log transformed for normalization and/or to reduce the heterogeneity in variance. If the transformations failed to normalize the data, a non-parametric Mann-Whitney U test was applied followed by a Bonferonni-Holm adjustment. For data that met the assumptions of a parametric test, one-way analysis of variance (ANOVA) was used to test for differences across treatment groups. A Tukey's post hoc test was used to determine which treatment(s) differed from the control. Statistical analyses were performed using IBM SPSS Statistics (Armonk, NY, USA). Differences were considered statistically significant at $p<0.05$. All graphs were generated using Prism 6 (Graphpad Software, La Jolla, CA, USA).

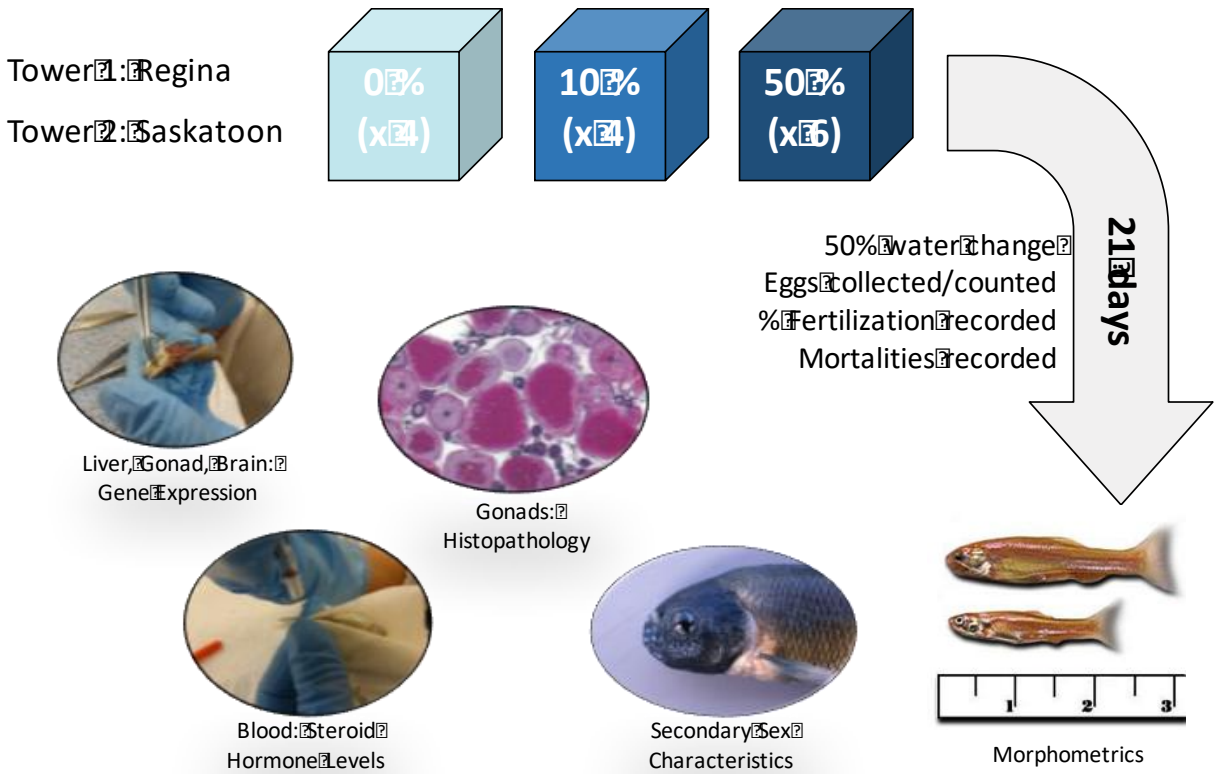

Figure 2.1. Schematic diagram of exposure regime with MWWE and endpoints analyzed during and after the fathead minnow 21-day short-term reproduction assay.

Table 2.1. Primers for qPCR assessment of target transcript levels in liver, gonads, and brain of fathead minnow.

Target Gene	Primer	Sequence (5'-3')	Tissue	Reference
17α-hydroxylase (CYP17)	Forward Reverse	CTGCCCATCATTGGAAGTCT GCATGATGGTGGTTGTTCAC	Gonad	He et al. 2012
17β-hydroxysteroid dehydrogenase (17βHSD)	Forward Reverse	ATCCAGAGTGTGCTGCCTTT AGGGAAATAGCCGTTGGTCT	Gonad	He et al. 2012
3β-hydroxysteroid delta dehydrogenase (3βHSD)	Forward Reverse	TAACTGGAGGATGCGGTTTT TGCACCACTACCACCTTCAC	Gonad	He et al. 2012
Androgen receptor (AR)	Forward Reverse	CAACGCGTCTAAATCCCATT TGTTCGAACTGACACGAAGC	Liver, Brain	He et al. 2012
Apoptosis-inducing factor 3 (AIF)	Forward Reverse	GCGTATCATGCTGGACTTCA TTAAACTGGCCCACTCCATC	Liver	Wiseman et al. 2013
Aromatase α (CYP19a)	Forward Reverse	GCTGCACAAGAAGCACAAG CGTGGCTCTGAGCGAATATC	Liver	He et al. 2012
Aromatase β (CYP19b)	Forward Reverse	AGGGTGTATCCTGGCAACTG ATCTGCACCCGTTTCATTTC	Brain	He et al. 2012
Cholesterol side-chain cleavage (CYP11A)	Forward Reverse	CACACTGATGTGGACGCTCT AGGGCTCCTTTAAGCAGAGG	Gonad	He et al. 2012
Cytochrome p450 1A (CYP1a)	Forward Reverse	CCTGCAGGGAGAACTGAG TCGACGTACAGTGAGGGA	Liver	He et al. 2012.
Cytochrome p450 3A (CYP3a)	Forward Reverse	CGACGAGACCTTCCCAAAT GTTTTCTTGCAGACCCGTT	Liver	He et al. 2012
Estrogen receptor alpha (ERα)	Forward Reverse	CGGTGTGCAGTGACTATGCT CTCTTCCTGCGGTTTCTGTC	Liver, Brain	He et al. 2012
Estrogen receptor beta (ERβ)	Forward Reverse	CGTTTTGGCATAACCATGTG TGCTGTCAGACTTCCGAATG	Liver, Brain	He et al. 2012
Follicle-stimulating hormone receptor (FSHR)	Forward Reverse	CACGTACTGCTGTCCAGACG GTGGCTGGGGTATGTCAGAT	Gonad	He et al. 2012
Glutathione-S-transferase (GST)	Forward Reverse	TCTGGTGCGCTCTTTGAATA TCTCGGGCTAAAAGTTGGTG	Liver	He et al. 2012
Heat shock protein 70 (HSP70)	Forward Reverse	TGGGCTCAATGTCCTCAGAAT CTGCTCCTTTGCCTTTGTCAA	Liver	Klapper et al. 2008
Luteinizing hormone receptor (LHR)	Forward Reverse	CTTTCAACCACCTTCCCAAG AGCATTTGGTGGGACTGAAC	Gonad	He et al. 2012
Ribosomal RNA 18s (18s)	Forward Reverse	GCCCTGTAATTGGAATGAGC TCCCGAGATCCAACTACGAG	All	He et al. 2012
Steroidogenic acute regulatory protein (StAR)	Forward Reverse	ATGCCCGAGAAGAAAGGATT CCCGGTTGATGACTGTTTTT	Gonad	He et al. 2012
Vitellogenin (VTG)	Forward Reverse	TTGCTCTCCAGACCTTTGCT GCAGAGCCTCCACCTTGTAG	Liver, Brain	He et al. 2012

2.4. Results

2.4.1. Water quality monitoring

Water quality parameters were compared among treatments during both pre-exposure and 21 d exposure period (Table 2.2). Measured water quality data revealed no significant difference in temperature or pH among treatments of either the Regina or Saskatoon MWWE during the pre-exposure and exposure periods of the assay. In the Regina effluent exposure, no significant difference in dissolved oxygen was observed between effluent treatments and control group. There was a significant difference in dissolved oxygen observed in the Saskatoon 50 % effluent relative to control. However, the oxygen levels remained above 80 % saturation, which is the benchmark for the exposure (US EPA, 2009). A spike in both N-NH3 and N-NO2 was observed in the 50 % Regina treatment after effluent was introduced (Figure 2.2A; 2.2B). There was no such increase in the 10 % effluent exposure. In the Saskatoon MWWE exposure, an increase in both N-NH3 and N-NO2 after effluent introduction occurred in both the 10 % and 50 % effluent treatments (Figure 2.2C; 2.2D). After these initial spikes, N-NH3 and N-NO2 levels decreased to <0.6 mg/L and <0.4 mg/L by the third day of exposure in both Regina and Saskatoon effluent treatments.

2.4.2. Fecundity and fertilization

At the end of the exposure period (21 days), overall cumulative egg production was significantly lower in the fathead minnows exposed to 10 % (n=771 ± 76.7) and 50 % (n=871 ± 101.6) Regina MWWE compared to the controls (n=1996 ± 131.3) ($p \leq 0.05$) (Figure 2.3). Conversely, there was a significant increase in cumulative egg production in fathead minnows exposed to 50 % Saskatoon MWWE (n=2585 ± 165.4) compared to the control (n=1996 ± 131.3) and a slight decrease in the 10 % effluent treatment (n=1498 ± 190.4). There was no significant change in fertilization of eggs between treatment exposed to 10 % and 50 % effluent from either Regina of Saskatoon MWWE with percent of fertilized eggs remaining constant between 85-95 % throughout the 21 d exposure period (Figure 2.4).

2.4.3. Morphometrics and secondary sex characteristics

No significant mortality was observed in either the Regina or Saskatoon MWWE exposures. There was no significant difference in wet weight, length, or condition factor in either

males or females exposed to MWWE for 21 days (Table 2.3). There were no significant differences among the treatments for male HSI but a significant decrease in HSI of female fish (2.69 ± 0.88) exposed to 50 % Saskatoon MWWE compared to the controls ($p=0.031$). There were no significant differences in GSI among males or females exposed to Regina or Saskatoon MWWE. There were no significant differences in tubercle scores between males exposed to Regina or Saskatoon MWWE (Figure 2.5). Similarly, in Regina and Saskatoon MWWE exposures, no incidence of nuptial tubercles was observed on exposed female fathead minnows.

2.4.4. Histopathology of the gonads

Gonad histopathology was assessed in male and female fish exposed to the control water and 50 % MWWE concentration. Histopathological analysis revealed a significant increase in proportion of spermatogonia in males exposed to 50% Regina and Saskatoon MWWE (Figure 2.6A) as indicated by a significant increase in frequency of grade 1 proportion of spermatogonia in testes from fish exposed to 50 % MWWE from Regina (n=6) and Saskatoon (n=4) (Figure 2.6A). An increased proportion of spermatogonia (grade 2) was observed in testes of male fish exposed to the 50 % Saskatoon MWWE (Figure 2.6A). An increased instance of grade 1 testicular degeneration was also observed in males exposed to 50 % Regina MWWE (n=3) and Saskatoon MWWE (n=6) (Figure 2.6B). There were no instances of testicular oocytes in any of the examined testes from fish exposed to Regina or Saskatoon MWWE. Similarly, no remarkable occurrences of interstitial cell hyperplasia/hypertrophy were observed in testes.

There was a significantly increased frequency of grade 1 oocyte atresia in female minnows exposed to 50 % Regina MWWE treatment (45.5%) when compared to the control (10 %) (Figure 2.8A). There was also one observed instance of grade 2 oocyte atresia in the 50 % effluent treatment. Similarly, histopathological examination of female minnow ovaries exposed to 50 % Saskatoon MWWE treatment showed a significantly increased incidence of grade 1 oocyte atresia compared to the control minnows (Figure 2.8A). However, one instance of grade 2 atresia was observed in the control minnows, where no instances were observed in the 50 % treatment. No significant increase or decrease in gonadal staging was observed in female ovaries exposed the 50 % effluent concentrations from both Regina and Saskatoon (Figure 2.8B). No instances of perifollicular cell hyperplasia or decreased yolk formation were observed in female minnows exposed to Regina and Saskatoon wastewater effluents.

2.4.5. Plasma hormone analysis

Analysis of the circulating sex steroids revealed no statistically significant difference in 11-KT in males among treatments within the Regina and Saskatoon MWWE exposures (Figure 2.10A; 2.10B). There were no statistical differences in plasma E2 concentration in female fish across treatments within the Regina and Saskatoon MWWE exposures (Figure 2.11A; 2.11B).

2.4.6. Gene expression analysis

Male and female fish exposed to Regina effluent did not exhibit any change expression of any of the target genes in the liver relative to the control group (Fig. 2.12A; 2.12B). There was a significant increase in ERα in liver of males exposed to 10 % Saskatoon effluent ($p=0.005$; Fig. 2.12A); however, there was no change with exposure to 50 % effluent. Males also had increased expression of AR in liver with exposure to 10 % ($p=0.005$) and 50 % ($p=0.005$) Saskatoon effluent compared to the control (Fig. 2.12A). Females exposed to 50 % Saskatoon effluent had significantly higher expression of ERα ($p\leq0.05$; Fig. 2.12B) relative to the control.

In both male and female minnows exposed to Regina MWWE, no significant difference in expression of genes involved with general metabolism and oxidative stress was observed in any of the target genes relative to the control group (Fig. 2.13A; 2.13B). Exposure to Saskatoon effluent also had no effect on expression of genes in livers of female fish (Fig. 2.13B). A significant induction in CYP1a was observed in livers of male fish exposed to both 10 % and 50 % Saskatoon MWWE treatments ($p\leq0.05$; Fig. 2.13A) but no change in expression of CYP3a, HSP70 or GST (Fig. 2.13A).

Exposure to Regina MWWE had no effect on expression of follicle stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR), CYP19a, 17-beta hydroxysteroid dehydrogenase (17bHSD), and steroidogenic acute regulatory protein (StAR) in gonadal tissue of male and female fathead minnows (Fig. 2.14A). Female minnows exposed to the 50 % Saskatoon MWWE demonstrated a significant induction in FSHR in ovaries compared to the control group ($p\leq0.05$; Fig. 2.14B) but there was not effect of this effluent on any of the other target genes. Similarly, Saskatoon MWWE exposure had no effect on expression of any gonad target genes in male minnows.

There was no change in expression of any of the transcripts in the brain of male fathead minnows after 21-day exposure to Regina or Saskatoon MWWE (Fig. 2.15A). Both ERβ and AR

were significantly induced in the brain of females exposed to 10 % and 50 % Regina MWWE relative to the control ($p \leq 0.05$; Fig. 2.15B). There was no change in expression of ERα or CYP19b in female minnows exposed to Regina MWWE or in any of the target genes in females exposed to Saskatoon MWWE (Fig. 2.15B).

Table 2.2. Temperature, pH, and dissolved oxygen (DO) measurements taken during the pre-exposure and exposure periods of both the Regina and Saskatoon MWWE exposures. Data are means (± SEM). Data were statistically analyzed with one-way ANOVA followed by a Tukey post-hoc test. Asterisk (*) denotes statistically significant difference relative to site-specific control.

		Regina MWWE			Saskatoon MWWE		
	Parameters	**Control**	**10%**	**50%**	**Control**	**10%**	**50%**
Pre-exposure	**Temperature (°C)**	24.50 ± 0.18	24.52 ± 0.18	24.40 ± 0.18	24.57 ± 0.18	24.21 ± 0.18	24.45 ± 0.16
	pH	7.90 ± 0.09	8.29 ± 0.04	8.35 ± 0.02	8.48 ± 0.03	8.45 ± 0.02	8.43 ± 0.03
	DO (%)	90.9 ± 0.6	95.7 ± 0.7	88.8 ± 0.4	101.4 ± 0.6	102.1 ± 0.6	90.2 ± 0.9
Exposure	**Temperature (°C)**	24.83 ± 0.06	24.80 ± 0.06	24.75 ± 0.04	24.70 ± 0.06	24.86 ± 0.06	24.39 ± 0.09
	pH	8.12 ± 0.08	8.23 ± 0.04	8.09 ± 0.03	8.32 ± 0.03	8.29 ± 0.02	8.26 ± 0.03
	DO (%)	85.8 ± 0.4	93.8 ± 0.8	85.0 ± 0.6	96.0 ± 0.4	104.2 ± 0.7	86.0 ± 0.4*

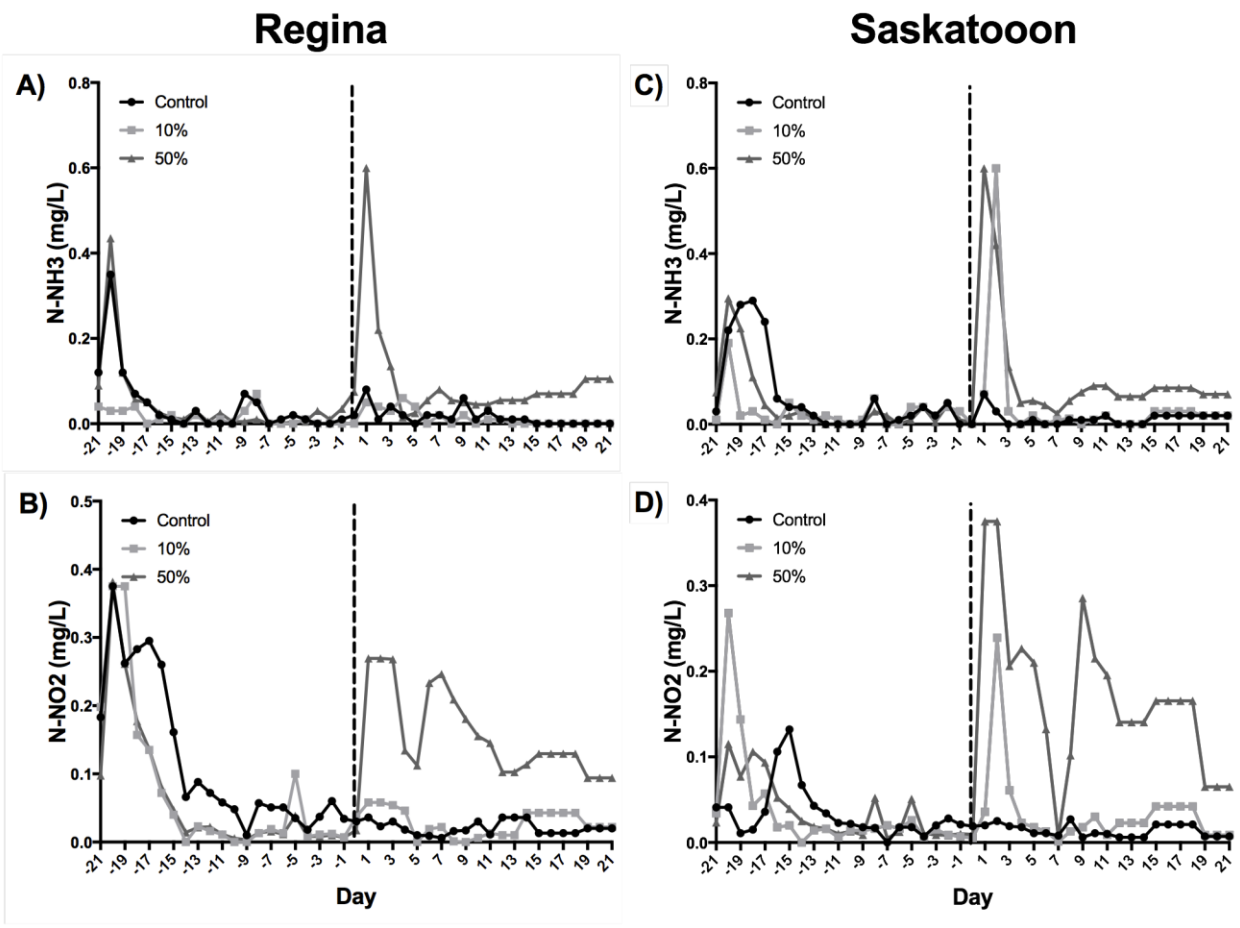

Fig. 2.2. Water quality parameters measured during pre-exposure period and 21-day effluent exposure for Regina MWWE showing (A) ammonia nitrogen (N-NH3) and (B) nitrate nitrogen (N-NO2) and Saskatoon MWWE showing (C) N-NH3 and (D) N-NO2 concentrations. Vertical dashed line indicates the end of pre-exposure and start of effluent exposure.

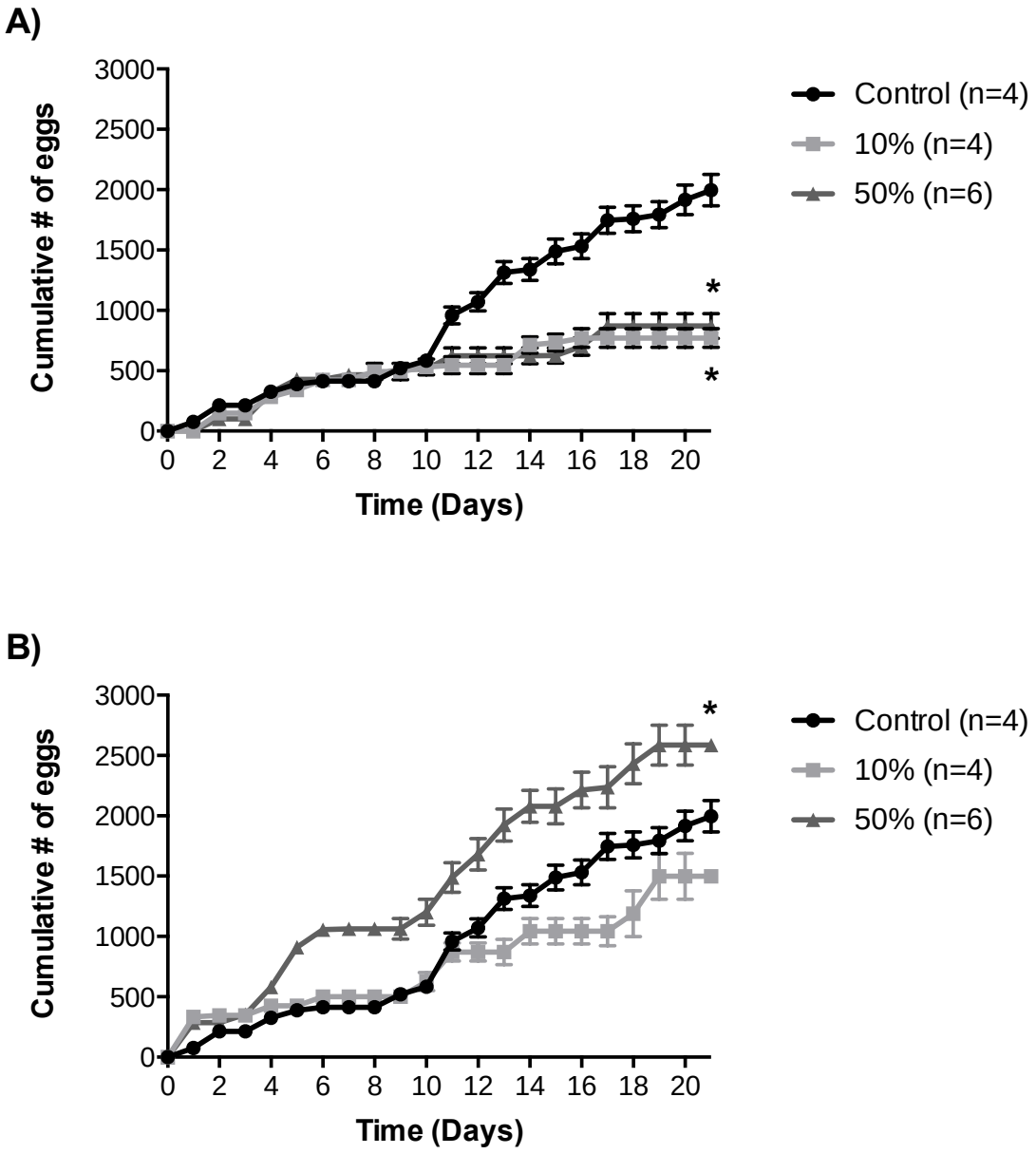

Figure 2.3. Cumulative number of eggs spawned by female fathead minnows during 21-day exposure to MWWE from (A) Regina and (B) Saskatoon at three concentrations (control, 10%, and 50%). Data points represent mean cumulative eggs produced per tank (± SEM) and were statistically analyzed with one-way ANOVA followed by a Tukey post-hoc test. Asterisk (*) indicates statistically significant difference ($p<0.05$) in mean cumulative # of eggs on day 21 compared to the control.

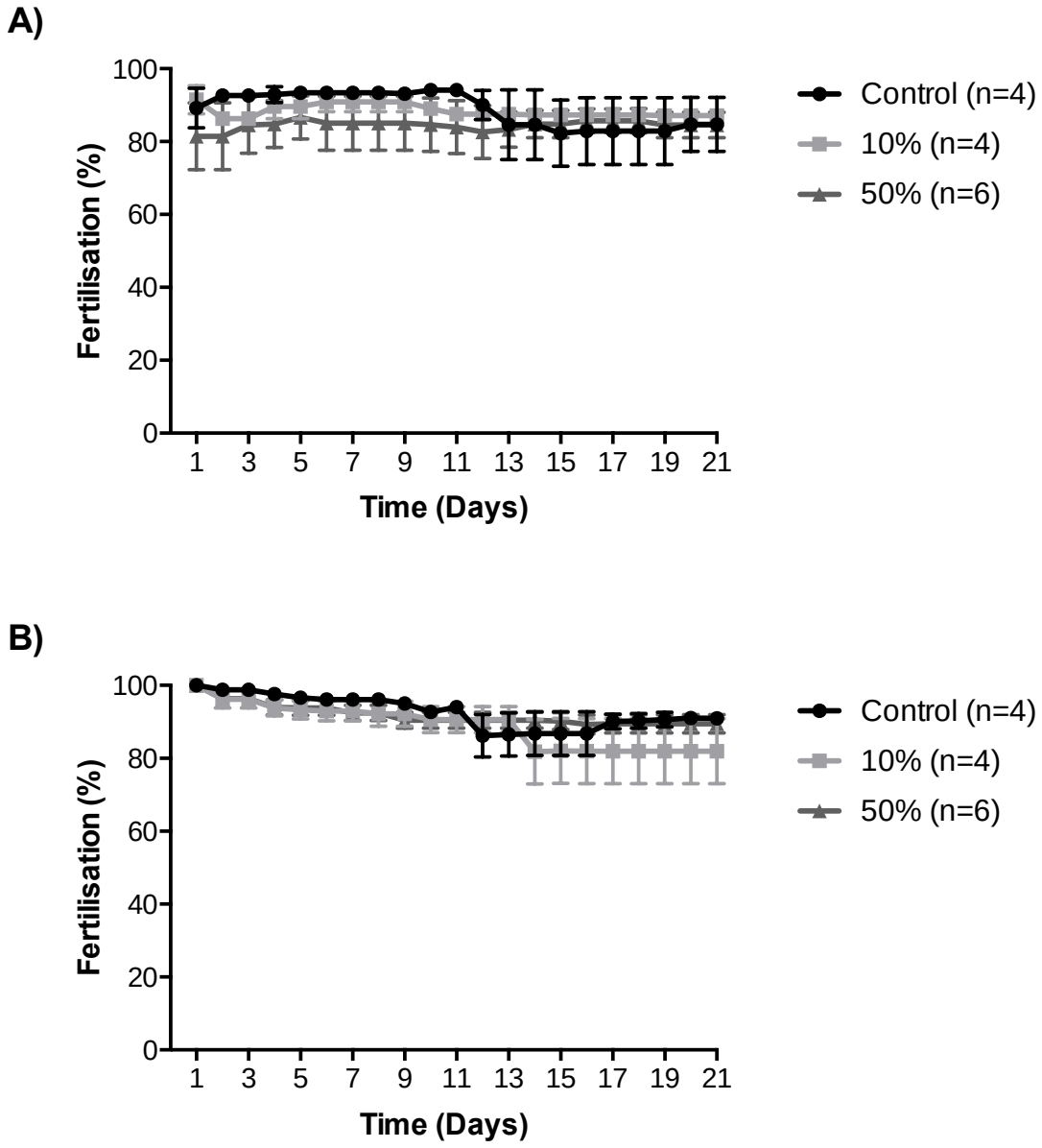

Figure 2.4. Fertilisation of eggs (%) spawned by female fathead minnows during 21-day exposure to municipal wastewater effluent (MWWE) from (A) Regina and (B) Saskatoon at three concentrations (control, 10%, and 50%). Data points represent mean % of eggs fertilised per tank (± SEM) and were statistically analyzed with one-way ANOVA followed by a Tukey post-hoc test

Table 2.3. Total length (cm), weight (g), hepatosomatic index (HSI, %), gonadosomatic index (GSI, %), and condition factor (K) for male and female fathead minnow (*Pimephales promelas*) exposed to Regina or Saskatoon municipal wastewater effluent (MWWE) for 21 days.

		Regina MWWE		
Sex	**Variable**	**Control**	**10% Effluent**	**50% Effluent**
Females	*n*	14	17	22
	Length	4.52 ± 0.13	4.62 ± 0.11	4.54 ± 0.06
	Weight	1.04 ± 0.05	1.07 ± 0.07	1.18 ± 0.06
	HSI (%)	1.84 ± 0.17	1.32 ± 0.15	1.66 ± 0.19
	GSI (%)	8.60± 0.10	9.43 ± 0.13	10.50 ± 0.12
	K	1.17 ± 0.05	1.10 ± 0.07	1.25 ± 0.06
Males	*n*	9	7	12
	Length	4.91 ± 0.12	5.83 ± 0.13	5.10 ± 0.14
	Weight	1.62 ± 0.07	1.97 ± 0.15	1.85 ± 0.14
	HSI (%)	1.94 ± 0.31	1.70 ± 0.26	1.98 ± 0.28
	GSI (%)	1.54 ± 0.54	1.04 ± 0.13	1.01 ± 0.10
	K	1.39 ± 0.046	0.99 ± 0.052	1.37 ± 0.070
		Saskatoon MWWE		
		Control	**10% Effluent**	**50% Effluent**
Females	*n*	13	15	20
	Length (cm)	4.50 ±0.07	4.59 ± 0.12	4.44 ±0,07
	Weight (g)	1.14 ±0.06	1.10 ± 0.07	0.98 ± 0.07
	HSI (%)	1.54 ± 0.27	1.76 ± 0.30	2.69 ± 0.81*
	GSI (%)	10.23 ± 0.11	9.01 ± 0.73	12.30 ± 0.14
	K	1.38 ± 0.05	1.23 ± 0.05	1.31 ± 0.05
Males	*n*	11	9	14
	Length (cm)	4.90 ±0.13	5.26 ± 0.13	5.20 ± 0.08
	Weight (g)	1.62 ± 0.10	1.80 ± 0.13	1.87 ± 0.12
	HSI (%)	1.71 ± 0.26	1.70 ± 0.20	1.62± 0.21
	GSI (%)	1.02 ± 0.80	0.94 ± 0.93	1.23 ± 0.30
	K	1.24 ± 0.04	1.14 ± 0.05	1.10 ± 0.07

Note: Values are means ± SEM. Values with an asterisk (*) are significantly different ($p < 0.05$) when compared with control.

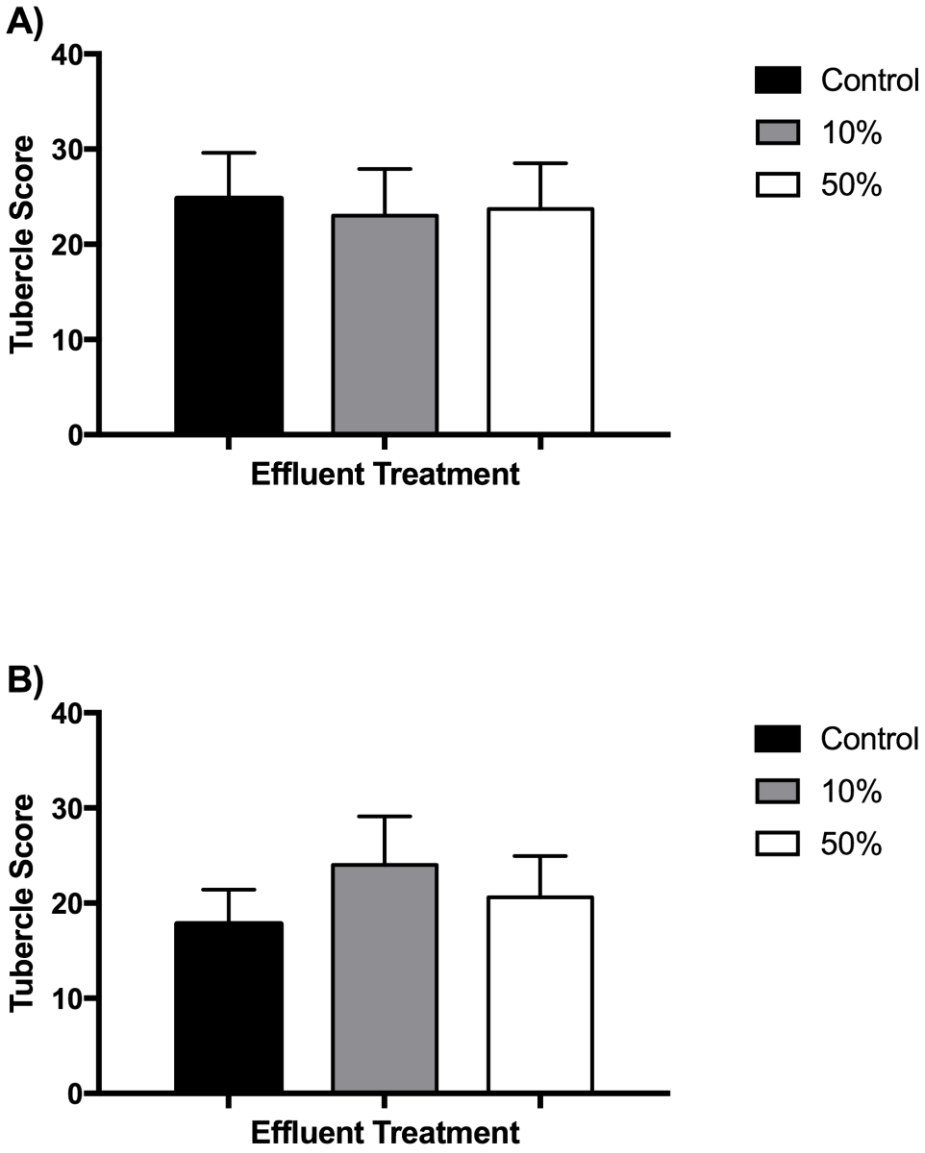

Figure 2.5. Tubercle score (mean ±SEM) of male fathead minnow (*Pimephales promelas*) exposed to (A) Regina and (B) Saskatoon municipal wastewater effluent for 21 days. Bars represent mean (± SEM). Data were statistically analyzed with one-way ANOVA followed by a Tukey post-hoc test.

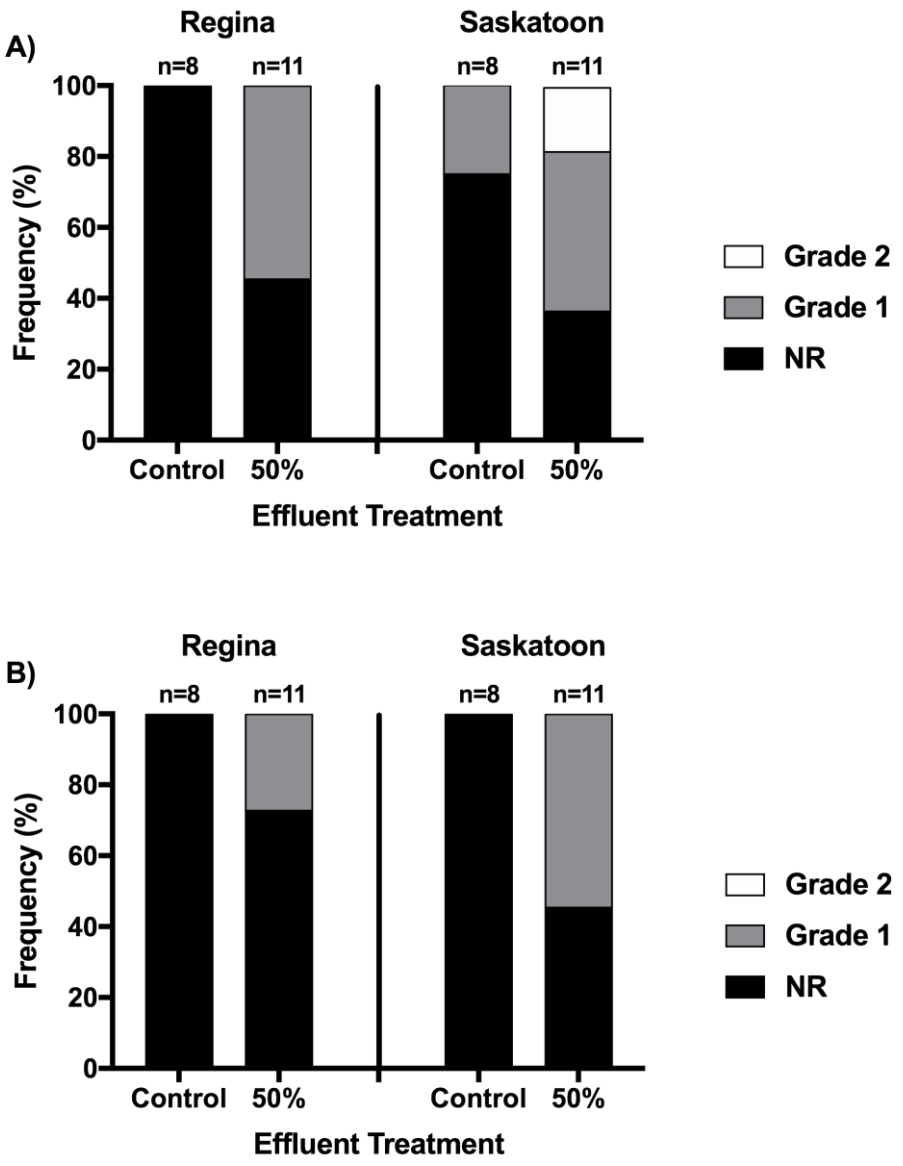

Figure 2.6. Frequency of (A) proportion of spermatogonia and (B) testicular degeneration for testes of male fathead minnows (*Pimephales proemelas*) after 21-day exposure to Regina and Saskatoon municipal wastewater effluent. NR = non-remarkable

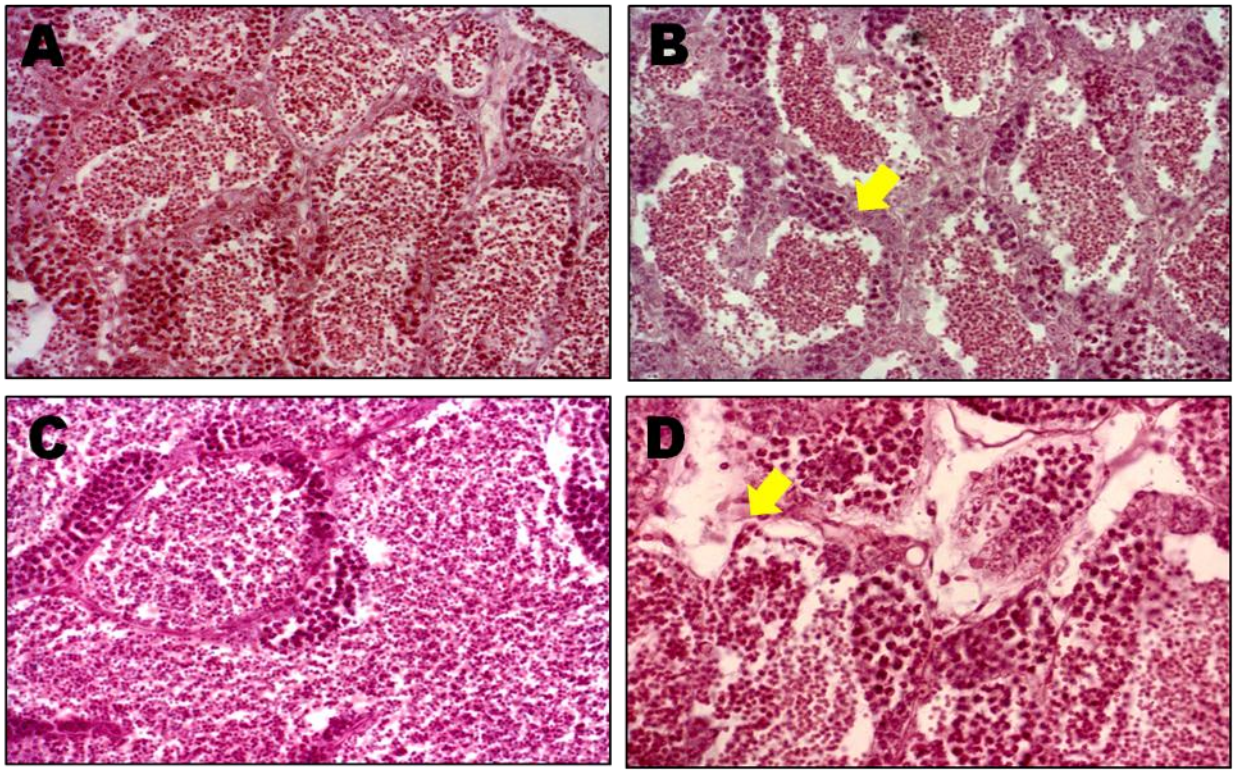

Figure 2.7. Representative photos of histopathological criteria observed in testicular tissue sections from male fathead minnow following 21-day exposure to Regina municipal wastewater effluent (MWWE). Testicular tissue from males exposed to (A) 0% MWWE compared to (B) 50% MWWE, which exhibit increased incidence of spermatogonia (Grade 1). Testicular tissue from males exposed to (C) 0% MWWE compared to (D) 50% MWWE, which show increased testicular degeneration (Grade 1).

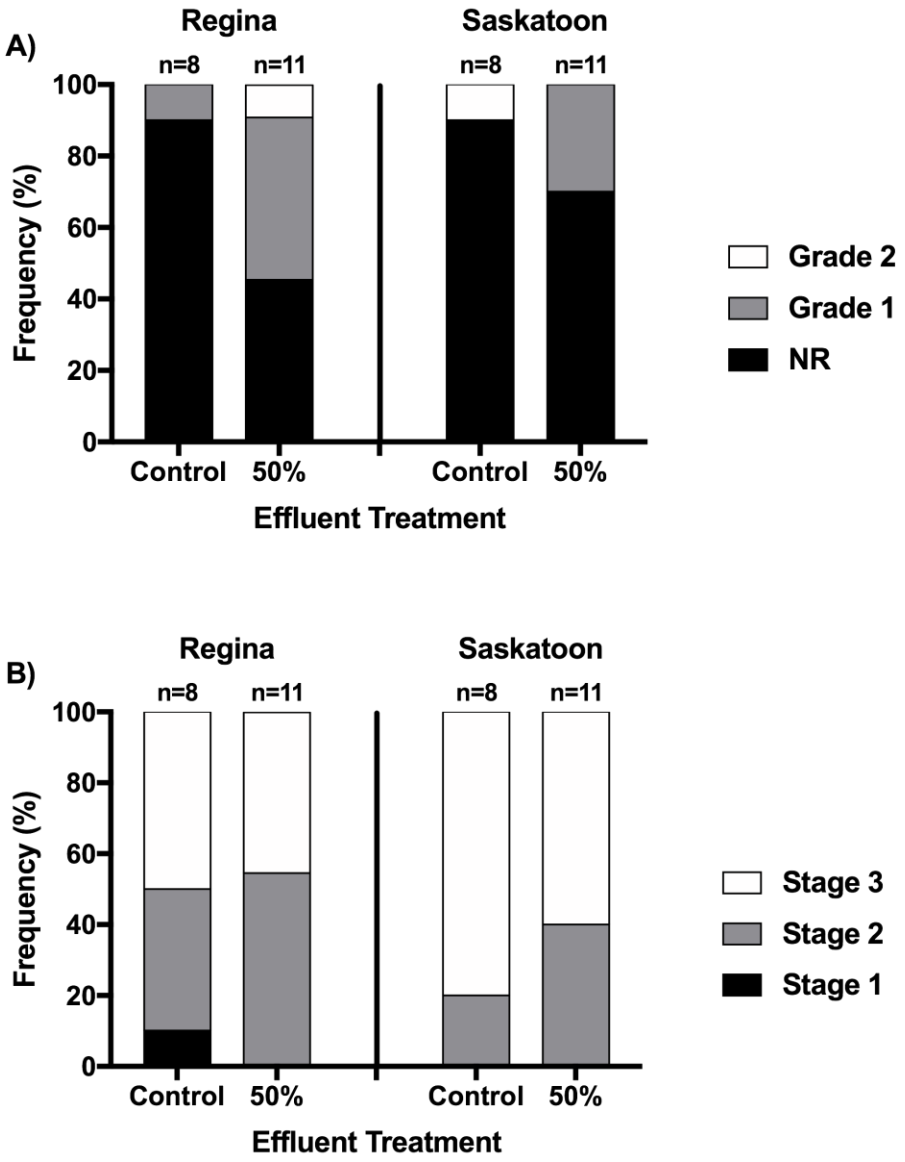

Figure 2.8. Frequency of (A) oocyte atresia and (B) gonadal staging for ovaries of female fathead minnows (*Pimephales proemelas*) after 21-day exposure to Regina and Saskatoon municipal wastewater effluent. NR = non-remarkable

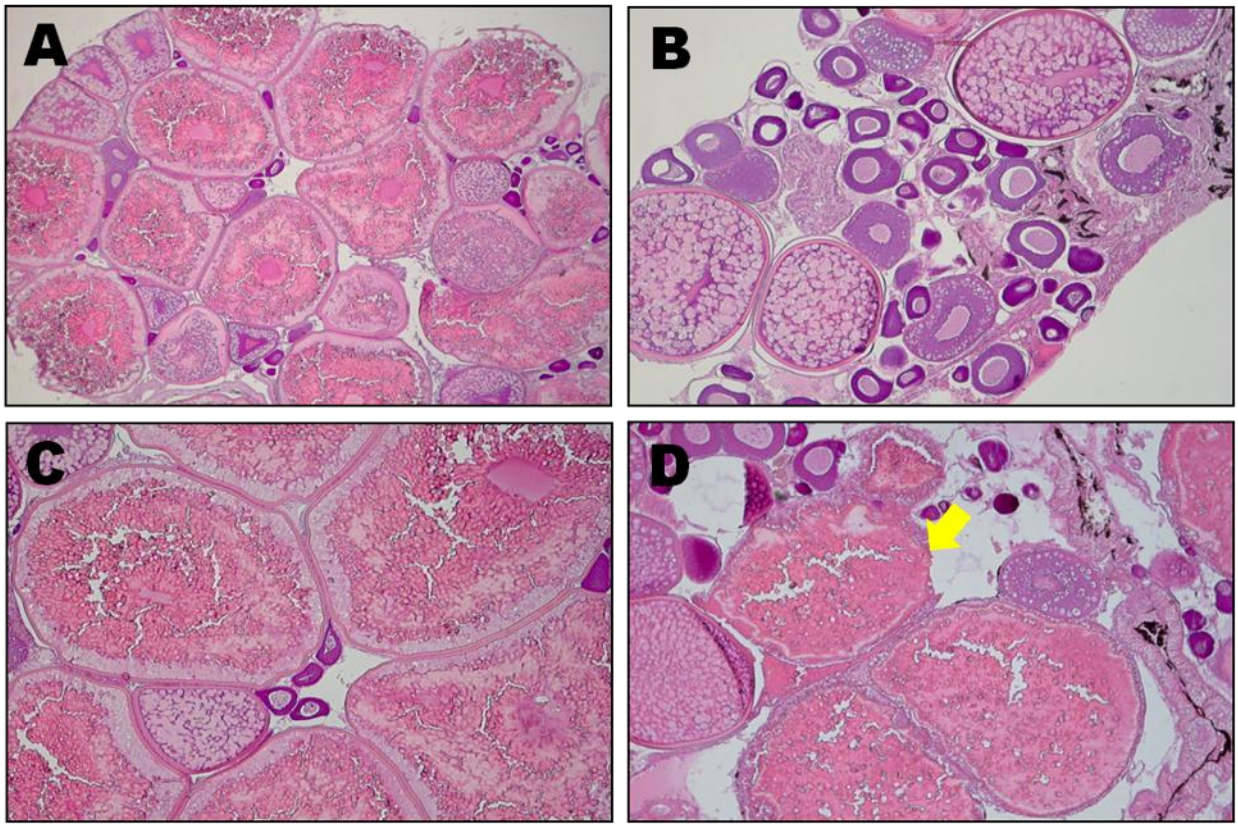

Figure 2.9. Representative photos of histopathological criteria observed in ovarian tissue sections from female fathead minnow following 21-day exposure to Regina municipal wastewater effluent (MWWE). Ovarian tissue from females exposed to (A) 0% MWWE showing stage 3 gonadal stage compared to (B) 50% MWWE showing stage 1 gonadal staging. Ovarian tissue from females exposed to (C) 0% MWWE showing non-remarkable oocyte atresia compared to (D) 50% MWWE showing Grade 1 oocyte atresia.

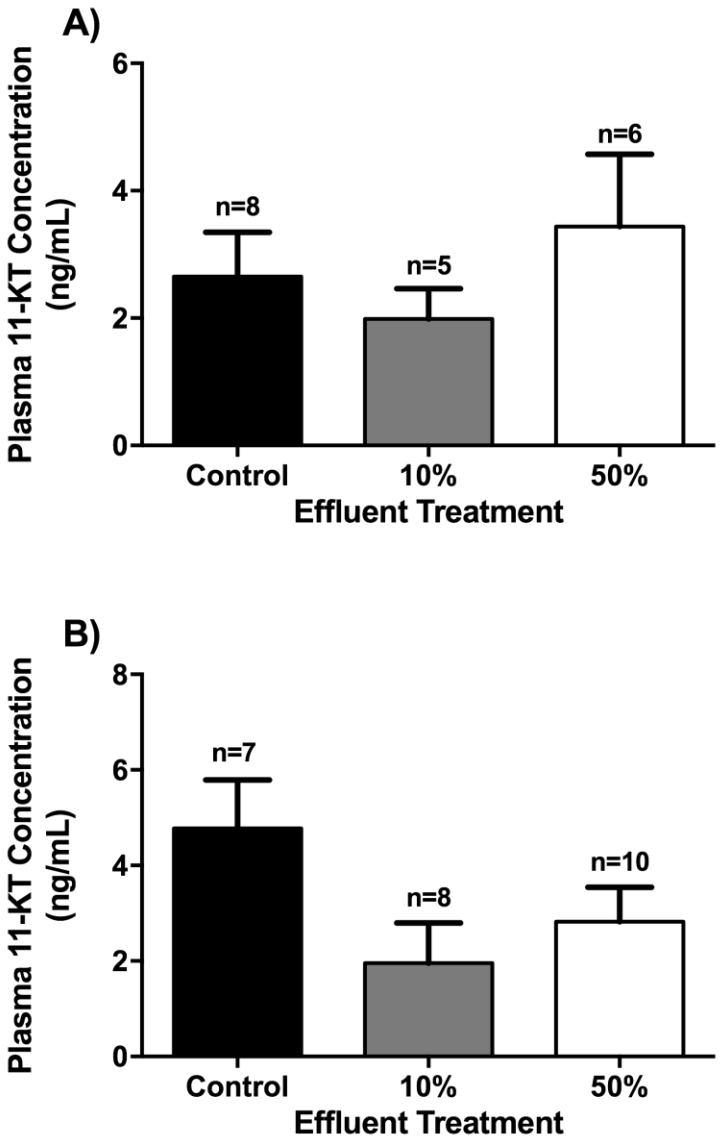

Figure 2.10. Plasma 11-ketotestosterone (11-KT) concentration in male fathead minnows *(Pimephales promelas*) exposed to (A) Regina and (B) Saskatoon municipal wastewater effluent (MWWE) for 21 days. Bars represent mean (± SEM). Data were statistically analyzed with one-way ANOVA followed by a Tukey post-hoc test.

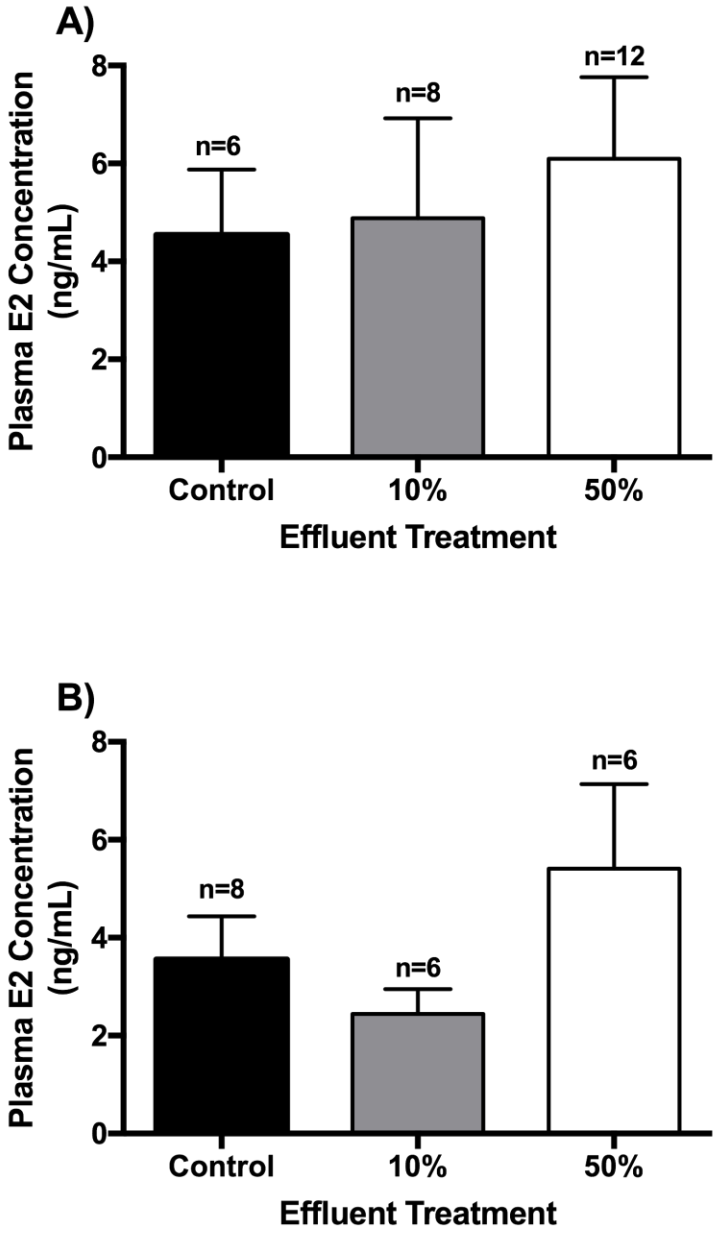

Figure 2.11. Plasma estradiol (E2) concentration in female fathead minnows *(Pimephales promelas*) exposed to (A) Regina and (B) Saskatoon municipal wastewater effluent (MWWE) for 21 days. Bars represent mean (± SEM). Data were statistically analyzed with one-way ANOVA followed by a Tukey post-hoc test.

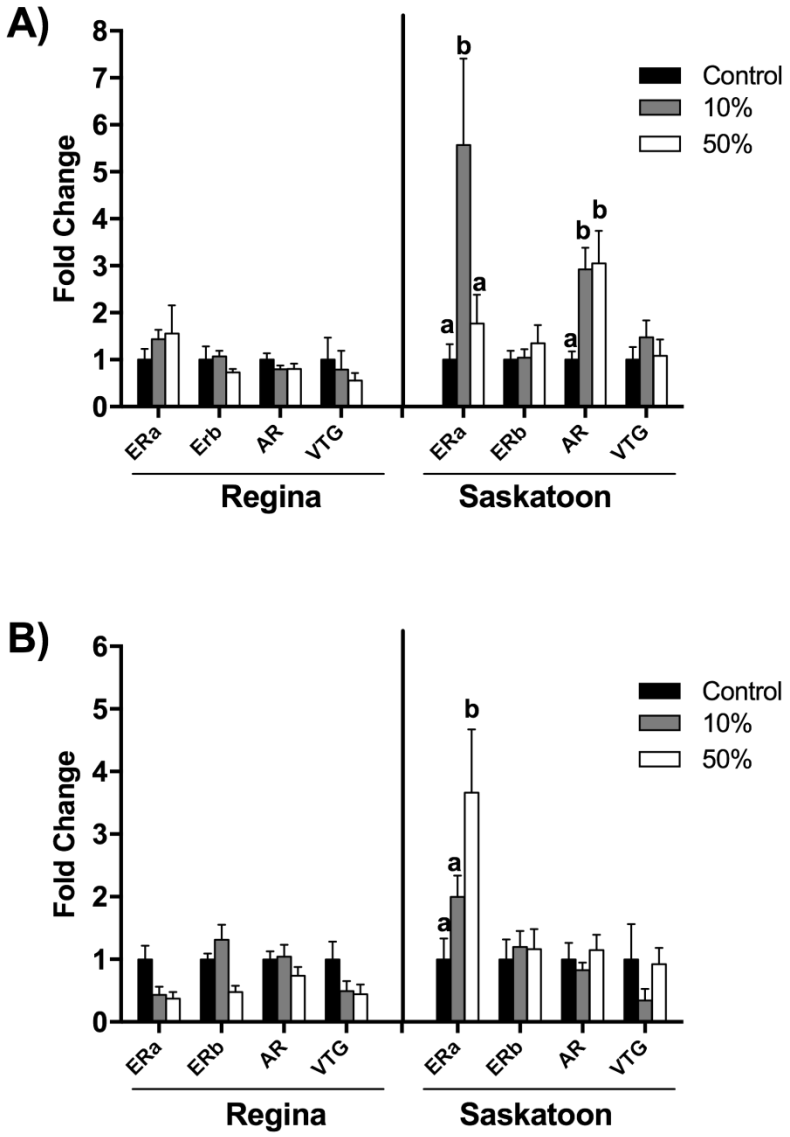

Figure 2.12. Relative expression of transcripts associated with endocrine function in livers of (A) male, and (B) female fathead minnows *(Pimephales promelas*) exposed to municipal wastewater effluent (MWWE) for 21 days. Bars represent mean (± SEM) of 8 individuals per treatment and are expressed as fold change relative to control. Within each transcript, differing letter labels represents a significant difference between treatment groups (one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). ERa, estrogen receptor alpha; ERb, estrogen receptor beta; AR, androgen receptor; VTG, vitellogenin.

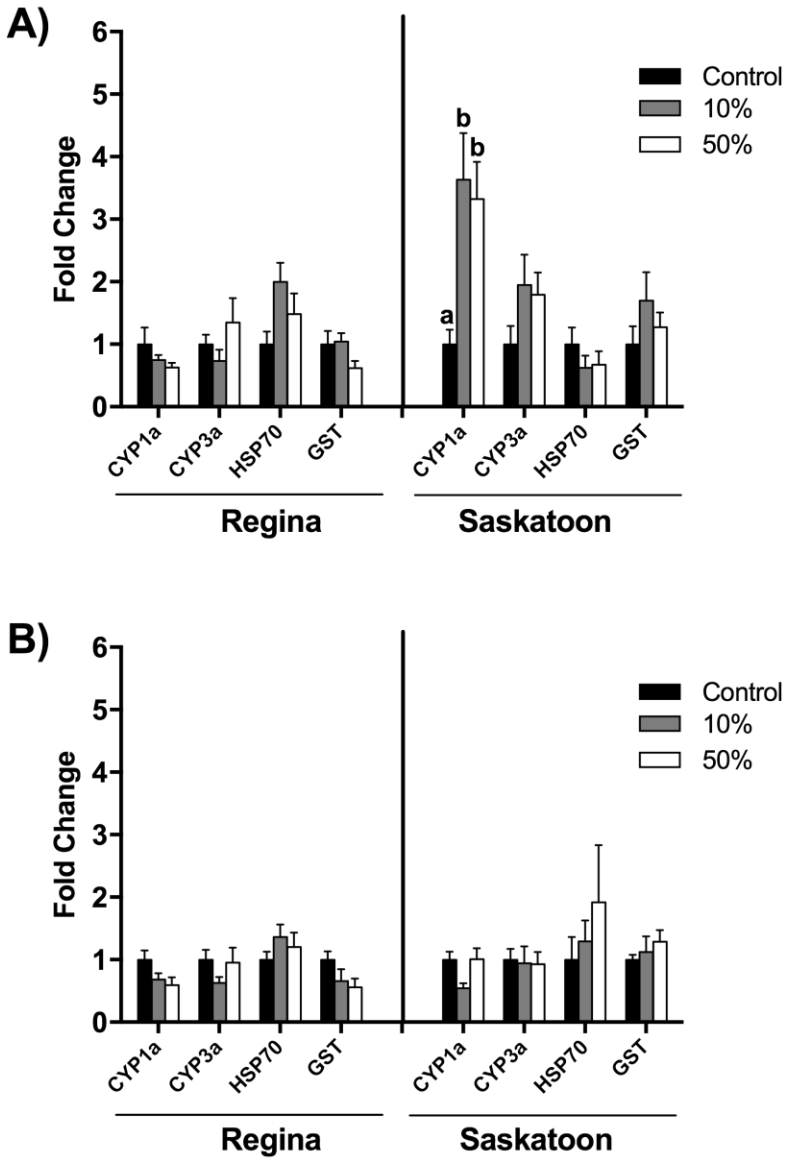

Figure 2.13. Relative expression of transcripts associated with metabolism in livers of (A) male, and (B) female fathead minnows *(Pimephales promelas*) exposed to municipal wastewater effluent (MWWE) for 21 days. Bars represent mean (± SEM) of 8 individuals per treatment and are expressed as fold change relative to control. Within each transcript, differing letter labels represents a significant difference between treatment groups (one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). CYP1a, cytochrome p450 1A; CYP3a, cytochrome p450 3A; HSP70, heat-shock protein 70; GST, glutathione-s-transferase.

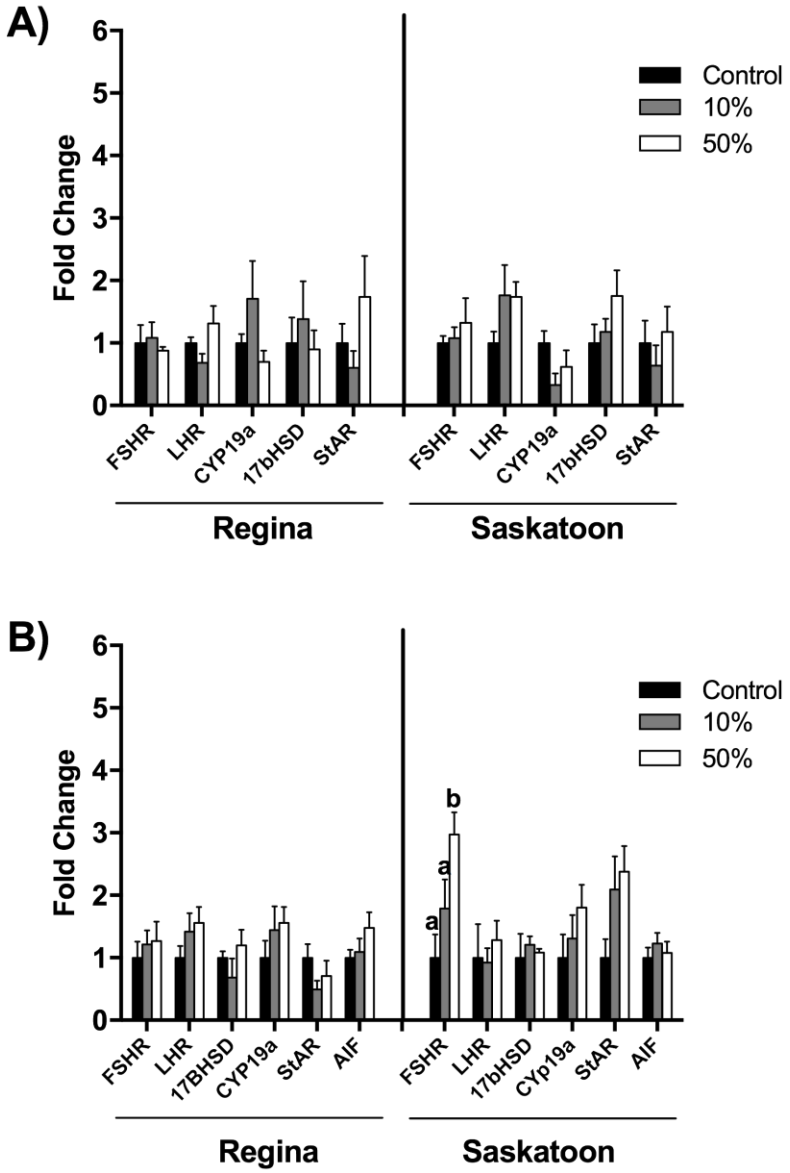

Figure 2.14. Relative expression of transcripts associated with endocrine function in gonads of (A) male, and (B) female fathead minnows *(Pimephales promelas*) exposed to municipal wastewater effluent (MWWE) for 21 days. Bars represent mean (± SEM) of 8 individuals per treatment and are expressed as fold change relative to control. Within each transcript, differing letter labels represents a significant difference between treatment groups (one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). FSHR, follicle-stimulating hormone receptor; LHR, luteinizing hormone receptor; 17BHSD, 17β-hydroxysteroid dehydrogenase; CYP19a, aromatase α; StAR, steroidogenic acute regulatory protein; AIF, apoptosis-inducing factor 3.

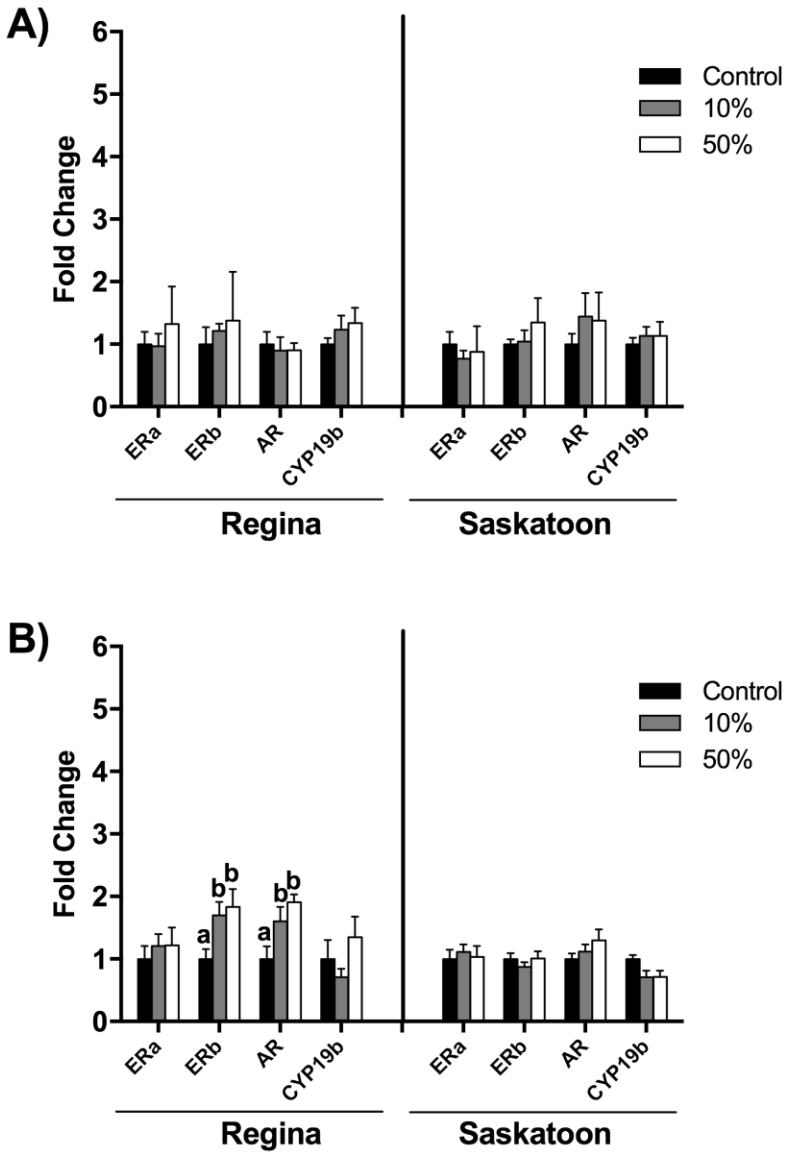

Figure 2.15. Relative expression of transcripts associated with endocrine function in brains of (A) male, and (B) female fathead minnows *(Pimephales promelas*) exposed to municipal wastewater effluent (MWWE) for 21 days. Bars represent mean (± SEM) of 8 individuals per treatment and are expressed as fold change relative to control. Within each transcript, differing letter labels represents a significant difference between treatment groups (one-way ANOVA with Tukey's post-hoc test, $p \leq 0.05$). Era, estrogen receptor alpha; ERb, estrogen receptor beta; AR, androgen receptor; CYP19b, Aromatase β.

2.5. Discussion

Municipal wastewater effluent is a complex mixture of biological and chemical compounds, particularly emerging contaminants, and exposure to such contaminant mixtures has the potential to disrupt reproduction in fishes. This study was conducted to identify potential reproductive effects of MWWEs on adult fathead minnows following a 21-day exposure to dilutions of MWWE collected from the Regina and Saskatoon WWTPs. The degree of the reproductive disruption and the specific biological measures affected did vary between effluent source and sex of fish. Exposure to Regina MWWE resulted in a significant decrease in egg production while tissue-level effects, specifically oocyte atresia in the ovaries of females and increased spermatogonia in males, was observed following exposure to both effluent sources. However, we were unable to associate higher level, apical effects with changes at lower levels of the biological scale (e.g. hormone levels, gene expression) that would indicate specific endocrine activity (estrogenic or androgenic) of the effluent.

Fecundity represents one of the most ecologically-relevant endpoints to evaluate the toxicological impacts of chemicals, as the ability of fish to spawn can directly affect whole populations. We found that female fish had significantly decreased egg production with exposure to Regina MWWE concentrations as low as 10 %. In contrast, there was no clear effect of Saskatoon MWWE on fecundity as increased egg production was noted following exposure to 50 % effluent, while exposure to 10 % effluent resulted in decreased egg production. Previous studies have reported decreased egg production with MWWE exposure in a number of species, including zebrafish (Lister et al., 2009) and fathead minnows (Thorpe et al., 2009; Cavallin et al., 2016). Similarly, we conclude that exposure to Regina MWWE negatively impacts fish reproduction and could result in changes at the population level. Reduction in fecundity was also reported in fathead minnows following exposure to a number of contaminants including ammonia (Armstrong et al. 2012), the hormone receptor agonists EE2 (Lange et al., 2001) and 17β-trenbolone (Ankley et al., 2003), as well as the plasticizer bisphenol A (Sohoni et al., 2001). In particular, elevated ammonia levels are often reported in MWWE (Servos et al., 2005; Guo et al., 2010; Waiser et al., 2011) and exposure to un-ionized ammonia (NH_3-N) at levels as low as 0.06 mg/L can increase mortality, reduce growth, and impair reproductive output in fathead minnow with 20-day exposure (Armstrong, et al. 2012). Concentrations of ammonia in Wascana Creek have been measured as high as 0.45 mg/ L, above CCME guidelines and high enough to

elicit the reported reproductive impacts (Waiser et al., 2011). In the present study, NH_3-N levels spiked to 0.6 mg/ L when 50 % effluent from Regina and Saskatoon was introduced to the flow-through system at the start of the 21-day exposure. After this initial spike, NH_3-N in both Regina and Saskatoon effluent exposures decreased in concentration to below 0.1 mg/L. While the initial peak in measured ammonia concentration occurred with both effluents at 50 % exposure, decreased fecundity was only observed with exposure to Regina effluent at both 10 % and 50 %; therefore, it is unclear whether impaired reproductive output can be attributed to increased ammonia levels. Ours and other studies demonstrate that fecundity is a useful endpoint to assess the impact of contaminants or complex mixtures on reproductive fitness. However, decreased fecundity is a reproductive impairment that is not specific to any single mode or mechanism of action, and thus does not allow us to identify the specific mode of action or, in case of mixtures, the specific compound responsible for the effect.

In contrast to the source-specific effects on fecundity, exposure to both Regina and Saskatoon effluents resulted in histopathological changes in the gonads of male and female fathead minnow. There was a significant increase in proportion of spermatogonia in testes of fish exposed to 50 % Saskatoon and Regina MWWEs. Spermatogonia are less developed gonadal cells in the testes, and an increased proportion indicates delayed development (Jensen et al., 2001), which has been previously observed in minnows exposed to MWWEs (Vadja et al., 2011; Tetreault, et al., 2012). Histopathological changes observed in males did not appear to impact fertility, as there were no differences in fertilization success across treatments. Increased oocyte atresia was also observed in female minnows following exposure to both 50 % Regina and Saskatoon MWWEs and could reflect a disruption in the normal processes of final maturation of oocytes with subsequent disturbances in ovulation and oviposition. Reduced fecundity in fathead minnows has been reported together with delayed oocyte maturation following exposure to EE2 (Lange et al., 2001) and exposure to bleached sulphite mill effluent (Parrott et al., 2004). However, differing response in fecundity with exposure to Regina effluent (decreased) and Saskatoon effluent (increased) does not correlate with increased oocyte atresia observed with exposure to either effluents. Regardless, alterations in gonadal tissue clearly demonstrate that MWWEs from both Regina and Saskatoon are impacting gonadal development in fathead minnows of both sexes.

As previously stated, fecundity is an apical endpoint that is not specific to any single mode or mechanism of action. The cause of impaired reproductive output in fathead minnows with exposure to Regina MWWEs is not apparent based on our suite of diagnostic endpoints. With the exception of gonadal alterations, no consistent estrogen-dependent endpoints were affected by exposure to either Regina or Saskatoon MMWE, indicating either a lack of estrogenic compounds present in the effluent or perhaps that estrogenic effects were masked by other impacts or types of toxicities. Exposure to estrogenic compounds in wastewater have been previously reported to reduce of male nuptial tubercle size (Barber et al., 2007; Vadja et al., 2011), altered concentration of circulating sex steroid hormones (Salierno and Kane, 2009), and induce of hepatic VTG mRNA (Lattier et al., 2002; Flick et al., 2014) in fathead minnow. No such effects were observed with effluent exposure in the present study nor were there changes in the expression of most of the selected genes of interest related to endocrine signalling, steroidogenesis and oxidative stress. The measurement of target genes is a useful tool to aide in determining a specific mode of action. When it comes to the molecular markers of exposure to xenoestrogenic compounds in fish, the hepatic up-regulation of vitellogenin genes is most widely used (Arukwe and Goksøyr, 2003; Brander, 2013). Notably, there was no change in VTG expression in male and female minnows exposed to MWWE from Regina and Saskatoon. Exposure to Saskatoon effluent did increase expression of steroid hormone receptors, specifically ERα in both males and females along with AR in males. Induction of ERα is often indicative of estrogenicity and has been reported with exposure to an estrogenic compound such as EE2 (Filby et al., 2006). However, without changes in plasma 17β-estradiol or VTG expression in response to Saskatoon effluent, this induction of ERα is not likely a physiologically relevant response to effluent exposure. It is worth noting that changes in transcript levels associated with exposure are also influenced by timing in relation to length of exposure. Sampling tissue in the first few days of an exposure offers a better representation before the endocrine pathways involved in steroidogenesis and neuroendocrine signaling are able to compensate for disruption by effluent exposure (Villeneuve et al., 2009). It's possible that the induction of AR and ERα observed in our study might be the result of a compensatory response to the potential anti-androgenic and anti-estrogenic potentials of the effluent. In our study, sampling was conducted at the conclusion of a 21-day exposure, leaving the possibility for the compensatory response to mask transcriptional responses in the HPG-L axis of fish to effluent exposure.

Environmental androgens can also impact fathead minnows on an apical and physiological level, although a specific molecular biomarker of androgen exposure has yet to be established (Leet et al., 2012). Reported effects following exposure to androgens, such as 17β-trenbolone and 17α-methyltestosterone, include development of nuptial tubercles in females and increased size in males, impaired ovary growth in females, and alterations to sex steroid hormones (E2, 11-KT) in both male and female fish (Ankley et al., 2003; Pawlowski et al., 2004). While no significant changes in tubercle size or plasma sex steroid concentration were observed in the present study, there was increased oocyte atresia observed in female minnows with exposure to both Regina and Saskatoon effluents. There was also an increased expression of AR in tissues collected from the brains of female fathead minnows exposed to Regina MWWE, suggesting that these changes may be the result of some androgenic compound present in the effluent. However, these changes cannot be definitively linked to androgenic exposure and no change was observed in other androgen-responsive endpoints (e.g. increased tubercle size).

The lack of specific estrogenic or androgenic effects with exposure to these effluents is further supported by chemical analysis and *in vitro* bioassays conducted by Bagatim et al. (2018) in efforts to characterise both Saskatoon and Regina MWWE. As part of this research, effluents were collected during a similar time (Spring 2014) and from the same outfall source (Regina and Saskatoon WWTPs) as in the present study. The MVLN and MDA cell line assays were used to evaluate the effluents in terms of their estrogenic and androgenic potency, respectively. Regina effluent collected in the spring of 2014 was found to be estrogenic, which is interesting given that no changes in common estrogen endpoints were observed in our study, suggesting that the effects are being masked by another property of the effluent (anti-estrogenic), or the estrogenic potential is not high enough to illicit a physiological effect in fish. Conversely, Saskatoon effluent did not demonstrate estrogenicity in the *in vitro* assay. Neither effluent displayed significant androgenic activity as determined by MDA cell line assays (Bagatim et al. 2018), suggesting that the observed changes in ovarian tissue and AR induction in female minnows following Regina effluent exposure are not due to direct androgen agonist activity of the effluent. While significant anti-androgenic activity was observed in effluents collected from both Regina and Saskatoon (Bagatim et al., 2018), we did not observe changes in androgen-dependent tubercle score, which is one of the few endpoints for measuring anti-androgenic activity in male fathead minnows (Panter et al., 2004; Matrinovic et al., 2008). It is possible that the anti-

androgenic activity demonstrated *in vitro* was not elevated enough to result in apical changes to fish in the 21-day reproductive bioassay. Chemical analyses of the effluents did not detect reproductive hormones or hormone agonists often reported in wastewater effluents (e.g. estradiol, testosterone, estrone, estriol, ethynylestradiol), although there was elevated concentration of DEET and triclosan present in the Regina MWWE (Bagatim et al., 2018). Taken together, the fish reproductive bioassay along with chemical analysis and *in vitro* bioassays of the effluents indicate that Regina and Saskatoon MWWEs do not possess any significant agonist activity that could explain the observed impairments to fecundity and gonadal development in exposed fathead minnows.

A related wild-fish study was conducted by Hanson et al. (2018) where fathead minnows were collected upstream and downstream of the Regina WWTP during the summer of 2014 and 2015. Male fish collected downstream exhibited increased spermatogonia as determined by histological examination of the testes while females had increased oocyte atresia (Hanson et al., 2018). These findings parallel those in the present study and similarly, Hanson et al. (2018) found no change in plasma sex steroid concentration nor induction of VTG in male fathead minnows. While the wild-fish and laboratory studies present two different exposure scenarios, the accordance in their findings demonstrate that the short-term fish bioassay is predictive of responses in fish inhabiting an effluent-contaminated aquatic system.

The complex chemical composition of MWWEs makes determining the precise cause for any observed effects quite challenging. Our results, coupled with the findings of both Bagatim et al. (2018) and Hanson et al. (2018) indicate that altered fecundity and gonadal histopathology (increased spermatogonia and oocyte atresia) are not likely due to direct agonist activity of compounds in the effluents. These effects on egg production and gonadal development may be due to other physio-chemical properties of the effluents or specific constituents acting through mechanisms not mediated by interaction with estrogen or androgen receptors. Non-steroidal pharmaceuticals, such as carbamazepine, have been shown to cause similar histopathological changes in testes of zebrafish (Madureira et al., 2011) and these compounds were detected in our effluents, albeit at 7.62 ng/L (Bagatim et al., 2018), which is lower than those reported to elicit effects. Behaviour is also an important part of successful spawning in fathead minnow as males need to attract females and defend their nests. Changes in normal courting and territorial behaviours have been linked to decreased successful spawning events in laboratory studies with

fathead minnows, including decreased aggression in males (Martinovic et al, 2007). Several fish behaviours, including mate selection, predator avoidance and aggression, have been altered following exposures to environmentally relevant concentrations of pharmaceuticals (reviewed in Overturf et al. 2015). In the present study, an elevated concentration of triclosan (28.95 ng/ L) was measured in Regina MWWE (Bagatim et al., 2018). Fathead minnows exposed to triclosan at concentrations as low as 75 μg/ L exhibited decreased swimming activity (Fritsch et al., 2013) while mixtures of triclosan (560 ng/ L) and triclocarbon (179 ng/ L) had decreased aggression (Schultz et al., 2012). Lowered aggression could result in decreased nest holding ability, which may offer an explanation for the decrease in fecundity with exposure Regina MWWE, but not in Saskatoon MWWE. It is also interesting to note that elevated levels of DEET (345.5 ng/L) were measured in Regina effluent (Bagatim et al., 2017); however, concentrations fell below reported 96 h LC50 (110 mg/ L) and EC50 (75.7 mg/ L) values (Weeks et al., 2012).

The exposures conducted in this study used effluents collected from two separate WWTPs that utilize differing treatment technology. The manner in which the effluent is processed may be responsible for the differences in effects observed in fathead minnow. One of the main findings was that Regina MWWE caused significant decrease in fecundity of exposed female minnows while no dose-dependent effects were seen following Saskatoon MWWE exposure. The Saskatoon WWTP utilizes an activated sludge and biological nutrient reactor as part of the treatment process (City of Saskatoon, n.d.). An activated sludge process similar to that employed by Saskatoon WWTP (i.e. anaerobic, anoxic, and aerobic) has demonstrated efficient removal of environmental and synthetic estrogens (Li et al., 2011). However, removal of estrogens from wastewater has also been observed in aerated lagoon treatment technologies similar to those used at the Regina WWTP (Li et al., 2013). The difference in observed effects may in fact be due to the inefficient removal of other PPCPs present in greater concentration in Regina effluent than in Saskatoon MWWE. As previously mentioned, Bagatim et al. (2018) found that certain contaminants, such as triclosan and DEET, were measured in much greater concentration in the Regina effluent compared to Saskatoon. PPCP contamination in Wascana Creek has also been documented by Waiser et al. (2011), supporting the likelihood that the observed differences in effects to fathead minnows in the present study could be associated such contaminants.

The present study used multiple lines of evidence to evaluate the effects effluents have across many levels of biological organization. While both Regina and Saskatoon effluents impacted minnows on an apical level (histopathology), exposure to Regina MWWE caused impairment of reproductive output (i.e. fecundity), which is environmentally relevant as it can cause declines in population. Potential risk is increased in semi-arid regions such as South Saskatchewan where receiving water bodies, such as Wascana Creek, experience periods of seasonal low-flow and effectively concentrate the effects of the effluents as a result of low dilution. Therefore, the dilutions chosen for this study (eg. 10 and 50 %), are environmentally relevant as 100 % effluent composition in Wascana Creek was possible before the upgrades to Regina's WWTP in 2016. Larger water bodies, such as the South Saskatchewan River in Saskatoon, reduce the risk of exposure to aquatic life due to the greater dilution that occurs and aquatic organisms will not likely be exposed to the concentrations of effluent used in this study. Molecular and biochemical biomarkers demonstrated no mechanism or trend following effluent exposure so it does not appear that apical effects were a result of direct disruption to the HPG-L axis. Furthermore, since effluents are a mixture of many chemicals, it is difficult to discern which chemicals are responsible for reproductive impacts on fathead minnows (and other resident fish species) and whether other constituents (e.g. ammonia, nitrate) or physical characteristics of the effluent are impacting the fish. However, additional endpoints could be incorporated into the 21-day reproductive bioassay to further elucidate mechanisms underlying effects of South Saskatchewan MWWE. This study demonstrated that exposure to high concentrations of municipal effluent can cause adverse biological effects in sentinel fish species and represent a risk to the health of populations at and near effluent outfall.

CHAPTER 3

3. GENERAL DISCUSSION

3.1 Project Focus and Objectives

As the human population continues to increase, particularly in urban centres, there is increasing pressure on wastewater treatment facilities to effectively treat the municipal waste. Quite often there is a need for improved capacity and upgraded infrastructure within these facilities in order to ensure the water quality of receiving environments is maintained when the effluents are released. In addition to the growing demand placed on WWTP due to increased influent load, there is an increased amount of chemicals being introduced into these environments. These chemicals present a risk to aquatic environments as they can cause adverse effects to the organisms living within these systems. A subset of these chemicals, including pharmaceuticals and personal care products (PPCP), are designed to work at very low concentrations and can persist and potentially accumulate in the environment. Chronic exposure to PPCPs can impair reproduction in fish – effects that are more subtle and often more difficult to detect compared to responses such as deformities and lethality. Reproductive effects, such as reduced fecundity and altered gonadal development in male and female fish can result in decline of populations and compromise ecosystem health. Saskatchewan aquatic systems are affected by other factors including input from agricultural activity, which can lead to eutrophication, as well as seasonal low flow conditions, which decreases the dilution of the wastewater effluents being released. Given that there is already risk associated with these other stressors, it is important to understand the additional effects of contaminants contained within wastewater effluent on aquatic life. Therefore, the overall objective of this study was to determine the impacts of South Saskatchewan MWWEs on fish reproduction.

The experiment conducted as part of this thesis was designed to evaluate the effects of effluent taken directly from Saskatoon and Regina WWTPs on the reproductive output and endocrine responses in the model fish species, the fathead minnow, using a standardised 21-day reproductive bioassay. The effluent was collected end-of-pipe from these two locations as they represent two separate exposure scenarios: Regina having fewer treatment technologies and low volume receiving environment, while Saskatoon featured more advanced technologies as well as a larger volume aquatic system. The first objective was to determine the effects of effluent on the reproductive success of fathead minnows, which involved recording fecundity and fertility over the duration of the exposure. The second objective was to identify potential underlying cause for effects on reproductive output by examining reproductive endpoints, such as expressed

secondary sex characteristics, histopathological examination of the gonads, and measurement of circulating sex steroids. The final objective was to determine the potential mechanisms of action underlying the effects manifesting at the apical and physiological levels by evaluating transcriptional changes in key target genes collected from multiple tissues. This research was aimed at determining whether Regina and Saskatoon effluents have the potential to cause adverse reproductive effects in fathead minnows, and therefore pose a risk to aquatic organisms in receiving environments. Furthermore, this study was a portion of the Aquatic Impact Assessment of Municipal Effluents (AIME), a larger research effort that used a tiered system with various testing approaches, including (1) *in vitro* bioassays to identify biological activity of effluent, (2) a short-term fish reproductive bioassay with effluent exposures in laboratory, and (3) wild-fish surveys at effluent-impacted sites. The overall goal of this research initiative was to establish effective endpoints and develop a set of techniques or a "toolbox" for assessing the potential impacts of MWWEs to biota in aquatic ecosystems.

3.2 Summary of Findings

This study used multiple lines of evidence to evaluate the effect of MWWE from two sources, Regina and Saskatoon wastewater treatment plants, from the molecular level to whole organism level. The totality of these apical and sub-organismal response data described in text below and summarised in Table 3.1, support the conclusion that short-term exposure to high concentrations of effluent can impair fish reproduction and that these responses varied between effluent source and sex of fish.

3.2.1 Effects of MWWE on Reproductive Output

Exposure to both 10 % and 50 % Regina effluent resulted in a 67 % and 63 % decrease, respectively, in egg production as compared to the control. Fertilization rate was not affected by exposure to either Regina or Saskatoon effluent. From these results, it is clear that Regina effluent can impact the reproductive output of female fathead minnows, a response reported in other studies with fathead minnows exposed to effluent (Thorpe et al., 2009; Cavallin et al., 2016). The fact that fecundity was affected and fertility remained unchanged in the present study suggests that that the effluents, especially from Regina, are having a more targeted effect on

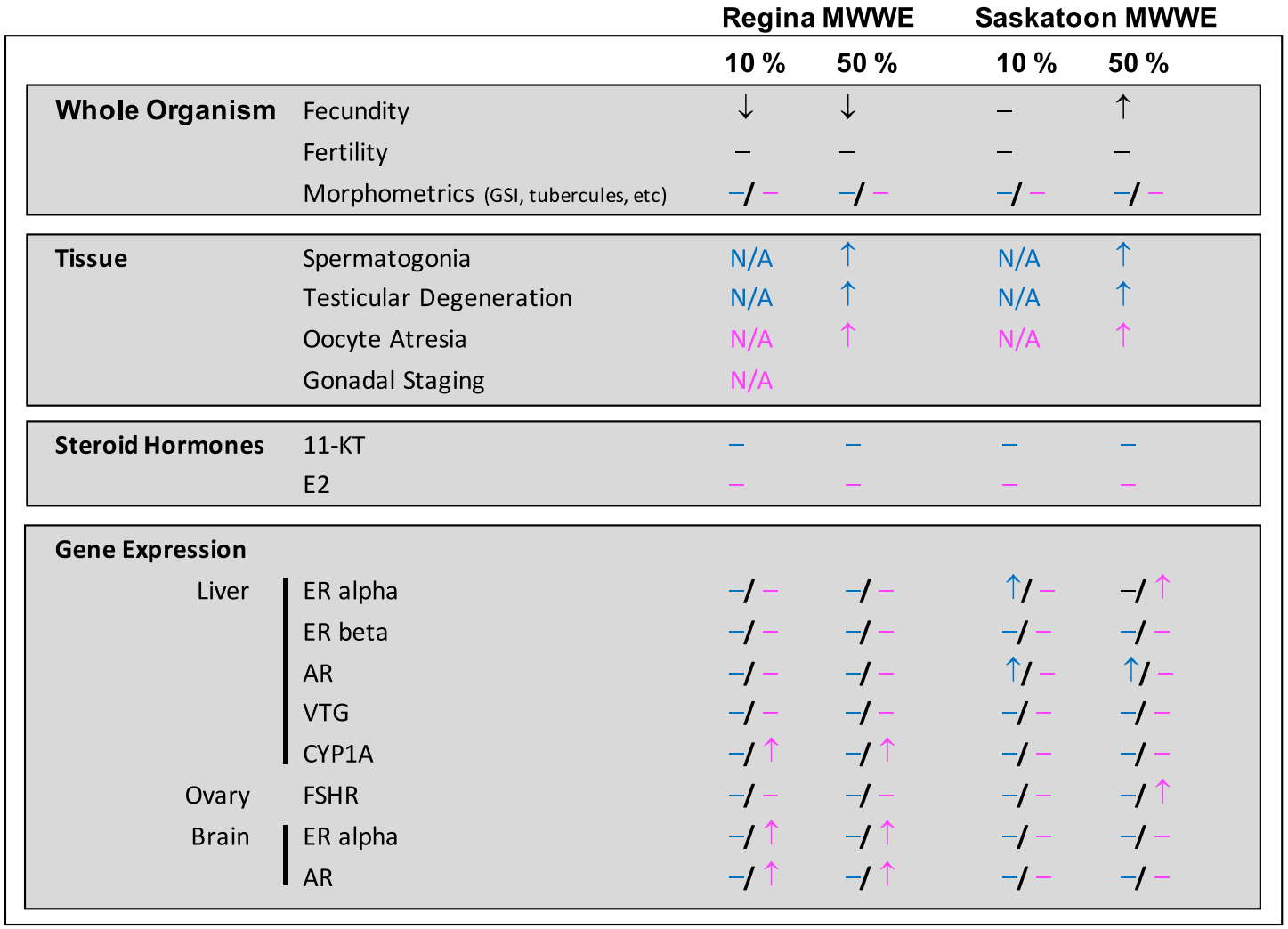

Figure 3.1. Summary of responses (whole organism, tissue, hormone, gene expression) for female (pink) and male fathead minnows (blue) following 21 d exposure to MWWE from Regina and Saskatoon WWTP. (↑) statistically significant increase, (↓) statistically significant decrease, (-) difference from control group is not statistically detectable.

female minnows. In a recent study, FHM exposed to MWWE with confirmed estrogenic activity exhibited similar effects on fecundity with no effect on fertility (Cavallin et al., 2016). Similar to fathead minnow fertility, there were also no significant changes observed in tubercle score in male fathead minnow. The lack of change in tubercule number in males and females suggests that while the effluent is causing reproductive impairment, it is not the result of contaminants in wastewater effluent possessing androgenic or estrogenic potential.

3.2.2 Impact of MWWEs on Gonads and Hormone Profiles

Fish exposed to effluent exhibited significant changes in gonadal morphology and development which may have implications for reproductive output. In female minnows, exposure to the 50 % Regina and Saskatoon effluent resulted in increased frequency of oocyte atresia, while ovarian stage remained consistent following effluent exposure. Changes in gonadal tissue structure or altered development of ovarian tissues may have long-term consequences for the individual (i.e. reproductive fitness). Several studies report altered gonadal development and sex steroid concentrations in wild fish exposed to wastewater effluent (Jobling et al., 2002, Hecker et al., 2002, Douxfils et al., 2007, Tetreault et al., 2011). Conversely, in a recent study, exposure to MWWEs with confirmed estrogenic potential did not result in altered ovarian development in fathead minnows, highlighting that estrogenicity will not always manifest histopathologically (Cavallin et al., 2016). It is possible that organ and tissue-level changes to the gonads in response to effluent exposure may be associated with poor water quality rather than individual chemicals in the effluent. Spencer et al. (2008) found that GSI of male and female slimy sculpin (Cottus cognatus) increased with a 21-day exposure to 1.76 ppm un-ionized ammonia (NH_3), a common contaminant in municipal and industrial effluents. In male fish, increased proportions of spermatogonia were observed in the 50 % effluent treatments for both Regina and Saskatoon effluents. Increased testicular degeneration (abnormal or degenerating male germ cells) was also observed in male minnows following exposure to 50 % effluent from both Regina and Saskatoon. It is interesting to note that while in females histopathological changes were accompanied by altered fecundity, changes to male gonadal tissue did not appear to affect fertility. While histopathological changes were observed in the gonads of fathead minnow following effluent exposures, there were no changes in plasma concentrations of both estradiol and 11-ketotestosterone. This further suggests that the higher level biological effects (i.e. fecundity,

histopathology) of effluent exposure in this study may not be driven by changes in plasma hormone levels.

3.2.3 Impact of MWWEs on Transcription of Genes along the HPGL

While exposure to Regina and Saskatoon effluents resulted in altered expression of a number of target genes along the HPGL, transcriptional responses did not reveal a clear pattern or mechanism by which the wastewater effluents are causing apical effects in fathead minnow, such as fecundity or gonadal alterations. Notably, there was no induction of VTG mRNA, a key indicator of exposure to estrogenic compounds (Barber et al., 2012). This is in contrast to previous studies that have shown VTG induction in fish exposed to MWWE or in wild fish sampled downstream of WWTPs (Barber et al., 2007, Barber et al., 2011, Harries et al., 1999, Ings et al., 2011). These results suggest that the effluents tested had low estrogenic activity that was insufficient to stimulate VTG mRNA levels. Exposure to Saskatoon effluent induced AR expression in male fish and ERα expression in female fish. However, no other endpoints (e.g. tubercle score) measured in the present study suggest any androgenic potential of the Saskatoon effluent. Induction of AR was also observed following exposures to anti-androgen, such as vinclozin, indicating the possible presence of anti-androgenic compounds in the effluent (Martinovic et al., 2008). In females, FHSR in the ovary was induced following exposure to 50 % Saskatoon effluent. In a study by Skolness et al. (2013), fathead minnows exposed to propiconazole (steroidogenesis inhibitor) demonstrated induction of gonadal transcripts, including FSHR; however, they also reported increases in CYP11a, StAR, and CYP17 mRNA, which did not occur in the present study. A significant induction of ERβ and AR mRNA was observed in brain tissue of females exposed to 50 % Regina effluent.

An attempt was made in this study to broaden the scope of the effects by adding an additional set of target genes in the liver related to general metabolism and oxidative stress. Among these additional targets, only a significant induction of CYP1a was observed in female fathead minnows following exposure to 10 % and 50 % Saskatoon effluent. CYP1a is an enzyme involved in xenobiotic transformation mediated by the aryl-hydrocarbon receptor (AHR) and is used as a biomarker for the presence of such compounds (Notch and Mayer, 2009). Induction of CYP1a was reported following exposure to effluents (Filby et al., 2007; Chen et al., 2016) and

increased expression with exposure to Saskatoon effluent could indicate the presence of AHR agonists.

3.3 Effective Endpoints for Assessment of Effluent Impacts in Fish

The findings from this study were used as part of a larger project known as the Aquatic Impact Assessment of Municipal Effluents (AIME). The overall goal of the AIME project was to use a tiered approach to establish effective endpoints to create a toolbox for assessing the impacts of MWWEs on aquatic organisms. Two additional parallel projects were conducted that examined potential impacts of the same effluents considered in this thesis research by (1) characterizing the specific endocrine activity of both effluents using *in vitro* assays (Bagatim et al., 2018) and (2) determining *in situ* effects of the Regina effluent by collecting wild fathead minnows upstream and downstream of the Regina WWTP during the summer of 2014 and 2015 (Hanson et al., 2018). Although not all of the findings from the three studies are in complete accordance with each other with regards to potential impacts of effluent exposure and underlying mechanism in fish, there are some supporting lines of evidence across studies that deserve mention.

Reduction in fecundity is one of the more consistent responses with regards to effluent exposure. Studies with pulp and paper mill effluents (Rickwood et al., 2006, Werner et al., 2010) and MWWEs (Thorpe et al., 2009) have reported reduced fecundity in fish at various effluent concentrations, while increased fecundity is reported in other studies (Parrott et al., 2010; Chen et al., 2016). Exposure to Regina effluent resulted in decreased fecundity measured in female fathead minnows, while no dose-dependent change was observed in fathead minnows following exposure to Saskatoon effluent. A parallel AIME study conducted by Bagatim et al. (2018) measured significant anti-androgen potency in both Regina (0.8 fold) and Saskatoon (0.6 fold) effluents, while Regina effluents showed weak but significant estrogenic potential (2.3 fold). Exposure to model estrogens, such as EE2 (Lange et al., 2001) and model anti-androgens such as flutamide and vinclozin (Jensen et al., 2004; Martinovic et al., 2008) reduced fecundity in fathead minnows, which suggests that decreased fecundity following effluent exposures may be driven by endocrine disruption. The contrasting changes in fecundity following effluent exposure (i.e. increase with Regina effluent, no change with Saskatoon effluent) could be attributed to lower

estrogenic potential in the Saskatoon effluent, and increased efficiency in removal of anti-androgens (56 %) compared to Regina (negative or zero).

In terms of the gonads, similar tissue-level changes (increased spermatogonia in males and increased oocyte atresia in females) were observed in fish exposed to Regina effluent in the laboratory (this thesis research) and collected downstream of the Regina WWTP (Hanson et al., 2018). Additionally, no change in plasma sex steroid concentration and no induction of VTG were reported in either study. While the wild-fish and laboratory studies present two different exposure scenarios, Hanson et al. (2018) hypothesized that the effects they observed were not likely due to estrogenic compounds in the effluent, a conclusion supported by the present study and also demonstrates the environmental relevance of conducting laboratory MWWE exposures. Conversely, Bagatim et al. (2018) observed an induction in *in vitro* E2 production with exposure to Regina effluent and an inhibition in E2 production with Saskatoon effluent using the H295R steroidogenesis assay. Although there was no evidence of estrogenicity based on whole organism responses (wild fish and laboratory exposures), apical effects (fecundity, gonadal development) observed may be the result of an estrogenic mechanism that is masked by anti-estrogenic or anti-androgenic compounds contained in the MWWEs.

Both lab-exposed and wild-caught fish exposed to Regina effluent demonstrated no evidence of androgenicity, such as masculinization of female fish (i.e. nuptial tubercles), but revealed significant anti-androgenic responses, such as delayed gonadal maturation (lab-exposed and wild-caught fish) and decreased nuptial tubercles (wild-caught fish only). These responses suggest the presence of AR antagonists in effluents that could impact normal reproductive functioning of male fish downstream of the Regina WWTP. According to *in vitro* assessment of endocrine activity, significant anti-androgenic potential was observed in influent and effluent samples collected during the spring from Regina and Saskatoon (Bagatim et al., 2018). In addition, the Saskatoon WWTP showed higher removal efficiency of antagonists (56 %) compared to Regina with negative or no removal, suggesting that the advanced Saskatoon treatment system was more efficient at removing AR antagonists from raw wastewater (Bagatim et al., 2018). There was a significant increase in AR expression in male fish exposed to Saskatoon effluent, similar to that reported in fathead minnows following exposure the anti-androgen flutamide (Martinovic et al., 2008). Based on the results of all three AIME studies,

Regina effluent appears to have greater anti-androgenic potential and thus, may pose a greater risk to fish inhabiting effluent-contaminated sites.

Wastewater effluents are complex mixtures with a multitude of chemicals and variable water qualities. Eutrophication of aquatic environments leads to hypoxic conditions and can be attributed to increased nutrient load downstream of wastewater treatment plants (Jarvie et al., 2006). Hypoxia can significantly impact the health of fish with examples such as decreased growth and fecundity in gulf killfish (Landry et al., 2007), and decreased spawning in fathead minnows (Corsi et al., 2011). Increased ammonia is often reported in MWWE and specifically the receiving environments downstream of Regina's WWTP (Servos et al., 2005; Guo et al., 2010; Waiser et al., 2011). Increased ammonia (0.17 and 0.34 mg/L) decreased fecundity in fathead minnow (Armstrong et al. 2012) and a 21-day exposure to 1.76 ppm un-ionized ammonia (NH_3) increased GSI of male and female slimy sculpin (Cottus cognatus) (Spencer et al. 2008). In the context of the AIME project, Bagatim et al. (2018) measured increased ammonia in both Saskatoon (4.5 – 42.6 mg/L) and Regina (1.2 – 41.2 mg/L) effluents. During our exposure, ammonia levels peaked to 0.6 mg/L before decreasing over the remainder of the 21-day exposure. Therefore, it is possible that ammonia, and not endocrine active compounds, in the effluents contributed to the decreased fecundity and altered gonadal development. In fact, in an overview of how to interpret the results of a reproductive bioassay, Ankley and Jensen (2014) state that both fecundity and gonad histopathology represent the two endpoints with the least diagnostic utility for assessing endocrine potential. Endpoints more indicative of endocrine disruption (VTG, tubercles, sex steroids) were evaluated in the present study and the wild fish study and these did not clearly identify androgenic or estrogenic actitivty in the effluent. Overall, the complexity of MWWEs highlights the importance the AIME project using multiple endpoints and a tiered diagnostic approach when assessing overall reproductive effects to aquatic wildlife.

3.4 Recommendations for Future Work

This thesis research features a comprehensive study examining the effects of two distinct MWWE at multiple physiological levels in a model fish species. However, there are some limitations of the study that could be overcome with additional research. One relatively conclusive finding was that while effluent exposure affected reproductive output and gonadal

development, the observed effects are likely not mediated by estrogenic or androgenic mechanisms. One advantage of fathead minnows in exposure studies is their well-understood breeding patterns. Changes in spawning behavior have been observed in fish with exposure to various chemicals such as anti-depressants (Weinberger and Klaper, 2014), anti-microbials like triclosan (Fritsch et al., 2013), and synthetic hormones such as 17α-ethinylestradiol (Salierno and Kane, 2009). Following a four-week exposure to the anti-depressant fluoxetine, Weinberger and Klaper (2014) reported decreased male behaviour such as nest building and nest protection while others, such as aggression, were increased. This increased male aggression resulted in female mortality and subsequently decreased fecundity at the highest concentration. The addition of a behavioural endpoint to MWWE studies would provide an additional method to assess the mechanism of the observed reproductive impacts in fish.

When sampling effluents, it is also important to consider that season to season variation affects the composition of MWWEs and can influence the observed effects in fish. For example, a short-term fish reproductive assay conducted in a laboratory setting was the method used in the present study and due to limited effluent storage, a single seasonal sampling time (spring) was selected. Previous studies have reported seasonal variation in chemical composition of effluents (Vienno et al., 2005, Yu et al., 2013). Bagatim et al. (2018) also observed seasonal variation in the endocrine potency of Regina and Saskatoon effluents across season using *in vitro* assays. Regina effluent sampled during the spring and summer months exhibited estrogenic potential, while effluent sampled in early and late winter resulted in no-change and a decrease, respectively. Similarly, there was a significant increase in androgenicity in early summer Regina effluent that was not observed in effluent collected during other sampling dates. For Saskatoon effluent, anti-androgenicity was only observed during the spring sampling period, while anti-estrogenicity was only observed in spring and summer. The seasonal differences in potency could be attributed to cold temperatures affecting treatment efficacy. Vieno et al. (2005) found increased pharmaceutical concentration in treated effluents collected in the winter compared to spring and summer. Additionally, warm weather evaporation could concentrate the compounds present in the effluent (Pessoa et al., 2014). Further work should incorporate exposures with effluents sampled across seasons to account for variation and provide a more comprehensive understanding of the risks present to aquatic organisms following exposure to MWWEs.

In the aquatic environment, fish can be exposed to contaminants at different periods during their development (embryo, larval, juvenile adult). In laboratory bioassays, the specific life stage chosen for the exposure will influence what data can be collected, as well as the severity of the observed effects. Fathead minnows have been used for a number of tests designed for different regulatory applications – these encompass simply evaluating lethality as an endpoint in 48/96-h exposures to life-cycle tests involving a battery of both apical and mechanistic/diagnostic endpoints (Ankley et Villeneuve, 2006). The 21-day short term reproductive bioassay assay used in this thesis research used sexually mature fathead minnows (4-6 months old). While mature fish are required to assess responses and endpoints relevant to reproductive effects (e.g. fecundity, tubercle score, gonadal development), conducting a life-cycle or transgenerational study using MWWE would provide information on the long-term effects of effluent, while also representing a more environmentally relevant exposure scenario. Life-cycle studies involving exposure to individual constituents in effluent such as EE2 (Parrott et al., 2005) and mixtures such as pulp and paper mill effluents (Parrott et al., 2004) have demonstrated lower response thresholds with longer exposure. Early development and active reproduction are both sensitive biological "windows" in terms of manifesting chemical or mixture toxicity and life-cycle studies encompass both of these windows. However, full life-cycle tests are both costly and time consuming. In an overview of fathead minnow usage in aquatic toxicology, Ankley and Villenueve (2006) suggested conducting a 21-day reproductive assay followed by a 30 day early life-stage assay with resulting embryos to maximize both cost and breath of measured enpoints, as was done in a study with fathead minnows and perfluoroocatanesulfnate (Ankley et al, 2005). From an environmental perspective, it is also important to understand the effects of effluent on multiple generations. Schwindt et al. (2014) examined transgenerational effects in fathead minnows with a year-long exposure to EE2 and reported decreased survival and reproduction in the F0 and F1 generation (both continuously exposed and removed from EE2), and decreased survival of F2 embryos (exposed only as germ cells in parents). With regards to wastewater effluents. Sowers et al. (2009) found an increased expression of secondary sex characteristics in male fathead minnows of the F1 generation and a positive relationship between parental exposure to wastewater and advanced onset of reproductive activity. Future studies should involve life-cycle exposure or consider multi-generational studies, as they would include environmentally

realistic exposures periods and therefore provide a more relevant assessment of the potential effects of MWWE on fish populations.

3.5 Conclusion

This thesis research was conducted to examine the potential reproductive effects of South Saskatchewan MWWE on fathead minnows. The results demonstrated that short-term exposure to effluents from both Regina and Saskatoon can affect reproductive output in fathead minnows, although in separate ways, with Regina effluent impairing fecundity while Saskatoon effluent increasing egg production at the highest effluent concentration. This suggests that effluents from these WWTP pose a risk to populations of fish occupying the receiving environments. Regina and Saskatoon effluent exposure also impacted normal gonadal development in both male and female fathead minnows. One of the principal hypotheses of this project was that any reproductive effects arising from effluent exposure would be largely mediated by changes in gonadal tissue, plasma hormone level, and/or transcript expression in key tissues. Although changes in reproductive output and gonadal tissue morphology were observed, lack of change to endpoints more definitively tied to estrogenic or androgenic potential of the effluents (e.g. tubercles, sex steroids, VTG) suggests that the observed adverse effects were the result of other chemical/physical properties of the effluent. Overall, this research highlights the complex nature of effluents and the challenges in determining the precise mechanisms underlying impacts in the exposed organisms.

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APPENDIX

Mortalities of fathead minnow following 96-hour exposure to three concentrations of effluent (50%, 75%, 100%) from Regina and Saskatoon municipal wastewater treatment plants (MWTP).

	50%		**75%**		**100%**	
	Total Fish	Mortalities	Total Fish	Mortalities	Total Fish	Mortalities
Regina	6	0	6	2	6	6
Saskatoon	6	0	6	0	6	0