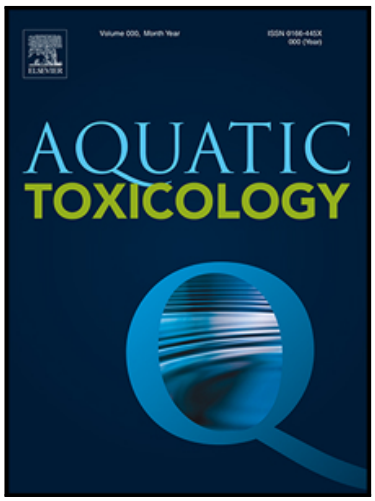

Health status of fathead minnow (Pimephales promelas) populations in a municipal wastewater effluent-dominated stream in the Canadian prairies, Wascana Creek, Saskatchewan

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Health status of fathead minnow (*Pimephales promelas*) populations in a municipal wastewater effluent-dominated stream in the Canadian prairies, Wascana Creek, Saskatchewan

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Highlights

- There was general deterioration of the health and reproductive status of fish collected downstream of the effluent fallout compared to upstream populations
- Expression of the majority of transcripts measured in fathead minnows downstream of the effluent fallout was significantly downregulated, which supports observations of general health deterioration
- Exposure was characterized by complex mixtures of contaminants including several pharmaceuticals and personal care products as well as ammonia and nutrients
- No significant exposure to estrogenic compounds occurred, which was in accordance with a lack of estrogenic effects in exposed fish
- Fish in small, semi-arid stream ecosystems are at elevated risk from exposure to contaminants released from municipal wastewater effluents

Abstract

Their unique hydrological and climatic conditions render surface water systems in the southern Canadian Prairies at an elevated risk from exposure to contaminants released from municipal wastewater effluents (MWWEs). The aim of this study was to characterize the potential health effects

and their underlying molecular mechanisms in populations of fathead minnow (*Pimephales promelas*; FHM) in Wascana Creek, an effluent dominated stream in Southern Saskatchewan, Canada. Studies were conducted during the spawning season in 2014 and 2015 to assess responses in terms of overall health, reproductive functions, plasma sex steroid hormone levels, and expression of selected genes along the hypothalamus-pituitary-gonadal axis. FHM downstream of the effluent fallout had lower gonadosomatic indices and significantly greater hepatosomatic indices compared to upstream populations. In both male and female FHMs, significantly greater occurrence and severity of gonadal degradation and delayed maturation were observed in downstream fish compared to upstream fish. Downstream males also displayed lower scores of secondary sexual characteristics and a decreasing trend in plasma 11-ketotestosterone levels. Interestingly, no indications of exposure to estrogenic compounds, such as occurrence of testicular oocytes were observed, which was in accordance with the lack of presence of key biomarkers of estrogenic exposure, such as induction of vitellogenin. In general, expression of the majority of transcripts measured in FHMs downstream of the effluent fallout was significantly downregulated, which supports observations of the general deterioration of the health and reproductive status of these fish. Chemical analysis indicated that 10 pharmaceuticals and personal care products (PPCPs) were present at the downstream site, some at sufficiently great concentrations that may present a risk to aquatic organisms. With continuous exposure to a diverse number of stressors including high nutrient and ammonia levels, the presence of a variety of PPCPs and other contaminants, Wascana Creek should be considered as an ecosystem at risk.

Keywords

Endocrine disruption; emerging contaminants; fish; in situ; morphometric; histology; municipal effluent

Introduction

Concern has been raised surrounding the occurrence of emerging contaminants (ECs) in our water resources and their potential effects on resident organisms. ECs are a diverse class of compounds including, but not limited to, pesticides, plasticizers, nanoparticles, flame retardants, personal care products, and pharmaceuticals (PPCPs), as well as hormones and chemicals released from livestock operations (Folmar et al. 1996; Hoffman and Oris 2006**)**. They enter aquatic environments through many routes such as agricultural runoff, industrial effluents, and municipal wastewater effluents (MWWE), with the latter representing one of the most prominent sources of ECs (CCME 2006). Although concentrations of ECs are generally low in treated sewage effluents, environmental risks are still present due to the pseudo-persistence of these chemicals that is a result of their continuous influx (Nilsen et al. 2019). This is of particular concern in semi-arid environments, like the Canadian prairies, where receiving water bodies are often characterized by low flow and minimal dilution of effluents (Waiser et al. 2011a).

Many ECs present in MWWEs have been identified as endocrine disrupting compounds (EDCs) that affect growth, development, and reproduction of aquatic organisms, particularly fish, through disruption of endocrine homeostasis, sometimes at very low concentrations (Goksøyr 2006; Matthiessen et al.

2018). One of the most frequently reported endocrine disrupting effects in male fishes downstream of WWTPs is the presence of female germ cells in testicular tissue (Kidd et al. 2007; Tyler and Jobling 2008). Together with other impairments such as delayed gonadal maturation and development as well as impacts on concentrations of sex steroids, this intersex condition has been observed globally in wild fish exposed to MWWEs (Hecker et al. 2002; Tyler and Jobling 2008). For example, a 7-year whole-lake study showed that chronic exposure of fathead minnows (*Pimephales promelas*; FHM) to low environmentally relevant concentrations (5 ng/L) of 17α-ethinylestradiol (EE2) led to feminization of males, altered oogenesis in females and population changes with a near extinction of the species from the experimental lake (Kidd et al. 2007). In addition to environmental estrogens, exposure to EDCs with anti-estrogenic, (anti)-androgenic and steroidogenesis-disrupting properties needs to be considered for their potential to contribute to disruption of the reproductive system. Varying responses have been observed for several compounds often found in MWWEs. For example, decreases in plasma vitellogenin as well as fecundity and fertility were observed in female Japanese medaka (*Oryzias latipes*) exposed to the anti-estrogen, tamoxifen (Sun et al. 2007). Furthermore, reduced testosterone concentrations occurred in male FHMs exposed to the herbicide linuron and the plasticizer DEHP (anti-androgens; Crago and Klaper 2012), and reduced fecundity and formation of secondary sexual characteristics were reported in female FHMs exposed to 17β-trenbolone (androgen; Ankley et al. 2003). In addition, exposure of fish to EDCs has been shown to alter organosomatic indices or condition (Diniz et al. 2005; Lavado et al. 2003).

While the physiological responses listed above are commonly assessed endpoints in fish exposed to MMWEs, responses can vary due to different interactions of the diversity of chemicals in these effluents with a variety of molecular targets, causing disruption of downstream processes. Therefore, it is important to characterize specific molecular mechanisms driving these biological alterations, provided that molecular effects are indicative of apical outcomes. In the literature, focus has been on EDCs that mimic hormones resulting in agonistic effects on cellular processes, or alternatively that act antagonistically by blocking hormone receptor binding sites, thereby inhibiting the transcriptional activation triggered through these receptors (Arukwe, 2001). The activity of these receptors, such as estrogen receptor (ER) and androgen receptor (AR), can be altered if changes occur in the abundances of mRNA or protein of these receptors and/or co-regulators (Tabb and Blumberg, 2006). Due to the regulation of VTG, an egg-yolk precursor protein, by the ER, its induction is indicative of estrogenic stimulation, making it an excellent biomarker of exposure to estrogenic chemicals. As discussed above, EDCs can also elicit effects through modulation of sex steroid hormone production, or steroidogenesis (Craig et al., 2011). Steroidogenesis is a biosynthesis pathway involving a series of enzyme mediated steps converting cholesterol into sex steroid hormones such as estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT), the most active reproductive androgen specific to fish, and is controlled by the hypothalamus-pituitary-gonadal-liver (HPGL) axis (Hogan et al., 2010). This pathway is tightly regulated by feedback loops that up- and down-regulate development, growth, and reproductive processes. Disruption of steroidogenesis can alter the transcription, expression, or activity of enzymes and target genes involved in mediating homeostasis of sex steroid concentrations, which ultimately leads to disruption of the reproductive endocrine system (Ankley and Johnson, 2004). Changes in sex steroid hormones have been shown to impact other key systems involved in immune

function, brain development, and behaviour (Arcand and Benson, 1997). Therefore, disruption of the activity and expression of genes along the HPGL axis and/or circulating levels of these hormones is likely to impact sexual development, reproduction, growth, and overall health status.

Even though potential risks of MWWEs on aquatic systems have been identified, little is known regarding their potential impacts to surface water systems in the Canadian prairies (Waiser et al. 2011a,b; Tetrault et al. 2012). In this study, Wascana Creek, SK, Canada, was chosen as an aquatic ecosystem that is representative of small semi-arid and cold prairie environments, as it is an effluent dominated prairie stream that received MWWEs from the City of Regina's outdated lagoon-based treatment facility. The treatment plant discharged effluent into Wascana Creek at a rate of 0.90m^3/s, and in winter, treated sewage effluent can make up to 99% of the creek flow until it connects with the Qu'Appelle River, 60km downstream (Waiser et al. 2011b). Previous studies of Wascana Creek have shown the presence of 25 PPCPs and their metabolites at concentrations in the ng/L to μg/L range downstream of the effluent fallout, as well as excessive nutrients being released within the effluents, causing it to be a nitrogen hypersaturated system (total dissolved N > 3 mg/L; Waiser et al. 2011b). Additional studies observed delayed spawning and altered gonadal development in FHMs downstream of the City of Regina's effluent fallout (Tetreault et al. 2012).

The overarching aim of the present study was to investigate the overall health and reproductive status as well as the potential molecular mechanisms of any disruptions in populations of FHMs that inhabit Wascana Creek, SK, Canada, eight years after an initial study by Tetrault et al. (2012) that reported significant impacts on reproductive fitness of resident fishes in the creek. Although there were upgrades to the Regina WWTP since the study by Tetrault et al. (2012), there were significant remaining concerns regarding the health of resident aquatic wildlife due to the significant proportion of effluent that comprises the downstream flow. Specifically, the present study assessed 1) overall health of FHM populations in Wascana Creek, SK, up- and downstream of the City of Regina's WWTP outfall; 2) alterations of gonad histopathology in both males and females; 3) changes in plasma sex steroid hormone levels; 4) alterations in abundances of key genes along the hypothalamus-gonad-liver axis; 5) stable isotope concentrations in fish to determine if movement between upstream and downstream populations was occurring; 6) chemical analysis of in stream samples to determine concentrations of EC's; and 7) whether any observed effects could be attributed to the ECs present.

Materials and Methods

Site Location and Field Collections

FHMs were collected in 2014 and 2015 during spawning season (late July) from two locations in Wascana Creek, SK, Canada; a reference site upstream of the city (RUS; 50.40, -104.49), and directly downstream of the effluent fallout (RDS; 50.48, -104.75) (Figure 1). Seine nets were pulled from shore to shore and approximately 30 FHMs from each site were collected, removed, and transported in aerated pails to an on-site mobile laboratory (numbers of males and females collected during each season are provided in Table S1). Temperature, dissolved oxygen, and pH of surface water was measured during each collection event at both sites, by use of a YSI Professional Plus meter with YSI

Quatro ISE-ISEDO-COND-T probes (YSI, Yellow Springs, OH, USA). Ammonia nitrogen (mg/L), unionized ammonia at 15±1°C (mg/L), total phosphorus (mg/L) and total nitrogen (mg/L) in final effluents were measured and provided by the City of Regina as part of their effluent monitoring program.

Tissue Sampling and Morphometrics

Fish were anesthetized in Aquacalm (5-10 mg/L; Syndel Laboratories, Nanaimo, BC, Canada). Mass (to the nearest 0.01 g) and fork length (to the nearest 0.1 cm) were determined, and condition factor (K, Eq. 1) was calculated. When possible, blood was collected from the caudal vein, plasma was separated via centrifugation (9,000 x g for 5 min), and stored at -80 °C until hormone analysis. Next, fish were euthanized by spinal severance and liver, gonads, and brain tissues were excised and weighed. Livers, brains, spleens, and half of each gonad were flash frozen in liquid nitrogen and stored at -80 °C until analysis. If sufficient tissue was available, the second half of each gonad was fixed in 10% buffered formalin for histopathological analysis. Using the carcass, secondary sexual characteristics were scored based on the U.S. EPA Fish *Short-Term Reproduction Assay Test Protocol – Fathead Minnow*, and pictures were taken of each individual (USEPA 2009). Livers and gonads were weighed and hepatosomatic index (HSI, Eq. 2) and gonadosomatic index (GSI, Eq. 3), respectively, were calculated.

$$K = (\text{body weight}/\text{length}^3)*100 \quad (1)$$

$$HSI = (\text{liver weight}/\text{total body weight})*100 \quad (2)$$

$$GSI = (\text{gonad weight}/\text{total body weight})*100 \quad (3)$$

Details on the number of samples that were subjected to the different analyses described below are given in Table S1.

Histological Analysis

Gonads were fixed in 10% buffered formalin for 24 hours, and then transferred to and stored in 70% ethanol at room temperature. Tissues were processed following standard histological methods (Supplemental Materials, S1) using an automatic tissue processor (Leica Microsystems Inc., Concord, ON, Canada) and embedded in paraffin wax. Processing was conducted by Prairie Diagnostic Services, University of Saskatchewan (Saskatoon, SK, Canada). Paraffin blocks were serially sectioned at 4 and 6 µm for testes and ovaries, respectively, on a Microm HM model 310 microtome (GMI, Ramsey, MN, USA). Slides were stained with hematoxylin-2 and eosin-y (H & E) following standard histological methods (Supplemental Materials, Section A) as outlined in the Fish Short-Term Reproduction Assay (US EPA 2011).

Six and seven males, and ten and five females from RUS and RDS, respectively, were randomly selected for analysis (lower number of males was due to limited number of mature males collected at each site). From each of these fish, three slides with five sections each were analyzed using a Zeiss Axiostar Plus light microscope 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). Primary criteria were scored for both sexes, using either a severity or staging index (see Supplemental Materials S2 and Table S2) in accordance with the *Diagnosis of Endocrine-Related*

Histopathology of Fish Gonads (USEPA 2009). Unfortunately, due to the low number of males collected in 2015 at RDS, there were insufficient tissues available for histological analysis of males during that year, so only 2014 data are presented.

Plasma Hormone Analysis

E2 and 11KT were quantified using enzyme-linked immunosorbent assays (ELISA) purchased from Cayman Chemical (Ann Arbor, MI, USA). As plasma volume was limited, only E2 was measured for females and 11-KT for males. Extractions were done using a liquid-liquid extraction method in accordance with Chang et al. (2009) with minor alterations detailed in Beitel et al. (2014) (Supplemental Materials S3). Quantification of steroid hormones was conducted in triplicate for each sample following protocols provided by the manufacturer (Cayman Chemical). Steroid hormone concentrations were expressed as ng per mL (ng/mL).

Gene Expression Analysis

Total RNA was extracted from approximately 20 mg of tissue using the RNeasy Mini Kit as described by the manufacturer (Qiagen, Missassauga, ON, Canada). Concentrations of RNA were determined using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE, USA). Reverse transcription was performed using 1 µg of RNA from each sample using a Quanti-tect Reverse Transcription Kit (Qiagen) according to the manufacturer's directions to obtain cDNA. Quantitative real-time PCR (qPCR) was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 50 µL master mix consisting of 2.5 µL of cDNA, 2.5 µL of sense/antisense gene-specific primer, 25 µL 2x QuantiFast SYBR Green Master Mix (Qiagen), and 20 µL of RNase-free water (Qiagen) was prepared for each sample of cDNA and primer combination. All reactions were all run in duplicate. The reaction mixture for PCR was denatured at 95 °C for 10 min followed by a thermal cycle profile consisting of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. After amplification, melt curves were generated to ensure amplification of a single product by increasing 0.5 °C every 5 s from 65 °C to 95 °C. Transcript abundances of target genes were quantified by normalizing to 18s according to the method of Simon (2003) and correcting for differences in PCR efficiency (Table S3). Fourteen genes involved in reproduction pathways and processes along the hypothalamus-pituitary-gonadal-liver (HPGL) axis were selected. Genes and nucleotide primer sequences were ordered from Invitrogen (Burlington, ON, Canada; Table S3).

Stable Isotope Analysis

Spleen samples were analysed for stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) by isotope ratio mass spectrometry and expressed as δ values (i.e., $\delta^{13}C$ and $\delta^{15}N$). EM Protein 1 and atmospheric nitrogen ratios (heavy to light) were used to derive standards for $\delta^{13}C$ and $\delta^{15}N$, respectively. Samples were dried, weighed, homogenized using a mortar and pestle, and analyzed using a Costech ECS4010 elemental analyzer coupled to a Thermo Scientific Delta V mass spectrometer. Analysis was conducted by Miles Stocki Stable Isotope Facilities at the University of Saskatchewan.

Chemical Analysis

Water samples were collected at the same time fish sampling took place in 2014. For each sample, water was collected into two 1 L amber bottles (total volume 2 L) with two drops of chloroform added for preservation purposes and stored in the dark at 4°C until analysis. Prior to analysis, samples were filtered (0.45 micron) and transferred to a new clean bottle. Solid phase extraction was conducted using 2 L of sample and air dried following the addition of 5 mL of each MiliQ water and 0.1% acetic acid. Cartridges were extracted using 5mL of 1:1 Hexane:DCM, dried using nitrogen. Samples were reconstituted by adding 400 µL of isooctane for each 2 L sample.

Extracts were analyzed in house using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON). Separation of chemicals was achieved with a Betasil C18 column (5 µm; 2.1 mm × 100 mm; Thermo Fisher Scientific, Toronto, ON) with an injection volume of 5 µl. Details of analytical procedures are provided in the Supplemental Materials (S3).

Statistical Analysis

Statistical evaluation of the data was conducted using SPSS Version 20 (IBM Corp., Armonk, NY, USA). Male and female fish were analyzed separately. Normality of the data was tested using Shapiro-Wilk test and homogeneity of variance was tested using Levene's test. Any data that did not meet the assumptions of a parametric test were logarithmically transformed and re-analyzed for use in parametric tests. Normally distributed data was analyzed using analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine if there was significant variation among and within the sampled sites for all parameters investigated. Where no normalizing transformation could be found, the Kruskal-Wallis test followed by the Mann Whitney U post-hoc test was applied. All statistical tests were performed using an alpha value of 0.05.

Results

Water Quality

Surface water quality parameters measured at both sites were comparable between 2014 and 2015, with slightly higher temperatures and lower DO and pH values at the downstream site during both seasons. The highest recorded temperature during the sampling season was in 2014 at the downstream site (25.4°C) and the lowest in 2014 at the upstream site (23.8°C). Dissolved oxygen ranged between 11.1 and 12.1 mg/L at the upstream site, and 10.4 and 10.8 mg/L at the downstream site in 2014 and 2015, respectively. pH varied slightly between sites, ranging between 8.45 and 8.49 at the upstream and 8.23 and 8.31 at the downstream site. Unionized ammonia concentrations in the effluents discharged into the creek averaged 0.241 and 0.102 mg/L for July 2014 and 2015, respectively, with a maximum concentration of 0.511 mg/L detected in 2014 (Table 1). Total phosphorus concentrations in the effluents released ranged from 0.46 to 1.99 and 0.47 to 1.08 mg/L in July 2014 and 2015 respectively (Table 1). Average concentrations of nitrogen in final effluents were lower in July 2014 (27.1 mg/L) compared to 2015 (38.5 mg/L) (Table 1). Ammonia, phosphorous, and nitrogen concentrations are

based on measurements made over n = 25 and 30 days during the 2014 and 2015 sampling seasons, respectively.

General Fish Health

The overall health status of fish collected downstream of the Regina WWTP effluent fallout differed significantly from those collected at RUS (Table 2). There were no differences with respect to length, weight, and K (p=0.167) of female FHMs between sites in 2014, while females collected in 2015 at RDS were significantly longer and heavier (p<0.001) and had lower K (p<0.001) compared to females collected at RUS. In contrast, RDS males were shorter and lighter, with significantly greater K (p<0.01) when compared to upstream males in 2014 but no difference between sites were found for any of these endpoints in 2015 (p=0.117) (Table 2). During both sampling seasons, female and male fish from RDS had significantly elevated HSIs (p<0.001), and, with the exception of males in 2015, significantly lower GSIs when compared to RUS (Table 2).

In both 2014 and 2015, males collected at RDS had significantly lower average nuptial tubercle scores compared to males collected at the upstream site (2014: p<0.05; 2015: p<0.01; Figure S1; Table S4). The presence of nuptial tubercles on female FHMs was not observed at any site during either year.

Gonad Histopathology

There were significant differences in histological phenotypes of FHMs sampled from RDS compared to RUS (Figures 2 and 3). In 2014, males sampled downstream of the effluent fallout displayed a significant increase in the proportion of earlier cell type spermatogonia and fewer mature spermatozoa when compared to males collected upstream with a minimal severity grade (0.83 ± 0.37) upstream and a moderate to severe severity grade (3.57 ± 0.16) downstream (p<0.001). There was no incidence of testicular oocytes in any of the testis samples analyzed in either year. Testicular degeneration, characterized primarily by apoptotic germ cell formation, was significantly increased in males at RDS compared to RUS (p<0.001). RDS males also had significantly greater incidences of interstitial cell (Leydig) hypertrophy/hyperplasia compared to males collected upstream (p<0.01).

Histopathological analysis of ovaries in both 2014 and 2015 did not demonstrate any differences between the upstream and downstream sites for perifollicular cell hyperplasia/ hypertrophy or decreased yolk formation (Figures 2 and 3). However, in both years, females at RDS did display significant increases in oocyte atresia compared to RUS (2014: p<0.001, 2015: p<0.01). Also, females collected from RDS were at a significantly lower gonadal maturation stage (stage 1) compared to specimens from RUS (stage 2.5) in both 2014 (p<0.05) and 2015 (p<0.01).

Plasma Sex Steroid Concentrations

E2 concentrations in blood plasma of females were not significantly different between RUS and RDS for either year (2014: p=0.475; 2015: p=0.392) despite a trend of increasing concentrations in fish downstream of the effluent fallout (Figure 4). There was a decreasing trend of 11-KT concentrations in males at RDS in both 2014 and 2015; however, no significant differences were found (2014: p=0.190; 2015: p=0.173).

Gene Expression

Exposure to MWWEs at RDS significantly affected the transcript abundance of target genes expressed in all tissues of female FHMs. In brains, the levels of ERα, ERβ, and CYP19β at RDS were 0.42-, 0.07-, and 0.20-fold less abundant, respectively, than in in females at RUS in 2014 ($p<0.05$; Figure 5A). Similarly, in 2015, abundances of ERα and CYP19β were significantly lower by 0.50 and 0.42-fold, respectively, in females at RDS compared to RUS. No effects were seen on expression of AR in brain tissue of fish from downstream compared to upstream fish in either 2014 or 2015. In gonads, the abundance of StAR was significantly greater by 4.34- and 2.58-fold in 2014 and 2015, respectively, in females downstream compared to those upstream (Figure 5B). In 2014, the abundance of LHR was approximately 4 times greater in females at RDS relative to upstream fish. In 2015, no change was observed in LHR, but abundance of FSHR was significantly increased by 4.43-fold in females collected from the downstream site relative to RUS. Abundances of transcripts 17βHSD and CYP19α were not different in the gonads of female fish at RDS. Female fish from RDS in 2015 had lower abundances of transcripts of ERα and ERβ in liver by 0.06- and 0.40-fold, respectively, while there was no difference in abundance of ER transcripts in females collected during 2014 (Figure 5C). There were no differences in AR transcript abundance between sampling sites in liver in 2014; however, abundance of AR was 0.34-fold less in 2015 in females at RDS ($p<0.05$). In 2014, abundance of VTG transcripts in liver was not different between fish collected from RDS and RUS. Conversely, abundance of VTG in 2015 was significantly reduced by 0.01-fold in livers of females at RDS.

Male FHMs collected at RDS also displayed differences in the abundances of transcripts of target genes expressed in all tissues. In brain, the abundances of ERβ and CYP19β were down-regulated by 0.17- and 0.10-fold, respectively, in males at RDS compared to males collected at RUS in 2014 (Figure 6A). In 2015, expression of both estrogen receptors, ERα and ERβ, were 0.35- and 0.45-fold less, respectively, in downstream fish compared to RUS. No changes in the abundance of transcripts of AR were observed in either year. In the gonads, exposure to effluents at RDS significantly increased abundances of 17βHSD transcripts by 3.89- and 9.18-fold in 2014 and 2015, respectively, compared to RUSs, while no changes occurred in transcript abundance of CYP19α (Figure 6B). Furthermore, in 2014, a significant increase in abundance of transcripts of LHR was observed; however, abundances were not different for StAR or FSHR at RDS. In 2015, abundances of StAR and LHR were significantly less by 0.01- and 0.0002-fold, respectively, but abundance of FSHR was significantly greater by 4.43-fold in male gonad tissue at RDS compared to RUS. Finally, in liver, abundances of transcripts ERα and ERβ were significantly less in 2014 by 0.55- and 0.45-fold, respectively, and no change was seen in AR (Figure 6C). Significantly lowered abundances of target genes in the liver were also found in 2015, with greatly reduced transcript levels of ERα and AR, but no differences measured in ERβ in males at RDS. The expression of VTG in male livers was not different in 2014; however, in 2015, VTG levels were reduced by 0.46-fold in males from RDS.

Stable Isotopes

Stable nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotope data obtained from spleens of FHMs differed significantly between sites and years (Figure S2). In 2014, nitrogen isotope analysis of the spleen tissues showed a significant enrichment at RUS compared to RDS ($p\leq0.0005$). The opposite trend was seen in

2015 with the upstream site having a significantly lower signature compared to downstream ($p<0.01$). Significant differences in enrichment between years were determined at RUS ($p<0.001$), but not at RDS ($p=0.481$).

Carbon isotope analysis for 2014 showed a significant enrichment at RUS compared to RDS ($p\leq0.05$). In 2015, the opposite trend was observed with the upstream site having a significantly greater ^{13}C signature compared to downstream ($p\leq0.05$). Between years, there were significant differences at RUS ($p\leq0.001$), but not at RDS ($p=0.110$).

Chemical Analysis

All chemicals detected were at higher concentrations at RDS compared to RUS with the exception of clofibrate (Table 3). A wide variety of antibiotics, prescription and non-prescription drugs, as well as other wastewater related compounds were detected downstream of the effluent fallout. DEET, an insecticide, gemfibrozil, an antihyperlipidemic, and triclosan, an antimicrobial disinfectant, were detected at maximum concentrations of 172, 46.2, and 33.2 ng/L, respectively. Reproductive hormones, E2, estrone (E1), progesterone, and T were all below their detection limits at both sites. Similarly, the synthetic ovulation inhibitor EE2 was not-detected upstream or downstream of the effluent fallout.

Discussion

The current study demonstrated that the health status of native population of FHMs downstream of the effluent fallout (RDS) in Wascana Creek, SK, Canada was significantly impacted. Effluents from WWTPs are comprised of complex mixtures whose composition may vary both seasonally and temporally as demonstrated in this and other studies (Sui et al. 2011). Interestingly, while a variety of PPCPs and other contaminants were identified downstream of the effluent outfall, estrogenic chemicals that have been frequently reported by other studies downstream of municipal effluent fallouts were not detected. This observation was reflected by the lack of specific estrogenic responses in any of the individuals analyzed. In fact, the general deterioration in health was confirmed by the predominantly inhibitory effects on the expression of genes along the HPGL axis.

Analysis of stable isotopes confirmed site fidelity of fish at RDS for both 2014 and 2015, with no differences in $\delta^{15}N$ and carbon $\delta^{13}C$ signatures. The increased $\delta^{15}N$ values in tissues of RDS fish downstream of the effluent fallout in 2015 were similar to previous studies reporting that increased human inputs of nitrogen led to increasing nitrogen content values in aquatic organisms (Dube et al. 2005; Loomer et al. 2014). Interestingly, a shift in $\delta^{15}N$ and carbon $\delta^{13}C$ signatures was observed at RUS, suggesting differences in either site fidelity or contributions of nutrients between years. Regardless, the similarities between sampling years at RDS may support that MWWEs dominate this portion of the stream, and thus, other factors, such as fertilizer runoff, might not influence as much as at sites that lack the effluent input.

Site-specific responses of fish with regard to K varied year to year. The minimal changes in K observed in this study are similar to findings in which no changes, or similar energy allocations were

observed in wild roach (*Rutilus rutilus*) (Tyler and Jobling 2008) and flounder (*Platichthys flesus*; Allen et al. 2009) exposed to MWWE compared to fish from reference sites. In contrast, increases have been observed in several other fish species, including longnose suckers (*Catostomus catostomus)* and longear sunfish (*L. megalotis*) likely due to increases in nutrients released within the treated effluents (McMaster et al. 2005; Porter and Janz 2003). This hypothesis may explain the relatively high K observed in male FHMs at RDS in 2014; however, it does not explain the differences between sexes. Alternatively, decreases in condition observed in male feral carp (*Cyprinus carpio*) exposed to EE2, were hypothesized to be due to high estrogenic exposure having a significant impact on the physiological state of the fish (Solé et al. 2002). However, based on the absence of estrogenic compounds in our analytical findings as well as the lack of responses that are typical for the exposure of male fish to estrogens, for example greater mRNA abundance of VTG, this was unlikely to be the reason for the observed decrease in K.

HSI provides an indication of energy status, and therefore, an increase in liver size as observed in RDS fish could be a sign of increased metabolic activity due to contaminant exposure and overall increase in energy storage. Hypertrophy of the liver due to increased metabolic activity has been reported as a hypothesis for increases in liver size with exposure to MWWEs (Porter and Janz 2003). Previous studies have also shown that higher HSI, indicating increased energy stores, may be due to the elevation of nutrients associated with MWWEs resulting in changes in lipid or glycogen storage and food supply (Porter and Janz 2003; McMaster et al. 2005; Tetreault et al. 2011). Additionally, fish livers, specifically in females, are highly responsive to estrogens and contain several forms of estrogen receptors allowing them to produce great amounts of VTG when stimulated (Tyler and Jobling 2008). In this study, however, the increase in liver sizes of fish collected downstream of the effluent fallout was unlikely due to greater expression of VTG as some studies have reported, because of the consistent absence of estrogenic effects as well as the lack of detection of estrogenic compounds in the surface water. The increases in HSI observed here may instead have been influenced by the elevated concentrations of nutrients such as nitrogen (N) and phosphorus (P) downstream of the effluent fallout in July of 2014 and 2015, which were in accordance with an earlier study by Waiser et al. (2011). However, changes in lipid or glycogen storage and food supply were not assessed in this study, and therefore, further investigation of liver enzymatic activity and energy stores would help to determine the cause of the greater HSI.

GSI provides an indication of the sexual maturity of fishes and has been widely used as a reliable indicator of exposure to compounds causing deleterious effects on reproduction, specifically EDCs. Reductions in GSI of fish exposed to MWWEs, as observed in our study, has been widely reported in a variety of species with effects related to inhibition of maturation (*Abramis brama*: Hecker et al. 2002; *Catostomus commersonii*: Vajda et al. 2008; *Etheostoma blennioides*: Tetreault et al. 2011), metabolic disruption (several freshwater species: Munkittrick et al. 2002), and decreased hypothalamic, pituitary, or gonadal activity (*Pimephales promelas*: Hachfi et al. 2012). Alternatively, increases in GSI of fish exposed to industrial and municipal effluents have also been reported and associated with increased nutrients or food supply (McMaster et al., 2005; Munkittrick et al., 2002) or reabsorption of gonadal material due to delayed spawning (Tetreault et al., 2011). While Hachfi et al. (2012) stated that GSI values typically are reduced after exposure to estrogenic or antiandrogenic contaminants, in this study,

it is likely the effects observed are due to a number of different stressors leading to delayed or inhibited maturation of gonads. These potentially include exposure to high concentrations of nutrients, a complex mixture of ECs in the effluents, and most importantly, high concentrations of ammonia in the effluents released into the system during the sampling period. While not necessarily endocrine specific, ammonia at concentrations as low as 0.06 mg/L have been shown to negatively impact gonadal development (Armstrong et al. 2012), which is significantly lower than the average concentrations of 0.241 and 0.102 mg/L in the City of Regina effluents discharged in July 2014 and 2015, respectively.

Lower secondary sexual characteristic scores observed in males at RDS compared to RUS for both years suggest demasculinization of males exposed to MWWEs, as development of these characteristics are androgen dependent (Harries et al. 2000). Losses of nuptial tubercles and dorsal fatpad formation in male FHMs have been associated with exposure to wastewater effluents (Vajda et al., 2011) and its common constituent E2 (Miles-Richardson et al. 1999). This reduction in the expression of secondary sex characteristics was also reflected by the decreasing trend in 11-KT plasma concentrations, as well as the decrease in mRNA abundances of several genes involved with mediating normal reproductive functioning along the HPGL axis, including CYP19β, ERα/β, and AR that is discussed below. As secondary sexual characteristics play a key role in FHM spawning behaviours, their disruption may adversely impact the fitness of the fish in territorial contests, access to mates, or quality of parental care (Lange et al., 2011).

In addition to effects on somatic indices and secondary sex characteristics, there were significant differences in gonadal histopathology in fish between the up- and downstream site. The significant increase in proportions of spermatogonia and decrease in spermatocytes in males at RDS were similar to findings from earlier studies by Tetreault et al. (2011) and Kidd et al. (2007) with FHMs exposed to MWWE and EE2, respectively. This shift in cell type between the up- and downstream site suggests a delay in gonadal maturation of male fish at RDS. Similar delays in spermatogenesis have been documented in wild fish exposed to treated sewage effluent (Diniz et al. 2005). One explanation for this reduced maturation stage could be that males collected at RDS during 2014 were slightly smaller (~ 17%) in size than those collected at RUS, and it is known that body size is related to gonadal maturation in fish. However, all fish were of reproductive age with representation of diverse germ cell stages including spermatozoa. Together with the parallel severe degeneration of testicular tissue structure it is therefore considered unlikely that the observed histopathologies were a function of different size of the fish.

This delay in testicular maturation may be explained by the decreasing trend in plasma 11-KT concentrations as well as the inhibition of expression of several genes encoding for enzymes involved with steroidogenesis and other processes along the HPGL in males. 11-KT is crucial for regulating reproductive physiology in male fish and correlations have been made between increasing 11-KT levels and maturation state in male teleosts (Beitel et al., 2015). In steroidogenesis, the rate-limiting step of supplying cholesterol, the precursor for steroids, to the inner mitochondrial membrane is mediated by steroidogenic acute regulatory protein (StAR). Expression of the gene encoding for this protein was decreased in males, which is thought to have contributed to the decreasing trend in plasma 11-KT as described above. Interestingly, expression of 17β-HSD, an enzyme involved in regulating the levels of

active androgens and estrogens, was up-regulated (9-fold) in males collected from RDS. Similar findings of increased expression of 17β-HSD are not prevalent in the literature; however, it is possible that exposed fish upregulated 17β-HSD as a compensatory mechanism to increase hormone production. Furthermore, the significant dysregulation of the expression of hormone receptors (ER, AR, FSHR and LHR) in males further indicates disruption of normal reproductive functioning and maturation. However, no clear patterns with regard to the direction of changes were observed, which would support the hypothesis that the changes observed may have been compensatory in nature. In summary, normal reproductive functioning of RDS males was likely to have been negatively affected due to the disruption of several molecular processes along the HPGL axis leading to inhibited maturation and testicular degeneration that was observed in the same fish. General degeneration of testicular tissue has been reported in fish exposed to treated sewage effluent (Diniz et al. 2005), the xenoestrogen EE2 (Kidd et al. 2007), as well as a variety of other contaminants such as metals and pesticides (reviewed in Kime 1995). It is hypothesized that the here-observed impairment of normal gonadal morphology and structure was not likely caused by exposure to estrogenic contaminants but rather other stressors such as ammonia or other contaminants associated with MMWEs due to the lack of typical effects of estrogens and their absence from the effluent at the time of sampling.

One condition that has been widely documented in fish exposed to MWWE is testicular oocytes or intersex (Diniz et al. 2005; Kidd et al. 2007; Tyler and Jobling 2008; Tetreault et al. 2011). Interestingly, there was no evidence of the occurrence of testicular oocytes or mixed sex conditions in males collected from RDS, nor was there an increase in the expression of genes associated with estrogen signalling. In this study, FHM populations in both 2014 and 2015 at RDS had decreased expression of both estrogen receptors measured in brain and liver tissues, which is opposite to responses previously reported for exposure to estrogens and the anti-androgen flutamide (Bowman et al., 2000; Filby et al., 2007). This suggests that the changes observed are unlikely due to the direct action of estrogenic or anti-androgenic compounds, which is supported by the lack of analytical detection of natural or synthetic hormones at RDS. Expression of VTG, an estrogen responsive gene in the liver that is known to increase with estrogen exposure, was significantly downregulated in females at RDS in 2015, with a similar but non-significant trend in 2014, suggesting further that exposure to MWWE discharged into Wascana Creek did not stimulate estrogenic responses. Decreases in VTG have been reported in female FHMs exposed to androgen agonists (17α-trenbolone and 17β-trenbolone), an estrogen antagonist (fenarimol) and inhibitors of steroidogenesis including fungicides (prochloraz and fenarimol) and certain pharmaceuticals (fadrozole; Miller et al., 2007). Furthermore, VTG induction in males is a highly sensitive biomarker of estrogenic exposure in fish and is used extensively in both laboratory and field settings (Vajda et al., 2008; Folmar et al., 1996; Palace et al., 2002; Kidd et al., 2007). However, in this present study, abundance of VTG mRNA was not increased in males at RDS further suggesting that populations downstream were not exposed to estrogenic compounds. This is further supported by the lack of detection of reproductive hormones or hormone agonists (e.g. E2, T, E1, estriol, EE2) in the surface water samples from RDS. In addition, Filby et al. (2007) observed decreases in expression of the AR in liver and gonad tissues exposed to the anti-androgen, flutamide. Similarly, in this study, we also found down-regulation of AR in the livers of both females and males, which may indicate the presence of chemicals with anti-androgenic properties.

Females downstream of the effluent discharge site were less mature with smaller gonads, significantly fewer vitellogenic follicles, and an increase in atresia of oocytes. Suppression of ovarian development has been observed in FHMs ws exposed to a variety of endocrine active compounds such as androgens (Ankley et al. 2001; Ankley et al. 2003), pesticides (Makyen et al. 2000), estrogens (Miles-Richardson et al. 1999), and industrial chemicals (Ankley et al., 2001) among others, all of which are compounds potentially found in MWWEs. While a significant proportion of the genes assessed was dysregulated in the same fish, no clear patterns that point towards a specific endocrine mechanism of action for the observed inhibition of maturation could be determined. In general, expression of most of the genes measured along the HPGL axis was downregulated in females. In particular, both ERs as well as VTG were significantly lower, further confirming the general inhibitory effects on gonadal maturation and other reproductive functions in female fish downstream of the Regina WWTP. Interestingly, significant increases in LHR and FSHR expression were observed in downstream fish, which may be a compensatory response to the general inhibitory effects. Many studies have shown FSHR- and LHR-activating properties by FSH and LH, respectively (Han et al., 1996; He et al., 2012; Grossman et al., 1997). In addition to effects on specific hormone receptors, there also was evidence of inhibition of steroidogenesis in females. Specifically, aromatase, a cytochrome P450 enzyme responsible for the production of E2 by catalyzing its conversion from T, was suppressed in brain tissue (CYP19β), but unchanged in the gonads (CYP19α). Differences in expression of CYP19 genes between tissues has been previously reported (Govoroun et al., 2001; Cheshenko et al., 2008). Aromatase inhibitors include a variety of chemicals found in the environment such as pesticides, organochlorines, and organotins, some of which have been shown to lead to prevalence of male phenotypes in exposed populations and inhibit ovarian and testicular growth in FHM s (Ankley et al., 2002; Villeneuve et al., 2006; Cheshenko et al., 2008). Decreased expression of CYP19β in the brain found in this study paired with lowered GSI and inhibited maturation of gonads found in populations at RDS suggests inhibition of steroidogenesis potentially due in part to aromatase inhibitors found in the MWWEs. In contrast to males, there was no significant effect on 17β-HSD in female fish RDS but there was a significant increase in expression of StAR, suggesting sex-specific disruption of steroidogenesis in FHMs exposed to MWWEs.

Alterations observed in wild FHMs collected downstream of the WWTP of the city of Regina were similar to those that were observed in laboratory reared male and female FHMs exposed to 50% Regina MWWE in a parallel study (Steeves, 2018), which confirmed that MWWE was likely responsible for the observed effects. Overall, the current study demonstrated major alterations in the histopathology of both male and female FHMs downstream of the City of Regina WWTP that were likely due to the downregulation of key molecular processes along the HPGL axis, and which indicated likely impacts on the reproductive fitness of these animals.

Of the 22 compounds analyzed, only 10 PPCP's and their metabolites were detected above the method detection limit in the ng/L to μg/L range downstream of the effluent fallout. There were five categories of use for chemicals found in surface water collected at RDS; pesticides (atrazine, DEET, and pentachlorophenol), a stimulant (caffeine), antimicrobials (triclocarban and triclosan), anti-convulsant (carbamazepine), and lipid regulators (gemfibrozil). While Waiser et al. (2011a) detected the presence of 25 PPCP's and their metabolites downstream of the City of Regina WWTP in water surveys

throughout the year, only 13 were detected at sampling times and locations (July 2006, Site 2) comparable to this study. Compounds detected varied between studies, but carbamazepine, DEET, gemfibrozil, and triclosan were consistently found. However, with the exception of DEET, concentrations of these compounds detected at RDS in the current study were all lower compared to those reported by Waiser et al. (2011a). Interestingly, concentrations of the estrogens, E1, E2, and EE2, were all below the detection limit at RDS and well below the median concentrations in sewage effluents of 19, 5.5, and 9.0 ng/L, respectively, reported across Canada (Sun et al., 2014). In the current study, chemical analysis was only conducted on surface waters collected during the sampling period of 2014, but it has been shown in previous studies that concentrations vary throughout the year, with the highest concentrations occurring most often during winter months (Waiser et al. 2011a). This allows for changes in exposure of aquatic organisms throughout the year. In general, chemicals detected at RDS are similar to those reported in other studies, and concentrations found here are within the lower range of previous contaminant reporting. However, concentrations of the majority of chemicals detected in this study were significantly lower than their previously reported biologically active concentrations. Of the 10 chemicals detected at RDS, only triclosan was measured at concentrations that were reported to have endocrine disrupting properties such as increases in HSI and synthesis of VTG and decreases in sperm counts in male mosquitofish (*Gambusia holbrooki*) (Raut and Angus, 2010). However, some of these chemicals have been shown to elicit endocrine effects at greater concentrations. For example, DEET has been shown to have anti-androgenic activity in female FHMs exposed to 600 ng/L (Zenobio et al. 2014), which was within the same order of magnitude of concentrations detected at RDS (172.2 ng/L). Ultimately, while many of these compounds were present at low concentrations in Wascana Creek, SK, they exist in a complex mixture, with the potential for cumulative effects and increased toxicity to aquatic organisms. Therefore, further investigation of these complex mixtures is needed.

Concentrations of un-ionized ammonia (NH_3-N) ranging from 0.066 to 0.511 mg/L in the final effluents released just upstream of RDS at the time of our sampling also raised significant concerns as exposure to un-ionized ammonia at levels as low as 0.06mg/L have been shown to increase mortality, reduce growth, and impair reproductive output in FHMs with a 20-day exposure (Armstrong et al. 2012). The elevated concentrations of ammonia present in Wascana creek may allow FHM populations at RDS to have a net uptake of ammonia, potentially causing some of the adverse biological effects that were observed. Sub-lethal effects in FHMs exposed to ammonia include growth reduction, inhibited reproductive success (Thurston et al. 1986; Armstrong et al. 2012), kidney malformations, irregular gill ventilation, and decreased brain monoamines (Tetreault et al. 2011; Armstrong et al. 2012). In conclusion, it remains difficult to pinpoint the observed effects in wild FHMs downstream of the City of Regina's WWTP and exposure with specific chemicals or mixtures; however, this study adds to the growing evidence of that exposure to MMWEs can have adverse effects on wild fish populations.

Conclusions

MWWEs released into Wascana Creek, SK, from of the City of Regina's WWTP, caused adverse biological effects such as altered K, increased liver sizes, delays in maturation, and degradation of

reproductive tissues in resident FHM populations, all of which are likely to weaken their ability to reproduce and survive. Interestingly, molecular mechanistic investigations revealed a lack of responses typical for the exposure to estrogens that have been previously hypothesized to be one of the main concerns for male fish downstream of MWWEs. This lack of estrogenicity is supported by the absence of estrogenic compounds in water chemistry samples at RDS and with non-significant estrogenicity reported in a parallel *in vitro* study (Bagatim et al., personal communication). This is in contrast to the common assumption that one of the main risks to wild fish populations associated with MWWEs is the release of chemicals with estrogenic properties. Alternatively, samples of effluents showed significant anti-androgenic and anti-estrogenic activity (Bagatim et al., personal communication), which is in accordance with the impacts on reproductive parameters and the general decrease in the expression of genes involved with normal reproductive functioning and maturation observed in this study. Ultimately, overall toxicity and suppression of reproductive systems in the exposed populations was likely occurring due to a cumulative effect of a diverse number of stressors including high nutrient and ammonia levels and presence of wide ranging PPCPs. Furthermore, we could demonstrate that mechanistic endpoints such as characterization of gene expression patterns present a promising approach to further our understanding of biological causes for apical outcomes in wild fish exposed to MWWEs. Future studies should be conducted to investigate if the more recent upgrades completed at the Regina WWTP in 2018 help to improve the overall quality of Wascana Creek and health of its aquatic life.

Creadit author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Figure and Table Legends

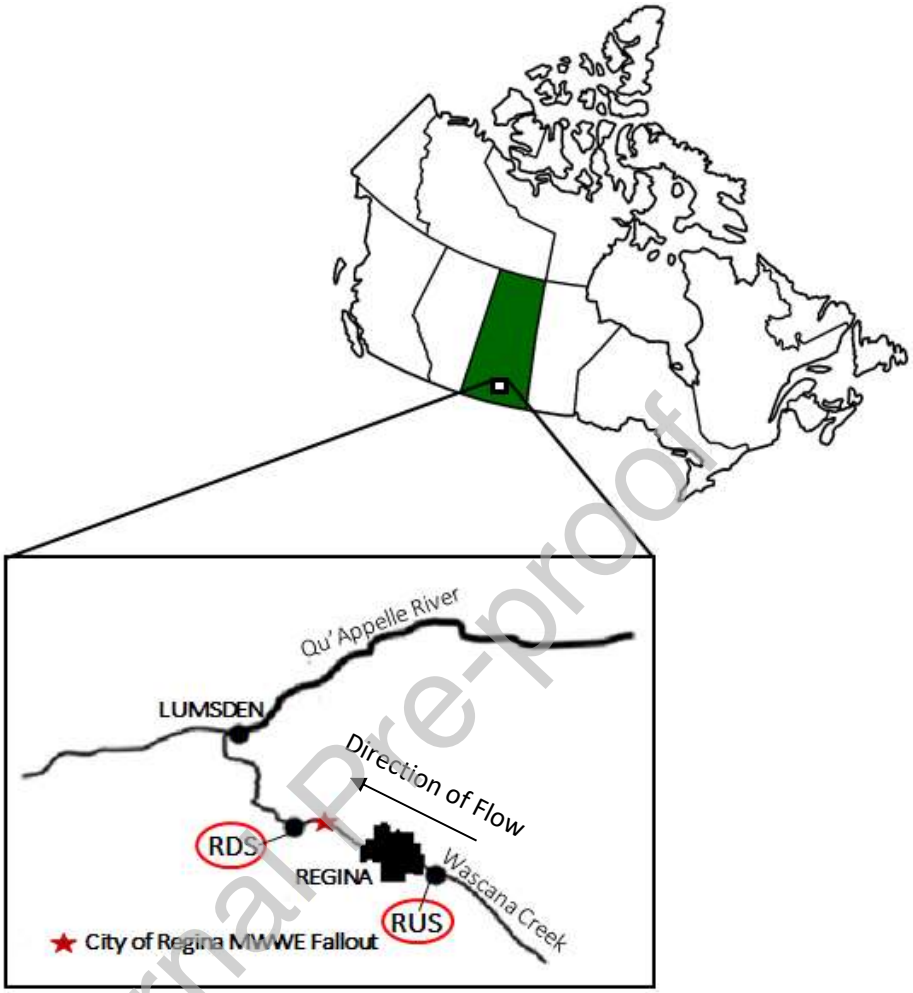

Figure 1: Site locations for sampling of fathead minnows in Wascana Creek, Regina, Canada. RUS (Regina Upstream Site); RDS (Regina Downstream Site); MWWE (Municipal Wastewater Effluent).

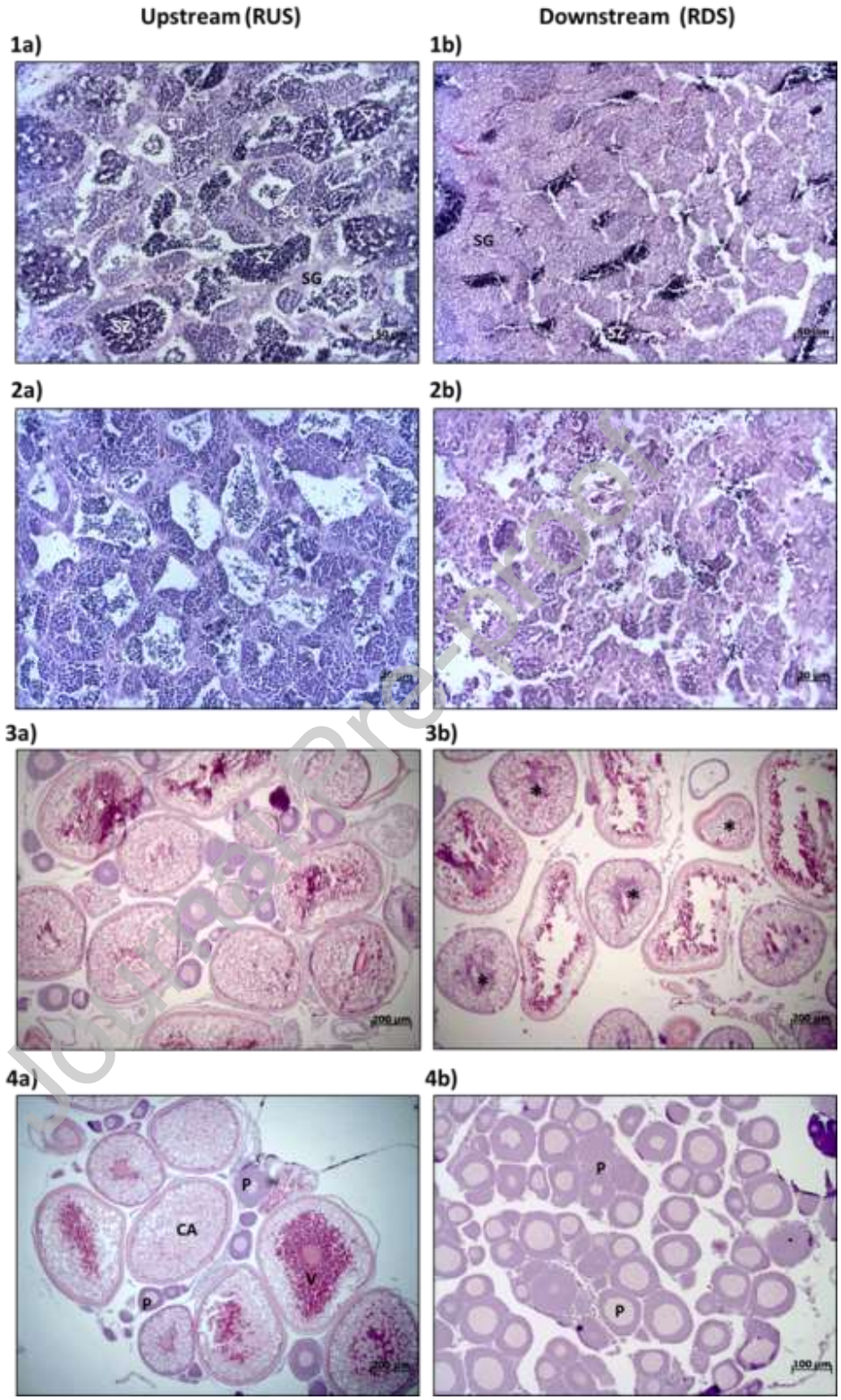

Figure 2. Micrographs of hematoxylin and eosin stained sections of gonad tissues of adult male and female fathead minnows collected in Wascana Creek upstream (left; RUS) and downstream (right; RDS) of the City of Regina in 2014 and 2015 (only female data available for 2015 as insufficient male fish were collected). Adult male testes (1) containing spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ). RDS male (1b) showing moderate (grade 3) increase in proportion of spermatogonia (SG) compared to RUS male (1a). Male upstream (2a) displaying non-remarkable degeneration, with RDS male (2b) showing a mild (grade 2) increase in testicular degeneration with losses of cell architecture, apoptotic germ cell formation and cell shrinkage and fragmentation. Ovarian tissue showing non-remarkable atresia in RUS females (3a) and a moderate (grade 3) increase in oocyte atresia in RDS females (3b). Asterisks (*) indicate the relatively few non-atretic oocytes in the tissue form RDS females. (4) Mid-development (stage 2) was observed in RUS females (4a) with vitellogenic (V) and cortical alveolar (CA) follicles, while undeveloped (stage 0) was observed in RDS (4b) with the tissue consisting of entirely primary (P) follicles (paraffin, H&E).

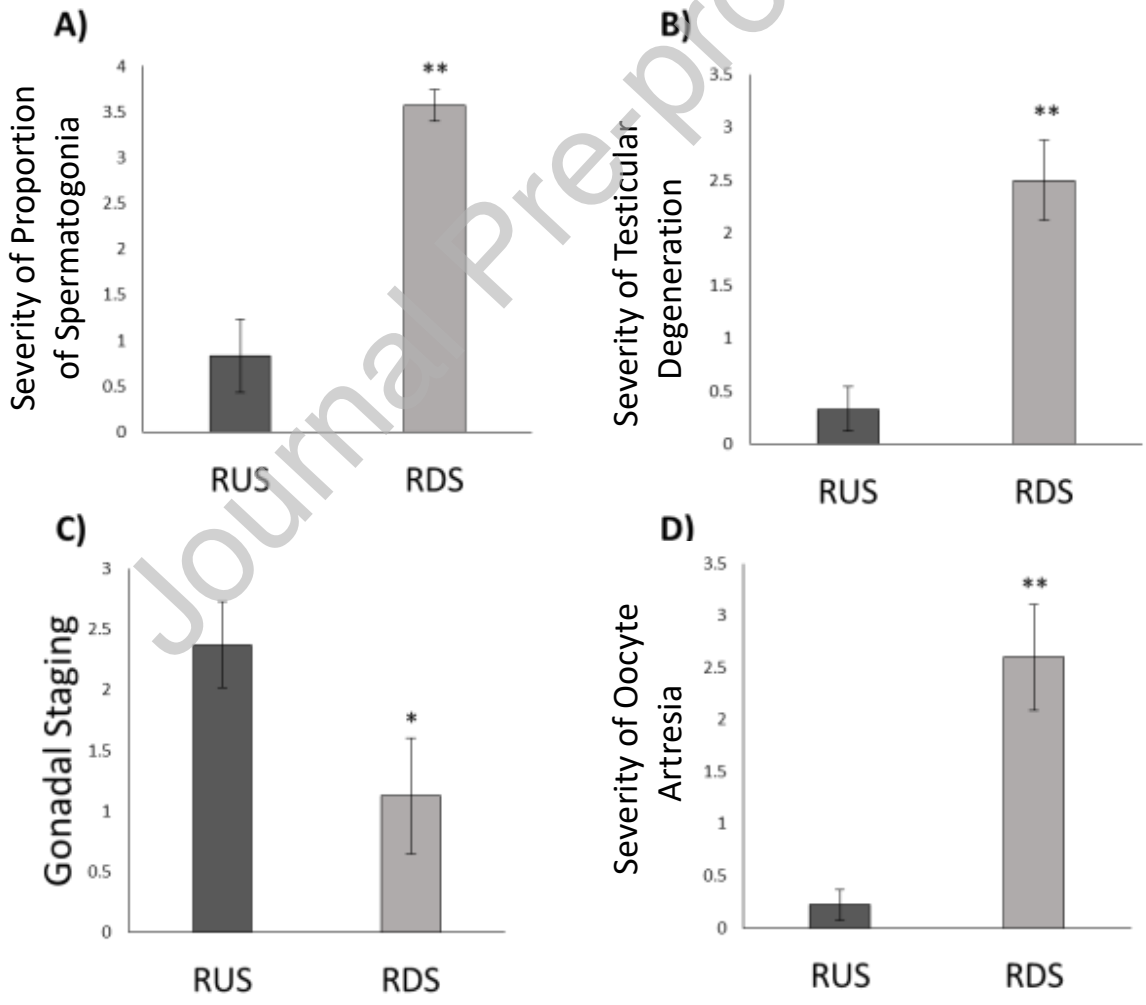

Figure 3: Average severity grading (±SEM) for associated histopathological primary criteria of males (A and B), and females (C and D) at sampling locations upstream (RUS; males: n= 6, females: n=10) and downstream (RDS; males: n=7, females: n=5) of the City of Regina in 2014. Average gonadal maturity grade of females (C). * = $p<0.05$ and ** = $p<0.001$.

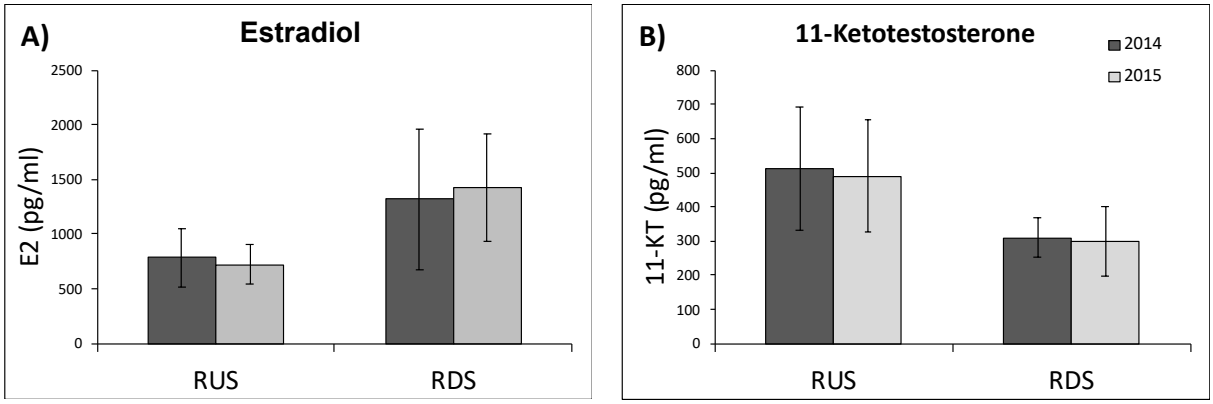

Figure 4. Mean (± SEM) concentrations of plasma hormones (A) 17β-estradiol (E2) in female, and (B) 11-ketotestosterone (11-KT) in male fathead minnows collected upstream (RUS; females: n=6 [2014 and 2015]; males: n=6 [2014 and 2015]) and downstream (RDS; females: n=6 [2014 and 2015]; males: n=14 [2014], n=4 [2015]) of the effluent fallout in Wascana Creek, SK in 2014 (solid bars) and 2015 (dashed bars). No significant differences in the concentrations of plasma hormone were observed between sites in each year ($p > 0.05$).

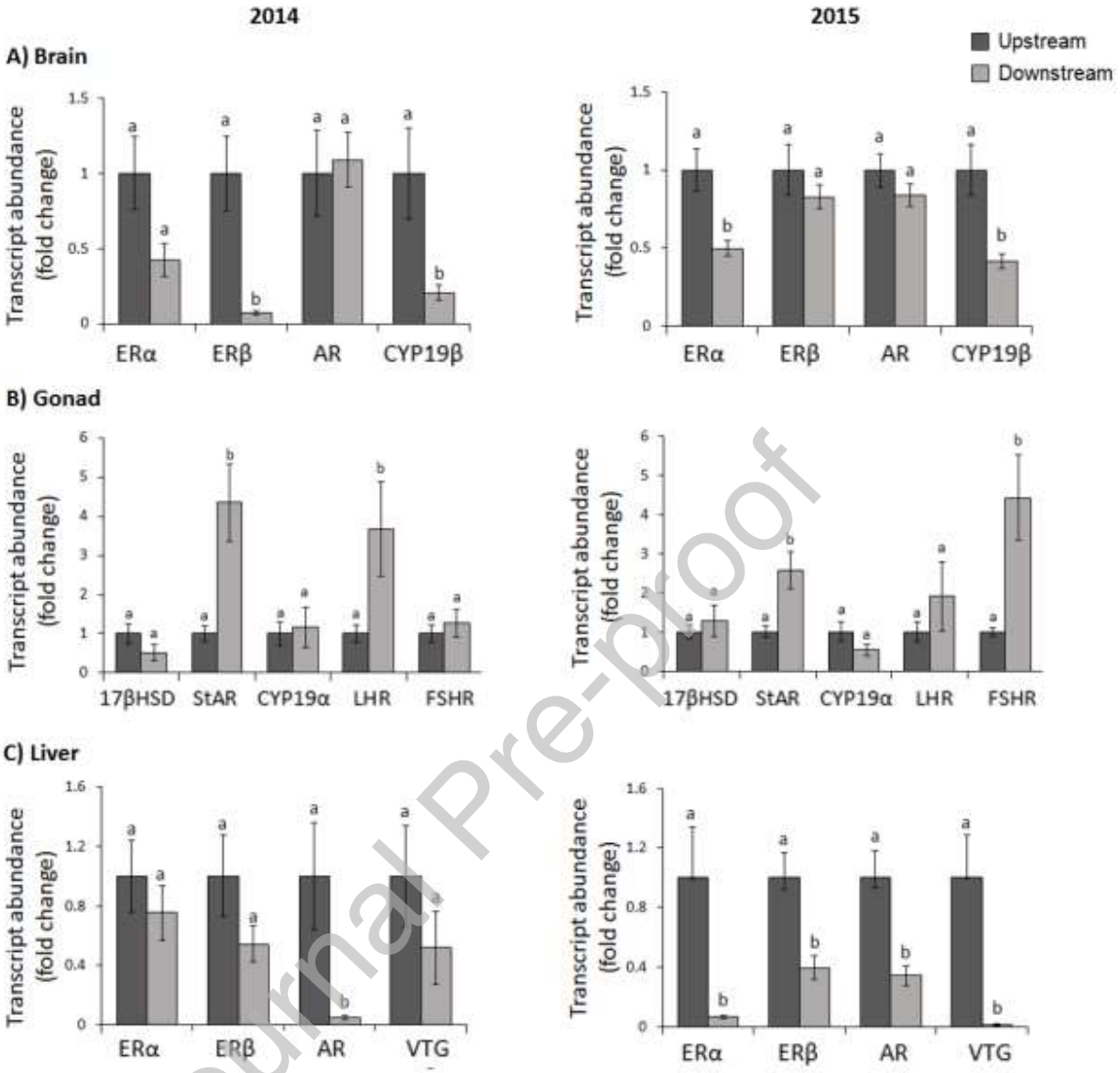

Figure 5. Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in female fathead minnows upstream (RUS; dark bars) and downstream (RDS; light bars) of the effluent fallout in Wascana Creek, SK in 2014 (left; RUS: n = 17, RDS: n = 10) and 2015 (right; RUS: *n* = 17 [except gonad where n=16], RDS: *n* = 26). (A) brain, (B), gonad, (C) liver. For each transcript, data are expressed relative to RUS group. Bars represent mean ± SEM. Different letters indicate significant difference in the concentrations of plasma hormone between sites (ANOVA; $p \leq 0.05$).

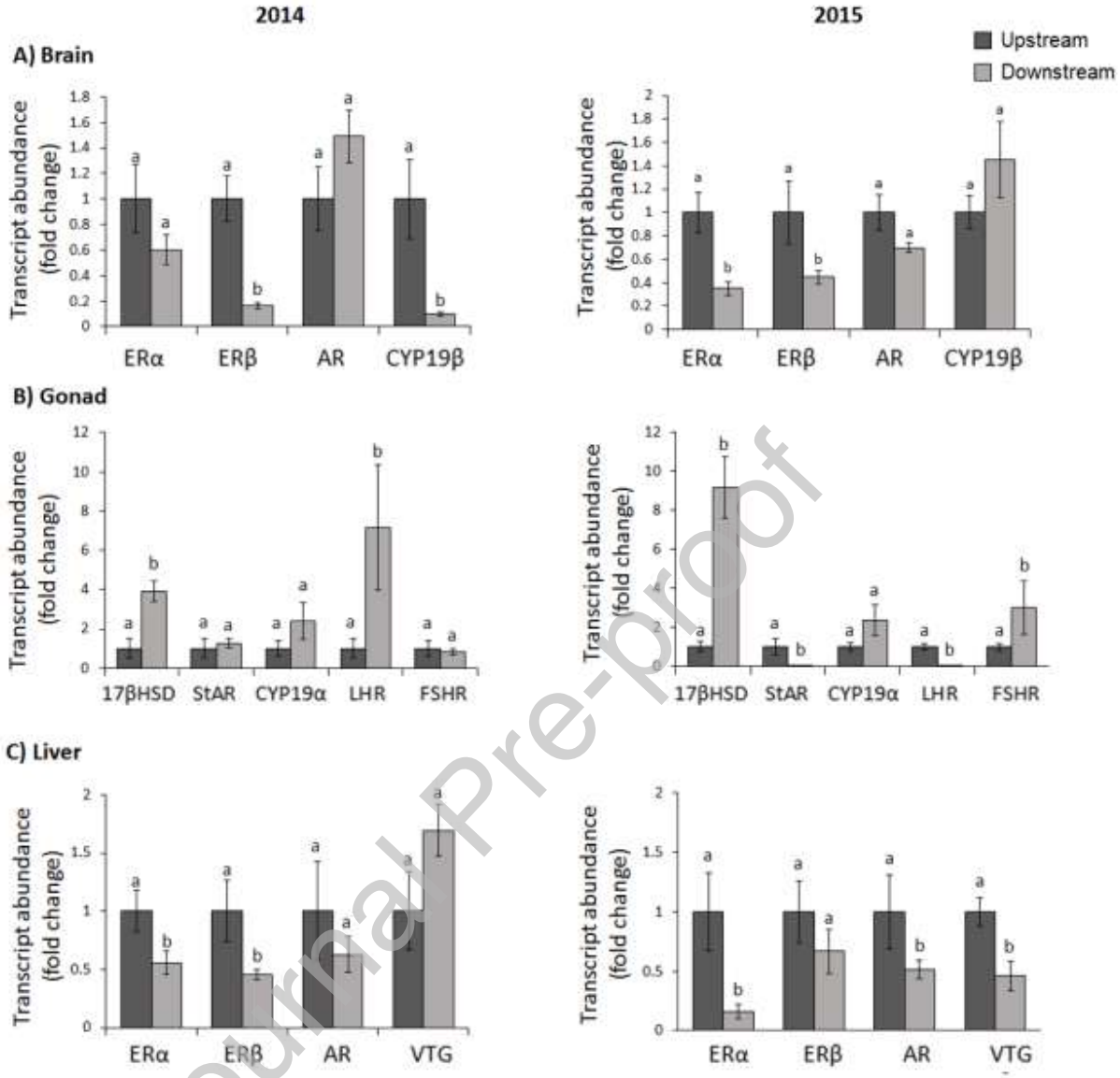

Figure 6. Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in male fathead minnows upstream (RUS; dark) and downstream (RDS; light) of the effluent fallout in Wascana Creek, SK in 2014 (left; RUS: n = 10, RDS: n = 25 [except gonad where n=19]) and 2015 (right; RUS: n = 6 , RDS: n = 4). (A) brain, (B), gonad, (C) liver. For each transcript, data are expressed relative to RUS group. Bars represent mean ± SEM. Statistical significance denoted by * = $p \leq 0.05$ (ANOVA).

Table 1. Average, maximum and minimum concentrations of ammonia nitrogen (mg/L), unionized ammonia at 15±1°C (mg/L), total phosphorus (mg/L) and total nitrogen (mg/L) in final effluents at the City of Regina's WWTP in July for both 2014 and 2015. Data was provided as a courtesy by the City of Regina.

		Ammonia Nitrogen (mg/L)	**Unionized Ammonia at 15±1°C (mg/L)**	**Total Phosphorus (mg/L)**	**Total Nitrogen (mg/L)**
July 2014	Average	16.6	0.241	0.87	27.1
	Max	24.1	0.511	1.99	33.0
	Min	12.0	0.101	0.46	23.8
July 2015	Average	17.9	0.102	0.74	38.5
	Max	13.6	0.179	1.08	47.1
	Min	24.9	0.066	0.47	34.2

Table 2. Mean (± SEM) length, body weight, condition factor (k), hepatosomatic index (HSI), and gonadosomatic index (GSI) of fathead minnows collected in Wascana Creek, SK. Differences among sites ($p<0.05$) are denoted by different lowercase letters. Numbers in parenthesis represent numbers of individuals. n.o.: not observed

Year	Sex	Site	Length (cm)	Weight (g)	*k*[a]	HSI[b]	GSI[c]	Tubercle Score
2014	Female	RUS	4.59 ± 0.09 (17)[a]	0.98 ± 0.06 (17)[a]	0.99 ± 0.04 (17)[a]	1.70 ± 0.84 (16)[a]	9.92 ± 3.18 (10)[a]	n.o.
		RDS	4.92 ± 0.31 (10)[a]	1.33 ± 0.18 (10)[a]	1.10 ± 0.07 (10)[a]	3.74 ± 0.38 (9)[b]	2.21 ± 0.87 (9)[b]	n.o.
	Male	RUS	5.37 ± 0.19 (10)[a]	1.63 ± 0.19 (10)[a]	1.02 ± 0.02 (10)[a]	1.57 ± 0.24 (9)[a]	0.96 ± 0.25 (8)[a]	10 ± 6.5 (10)[a]
		RDS	4.47 ± 0.16 (25)[b]	1.1 ± 0.11 (25)[b]	1.17 ± 0.03 (25)[b]	3.76 ± 0.24 (25)[b]	0.60 ± 0.04 (19)[b]	0.6 ± 0.4 (25)[b]
2015	Female	RUS	4.56 ± 0.10 (17)[a]	1.11 ± 0.06 (17)[a]	1.16 ± 0.04 (17)[a]	1.95 ± 0.18 (13)[a]	10.4 ± 1.03 (13)[a]	n.o.
		RDS	5.42 ± 0.08 (26)[b]	1.5 ± 0.07 (26)[b]	0.93 ± 0.02 (26)[b]	2.69 ± 0.23 (26)[b]	3.99 ± 1.97 (7)[b]	n.o.
	Male	RUS	4.97 ± 0.17 (6)[a]	1.64 ± 0.06 (6)[a]	1.38 ± 0.14 (6)[a]	1.30 ± 0.19 (6)[a]	2.21 ± 0.40 (5)[a]	42 ± 8.30 (6)[a]
		RDS	5.43 ± 0.37 (4)[a]	1.73 ± 0.36 (4)[a]	1.05 ± 0.10 (4)[a]	2.43 ± 0.37 (4)[b]	0.81 ± 0.66 (2)[a]	0.0 ± 0.0 (4)[b]

Table 3. Summary of analytical results of in-stream water samples from RUS and RDS in 2014 for 22 organic wastewater contaminants. Chemical concentrations are shown in ng/L. Concentrations below the method detection limit are denoted by <MDL.

Chemical (ng/L)	Regina Upstream Site (RUS)	Regina Downstream Site (RDS)	Use
Atrazine	0.2	0.3	Herbicide
Caffeine	0.5	3.4	Stimulant
Carbamazepine	0.2	19.4	Anticonvulsant
Chlorpyrifos	<MDL	<MDL	Insecticide
Clofibrate	74.3	<MDL	Antihyperlipidemic
DEET	37.2	172.2	Insecticide
Diazepam	<MDL	0.2	Benzodiazepine
EE2	<MDL	<MDL	Ovulation inhibitor
Estradiol	<MDL	<MDL	Reproductive hormone
Estrone	<MDL	<MDL	Reproductive hormone
Gemfibrozil	<MDL	46.2	Antihyperlipidemic
Ibuprofen	<MDL	<MDL	Anti-inflammatory
Miconazole Nitrate	<MDL	<MDL	Antifungal
Naproxen	<MDL	<MDL	Anti-inflammatory
Pentachlorophenol	<MDL	4.2	Pesticide
Progesterone	<MDL	<MDL	Reproductive hormone
Sulfamethoxazole	<MDL	<MDL	Antibiotic
Testosterone	<MDL	<MDL	Reproductive hormone
Triclocarban	<MDL	0.1	Antimicrobial disinfectant
Triclosan	2.0	33.2	Antimicrobial disinfectant
Trimethoprim	<MDL	<MDL	Antibiotic

SUPPLEMENTAL MATERIALS

Table S1: Summary of fish collected in 2014 and 2015 at the upstream (RUS) and downstream (RUS) sites in Wascana Creek, SK, Canada and endpoints that were assessed for each individual.

Year of Sample	Fish ID	Sex (F=female; M=male; I=Immature	Somatic Indices			Steroid Analysis	Gene Analysis			Histology	Stable Isotope Analysis
			Weight [g]	Length [cm]	Condition [K]	Plasma	Liver	Gonad	Brain	Gonad	Spleen
2014											
	RUS - 2	F	0.673	4.4	0.790	no	yes	yes	yes	no	yes
	RUS - 3	F	0.706	3.9	1.190	no	yes	yes	yes	yes	yes
	RUS - 4	F	1.284	5.1	0.968	yes	yes	yes	yes	no	yes
	RUS - 8	F	1.107	4.6	1.137	no	yes	yes	yes	yes	yes
	RUS - 12	F	0.901	4.9	0.766	yes	yes	yes	yes	no	yes
	RUS - 13	F	1.033	4.3	1.299	no	yes	yes	yes	no	yes
	RUS - 14	F	0.571	4	0.892	yes	yes	yes	yes	no	yes
	RUS - 15	F	0.892	4.9	0.758	no	yes	yes	yes	yes	yes
	RUS - 17	F	0.851	4.7	0.820	no	yes	yes	yes	no	yes
	RUS - 22	F	0.933	4.5	1.024	no	yes	yes	yes	yes	yes
	RUS - 24	F	1.388	4.9	1.180	no	yes	yes	yes	no	yes
	RUS - 26	F	1.131	4.8	1.023	yes	yes	yes	yes	yes	yes
	RUS - 27	F	1.12	4.8	1.013	no	yes	yes	yes	yes	yes
	RUS - 28	F	0.971	4.7	0.935	yes	yes	yes	yes	yes	yes
	RUS - 30	F	0.859	4.3	1.080	yes	yes	yes	yes	yes	yes
	RUS - 31	F	1.373	5.1	1.035	no	yes	yes	yes	yes	yes
	RUS - 32	F	0.794	4.2	1.072	no	yes	yes	yes	yes	no
	RDS - 1	F	1.37	5.1	1.033	yes	yes	yes	yes	no	yes
	RDS - 3	F	2.044	6.4	0.780	yes	yes	yes	yes	yes	yes
	RDS - 6	F	2.194	6.2	0.921	yes	yes	yes	yes	yes	yes
	RDS - 7	F	1.553	5.8	0.796	yes	yes	yes	yes	yes	yes
	RDS - 8	F	1.782	5.1	1.343	yes	yes	yes	yes	yes	yes
	RDS - 26	F	0.98	4.4	1.150	no	yes	yes	yes	no	yes
	RDS - 27	F	1.192	4.6	1.225	yes	yes	yes	yes	no	yes
	RDS - 28	F	0.735	4	1.148	no	yes	yes	yes	no	yes
	RDS - 29	F	0.66	3.7	1.303	no	yes	yes	yes	yes	yes
	RDS - 36	F	0.77	3.9	1.298	no	yes	yes	yes	no	yes
	RUS - 1	M	2.474	6.5	0.901	no	yes	yes	yes	yes	yes
	RUS - 5	M	1.943	5.7	1.049	no	yes	yes	yes	yes	yes
	RUS - 6	M	1.137	4.9	0.966	yes	yes	yes	yes	yes	yes
	RUS - 9	M	1.274	4.9	1.083	no	yes	yes	yes	yes	yes
	RUS - 10	M	1.473	5.1	1.110	yes	yes	yes	yes	yes	yes
	RUS - 19	M	2.842	6.4	1.084	no	yes	yes	yes	yes	yes
	RUS - 21	M	1.442	5.2	1.026	yes	yes	yes	yes	no	yes
	RUS - 23	M	1.222	4.9	1.039	yes	yes	yes	yes	no	yes
	RUS - 25	M	1.202	5	0.962	yes	yes	yes	yes	no	yes
	RUS - 29	M	1.247	5.1	0.940	yes	yes	yes	yes	no	yes
	RDS - 2	M	0.573	4	0.895	no	yes	yes	yes	no	yes
	RDS - 4	M	1.771	5.6	1.008	no	yes	yes	yes	yes	yes
	RDS - 5	M	0.494	3.4	1.257	no	yes	yes	yes	no	yes
	RDS - 9	M	2.73	6.5	0.994	yes	yes	yes	yes	no	yes
	RDS - 10	M	2.31	6.4	0.881	yes	yes	yes	yes	yes	yes
	RDS - 12	M	0.816	4.1	1.184	no	yes	yes	yes	no	yes
	RDS - 13	M	0.712	4	1.113	no	yes	yes	yes	no	yes
	RDS - 14	M	0.71	3.5	1.656	yes	yes	yes	yes	no	yes
	RDS - 15	M	0.8	4.1	1.161	yes	yes	yes	yes	no	yes
	RDS - 16	M	2.11	5.5	1.268	no	yes	yes	yes	yes	yes
	RDS - 17	M	1.05	4.4	1.233	no	yes	yes	yes	no	yes
	RDS - 18	M	1.67	5.4	1.061	yes	yes	yes	yes	yes	yes
	RDS - 19	M	1.035	4.5	1.136	no	yes	yes	yes	yes	yes
	RDS - 20	M	0.822	4.2	1.109	yes	yes	yes	yes	no	yes
	RDS - 21	M	0.801	4.1	1.162	no	yes	yes	yes	no	yes
	RDS - 22	M	0.74	3.9	1.247	yes	yes	yes	yes	no	yes
	RDS - 23	M	0.955	4.3	1.201	yes	yes	yes	yes	no	yes
	RDS - 25	M	0.795	4	1.242	yes	yes	yes	yes	no	yes
	RDS - 30	M	1.009	4.3	1.269	no	yes	yes	yes	no	yes
	RDS - 32	M	1.245	4.8	1.126	yes	yes	no	yes	no	yes
	RDS - 33	M	0.933	4.2	1.259	yes	yes	no	yes	yes	yes
	RDS - 34	M	0.83	4.2	1.120	yes	yes	no	yes	no	yes
	RDS - 35	M	0.725	3.9	1.222	no	yes	no	yes	no	yes
	RDS - 37	M	1.03	4.3	1.295	yes	yes	no	yes	yes	yes
	RDS - 38	M	0.75	4.1	1.088	yes	yes	no	yes	no	yes

Year of Sample	Fish ID	Sex (F=female; M=male; I=Immature	Somatic Indices			Steroid Analysis	Gene Analysis			Histology	Stable Isotope Analysis
			Weight [g]	Length [cm]	Condition [K]	Plasma	Liver	Gonad	Brain	Gonad	Spleen
2015											
	RUS-36	F	1.35	5	1.080	yes	yes	yes	yes	no	yes
	RUS-37	F	0.967	4.4	1.135	no	yes	yes	yes	yes	yes
	RUS-38	F	0.848	4.3	1.067	no	yes	yes	yes	yes	yes
	RUS-40	F	1.845	5.5	1.109	yes	yes	yes	yes	no	yes
	RUS-41	F	0.971	3.8	1.770	no	yes	yes	yes	yes	yes
	RUS-43	F	1.266	4.8	1.145	yes	yes	yes	yes	yes	yes
	RUS-46	F	1.16	4.6	1.192	no	yes	yes	yes	yes	yes
	RUS-47	F	1.48	5.2	1.053	no	yes	no	yes	yes	yes
	RUS-48	F	1.174	4.9	0.998	yes	yes	yes	yes	yes	yes
	RUS-50	F	0.865	4.3	1.088	no	yes	yes	yes	yes	yes
	RUS-51	F	1.07	4.4	1.256	no	yes	yes	yes	yes	yes
	RUS-52	F	1.091	4.7	1.051	yes	yes	yes	yes	yes	yes
	RUS-53	F	0.918	4.1	1.332	no	yes	yes	yes	yes	yes
	RUS-57	F	0.836	4.2	1.128	no	yes	yes	yes	yes	yes
	RUS-58	F	0.961	4.4	1.128	no	yes	yes	yes	yes	yes
	RUS-59	F	1.093	4.4	1.283	yes	yes	yes	yes	yes	yes
	RUS-60	F	0.917	4.6	0.942	no	yes	yes	yes	yes	yes
	RDS-44	F	1.43	5.5	0.860	no	yes	yes	yes	no	yes
	RDS-45	F	1.417	5.3	0.952	no	yes	yes	yes	no	yes
	RDS-47	F	1.515	5.3	1.018	no	yes	yes	yes	no	yes
	RDS-48	F	1.73	5.3	1.162	no	yes	yes	yes	no	yes
	RDS-55	F	1.41	5.1	1.063	yes	yes	yes	yes	yes	yes
	RDS-56	F	1.45	5.4	0.921	no	yes	yes	yes	no	yes
	RDS-58	F	1.145	5.3	0.769	yes	yes	yes	yes	yes	yes
	RDS-59	F	1.545	5.4	0.981	no	yes	yes	yes	no	yes
	RDS-60	F	1.475	5.8	0.756	no	yes	yes	yes	no	yes
	RDS-62	F	1.525	5.5	0.917	no	yes	yes	yes	no	yes
	RDS-64	F	1.941	6.1	0.855	no	yes	yes	yes	no	yes
	RDS-67	F	0.978	4.6	1.005	no	yes	yes	yes	no	yes
	RDS-68	F	1.759	5.6	1.002	yes	yes	yes	yes	yes	yes
	RDS-69	F	1.385	5.2	0.985	no	yes	yes	yes	no	yes
	RDS-70	F	1.696	5.7	0.916	no	yes	yes	yes	no	yes
	RDS-71	F	1.539	5.5	0.925	no	yes	yes	yes	no	yes
	RDS-72	F	1.898	5.6	1.081	yes	yes	yes	yes	yes	yes
	RDS-74	F	1.749	5.6	0.996	yes	yes	yes	yes	no	yes
	RDS-49	F/I	2.029	6	0.939	no	yes	yes	yes	no	yes
	RDS-50	F/I	2.045	6	0.947	no	yes	no	yes	no	yes
	RDS-51	F/I	1.206	5.1	0.909	no	yes	yes	yes	no	yes
	RDS-52	F/I	0.951	5.6	0.542	no	yes	no	yes	no	yes
	RDS-61	F/I	0.833	4.6	0.856	yes	yes	yes	yes	no	yes
	RDS-63	F/I	0.94	4.8	0.850	no	yes	no	yes	no	yes
	RDS-65	F/I	1.338	5	1.070	no	yes	yes	yes	no	yes
	RDS-75	F/I	2.062	6	0.955	no	yes	no	yes	no	yes
	RUS-33	M	1.483	4.8	1.341	yes	yes	yes	yes	yes	yes
	RUS-39	M	1.569	5	1.255	yes	yes	yes	yes	yes	yes
	RUS-45	M	1.77	5.2	1.259	yes	yes	yes	yes	yes	yes
	RUS-49	M	1.514	4.2	2.044	yes	yes	yes	yes	yes	yes
	RUS-55	M	1.729	5.3	1.161	yes	yes	yes	yes	yes	yes
	RUS-56	M	1.795	5.3	1.206	yes	yes	yes	yes	yes	yes
	RDS-53	M	0.975	4.5	1.070	yes	yes	yes	yes	no	yes
	RDS-54	M	2.038	6.2	0.855	yes	yes	yes	yes	no	yes
	RDS-57	M	1.325	5.2	0.942	yes	yes	yes	yes	no	yes
	RDS-73	M	2.584	5.8	1.324	yes	yes	yes	yes	no	yes

S1: Histological Processing and Staining

Processing Sequence

a. 10% neutral buffered formalin – 1 hour
b. 10% alcoholic formalin – 1 hour
c. 90% ethanol – 1 hour
d. Absolute ethanol – 1 hour
e. Absolute ethanol – 1 hour
f. Absolute ethanol – 1 hour
g. Absolute ethanol – 1 hour
h. Xylene – 1 hour
i. Xylene – 1 hour
j. Xylene – 1 hour
k. Paraplast wax – 1 hour
l. Paraplast wax – 1 hour
m. Paraplast wax – 1 hour
n. Paraplast wax – 1 hour

Hematoxylin and Eosin Staining

a. Xylene (1) – 2 min
b. Xylene (2) – 2 min
c. 100% Ethanol (1) – 2 min
d. 100% Ethanol (2) – 2 min
e. 95% Ethanol – 2 min
f. 70% Ethanol – 2 min
g. Dip into running water – 5 sec
h. Dip into distilled water – 5 sec
i. Hematoxylin – 2 min
j. Rinse in running water
k. Dip into acid alcohol – 5 sec
l. Running water – 10 min
m. Dip into distilled water – 5 sec
n. Eosin – 1 min
o. 2 dips into running water – 5 sec
p. Dip into 70%, 95% and 100% Ethanol – 5 sec
q. 100% Ethanol (2) – 1 min
r. Xylene – 2 min

S2: Histological Grading and Staging Scale

Severity grading's (0 = non-remarkable, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) were assessed for each specimen and used for all primary criteria. Staging criteria (juvenile, stage 0 = undeveloped, stage 1 = early development, stage 2 = mid-development, stage 3 = late development, stage 4 = late development/hydrated, stage 5 = post-ovulatory) were used to compare the relative maturational stage of the ovaries.

S2.1: General severity grading scale

Non Remarkable: This grade is used if there are no findings associated with a particular diagnostic criterion.

Grade 1: Minimal. Ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For discrete changes, grade 1 is used when there are fewer than 2 occurrences per microscopic field, or 1-2 occurrences per section.For multifocal or diffusely-distributed alterations, this grade is used for processes where ≤20% of the tissue in the section is involved.

Grade 2: Mild. A noticeable feature of the tissue. For discrete changes, grade 2 is used when there are 3-5 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 20-50% of the tissue in the section are involved.

Grade 3: Moderate. A dominant feature of the tissue. For discrete changes, grade 3 is used when there are 6-8 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 50-80% of the tissue in the section are involved.

Grade 4: Severe. An overwhelming feature of the tissue. For discrete changes, grade 4 is used when there are more than 9 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where >80% of the tissue in the section are involved.

S2.2: Criteria for Staging Ovaries

Juvenile: gonad consists of oogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals

Stage 0 – Undeveloped: entirely immature phases (oogonia to perinucleolar oocytes); no cortical aleoli.

Stage 1 – Early Development: vast majority (e.g., >90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.

Stage 2 – Mid-Development: at least half of the observed follicles are early and mid-vitellogenic.

Stage 3 – Late Development: majority of developing follicles are late vitellogenic

Stage 4 – Late Development/hydrated: majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.

Stage 5 – Post-ovulatory: predominately spent follicles, remnants of theca externa and granulosa.

Table S2. Summary of primary histological diagnostic criteria in male and female fathead minnows adapted from the Diagnosis of Endocrine-related Histopathology of Fish Gonads (US EPA 2009).

Primary Criteria	Males	Females
1.	Increased proportion of spermatogonia	Increased oocyte atresia
2.	Presence of testicular oocytes	Perifollicular cell hyperplasia
3.	Increased testicular degeneration	Decreased yolk formation
4.	Interstitial cell hyperplasia	Change in gonadal staging

S2: Gene Expression Analysis

Table S3. Nucleotide sequence, efficiencies and GenBank accession numbers of primer pairs used in qPCR for brain, gonad and liver tissues.

Target Gene	Primer	Sequence (5'-3')	Tissue	Efficiency	GenBank
17β-hydroxysteroid dehydrogenase (17bHSD)	Forward	ATCCAGAGTGTGCTGCCTTT	Gonad	98%	DT161033
	Reverse	AGGGAAATAGCCGTTGGTCT			
Androgen Receptor (AR)	Forward	CAACGCGTCTAAATCCCATT	Brain, liver	93, 95%	AY727529
	Reverse	TGTTCGAACTGACACGAAGC			
Aromatase α (CYP19α)	Forward	GCTGCACAAGAAGCACAAG	Gonad	94%	AF288755
	Reverse	CGTGGCTCTGAGCGAATATC			
Aromatase β (CYP19β)	Forward	AGGGTGTATCCTGGCAACTG	Brain	98%	AJ277866
	Reverse	ATCTGCACCCGTTTCATTTC			
Estrogen Receptor α (ERα)	Forward	CGGTGTGCAGTGACTATGCT	Brain, liver	94, 84%	AY775183
	Reverse	CTCTTCCTGCGGTTTCTGTC			
Estrogen Receptor β (ERβ)	Forward	CGTTTTGGCATAACCATGTG	Brain, liver	105, 96%	AY566178
	Reverse	TGCTGTCAGACTTCCGAATG			
Follicle-stimulating hormone receptor (FSHR)	Forward	CACGTACTGCTGTCCAGACG	Gonad	90%	EF219401
	Reverse	GTGGCTGGGGTATGTCAGAT			
	Forward	CTTTCAACCACCTTCCCAAG	Gonad	105%	DT281016

luteinizing hormone receptor (LHR)	Reverse	AGCATTTGGTGGGACTGAAC			
ribosomal RNA 18S (18S)	Forward	GCCCTGTAATTGGAATGAGC	Brain, liver, gonad	97%	AY855349
	Reverse	TCCCGAGATCCAACTACGAG			
Steroidogeneic acute regulatory protein (StAR)	Forward	ATGCCCGAGAAGAAAGGATT	Gonad	80%	DQ360497
	Reverse	CCCGGTTGATGACTGTTTTT			
Vitellogenin (VTG)	Forward	TTGCTCTCCAGACCTTTGCT	Liver	98%	AF130354
	Reverse	GCAGAGCCTCCACCTTGTAG			

S3: Extraction of Plasma for Hormone Analysis

Plasma samples were extracted twice with 2 mL of a 1:1 Hexane:Ethyl Acetate mixture by vortexing the sample mixture for one minute, followed by centrifugation at 2000 x g for three minutes. The supernatant was collected, dried under a stream of nitrogen and brought up into a buffer provided by the manufacture (Cayman Chemical, Anne Arbor, MI, USA) totalling the required volume (250 µL) for quantification.

S4: Chemical Analysis Using Liquid Chromatography-Mass Spectroscopy

Samples were filtered and solid phase extraction was conducted using two litres of filtered sample. After extraction, samples were air dried following the addition of 5mL of MiliQ water and 0.1% acetic acid. Cartridges were stored at -20°C. Cartridges were extracted using 5mL of 1:1 Hexane:DCM, dried using nitrogen and glass tubes stored at -20°C. Samples were reconstituted by adding 400µL of isooctane for each 2L sample. Following reconstitution, extracts were transferred into a clear insert and placed into an amber vial and stored at -20°C until analysis.

Extracts were analyzed in house using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON). Separation of chemicals was achieved with a Betasil C18 column (5 µm; 2.1 mm × 100 mm; Thermo Fisher Scientific, Toronto, ON) with an injection volume of 5 µlUltrapure water (A) and methanol (B) were used as mobile phases. Initially 10% B was increased to 50% in 5 min, then increased to 100% at 20 min and held static for 6 min, followed by a decrease to initial conditions of 10% B and held for 3 min to allow for column re-equilibration. The flow rate was 0.20 mL/min. The column and sample chamber temperatures were maintained at 40 °C and 10 °C, respectively. Data was acquired using full scan mode and selected ion monitoring (SIM). Briefly, MS scans (100 - 1000 *m/z*) were recorded at resolution R = 70000 (at m/z 200) with a maximum of 3×10^6 ions collected within 200 ms, based on the predictive automated gain control. SIM scans (*m/z* = 227.1072, 271.1698, 269.1542, 295.1698) were recorded at a resolution R = 35000 (at *m/z* 200) with a maximum of 5×10^4 ions collected within 80 ms, based on the predictive automated gain control, with the precursor isolation width set at 2.0 *m/z*. General mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375 °C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375 °C. Similarly, settings applied for positive ion mode were: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350 °C.

S4: Secondary Sex Characteristics

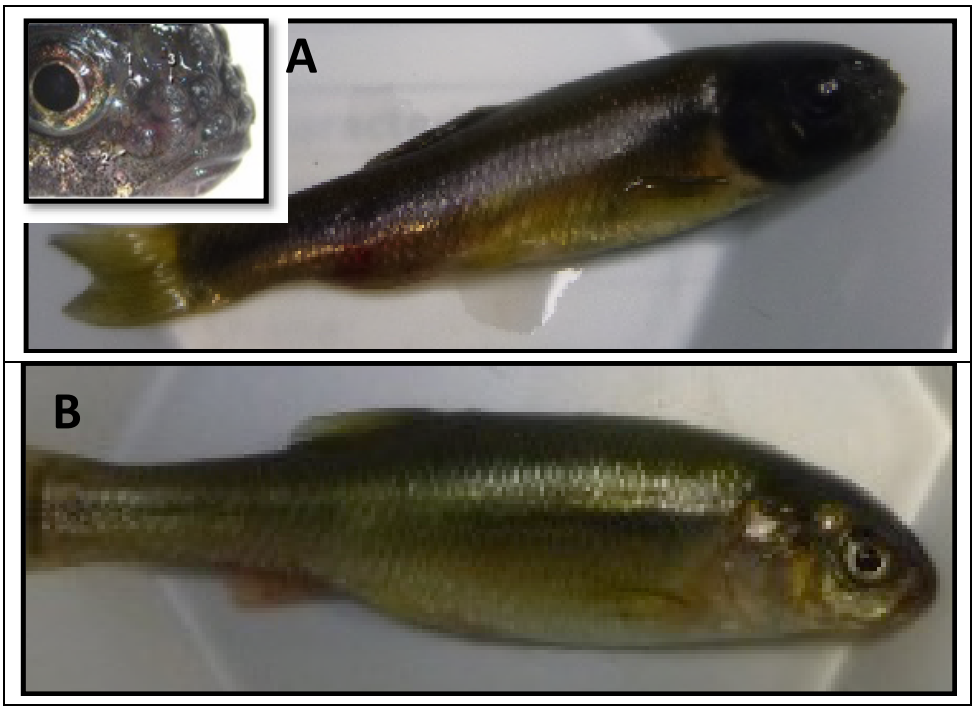

Figure S1. A) Typical male fathead minnow collected upstream of the city of Regina (RUS) with marked secondary sex characteristics displaying a tubercle score of 57, distinct banding and a dorsal fatpad; B) Typical male fathead minnow collected downstream of the city of Regina (RDS) displaying loss of tubercle formation, banding, fatpad formation and overall colour.

Table S4. Percent of male fathead minnows with a tubercle score of greater or equal to 5 collected at the up- (RUS) and down-stream (RDS) sampling sites.

	2014	2015
RUS	40%	85%
RDS	12%	6%

S5: Stable Isotope Analysis

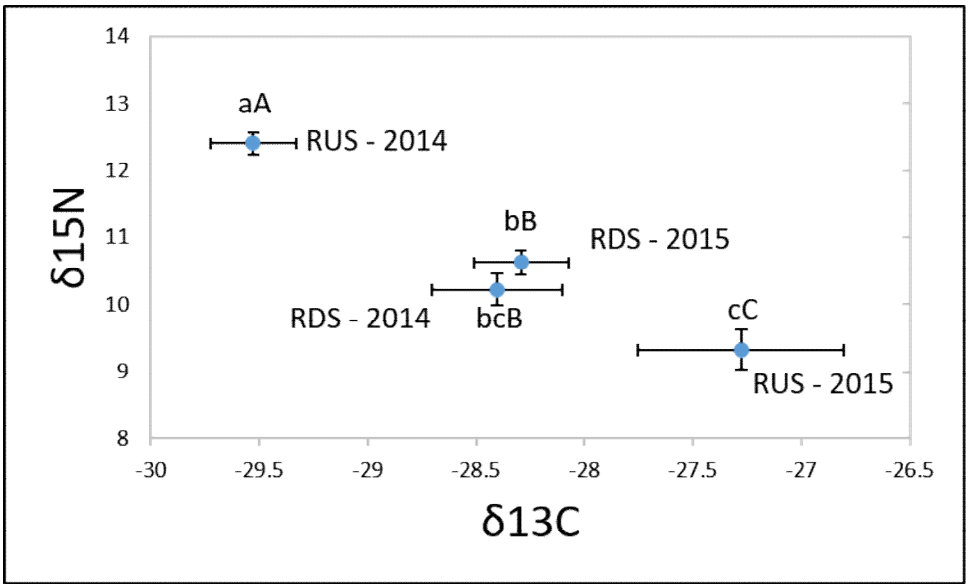

Figure S2. Stable isotope bi-plot showing nitrogen $\delta^{15}N$ and carbon $\delta^{13}C$ values (mean ± SEM) for sampling locations upstream (RUS) and downstream (RDS) of the City of Regina in 2014 and 2015. Statistically significant differences between sites are denoted by lower case letters for δ15N and uppercase letters for δ13C.