Biological responses in rainbow darter (*Etheostoma caeruleum*) to changes in municipal wastewater quality in the Grand River

by

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AUTHOR'S DECLARATION

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

This thesis contains three chapters that are written in a manuscript format (Chapter 2 - 4). At the time of thesis submission, the first two chapters were published in refereed journals, whereas the third chapter is currently in press. The manuscripts here as chapters are modified slightly for formatting consistency. I am the first author of all three manuscripts; Mark Servos is the anchor author on two manuscripts, Chapter 3 and 4, and Christopher Martyniuk is the anchor author on Chapter 2. The manuscripts were written by me, although there are several other individuals recognized as coauthors who provided intellectual contribution as explained below:

Chapter 2

Marjan, P., Bragg, L. M., MacLatchy, D. L., Servos, M. R., & Martyniuk, C. J. (2017). How does reference site selection influence interpretation of omics data? Evaluating liver transcriptome responses in male rainbow darter (*Etheostoma caeruleum*) across an urban environment. *Environmental Science and Technology, 51*(11), 6470–6479; doi: 10.1021/acs.est.7b00894

The key ideas in this manuscript were generated by me and Dr. Mark Servos who assisted with the study design and provided logistics for sample collection. I analyzed the samples while Christopher Martyniuk trained me in data analysis (bioinformatics) and data interpretation. Leslie Bragg analyzed surface water and effluent chemistry data. All authors participated actively in the discussion and the review process of the manuscript.

Chapter 3

Marjan, P., Martyniuk, C. J., Fuzzen, M. L., MacLatchy, D. L., McMaster, M. E., & Servos, M. R. (2017). Returning to normal? Assessing transcriptome recovery over time in male rainbow darter (*Etheostoma caeruleum*) liver in response to wastewater treatment plant upgrades. *Environmental Toxicology and Chemistry*, *36*(8), 2108-2122; doi: 10.1002/etc.3741.

The ideas for this manuscript were derived from Chapter 2 and were mostly generated by me although Dr. Mark Servos and Dr. Christopher Martyniuk provided great intellectual contribution. Meghan Fuzzen provided archived samples. I conducted sample analysis while Christopher Martyniuk helped with bioinformatics analysis and data interpretation. All authors participated in the discussion and the review process of the manuscript.

Chapter 4

Marjan, P., Van Der Kraak G.T., MacLatchy, D. L., Fuzzen, M. L., Bragg, L. M., McMaster M.E., Tetreault G.R., & Servos, M. R. (2017). Assessing recovery of *in vitro* steroid production in male rainbow darter (*Etheostoma caeruleum*) in response to municipal wastewater treatment plant infrastructure changes. *Environmental Toxicology and Chemistry,* doi: 10.1002/etc.3986 (in press).

The ideas in the final manuscript leverage previous work conducted by other students from Dr. Servos' lab that focus on the post-upgrade responses in fish. Meghan Fuzzen provided historical data sets. Dr. Mark Servos participated in the study design, discussion and the review process. Leslie Bragg was responsible for providing chemical and estrogenicity data. The Region of Waterloo provided data on effluent quality parameters. I analysed the samples, performed statistical analysis, and interpreted the data with assistance from Dr. Glen Van Der Kraak. All authors participated in the discussion and the review process of the manuscript.

Abstract

This thesis is a synthesis of studies that explore the effects of urbanization on a small-bodied fish species, rainbow darter (*Etheostoma caeruleum*), in the central Grand River (Ontario, Canada) over time. This watershed has been exposed to various human activities that have deteriorated water quality and adversely affected aquatic organisms. Point-source contaminants are discharged through two municipal wastewater treatment plants (MWWTP) associated with major urban centres (Waterloo and Kitchener). It is well documented that municipal effluents contain contaminants with endocrine disrupting potency. Effluents discharged in the Grand River contain a diversity of estrogenic, anti-androgenic compounds and pharmaceuticals, with various therapeutic activities. Previous research has shown an association between biological responses indicative of endocrine disruption and exposure of rainbow darter to municipal effluents in the Grand River. These responses range from the molecular level all the way to whole organism responses. To improve river water quality and meet new federal effluent quality guidelines, the local municipality (Region of Waterloo) decided to invest in upgrading the two treatment plants. The major upgrades (e.g., nitrification) were initiated at the Kitchener plant in late 2012 while the upgrades at Waterloo were delayed. This created a unique opportunity to design a series of experiments with the main goal to investigate whether responses in rainbow darter previously detected with effluent exposure (e.g., suppressed sex steroid production) would return to reference levels following upgrades in wastewater treatment.

Building on multiple years of studies, changes in these endpoints were assessed in the context of spatial and temporal variability in the watershed. The hepatic transcriptomics in rainbow darter was assessed using a custom microarray to determine molecular responses across an urban environment, with emphasis on changes in MWWTP effluent quality and exposure. The importance of reference site selection on interpretation of omics data was also evaluated. Rainbow darter were collected from nine sites located at different distances from the Waterloo and Kitchener MWWTPs in the central Grand River during the fall season of 2013. Hepatic transcriptome responses indicated that fish collected below Waterloo had the most distinct gene expression profile compared to fish from other sites (including those collected below the outfall of the recently upgraded Kitchener MWWTP). Further, this study concluded that reference site selection matters more when fish are exposed to minor anthropogenic pollution. In contrast, reference site selection has less impact on transcriptomic responses in fish exposed to highly polluted sites. Anthropogenic inputs from

MWWTPs can thus be captured in the rainbow darter liver transcriptome and these responses can inform future assessments of stressors.

The hepatic transcriptome responses in rainbow darter below the two MWWTPs were also examined over time encompassing a pre-upgrade period (2011 - 2012) and a post upgrade period (2013 - 2014). Conducting transcriptomic studies in long-term field-based settings is quite challenging due to uncertainties arising from changes in the ecosystem structure and environmental factors that confound the potential effects of the stressor of interest (wastewater outfalls). Quantitative gene expression analyses (number of significantly different transcripts) were not always reflective of the changes that occurred in effluent quality (upgrades). In contrast, qualitative analysis of selected transcripts and functional analysis of genes involved in different biological pathways suggested that fish responded to the implemented upgrades and improved effluent quality. Following the upgrades, rainbow darter below the Kitchener MWWTP had fewer pathways that were significantly altered in the liver. Moreover, the pathways become more divergent from the Waterloo fish with which they shared great similarities in functional pathways prior to upgrades (2011 - 2012).

In the final study, changes in *in vitro* steroid production in rainbow darter males was compared at sites over six years covering the period of time when the Kitchener MWWTP upgrades were implemented. Rainbow darter were collected from ten sites in the central Grand River located at different distances from the Waterloo and Kitchener MWWTPs in the fall season from 2013 to 2016 (post-upgrades) and compared with samples collected and analysed from 2011 to 2012 (pre-upgrades). Despite high variability in the ability of individual fish to produce sex steroids, the results of this study suggested that 11-ketotestosterone in fish collected below Kitchener MWWTP following the upgrades gradually recovered and 11-ketotesosterone returned to comparable levels to that measured in fish collected at reference sites. In contrast, fish collected below the Waterloo MWWTP, a site that underwent minor changes, displayed an apparent recovery in 11-ketotestosterone and still remained significantly below the reference level in the final years of the study. Testosterone production showed less consistent patterns compared to 11-ketotestosterone and it was more difficult to determine whether this steroid recovered in response to the upgrades.

The results in this thesis advanced our knowledge regarding rainbow darter responses in a system that went through dynamic changes that resulted in an overall quality improvement. However, these results were also critical to the general understanding of the application of

(transcript)omics information into field-based ecotoxicology and provide substance for transcriptomic advancement in regulation. Studying transcriptomic responses across an environmental gradient with multiple reference sites allowed for deeper assessment and quantitation of the transcriptome, and in highly impacted sites, there was considerable consistency in interpretation of responses associated with anthropogenic activities. Moreover, application of transcriptomics methods in a longer-term field-based setting demonstrated that qualitative gene expression analyses (Gene Set Enrichment Analysis and Sub Network Enrichment Analysis) may be more informative than quantitative analysis for the interpretation of responses associated with changes in the environment. Despite considerable natural variability, *in vitro* steroid production was found to be a useful endpoint that responded to wastewater treatment plant effluent quality. Changes in gene expression and steroid production can be viewed as key events responsive to stressors in fish and have a great potential to be studied within the context of an adverse outcome pathway. Taken together, the findings and conclusions derived from these studies have implications beyond the Grand River watershed, and they provide strong foundation for understanding that can be transferred to other fish species similar to rainbow darter.

Acknowledgements

The process that led to the completion of this PhD dissertation has been an incredible journey that helped me become a better scientist but it also helped me grow personally. I am very proud to say that one of the most valuable things that I have learned during these five years is the ability to think critically. Although the thesis bears my name the work that resulted in its finalization involves many remarkable people who helped me along the way and who I am pleased to acknowledge.

First and foremost, I would like to express my gratitude to my advisor, Dr. Mark Servos for guiding me and for providing constant support, both scientific and moral. Mark was very patient and brave to take someone like me, who barely spoke English under his supervision. He gave me the opportunity to do great science, meet and work with top professionals in the field of aquatic toxicology, and in the meantime, I managed to improve my English. Mark would encourage me by constantly reminding me that I am in a good spot, and he was right about that. Therefore, I would like to thank my co-supervisor Dr. Deborah MacLatchy and other committee members Dr. Christopher Martyniuk, Dr. Glen Van Der Kraak, and Dr. Brian Dixon who all contributed with their expertise and who kept me in that good spot throughout all this time. Special thanks to Dr. Christopher Martyniuk who has been a great host several times, an excellent alligator guide, and provided an unforgettable experience at University of Florida.

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my analyses was conducted in Dr. MacLatchy's lab at the Center for Cold Regions and Water Science, Wilfrid Laurier University, and I would like to thank her students for making me feel as a part of their lab and special thanks to the lab manager Dr. Andrea Lister for assisting me with data interpretation and troubleshooting.

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Dedication

Dedicated to the memories of my father Lucian Marjan.

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Chapter 1

General introduction

1.1 Endocrine disrupting compounds

Anthropogenic chemicals that pollute aquatic environments arise from almost all current human activities and are released through various non-point (agricultural runoff, road runoff, etc.) and point-sources (e.g., effluents). Currently, there are over 145,000 unique registered chemicals and an undefined number of their degradation products (European Chemical Agency 2016). The large number of chemicals used in the developed world has outpaced our capacity to thoroughly investigate their risks to the environment. A major point source of anthropogenic chemicals is municipal wastewater treatment plants (MWWTP). In Canada, municipal wastewater effluents represent the largest volume of effluent released into the aquatic environment (Chambers et al., 1997).

It has been well documented that MWWTP effluents contain a variety of contaminants that interfere with the endocrine systems of aquatic organisms (Vajda and Norris, 2011; Tyler et al., 1998). Aquatic vertebrates, including fish, are particularly sensitive to these chemicals that can alter many biological functions compromising their overall fitness, reproductive success and survival (Mills and Chichester, 2005). Endocrine disrupting compounds (EDCs) detected in municipal effluents represent a variety of natural and synthetic hormones, pharmaceuticals, and personal care products (Vajda et al., 2015; Barber et al., 2013; Martinović et al., 2007). Many EDCs have chemical structures that are similar to endogenous hormones and can, therefore, bind to nuclear receptors. These induced changes in gene expression that control different functions in organisms such as sex determination, sex differentiation and development (Leet et al., 2011). EDCs can also cause alterations in signaling along the hypothalamus-pituitary-gonadal axis (HPG), subsequently affecting hormone synthesis, release, transport, metabolism, binding, and degradation processes, which can lead to changes in endocrine function (Norris and Lopez, 2010). There are alternative routes, such as receptor-independent pathways, that EDCs exert their actions, however these pathways have received less attention. Responses in organisms exposed to EDCs can occur across various levels of biological organization depending upon multiple factors including the dose, exposure duration, developmental window at which the exposure occurred, and species sensitivity (Connon et al., 2012). Endocrine disrupting compounds can target different types of hormones, as well as their synthesis, and downstream mechanisms of action. This includes reproductive hormones, (e.g., estrogens, androgens), corticosteroid hormones, thyroidal hormones, growth hormones, and hypothalamus-

pituitary releasing and stimulating hormones (Söffker and Tyler, 2012). Despite this diversity, research efforts have primarily focused on studying the effects of EDCs on reproductive hormones (Söffker and Tyler, 2012), especially EDCs with estrogen agonistic activity. Reproductive hormones are very important as they are involved in sexual differentiation and development (Devlin and Nagahama, 2002), and they control all aspects of reproduction. Reduced reproductive success is considered to be an adverse effect of exposure to EDCs with potential implications at the population level of biological organization (Jobling et al., 2002; Arukwe, 2001).

1.2 Chemicals that are frequently detected in municipal effluents

The purpose of wastewater treatment facilities is to improve water quality prior to discharge. However, most of the existing treatment systems worldwide were not designed to address the removal of the diversity of emerging contaminants that have a broad spectrum of properties and are capable of causing endocrine disruption via various mechanisms. There is substantial evidence that treated municipal wastewater effluents contain estrogenic EDCs. The main contributors to effluent estrogenicity are natural hormones (17β-estradiol, estrone, estriol); synthetic estrogens, common constituents of birth control pills (17α-ethinylestradiol); phenolic compounds (alkylphenols); and phthalate esters (Sun et al., 2014; Sun et al., 2013; Servos et al., 2005; Andersen et al., 2003). Estrogenic EDCs in municipal effluents are associated with biological responses in fish such as vitellogenin induction (mRNA or circulating protein) in male individuals (Folmar et al., 2001; Solé et al., 2001), impairment in development of reproductive ducts (Rodgers-Gray et al., 2001), male fish feminization (Woodling et al., 2006; Nolan et al., 2001; Van Aerle et al., 2001), reduced egg production in female fish (Thorpe et al., 2009), reduced secondary sex characteristics, and female-skewed sex ratios (Vajda et al., 2008; Filby et al., 2007a). In addition, to estrogenic EDCs, there are many other contaminants detected in municipal effluents such as pharmaceuticals (Mennigen et al., 2011; Kolpin et al., 2002), personal care products (Brausch and Rand, 2011), and pesticides (Khan and Law, 2005); however, the effects for the majority of these chemicals remain unknown (Caliman and Gavrilescu, 2009). For instance, many pharmaceuticals are measured at orders of magnitude (μg/L) higher in concentration than estrogenic compounds (ng/L). However, only around 10% have been studied in detail for their environmental effects (Brausch et al., 2012). One of the reasons for the limited studies on these emerging chemicals, including pharmaceuticals, is the lack of ecologically-relevant endpoints to measure. The majority of studies investigating pharmaceutical

effects in fish have assessed endpoints such as acute mortality or sub-chronic responses likely resulting from narcosis (Sanderson and Thomsen, 2009), instead of evaluating endpoints associated with therapeutic characteristics these pharmaceuticals exert in mammalian species (Ankley et al., 2007). In addition, chemicals that are identified in the municipal effluents are usually tested individually to determine their toxic effects, in concentrations that usually exceed environmental levels, even though these chemicals are present as complex mixtures which can influence activity in a different way compared to individual activity. Although chemicals present in multicomponent mixtures may be at concentrations below their (individual) no observed effect concentration (NOEC), they can act in an additive fashion to cause an effect (Silva et al., 2002). Chemicals found in complex mixtures (e.g., municipal effluents) belong to many different classes, thus their effects can be additive, synergistic or antagonistic (Kortenkamp, 2007). In addition, one of the disadvantages of testing chemicals under controlled conditions is that it does not account for physical and biological factors that can change how organisms are exposed and respond (Connon et al., 2012). Therefore, monitoring responses in the field, especially using wild fish populations is very powerful because it provides ecological relevance that captures multiple stressors and natural variability in dynamic environments (Connon et al., 2012). This can not be achieved with laboratory experiments.

1.3 Selection of ecologically relevant endpoints – a major dilemma

EDCs exert their effects across multiple levels of biological organization. This can range from molecular initiating events that may lead to changes in the lower biological organization endpoints, such as gene expression, protein synthesis, and biochemical alterations, to higher levels of organization (organism and population). Due to the complexity of effects that can manifest at various levels, it is difficult to select appropriate endpoints that will be indicative of exposure to specific EDCs. For years, risk assessment programs have focused on investigating apical endpoints such as reproduction, growth and development, although these endpoints are not very informative in terms of the mechanisms of action of EDC exposure (Hutchinson et al., 2006). Despite the fact that effects caused by municipal effluent exposure in teleost species have been researched for several decades, there is still no general consensus nor well-developed guidance for biomonitoring programs that would contain recommendations on endpoints and responses that should be assessed. As pointed out in the International Bioassay Use for Effluent Management developing regulatory guidance is difficult because different jurisdictions have different environmental protection goals (Power and

Boumphrey, 2004). For example, the primary goal of the Canadian Environmental Effects Monitoring Program was to ascertain whether fish or macroinvertebrates are impacted by stressors from effluents discharged from treatment plants that are in compliance with their effluent discharge regulations. The program was mainly designed for monitoring industrial discharges from pulp and paper mills and mining site operations (Kilgour et al., 2007). One of its components recommends surveys on adult fish and the endpoints included are sex, age, length, liver size, gonad size, fecundity, egg size, and external appearance as these endpoints provide comprehensive assessment of effluent effects on survival, energy storage, and energy use (Walker et al., 2002). A concept introduced by (Ankley et al., 2010), termed adverse outcome pathway (AOP), identifies effects at multiple levels of biological organization, by incorporating changes in molecular initiating events, and determining key biological events that lead to adverse outcomes that manifest at the organism or population levels which is more relevant for risk assessment. Applying such a concept in environmental biomonitoring of municipal effluent effects is desirable as it gives an overview of the overall responses of an exposed organism. However, there are certain limitations and challenges when it comes to extending the AOP application to include the complexities of exposure in aquatic environments. This concept incorporates compound-specific risk assessment and it is based on data gathered under controlled or semi-controlled conditions (e.g., caging) which increases its application as a predictive tool for adverse outcomes. Municipal effluents, on the other hand, are complex mixtures and it is difficult to predict their effects especially for non-model species for which there is limited information on molecular responses (e.g., genome sequencing).

1.4 Gene expression – transcriptomics

Changes in gene expression are the first in a line of responses and they are considered to be sensitive indicators of an organism's interaction with its surrounding environment. The rapid growth in the field of molecular biology and genomics has given rise to a variety of techniques and tools that allow measurements of molecular responses. Transcriptomics, using microarray cDNA oligonucleotides spotted on glass slides, is a powerful method that allows for the simultaneous quantification of a multitude of gene transcripts that can potentially reveal underling changes in physiology and behavioral responses in an organism (Figure 1.1) (Bartosiewicz et al., 2000). Further, this technique can be used to identify individual molecular transcripts and pathways that can be used as biomarkers of contaminant exposure (Snape et al., 2004; Waters and Fostel, 2004). Examining

transcriptional expression profiles can allow determination of functional networks of the biochemical process and specific modes of action (MOA) of environmental stressors (Miracle and Ankley, 2005).

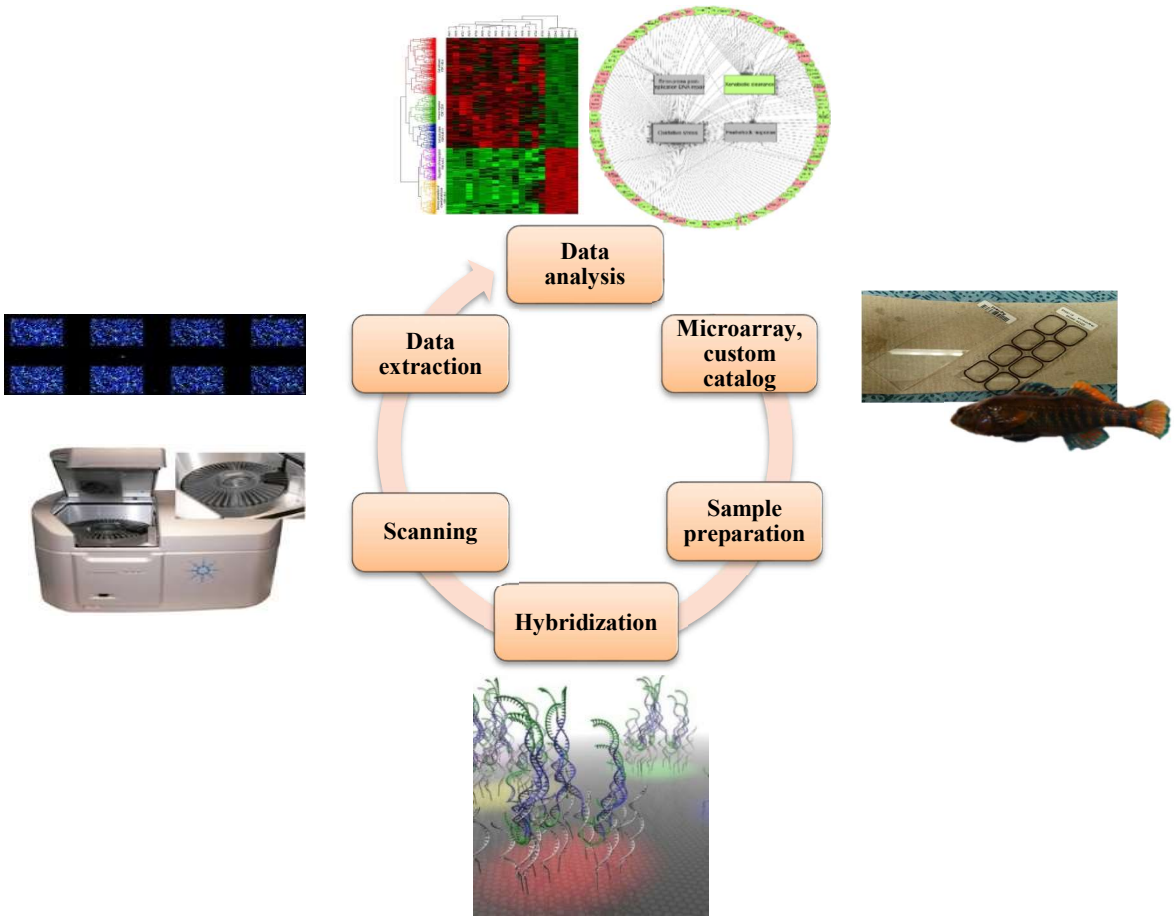


Figure 1.1 Diagram of a custom-made microarray slide for rainbow darter (*Etheostoma caeruleum*) and the key steps involved in the transcriptomic analysis.

Functional expression of genes is carried out by the proteins, thus expression of messenger ribonucleic acid (mRNA) is subjected to translational processes that are susceptible to various alterations caused by endogenous factors (Maier et al., 2009) as well as exogenous factors (e.g., treatment with 17β-estradiol), which can substantially modify the final product(s) (Denslow et al., 2005). This also results in regulation changes at both transcription and post-translational level and this can cause differences in transcript and protein abundance. For example, correlation between mRNA level and protein expression was reported to be r^2= 0.61 (Denslow et al., 2005). Molecular signatures have varying potentials to be translated into more meaningful responses at higher levels of biological organization. Therefore, detecting changes at the molecular level can serve as an early warning sign of exposure to compromised environments and allow detection of potential adverse outcomes. Transcriptomic analysis has proven a useful diagnostic tool for single compounds (Martyniuk et al., 2013; Martyniuk et al., 2011; Martyniuk et al., 2010; Jia et al., 2011; Knoebl et al., 2006) and for identifying specific stress-responsive transcripts. Recently, this technique has become more applicable in field-based environmental toxicology and it was shown to be useful in differentiating clean sites from contaminated sites (Asker et al., 2013; Osachoff et al., 2013; Vidal-Dorsch et al., 2013; Falciani et al., 2008).

Recent studies demonstrated that transcriptomics can bridge the gap between molecular responses to apical endpoints (Bahamonde et al., 2015a; Vidal-Dorsch et al., 2013; Garcia-Reyero et al., 2009). Although transcriptomics has not yet found wide acceptance as a monitoring tool in regulation, there have been efforts to address issues with managing high-throughput data to increase reproducibility, minimize variability, and standardize interpretation in order to move omics from proof of concept to a functional tool that can be applied in standardized ecosystem health assessment (Feswick et al., 2017; Bahamonde et al., 2016). Genomic approaches hold promise to become a biomarker tool because they enable a broad spectrum of response parameters to be assessed and methods are transferable to different species (Connon et al., 2012). Hence, their potential has been widely recognized in systems biology which is designed to systematically collect mechanistic information that can be used in diagnostic and predictive assessments of the risk of toxic chemicals and their effects in fish or other model species used for ecological risk assessment (Miracle and Ankley, 2005).

1.5 Steroidogenesis

Steroidogenesis can be defined as sex steroid production and this process has been best-studied in mammalian species. Despite the phylogenetic distances between teleost fishes and mammals, steroidogenesis and the neuroendocrine control of reproduction share a significant number of common features (Norris and Lopez, 2010). The production of androgens, estrogens and progesterones by the ovarian and testicular tissues, is controlled by gonadotropin hormones that are released from the pituitary gland under stimulation of gonadotropin releasing hormone (Kime, 1995) (Figure 1.2). Photoperiod and temperature also play important roles in steroidogenesis as they represent the main cues that the hypothalamus interprets as signals to start producing gonadotropin releasing hormone (Pankhurst and Porter, 2003). There are several types of gonadotropin hormones that are present in teleost fish species, formally called GTH I and GTH II (Querat et al., 2000), which are analogous to the mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH), and now termed as such, respectively. There are two main androgen hormones in male fish, testosterone and 11-ketotestosterone, and they are mainly produced in the testis tissue, and have affinity to bind to androgen receptors (AR) in various tissues and affect gene expression. However, 11-ketotestosterone is considered to be more biologically active than testosterone (Evans et al., 2014; Norris and Lopez, 2010). In female fish, 17β-estradiol is the main estrogen produced by the ovaries which exerts its activity in various tissues through binding to the estrogen receptor (ER) (Evans et al., 2014; Norris and Lopez, 2010).

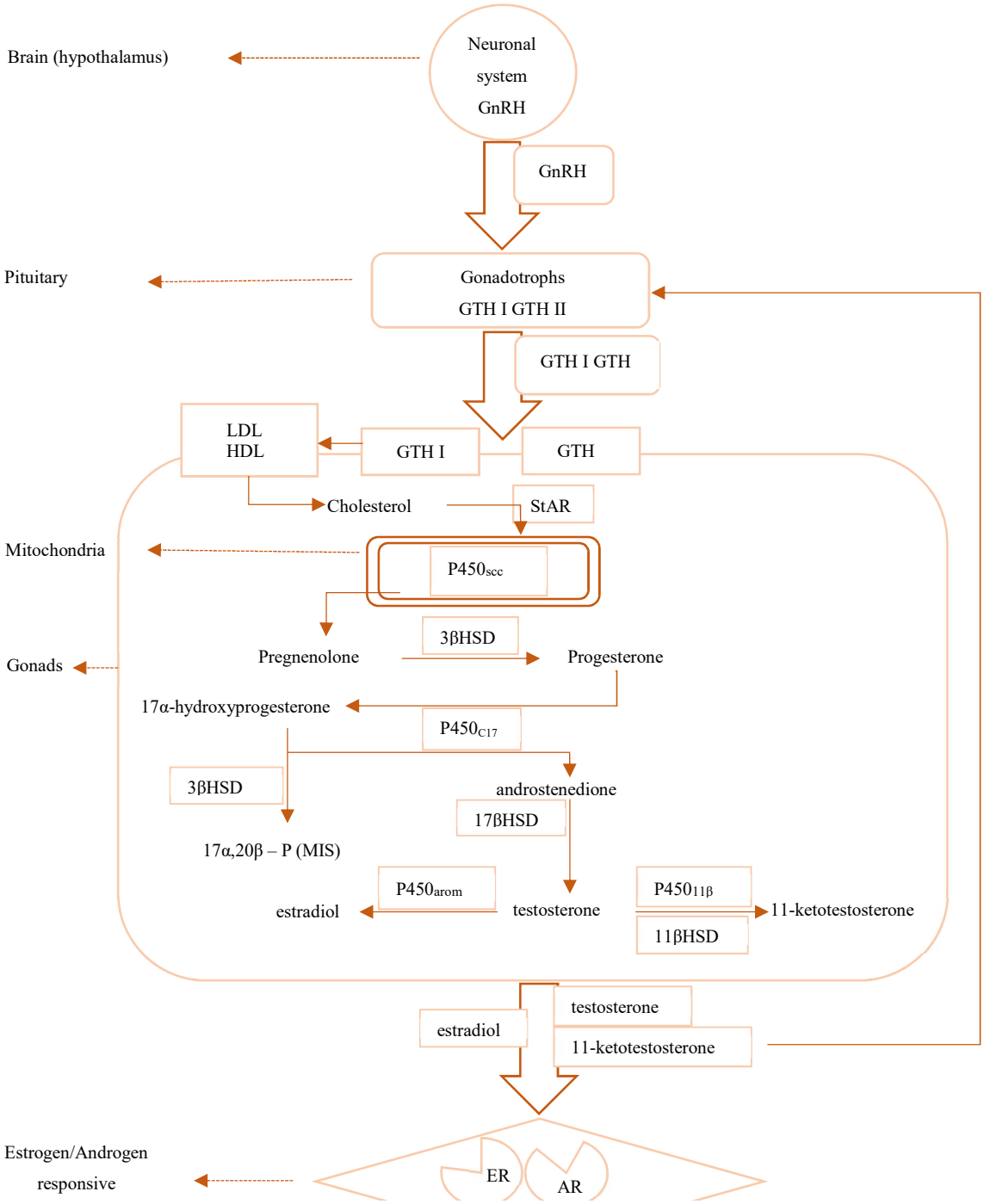


Figure 1.2 Diagram of hypothalamus-pituitary-gonadal (HPG) axis with key factors controlling the steroidogenic pathway that involve gonadotropin releasing hormone (GnRH) synthetized in the hypothalamus and gonadotropin hormones (GTH I, GTH II) produced in the pituitary gland. Steroidogenesis occurs in the gonadal tissue (theca and granulosa cells/Leydig cells) and it starts with cholesterol which serves as a precursor for synthesis of progesterones, androgens, and estrogens through a series of reactions mediated by various steroidogenic enzymes. Diagram adapted (simplified) from Ankley et al., (2009).

Environmental contaminants can act at various sites along the HPG axis and consequently alter the production and ratios of sex steroids in fish plasma (Ankley et al., 2009). However, one of the most typical ways EDSc act as naturally occurring sex steroids is through effects on key enzymes that regulate steroidogenesis in either ovaries or testes (Ankley et al., 2009; Filby et al., 2007b; Filby et al., 2007c; Hinfray et al., 2006). Sex steroids are important because they regulate gametogenesis and play a central role in mediating reproduction. Their role in sex differentiation during early development is debatable with studies showing inconsistent results, some arguing that they have an important role in sex differentiation, while others suggesting that sex steroids are synthetized only after sex differentiation (Strüssmann and Nakamura, 2002). Further, optimal production of estrogen and androgen hormones impacts the development of secondary sex characteristics, and they are also involved in brain differentiation, hence they can regulate sexual behaviour (Pradhan and Olsson, 2015; Gonçalves and Oliveira, 2010; Nelson, 2005). Measuring sex steroids in fish exposed to EDCs can provide evidence of endocrine disruption that may be related to reproductive parameters such as fecundity which can subsequently have implications at the population level (Ankley et al., 2009; Ankley et al., 2001). In a life cycle toxicity test and early life stage toxicity test on Japanese medaka (*Oryzias latipes*) developed by the United States Environmental Protection Agency, steroid production is assessed as an exposure endpoint that can be associated with various reproductive endpoints (e.g., time of spawning, egg size, larval survival) as well as to histological endpoints (e.g., gonadal reproductive state/abnormalities). Alterations in sex steroid production was used as an endocrine response in a number of studies examining environmental effects of bleached kraft mill effluents in wild populations of white sucker in the Lake Superior (Bowron et al., 2009; McMaster et al., 1995; Munkittrick et al., 1994; McMaster et al., 1991; Munkittrick et al., 1991). Steroid production was also used as a relevant endpoint in assessing exposure effects of wastewater effluents on fish (Folmar et al., 2001; Folmar et al., 1996), or contaminated water streams (Hecker et al., 2002; Goodbred et al., 1997). The above cited studies reported decreased production of sex steroids in fish caught downstream of the municipal outfalls or highly-polluted areas. Changes in concentrations of sex steroids are often confounded by natural factors such as photoperiod and water temperature that mostly depends upon rain and runoff events (Jobling, 1994). In addition, individual differences in fish capacity to produce sex steroids are contributing to their relatively high variability. Thus, to reduce the effects of confounding factors and individual differences on steroid production it is of

great importance to conduct multi-year and multi-season data collections to better interpret the significance of different hormone concentrations (Iwanowicz et al., 2009).

1.6 Recent research in the Grand River

The Grand River watershed in southern Ontario is the largest drainage basin, covering an area of 6800 km^2 with an estimated one million inhabitants. The watershed receives various inputs that represent a mixture of domestic and industrial wastes from 30 MWWTPs, urban storm water and run-off from non-point pollution sources such as agriculture, which influences 71% of its extent. The central part of the watershed has been under intense urban development that has compromised the quality of its receiving body as reflected in increased nutrient levels such as non-ionized ammonia (Loomer and Cooke, 2011), and hypoxic conditions (Jamieson et al., 2013; Loomer and Cooke, 2011). Effluents discharged in the Grand River contain pharmaceuticals and personal care products (Metcalfe et al., 2010; Servos et al., 2005), and endocrine disrupting chemicals (Hicks et al., 2017), some of which can be traced in the surface water downstream of major municipal effluent outfalls (Arlos et al., 2015).

Biological effects associated with municipal effluents have been monitored in two fish species, the greenside darter (*Etheostoma blennoides*), and more intensively the rainbow darter (*Ethestoma caeruleum*). Both species have very similar biology, however, rainbow darter are more abundant and have a more even distribution across the watershed (Hicks, 2017; Tetreault et al., 2011), and their sexual dimorphism is more obvious. In addition, the relatively short life span and a small home range make this small-bodied species a suitable model for studying effects of municipal effluent exposure. Effluent exposure has been associated with changes at various levels of biological organization in rainbow darter from molecular responses to whole organism levels. Increased expression in vitellogenin mRNA was observed in male rainbow darter downstream of the major outfalls in the central Grand River (Fuzzen et al., 2016; Bahamonde et al., 2014). Molecular signatures were specific and discernable in fish that were severely intersex (Bahamonde et al., 2014). Intersex was one of the most consistent endpoints observed in rainbow darter to wastewater effluent with very high prevalence and severity downstream of the Kitchener and the Waterloo MWWTPs (Fuzzen et al., 2015). In addition, rainbow darter males showed decreased levels of 11-ketotestosterone and delayed sperm development (Fuzzen et al., 2016; Bahamonde et al., 2015b). A

study conducted by Fuzzen et al. (2015) demonstrated decreased fertilization success of rainbow darter collected downstream of the Kitchener MWWTP, indicating potential population-level effects.

The municipality of Kitchener-Waterloo, which is located in the central stretch of the Grand River watershed, is serviced by two major treatment plants (Waterloo and Kitchener MWWTPs). These two MWWTPs are considered to be major contaminant contributors in the Grand River watershed and as such, have raised concerns about environmental impacts. With the prediction that the population of the watershed will increase by 38% by 2036 (Environmental Commissioner of Ontario, 2010) and the need to meet new national effluent quality regulations, the Region of Waterloo decided to invest in infrastructure upgrades of these two MWWTPs. The major infrastructure upgrades include adding nitrifying activated sludge to improve the removal of ammonia and consequently improve the removal of many other contaminants. In 2012, infrastructure upgrades were initiated at the Kitchener MWWTP and upgrades continue to date (Figure 1.3).

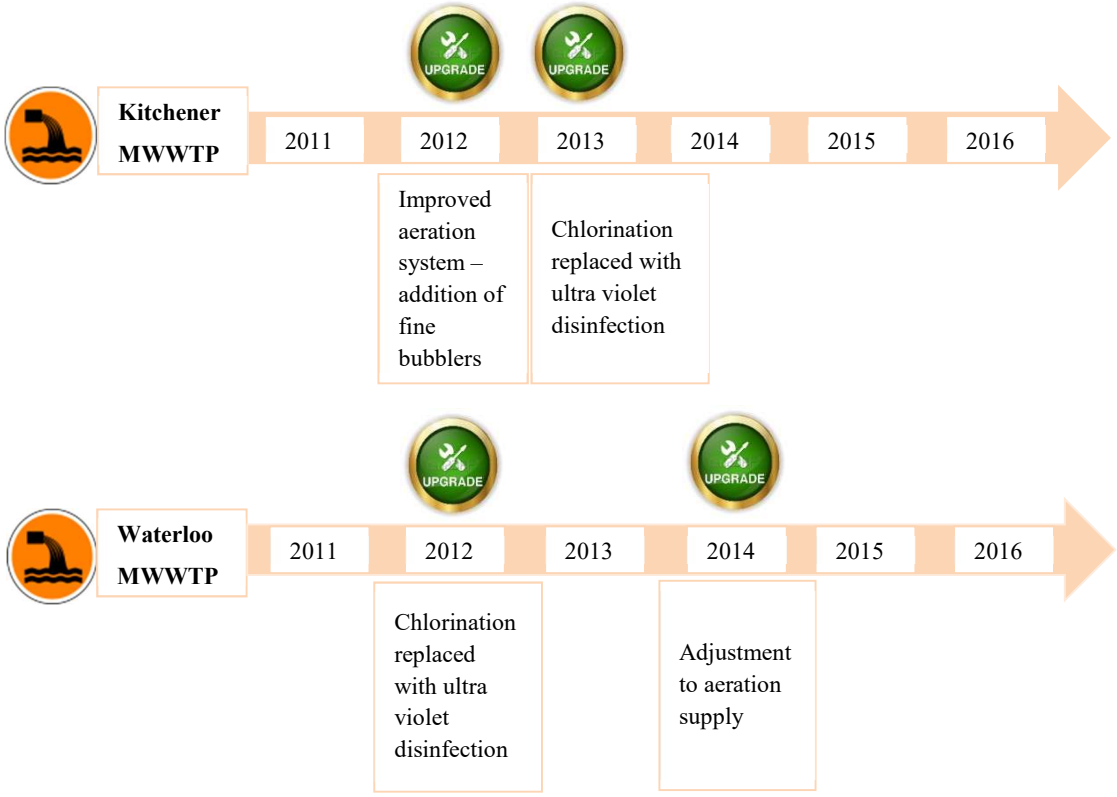


Figure 1.3 Timeline of major process changes implemented at the Kitchener and Waterloo municipal wastewater treatment plants from 2011 to 2016.

However, due to contractual issues, there were only minor infrastructure upgrades at the Waterloo MWWTP. Although completion of the upgrades is expected by 2022, major improvements in effluent quality in the Kitchener effluent have already been observed after the first phase of upgrades (Bicudo et al., 2016).

As discussed above, abnormalities in the rainbow darter gonadal tissue structure (e.g., intersex, delayed spermatogenesis) and their function (e.g., sex steroid production) have been well documented. Therefore, it was of great interest to determine whether changes in effluent quality were reflected in improved health of the receiving environment, including recovery in biological responses in fish that have previously shown to be impaired. Some of these questions have already been addressed in the work of Hicks (2017) who reported significant improvement in stable isotope signatures and reduced intersex occurrence and severity in rainbow darter males following the treatment plant upgrades. Sex steroid production in rainbow darter males was one of the endpoints shown to be supressed at sites immediately below the MWWTP outfalls in the Grand River in the pre-upgrade period (2011 - 2012). In addition, reduction of androgens was previously associated with severely intersex rainbow darter males, with altered sexual behaviour (reduced territorial defense, aggressiveness), and secondary sex characteristics (reduced colour intensity) (Fuzzen, 2016). Thus, it was important to continue to monitor this endpoint that is indicative of reproductive health impairment. Moreover, it was important to determine if sex steroid production (11-ketotestosterone and testosterone) returned to that measured in fish collected from reference sites (reference level) following the treatment plant upgrades (2013 - 2016). Reduced reproductive success is considered to be a relevant endpoint in risk assessment and can have implications at the population level. For example, the site of action for many environmental chemicals is the liver. Bahamonde et al. (2014) and Fuzzen et al. (2016) explored the potential of estrogenic compounds in the effluents to induce vitellogenin mRNA in the liver of adult rainbow darter males and females. Hepatic transcriptome responses in the liver of rainbow darter males in the Grand River have not been examined before although they can elucidate toxicity mechanisms associated with exposure to MWWTP effluents and can be used to discover novel responses in addition to those related to reproduction. Thus, investigating hepatic transcriptome responses in rainbow darter males allows for the identification of pathways indicative of stress-related exposure, but also provides ways to determine potential recovery associated to effluent quality improvement across time (2011 - 2014).

1.7 Thesis objectives

The overall objective of this thesis was to assess the impacts of MWWTP effluent exposure on spatial and temporal variability of biological responses in wild rainbow darter. To achieve this goal, a series of field-based studies were conducted in the fall season from 2013 to 2016 in the central Grand River at ten sites located at different spatial distances from the Waterloo and the Kitchener MWWTPs. One of the major objectives of the assessment of temporal variability in biological responses was to investigate if the upgrades implemented at the Kitchener MWWTP in 2012 resulted in recovery of the selected endpoints in fish including hepatic gene expression and sex steroid production (11-ketotestosterone and testosterone). To address this specific objective, this thesis used data collected from previous studies, conducted in 2011 and 2012 prior to upgrades and new data that were collected in fish following the upgrades from 2013 to 2016 to assess the changes in *in vitro* sex steroid production and gene expression. Specific objectives of this thesis were accomplished through the individual projects presented in three chapters:

Objective 1: The first chapter examines spatial variability in the rainbow darter hepatic transcriptome across an urban environment in the central Grand River. One of the goals of the study was directed to investigating how much variation observed in the transcriptome is due to reference site selection. To address this specific question transcriptomic expression in fish collected from various sites across the urban environment were compared to transcriptomic responses in fish from three reference sites (in three independent data analyses scenarios). The second goal was to determine specific pathways associated with exposure to two wastewater treatment plant outfalls (Waterloo and Kitchener).

Objective 2: In addition to the spatial responses in the transcriptome this thesis addressed temporal changes in the hepatic transcriptome responses in rainbow darter males collected downstream of the MWWTPs. The main objective of this chapter was to investigate the hepatic transcriptome responses in fish associated with changes occurring in two MWWTPs (Waterloo and Kitchener) in the Grand River from 2011 to 2014 and to determine whether upgrades to the Kitchener MWWTP resulted in transcriptome responses indicative of changes in contaminant exposure.

Objective 3: The third chapter investigated changes in *in vitro* sex steroid production by testis tissue of rainbow darter across the urbanized environment over time (2011 - 2016). Moreover, the specific goal in this chapter was to assess if there was recovery of *in vitro* sex steroid production following the upgrades at the Kitchener MWWTP (2013 to 2016).

Chapter 2

How does reference site selection influence interpretation of omics data? Evaluating liver transcriptome responses in male rainbow darter (*Etheostoma caeruleum*) across an urban environment

2.1 Chapter summary

Studies quantifying the influence of reference site selection on transcriptomic profiles in aquatic organisms exposed to complex mixtures are lacking in the literature, despite the significant implications of such research for the interpretation of omics datasets. We measured hepatic transcriptomic responses in fish across an urban environment in the central Grand River watershed (Ontario, Canada). Adult male rainbow darter (*Etheostoma caeruleum*) were collected from nine sites at varying distances from two major municipal wastewater treatment plants (MWWTPs) (Waterloo, Kitchener), including three upstream reference sites. The transcriptomic response in rainbow darter was independently compared with that of fish from each of the three reference sites. Data collected in fish downstream of the Waterloo MWWTP (poorest effluent quality) suggested ~15.5 % of the transcriptome response was influenced by reference site selection. In contrast, at sites where the impact of MWWTPs was less pronounced and fish showed less of a transcriptome response, reference site selection had a greater influence (e.g., ~56.9% of transcripts were different depending on the site used). This study highlights the importance of conducting transcriptomic studies that leverage more than one reference site and it broadens our understanding of the molecular responses in fish in dynamic natural environments.

2.2 Introduction

Over the last several decades, global efforts have been made to identify mechanisms by which contaminants from municipal wastewater treatment plants (MWWTPs) disrupt the endocrine system of aquatic species. Studies have demonstrated a relationship between male feminization in wild fish and exposure to MWWTP effluents (Vajda et al., 2008; Bjerregaard et al., 2006; Woodling et al., 2006; van Aerle et al., 2001). It has also been well documented that exposure to municipal effluents impairs steroid production in fish (Fuzzen et al., 2016; Blazer et al., 2012; Jobling et al., 2002), alters secondary sex characteristics (Vajda et al., 2011), disrupts reproductive behaviour (Garcia-Reyero et al., 2011), and can lead to delayed ovulation and spermatogenesis (Bahamonde et al., 2015b; Tetreault et al., 2011; Johnson et al., 2008). Contaminants detected in municipal effluents display a wide range of estrogenic and androgenic effects, and each can act as both receptor agonists and/or antagonists. These compounds affect fish at various levels of biological organization, from molecular to apical endpoints. Natural and synthetic estrogens (e.g., 17α-ethinylestradiol) and several industrial chemicals (e.g., nonylphenol, bisphenol A) that have been commonly found in municipal effluents have been identified as the main culprits responsible for modulating estrogen-receptor dependent processes (Thorpe et al., 2003; Loomis and Thomas, 1999; Desbrow et al., 1998; Routledge et al., 1998), such as the induction of vitellogenin protein and gene expression in male fish (Fuzzen et al., 2016; Sanchez et al., 2011; Hinfray et al., 2010; van Aerle et al., 2001). Contaminants from MWWTPs are also known to disrupt the expression of transcripts involved in the hypothalamic-pituitary-gonadal axis and hypothalamic-pituitary-adrenal axis that control a variety of regulatory pathways (Liang et al., 2015; Villeneuve et al., 2007). Identification of these regulatory pathways at the molecular level can provide important insights into the physiological and behavioural responses of organisms chronically exposed to chemical stressors.

The field of transcriptomics includes widely used approaches that allow regulatory pathways to be thoroughly investigated and subsequently the overall biological state of the organism to be assessed (Denslow et al., 2007). Using transcriptomic signatures, it can be possible to identify responses to individual stressors (or mechanisms) in fish exposed to complex mixtures (Denslow et al., 2007). However, variation in biotic and abiotic factors can confound interpretation of transcriptomic responses, making it difficult to distinguish the effects of specific stressors, which limits the use of transcriptomics in environmental biomonitoring and risk assessment. Nevertheless,

studies in the literature have demonstrated that transcriptomics can be useful in field-based studies to differentiate individuals collected at contaminated sites from those collected at pristine sites (Christiansen et al., 2014; Baker et al., 2013; Cuklev et al., 2012; Garcia-Reyero et al., 2009), in addition to providing insight into the mechanisms of action underlying changes at higher levels of biological organization (Vidal-Dorsch et al., 2013; Shelley et al., 2012; Duan et al., 2010; Ankley et al., 2009; Tilton et al., 2008).

The central part of the Grand River, in southern Ontario, which flows through a densely-populated area, has historically had degraded water quality, primarily because of discharges from two major MWWTPs (Waterloo and Kitchener). Rainbow darter (*Etheostoma caeruleum*) have proven to be a useful sentinel species for biomonitoring in the Grand River as they are well distributed throughout the watershed, are sexually dimorphic, have a relatively short lifespan, and exhibit a small home range (Paine, 1990). Exposure of rainbow darter, especially those with a high level of intersex, to wastewater effluent has also been associated with changes in the gonadal transcriptome (Bahamonde et al., 2015a; Bahamonde et al., 2014). Adverse effects associated with wastewater exposure have been reported on gonadal development, histology (e.g., intersex), *in vitro* steroid production, reproductive behaviour, and fertilization (Fuzzen et al., 2016; Fuzzen et al., 2015). Although these studies have demonstrated a variety of negative effects on rainbow darter, there is limited information on how regulatory pathways are impacted at the transcriptome level in rainbow darter exposed to municipal effluents (Bahamonde et al., 2014). Changes at the transcriptome level are argued to be more sensitive than changes in higher level endpoints and theoretically allow for earlier detection of adverse effects. Moreover, high-throughput molecular analyses can uncover previously unidentified pathways that may be related to adverse effects, such as impaired growth or metabolism. Analysis of molecular endpoints may therefore inform and support risk assessments for rainbow darter exposed to wastewater effluents. As the liver has a major role in metabolism, it is the site of action for many environmental contaminants (e.g., detoxification). Assessing the rainbow darter hepatic transcriptome can be useful in elucidating toxicity mechanisms associated with exposure to MWWTP effluents.

The objective of this study was to determine if there were specific molecular pathways in rainbow darter liver that were associated with exposure to two MWWTP outfalls (Waterloo and Kitchener) in the Grand River. To determine how much of the variation observed in the hepatic

transcriptome response was associated with reference site selection, male rainbow darter from three reference sites were compared with fish collected downstream of the outfalls. Transcriptomic data were supported with measurements of river water quality in terms of nitrogenous products, selected indicator chemicals, and total estrogenicity of the effluent samples. The chemicals were selected because they act as indicators of effluent quality.

2.3 Materials and methods

2.3.1 Study design and site description

Rainbow darter adult males were collected from nine river sites across an urbanized environment that includes two major MWWTP outfalls in the central Grand River watershed: three reference sites (US1, US2, and US3) in the upstream non-urbanized reaches, a site located downstream of the Waterloo MWWTP (DSW), an intermediate site between the two MWWTPs (INT), a site immediately downstream of the Kitchener MWWTP (DSK1), and three sites further downstream (DSK2, DSK3, and DSK4) (Figure 2.1). Fish were sampled over six days in late November 2013. There were 15 - 20 male rainbow darter that were captured at each site by backpack electrofishing. Immediately following capture, morphometric characteristics were recorded (length ± 1 mm and weight ± 0.001 g), and a portion of liver tissue was frozen in liquid nitrogen and stored at -80^{o}C until analysis. A subset of 63 (7 samples × 9 sites) liver samples were analyzed using a rainbow darter microarray (8 × 15 K format GPL18038, Agilent Technologies Inc.). The fish sampled were similar across the sites, with only minor differences ($p < 0.05$) in length, weight, liver somatic index (LSI), and condition factor (k); there were also minor differences in gonad somatic index (GSI) in the fish from reference site US2 compared with the fish from all other sites (Table S2.1).

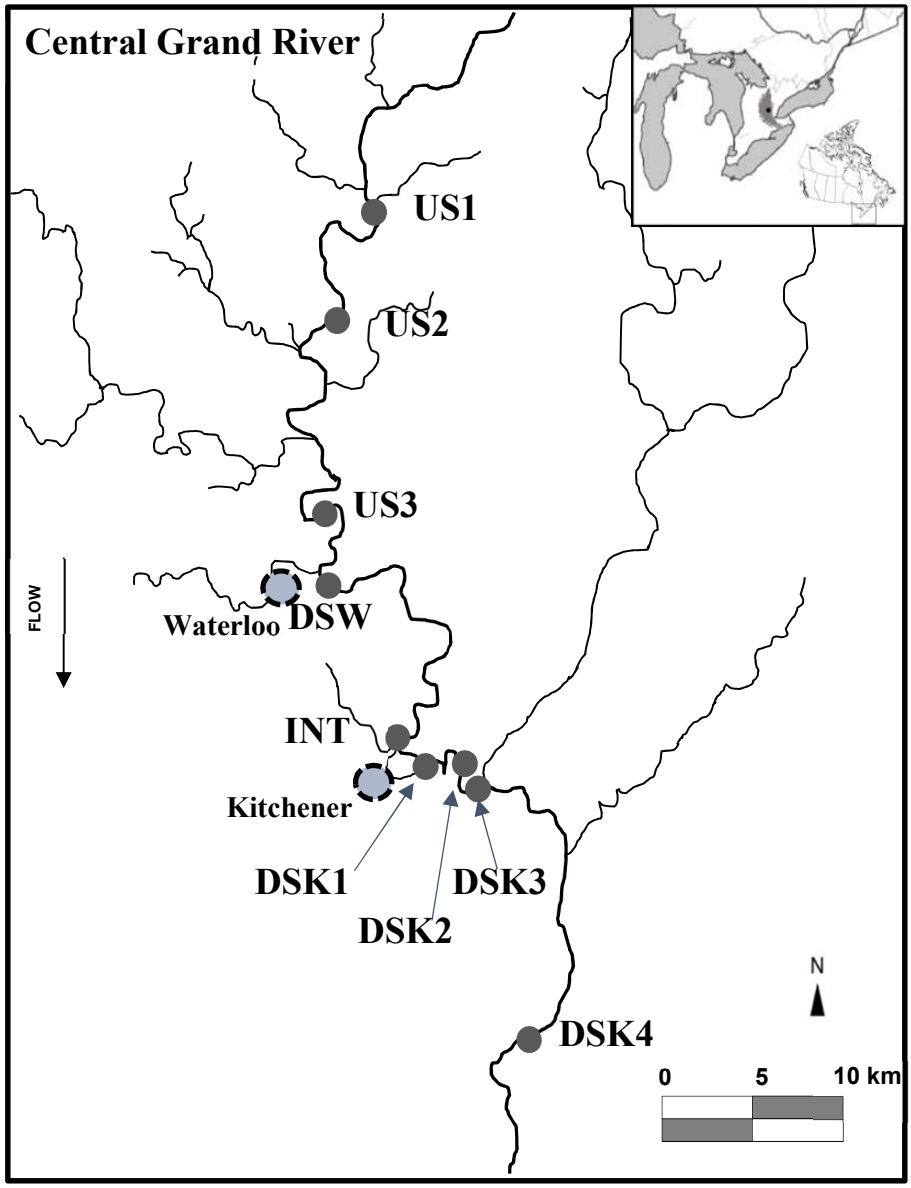


Figure 2.1 Geographical distribution of sampling sites in the central part of the Grand River. The study design incorporated nine sampling sites: three reference sites (US1, US2, and US3), a site located downstream of the Waterloo MWWTP (DSW), an intermediate reference site located in the urbanized area (INT), a site located downstream of the Kitchener MWWTP (DSK1), and three downstream river sites (DSK2, DSK3, and DSK4).

2.3.2 Chemical analysis

Specific chemicals (the pharmaceuticals ibuprofen, naproxen, triclosan, carbamazepine, and venlafaxine and the herbicide atrazine) were selected because they are good indicators of effluent quality and wastewater processes, not because they are directly linked to specific biological processes. Chemical analyses of surface water samples (500 mL) and final effluent samples (125 mL) were conducted as previously described (Couperus et al., 2016). After extraction with Agilent Bond Elut Plexa cartridges and spiking with surrogate standards, the indicator chemicals were separated by liquid chromatography (Agilent 1200; Eclipse XBD-C18) and quantified using a Sciex API 3200 QTRAP mass spectrometer with electrospray ionization in multiple reaction monitoring mode (Couperus et al., 2016; Wang et al., 2011). A separate 500 mL sample of effluent was extracted and analyzed for total estrogenicity using the yeast estrogen screen (YES) method outlined in Appendix A. Total ammonia, nitrite, and nitrate concentrations were measured in the samples by Maxxam Analytics (Mississauga, ON). Additional river water data were provided by the Region of Waterloo under a data-sharing agreement (Table S2.2).

2.3.3 Transcriptomics analysis

Total ribonucleic acid (RNA) was isolated from liver samples using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. In brief, a portion of liver tissue (<30 mg) was homogenized with 800 µL of QIAzol reagent (provided in the kit). The aqueous phase containing nucleic acids was separated with chloroform and subsequently loaded onto RNeasy Mini columns. RNA was eluted from the columns with ~30 µL of nuclease-free water. The concentration and A260/A280 ratio of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada), whereas RNA integrity was determined with an Agilent R6K ScreenTape System 2200 TapeStation (Agilent Technologies). Samples with A260/A280 ratios of 1.8 - 2 and RNA integrity scores (RIN) ≥ 8 were selected for labelling in the subsequent steps. The mean RIN score for all samples was 9.0 ± 0.4 (average ± SD). Isolated RNA served as a template for complementary DNA synthesis and subsequently for complementary RNA (cRNA) *in vitro* transcription using a Low Input Quick Amp Labelling Kit (Agilent Technologies) as per instructions provided in the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with cyanine 3 (Cy3). All samples were labelled using 50 ng of RNA input. Labelled cRNA was purified with buffer RLT, molecular grade ethanol, and buffer RPE (QIAGEN). Purified cRNA

was eluted from the columns with ~25 µL of nuclease-free water. Samples with specific activity higher than 6 pmol of Cy3 per microgram of cRNA were used for hybridization. Samples were adjusted to 600 ng of labelled RNA and subsequently subjected to fragmentation, followed by hybridization for 17 h at 65ºC in the microarray oven. A 15,000-oligonucleotide microarray was used for sample analysis (GEO accession number: GSE95082). The rainbow darter microarray contains 14 948 target sequences of which 8862 are unique sequences. There are 6087 duplicated sequences on the microarray. The gene sequences were annotated to the zebrafish (*Danio rerio*) genome sequences using UniProt. Microarray slides were washed according to instructions provided by Agilent (Gene Expression Hybridization Kit) and scanned with an Agilent G2505C microarray scanner at 5 µm density. Raw signal intensities were extracted using the Agilent Feature Extraction software (v. 10.1.1.1). Microarray reports for each sample were generated following array scanning and were individually inspected for quality. All samples were deemed to be of high quality (background levels < 5%, linear relationship of spike-in standards).

2.3.4 Bioinformatics

Microarray data were normalized by the quantile normalization method and analyzed in JMP Genomics (v 7.0, SAS Institute Inc.). Data were filtered to a value of 2.33, which was reasoned to be the detection limit of the arrays on the basis of the control spots and the dynamic range of the Agilent spike-in (which was no longer linear). Probes with intensity below 2.33 were assigned the limit of detection (log2 intensity = 2.33). Control spots were removed from the dataset and excluded from analysis. To identify differently expressed genes, normalized and filtered data were subjected to one-way analysis of variance (ANOVA) with a false discovery rate set at 5% (FDR = 0.05) (JMP Genomics).

The first part of data analysis focused on determining the impact of reference site selection (US1, US2, and US3) on gene expression patterns. Rainbow darter from each reference site were used as a control in three independent analyses (scenarios). To visualize differences in transcriptomic patterns among the nine groups (sites) on the basis of reference site selection, heat maps were generated to determine clustering of samples per treatment group by using the least square mean of gene probes for each site.

The second part of analysis focused on identifying differentially expressed pathways at the high-impact sites. Gene set enrichment analysis (GSEA) and subnetwork enrichment analysis

(SNEA) were conducted using rainbow darter from reference site US2 as a control. Site US2 is upstream of the major urbanized areas that are the focus of this study, and biological responses and water quality at this site have been well characterized (Fuzzen et al., 2016; Bahamonde et al., 2015a; Fuzzen et al., 2015) in previous studies. Thus, we reasoned this was the most appropriate reference site for such comparisons.

Parametric Analysis of Gene Set Enrichment (PAGE) (Kim and Volsky, 2005) was conducted in JMP Genomics. This statistical method was used to determine significantly upregulated or downregulated gene sets in rainbow darter liver that share common biological processes, molecular functions, or cellular components. Unique gene sets differentially expressed in rainbow darter livers from exposure sites (DSW and DSK1) and downstream river sites (DSK2, DSK3, DSK4) were identified with Venny 2.1.0 - BioninfoGP - CSIC (Oliveros, 2007 - 2015). It is useful to identify unique gene sets significantly enriched in a group of fish compared with the entire dataset because this may indicate that the gene sets are responsive to MWWTP effluent exposure or that recovery is occurring at the sites further downstream.

Subnetwork enrichment analysis was conducted in Pathway Studio 9.0 (Elsevier) with ResNet 9.0. This software uses known relationships (i.e., based on expression, binding, common pathways) between genes to create networks focused around gene hubs. Annotated pathways in Pathway Studio were expanded to include cell processes. The function "Name + Alias" was used to map rainbow darter genes to mammalian homologs in Pathway Studio, and 5968 probes were successfully mapped. Duplicate genes were controlled for in the pathway analysis by using the "highest magnitude, best *p*-value" function. After we determined the most enriched gene subnetworks, we worked to identify unique (SNEA) cell processes in rainbow darter at the effluent-exposed sites (DSW and DSK1) and at the downstream sites (DSK2, DSK3, and DSK4).

Although GSEA is more widely used in microarray analysis, conducting SNEA in the present study may provide additional insight into patterns of hepatic gene expression in rainbow darter males across the urban environment. While GSEA determines if a specific predefined pathway is enriched, SNEA allows for the subnetworks to be built on the basis of the relationship of the genes in the same biological process. These expression networks are not known a priori (as in the case of a defined signaling pathway) but rather are built upon relationships reported from the literature, thus providing a different perspective.

2.4 Results and discussion

2.4.1 Chemical profiles

In the fall of 2013, water quality declined across the urbanized environment, especially at the sites downstream from the first major outfall (Waterloo MWWTP) (Figure S2.1). Elevated concentrations of ammonia and the indicator chemicals were observed at the site immediately downstream of the Waterloo MWWTP outfall. The concentrations of ibuprofen and naproxen were ~50% lower downstream (INT) than at the site immediately below the Waterloo outfall (DSW). Their concentrations remained relatively consistent at sites further downstream, including the sites below the Kitchener outfall (DSK1, DSK2, DSK3) and the far field site (DSK4). The contaminant profiles of the Waterloo effluent were indicative of poor effluent treatment with minimal nitrification. The total estrogenicity in the Waterloo effluent was much higher than in the Kitchener effluent: 16.6 ± 1.6 versus 0.9 ± 0.5 estradiol equivalents/L (E2eq ng/L) (Table S2.3). The Kitchener MWWTP underwent major upgrades in the fall of 2012; the Waterloo MWWTP continued to operate with minimal aeration and nitrification during the study period (Bicudo et al., 2016; Hicks et al., 2016).

2.4.2 Magnitude of change in differently expressed probes

The number, type, and magnitude of change in differently expressed probes (DEPs) in rainbow darter collected across the urban environment (eight sites) changed depending upon reference site selection (Figure 2.2). Rainbow darter collected from downstream of the Waterloo MWWTP outfall (DSW) showed greater transcriptomic response than those from all other sites, which clearly distinguished them from other groups of fish. Similarly, the heat maps showed that independent of the reference site selected, DSW fish differed most from the other groups on the basis of the transcriptome (Figure 2.3). There were 3340 probes that were differently expressed when DSW rainbow darter transcriptomic changes were compared with those of US1 rainbow darter, 3952 DEPs when compared with those of US2 rainbow darter, and 3940 DEPs when compared with those of US3 rainbow darter. To illustrate this further, up to 15.5% of the probes identified as differently expressed responded differently depending upon reference site selection. Rainbow darter from DSK1 showed consistent numbers of DEPs, regardless of the reference site they were compared with. The number of probes that were differently expressed ranged from 1893 to 2055. In other words, up to

7.9% of probes identified as differently expressed were different depending upon reference site selection.

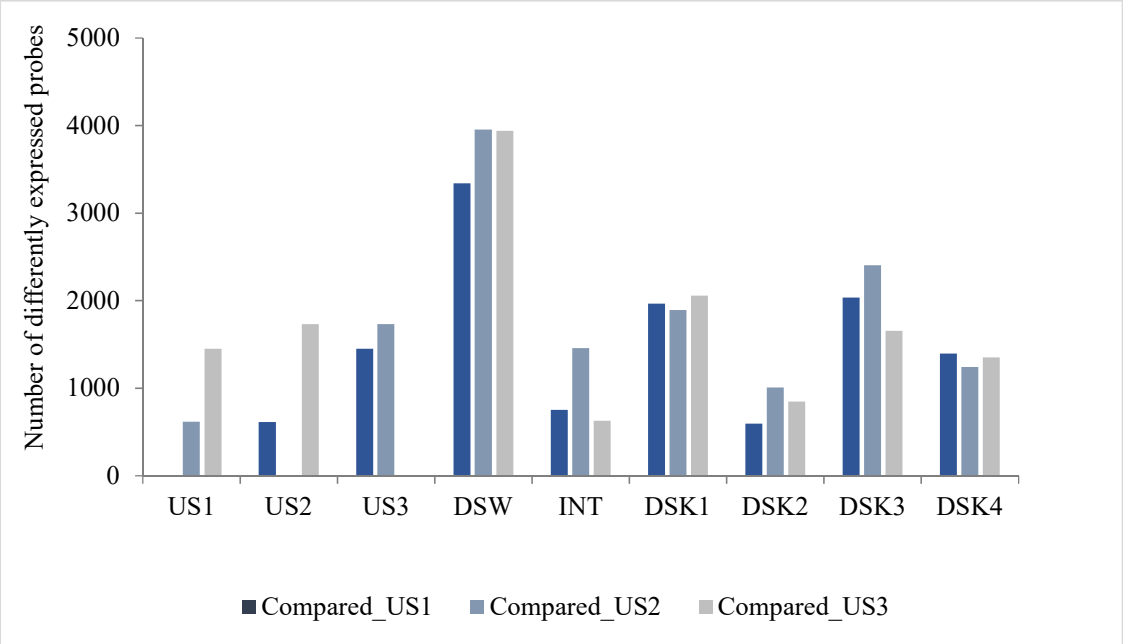


Figure 2.2 Number of differently expressed probes (DEPs, using unadjusted *p*-values) in rainbow darter across the urban environment in the central Grand River depending on the reference site selected. Dark blue bars indicate the number of DEPs in rainbow darter compared with US1 fish, medium gray bars indicate the number of DEPs in rainbow darter compared with US2 fish, and light gray bars indicate the number of DEPs in rainbow darter compared with US3 fish.

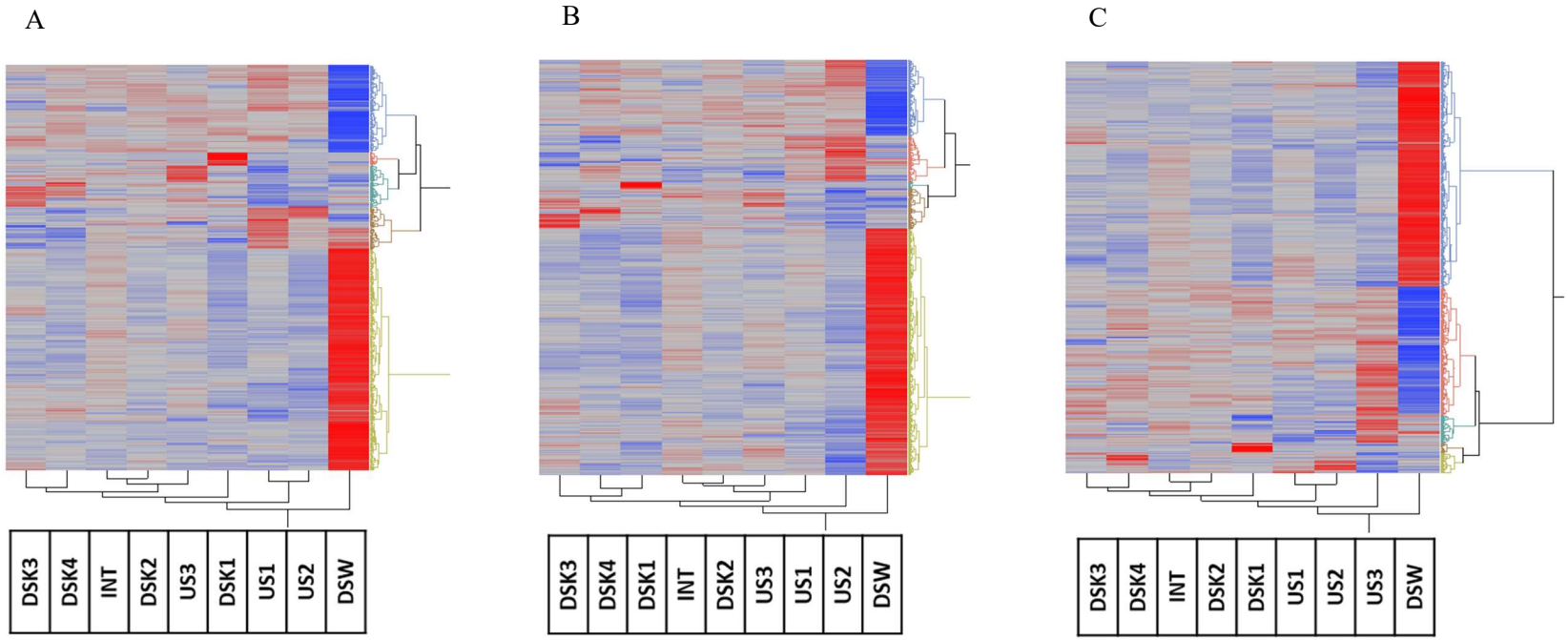


Figure 2.3 Heat maps that contrast rainbow darter transcriptomic signatures across the urban environment using three reference sites as independent baselines for comparison: the furthest upstream reference site (US1) (A), the second upstream reference site (US2) (B), and the reference site immediately upstream of the Waterloo MWWTP (US3) (C).

Fish collected from the furthest downstream site (DSK4) showed consistent responses despite reference site selection, and only ~11.1% of the probes showed dependence upon the reference site selected. In contrast, reference site selection had a greater effect on the transcriptome of rainbow darter collected from sites INT, DSK2, and DSK3. This was reflected in the greater fluctuation in the number of DEPs identified when the transcriptome of these fish was compared with those of each reference site individually. The greatest fluctuation in DEPs was observed for rainbow darter from the intermediate site (INT). For instance, only 628 probes were differently expressed when INT rainbow darter were compared were US3 rainbow darter, whereas 1456 were different when they were compared with US2 rainbow darter. Thus, ~56.9% of the probes identified as differently expressed depended upon which reference site was selected as a control. Rainbow darter from DSK2 and DSK3 also showed high variability in DEPs, with up to 40.7% and 45.3% of DEPs depending on reference site selection, respectively.

Although reference site selection had some influence on the identification of gene expression changes, it did not have a major effect on the interpretation of the spatial patterns in the current study, especially where there were major responses to point sources of pollution. This was evident in the transcriptome of the rainbow darter downstream of the Waterloo MWWTP outfall (DSW). Although rainbow darter downstream of the Kitchener MWWTP (DSK1) and those from the far field downstream site (DSK4) did not show responses as dramatic as those seen in fish from DSW, there was consistency in the number of probes that differed in expression. Sites DSW and DSK1 are located immediately below the Waterloo and Kitchener MWWTPs, respectively. Therefore, it is possible that the consistent responses seen as low fluctuation in the numbers of DEPs were due to MWWTP-originating contamination. In 2013, the Waterloo MWWTP was discharging effluent of poor quality, indicated by the low concentrations of nitrate and relatively high concentrations of ammonia, ibuprofen, and naproxen (Figure S2.1) (Hicks et al., 2016). In contrast, in 2013 the Kitchener MWWTP was discharging effluent of higher quality as a result of infrastructure upgrades that were initiated in late 2012 (Bicudo et al., 2016; Hicks et al., 2016). Despite the improvement in effluent quality, numerous contaminants were still detectable downstream of the Kitchener MWWTP (i.e., DSK1) at concentrations that were possibly sufficient to cause transcriptomic responses in rainbow darter. It is noteworthy that the concentrations of indicator chemicals remained relatively consistent at the further downstream river sites (Figure S2.1). Despite the consistency in water quality, there was no consistency in the fluctuation of DEPs at the downstream river sites except at the furthest downstream site (DSK4), which is spatially separated (20 km downstream of DKS2)

from the other core sites. The geographical distance may have influenced site characteristics, resulting in reduced responsiveness to reference site selection. It is also worth mentioning that there are several additional wastewater treatment plant inputs and other possible contaminant sources between the Kitchener outfall and the furthest downstream site (DKS4) that may have influenced the transcriptomic response of fish downstream.

2.4.3 Changes in some transcripts are not dependent upon reference site selection

Analysis of transcripts in DSW and DSK1 rainbow darter that were different than the transcripts in rainbow darter from all three reference sites further confirmed that selection of reference site had no major effect on identification of site differences below MWWTP outfalls. There were 670 transcripts (39.4%) identified as differently expressed in DSW rainbow darter that were not affected by reference site selection (Figure 2.4A). The direction of expressed genes (in terms of upregulation or downregulation) was consistent, with 36 transcripts (5.4%) showing a change in direction (that is, a specific gene that was downregulated relative to US1 fish but upregulated relative to US2 and US3 fish). In the liver of rainbow darter from DSK1, 389 transcripts (27.7%) were not affected by reference site selection (Figure 2.4B). Within this group, 78 transcripts (20.1%) had a different degree and fold change direction in two or more sites. These results suggest that during the study period, reference site selection played less of a role in determining the magnitude of changes in differently expressed transcripts in fish from the highly-impacted site (DSW) than it did on the transcriptome of rainbow darter from other sites in the urban area (Figure S2.2).

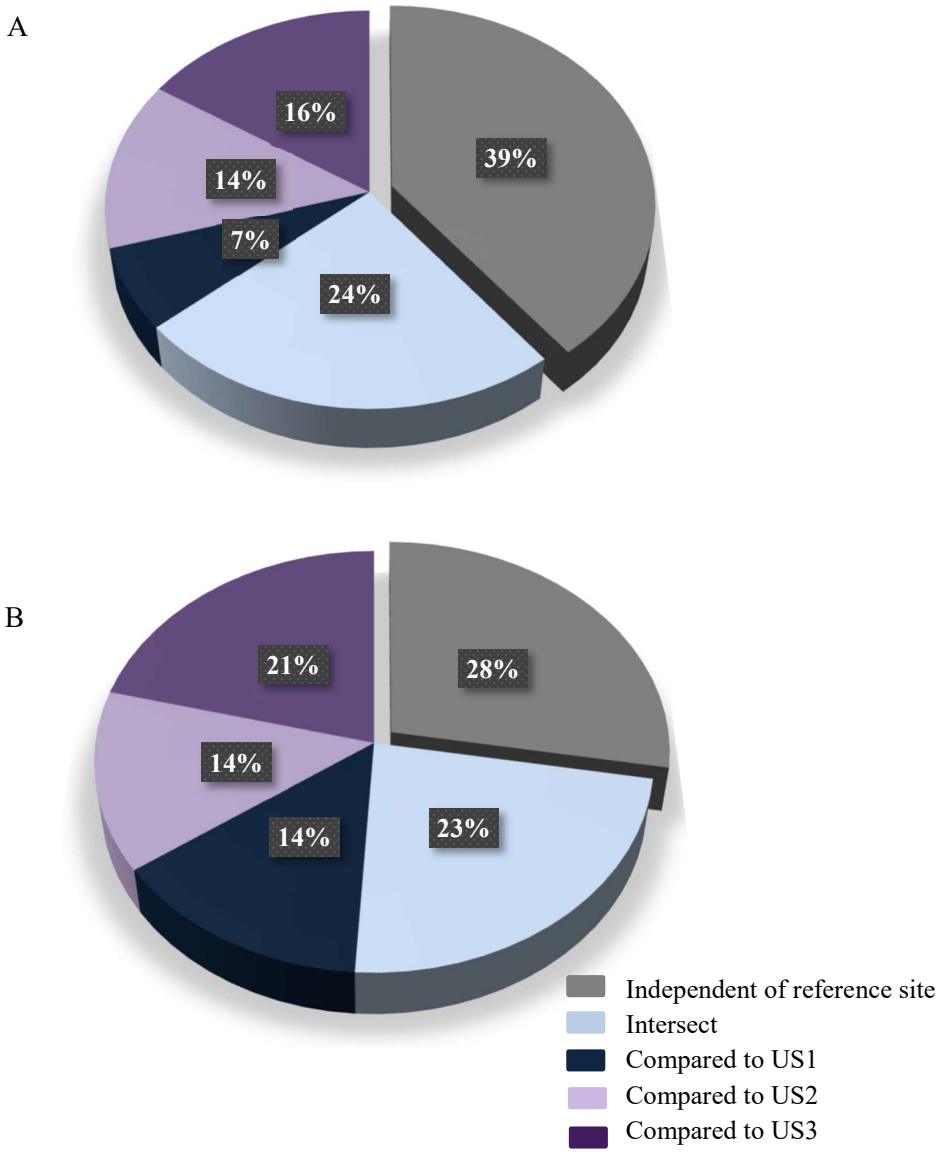


Figure 2.4 Percentage distribution of genes altered with reference site selection in DSW rainbow darter (A) and in DSK1 rainbow darter (B). The portion of the pie chart labelled as "*Independent of reference site*" indicates the percentage of differently expressed transcripts that are not affected by reference site selection, whereas the "*Intersect*" portion indicates the percentage of differently expressed transcripts that are in common for any two of the reference sites. The "*Compared to US1,*" "*Compared to US2,*" and "*Compared to US3*" portions of the chart are for transcripts that are differently expressed in US1, US2, and US3 rainbow darter, respectively.

2.4.4 Effects of reference site selection on expression of the top three transcripts

Choriogenin H minor was one transcript that was consistently upregulated in DSW fish compared with all three groups of reference rainbow darter (US1, US2, and US3) (Table S2.4). This transcript has been used as a biomarker responsive to estrogen exposure in males of several fish species (Fujita et al., 2004; Kurauchi et al., 2005); in some instances it is even regarded as more sensitive than the conventionally used estrogen-responsive vitellogenin messenger RNA (mRNA) transcript (Lee et al., 2002). In a separate study, this transcript was also classified among the top three transcripts in fish collected downstream of the Waterloo outfall in three consecutive years: 2011, 2012, and 2013 (Marjan et al., 2017). Total estrogenicity of the Waterloo effluent was variable from 2009 to 2015, with the lowest level being 2.3 ± 0.6 ng/L E2eq (2014) and the highest being above 20.0 ng/L E2eq (2009). Although the 2013 level (16.6 ± 1.6 ng/L E2eq) was not the highest estrogenicity recorded, it was in the higher range, suggesting poor effluent treatment (McCann, 2016). At the same time as fish were sampled in 2013, effluent samples from the Waterloo MWWTP (1 L) were collected for effect directed analysis; samples were fractioned by high-performance liquid chromatography and tested for estrogenic activity using YES (methods outlined in Appendix A). These analyses indicated that the main contributors of estrogenicity were 17α-ethinylestradiol, 17β-estradiol, and estrone (Marjan, unpublished data). Thus, it is plausible that the estrogenic contaminants in the river downstream of the Waterloo MWWTP were responsible for the upregulation of choriogenin H minor.

Type-4 ice-structuring protein LS12 was also among the top three upregulated transcripts identified in DSW fish compared to its relative expression in US2 and US3 fish but not to US1. In Atlantic cod (*Gadus morhua*) this transcript participates in the antifreeze response through absorption to the surface of ice crystals, which prevents their growth and thereby lowers the blood freezing point (UniProt). Fish collections were delayed until late November because of adverse weather conditions earlier in the season; collections ended up being extremely difficult for sites DSW and US3 in particular because of ice formation in the river on collection days. In DSK1 fish, UPF085 protein Clorf144 homolog was upregulated in comparison with its expression in rainbow darter from all three reference sites. Information on this transcript, other than the fact that it has been identified in *Xenopus laevis* as one of the transcripts involved in cell signaling during lens regeneration (Malloch et al., 2009), is lacking. Thus, we are unable to comment on the reason why this transcript showed upregulation in rainbow darter at this site.

The top three downregulated transcripts shared functional commonalities. In rainbow darter from DSK1, transcripts that were not necessarily identical but were functionally related, such as trypsinogen 1 and trypsinogen 2, and carboxypeptidase A1 and carboxypeptidase B, were among the top three downregulated transcripts. Carboxypeptidase A1 and B and trypsinogen 1 and 2 code for enzymes involved in protein digestion in teleost fish (Hoar et al., 1979). Environmental factors (such as temperature and light) have been shown to control the levels of trypsin, which in turn controls the food influx and subsequently the growth ratio and indirectly the immune system (Niemuth et al., 2015; Rungruangsak-Torrissen et al., 2006). Contaminants in the effluent, such as metformin, may also be capable of altering fish metabolism (Niemuth et al., 2015). The condition factor of DSK1 fish (1.13 ± 0.07) was significantly lower that of US1 and US2 fish (1.33 ± 0.06 and 1.28 ± 0.08, respectively). It has been reported that fish collected downstream of effluent discharges display alteration in health indices (GSI, LSI, k) (Iwanowicz et al., 2009) because of nutrient sources that promote algae/biofilm growth (i.e., food availability) or toxicity. The consistent downregulation of trypsinogen and carboxypeptidase genes in the present study, independent of reference site selection, suggests that the stressor was specific to the downstream effluent-exposed site (i.e., Kitchener). It can only be speculated that decreased levels of these genes may lead to impaired energy assimilation and utilization in wastewater-exposed fish. However, this needs to be further investigated.

2.4.5 Unique gene set enrichment analysis (GSEA) entities

Identification of the perturbed gene sets (using GSEA or SNEA) that are unique to fish from a particular site enhances interpretation of the liver cell processes affected by exposure to pollution (gene sets that are constantly enriched in the liver across the urban environment are not very informative as they show no pattern with pollution exposure) (Figure S2.3). Rainbow darter downstream of the Waterloo MWWTP (DSW) had the highest number of unique gene sets. This is not surprising, as over 3000 transcripts in this group of fish showed differential expression. The extent to which quantitative transcriptomic information can be indicative of exposure to stressors is not clear, so caution should be taken to not over-interpret these data. Therefore, focusing on qualitative GSEA responses could provide better insight into the association of transcriptomic information with responses to stress (e.g., municipal effluent exposure). Molecular functions in DSW rainbow darter were related to DNA processing: “DNA-directed RNA polymerase activity” and “sequence-specific DNA binding” ($p < 0.001$). Proper processing of DNA is important because it

establishes the basis for unimpeded downstream transcriptional and translational processes. The following cell processes were also unique: “mannose binding” and “mannose transmembrane activity,” as well as “o-methyltransferase activity” and “calcium binding” ($p < 0.001$). Molecular functions involving mannose are important for optimal functioning of the innate immune system. For instance, mannose-binding lectin gene was found to be highly expressed in the liver of channel fish (*Ictalurus punctatus*); however, when these fish were exposed to a pathogen challenge, the greatest transcriptional response was detected in the spleen, and this was explained as a downstream immune-activation response (Zhang et al., 2012). Changes in mannose binding suggested that fish from DSW may have had difficulties in properly activating their immune defense responses, although this would need to be confirmed. Nevertheless, this response is indicative of fish responses to chronic exposure to stress, a finding that was further supported by the SNEA results. Garcia-Reyero et al., (2008) reported that the innate immune system response was one of the main pathways downregulated in fish exposed to effluent downstream of a sewage treatment plant. In the present study rainbow darter from DSW had a variety of enriched and unique gene sets that were involved in subcellular responses. However, it is difficult to estimate the relevance of changes at the subcellular level and whether they translate to responses at higher levels of biological organization.

DSK1 rainbow darter were more variable than DSW rainbow darter, with 13 unique molecular functions, six unique biological processes, and only two unique cellular components. Upgrades at the Kitchener MWWTP were initiated in 2012, which means that the fish collected in 2013 were exposed to improved effluent for over a year. In terms of quantitative interpretation of the gene sets involved in the functional analyses, there was little consistency among sites. Transcriptome profiles were diverse and site specific, with no discernable pattern that followed the gradient. However, some gene sets were indicative of stress-driven responses, such as those involved in the molecular functioning of antioxidant activity or protein tyrosine kinase activity. Kinase cascade has been identified as one of the processes that shows upregulation in response to sewage exposure (Garcia-Reyero et al., 2008).

Gene sets identified as unique to a site increased in number at the downstream sites, with DSK2 showing the least unique gene sets and DSK4 showing the greatest uniqueness. Most of the molecular functions in DSK4 rainbow darter were related to histone processing such as “histone deacetylase activity (h3-k16 specific),” “NAD-dependent histone deacetylase activity” with three

different specificities (h3 – k14 specific, h3 – k9 specific, and h4 – 16 specific), and "histone pre-mRNA DCP binding." Histones are DNA structural units that play an important role in regulating gene expression. Histones have received increasing attention in recent studies that explore epigenetic mechanisms of action (MOA). One of the MOA of epigenetics is mediated through post-translational modifications to the core histones. Environmental contaminants can act as epigenetic vectors and can silence or activate target genes (Manikkam et al., 2012). The far field downstream site (DSK4) in the present study was geographically separated from the sites in the urbanized area, and it is reasoned that the unique gene sets in fish collected from this site may have played a role in the recovery of these fish.

2.4.6 Unique subnetwork enrichment analysis (SNEA) cell processes

Sixteen cell processes were exclusively present in DSW rainbow darter. A subset of these processes exhibited a relationship to RNA processing/metabolism: "mRNA processing, "poly(A) + mRNAnucleus export," and "rRNA processing" as well as "ribosome biogenesis and assembly." Enriched subnetworks of genes related to RNA metabolism/processing corroborated the GSEA data analysis discussed above. Ribosomal biogenesis and assembly is a very complex process and it can involve up to 170 factors, mostly proteins (Fromont-Racine et al., 2003). Improper biogenesis and the assembly of the ribosomal subunits can lead to translational impairments and deregulated protein synthesis. Unique cell processes were also related to intracellular transport ("nucleocytoplasmic transport" and "mitochondrial protein transport"), lipid metabolism ("lipid peroxidation," "LDL oxidation," and "glycerol metabolism"), and cell fate ("cell proliferation" and "apoptosis"). Lastly "xenobiotic clearance," "growth rate," and "sperm cell adhesion" were also unique for DSW rainbow darter (Table S2.5). Responses such as xenobiotic clearance, LDL oxidation, regulation of the cell cycle (DNA replication), and apoptosis have been shown to be related to stress (Martyniuk and Denslow, 2012) and may be attributed to MWWTP effluent exposure.

Rainbow darter downstream of the Kitchener MWWTP (DSK1) exhibited most of the cell processes related to immunity, and the following subset was unique: "immune cell chemotaxis," "leucocyte cell adhesion," "monocyte recruitment," "establishment of T-cell polarity," "T-cell tolerance," "eosinophil chemotaxis," and "leucocyte migration" (Table S2.5). Immune system response relies profoundly on chemotaxis, which generates movement of cells, such as leucocytes, in response to a chemical gradient (Luster, 2001). Leucocytes play a key role in homeostasis and

inflammation by bringing together factors that participate in the immune response, such as T-cells (Luster, 2001). Immune cell chemotaxis, leucocyte migration and adhesion, T-cell tolerance, and the establishment of T-cell polarity were all found to be downregulated in this group of fish. Downregulation of the essential features of immunity suggests that rainbow darter downstream of Kitchener may not have been able to adequately respond to various stressors. The effectiveness of the immune response depends on the body's energy reserves; this was demonstrated in a starvation study in which Atlantic salmon (*Salmo salar*) exhibited decreased transcription of immune genes (Martin et al., 2006). Fish from DSK1 had a decreased condition factor, which may indicate that they could not allocate sufficient body energy reserves to secure normal activation of the immune response.

It was noteworthy that none of the cell processes identified as differently regulated in rainbow darter collected from DSK2 and DSK3 were unique. In contrast, DSK4 rainbow darter exhibited exclusive cell processes such as "osmotic stress," "triglyceride storage," "triacylglycerol's biosynthesis," "vitellogenesis," and "cell process of regulated secretory pathway" (Table S2.5). We raised the possibility earlier in this chapter that there were additional stressors upstream of the furthest downstream site (DSK4) that potentially could influence recovery. Unique cell processes identified by SNEA were not consistent with a system that had recovered from wastewater exposure.

Transcriptomic studies have proven useful in identifying gene expression changes in response to exposure to complex mixtures discharged in municipal effluents. Studies have varied in their experimental approach, from ones conducted on model fish species in the laboratory (Hasenbein et al., 2014; Osachoff et al., 2013; Vidal-Dorsch et al., 2013; Garcia-Reyero et al., 2011) or in semi-controlled conditions (caging) (Berninger et al., 2014; Martinović-Weigelt et al., 2014; Ings et al., 2011; Garcia-Reyero et al., 2009) to ones using wild fish caught in polluted environments (Bahamonde et al., 2015a). Gene expression changes in fish exposed to estrogenic effluents do not necessary parallel the changes in fish exposed to model estrogens (Garcia-Reyero et al., 2011). This illustrates the diversity of effluent composition, the variety of MOA, and the importance of conducting experiments with complex mixtures. Although field-based studies are challenging to conduct, they provide the most realistic assessment of the impacts of complex mixtures. The use of multiple sites within a gradient approach provides a means to (1) understand variability, (2) account for background contamination, and (3) assess the potential for responses to return to reference

conditions (Berninger et al., 2014; Martinović-Weigelt et al., 2014). A common experimental approach in field-based studies of ecotoxicogenomics is to select a single reference site located upstream of the polluted area of interest. However, it is well recognized that there is considerable spatial variability in habitat and ecosystem structure across watersheds, which can influence fish responses to stressors. None of the studies mentioned above used more than one reference site for comparison in its analyses of gene expression profiles. The use of multiple reference sites is expected to increase confidence in the interpretation of changes detected and their potential association with anthropogenic stressors (i.e., rather than with other uncontrolled natural factors). For example, Collí-Dulá et al., (2016) accounted for some natural variability in the transcriptomic responses of largemouth bass (*Micropterus salmonids*) exposed to perfluorinated compounds by using several reference lakes for comparisons. Although it is a very powerful tool, transcriptomic analysis involves numerous comparisons and therefore considerable uncertainty. It is critically important to reduce this uncertainty and increase the reproducibility of results in field-based studies of (transcript)omics to enhance the confidence with which data are interpreted, especially if these tools are to be useful for environmental assessment and monitoring.

Does reference site selection matter? Transcriptomic responses in rainbow darter that were collected from a highly-impacted site downstream of the Waterloo MWWTP were less sensitive to reference site selection than the responses in fish collected from other sites. In contrast, the expression profiles in fish from a site with lower anthropogenic impacts (e.g., INT) were more dependent on reference site selection. It was difficult to identify clear patterns of transcriptome changes in fish collected below the Kitchener effluent outfall following the treatment upgrades. Various analyses of transcriptomic signatures in rainbow darter fish from the far field site (DSK4) suggested that these fish continued to show stress-related responses. Currently, there is no consensus on which molecular responses are relevant indicators of exposure or recovery, especially in field-based transcriptomic studies where the influence of confounding factors cannot be easily accounted for. Therefore, it is important to develop guidelines for interpreting field transcriptomic data that will allow researchers to better isolate transcriptomic signatures responsive to confounding environmental factors from those responsive to contaminants. Use of multiple reference sites should enable researchers to better define the baseline and should increase the probability of unambiguously identifying stress-related molecular signatures. Use of multiple reference sites in independent transcriptomic analyses could improve the reliability of the interpretation of the (transcript)omics

results. Combining multiple reference sites may be a good way to overcome the problems associated with establishing relevant baselines for comparison. Before using such an approach, it would be important to understand the similarities among reference sites; this could be quite challenging with field-based study designs, but it would be of great value.

Chapter 3

Returning to normal? Assessing transcriptome recovery over time in male rainbow darter (*Etheostoma caeruleum*) liver in response to wastewater treatment plant upgrades

3.1 Chapter summary

The present study measured hepatic transcriptome responses in male rainbow darter (*Etheostoma caeruleum*) exposed to two municipal wastewater treatment plants (MWWTPs) (Kitchener and Waterloo) over four fall seasons (2011 - 2014) in the Grand River, Ontario. The overall goal was to determine if upgrades at the Kitchener MWWTP (in 2012) resulted in transcriptome responses indicative of improved effluent quality. The number of differentially expressed probes in fish downstream of the Kitchener outfall (904 - 1223), remained comparable to that downstream of Waterloo (767 - 3867). Noteworthy was that the year, and the interaction of year and site, explained variability in more than twice the number of transcripts than site alone. This suggests that year, and the interaction of year and site, had a greater effect on the transcriptome than site alone. Gene set enrichment analysis revealed a gradual reduction in the number of gene ontologies over time at exposure sites, which corresponded with lower contaminant load. Subnetwork enrichment analysis revealed that there were noticeable shifts in the cell pathways differently expressed in the liver pre- and post-upgrade. The dominant pathways altered in the fish liver pre-upgrades were related to genetic modifications and cell division whereas, post-upgrades, the differently expresses pathways were associated with the immune system, reproduction, and biochemical responses. Molecular pathways were dynamic over time, and following the upgrades, there was little evidence that gene expression profiles in fish collected from high impact sites post-upgrade were more similar to fish collected from the reference site.

3.2 Introduction

It has been well established that municipal wastewater effluents can disrupt multiple biological processes in fish, ranging from molecular signaling cascades to the whole organism level. Studies conducted in rivers throughout the United Kingdom demonstrated the feminization of male fish and the presence of intersex (ova-testes) in wild populations of roach (*Rutilus rutilus*), which was associated with the proximity to municipal wastewater treatment plants (MWWTPs) (Tyler and Jobling, 2008; Jobling et al., 2006; Jobling et al., 1998). Intersex has become a widely reported biological response in fish associated with exposure to MWWTP effluents worldwide (Bahamonde et al., 2015b; Blazer et al., 2014; Bjerregaard et al., 2006). In addition to intersex, MWWTP effluents have also been associated with vitellogenin induction in male fish (Hoger et al., 2006; Harries et al., 1997), altered steroid hormone production (Hogan et al., 2010; Folmar et al., 2001), reduced secondary sex characteristics (Vajda et al., 2011; Filby et al., 2007a), disrupted behaviour (Garcia-Reyero et al., 2011; Garcia-Reyero et al., 2009), impaired gametogenesis (Fuzzen et al., 2015; Vajda et al., 2008), and effects on somatic indices, including gonad somatic index (GSI) and liver somatic index (LSI) (Schoenfuss et al., 2009; Hoger et al., 2006; Allen et al., 1999). Thus, there is a multitude of documented responses that can manifest across many levels of biological organization following MWWTP effluent exposure.

Due to the complexity of MWWTP effluents, contaminant exposure pathways, and the diverse chemical modes of action, it is difficult to predict the effects on ecologically relevant responses, such as growth and reproduction, in fish and other organisms in the receiving environment. At the molecular level, there is considerable evidence that contaminants found in MWWTP effluents can alter the expression of genes in tissues of the hypothalamus-pituitary-gonadal axis as well as those transcripts related to sexual differentiation and reproduction (Bahamonde et al., 2014; Garcia-Reyero et al., 2011). Application of transcriptomics in aquatic ecotoxicology represents a powerful genome-based tool that allows one to obtain detailed insight into toxicological responses (Snape et al., 2004). Hence, toxicogenomic approaches can broaden understanding as to the mechanisms of action of individual chemicals or chemical mixtures in an organism, such as those encountered in freshwater ecosystems exposed to MWWTP effluents. Oligonucleotide microarrays are a tool that can provide insights into transcriptome patterns, allowing one to examine thousands of genes in unison. Transcriptomics has been used to study the effects of MWWTP effluents on fish

molecular responses (Denslow and Sabo-Attwood, 2015). However, only a small subset of these studies have examined molecular responses in wild fish populations residing downstream of MWWTP discharges (Cavallin et al., 2016; Bahamonde et al., 2015b; Baker et al., 2013; Vidal-Dorsch et al., 2013). In addition, studies that address the question of temporal (annual or seasonal) effects of highly urbanized aquatic environments on expression patterns are rare.

The Grand River is the largest watershed in southern Ontario, Canada, with a population of 925,000 inhabitants. This system receives discharges from 30 MWWTPs. The central stretch of the Grand River is the most densely populated with many anthropogenic inputs, including discharges from two major MWWTPs (Waterloo and Kitchener). In 2007, the local government (i.e., Region of Waterloo) initiated a plan to upgrade both of these conventional activated sludge treatment plants. The first phase of upgrades at the Kitchener MWWTP started in late 2012 with the construction of a dewatering facility, UV disinfection and more fine bubble diffusers that resulted in improved aeration and additional nitrification. Upgrades to the Waterloo MWWTP have only been partially completed due to a construction delay and the plant continues to operate with minimal aeration leading to only partial nitrification.

Rainbow darter (*Etheostoma caeruleum*), a native, small bodied fish that inhabits riffle habitats of the Grand River watershed has been used as a sentinel species in many recent studies. This species is highly abundant throughout the Grand River watershed and it has a relatively small home range, moving only a few meters, mostly in the spring during spawning (Loomer et al., 2015; Tetreault et al., 2011). Rainbow darter become sexually mature after one year (Crichton, 2016; Paine, 1990). They are seasonal asynchronous spawners that typically reproduce in late March, and continue spawning until mid-June (Fuller, 1998). Recent studies have shown that complex mixtures of pollutants released from the two major municipal wastewater outfalls in the central reaches of the Grand River (i.e., Kitchener and Waterloo) have resulted in endocrine disruption in rainbow darter. High incidence of intersex condition (testes-ova) (Tanna et al., 2013; Tetreault et al., 2011), decreased production of androgens (Bahamonde et al., 2015b; Tetreault et al., 2011), and reduced reproductive performance (Fuzzen et al., 2015), have been observed in rainbow darter downstream of the wastewater outfalls. Studies with rainbow darter have also shown that there are changes in the expression of transcripts related to reproduction (Bahamonde et al., 2014), and unique transcriptome responses in intersex males (Bahamonde et al., 2015b). Thus, the activities in the Grand River create

a unique opportunity to explore the effects of major infrastructure investments in wastewater treatment plant upgrades on biological responses in fish exposed to the effluents.

The objective of the present study was to investigate the hepatic transcriptome response in male rainbow darter associated with the outfalls of two MWWTPs (Waterloo and Kitchener) in the Grand River. Rainbow darter males were collected near the effluent outfalls before and after the upgrades, every fall season from 2011 to 2014. In addition, we aimed to determine if the upgrades at the Kitchener MWWTP in 2012 resulted in transcriptome responses in the rainbow darter over time, that indicated changes in effluent quality. To better place the transcriptome profiles into context, the general quality of effluent and exposure at collection sites (surface water) were characterized over time (2011 - 2014) using nutrients (ammonia, nitrate, nitrite), water parameters (conductivity, dissolved chloride), and major pharmaceuticals (ibuprofen, naproxen, venlafaxine, and carbamazepine), reported to be present in the effluent.

3.3 Materials and methods

3.3.1 Experimental design and study sites

Wild, adult male rainbow darter were collected from three sites in the Grand River (Figure 3.1) over four consecutive fall seasons (October/November) from 2011 to 2014. Rainbow darter were sampled from sites located downstream of the Waterloo MWWTP (DSW) and the Kitchener MWWTP (DSK1), as well as from a site located upstream of the urbanized area that was used as a reference site (US2). Approximately 15 - 20 rainbow darter males were collected per site using an electrofishing backpack unit and were sampled immediately following capture. Morphometric characteristics including length (± 1 mm) and weight (± 0.001 g) were recorded. Liver and gonad tissues were weighed (± 0.001 g), and a portion of each tissue was frozen in liquid nitrogen, and then stored at -80ºC for further analyses. A subset of 84 liver samples were processed (3 sites; 4 years with n = 7/group), and were analysed using a rainbow darter microarray (8 × 15 K format GPL18038, Agilent Technologies Inc.). Following hybridization, the transcriptome profiles of 79 liver samples were subjected to bioinformatics analysis. Five samples were excluded from the analysis as they showed high background levels, and did not meet the quality standards. Table 3.1 summarizes information on sample sizes and meristic endpoints of rainbow darter sampled (per site

and year). All procedures involving fish were approved by University of Waterloo's Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

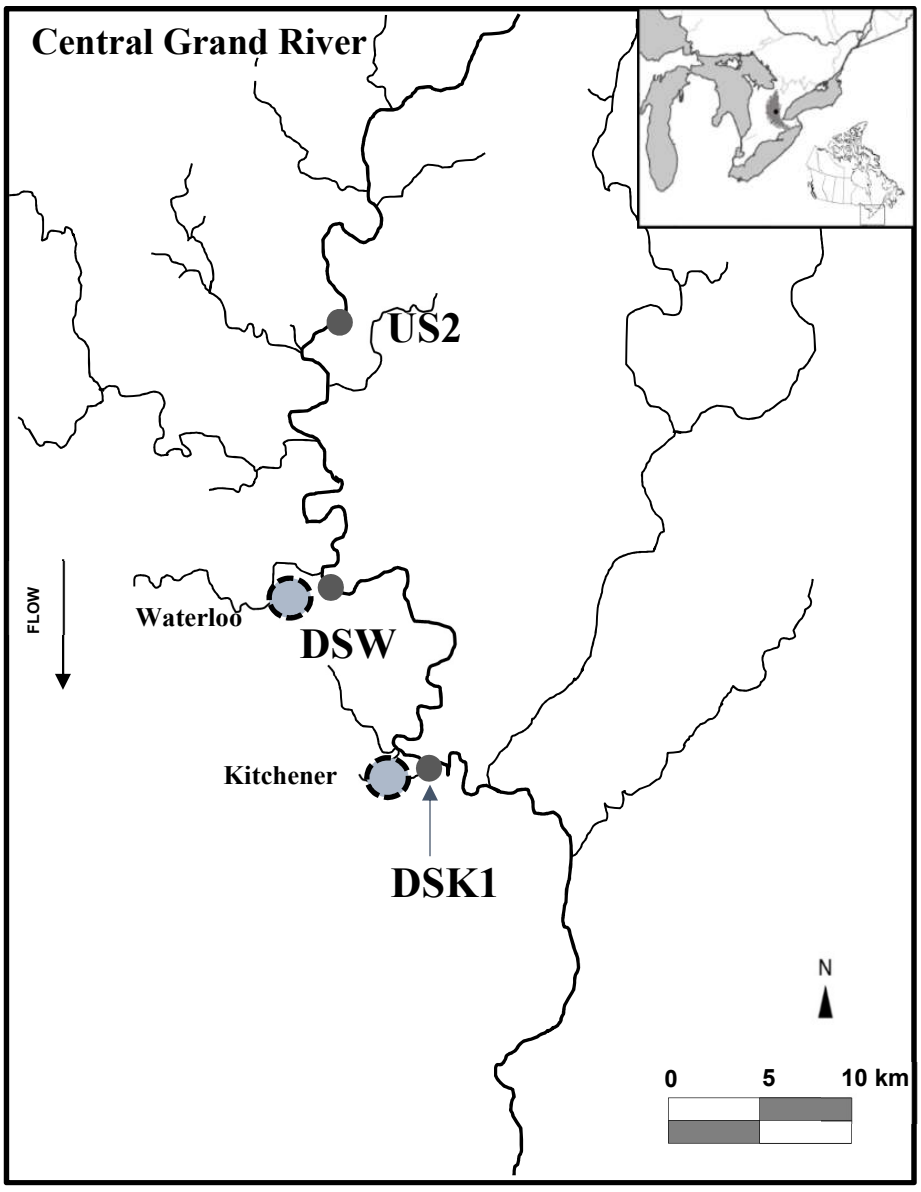


Figure 3.1 Sampling sites map. Grand River watershed in southern Ontario – enlarged is the central part of the Grand River displaying the location of sampling sites: (1) reference site located in a non-impacted area (US2); (2) DSW located downstream of the Waterloo municipal wastewater treatment plant (MWWTP) discharge; (3) DSK1 located downstream of the Kitchener MWWTP discharge.

3.3.2 RNA isolation

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN; Valencia, CA) following the manufacturer's protocol. Briefly, liver tissues weighing less than 30 mg were homogenized with 800 µL of QIAzol (provided in the kit). Nucleic acids were separated with chloroform, and loaded onto RNeasy Mini columns as per the manufacturer's protocol. Samples were eluted with approximately 30 µL of nuclease free water. RNA concentration and A260/A280 ratio was determined using the NanoDropTM2000 (Thermo Scientific, Canada). Samples with A260/A280 ratio, ranging from 1.8 to 2.0 were selected for sample labelling. RNA integrity (RIN score) was determined using the Agilent R6K ScreenTape System and the 2200 TapeStation (Agilent Technologies, Canada). Samples with RIN scores greater or equal to 8 were deemed to be high quality RNA. RIN scores for all samples were 9.31 ± 0.68 (average ± SD).

3.3.3 Transcriptomics analysis using a rainbow darter 8 × 15 microarray platform

Total RNA was used for cDNA synthesis and cRNA *in vitro* transcription using the Low Input Quick Amp Labelling Kit (Agilent Technologies, Canada) as outlined in the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with cyanine 3 (Cy3). Four samples contained low amounts of RNA and were labelled with 25 ng input while all other samples were labelled using 50 ng. Labeled cRNA was then purified through RNeasy Mini Kit columns with buffer RLT, molecular grade ethanol and buffer RPE (QIAGEN, Valencia, CA). Columns were then eluted with approximately 24 µL of nuclease free water to collect the labeled cRNA. Samples that had a specific activity higher than 6 pmol of Cy 3 per µg of cRNA were used in the hybridization step. Prior to hybridization, every sample was adjusted to a mass of 600 ng of labeled cRNA for the 8 × 15 K microarray format, subjected to fragmentation, and hybridized for 17 h at 65ºC. Slides were washed according to instructions provided by Agilent (Gene Expression Hybridization Kit).

Microarray slides were scanned with the Agilent G2505C Microarray Scanner at 5 µm density. Raw signal intensities were extracted using the Agilent Feature Extraction Software (v. 10.1.1.1) (Agilent Technologies). Microarray reports were generated following array scanning, and were manually inspected for quality. Samples that had low background levels (< 5%), and those for which spike-in standards showed a linear relationship were deemed to be high quality arrays.

3.3.4 Bioinformatics

Raw intensity data were imported into JMP Genomics (v. 7.0, SAS Institute Inc.) and were normalized using Quantile Normalization. Following normalization, data were filtered to an intensity value of 4.0 (determined to be the detection limit based on control spots and Agilent spike-in). Spots that showed an intensity below this value were assigned the limit of detection (log2 intensity = 4.0). Control spots were excluded from further analysis. One-way analysis of variance (ANOVA), and a false discovery rate of 5% (FDR = 0.05) (JMP Genomics) was used to identify differentially expressed genes. Reference (US2) rainbow darter collections were regarded as the baseline environmental condition, and changes in gene expression in fish collected from the exposed sites (DSW and DSK1) were compared to the US2 rainbow darter within the same year. Normalized and filtered non-FDR corrected data were subjected to two-way hierarchical clustering using the Fast Ward algorithm. Rows were centered to a mean of zero prior to clustering and were also scaled to a variance of one prior to clustering. In order to discriminate between the site and year effects on the liver transcriptome, a two-way ANOVA was performed.

Selected transcripts that were differently expressed in rainbow darter over time were identified. We highlighted the top three genes that were significantly upregulated, and the top three genes that were significantly downregulated in the liver of rainbow darter. The focus was on transcripts that were annotated, as well as those that showed a relatively high fold change (≥ 3 and ≤ -3) when compared to fish from the reference site. This approach was applied to determine whether these transcripts showed site-specific or time-specific (year) patterns of expression. In addition, differentially expressed probes (DEPs) were examined to identify selected transcripts that are known targets of the chemicals typically measured in municipal effluents, such as the indicator compounds (i.e., ibuprofen, naproxen and ammonia) in our study. We used Search Tool for Interaction of Chemicals (STITCH) (v. 5.0) to determine the key molecular targets (transcripts) of the above-mentioned compounds, and we searched our data sets to identify the same transcripts (with $\alpha < 0.05$).

Parametric Analysis of Gene Set Enrichment (PAGE) (Kim and Volsky, 2005) was conducted in JMP Genomics. This statistical approach identifies significantly upregulated or downregulated gene sets that share common biological process, cellular component, or molecular function. Pathway Studio 9.0 (Elsevier) and ResNet 9.0 were utilized for subnetwork enrichment analysis (SNEA) that was conducted with non-FDR corrected DEPs. SNEA uses known

relationships (i.e., based on expression, binding, common pathways) between genes to build networks focused around gene hubs. For all analyses, annotated pathways in Pathway Studio were expanded to include cell processes related to the temporal and spatial exposure. “Name + Alias” was used for mapping rainbow darter genes to mammalian homologs in Pathway Studio, successfully mapping 5968 probes.

3.3.5 Statistical analysis

One-way ANOVA within each individual sampling group (2011, 2012, 2013, and 2014) was used for higher level endpoints including condition factor (k), gonad somatic index (GSI), and liver somatic index (LSI). A Holm-Sidak multiple comparison test was performed in order to detect which level(s) within a group had a significantly different response.

3.3.6 Indicators of effluent quality and exposure

In the current study, pharmaceuticals and water parameters such as inorganic nitrogen products, chlorides and conductivity were measured to characterize the effluent quality, and exposure. We point out that this was not conducted to link gene expression with measured parameters, but rather to show that effluent quality changed over time (especially at the upgraded Kitchener MWWTP).

Detailed information regarding sample collection, preparation, processing, and analysis can be found in Arlos et al., (2014). Wastewater effluent and river water sample collections coincided with fish fall samplings except for samples taken at the Waterloo outfall in 2012 when samples were collected in July. Wastewater effluent (125 mL collected; 100 mL extracted) and 500 mL of surface water were collected and extracted using solid phase Oasis HLB cartridges (6 cc, 500 mg). Cartridges were eluted with methanol and methanol:methyl tert-butyl ether (10:90 v/v), dried with a gentle stream of nitrogen gas and reconstituted with 500 µL of methanol. Contaminants of interest included several pharmaceuticals: ibuprofen, naproxen, venlafaxine, and carbamazepine. They were separated using a 1200 Agilent LC with an Agilent Eclipse XBD-C18 column (5 µm × 4.6 mm × 150 mm), and quantified with a Sciex API 3200 QTRAP MS with electrospray ionization using multiple reaction monitoring mode (Arlos et al., 2014). Recovery of the measured pharmaceuticals in the effluent samples, and river water samples over a four-year period (2011 - 2014) averaged between

70% and 130%. Selected nutrients and water quality analyses were conducted by Maxxam Analytical (Mississauga, ON).

3.4 Results

3.4.1 Fish health

Fish analyzed in the present study (84 samples in total) were adult rainbow darter males with an average body length of 5.7 ± 0.8 cm (ranging from 3.7 - 7.3 cm) (Table 3.1). GSI ranged from 0.93 ± 0.31 to 1.40 ± 0.24 and was not significantly different among any of the sampling collections conducted across years. Conversely, rainbow darter males collected from below Waterloo (DSW) in 2011 had a significantly higher LSI when compared to the fish collected at the reference site (US2). Rainbow darter males from US2 in 2013 had a higher condition factor (1.30 ± 0.062) than those collected below both wastewater outfall sites (DSW and DSK1, 1.22 ± 0.074 and 1.13 ± 0.075, respectively; $p < 0.005$).

Table 3.1 Summary table for rainbow darter analysed in the present study, including sample size, length (cm), weight (g), condition factor (k), liver somatic index (LSI), and gonad somatic index (GSI).

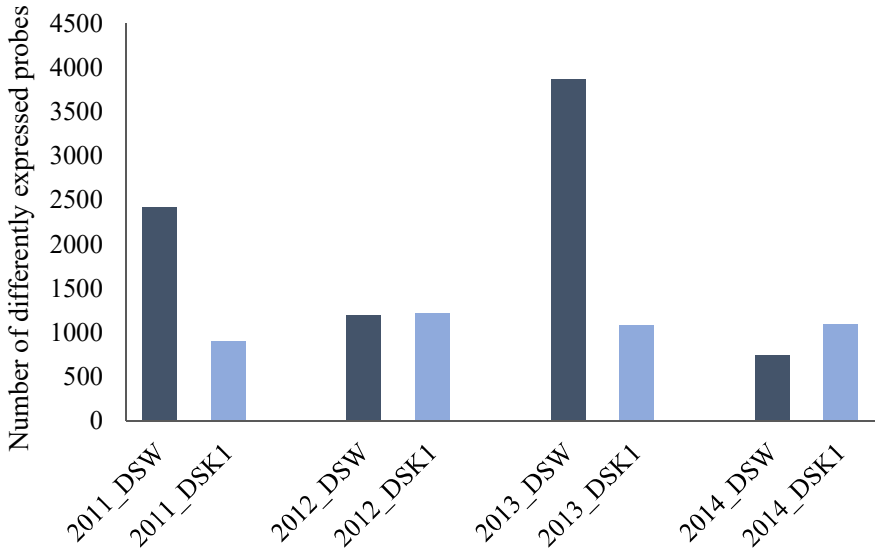
Treatment	Sample size	Length (cm)	Weight (g)	k	LSI	GSI
US2 2011	6	5.70 ± 0.84	2.30 ± 1.07	1.16 ± 0.08	1.62 ± 0.16[b]	1.23 ± 0.16
DSW 2011	6	5.15 ± 1.34	2.11 ± 1.88	1.23 ± 0.12	2.5 ± 0.82[a]	1.02 ± 0.27
DSK1 2011	6	5.25 ± 0.93	1.85 ± 1.04	1.16 ± 0.09	1.65 ± 0.41[ab]	0.94 ± 0.44
US2 2012	7	5.64 ± 0.58	2.26 ± 0.81	1.21 ± 0.08	1.98 ± 0.10	1.36 ± 0.28
DSW 2012	7	6.06 ± 0.73	2.74 ± 1.05	1.18 ± 0.10	2.12 ± 0.28	0.93 ± 0.31
DSK1 2012	7	5.43 ± 0.64	1.98 ± 0.85	1.17 ± 0.08	1.93 ± 0.39	1.32 ± 0.59
US2 2013	7	5.89 ± 0.50	2.70 ± 0.72	1.30 ± 0.06[a]	2.33 ± 0.29	1.17 ± 0.20
DSW 2013	7	5.69 ± 0.80	2.38 ± 0.97	1.22 ± 0.07[b]	2.03 ± 0.25	1.15 ± 0.43
DSK1 2013	6	5.02 ± 0.53	1.48 ± 0.56	1.13 ± 0.08[b]	2.09 ± 0.27	1.17 ± 0.28
US2 2014	6	5.88 ± 0.54	2.78 ± 0.71	1.34 ± 0.10	2.02 ± 0.28	1.17 ± 0.31
DSW 2014	7	6.4 ± 0.76	3.54 ± 1.32	1.28 ± 0.13	2.08 ± 0.23	1.40 ± 0.24
DSK1 2014	7	5.87 ± 0.89	2.58 ± 1.14	1.22 ± 0.19	2.17 ± 0.38	1.36 ± 0.27

Significant differences are indicated with [a] and [b]

3.4.2 Liver transcriptomics in rainbow darter males

To determine liver transcriptome changes in rainbow darter exposed to urbanized sites receiving MWWTP effluents compared to the reference site, the number of unadjusted DEPs, and those that were FDR adjusted were determined. The number of DEPs (unadjusted *p*-value) in rainbow darter male liver varied depending on both exposure site and sampling year. Rainbow darter males collected downstream of the Waterloo MWWTP (DSW) showed higher variability in the number of DEPs over time when compared to rainbow darter males collected downstream of the Kitchener MWWTP outfall (DSK1). In 2011, DSW rainbow darter had 2423 DEPs (987 upregulated and 1436 downregulated). In the following year (2012) the number of DEPs was 1202 (766 upregulated and 436 downregulated), followed by an increase in 2013 when rainbow darter males showed a total of 3867 DEPs in the liver (2265 upregulated and 1602 downregulated). The fewest number of DEPs was observed in rainbow darter collected in 2014 when there were 767 DEPs (314 upregulated and 433 downregulated) (Figure 3.2A). As stated above, rainbow darter males exposed to the effluent discharged from the Kitchener MWWTP (DSK1) did not show a significant response as reflected in the numbers of DEPs over the course of four years. In 2011, the number of DEPs was 904 (424 upregulated and 480 downregulated). Rainbow darter from this site had the highest number of DEPs at 1223 (568 upregulated and 655 downregulated) in 2012. In the following consecutive years, 2013 and 2014, there was a decrease to 1086 (591 upregulated and 495 downregulated), and 1093 DEPs in the final sampling year (285 upregulated and 808 downregulated) (Figure 3.2A). In 2012 and 2014, no transcript passed an FDR adjusted *p*-value from either of the exposed sites compared to reference fish (Figure 3.2B).

A



B

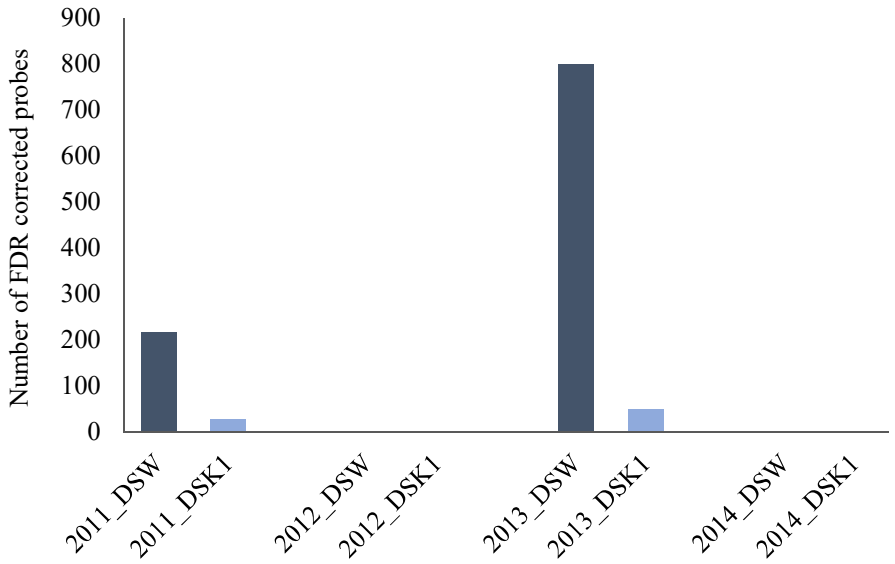


Figure 3.2 Differentially expressed probes (DEPs). Number of DEPs using unadjusted *p*-values (A), and false discovery rate (FDR) corrected *p*-values (B) in rainbow darter collected from the Waterloo MWWTP (DSW) and the Kitchener MWWTP (DSK1) compared to the upstream reference site (US2) over time.

3.4.3 Selected transcripts that were differentially expressed in rainbow darter over time and sites

The top three genes that were significantly upregulated, and the top three genes that were significantly downregulated are presented in Table 3.2. These transcripts also showed site-specific and/or time-specific patterns in the liver of rainbow darter from the two polluted sites over time. The highest downregulated transcript in the liver was proto-oncogene protein c-Fos in males from DSW in 2011, which showed a 138.79-fold decrease in expression ($p < 0.001$). This transcript was also differentially expressed in rainbow darter from both DSW and DSK1 in 2014 but in this year, the transcript was significantly upregulated in fish from both sites (5.70, $p = 0.008$ and 5.73, $p = 0.008$, respectively). The expression of hemoglobin beta was also differentially expressed in males at different sites and in different years. Rainbow darter from DSK1 in 2011 and in 2012 showed a significant upregulation of this transcript (5.44, $p = 0.031$ and 4.02, $p = 0.002$, respectively), whereas rainbow darter from DSW in 2014 showed a downregulation of this gene (-3.48, $p = 0.017$). Choriogenin H minor also showed differential expression over time. This transcript was upregulated in rainbow darter from DSW compared to males collected at US2 in three consecutive years, 2011 (9.26; $p = 0.003$), 2012 (6.55; $p = 0.016$) and 2013 (63.92; $p < 0.001$). Thus, choriogenin H minor showed a consistent response in the liver over time at this particular site. Compared to rainbow darter from the control site, carboxypeptidase A1 was decreased in rainbow darter from both DSW and DSK1 in 2013. Rainbow darter from DSW expressed a 7.96-fold downregulation ($p = 0.043$) while in the DSK1 fish the same transcript had a 20.70-fold downregulation ($p = 0.007$). Thus, there were some transcripts that consistently showed significant changes over time and across sites.

Table 3.2 Genes with a fold change larger than three positive, and lower than three negative between the reference site, and each of the two downstream sites (DSW and DSK1) from 2011 to 2014. Selected are those transcripts that showed site fidelity across time, and they are indicated with superscript "a"; those that showed expression across sites, indicated with "b"; and those that showed site fidelity, and expression across time, indicated with superscript "ab".

Year	DSW	DSK1	Fold change	p-value	Gene function
2011	Proto-oncogen protein c-Fos[ab]		-138.79	3.66E-06	DNA binding; cell signaling is the function; nucleus
	Choriogenin H minor[a]		9.26	0.003	Zona pellucida sperm-binding protein 4-like
		Hemoglobin β[ab]	5.44	0.031	Heme binding; oxygen binding; oxygen transporter activity; hemoglobin complex
2012	Choriogenin H minor[a]		6.55	0.016	Zona pellucida sperm-binding protein 4-like
2013	Choriogenin H minor[a]		63.92	4.71E-05	Zona pellucida sperm-binding protein 4-like
	Carboxypeptidase A1[b]	Carboxypeptidase A1[b]	-7.96; -20.70	0.043; 0.007	Metallocarboxypeptidase activity; zinc ion binding; proteolysis
	Type-4 ice-structuring protein LS12[b]		11.57	0.008	Lipid binding; lipid transport; lipoprotein metabolic process
		Hemoglobin β[ab]	4.02	0.002	Heme binding; oxygen binding; oxygen transporter activity; hemoglobin complex;
2014	Hemoglobin β[ab]		-3.48	0.017	Heme binding; oxygen binding; oxygen transporter activity; hemoglobin complex
	Proto-oncogen protein c-Fos[ab]	Proto-oncogen protein c-Fos[ab]	5.70; 5.73	0.008; 0.008	DNA binding; cell signaling is the function; nucleus
		Type-4 ice-structuring protein LS12[b]	8.93	0.000	Lipid binding; lipid transport; lipoprotein metabolic process

Using STITCH, we first identified transcripts that are known targets of both non-steroidal anti-inflammatory pharmaceuticals, ibuprofen and naproxen, and these included prostaglandin-endoperoxide synthase 1 and prostaglandin-endoperoxide synthase 2, arachidonate 5-lioxygenase, and albumin. Rainbow darter in our study did not show differential expression for any of these transcripts, except prostaglandin E synthase 3. In 2011, rainbow darter from DSW and DSK1 showed that this transcript was downregulated, whereas in 2013, rainbow darter collected from DSW showed an upregulation of this transcript. STITCH identified several transcripts to be targets of ammonia, and with support from literature (Wright et al., 2007), we narrowed this selection to the following transcripts: glutamate dehydrogenase 1b, glutamate dehydrogenase 1a, glutamine synthase a and glutamine synthase b. None of these transcripts were identified in the rainbow darter from our study. Thus, expression patterns observed are more likely the result of multiple stressor interactions or due to unmeasured chemical/environmental factors.

3.4.4 Two-way ANOVA

Two-way ANOVA was conducted in order to expand our understanding of the main factor affecting the hepatic transcriptome (e.g., site or year-specific). Following the two-way ANOVA, it was determined that the effect of year was more dominant than that of site (Figure 3.3). There were approximately twice as many probes responding to year (i.e., time) compared to site. The interaction of both year and site also explained variation in a significant number of probes, more so in this case than site alone.

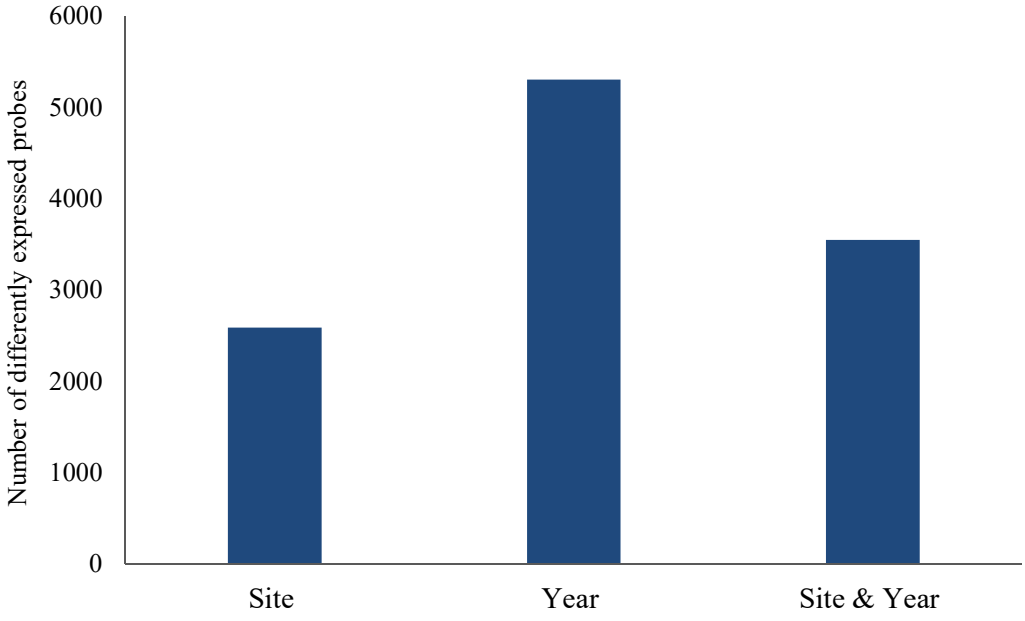


Figure 3.3 Transcriptome responses analyzed with a two-way ANOVA. The two-way ANOVA reveals separate effects of site-specific and year-specific factors, and their combined effect on the rainbow darter liver transcriptome.

3.4.5 Gene set enrichment analysis (GSEA)

Gene set enrichment analysis was used to determine if there were over-represented gene ontologies in rainbow darter livers following collections at sites DSW and DSK1 over the four years. GSEA focuses on identifying gene sets that are associated with either biological or biochemical pathways, which are defined a priori. There were three categories of functional analyses that were evaluated, that included biological processes, cellular components, and molecular functions. It was observed that the number of affected gene sets in all three categories displayed, in general, a decreasing trend from 2011 to 2014, in rainbow darter from both sites (DSW, DSK1) (Figure 3.4A). In addition, rainbow darter males originating from site DSW consistently showed (over the four-year course) a greater number of impacted gene sets when compared to rainbow darter males from DSK1 (Figure 3.4B).

To assess overlap in expression profiles in rainbow darter collected from the two sites, Venn diagrams were used, which were generated by Venny (Oliveros, 2007 - 2015). This program identifies common sets of genes related to identical biological processes, cellular components or molecular functions, and determines interactions between them. Venn diagrams showed that DSW and DSK1 rainbow darter in 2011 shared 26.8% of the biological processes that were differently expressed from the US2 rainbow darter. In the following years, the percentage of shared biological processes decreased to 22.8% in 2012, 12.7% in 2013 and 13.6% in the final 2014 sampling year (Figure 3.5A). It was also observed that there was a decrease in the percentage of shared cellular component and molecular function entities between DSW and DSK1 rainbow darter over time (Figure 3.5B and C).

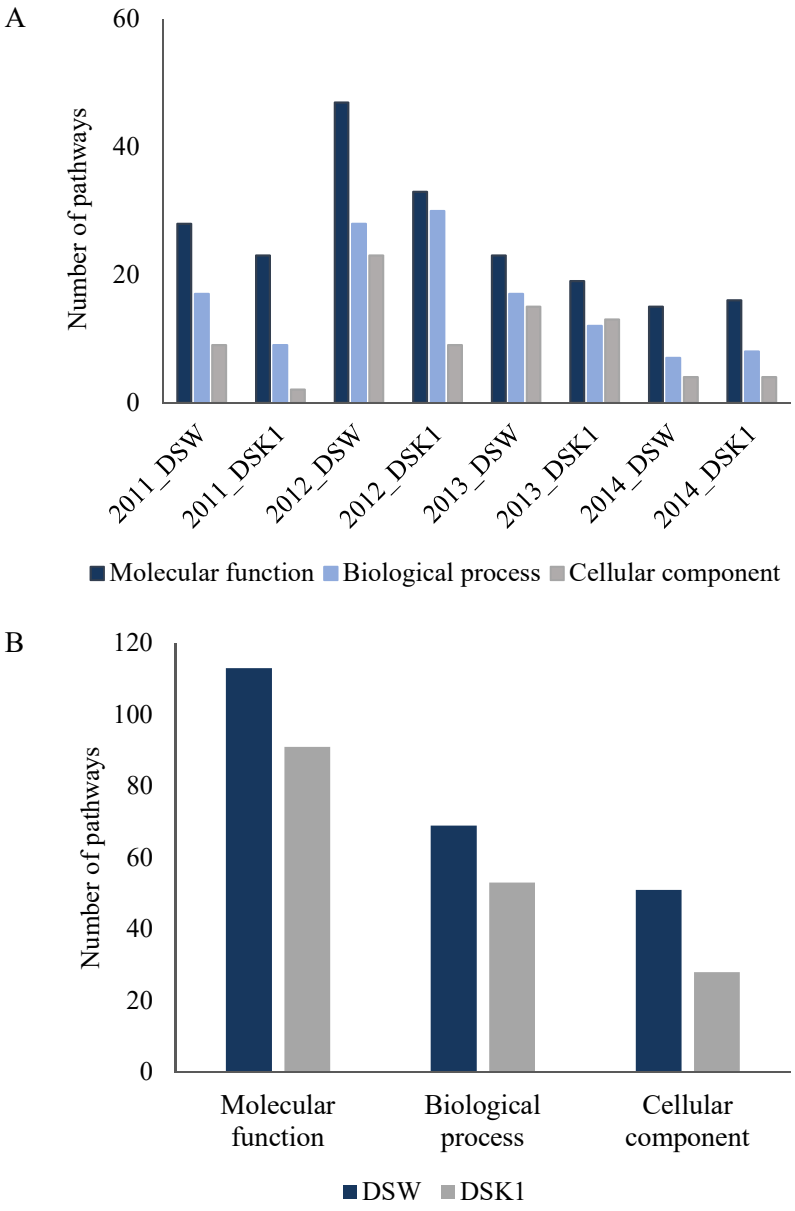


Figure 3.4 Gene set enrichment analysis (GSEA). Shown are sets of genes involved in various biological processes, cellular components and molecular functions using adjusted *p*-values of transcripts in rainbow darter males collected from the Waterloo MWWTP (DSW) and the Kitchener MWWTP (DSK1) throughout four sampling seasons (2011 - 2014) (A); GSEA components based on adjusted *p*-values of enriched gene sets in rainbow darter males collected downstream of the Waterloo MWWTP (DSW) compared to the rainbow darter males collected at the Kitchener MWWTP (DSK1) (B).

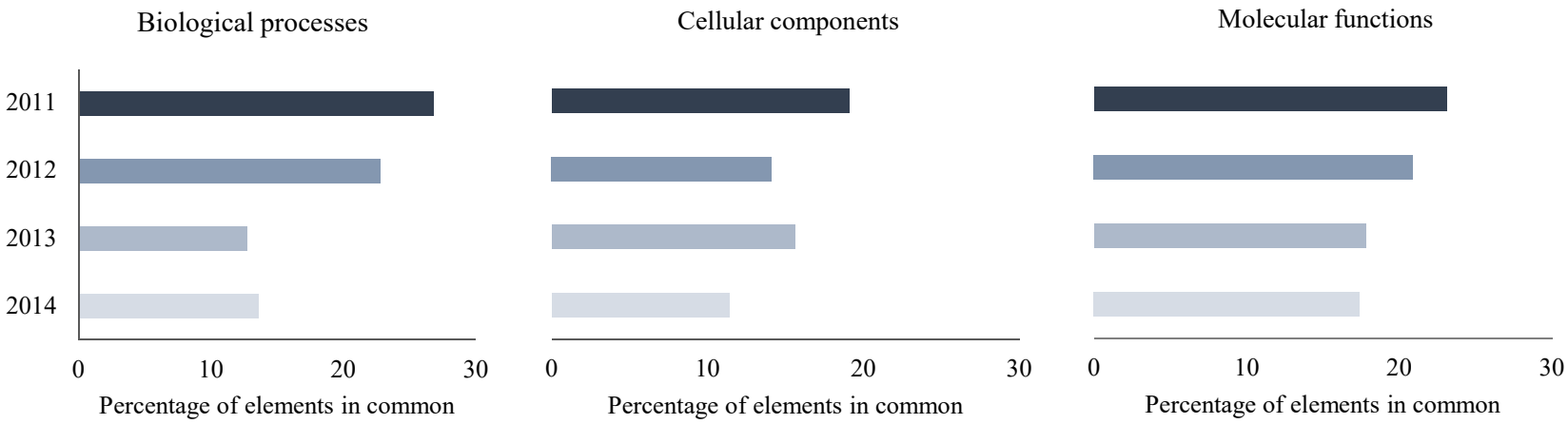


Figure 3.5 Gene set enrichment analysis (GSEA) ontologies shared between DSW and DSK1 rainbow darter. Percentage of GSEA biological processes, cellular components and molecular functions in common for the Waterloo MWWTP (DSW) exposed rainbow darter and the Kitchener MWWTP (DSK1) exposed rainbow darter in 2011, 2012, 2013 and 2014.

3.4.6 Subnetwork enrichment analysis (SNEA)

Differentially expressed cell processes identified by SNEA were individually inspected and manually categorized based on their relationship to other cell processes, and their mutual involvement in known biological functions (i.e., grouped by prevailing themes such as processes related to cardio-vascular system, reproduction, immunity, cell signaling and many others). Among the eight levels of comparison (2 sites × 4 years), there were 20 different categories, each containing a minimum of two or more cell processes. There were also cell pathways that remained uncategorized as they did not fall in any of the 20 categories (Figure S3.1). Special attention was devoted to identifying cell processes that were unique and prevalent in rainbow darter from both DSW and DSK1 (Table S3.1 and S3.2).

In 2011, unique and dominant cell processes that were affected in rainbow darter collected downstream of the Waterloo MWWTP were related to DNA replication and DNA repair, namely: "DNA double-strand break formation," "DNA metabolism," and "error-prone post replication DNA repair." Further, "cell aging," "intrinsic pathway of apoptosis," and "necrotic cell death," were also affected and unique to individuals at this site. The following year (2012) was dominated by cell processes related to the immunity: "establishment of T-cell polarity," "lymphocyte adhesion," "natural killer cell proliferation," "granulocyte function," and "Th1 immune response." In 2013, DSW rainbow darter expressed uniqueness in "mRNA elongation," "mRNA stabilization," "mRNA processing," "poly(A) + mRNA-nucleus export," "mRNA metabolism and degradation." (Figure S3.2). Rainbow darter from DSW in 2014 had unique immunity related cell processes (Table S3.1).

Compared to rainbow darter from the reference site, those males exposed downstream of the Kitchener MWWTP in 2011 had enriched and unique cell cycle processes preceding DNA replication ("S-G2 transition," "G2/M transition," "M phase," and "chromosome condensation"). Pathways related to "meiosis," "exit from mitosis," and "mitotic checkpoint" may indicate that cell division was affected in both somatic and sex cells of these individuals. DNA replication was also compromised at the transcriptome level, based on enriched processes such as "DNA replication checkpoint" and "DNA metabolism." Further, overall genome integrity was affected ("genome stability" and "genetic instability"), in addition to "cell aging" and "autolysis" (Figure 3.6). In 2012, rainbow darter from DSK1 were unique in the sense that most of the processes affected were related to immunity (Figure 3.7).

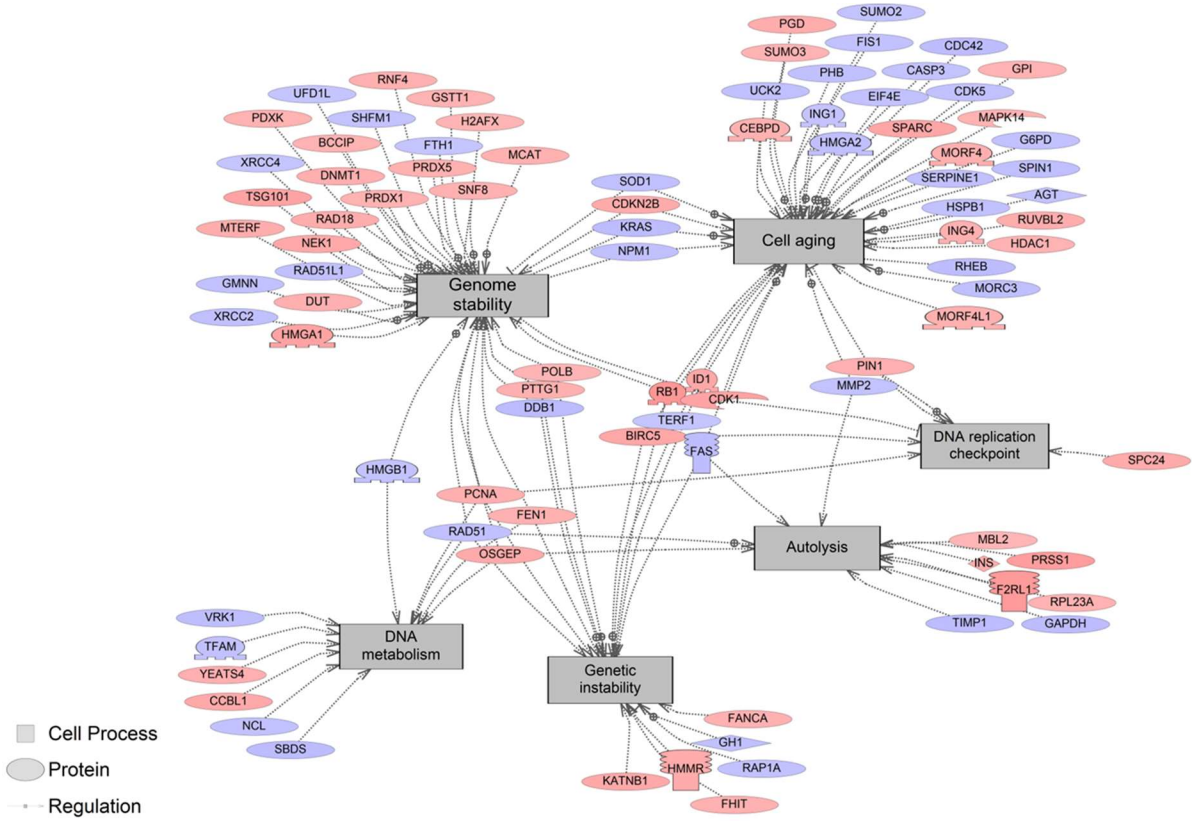


Figure 3.6 Pathway analysis of cell process differently regulated by the Kitchener MWWTP effluent in 2011. The majority of transcripts were associated with cell aging which was increased, and genome stability which was decreased. DNA processing involving DNA metabolism and DNA replication checkpoint were down-regulated, and up-regulated, respectively. Both autolysis and genetic instability were up-regulated at the transcriptome level. Transcripts colored in red are upregulated whereas those in violet are downregulated. Details on specific entities involved in the presented pathways can be found in Table S3.3.

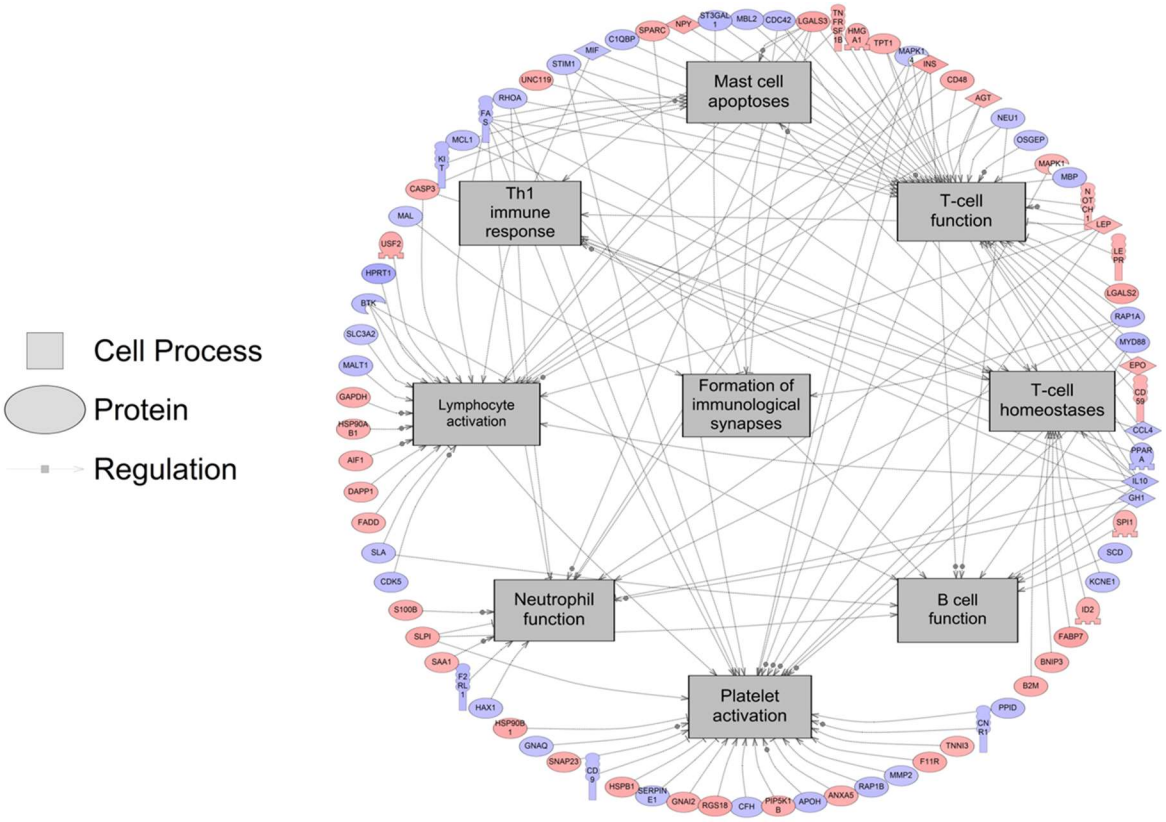


Figure 3.7 Pathway analysis of cell process differently regulated by the Kitchener MWWTP effluent in 2012. The majority of transcripts were associated with innate immune system responses and most of them were down-regulated except T-cell homeostasis, platelet activation and lymphocyte activation which were up-regulated. Transcripts colored in red are upregulated whereas those in violet are downregulated. Details on specific entities involved in the presented pathways can be found in Table S3.4.

Processes involving T-cells, which play an important role in the immune response, showed changes at the transcriptome level, and these included “T-cell homeostasis establishment” as well as “establishment of T-cell polarity”. Along with T-cells, function of B-cells was also affected, as well as “platelet function” and “complement activation”. Rainbow darter from DSK1 in 2013 had approximately 47% of unique cell processes, with immune responses showing prevalence over others. “Complement activation,” “lymphocyte chemotaxis,” “monocyte response,” and “T-cell tolerance” were unique to this particular group. Rainbow darter collected in 2014 had 12 unique cell processes compared to all years and datasets (Table 3.3). These included cell processes such as “macro autophagy,” “nervous system physiology,” “sex maturation,” and “apoptosis of neutrophils.” These may be viewed as processes that are responsive in the liver to upgrades since they are not enriched in any other year nor were observed in the DSW fish.

Table 3.3 Subnetwork enrichment analysis (SNEA) for cell pathways that were unique for rainbow darter exposed to the Kitchener MWWTP effluent in 2014. These transcriptome responses are hypothesized to be related to the upgrades.

SNEA cell process pathways	Median change	*p*-value
Macro autophagy	1.78	0.018
Receptor clustering	1.66	< 0.001
Nervous system physiology	1.55	0.046
Sex maturation	1.55	0.032
Osteoclast function	1.26	0.049
Macrophage chemotaxis	1.19	< 0.001
Apoptosis of neutrophils	1.08	0.015
Artery blood flow	-1.04	0.037
Acid secretion	-1.04	0.031
Phosphate import	-1.25	0.044
Tropism	-1.27	0.033
Mitochondrial translocation	-1.28	0.029

3.4.7 Clustering

Hierarchical clustering of expression data did not result in any clear pattern for site or year. Clear sample segregation did not exist except for a tendency of reference sites and/or polluted sites, regardless of year, to cluster more often (Figure S3.3).

3.4.8 Chemistry data

Waterloo MWWTP had gone through minor treatment changes that partially improved the effluent quality (2011 - 2014). Pharmaceutical concentration in effluent samples decreased significantly over time but still remained relatively high in the final 2014 sampling year (e.g., ibuprofen = 635 ng/L, naproxen = 482 ng/L). In spite of its gradual decrease (2011 - 2014), ammonia concentration still remained elevated, with no evident increase in nitrate concentration (Table 3.4). Concentration of pharmaceuticals in river samples did not decrease significantly over time. Ammonia concentration in the river started to decrease in 2013 but this was not significant. Concentration of nitrate dramatically increased only in 2014 (Table 3.5).

Chemical analyses suggested that the Kitchener MWWTP effluent quality improved over time 2011 - 2014. Based on a significant decrease in concentration of conservative pharmaceuticals (i.e., ibuprofen and naproxen) in the effluent samples, particularly after 2012, it can be concluded that the implemented upgrades had a positive outcome (Table 3.4). Significant decreases in total ammonia concentration, especially in 2013, resulted in a significant nitrate increase in the effluent, in the same time period, which further confirms that the upgrades had improved the effluent quality. Ammonia dropped significantly in the river after 2012 (Table 3.5), with a simultaneous significant increase in nitrate concentration from 2012 - 2014.

Table 3.4 The analyte list represents a diversity of pharmaceuticals that were measured in the river water samples collected at the studied sites and in the effluent samples originating from the Waterloo MWWTP and the Kitchener MWWTP over four fall seasons (2011 - 2014). Values in the table represent mean concentration in ng/L and corresponding standard deviation. Pharmaceutical data collection for US2, DSW and Waterloo MWWTP in 2012 did not coincide with the fish sampling conducted in the fall. Instead, it corresponded to a sampling event conducted in July 2012.

Pharmaceutical	US2	DSW	DSK1	Waterloo MWWTP	Kitchener MWWTP
			2011		
Ibuprofen	5.3 ± 0.7	832.3 ± 187.5	231.0 ± 45.9	4543.0 ± 244.0	1742.0 ± 42.5
Venlafaxine	9.3 ± 0.5	159.0 ± 44.0	130.6 ± 36.0	1033.0 ± 65.1	1585.0 ± 17.3
Naproxen	9.5 ± 0.6	534.7 ± 111.8	191.7 ± 48.0	3377.0 ± 714.1	1718.0 ± 77.5
Carbamazepine	59.6 ± 1.5	126.0 ± 12.8	95.5 ± 36.8	426.7 ± 14.7	676.7 ± 7.6
			2012		
Ibuprofen	134.0 ± 25.2	201.7 ± 13.3	112.0 ± 19.7	803.0 ± 261.0	376.0 ± 68.4
Venlafaxine	11.8 ± 0.8	190.3 ± 28.2	173.0 ± 44	1053.0 ± 63.0	1045.0 ± 234.4
Naproxen	4.5 ± 1.2	241.3 ± 52.5	122.0 ± 9.5	1622.0 ± 495.0	630.0 ± 52.9
Carbamazepine	13.4 ± 2.3	16.4 ± 2.5	61.5 ± 6.9	39.0 ± 2.0	407.8 ± 132.6
			2013		
Ibuprofen	0.0 ± 0.0	112.0 ± 105.3	41.5 ± 5.57	2318.33 ± 164.34	72.5 ± 15.60
Venlafaxine	9.58 ± 1.7	17.8 ± 4.4	55.7 ± 4.76	846.67 ± 102.02	595 ± 37.75
Naproxen	3.4 ± 1.0	75.3 ± 65.9	25.63 ± 6.26	1711.67 ± 252.70	346.5 ± 49.37
Carbamazepine	4.6 ± 1.2	27.1 ± 6.1	21.6 ± 2.72	202.83 ± 15.33	275.67 ± 17.50
			2014		
Ibuprofen	10 ± 5.7	719.0 ± 642.7	51.0 ± 20.1	635.0 ± 141.4	26.0 ± 23.3
Venlafaxine	8 ± 1.2	4.0 ± 0.3	95.0 ± 44.5	587.0 ±0.0	847.0 ± 28.9
Naproxen	12 ± 7.1	26.0 ± 6.3	36.0 ± 15.6	482.0 ± 89.1	111.0 ± 52.6
Carbamazepine	38 ± 13.8	105.0 ± 50.6	97.0 ± 74.7	277.0 ± 19.2	346.0 ± 19.6

Table 3.5 Selected nutrients measured in the river water samples at the studied sites and in MWWTP effluent samples (Waterloo and Kitchener MWWTP) collected over four fall seasons (2011 - 2014). Total ammonia, nitrite, nitrate, and dissolved chloride mean concentration is in mg/L, whereas conductivity is measured in µmho/cm.

Nutrient	US2	DSW	DSK1	Waterloo MWWTP	Kitchener MWWTP
			2011		
Total ammonia	0.1 ± 0.1	12.3 ± 10.2	2.1 ± 0.5	36.3 ± 1.1	16.9 ± 10.6
Nitrite	0.0 ± 0.0	1.1 ± 1.0	0.5 ± 0.2	1.8 ± 1.2	3.4 ± 0.0
Nitrate	2.6 ± 0.1	2.7 ± 0.3	3.6 ± 0.1	0.3 ± 0.1	1.0 ± 0.1
Conductivity	545.0 ± 3.0	884.0 ± 62.0	791.0 ± 60.0	2343.0 ± 60.0	2577.0 ± 6.0
Dissolved chloride	30.0 ± 1.0	104.0 ± 14.0	82.0 ± 9.0	447.0 ± 15	510.0 ± 0.0
			2012		
Total ammonia	0.0 ± 0.0	3.5 ± 0.8	1.5 ± 0.2	23.7 ± 0.6	23.3 ± 0.6
Nitrite	0.0 ± 0.0	1.5 ± 0.1	0.2 ± 0.0	2.5 ± 0.2	1.4 ± 0.1
Nitrate	0.7 ± 0.0	1.9 ± 0.1	1.5 ± 0.0	1.7 ± 0.2	1.9 ± 0.2
Conductivity	N/A	N/A	N/A	N/A	N/A
Dissolved chloride	22.0 ± 0.0	117.0 ± 15.3	79.0 ± 4.6	383.0 ± 5.8	427.0 ± 5.8
			2013		
Total ammonia	0.0 ± 0.0	2.8 ± 0.8	0.5 ± 0.0	24.3 ± 1.2	0.4 ± 0.0
Nitrite	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.4 ± 0.0
Nitrate	2.6 ± 0.1	3.2 ± 0.2	4.5 ± 0.2	0.6 ± 0.2	17.7 ± 0.6
Conductivity	N/A	N/A	N/A	N/A	N/A
Dissolved chloride	31.0 ± 0.0	87.0 ± 10.5	74.0 ± 6.2	393.0 ± 20.8	417.0 ± 5.8
			2014		
Total ammonia	0.1 ± 0.0	1.0 ± 0.7	0.2 ± 0.0	14.0 ± 0.0	1.1 ± 0.0
Nitrite	0.0 ± 0.0	0.4 ± 0.5	0.1 ± 0.0	2.2 ± 0.0	0.8 ± 0.0
Nitrate	1.8 ± 0.0	3.1 ± 1.0	3.3 ± 0.3	13.1 ± 0.1	16.5 ± 0.1
Conductivity	597.0 ± 5.8	807.0 ± 106.0	813.0 ± 40.4	2200.0 ± 0.0	2000.0 ± 0.5
Dissolved chloride	28.0 ± 0.6	73.0 ± 26.5	80.0 ± 9.1	413.0 ± 11.5	377.0 ± 5.8

3.5 Discussion

3.5.1 Over-time environmental conditions at the MWWTPs impacted sites

Major upgrades were planned for the Waterloo MWWTP, however a contractual issue delayed implementation. In spite of this issue, small process changes led to differences in the Waterloo effluent quality between 2011 and 2014. The highest (annual) ammonia load was reported in 2012 when nitrification of the effluent was minimal (Hicks et al., 2016). In contrast, the Kitchener MWWTP underwent major process upgrades (i.e., improved aeration and solids retention time (SRT)) which were initiated in August 2012, and came fully on line in early 2013. These upgrades resulted in a major effluent quality improvement with ammonia being greatly reduced (and nitrate increased) as a result of enhanced nitrification (Bicudo et al., 2016; Hicks et al., 2016). Nitrification and extended SRT have previously been shown to be associated with greater removal of a variety of trace contaminants including pharmaceuticals and environmental estrogens (Metcalfe et al., 2010; Clara et al., 2005a; Servos et al., 2005; Andersen et al., 2003). The treatment changes were also evident as the relative concentrations of the selected indicator pharmaceuticals, such as ibuprofen and naproxen, were greatly reduced in the final effluent. These chemicals are known to decrease with improved wastewater practices (Clara et al., 2005b; Heberer, 2002), and have been proposed as treatment indicators (Salveson et al., 2013; Dickenson et al., 2011). In contrast, it is well known that recalcitrant chemicals such as carbamazepine and venlafaxine are resistant to treatment (Rua-Gomez and Puttmann, 2012; Metcalfe et al., 2010), and their concentrations remained relatively consistent in the final effluent over the study period.

Changes in effluent quality are also modified by environmental conditions in the watershed across years and are likely to influence transcriptome responses. The year 2012 was unusually dry (low flow) and warm compared to 2013 that was unusually wet (high flows) (Hicks et al., 2016). Such conditions can alter the dilution of effluent, the distribution of the plume and exposure of organisms including rainbow darter to chemicals, as well as other environmental factors (temperature, eutrophication, habitat, etc.). The fish collections in 2013 were completed after a period of particularly high river flows. Altered exposure and environmental factors such as fluctuation in temperature and oxygen availability, are potentially important in modifying gene expression across years and sites. It has been documented that hypoxia can affect reproductive function, such as impaired sperm production and testicular growth (Thomas et al., 2007). It can also

affect biotransformation of lipids, steroids, vitamins, and environmental toxicants through changes in CYP1A mRNA expression (Rahman and Thomas, 2012). In addition, it alters stress-related proteins, and hypoxia inducible factor (HIFα) which controls the expression of multiple genes during hypoxia (Martínez et al., 2006; Semenza 2001). Hypothermic and hyperthermic conditions are reported to induce expression of stress-related genes and to affect the metabolic rate of organisms (Vergauwen et al., 2010). Rainbow darter have the potential to move in these open systems, however, stable isotope analysis has demonstrated that the majority of rainbow darter have a very small home range of a few meters (Hicks et al., 2016; Tetreault et al., 2011), suggesting that there can be prolonged exposure to chemical stressors. Although field-based samples are complicated by natural variability as well as anthropogenic factors (e.g., multiple stressors), some patterns in gene expression are discernible across the various sites in rainbow darter that are associated with effluent exposure. These results are particularly useful for advancing our understanding of the mechanisms of how effluent potentially impacts fish in watersheds and contributes to the generation of new hypotheses for further studies.

3.5.2 Differentially expressed probes across sites and years

Rainbow darter downstream of the Waterloo MWWTP (DSW) had a higher number of altered transcripts over time which was particularly evident in 2011 and 2013. Based on chloride, which is a conservative indicator in effluent exposure, the Waterloo site appears to have had similar or slightly higher effluent exposure (10 - 16%) across the years (except for 2012) compared to the site below the Kitchener outfall. Although Waterloo MWWTP did not have major upgrades during the study period, there were changes in the effluent quality (Hicks et al., 2016) that resulted in altered contaminant distribution in the effluent. Interestingly, the concentration of the more labile pharmaceuticals (ibuprofen, naproxen) in the Waterloo MWWTP effluent measured in the study periods was the greatest in 2011 and 2013, which corresponds to the lowest nitrate values (means 0.3 and 0.6 mg/L) in the effluent, and the elevated DEPs in fish. The river values are likely much more variable, and less indicative of longer term exposure prior to fish collections because of the effects of rapidly changing river flow. The ratio of ibuprofen to venlafaxine was considerably lower in 2012 (0.8) and in 2014 (1.1), compared to 2011 (4.3) or 2013 (2.7) suggesting poorer effluent treatment during that period. The Waterloo MWWTP effluent quality improved greatly in 2014 possibly due to the introduction of return activated sludge reaeration tanks that resulted in additional nitrification. It is also noteworthy that rainbow darter exposed downstream of the Waterloo MWWTP may have

been collected with different exposures depending on the plume distribution or small movement of the fish in different years.

The changes in treatment processes in the Kitchener MWWTP resulted in improved effluent quality but did not result in a reduced number of differentially expressed transcripts. The highest number of DEPs in rainbow darter from downstream of the Kitchener MWWTP (DSK1) was in 2012. These fish were exposed to partially improved effluent for at least two months (upgrades were initiated in August 2012) prior to being collected from the river. It is also possible that rainbow darter responded to the dramatic change in effluent quality in terms of the transcriptome, in addition to other site-specific variables. As mentioned, the year 2012 was unusually dry, which may have led to reduced effluent dilution and higher exposure to a variety of contaminants. Based on the relative concentrations of conservative chemicals (i.e., chloride, venlafaxine) in the effluent vs. river samples (used to calculate dilution), it was estimated that the exposure to the effluent was greatest in 2012 (possibly as high as 18% effluent below Kitchener).

In the present study, the transcriptome response was variable depending on the field sites and environmental conditions (e.g., exposure). It is also important to point out that the number of DEPs may not necessarily indicate negative impacts due to chemical exposure. A single comparison of sites within a year, as it is often done in environmental omics studies, may not lead to a sound conclusion without better understanding of exposure conditions in addition to improved knowledge about the genes involved.

3.5.3 Selected transcripts that were differentially expressed

There were several transcripts that were affected at multiple time points at one or both exposed sites, suggesting that these transcripts are potential molecular endpoints that may be useful for monitoring. One of these transcripts was choriogenin H minor, which showed upregulation in rainbow darter exposed downstream of the Waterloo MWWTP in three consecutive years (2011 - 2013). In teleost fish, choriogenin H minor is synthesized in the liver of adult females in response to estrogens, and then transported to the ovary (Arukwe and Goksoyr, 2003), where along with other liver synthetized proteins, it becomes incorporated into the egg envelope (zona pellucida or chorion). Expression of chorion proteins in male fish indicates exposure to xenoestrogens, and represents a sensitive indicator of endocrine disruption (Kurauchi et al., 2005; Fujita et al., 2004). Choriogenin mRNA was shown to be a responsive, and sensitive biomarker in early developmental stages, and

adult *Oryzias melastigma* exposed to 17β-estradiol, 17α-ethinylestradiol, nonylphenol, and bisphenol-A (Chen et al., 2008). Furthermore, chorion genes have been used as biomarkers of testis-ova condition in Japanese medaka (*Oryzias latipes*) exposed to 17α-ethinylestradiol (Hirakawa et al., 2012). Continuous upregulation of choriogenin H minor in rainbow darter from DSW may suggest estrogenic exposure of fish, most likely originating from the Waterloo MWWTP which persisted in the river water where these fish resided. Although choriogenin H minor is a biomarker of endocrine disruption, it is not as widely used as vitellogenin mRNA (*vtg*). The *vtg* transcript was not elevated in rainbow darter in the present study, even though *vtg* induction has been commonly seen as a response to estrogens in wastewater exposed sites (Jobling et al., 1998; Folmar et al., 1996). Bahamonde et al., (2014) found that *vtg* was increased in rainbow darter males collected below the Kitchener MWWTP in the spring of 2011 which also showed 67% of intersex incidence (Bahamonde et al., 2014). Based on yeast estrogen screen (YES) of samples collected from the Waterloo MWWTP in the summer of 2010, there was 4.32 ± 0.07 ng/L 17β-estradiol equivalents (Tanna et al., 2013). In several studies, it has been shown that the intersex occurrence in the rainbow darter collected downstream of the Waterloo MWWTP was more prevalent compared to the upstream sites (Fuzzen et al., 2015; Tanna et al., 2013). Considering the increased incidence of intersex in rainbow darter in the urbanized stretch of the Grand River, it is possible that the reported estrogenicity was sufficient to affect molecular endpoints indicative of (xeno)estrogenic exposure. It has been suggested that choriogenin H and L may be more sensitive than *vtg* (I and II) in *Oryzias latipes* exposed to 17α-ethinylestradiol (Lee et al., 2002), which implies that choriogenin H minor may provide improved sensitivity to estrogen exposures in rainbow darter males. This biomarker should be explored further, and its sensitivity rigorously compared to *vtg*.

Proto-oncogene protein c-Fos (*c-fos*) was by far the most downregulated transcript in rainbow darter from DSW in 2011 when the Waterloo MWWTP was discharging effluent of relatively poor quality. Proto-oncogenes including *c-fos*, *c-myc* and c-*jun*, encode nuclear proteins that function as transcription factors and are highly conserved in eukaryotic organisms. These transcription factors are mainly involved in cellular growth, proliferation, and differentiation (Weinberg, 1985). In a study examining hepatic transcriptomic responses in largemouth bass (*Micropterus salmoides*) exposed to the weakly estrogenic organochlorine pesticide methoxychlor (25 mg/kg) for 48 h, *c-fos* showed a downregulation whereas *esr1* and *ar* were upregulated (Martyniuk et al., 2011), thus this chemical can modify the expression of *c-fos*. In a study that

investigated *in vitro* hepatocyte exposure of male channel fish (*Ictalurus punctatus*) to 2000 μg/mL of pentachlorophenol for 48 h, c-fos protein was over-expressed along with vitellogenin protein (Dorsey and Tchounwou, 2004), suggesting that this transcript is estrogen responsive. Based on the regulation of these estrogen-responsive transcripts, xenoestrogens may be present in the Waterloo MWWTP effluent.

Hemoglobin β (*Hbβ*) was another transcript that showed variability over multiple years, as well as site-dependent expression. The major role of hemoglobin is carrying oxygen from the respiratory surface (gills) to the inner organs including the liver. Hemoglobin can be affected by hypoxia; however, studies investigating effects of low oxygen concentration on hemoglobin genes expression showed conflicting results (Nikinmaa and Rees, 2005). Hypoxia can result in adverse effects on hemoglobin gene expression and hemoglobin induction, and it is well known that it can compromise energetically expensive processes such as foraging or spawning. Hypoxia is also associated with MWWTP effluents. Prior to the upgrades at the Kitchener MWWTP, the areas downstream of the outfall exhibited reduced oxygen, especially in the early mornings during the summer (Cooke, 2006). Effluents originating from MWWTPs usually contain high concentrations of nutrients which leads to higher respiration of biofilms and plants, ammonia that is nitrified in the river (consuming oxygen), increased dissolved salts and higher temperature, all of which can decrease the amount of dissolved oxygen in the recipient system. *Hbβ* mRNA was upregulated in rainbow darter exposed downstream of the Kitchener MWWTP in 2011 and 2012 during the period when nutrient release and low flows caused oxygen depletion in the river (Grand River Conservation Authority online monitoring data). Rainbow darter exposed to the Waterloo MWWTP in 2014 displayed a downregulation of *Hbβ.* It is difficult to interpret why this transcript was downregulated considering that the effluent quality in 2014 was improved and the annual flow data are not indicative of any extremes (dry or wet years) compared to previous years, in particular 2011, and 2012 (Grand River Conservation Authority online monitoring data).

In field-based transcriptomics, one overarching goal is to somehow associate chemical exposures to expression profiles. However, this is no trivial task as there are hundreds of chemicals in the effluent mixtures which cannot be measured. In the present study, ibuprofen and naproxen are two non-steroidal anti-inflammatory drugs that were measured. Analytical methods are in place to assess these pharmaceuticals accurately and these compounds are considered to be surrogates for

how well a sewage treatment upgrade is performing overall. These chemicals also changed after the upgrades (compared to carbamazepine and venlafaxine that remained relatively constant). Using STITCH, we were able to identify transcripts that are commonly reported targets of these two drugs, but in our data set we identified only one transcript (prostaglandin E synthase 3) to be differentially expressed in rainbow darter from our study. The mode of action of non-steroidal anti-inflammatory drugs is inhibition of cyclooxygenases enzymes that catalyze the synthesis of prostaglandins via the oxidation of arachidonic acid (Jeffries et al., 2015; Flippin et al., 2007). This is a complex process, involving multiples genes; therefore, it is difficult to make any conclusions as to whether ibuprofen and naproxen affected the fish and were responsible for changes in the expression of prostaglandin E synthase 3. Although, it is worth mentioning that downregulation of this gene in the liver in 2011 (DSW and DSK1) coincided with the highest concentration of these two drugs during the study period.

3.5.4 Gene set enrichment analysis and municipal wastewater treatment plant upgrades

Although chemical exposure was not the sole factor controlling the changes in the number of DEPs across sites over time, it was noted that there was a decrease in the number of biological processes with time, molecular functions and cellular components that were affected over time as determined by GSEA. This also corresponded to a gradual decline in pharmaceutical and nutrient load (especially below Kitchener). This does not necessary imply that the decrease in concentration of selected pharmaceuticals was responsible for the decrease in the number of affected biological processes in rainbow darter, and further experiments are required to investigate this relationship. The four pharmaceuticals that were measured in the present study were assessed because they are relevant indicators for monitoring changes in wastewater effluent treatment (Salveson et al., 2013). For example, ibuprofen and naproxen are two pharmaceuticals that are typically removed when the plants include nitrifying steps in the treatment, which diminishes the amount of ammonia in the final effluent (Salveson et al., 2013). The reduced ammonia, ibuprofen and naproxen strongly reflect the improvements in effluent quality and this is also reflected in the concentrations observed downstream. Similarly, there was a decrease in the Waterloo MWWTP ammonia effluent concentrations in 2014 compared to 2013, when there was almost no nitrate in the effluent, suggesting that minimal nitrification/treatment was occurring. Treatment of the Waterloo MWWTP effluent appeared to be relatively poor in both 2011 and 2012 as well. Caution should be used to not

over interpret the effluent and river water data from the fall samples, as they are influenced by many spatial and temporal factors (e.g., dilution). However, they do provide a general indication of the relative exposure during the sampling period, and are generally consistent with more comprehensive annual loads (Hicks et al., 2016).

Although the number of overrepresented gene sets involved in various biological processes decreased over time (2011 - 2014), processes indicative of stress exposure persisted until 2013 in fish from both sites (DSW, DSK1). In 2011 DSW fish showed a downregulation in their "stress response," whereas in 2012, the liver was dominated by processes that were involved in apoptosis. For instance, "biological process of apoptosis" and "negative regulation of apoptosis" were upregulated, whereas the process of "induction of apoptosis" was downregulated. Previous studies showed that the process of apoptosis was associated with exposure of fish to municipal effluents (Vidal-Dorsch et al., 2013). In 2013, fish collected downstream of Waterloo MWWTP showed upregulation of gene groups related to "steroid biosynthetic processes," "regulation of apoptosis," and processes affecting mitochondria such as "respiratory electron transport chain," all of which were upregulated. "Response to stress" in fish downstream of Waterloo remained downregulated (as in 2011). Exposure of fish to municipal effluents can cause changes in sex steroid production, and decreased production of androgen steroids in particular has been reported in rainbow darter in the Grand River downstream of wastewater outfalls (Bahamonde et al., 2015a; Fuzzen et al., 2015). An upregulation in "steroid biosynthetic process" can be interpreted as a means to compensate for suppressed androgen production, but caution should be taken when interpreting this process which is mostly specific for the gonadal tissue, and not liver. Fish collected downstream of Kitchener MWWTP had biological processes of "steroid biosynthesis" upregulated in 2011, similar to fish downstream of Waterloo (in 2011 and 2013). Further, in 2012 DSK1 fish showed downregulation in "response to stress" and "immune response", whereas, an upregulation in "induction of apoptosis". In 2013 "immune response" remained downregulated. It was interesting to observe that in 2014, none of the significantly different biological processes were indicative of exposure to stress, at any of the sites. Thus, there was no strong consensus on any consistent biological responses that could be monitored for recovery strategies of rainbow darter. Degradation of damaged proteins was one of the responses/processes demonstrated to be associated with fish recovery following an exposure to a mixture of toxicants (Corcoran et al., 2010); however, this process was not observed as affected in rainbow darter.

It was expected that over time, there would be fewer pathways in common between fish from DSW and DSK1 due to the upgrades. Both the number and percentage of gene ontology terms and pathways that were shared between rainbow darter from DSW and DSK1 decreased over time, suggesting that molecular responses in rainbow darter from these sites diverged and became more different from each other. This is consistent with the water quality data where in 2011 both plants had very poor effluent. While the Kitchener MWWTP effluent improved greatly after 2012, Waterloo had the worst effluent in 2013. Without a good understanding of the effluent quality, as well as the context of the exposure, it is difficult to interpret how expression patterns change in response solely to the treatment process. Future studies should be directed at improved understanding as to how annual site-specific changes influence gene expression patterns at reference sites, prior to assessing any changes at the impacted sites (i.e., collection of baseline data on transcript variability).

3.5.5 Cell process pathways gradual transition over time

Waterloo MWWTP did not undergo any substantial infrastructure upgrades, however, the effluent quality changed over the course of time from 2011 to 2014 (reaeration of the returning sludge centrate was initiated in 2013). Rainbow darter collected in the same time period downstream of the Waterloo MWWTP showed a gradual decrease in the number of cell processes affected. In 2013, fish exposed to the Waterloo MWWTP effluent had 3867 differentially expressed transcripts. This was not fully reflected in the quantity of cell pathways, since fewer processes were affected in 2013 and 2014 (30 and 29, respectively) than in 2011 and 2012 (47 and 45, respectively). In 2013 dominant types of cell processes in rainbow darter were related to mRNA processing and function. Because microarrays are based on assessing mRNA steady state abundance, compromised mRNA metabolism may explain in part the high numbers of DEPs detected in rainbow darter in 2013, although this hypothesis must be rigorously evaluated.

Rainbow darter downstream of the Kitchener MWWTP were collected before and after the infrastructural upgrades that resulted in improved effluent quality. It was of great interest to examine differently regulated cell processes in 2011, before the upgrades. Cell processes that created a unique molecular fingerprint in DSK1 rainbow darter were related to DNA processing, namely “DNA replication checkpoint” and “DNA metabolism” and “genome integrity”. Cells have surveillance mechanisms that control DNA replication, with DNA replication checkpoint being one of the mechanisms that enables stalled or collapsed DNA replication forks to recover from replication

interruption caused by endogenous or exogenous agents (Boddy and Russell, 2001).The median change of cell process of DNA replication was -1.53, and based on this value it can be concluded that this entire process was downregulated by 53% in 2011, suggesting a high rate of DNA mechanism activation (Aguilera and Gomez-Gonzalez, 2008; Branzei and Foiani, 2008). Cells need to maintain nuclear DNA structure and function; otherwise failure to properly repair nuclear DNA contributes to cell aging (Lombard et al., 2005), among other adverse effects that can be reflected at the cellular, organ and even the organism level. Despite repair mechanisms being activated, rainbow darter experienced difficulties in coping with nuclear DNA maintenance and its proper repair, and as a consequence, "cell aging" showed a 15% downregulation. Further, "mitochondrial damage" was another process unique to rainbow darter from 2011, and this process plays a very important role in cell aging (Balaban et al., 2005). The central theory surrounding cell aging is based upon interaction of reactive oxygen species with cellular components. Reactive oxygen species in fish can be generated endogenously in response to waterborne contaminant exposure when detoxifying mechanisms are suppressed, which results in oxidative stress. Exposure to municipal effluents caused alterations in biochemical oxidative stress endpoints in longnose sucker (*Catostomus catostomus*) (Oakes et al., 2004), therefore it is plausible that the exposure to effluent from the Kitchener MWWTP had similar effects on the transcriptome, causing a downregulation of "cell aging" processes. In fathead minnow (*Pimephales promelas*) caged downstream of a wastewater outfall in the North Saskatchewan River for four weeks there were elevated ratios of oxidized to total glutathione (i.e., GSSG/TGSH), and an increase in the activity of antioxidant enzymes (i.e., glutathione reductase, glutathione-S-transferase) (Jasinska et al., 2015). Failure to repair damaged DNA, mitochondrial damage, and cell aging are all related to cell damage which manifests through changes in cell permeability, leads to essential enzyme loss and eventually results in cell autolysis. The following year (2012) rainbow darter had 23 cell pathways affected, which represented almost 50% less pathways than the previous year. In contrast to earlier years, the majority of these pathways were related to the immune response (12 out of 23) and more than 60% of these were downregulated. Unique cell processes involving "T-cell function", "T-cell homeostasis", "establishment of T-cell polarity" and "B-cell function" were altered which suggested that fish immune response could have been compromised. T-cells and B-cells participate in the cell-mediated immune responses and control of viral diseases (Nakanishi et al., 2002). Control of bacterial caused diseases has proven to be regulated by complement activation (Magnadóttir, 2006), a pathway that was also downregulated

in the liver of rainbow darter. It is not possible to determine whether immune system responses at the transcriptome level were suppressed due to changes associated to MWWTP effluent quality or some site-specific factors in 2012 (overall dry year). In 2013 and 2014 cell pathways and the categories that they were classified into, continued to increase in number compared to 2012. In 2013, immune responses were differentially affected in rainbow darter liver, but new pathways emerged that included muscular activation ("muscle metabolism" and "muscle function") and reproduction ("hatching" and "blood-testicular barrier"). It is evident that the affected cell pathways were altered in rainbow darter over time and pre-upgrade conditions showed a tendency to target basic molecular processes such as DNA replication, whereas post-upgrades caused a shift in the cell process types mostly targeting various cell pathways associated to organ or even organism responses.

Overall examination of cell processes in both DSW and DSK1 rainbow darter indicated that there was a dynamic transition over years. Specific cell processes, and the categories they belonged to, were no longer present after 2012 in DSW and DSK1, whereas new categories emerged in 2013 and 2014. This transition went from basic molecular processes involving DNA replication, DNA repair or cell fate, to more complex processes related to immunity, reproduction, or thyroid function.

3.5.6 Are transcriptomics responses driven by site-specific or year-specific factors?

Rainbow darter transcriptomes did not cluster together according to level of exposure, site, or year. Previous studies showed conflicting results when it comes to relying on clustering analysis in the field-based studies to determine site-specific effects on gene expression in fish. Strong clustering was obtained in a study conducted with 48 h caged fathead minnow (*Pimephales promelas*) at five sewage effluent exposed sites (Garcia-Reyero et al., 2009). This was also accomplished in a study with adult fathead minnow that were caged for seven days at four sites impacted by agricultural runoff in Nebraska watersheds (Sellin Jeffries et al., 2012). Falciani et al., (2008) conducted a study with wild European flounder (*Platichthys flesus*) collected at six estuarine sites in the North Sea that contained various industrial and domestic contaminants, and they found that only individuals from one of the sites, which was heavily contaminated, clustered together based upon the transcriptome response (Falciani et al., 2008). The experimental design of the present study allows for a two-way ANOVA statistical approach which can be used to determine whether gene expression patterns in the rainbow darter are more affected by site-specific or year-specific factors. The analysis revealed that year-specific factors affected 5308 DEPs, as opposed to 2591 DEPs that

were altered by site-specific factors. This suggests that transcriptome responses may be highly dependent on variables that change from year to year compared to those across sites in the same year. However, it is also necessary to point out that there were 3554 DEPs for which expression was affected by both site-specific and year-specific factors. The notion that a great portion of DEPs remains affected by both site-specific and year-specific factors, may partially explain the non-perfect clustering in the present study. Another point to make is that clustering of individuals based on expression profiles may be more successful for fish that are placed into the river system for short periods (e.g., cage experiments), as opposed to wild fish that are caught in the system. The transcriptome of caged fish may respond more dramatically to effluent than those of wild fish in the system, resulting in stronger clustering.

3.6 Conclusions

Chemical analyses suggested that the effluent quality had improved over time at the Kitchener MWWTP, while the Waterloo effluent remained poor during the study period. However, the number of DEPs was not affected by the changes in effluent quality at either of the studied sites. Our results indicated that factors characteristic of individual years are affecting the number of genes that are differently expressed. As pointed out by Bahamonde et al., (2016), environmental factors such as temperature, flows, microhabitat use and/or nutrient availability may be important in altering gene expression across years and sites. However, GSEA and SNEA may be more sensitive types of analyses (compared to the number of DEPs) when it comes to interpreting gene patterns changes in response to MWWTP effluent exposure. Reduction in the number of gene sets involved in biological processes, molecular functions and cellular components corresponded with the reduction of contaminant load in the effluent and river water. Data using SNEA appeared to correspond to the effluent quality, and effluent exposure was associated with a shift in the cell process types, a transition from cell processes related to DNA metabolism to cell processes related to higher level biological responses. Several transcripts (e.g., choriogenin H minor, proto-oncogene protein c-Fos, hemoglobin β) that were altered in the microarrays may represent potential candidates for monitoring effects of MWWTP effluents on rainbow darter. Additional studies could be initiated to determine if these genes are relevant indicators of estrogenic exposure, hypoxic environments or potential immune response transcripts in rainbow darter or closely related species. The present study is unique in that it explores the effects of two MWWTP effluents on rainbow darter liver transcriptome over

four years that encompasses process changes in municipal wastewater effluent treatment facilities that resulted in improved effluent quality. However, measuring additional chemical and environmental factors influencing exposure would be useful for better understanding, and interpreting the role of annual variability in transcript levels. Future studies should focus on determining whether molecular transcripts identified in the present study are useful as biomarkers for monitoring responses in rainbow darter that are associated with environmental stressors in the Grand River.

Chapter 4

Assessing recovery of *in vitro* steroid production in male rainbow darter (*Etheostoma caeruleum*) in response to municipal wastewater treatment plant infrastructure changes

4.1 Chapter summary

The present study examined *in vitro* 11-ketotestosterone and testosterone production by the testes of rainbow darter (*Etheostoma caeruleum*) collected from selected reference sites and downstream of two municipal wastewater treatment plants (MWWTPs, Waterloo and Kitchener) in the central Grand River (Ontario, Canada), over six years (2011 - 2016). The main objective was to investigate if infrastructure upgrades at the Kitchener MWWTP in 2012 resulted in a recovery of this response in the post-upgrade period (2013 - 2016). Two supporting studies demonstrated that the fall season is appropriate for measuring *in vitro* sex steroid production as it provides stable detection of steroid patterns, and that the sample handling practiced in this study did not introduce a bias. Infrastructure upgrades of the Kitchener MWWTP resulted in significant reductions in ammonia and estrogenicity. Following the upgrades, 11-ketotestosterone production by MWWTP-exposed fish increased in 2013 and it continued to recover throughout the study period 2014 – 2016 returning to levels measured in reference fish. Testosterone production was less sensitive and it lacked consistency. Waterloo MWWTP underwent some minor upgrades but the level of ammonia and estrogenicity remained variable over time. The production of 11-ketotestosterone and testosterone in rainbow darter below the Waterloo MWWTP was variable and without a clear recovery pattern over the course of the study. The results of the present study demonstrated that measuring production of sex steroids (especially 11-ketotestosterone) over multiple years can be relevant for assessing responses in fish to environmental changes such as those resulting from major infrastructure upgrades.

4.2 Introduction

Aquatic environments that receive municipal effluents contain detectable levels of a diversity of natural and synthetic estrogens (Johnson et al. 2005; Ternes et al. 1999), androgens (Chang et al. 2011; Kirk et al. 2002), pharmaceuticals, and industrial chemicals (Corcoran, et al. 2010; Metcalfe et al. 2004), that have the potential to alter endocrine function and subsequently lead to adverse outcomes in aquatic organisms (Kramer et al. 2011; Miller et al. 2007). It has been well documented that fish exposures to some municipal effluents are associated with intersex (Blazer et al. 2012; Bjerregaard et al. 2006; Jobling et al. 1998), reduced sperm abundance, elevated levels of plasma vitellogenin (Vajda et al. 2011; Vajda et al. 2008), and alterations in sex steroid production (Hecker et al. 2002; Jobling et al. 2002; Folmar et al. 1996).

The central part of the Grand River in southern Ontario, Canada, is a highly-impacted system that flows through an urbanized and densely populated area (~1 million inhabitants). It receives various non-point as well as point-source inputs, including effluents from two large municipal wastewater treatment plants (MWWTPs; Waterloo and Kitchener). The effluents discharged from both treatment plants contain various contaminants resulting in an estrogenic effluent (Hicks et al. 2017; Arlos et al. 2015). A variety of biological effects in the native rainbow darter (*Etheostoma caeruleum*) have been associated with exposure to these effluents including a high prevalence of intersex (Bahamonde et al. 2015a; Fuzzen et al. 2015; Tanna et al. 2013; Tetreault et al. 2011). Molecular alterations in rainbow darter, such as changes in vitellogenin mRNA abundance and specific gene expression in intersex rainbow darter have also been observed (Fuzzen et al. 2016; Bahamonde et al. 2015b; Bahamonde et al. 2014). Results suggest that there is reduced fecundity associated with exposure to effluents discharged from these two MWWTPs (Fuzzen et al. 2015) and decreased androgen steroid production in males (Fuzzen et al. 2016).

The Region of Waterloo is currently investing in upgrades to both the Waterloo and Kitchener MWWTPs to convert them from conventional activated sludge secondary treatment facilities into nitrifying activated sludge treatment plants. The Waterloo MWWTP upgrades were delayed and effluent quality declined during the study period from 2007 to 2015 (Hicks et al. 2017; Hicks et al. 2016). However, the first upgrades at the Kitchener MWWTP were initiated in mid-2012 and came on line in early 2013. Changes to the plant infrastructure at Kitchener resulted in greater nitrification capacity, and greater removal of contaminants due to increased solids retention time, resulting in improved effluent quality (Hicks 2017; Bicudo et al. 2016). Infrastructure upgrades at the

Kitchener MWWTP have resulted in reduction of intersex severity and prevalence in rainbow darter (Hicks 2017). Functional analyses of the rainbow darter liver transcriptome suggested that fewer stress related pathways were affected, and rainbow darter collected below the two MWWTP outfalls showed greater divergence in biological pathways after the upgrade implementation (Marjan et al. 2017) at Kitchener. Gonadal histology and molecular responses supported the hypothesis that recovery in rainbow darter in response to MWWTP upgrades was occurring, especially after 2013.

We were interested in determining whether endocrine system responses could be used to indicate the effectiveness of upgrades to the wastewater treatment plant. We focused on the production of sex steroids as this reflects the culmination of effects at multiple sites in the hypothalamic pituitary gonadal axis. The quantification of sex steroids at different stages of the reproductive cycle can be a powerful biomarker indicative of stress-induced perturbations in the reproductive process (Tetreault et al. 2014; Donaldson 1990). Small alterations in steroid production have the potential to be translated into important changes at higher levels of biological organization in both males and females. Sex hormones along with complex neuronal stimuli can also play a critical role in determining sexual behaviour in male fish during courtship and therefore affect reproductive success (Söffker and Tyler 2012). For example, reduced 11-ketotestosterone was associated with altered sexual behaviour, namely diminished aggressiveness (Martinović et al. 2007; Pankhurst and Barnett 1993) in the laboratory studies. Moreover, production of 11-ketotestosterone was found to be important in establishing a new territory for spawning (Danylchuk and Tonn 2001), as well as nest site preparation before spawning (Páll et al 2002). Rainbow darter males collected at the site below the Kitchener outfall displayed reduced color intensity and 11-ketotestosterone production, in comparison with males from a rural reference site (Fuzzen 2016). Male fish with intense coloration during courtship become more conspicuous which can have positive implications on their social and sexual interaction (Fuzzen 2016). It can be concluded that alterations in sex steroids at critical windows in the life cycle, especially during reproduction, can affect reproductive success, which can disrupt genetic structure and reduce genetic variability (Martinović et al. 2007). Reduction of plasma sex steroid production has been linked with reduced fecundity and reproductive success in female fathead minnow (*Pimephales promelas*) exposed to chemicals known to affect the steroidogenic pathway or through negative feedback of the hypothalamus pituitary axis (Ankley et al. 2008). Alterations in sex steroid production have also been used extensively as an endocrine response in fish in studies examining environmental effects of industrial effluents, such as bleached

kraft mill effluents (Dubé and MacLatchy 2000; McMaster et al. 1995b; Munkittrick et al. 1994; Van Der Kraak et al. 1992; Munkittrick et al. 1991). For small bodied fish, including rainbow darter, it is difficult to obtain blood plasma samples, so *in vitro* production has been used as an alternative method to determine the potential of gonadal tissues to produce steroid hormones basally and in response to anthropogenic chemicals. Earlier studies assessing steroid production in the rainbow trout (*Oncorhynchus mykiss*) found a close correlation between *in vitro* and *in vivo* steroid production of sex hormones (Manning and Kime 1985; Kime and Hyder 1983). Further, correlations between *in vitro* and *in vivo* production of 11-ketotestosterone has also been observed in walleye (*Sander vitreus*), northern pike (*Exos lucius*), and white sucker (*Catostomus commeroni*) (Beitel et al. 2014). This *in vitro* approach has been shown to be sensitive in detecting changes in sex steroid production between fish from polluted sites and those from reference sites (Fuzzen 2016; Tetreault et al. 2011; McMaster et al. 1995). Studies examining changes in sex steroid production in fish associated with environmental pollution exposure showed recovery in this response in wild fish following major upgrades or discontinuation of the pollution (Barber et al. 2012; Bowron et al. 2009). However, these studies, including the current one, are not able to determine directly if the recovery in steroid production is due to the removal of a chemical that acutely impacts steroid biosynthesis or if the removal of the chemical limits the organizational changes in testis that reflect a long term developmental alteration in steroid biosynthetic capacity.

In the present study, *in vitro* production of 11-ketotestosterone and testosterone by rainbow darter testes was assessed to determine whether MWWTP infrastructure changes resulted in improved endocrine responses (return to reference) of *in vitro* androgen production. Androgen production by the testes from rainbow darter collected prior to upgrades (fall of 2011 and 2012) was compared to 4 additional fall season collections (2013 - 2016). A supporting study was conducted to determine the seasonal response of *in vitro* steroid production and determine the consistency of the approach to detecting change. A second supporting study was conducted to determine if fish handling was a factor for interpretation of the *in vitro* androgen production assay. Data for nitrogenous products and estrogenicity (conducted with the yeast estrogen screen assay; YES) of the two effluents (Waterloo and Kitchener) encompassing the pre- (2011 - 2012) and post- (2013 - 2016) upgrade period were contrasted to demonstrate the changes in effluent quality following the upgrades of the Kitchener MWWTP and to facilitate interpretation of androgen steroid production responses in rainbow darter.

4.3 Materials and methods

4.3.1 Fish collection

Rainbow darter are distinctly sexual dimorphic and live for 4 - 6 years. They are clutch spawners in the spring and grow rapidly becoming sexually mature in the first year (Crichton 2016; Paine 1990). Adult male rainbow darter, were collected from riffle habitats across the Grand River watershed in southern Ontario using an electrofishing backpack unit (Smith Root LR-20/24; Figure 4.1). Individuals ≥ 4.5 cm were targeted as they are likely not young of the year and therefore were exposed to effluent conditions for at least a year or more. Fish were processed as soon as possible after capture (usually <1 h). Length (±0.1 cm) and weight (±0.001 g) were recorded and used to calculate condition factor ($k = \text{body weight} / [\text{fish length}]^3 \times 100$). Testes and liver were removed and used to calculate gonadal somatic index (GSI = testes weight / body weight × 100) and liver somatic index (LSI = liver weight / body weight × 100), respectively. Lengths, weights, k, GSI and LSI are provided to support interpretation of changes in gonadal steroid production (Table S1; S2 and S3; Supplemental Informatin). A portion of the testes was placed in Medium 199 (Sigma) containing 25 mM of HEPES (Sigma), 4.0 mM sodium bicarbonate, 0.1 % bovine serum albumin, and 0.01 % streptomycin, and stored in a cooler on ice. These samples were subsequently processed and analysed for *in vitro* steroid production. All procedures involving fish were approved by University of Waterloo's Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

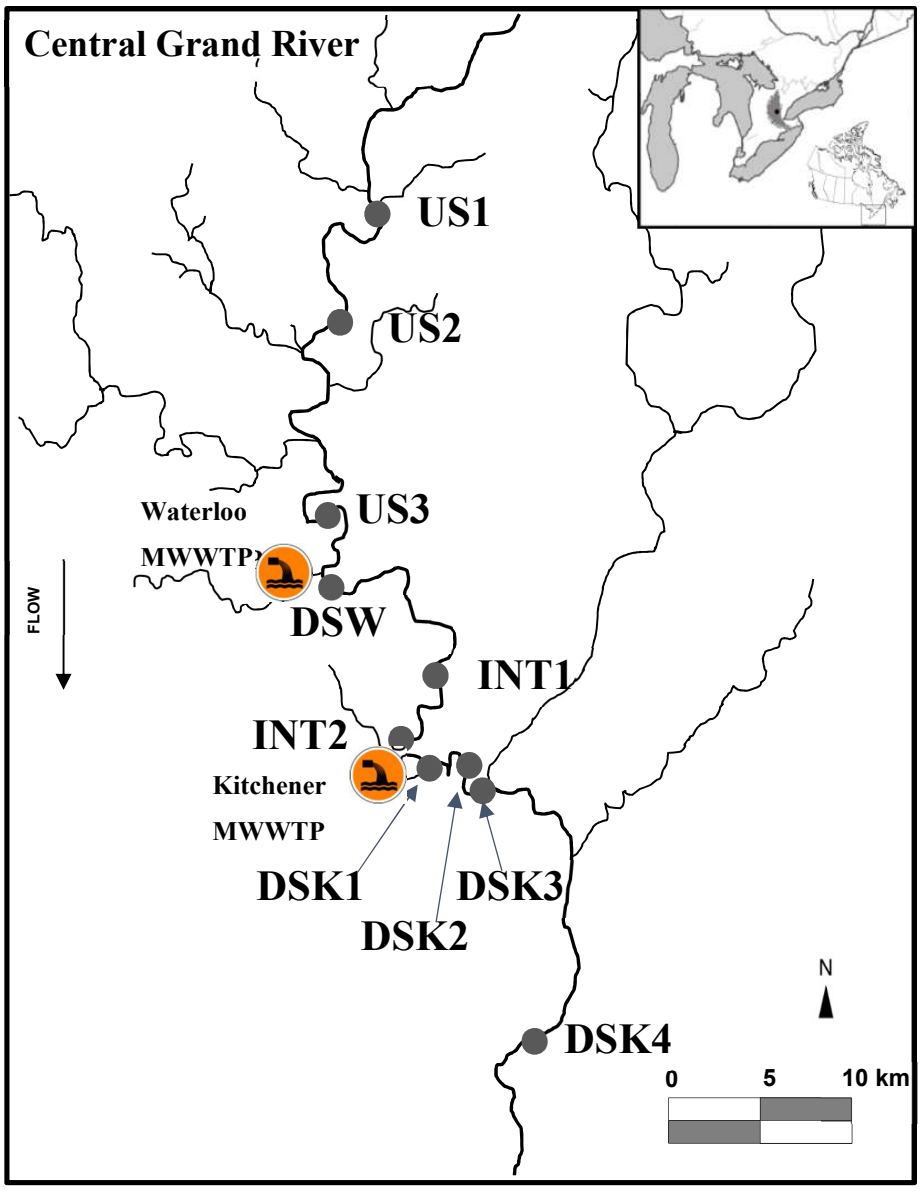


Figure 4.1 Map of sampling sites in the central Grand River located at varying distances from MWWTPs at Waterloo and Kitchener. Fish collections were conducted in the fall from 2011 to 2016 from two upstream sites in the non-urbanized area (US1, US2) and an urban reference site (US3), from a site located immediately downstream of the Waterloo MWWTP (DSW), two intermediate sites (INT1, INT2), a site downstream of the Kitchener MWWTP (DSK1) outfall, and three additional sites in the downstream reach (DSK2, DSK3, DSK4).

4.3.2 *In vitro* steroid production

Methods described in McMaster et al. (1995a) were adapted to measure *in vitro* production of 11-ketotestosterone and testosterone by rainbow darter testes. Briefly, approximately 10 mg of testis (exact weight recorded) were transferred to 1 mL of fresh Medium 199 (as above) and the tissue was stimulated with the addition of 10 µL of 1 IU/µL human chorionic gonadotropin (hCG) (an analogue to luteinizing hormone, the endogenous stimulator of gonadal steroidogenesis; Sigma), and then incubated at 16ºC for 24 h. Incubation temperature was selected on the bases of the work conducted by McMasster et al. (1995a; 1995b) who found that higher temperatures stimulate the gonadal tissue to produce more hormones (compared to lower temperature, e.g., 12ºC), do not affect the activity of hCG, and do not mask the site-specific effects. Medium was collected following incubation and stored at -80ºC until samples were analysed. Steroid analysis was done using a competitive enzyme-linked immunosorbent assay (ELISA) as per manufacturer's instruction (Cayman Chemical). To calculate inter-assay variation, samples from individual fall seasons were pooled (~3-4 samples per site), diluted (~40 times for 11-ketotestosterone and ~5 times for testosterone), and run on each plate, in either duplicate or quadruplet, and values were reported as the coefficient of variance (*CV*; standard deviation/mean × 100). If *CVs* of the pooled inter-assay samples was lower than 20%, assays (samples) were deemed to be good quality, whereas if this range was surpassed, samples were re-analysed. For *in vitro* steroid production methods from 2011 to 2012 refer to Fuzzen (2016).

4.3.3 Study design

4.3.3.1 Seasonal variability in steroid production

The annual cycle of testicular sex steroid production in male rainbow darter was evaluated to determine if the response to effluent was consistent across seasons and to confirm the selection of the most appropriate period to make comparisons of sex steroid production. Rainbow darter males (~20 per site) were collected from two reference sites (US2 – rural, and US3 – urban reference), and from an effluent exposed site downstream of the Waterloo MWWTP outfall (DSW) (Figure 4.1). Fish were collected approximately monthly during a full year (2015 – 2016; see Table S1).

4.3.3.2 Impacts of sample handling

It was important to determine that the time of sampling upon capture did not compromise the ability to detect differences in androgen production between the exposed (DSW) and the non-exposed fish (US2) (Figure 4.1). The processing of fish 1 h after capture was incorporated into our standard sampling methodology, and was consistently practiced throughout our study (from 2011 - 2016). However, on certain occasions, fish sampling was conducted up to 3 h post-capture (unusual situation). To confirm that time of sampling did not influence the production of sex steroids, male rainbow darter (on average 60 individuals per site) were collected from a rural reference site (US2) and from an effluent exposed site, immediately downstream of the Waterloo MWWTP during recrudescence period in November (2015). Three subsets of fish (~20 fish per subset) were held in aerated buckets and then processed for *in vitro* steroid production at 1 h, 6 h and 24 h post-capture (see Table S2). *In vitro* androgen production was determined as described above (Section 2.2).

4.3.3.3 Pre- and post-upgrade study design

To determine changes in androgen production in rainbow darter collected from sites across the urban environment in the central Grand River, and to investigate if there was recovery in sex steroid production associated with the treatment plant upgrades, sexually mature rainbow darter males (approximately 15 - 20 per site) were collected in the fall season (October - November) for six consecutive years (2011 - 2012 pre-upgrade period; 2013 - 2016 post-upgrade period) (Table S3). Fish were collected from sites located at different distances from two major MWWTPs (Waterloo and Kitchener) (Figure 4.1). In 2011, rainbow darter males were collected from 7 sites; in the following year (2012) an additional site was added, and in the period from 2013 to 2016, fish were collected from ten sites. Three sites were located upstream of the major urbanized area, and above the Waterloo MWWTP (DSW) outfall (US1, US2 and US3; 33 km upstream of the Waterloo outfall, 22 km, and 6 km from DSW). An effluent exposed site was located ~1 km below the Waterloo MWWTP outfall, and there were two intermediate sites (INT1, INT2; 13 km and 19 km, downstream of DSW) located between the two MWWTP outfalls. A site was located immediately downstream of the Kitchener MWWTP (DSK1; 20 km below DSW), and there were two additional sites downstream (DSK2, DSK3; 21 km and 25 km, below DSW), plus a far field site (DSK4; 44 km downstream of DSW).

4.3.4 Chemistry

Data on nitrogenous products in effluent samples (Waterloo and Kitchener) including total ammonia (mg/L) and nitrate (mg/L) were provided by the Region of Waterloo under a data-sharing agreement. Average monthly measurements from October to December, which coincided with fall sampling of the fish, were extracted from the annual reports to illustrate effluent quality change over time (2011 - 2016). To evaluate estrogenicity, effluent samples (500 mL) were collected from the two MWWTPs (Waterloo and Kitchener) over time (2011 - 2015) and the YES assay was conducted using a method adapted from Smith (2013) (Supplemental Information). Estrogenicity was expressed in ng of estradiol equivalents (E2eq) per liter. Collections of effluent samples were conducted at various time points within each year but not necessarily coinciding with fish fall sampling; thus, YES data in the study represents an annual mean.

4.3.5 Statistical analysis

Absolute values of 11-ketotestosterone and testosterone concentration (pg/mg of testicular tissue) were log10 transformed, tested for outliers using Tukey's boxplots method, and subsequently tested for normality with Kolmogorov-Smirnov test. Tukey's boxplots method is based on the 1.5 of the interquartile range (IQR) which is an absolute difference between the lower and the higher quartile. Values lower than 1.5 × IQR of the lower quartile, and those that are 1.5 × IQR higher than the higher quartile are identified as outliers. In addition, log10 transformed data distribution was visually assessed using vertical point plots which in conjunction with Tukey's boxplots aided in evaluating outlying values in data sets and their subsequent exclusion (or inclusion) from further analysis. There were 3.8% of 11-ketostesosterone data points and 5.4% of testosterone data points that did not meet the requirements (failed to pass the Tukey's boxplots testing, Kolmogorov-Smirnov test, and were visually outstanding in the vertical point plots**)** and were therefore excluded from the datasets.

Seasonal variability of androgen steroid production was determined using a one-way analysis of variance (ANOVA). Multiple comparisons with the Holm-Sidak post-hoc test was used to identify significant differences ($\alpha < 0.05$) in steroid production in rainbow darter within each month. When data failed to pass either normality or equal variance test, the Kruskal-Wallis test with Dunn's method were used and ANOVA was conducted on ranks. The impact of sample handling on steroid production was tested by two-way ANOVA.

Data of *in vitro* sex steroid production across the urban environment and in response to the MWWTP upgrades were analysed using three different approaches relying on one-way ANOVA and data normalization. Execution of two-way ANOVA was not possible because of unbalanced site numbers across years. Instead, one-way ANOVA was used to test for within year differences across the urban environment as well as across year differences within each individual site. In addition, normalization was done to emphasize differences within each individual site over time and to standardize the comparison between different sites (rather than using a multiple comparison method).

In the first approach, to determine differences in steroid production among rainbow darter from various sites in the urbanized stretch of central Grand River within each individual year (2011 - 2016), ANOVA was applied using multiple comparison with the Holm-Sidak post-hoc test or alternatively Kruskal-Wallis with Dunn's method was used for non-normally distributed data sets. In the second approach, to further evaluate the patterns of androgen steroid production in the pre-upgrade period (2011 - 2012), transition period (2013 - 2014) and the post-upgrade period (2015 - 2016), data (log10 transformed) were normalized to US2 rainbow darter according to each year and data were plotted for better visualization (these data were not subjected to statistical analysis). In the third approach to determine whether infrastructure changes at the Kitchener MWWTP resulted in recovery of steroid production (both 11-ketotestosterone and testosterone), data from the sites below the Kitchener MWWTP (DSK1, DSK2, and DSK4) across years (2011 - 2016) were subjected to one-way ANOVA with the Holm-Sidak test (alternatively Kruskal-Wallis and Dunn's test). Prior to ANOVA, data (log10 transformed) were normalized to the averaged (2011 - 2016) steroid production in US2 rainbow darter. In addition, across-year changes in steroid production were assessed in DSW rainbow darter, to contrast it with the production in fish exposed to the non-upgraded treatment plant.

Effluent chemistry data was analysed with one-way ANOVA and the Holm-Sidak post-hoc at each MWWTP over time (2011 - 2016). All statistical analyses were conducted in SigmaPlot (version 12.0; Systat Software, Inc.).

4.4 Results

4.4.1 Seasonal variation in *in vitro* steroid production

One of the objectives of the present study was to confirm that the fall season is an appropriate time for detecting differences in *in vitro* production between rainbow darter collected at exposed and reference sites. Production of both 11-ketotestosterone and testosterone in male rainbow darter showed relatively similar patterns over the fall months (October - December 2015, October 2016). This was observed through consistent significant suppression of 11-ketotestosterone and testosterone production in rainbow darter males below the Waterloo MWWTP outfall (DSW) compared to rainbow darter from the rural upstream site (US2) (Figure S4.1, A & B). In contrast, it was not always possible to detect significant differences in 11-ketotestosterone compared to fish collected from the immediate upstream site (US3). Fish from US3 also showed significantly lower testosterone production compared to US2 fish on two occasions during the fall sampling. Field sampling was not conducted in January through March due to ice cover or high flows. Rainbow darter spawn from March to mid-June when their gonads mature and the production of sex steroids is at its maximum. This was captured in the *in vitro* steroid production measured in our study as well. Both 11-ketotestosterone and testosterone production was elevated in April and May compared to the levels produced during recrudescence (Figure S4.1). However, the pattern of androgen production was variable especially towards the end of the spawning period (May) when it was no longer possible to detect differences between exposed (DSW) and non-exposed fish (US2). It was not possible to assess *in vitro* steroid production in June to August due to complete regression of the testes. A pattern similar to that in May was observed in September and no significant differences in either 11-ketotestosterone or testosterone production were detected between DSW, US2 and US3 fish at these times.

4.4.2 Influence of handling time

Rainbow darter males were sampled at three time points (1 h, 6 h and 24 h) after capture to determine if testicular steroid production was influenced by the time the fish were held before processing. The production of 11-ketotestosterone by the testes from fish collected below the Waterloo MWWTP was significantly lower than the levels produced by fish from the reference site at all three post-capture sampling periods (Figure S4.2 A). Further, there were no differences

between 11-ketotestosterone production sampled 1 h post-capture and those fish sampled 6 h upon capture. However, rainbow darter males sampled after 24 h had significantly lower levels of 11-ketotestosterone compared to the subset sampled 1 h post-capture. Testosterone production had an almost identical pattern to11-ketotestosterone with differences detected between sites in hormone production at 1 h and 6 h and reduced testosterone production at 24 h. Fish sampled 24 h after capture however did not show significant differences in testosterone production between DSW and US2 rainbow darter (Figure S4.2 B).

4.4.3 Individual year patterns of androgen steroid production across the urban environment

In the 2011 pre-upgrade period, 11-ketotestosterone production by rainbow darter males collected below the Kitchener MWWTP outfall (DSK1 and DSK2) was significantly reduced compared to males from both the non-urban and urban sites (Figure 4.2, Pre-upgrades). Testosterone was also significantly depressed in rainbow darter at the second site below the Kitchener MWWTP outfall (DSK2) compared to the further upstream site (US2) (Figure 4.3, Pre-upgrades). In 2012, rainbow darter from the urban sites showed an overall depression in both androgens compared to the upstream reference sites. Rainbow darter males from DSK1 collected in 2012 continued to display significantly lower production of 11-ketostesterone compared to rainbow darter from the two reference sites (US2 and US3) in the upstream reaches (Figure 4.2, Pre-upgrades). A significant reduction was also detected in testosterone production in males collected downstream of both Waterloo and Kitchener MWWTPs (DSW and DSK1) compared to the upstream sites (US2 and US3) (Figure 4.3, Pre-upgrades). Despite improved effluent quality following the upgrades at the Kitchener MWWTP, 11-ketotestosterone remained significantly depressed in DSK1 rainbow darter in 2013. Moreover, 11-ketotestosterone patterns in 2013 became more spatially variable (Figure 4.2, Transition). Rainbow darter below the Waterloo MWWTP (DSW) in 2013 showed a significant reduction of 11-ketotestosterone. In addition, males from the immediate upstream site (US3), the intermediate site (INT1), and the third downstream site (DSK3) from the Kitchener MWWTP, showed significantly depressed 11-ketotestosterone (Figure 4.2, Transition). The spatial pattern of testosterone production in 2013 was similar to that observed in 2012 (Figure 4.3, Transition). In the following year (2014), rainbow darter males collected immediately below the Kitchener MWWTP (DSK1) no longer showed significant reduction of 11-ketotestosterone. Recovery was also noticed in INT1 and DSK3 males that were previously significantly depressed. However, rainbow darter males from the urban reference site and the site below the Waterloo MWWTP (DSW) continued displaying significant suppression of 11-ketotestosterone production compared to fish collected from the upstream site (US2) (Figure 4.2, Transition). There were no significant differences in testosterone across the urbanized environment in 2014 (Figure 4.3, Transition). In the post-upgrade period from 2015 to 2016, there were no effects on 11-ketotestosterone (Figure 4.2, Post-upgrades) and testosterone (Figure 4.3, Post-upgrades) in fish downstream of the upgraded treatment plant in Kitchener. In contrast, rainbow darter downstream of the Waterloo MWWTP still showed significant depression in 11-ketotestosterone and testosterone in 2015 (compared to US2 and US3). In the final year of the study (2016), 11-ketotestosterone in rainbow darter collected below the Waterloo

MWWTP was recovered but not testosterone, which still showed significant reduction (compared to all three reference sites). Fish at DSK3 displayed significantly decreased production of both 11-ketotestosterone and testosterone compared to fish from the upstream sites and some of the neighbouring sites (Figure 4.2 & 4.3, Post-upgrades).

Overall, the patterns of *in vitro* 11-ketotestosterone and testosterone production became more consistent within the reference sites over time. Figure S4.3 (A & B) shows 11-ketotestosterone and testosterone in the rainbow darter across the urban environment that was normalized to production of these steroids in US2 rainbow darter. In the pre-upgrade period (2011 - 2012), it is noteworthy that there was more variation in the levels of 11-ketotestosterone and testosterone but both steroids showed very similar patterns during the two consecutive years. In the transition period (2013 - 2014) and post-upgrade period (2015 - 2016), the levels and patterns of sex steroid production became less variable and this is especially evident in the final two years of sampling, especially for testosterone that had almost identical patterns (in the final two years) (Figure S4.3 B).

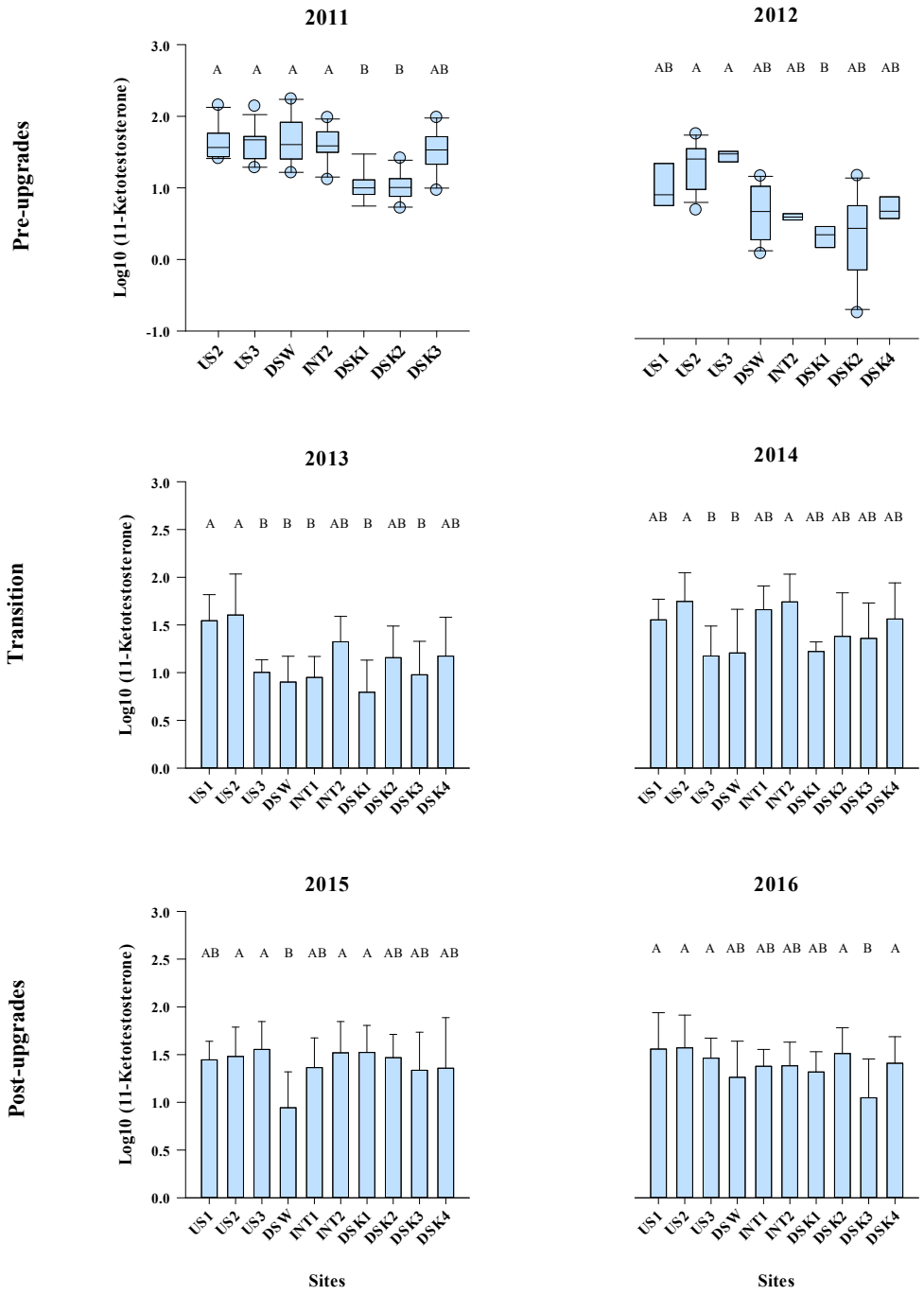


Figure 4.2 Time dependant changes in human chorionic gonadotropin stimulated production of 11-ketotestosterone by the testes from male rainbow darter collected across the urbanized environment in the central Grand River during the pre-upgrade (2011 - 2012), transition (2013 - 2014), and the post-upgrade period (2015 - 2016).

One-way analysis of variance (ANOVA) was conducted on log10 transformed data within each year. 2011 and 2012 (box plots) data was analysed using one-way ANOVA on ranks. Statistical differences are indicated by capital letters above box plots and vertical bars (mean ± standard error of the mean).

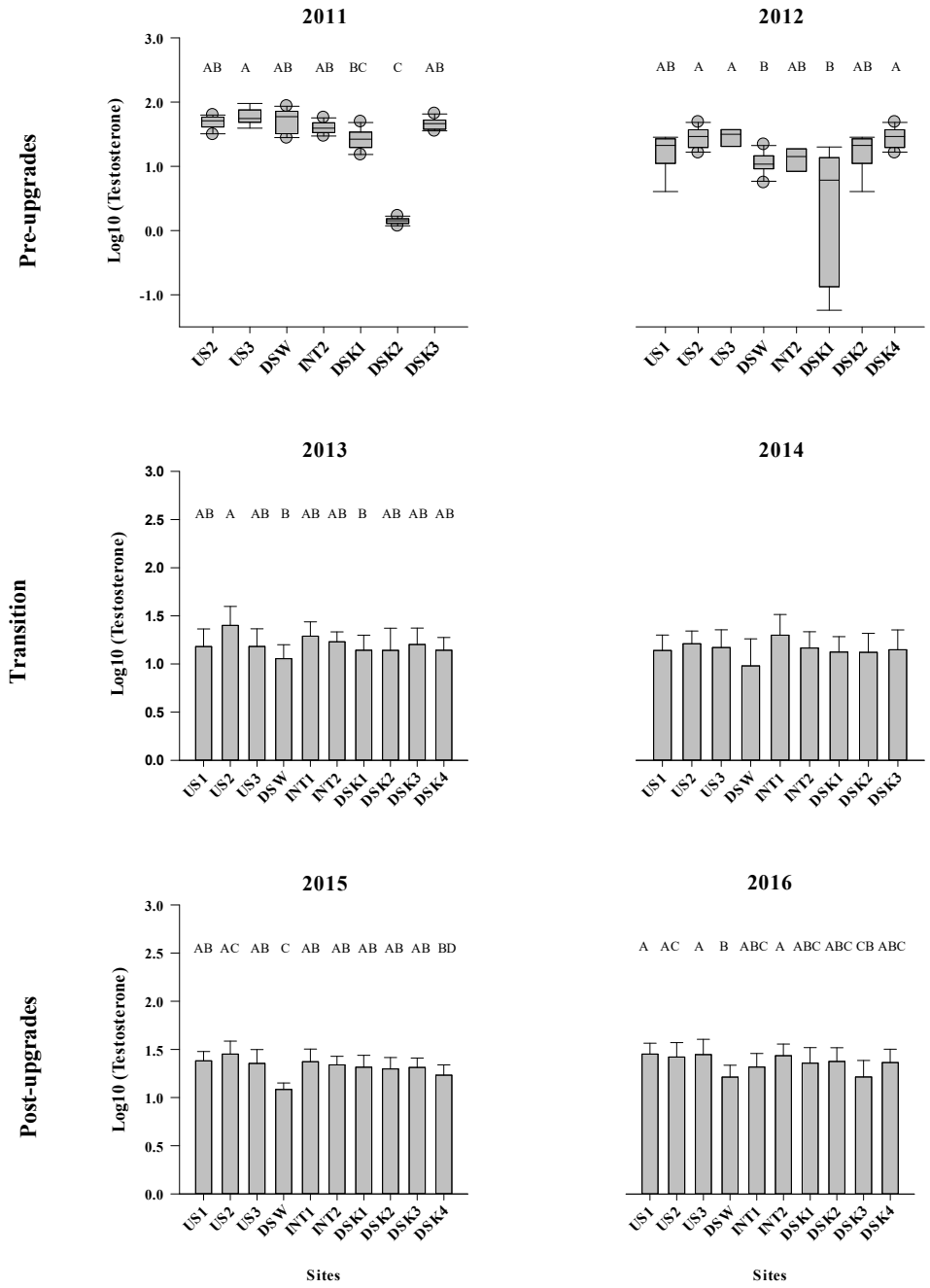


Figure 4.3 Time dependant changes in human chorionic gonadotropin stimulated production of teststerone by the testes from male rainbow darter collected across the urbanized environment in the central Grand River during the pre-upgrade period (2011 - 2012), transition period (2013 - 2014), and the post-upgrade period (2015 - 2016).

One-way analysis of variance (ANOVA) was conducted on log10 transformed data within each year. 2011 and 2012 (box plots) data was analysed using one-way ANOVA on ranks. Statistical differences are indicated by capital letters above box plots and vertical bars (mean ± standard error of the mean).

4.4.4 Across-year changes in gonadal steroids downstream of MWWTP outfalls

To further examine the temporal change in rainbow darter androgen production, individual sites (DSW, DSK1, DSK2 and DSK4) were compared across years (from 2011 to 2016). Data were normalized to a single value that was obtained by averaging androgen production in rainbow darter from the upstream reference site (US2) across years (2011 - 2016). Rainbow darter collected immediately below the Waterloo MWWTP outfall displayed a complex pattern of 11-ketotestosterone production over the course of six years (Figure 4.4A). Apparent recovery was observed after 2012 (low production of 11-ketotestosterone), when there was a gradual increase in 11-ketotestosterone production. However, these values remained well below that in 2011 when the Waterloo MWWTP was operating relatively well. Moreover, the trend lacked consistency as marked by a significant drop in 2015 compared to 2011 and 2012. Testosterone concentration over years within this site (DSW) also remained significantly decreased in the final year compared to 2011 and 2012 (Figure 4.4B).

In contrast, rainbow darter collected from three sites below the Kitchener MWWTP (DSK1, DSK2, and DSK4) following the upgrades showed a gradual increase in 11-ketotestosterone production over time (Figure 4.4A). In the final two years of the study (2015 and 2016) rainbow darter from all three sites (DSK1, DSK2, and DSK4) had significantly higher production than in 2011 and 2012. A clear recovery was observed in rainbow darter from the second (DSK2) site below the Kitchener outfall (Figure 4.4A). Although, apparent recovery trends in testosterone production were observed following the upgrades, this gradual increase was not statistically significant (Figure 4.4B).

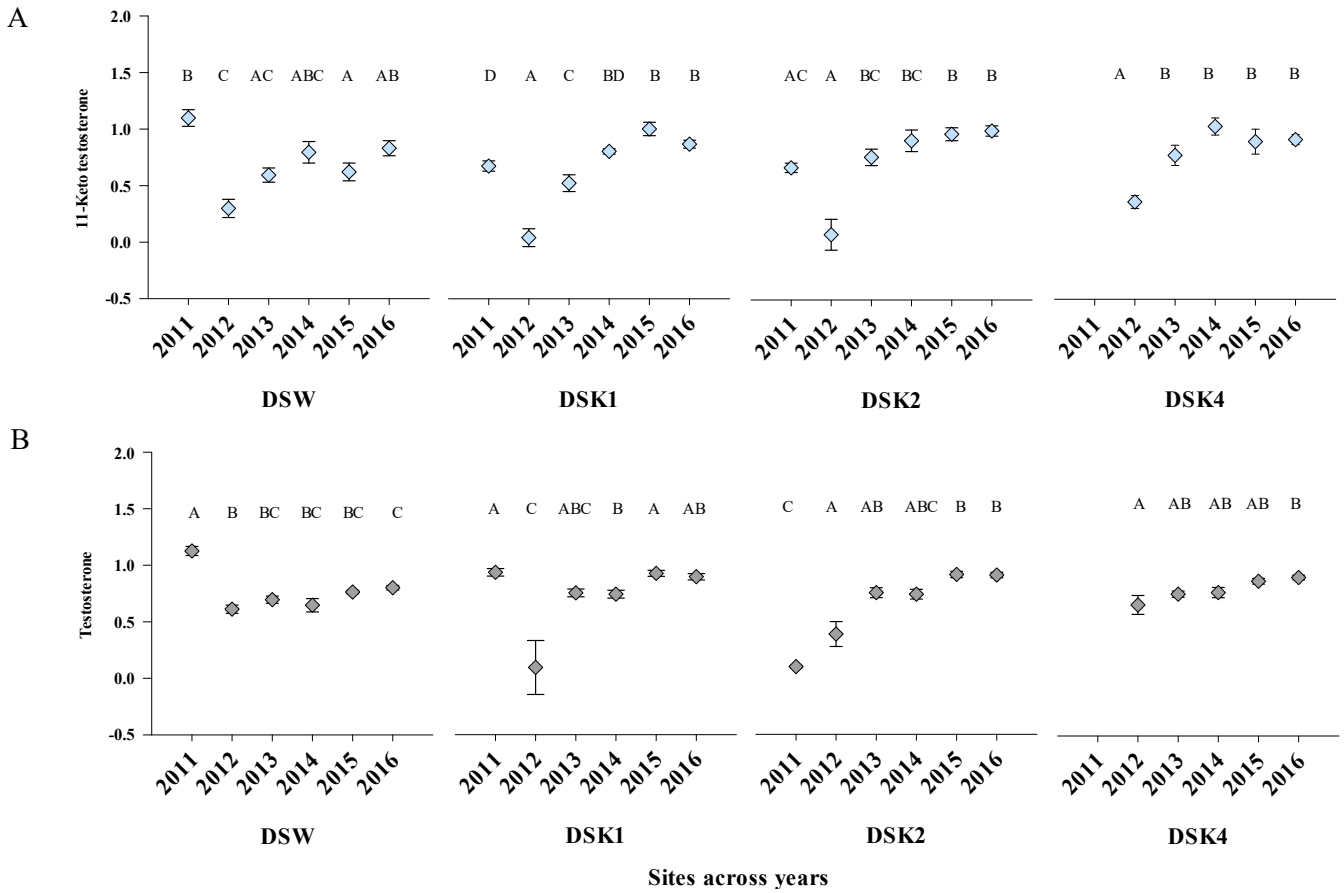


Figure 4.4 Across-year patterns (2011 - 2016) of human chorionic gonadotropin stimulated production of 11-ketotestosterone (A) and testosterone (B) by the testes from male rainbow darter collected downstream of the Waterloo MWWTP (DSW), below the Kitchener MWWTP outfall (DSK1), the second downstream site (DSK2) and the far field downstream site (DSK4). Presented are log10 transformed

androgen steroid concentrations that are normalized to US2 rainbow darter steroid concentrations across years (2011 - 2016). Statistical differences are indicated by capital letters above scatter plots (mean ± standard error of the mean).

4.4.5 Changes in effluent quality over time

The effluent discharged from the Waterloo MWWTP was variable and showed limited quality improvement over time (2011 – 2016). The highest level of ammonia was measured in 2012 and 2013 exceeding 27 mg/L and simultaneously the lowest nitrate concentrations were recorded (1.46 ± 0.41 mg/L and 0.88 ± 0.81 mg/L, respectively) (Figure 4.5A). In the final year (2016), the ammonia concentration dropped to 11.31 ± 4.68 mg/L while the nitrate concentration increased to 19.20 ± 8.39 mg/L; however, this was still considered to be lower nitrification compared to the Kitchener effluent in the same time period. Effluent estogenicity at Waterloo also showed high variability over time, ranging from as low as 1.83 ± 0.38 ng/L E2eq in 2014 to the highest level of 16.62 ± 1.59 ng/L E2eq in 2013 (Figure 4.5A). In 2015 estrogenicity increased (compared to its level in the previous year) reaching 8.78 ± 4.10 ng/L E2eq. In contrast, Kitchener effluent quality improved following MWWTP upgrades and this was first evident by an almost 50% reduction in ammonia from 22.18 ± 5.90 mg/L in 2011 to 12.76 ± 5.80 mg/L in 2012 (Figure 4.5B). Ammonia levels continued to drop in the post-upgrade period (2013 – 2016) remaining well below 5 mg/L. Reduction in ammonia was simultaneously followed with an increase in nitrate concentration ranging from 4.80 ± 1.43 mg/L in the pre-upgrade period (2011) to above 30 mg/L in 2016. Kitchener effluent estrogenicity showed a sharp drop immediately after the upgrades when it was reduced from 18.50 ± 1.51 ng/L E2eq (2011) to below 2 ng/L E2eq in 2015 (Figure 4.5B).

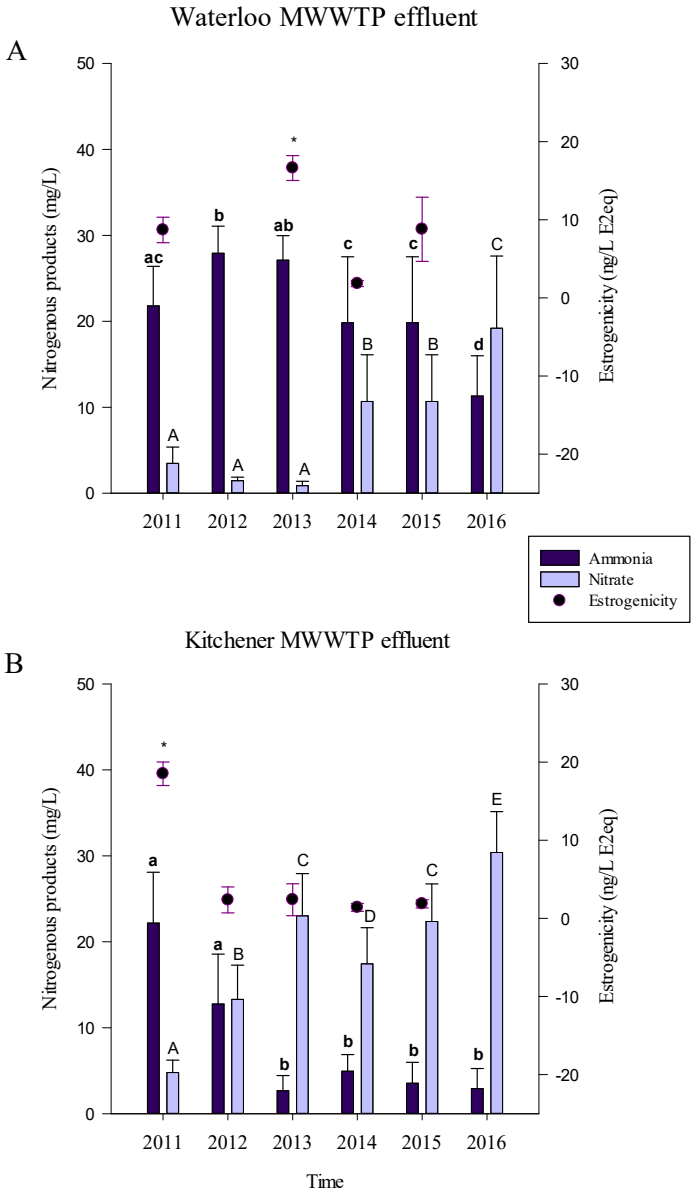


Figure 4.5 Time dependent changes in ammonia, nitrate and estrogenicity in the final effluents from the Waterloo (A) and Kitchener (B) MWWTPs. Estrogenicity was determined using the yeast estrogen screen. Ammonia and nitrogen concentrations (mg/L) represent the average values taken in the fall (October – December) across time from 2011 to 2016. Estrogenicity (ng/L E2eq) was measured in the effluent samples and it represents the annual mean (2011 - 2015). One-way analysis of variance was used to determine significant differences in ammonia, nitrate, and estrogenicity at each MWWTP over time.

4.5 Discussion

Studies conducted over a six-year period when the two MWWTPs on the Grand River were undergoing process changes demonstrated that *in vitro* production of androgens by the testes of rainbow darter was positively affected by improvements in effluent quality. Prior to the upgrades of the Kitchener MWWTP there was a reduction in the ability of rainbow darter to produce androgens (*in vitro*) at the downstream sites compared to upstream reference sites. In the years following the upgrades (2014 - 2016), that included increased nitrification of the effluent, *in vitro* androgen production in rainbow darter at the downstream sites was increased and it was comparable to the production in rainbow darter from the reference site(s). Below the Waterloo treatment plant outfall, there was also a suppression in the ability of rainbow darter to produce androgens (*in vitro*) after 2011 when the effluent quality declined due to process changes that reduced the level of nitrification and drastically increased ammonia. This suppression in androgen production in rainbow darter relative to the rural reference site was observed consistently immediately downstream of Waterloo across years and had no clear recovery pattern. However, there was considerable variability in the response spatially and temporally (e.g., annually) with some depression of androgen production across the urbanized environment including the urban reference site. Confounding factors (e.g., temperature, habitat, flow, other contaminant sources), could have modified the production of sex hormones in rainbow darter males across the study sites, thus complicating the interpretation of this response.

4.5.1 Effects of seasonal variability and fish handling on steroid production

Conducting field-based studies is important because they provide a realistic assessment of environmental state and organisms' health that integrates natural variability. However, field studies are confounded by other factors that are difficult to control. Therefore, long-term, consistent data collections on biological endpoints of interest are required with appropriate spatial and temporal resolution. Establishing appropriate and reproducible sampling protocols depends on a sound understanding of the biology of the sentinel species selected. In the current study, *in vitro* androgen production by rainbow darter testes was selected as a key endpoint, as it had been previously shown to be affected by wastewater exposure (Fuzzen 2016). In the Grand River, rainbow darter reach sexual maturity after 1 year and they reproduce asynchronously by releasing their gametes in clutches from mid-April to mid-June (Crichton 2016; Fuzzen et al. 2016). Following reproduction, their

gonads go through resorption during the summer months, from June to August. The recrudescence period starts in mid-September and continues to January. A study conducted in the Grand River on a related species, the greenside darter (*Etheostoma blennioides*), demonstrated that production of sex steroids was consistent and stable during the recrudescence period (October to January) and suggested that the fall season is appropriate for sampling and measuring *in vitro* sex steroid production (Tetreault et al. 2014) in that species. In the current study, it was confirmed that the fall season is an appropriate time for conducting the *in vitro* androgen production bioassay with the rainbow darter because this species goes through recrudescence in this period of the year which allows for detection of relatively stable levels of androgen production. There was consistent production of both 11-ketotestosterone and testosterone during the months of October, November, and December. In addition, during this period, it was possible to repeatedly and regardless of the fall month, detect a significant reduction in the production of androgen steroids in males collected downstream of the Waterloo MWWTP compared to the upstream rural reference site (US2). However, on certain occasions (November 2015 and December 2015) it was not possible to detect significant differences in 11-ketotestosterone and testosterone production between US3 (urban reference) and DSW males. The immediate upstream site is located in the urbanized area and is possibly affected by some confounding factors, or changes in habitat, that causes steroid production to be less consistent. Although steroid production was higher in the spring months, it was more variable especially towards the end of the reproduction period (mid-May) making it difficult to interpret comparisons among sites. In addition, early spring sampling is logistically difficult because of weather and unpredictable river flows. The late fall is therefore the best option for obtaining consistent responses that are comparable across sites.

Field-based studies tend to be challenging in that fish collections are dependent on a variety of external factors. Good practice and coordination of sampling is required to standardize sampling and reduce variability associated with confounding factors. In our studies, the dissection of the testicular tissue was typically completed in ~1 h after capture but in several occasions fish were kept in aerated buckets for prolonged periods of time (which did not exceed 3 h). This raised a concern that fish held in such conditions could have been subjected to additional stress, and that in turn may be an important source of bias when measuring production of androgens. An experiment was conducted to determine if the time fish were held prior to sampling affected androgen steroid production. Our study showed that androgen production by fish held for 6 h did not differ from those

held for 1 h post-capture. This suggests that a major bias was not introduced by holding the fish in the current study and at the targeted sampling sites (typically 1 h and rarely as long as 3 h). It is possible that the time of handling may have had different effects on steroid production in fish from other sites across the environmental gradient.

This study was not designed to control for possible effects of time of the day on sex steroid production. However, the fact that there were no differences in sex steroid production between the 1 h and 6 h post-capture sampling in the handling study and the consistency of multiple sampling occasions (fall) suggested that the time of the day did not introduce a major effect on the results but this potential bias should be further investigated in future studies. A decrease in steroid production after 24 h was observed which affected the detection of site differences. Our results indicate that sampling protocols that minimize stress are important for interpretation of androgen production studies. In a study with wild rainbow trout the levels of plasma testosterone and estradiol decreased in fish held over 24 h for recovery. The same study showed that cortisol levels increased in fish that were held for 1 h, and in some fish held for 24 h, possibly due to stress caused by confinement (Pankhurst and Dedualj 1994). Other studies showed that handling stress and confinement differentially affected sex steroid levels in white sucker at reference and bleached kraft pulp mill effluent exposed sites (McMaster et al. 1994). There is evidence of the inhibitory actions of stress on sex steroid production (Fuzzen et al. 2011; Milla et al. 2009). It is likely that rainbow darter in our study were also experiencing stress due to confinement over 24 h, and that the decrease in androgen production occurred as a consequence of stress. However, this is only speculation since cortisol was not measured in the present study.

4.5.2 Influence of upgrades on androgen production

Surface water directly below the two major MWWTPs in the central Grand River was of poor quality. However, following the upgrades to the Kitchener MWWTP the effluent quality improved while the effluent discharged from the Waterloo MWWTP remained variable and of poor quality. The Waterloo MWWTP was operating relatively well prior to 2012; however, the quality of the effluent declined during this study as indicated by increases in ammonia concentration and a decrease in nitrate concentration. Total effluent estrogenicity followed a similar pattern as that of nitrogenous products over time. Recent studies showed that there were various pharmaceuticals

detected in the Waterloo MWWTP effluent over time (2011 - 2015) further confirming that its quality remained extremely variable (Marjan et al. 2017; Hicks et al. 2016).

Infrastructure upgrades to the Kitchener MWWTP (2012), that included better aeration and a longer solids retention time, resulted in better removal of ammonia, and a simultaneous increase in the nitrates. Consequently, this was associated with ~10-fold reduction of total effluent estrogenicity in 2015 compared to the pre-upgrade period (2010 - 2011). Moreover, pharmaceuticals such as naproxen and ibuprofen, both of which are considered to be reliable indicators of effluent quality and process changes (e.g., nitrification), also showed a significant reduction following the upgrades (Marjan et al. 2017; Hicks et al. 2016). For instance, Hicks et al. (2016) reported a 135-fold reduction in ibuprofen and a 20-fold reduction in naproxen in the post-upgrades period, compared to the pre-upgrade period.

Androgen production in male rainbow darter revealed patterns that changed across the urbanized environment in the central Grand River over time (2011 - 2016). Despite the apparent increase in sex steroid production in rainbow darter below the Waterloo outfall over time, there was no clear recovery pattern associated with rainbow darter collected below this MWWTP. Waterloo MWWTP was operating relatively well in 2011 compared to the following years (2012, 2013) and there were no effects on androgen steroid production in fish at the site below the outfall. Subsequently in the following years of the study (2012 - 2016), levels of androgen production remained well below the level measured in 2011.

In the period following the upgrades at Kitchener (2014 – 2016), there was a clear recovery pattern, especially in 11-ketotestosterone, in the rainbow darter caught at the downstream sites (DSK1, DSK2 and DSK4) from the Kitchener MWWTP. This coincided closely with the improvements in effluent quality that were evident in 2013 but it appears to have taken an additional year for these changes to be reflected in hormone levels through a gradual increase in sex steroid production in rainbow darter males. Hicks et al. (2016) demonstrated a reduction of intersex prevalence and severity in rainbow darter males after the upgrades of the Kitchener MWWTP. They reported that fish from DSK2 started to recover in 2013 (similar to11-ketotestosterone in the present study), whereas DSK1 rainbow darter had a more gradual change from 2013 to 2015. Intersex severity was reduced and only primary oocytes were determined in the testes of fish that did not fully recovered following the upgrades. This change in the gonadal histology could have positively

affected *in vitro* steroid production. Reduction of effects on endocrine disrupting endpoints (vitellogenin, GSI, nuptial tubercles) were reported in male fathead minnow after major upgrades of the Boulder Colorado's MWWTP which resulted in better removal of 17β-estradiol and estrone, and reduced ammonia (Barber et al. 2012). Recent laboratory studies support the hypothesis that recovery in endocrine responses in fish is indeed possible after the removal of endocrine disruptors (Luzio et al. 2016; Baumann et al. 2014). Moreover, a whole lake study demonstrated recovery in vitellogenin concentration and absence of histopathology abnormalities in male fathead minnow three years after discontinuation of 17α-ethinylestradiol (~5 ng/L) dosing (Blanchfield et al. 2015).

4.5.3 Potential effects of effluent chemicals on androgen production

Effluents released from the MWWTPs in the central Grand River are estrogenic and contain natural hormones including 17β-estradiol, estrone, and estriol and synthetic estrogens such as 17α-ethinylestradiol as indicated following sample fractionation and its subsequent analysis using high performance liquid chromatography (Smith 2013). All of the exogenous estrogens have the potential to mimic naturally occurring 17β-estradiol and exert their action in low concentrations resulting in alterations of absolute and relative concentrations of sex steroids in circulation and in tissues (Hecker et al. 2006). For example, exposure of the marine hornyhead turbot (*Pleuronichthys verticalis*) to estradiol caused significant reduction in 11-ketotestosterone (Vidal-Dorsch et al. 2014). Reduction in 11-ketotestosterone was also observed in a study with male fathead minnow exposed to environmentally-relevant concentrations of 17α-ethinylestradiol for 21 days (Salierno and Kane 2009).

Despite the significant drop in the Kitchener MWWTP effluent estrogenicty following the upgrades, rainbow darter males still demonstrated significantly reduced ability to produce sex steroids in 2012 and 2013. However, at the same time rainbow darter from the second downstream site (DSK2) showed first signs of recovery as their sex steroid production was no longer significantly suppressed. This suggests that fish from the immediate site below the Kitchener outfall (DSK1) took an additional year to respond to upgrades based upon the return of their sex steroid to reference levels. In contrast, Waterloo MWWTP effluent had variable estrogenicity from 2011 to 2015 which could potentially explain the absence of specific patterns in androgen steroid production. The primary goal of treatment plant upgrades was to increase nitrification, but this had multiple positive effects on the overall quality of the effluent. It is possible that reduced estrogenicity had a positive effect on sex

steroid production in rainbow darter but it is more likely that the overall effluent quality improvement was responsible for sex steroid production recovery. Earlier it was mentioned that increased nitrification resulted in better removal of pharmaceuticals that subsequently enhanced the removal of other contaminants not measured in this study, and this process could have modified sex steroid production in the past.

4.5.4 Effects of potential confounding factors

As indicated above, androgen production changed dynamically over years responding to effects from urbanization but also to factors specific to individual years. In the post-upgrade years, rainbow darter collected from several sites that were not directly exposed to the MWWTPs effluents showed significant reduction in at least one of the androgen steroids (mostly 11-ketotestosterone) suggesting potential effects of confounding factors. These sites included the urban reference site (US3), displaying significant reduction in two consecutive years (2013 and 2014), and the third site below the Kitchener outfall (DSK3) in 2013 and 2016. In the seasonal cycle of androgen steroid production experiment, it was not always possible to detect differences between fish from the site immediately below the Waterloo MWWTP and those from the urban reference site (US3), suggesting that this site may have been under additional pressure (anthropogenic, habitat etc.). It was hypothesised that the highly agricultural Conestogo River located above the third reference site could be a potential source of contamination that causes reduction in 11-ketotestosterone. To test this, a separate study was conducted in fall 2015 that aimed to isolate potential effects from the Conestogo River (Figure S4.4; Supplemental Information). No differences in androgen production were detected at the sites associated with the Conestogo River in 2015 (Figure S4.5; Supplemental Information). Despite a depression in androgen production at US3 (relative to US2) in the previous two years, US3 fish in 2015 no longer showed significant reduction in 11-ketotestosterone (but there was still an effect below the Waterloo MWWTP in 2015). The effect detected at US3 in the two previous years appears to be temporally variable, making the cause difficult to determine.

The characteristics of the sites may also influence the variability in the responses. For instance, the effluent hugs the shore downstream of the Kitchener MWWTP and is not fully mixed until several kilometers downstream, which is estimated to be prior to DSK3. Changes in flow (high flows 2013, low flows 2016) can alter exposure to the effluent but also the influence of other confounding factors cannot be underestimated. It is interesting to note that after the upgrades Hicks et

al. (2016) found severe intersex in male rainbow darter males at this site (DSK3) to be relatively inconsistent (but high) compared to the severity in DSK1 and DSK2 fish after the upgrades.

Changes in river flow, temperature, oxygen availability, food availability, and the properties of the habitat can all indirectly contribute to alterations in gonadal steroid production. Temperature and photoperiod play a critical role in spermatogenesis which is dependent on steroid production (11-ketotestosterone and testosterone) that increases as spermatogenesis proceeds (Vlaming 1972). However, increased temperatures can be interpreted as "wrong" signals that can ultimately lead to delays in gonadal steroid production. Increased temperature can change the patterns of testicular and ovarian steroidogenesis by increasing conversion of sex steroids to their glucuronated forms (Pankhurst 1997), which are less biologically active (available). Elevated temperatures delay steroidogenesis and responsiveness to luteinizing hormone releasing hormone in rainbow trout (Pankhurst and Thomas 1998). It is known that effluent discharge can increase the temperature in the receiving environment, raising the possibility that this could contribute to alterations in steroid biosynthesis. For example, an overall suppression in androgen steroid production was observed at the sites in the urbanized area with significant reduction in fish downstream of the two MWWTPs in 2012. This year was extremely dry and warm with low flows, which decreased effluent dilution resulting in higher exposure and elevated temperature both of which could partially explain the overall suppression of 11-ketotestosterone and testosterone. This was evident at the site immediately downstream of the Waterloo MWWTP and remained relatively supressed until the second downstream site from the Kitchener MWWTP (DSK2) where a gradual increase was noted. Fish collected at the upstream sites (US1, US2) that are known to have lower water temperature due to groundwater inflow, had comparable levels of androgen steroids to those measured in fish from the upstream reference sites across years. Hicks (2017) reported that urban sites in the central Grand River (below the Kitchener MWWTP) had up to 2.5ºC higher mean daily summer temperature compared to the rural reference sites (above the Waterloo MWWTP). This further supports the idea that the increase in temperature in the urbanized area could affect sex steroids in rainbow darter.

Oxygen availability is usually decreased at the sites downstream of municipal discharges due to its high demand in biodegradation processes. Hypoxia can cause endocrine disruption at concentrations of oxygen below 2.0 mg/L (Thomas and Rahman 2009; Wu et al. 2003). Low oxygen levels affect various reproductive processes including effects at multiple steps in hypothalamus

pituitary gonadal axis (Lu et al. 2014; Rahman and Thomas 2013). Before the upgrade implementation, the site immediately below the Kitchener MWWTP experienced hypoxic conditions with (summer mean) daily dissolved oxygen levels as low as 1.2 mg/L which is well below the recommended 4 mg/ L (Hicks 2017; Loomer and Cooke 2011). Following the upgrades, dissolved oxygen was always above 5 mg/L (Hicks 2017). Beyond studies considering the effects of anthropogenic chemicals on reproductive function it will be important to understand the contribution of changes in the physical environment (temperature and oxygen availability) in efforts to establish cause and effect relationships.

4.6 Conclusions

The results of the present study show the potential for testicular steroid production to be used as a relevant and sensitive endpoint to assess anthropogenic inputs to the aquatic environment. Although there is considerable natural variability, *in vitro* steroid production responded to effluent inputs spatially and over time in response to treatment changes. Monitoring biological responses over long time scales (i.e., multiple years), as well as consideration of spatial variability, are important considerations when using this important endpoint. Moreover, the current study shows that infrastructure upgrades at MWWTPs (as demonstrated by the Kitchener MWWTP) can improve ecosystem health by reducing the exposure to multiple stressors, including a complex mixture of endocrine disruptors. Although the primary goal of the MWWTP upgrades was to reduce conventional parameters (e.g., ammonia), these changes have had a secondary positive benefit of reducing the effects on steroid production in a sentinel fish species in the receiving environment.

Chapter 5

General conclusions and recommendations

This thesis describes a series of research studies that were designed to investigate responses in rainbow darter to urbanization effects, reference site selection, and wastewater treatment changes in the central Grand River. Hepatic transcriptomic responses and *in vitro* sex steroid production in rainbow darter (males) were used as principal endpoints representative of different levels of biological organization, and their changes were monitored across temporal and spatial scales. Experiments that investigated temporal variation of the selected endpoints had a specific goal to evaluate if MWWTPs infrastructure upgrades resulted in fish responses returning to levels in fish collected from reference sites. To accomplish this goal, past results and analysis of archived samples collected prior to infrastructure upgrades (2011 - 2012) were compared to post-upgrade collections (2013 – 2016). Investigating several biological responses, including reproductive effects, across an urban environment (spatial scale) provided evidence for an association with MWWTP exposure but also highlighted the importance of the role that confounding factors play in field-based studies. Several major conclusions can be drawn from the work conducted in the thesis:

1. In the second chapter, hepatic transcriptomic responses in rainbow darter collected below the Waterloo MWWTP (that only underwent some minor changes) showed the most distinctive gene expression pattern when compared to fish collected from other sites across the urban environment. Moreover, this study suggested that selection of reference site does not affect gene expression in fish from a heavily polluted site (Waterloo MWWTP) that had ~26% of the probes differently expressed. In contrast, reference site selection matters in fish collected from sites that are not directly exposed to point sources and that showed ~10% of the total number of probes to have differential expression.

2. The third chapter determines whether upgrades at the MWWTPs and improved effluent quality were reflected in the rainbow darter hepatic transcriptome response. The results suggested that quantitative analyses of gene expression did not capture a significant change in the transcriptome and it indicated the significance of annual factors in driving molecular responses (e.g., high flows, temperature). However, qualitative functional analyses suggested that following the upgrades fewer pathways indicative of stress-exposure were affected, and rainbow darter collected below the two MWWTP outfalls showed greater divergence after the upgrade implementation.

3. The fourth chapter continued to investigate the effectiveness of the implemented MWWTP upgrades to remediate *in vitro* steroid production that showed reduction relative to reference sites in the years prior to upgrades (2011 - 2012) at the sites immediately below the effluent outfalls. 11-Keto testosterone was more consistent in its response to anthropogenic contamination from MWWTPs and it showed recovery following the upgrades (2013 - 2016). Testosterone on the other hand, was more difficult to interpret due to the lack of pattern consistency and its sensitivity as an appropriate endpoint to assess recovery after major upgrades remained unclear.

The experiments in this thesis improved our understanding of the suitability and sensitivity of the selected endpoints in rainbow darter to respond to changes to the environment including those coming from the upgrades of the wastewater treatment process. There have been significant efforts in the field of genomic technologies to standardize sample processing, preparation, and bioinformatic analysis in order to advance the use of this approach in regulation. Based on earlier transcriptomic studies that used various microarray platforms it was observed that gene expression responses change depending on the selection of the comparison baseline. However, to our knowledge there are no environmental transcriptomic studies that have quantified the effects that different baselines have on gene expression variation. Determining the magnitude of the variation in the transcriptome in response to reference site selection is of great importance as it can affect the interpretation of the data. This thesis advanced our knowledge about transcriptomic variation and it showed that subtler changes in the transcriptome are more prone to variation whereas dramatic changes in the transcriptome are more stable and tend to be less dependent of reference site (or control) selection. This does not necessarily mean that the method lacks sensitivity, it just means that caution should be taken not to overinterpret data. Comparisons against multiple reference sites provide an accurate and a more realistic insight in transcriptomic changes than comparisons against a single reference site. Conducting environmental transcriptomic studies that incorporate multiple reference sites in the experimental design and subsequent data analysis of future studies will provide a means to assess variability and to potentially identify or track its origin. As shown in this thesis, heavily polluted sites left a distinguishable imprint on the rainbow darter liver transcriptome. This may be particularly useful in (aquatic) systems that have never been researched before and where sources of pollution are unknown. Transcriptomic analyses are very informative as they evaluate early responses and therefore add insight into changes that organisms likely express as a consequence of direct

interactions with their surrounding environment. One of the limitations of transcriptomic analysis is that it requires extensive knowledge about the genome sequence. The rainbow darter genome is only partially sequenced, allowing relatively limited interpretation of gene expression and affected biological pathways. Therefore, integration of changes of the rainbow darter transcriptomic signatures in more complex cellular, organ or whole organism level remains a challenge. Rainbow darter with severe intersex showed discernable transcriptomic signatures compared to healthy males (and females) however the exact mechanisms underlying this condition have not been determined (Bahamonde et al., 2015a). Microarrays remain a valid tool in detecting early responses but this technology is being gradually replaced with more advanced ones such as RNA sequencing that allows an in-depth view of gene expression and regulation. This new technology is expected to lead towards a more comprehensive functional picture and establish comprehensive linkages between gene expression and phenotypic changes that have been historically showed to be responsive to anthropogenic contaminants originating from municipal effluents in the Grand River.

Transcriptomic studies in rainbow darter highlighted the importance of spatial and temporal variables in the ecosystem structure that influence molecular responses and their overall interpretation. Although, temporal assessment of the quantitative transcriptomic responses to changes in the wastewater treatment in the central Grand River were difficult to interpret it helped us understand the complexity of microarray utilization in long-term field-based studies. Although a great majority of studies on gene expression report quantitative changes, that is changes in numbers of differently expressed probes (genes), their relevance is still not well understood. In other words, large numbers of genes that are significantly altered in fish exposed to anthropogenic pollution do not necessarily indicate that the health of these fish is compromised or that they are stressed. In contrast, this can indicate a positive response that can be interpreted as activation of mechanisms that are involved in adaptation or recovery. Therefore, it is important to carefully analyse these transcripts and their involvement in biological processes or molecular functions that can elucidate mechanisms involved in adaptive or recovery responses. Qualitative gene expression analysis that focuses on identifying a suit of transcripts known to be responsive to a specific contaminant (e.g., 17α-ethinylestradiol) has shown to have high consistency in interlaboratory studies and it may be an important step in standardizing transcriptomic results (Feswick et al., 2017). In the present thesis, qualitative analysis identified relatively high consistency in expression of selected transcripts over time. For instance, choriogenin-H minor was a transcript that showed consistent upregulation in

response to effluent exposure. In addition, qualitative functional analysis (GSEA and SNEA) were more informative when rainbow darter responses were assessed over multiple years in a system that underwent major upgrades of the effluent treatment. This suggests that standardization of qualitative transcriptome analysis may be a way to move transcriptomics closer to their incorporation in risk assessment and regulation (or at least becoming more informative). The current thesis did not focus on elucidating mechanisms of action of specific pollutants with endocrine disrupting activity that were detected in the Grand River. It was estimated that total estrogenicity of the effluent was relatively high prior to upgrades which coincided with consistent expression of choriogenin-H minor. Future studies should examine choriogenin-H minor as an estrogen responsive biomarker and compare its sensitivity to the conventionally used vitellogenin (mRNA) expression in rainbow darter. However, this should be conducted in both liver and gonads because it is known that the abundance of choriogenin-H minor transcript is higher in the gonads. Gene expression provides a rapid and an early response, which can be detected within days or even hours, thus reflecting current changes in the surrounding environment. The time of sampling becomes a very important factor for detecting a response in gene expression. When exposed to stressors, organisms activate genes that are involved in adaptive changes but if the stress source is removed the organisms can return to a normal state which suggests that there is critical window for determining changes at the molecular level. Transcriptomics using microarrays have not been extensively used to monitor the effectiveness of remediation in environmental studies, but they show potential to be appropriate remediation indicators as also demonstrated in a study with mummichog (*Fundulus heteroclitus*) collected in an environment before and after significant reduction in chromium (Roling et al., 2007). The upgrades at the Kitchener MWWTP targeted removal of ammonia and enhanced nitrification. These basic changes in the treatment procedure resulted in an overall improvement in effluent quality and the quality of the receiving environment. Following the upgrades, there were fewer biological pathways in common between these two groups of fish and there was a greater divergence between fish from the upgraded treatment plant versus those from the treatment plant with minor changes. It can be speculated that rainbow darter below the MWWTP with minor changes were also recovering. However, considering that the two groups of rainbow darter were diverging over time, this suggests that they were using different strategies to recover or to adapt to recent changes. Further, this also suggests that microarray based transcriptomics can be utilised in long-term (multi-annual) scenarios. Gene expression changes reported in this thesis seemed to be greatly influenced by the variability in

environmental factors such as high annual flows or high temperatures but further research is needed to better understand the impacts of these factors on expression outcomes. Moreover, risk assessment programs usually rely on multi-season and multi-year studies which provide another reason for directing transcriptomic research toward a better understanding of their response and use in long-term studies that consider spatial variability.

It was suggested that following the upgrades rainbow darter below the MWWTP recovered their ability to produce 11-ketotestosterone and returned to levels observed in fish from reference sites. Increased 11-ketotestosterone corresponded to reduced intersex occurrence and severity in rainbow darter and with improved effluent quality reported in other studies that coincided with the current studies. One of the major challenges regarding the use of steroid production to detect changes associated with stressors is the high individual (natural) variabilities. Holding time (<6 h) of the fish prior to sampling showed negligible effect on the variability or ability to detect differences. Repeated collections in the fall season also demonstrated that consistent differences could be detected between sites. However, individual (natural) differences in the ability to produce *in vitro* steroids remained relatively high which indicated that further research on how to minimize these differences would be beneficial. To reduce natural variability within individual fish, future studies should consider increasing the number of samples that will subsequently increase the statistical power. Assuming that rainbow darter in the river show minimal movement, then they would be exposed to relatively similar conditions which could result in relatively unified responses. However, factors in environmental studies are less controllable subsequently leading to greater individual biological differences including gonadal maturation. It is known that levels of steroid hormones change during sexual maturation and they are lower at the onset of this process reaching their maximum prior to spawning. One of the options to consider in future studies that could potentially reduce variation is to conduct gonadal tissue staging prior to *in vitro* steroid production analysis. This method would allow comparison of steroid production between gonadal tissues that are within the same maturation stage. Further, it would be useful to find out whether fish age or size is correlated with the ability of the gonadal tissue in rainbow darter to produce sex hormones. These could be standardized in future studies to reduce individual sample variability providing more statistical power to detect changes between sites.

Linking molecular responses with changes at the higher level of biological organization is one of the main priorities in the field of environmental toxicology. One of the goals of this thesis was to assess fish responses to upgrades on the basis of examining endpoints at two levels of biological organization and to contribute to the greater context of the rainbow darter health assessment. This required a specific coordination of sample collections which reduced sample size for each endpoint and the type/amount of tissue available to study rainbow darter responses. This minimized the ability to make comparisons using individual fish across levels of biological organization and therefore linkages within an adverse outcome pathway. Some linkages can still be speculated based on the responses in the rainbow darter in two tissues that have different functions and roles. It is common to present these linkages as simple diagrams where a response (box) leads to another. However, it is known that these linkages are more complex in reality and they are actually a network of interrelated processes that occur simultaneously in various tissues. For example, gene expression in rainbow darter below the MWWTP outfalls was indicative of stress induction and immunity perturbations. At the same time these same fish were experiencing reduced ability to produce sex steroids. Immune system is controlled by steroid hormones that reduce inflammation and the overall immune response. Therefore, it is not surprising that rainbow darter with decreased sex steroid production had compromised immune responses (based on the liver transcriptomics). Although it would have been beneficial, the current approach did not allow us to determine if there were linkages between severely intersex rainbow darter and their reduced ability to produce 11-ketotestosterone. To address more specific questions that concern revealing mechanisms of action and establishing linkages between various biological responses future research should consider changing of sampling logistics to allow stronger correlations among endpoints using individual fish.

Taken together, changes in gene expression and sex steroid production were shown to be appropriate endpoints for evaluating responses in a system that was exposed to a diversity of anthropogenic inputs. These studies demonstrated the utility of using gene expression and steroid production to inform how stressors can impact fish responses especially within the context of an adverse outcome pathway. Gene expression can respond quickly and early to stressors providing indications of exposure and potential responses at higher levels of organization. However, these responses can be modified rapidly in the environment making them difficult to interpret without consideration and understanding of natural variability. In a similar way, *in vitr*o steroid production

was found to be a useful endpoint that responded to wastewater treatment plant effluent quality but was also prone to considerable natural variability.

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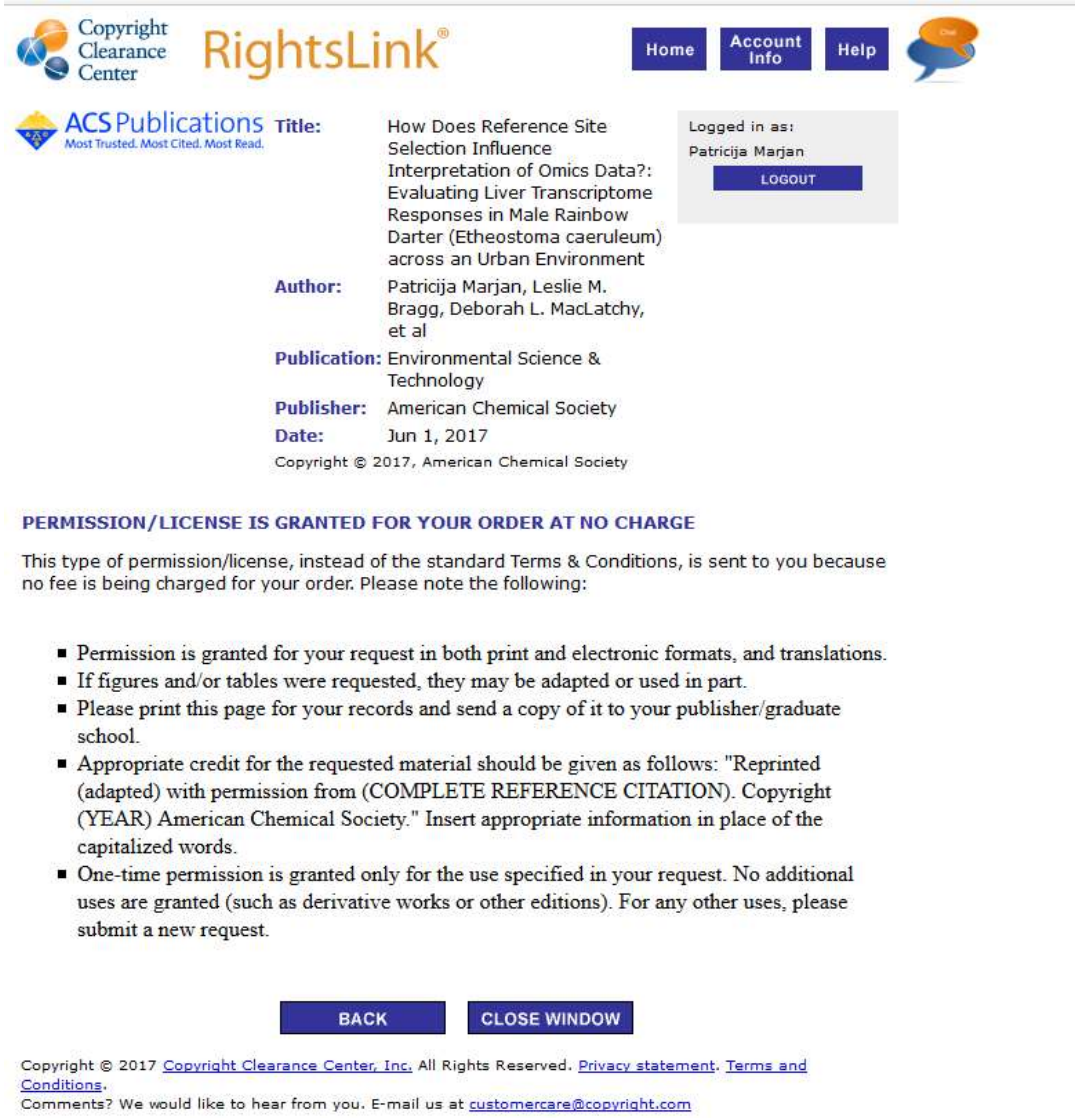

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Title: How Does Reference Site Selection Influence Interpretation of Omics Data?: Evaluating Liver Transcriptome Responses in Male Rainbow Darter (Etheostoma caeruleum) across an Urban Environment

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Appendix A
Supplemental information for Chapter 2

1. Site selection

The Grand River is a highly agricultural and urbanized watershed and there are no ideal reference sites that are free of anthropogenic influences. In the present study, 9 sampling sites were selected to isolate the influence of municipal wastewater treatment plants (MWWTP, Waterloo and Kitchener) and create context for the study. In addition to proximity to the wastewater outfalls, other considerations for sample site selection were the availability of suitable riffle habitat, accessibility, and the availability of historical data. Recent studies (Hicks et al., 2016, Fuzzen et al., 2016) have also investigated various biological responses in rainbow darter from these sites providing information that can be used for better interpretation and integration of data.

A site was selected immediately below the Waterloo and Kitchener MWWTP outfalls (DSW, DSK1) and one immediately upstream of each outfall (US3, INT). Additional sites further downstream from the urbanized area (DSK2, DSK3, and DSK4) were also included to replicate the observations and/or to detect potential recovery. Two additional upstream reference sites (US1, US2) were selected above the urbanized area of Waterloo to better characterize the upstream conditions. The site immediately above the Waterloo outfall (US3) is 6 km above the Waterloo MWWTP outfall and there is some urbanization in this reach. The reference site (US2) was added as it is well upstream of the major urbanized area; 22 km upstream of the Waterloo outfall. The third reference site (US1) was 11 km further upstream in a relatively rural area (i.e., 33 km upstream of the Waterloo outfall). Although US1 and US2 are well upstream of the major urbanized area there are several small wastewater treatment plants that discharge into the river further upstream.

Throughout the urban area there are numerous storm water outfalls and small creeks that join the main branch of the river. The upstream area also includes major agricultural activity. However, most of the wastewater indicator chemicals measured are below the detection limits or detected in much lower concentrations than those chemicals measured in the downstream urbanized area (Figure S2.1 and Table S2.2). There is a major tributary (Conestogo River) that enters the main branch (between US2 and US3) that drains a highly agricultural area that may also add a variety of contaminants and alter water quality. A major tributary joins the main branch just below site DSK3

(Speed River) and there are two additional wastewater outfalls upstream of DKS4. Therefore, there may be exposure to a variety of contaminants and stressors in the river that cannot be isolated or controlled.

None of the reference sites can be considered “pristine” and there are several possible confounding stressors in the watershed. This is the situation in most studies that examine the impacts of human activities on aquatic systems.

2. Effect Directed Analysis (EDA) coupled with Yeast Estrogen Screen (YES)

2.1 Effluent sample preparation and fractionation

Effluent samples (500 mL) were collected in triplicate and immediately preserved (with sodium azide and ascorbic acid). Upon bringing them to the lab, samples were filtered and extracted. Sample extraction was done with Waters Oasis HLB SPE 6cc, 500 mg cartridges. Preconditioning of the cartridges was done with 5 mL methyl tert-butyl ether, 5 mL methanol and rinsed with 5 mL of deionized water. Samples were eluted with 5 mL of 10 : 90 methanol : methyl tert-butyl ether. Samples were evaporated to dryness under a gentle stream of nitrogen and reconstituted in 80 µL of methanol. After this, samples were subjected to high performance liquid chromatography for separation which was done by Waters Alliance 2996 with a 2995 photodiode array detector, and subsequently fractioned using Gilson Fraction Collector. Samples were fractionated using a methanol/water gradient on an Agilent Eclipse XDB-C18 4.6 X 250 mm X 5µm (PN 990967 – 902) column.

2.2 Yeast cell preparation for yeast estrogen screen (YES)

Yeast cells were first streaked on minimal media agar and grown for 3 - 4 days until colonies were visible. A single colony was afterwards streaked for the second time. Following colony formation, a single colony was chosen and placed in the GOLD media for further development. Grown cells are then diluted to 1:10 in minimal media, incubated overnight and then diluted to 1:1 for the assay. A portion of this culture is preserved whereas a portion is further diluted to an optical density (OD_{600}) equivalent to 0.03 in minimal media and 50 µM of copper solution. Yeast cells prepared this way were dosed with sample (fractionated sample for EDA or effluent sample) or with a 17β-estradiol standard. The dosed cells were incubated overnight approximately 18 h at 30^{o}C in an orbital shaker set at 300 rpm. The following day cells were subjected to dilution to OD_{600} = 0.25. Diluted cells were distributed in triplicate to a 96-well plate, brought up to a volume of 100 µL with minimal media and dosed with 100 µL β-galactosidase solution. The absorbance was read at 420 nm every 15 s for 30 min. The β-galactosidase activity was calculated as outlined in the Yeast β-Galactosidase Microplate Assay kit from Thermo Scientific. In 1 L extraction, the lower limit of detection was 0.304 ± 0.088 ng/L estradiol equivalents (E2eEq) and the upper limit of detection was 22.3 ± 5.45 ng/L E2eq. For more detailed information on the method and the media composition refer to Smith (2013).

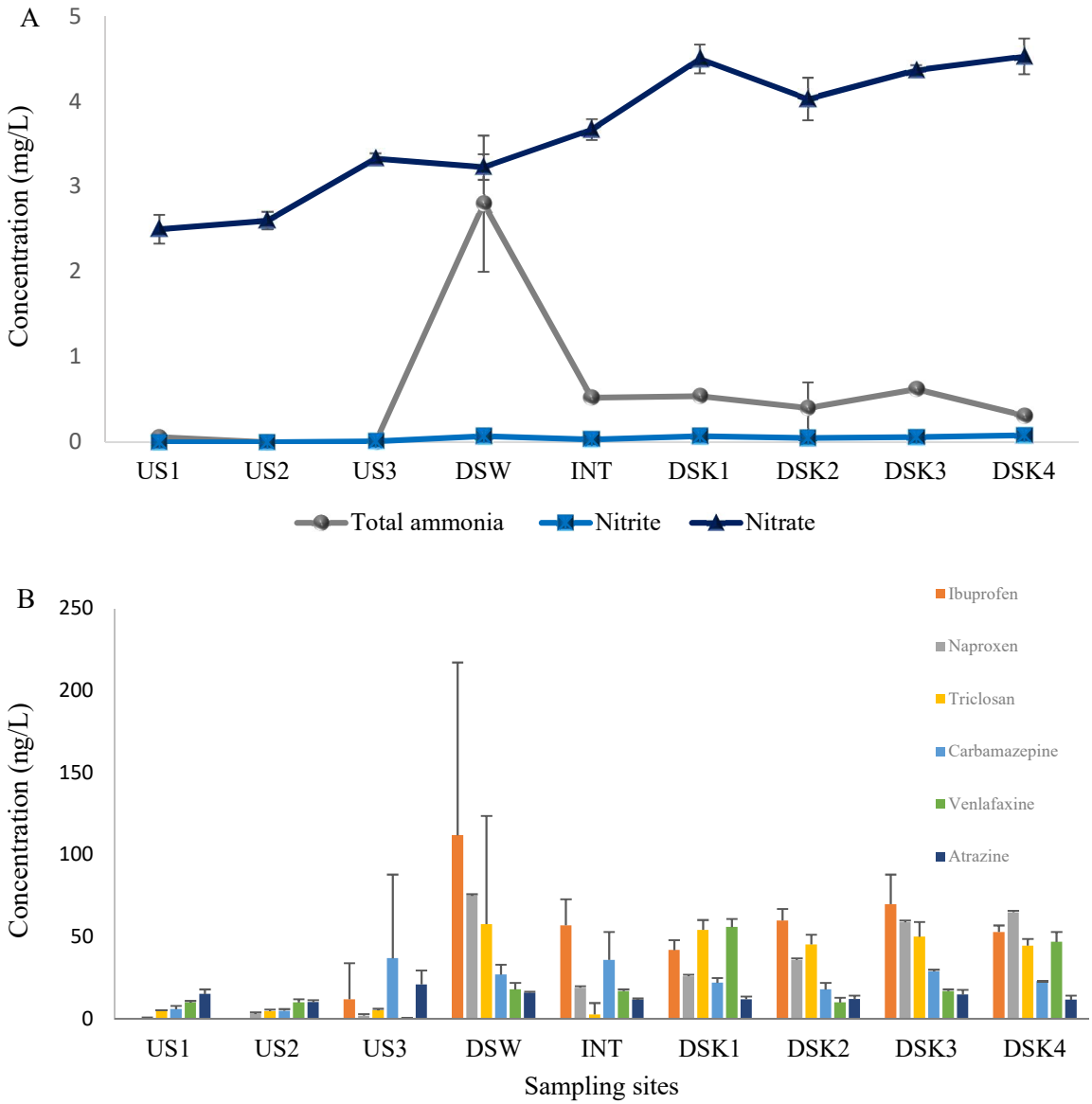


Figure S2.1 Concentration of total ammonia, nitrite and nitrate (mg/L) in the river at the sampling sites (A); concentration of various chemicals (ibuprofen, naproxen, triclosan, carbamazepine, venlafaxine and atrazine; ng/L) that are conventionally used as relevant indicators of the wastewater treatment quality, measured across the urbanized environment in the central Grand River (B).

3. Site selection effects on transcripts expression

(Approach #1) The 39.4% represent the total percentage of transcripts (differentially expressed genes) in DSW rainbow darter that were not influenced by reference site selection (common to all three reference sites). There was a total of 24.5% transcripts that were identified as differently expressed when compared to two out of the three reference sites. Thus, 45.2 - 49.8% of the transcripts identified as different were detected based upon 2 reference sites. The Venn diagram below depicts these percentages and it shows the unique genes. Example calculations are also provided.

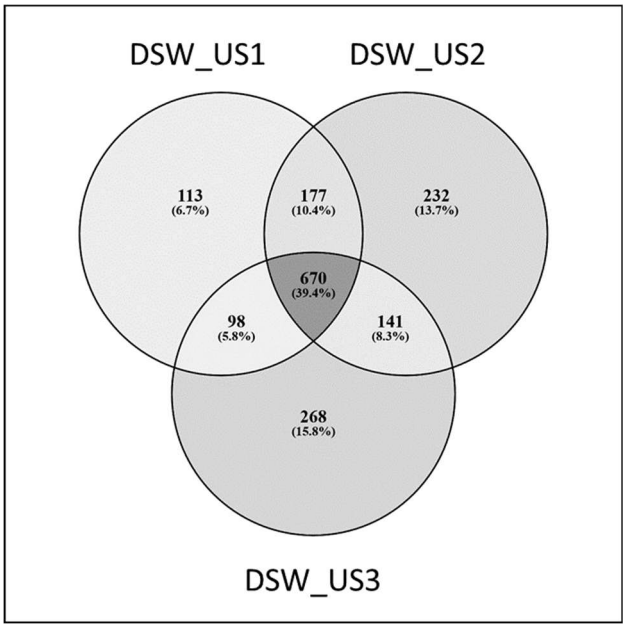
Example calculation:

Genes not affected by reference site selection = 39.4%.

Genes that were not affected by any two reference sites selected ranged from 42.5% (39.4% + 5.8%), to 49.8% (39.4% + 10.4%).

Unique genes in DSW fish in which expression was dependent upon reference site selection:

US1 = 6.7%; US2 = 13.7%; US3 = 15.8 %.



(Approach #2) The 56.9% represents the percentage of the differently expressed probes in INT rainbow darter as a representative group of fish (and site) where reference site seemed to matter and to show quite a dramatic effect on the magnitude on differently expressed transcripts.

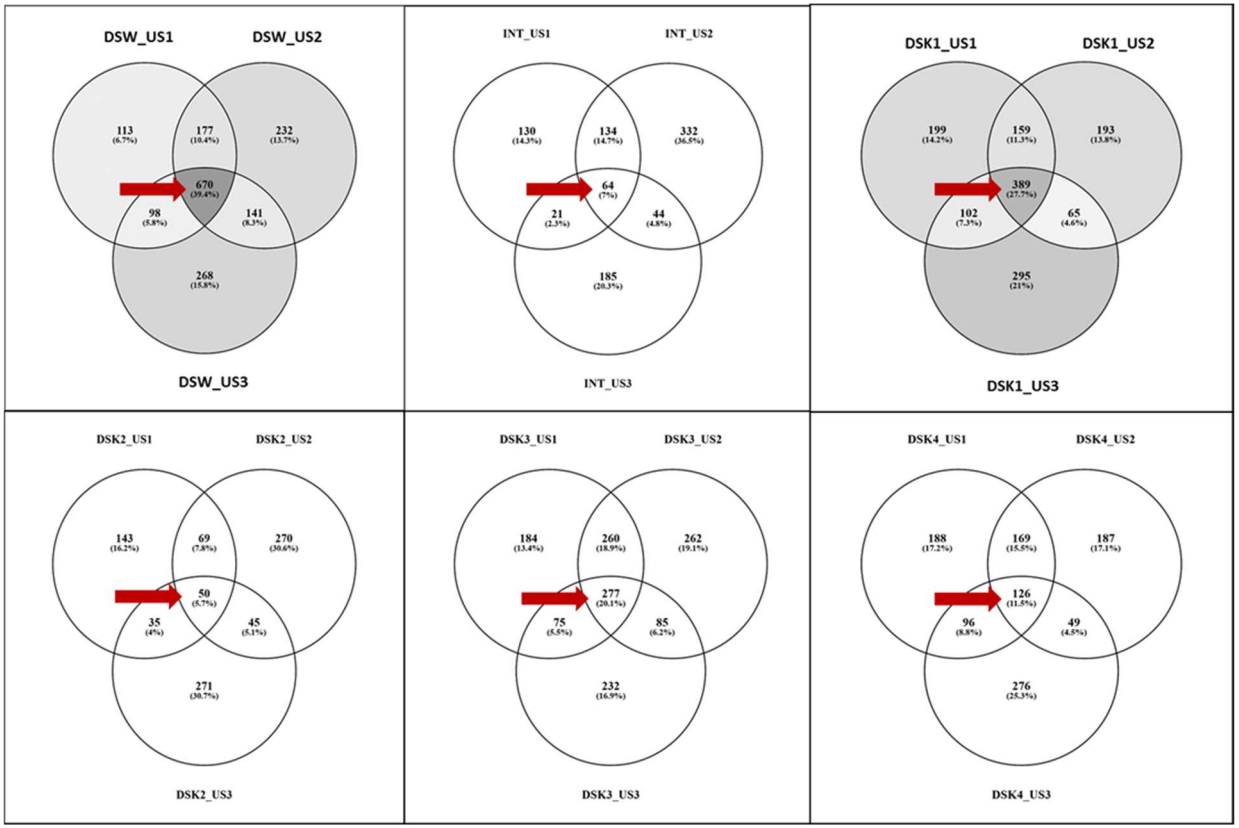


Figure S2.2 Venn diagrams depicting the effects that reference site selection had on differentially expressed genes in rainbow darter across the urban environment. Red arrows are pointing out on the number (and percentages) of transcripts that were not affected by reference site selection. Reference site selection had the lowest effect on the liver transcript expression in rainbow darter below the Waterloo municipal wastewater treatment plant (DSW) as 39.4% of transcripts remained unchanged, and the highest on fish from the second site below the Kitchener outfall (DSK2) with only 5.7% of transcripts that were in common.

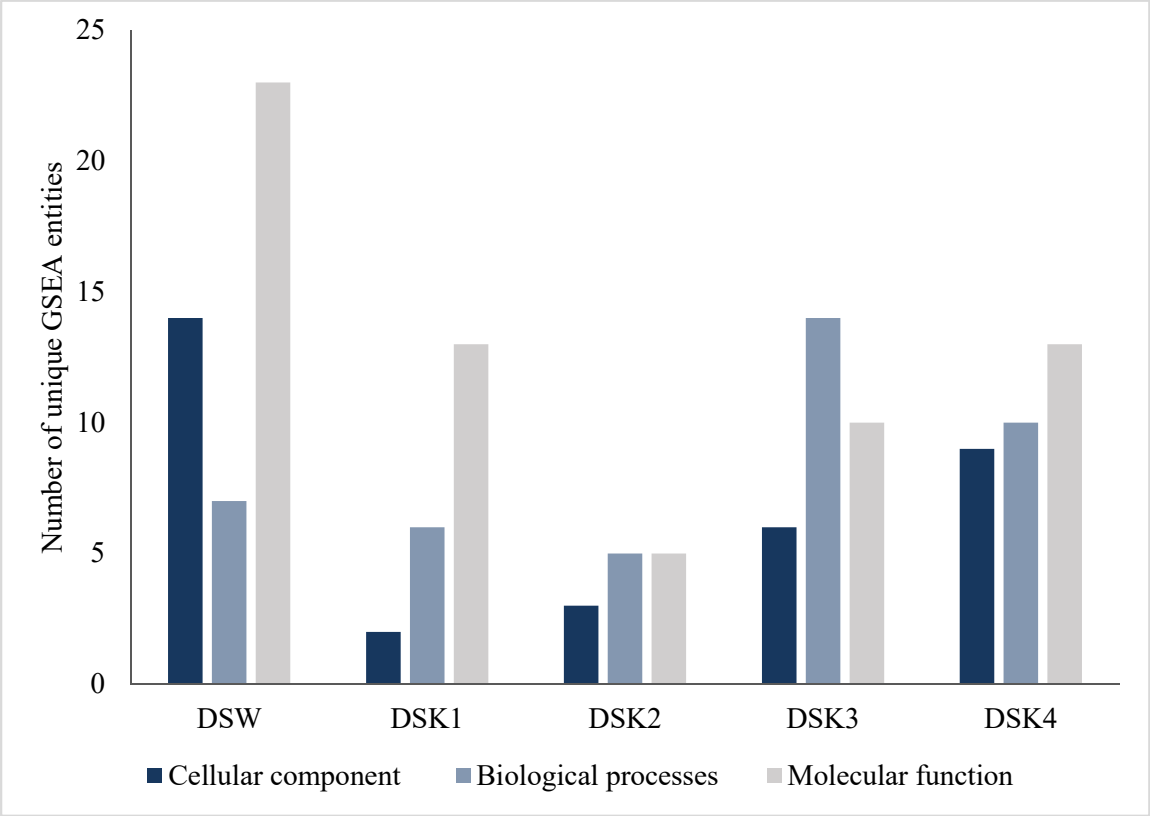


Figure S2.3 Gene set enrichment analysis using Parametric Analysis of Gene Set Enrichment (PAGE) algorithm (JMP Genomics, with unadjusted *p*-values) displaying different numbers of unique sets of genes involved in various cellular components, biological processes, and molecular functions in exposed rainbow darter (DSW and DSK1) and rainbow darter from downstream sites (DSK2, DSK3, and DSK4).

Table S2.1 Summary of morphometric characteristics of rainbow darter used in the analyses (n = 7 fish per site): total length (cm), total weight (g), liver somatic index (LSI), condition factor (k), and gonadal somatic index (GSI). One-way analysis of variance (ANOVA) with Dunnet's multiple comparison test was conducted to determine significant differences (US2 rainbow darter were used as control).

Site ID	Length (cm)	Weight (g)	LSI	k	GSI
US1	5.96 ± 0.52	2.88 ± 0.81	2.12 ± 0.17	1.33 ± 0.06	1.01 ± 0.20
US2	5.90 ± 0.48	2.70 ± 0.73	2.26 ± 0.37	1.28 ± 0.08	1.19 ± 0.23
US3	5.27 ± 0.55	1.87 ± 0.63	1.55 ± 0.49*	1.22 ± 0.10	1.15 ± 0.22
DSW	5.69 ± 0.80	2.38 ± 0.97	2.03 ± 0.25	1.22 ± 0.07	1.15 ± 0.43
INT	4.40 ± 0.13*	0.94 ± 0.11*	2.27 ± 0.17	1.11 ± 0.07*	1.12 ± 0.43
DSK1	4.97 ± 0.50*	1.44 ± 0.53*	2.10 ± 0.25	1.13 ± 0.07*	1.11 ± 0.28
DSK2	5.14 ± 0.75	1.72 ± 0.89	2.16 ± 0.34	1.19 ± 0.07	1.19 ± 0.30
DSK3	4.91 ± 0.49*	1.42 ± 0.46*	2.23 ± 0.63	1.17 ± 0.05	1.42 ± 0.31
DSK4	5.40 ± 0.73	2.26 ± 1.08	2.15 ± 0.37	1.34 ± 0.11	1.26 ± 0.18

Significant differences are indicated with *

Table S2.2 Additional surface water quality parameters (hardness, dissolved chloride, total organic carbon, and total phosphorus; mg/L) across the central Grand River.

	US1	US2	US3	DSW	INT	DSK1	DSK2	DSK3	DSK4
Hardness (mg/L)	310 ± 10.0	313 ± 5.8	336 ± 11.5	346 ± 5.8	333 ± 11.5	340 ± 10.0	340 ± 0.0	336 ± 5.8	336.7 ± 15.2
Dissolved chloride (mg/L)	28.7 ± 0.0	31 ± 0.0	30.7 ± 0.0	87 ± 0.0	45 ± 0.0	74 ± 0.0	70.3 ± 0.0	74.7 ± 0.0	85.3 ± 0.0
Total organic carbon (mg/L)	6.4 ± 0.3	6.1 ± 0.2	5.2 ± 0.1	6.0 ± 0.3	5.1 ± 0.3	5.4 ± 0.1	5.3 ± 0.2	5.5 ± 0.1	5.4 ± 0.1
Total phosphorus (mg/L)	ND	ND	0.04 ± 0.01	0.08 ± 0.02	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00

Table S2.3 Characterization of effluent samples form Waterloo and Kitchener MWWTPs. The indicator chemicals measured included ibuprofen, naproxen, triclosan, carbamazepine, venlafaxine, and atrazine (ng/L). Effluent samples were analyzed for total estrogenicity, concentrations are expressed in ng of estradiol (E2) equivalents (E2eq) per liter using the yeast estrogen

	Ibuprofen	Naproxen	Triclosan	Carbamazepine	Venlafaxine	Atrazine	YES (ng E2eq/L)
Waterloo MWWTP	2318.3 ± 164.3	1711.7 ± 252.7	1135 ± 137	202.83 ± 15.33	846.7 ± 102.0	11.6 ± 6	16.6 ± 1.6
Kitchener MWWTP	72.5 ± 15.6	346.5 ± 49.4	690 ± 52.2	275.67 ± 17.5	595 ± 37.8	10.7 ± 4	0.9 ± 0.5

Table S2.4 Top three genes with a fold change greater than three positive and lower than three negative in DSW and DSK1 rainbow darter compared to their expression in US1, US2 and US3 rainbow darter.

	US1		US2		US3	
	Upregulated	Downregulated	Upregulated	Downregulated	Upregulated	Downregulated
DSW	Choriogenin H minor*	Proto oncogen c-Fos**	Choriogenin H minor*	High choriolytic enzyme 1	Choriogenin H minor*	Suppressor of cytokine signaling
	LYRmotif – containing protein 7	Mannan-binding lecitin***	Type-4-ice-structuring protein LS12*	Trypsinogen 1	Chromosome 13 SCAF14555	C1q-like protein
	Chromosome 11 SCAF14642	Chromosome 10 SCAF10606	Chromosome 20 SCAF14749	Chromosome 11 SCAF 14979	Type-4-ice-structuring protein LS12*	Chromosome 18 SCAF14786, whole genome shotgun
DSK1	Novel ubiquitin protein ligase protein*	Proto-oncogen c-Fos**	Novel ubiquitin protein ligase protein*	Proproteinase E	Mennan binding lecmitin H2***	Chromosome undetermined SCAF6169, whole genome shotgun
	UPF0485protein Clorf144 homolog*	Trypsinogen 1***	UPF0485 protein Clorf144 homolog*	Carboxypeptidase A1***	Actin-related protein 2/3 complex subunit 3	Cytosolic sulfotransferase (< -3)[a]
	Epithelial membrane protein 2	Trypsinogen 2***	Chromosome 20 SCAF14744, whole genome shotgun	Carboxypeptidase B***	UPF0485 protein Clorf144 homolog	Dexamethasone-induced protein homolog (<-3)[a]

*genes that show consistent expression independent of reference site used (e.g. choriogenin H minor in DSW, and UPF0485 protein Clorf144 homolog in DSK1 fish)

**genes that show inter-site expression

***genes that show functional relationship (e.g. trypsinogen 1 and 2; carboxypeptidase A1 and B in DSK1 fish) and reference site selection dependence

[a] genes that had a fold change lower than three negative but were still among the top three downregulated

Table S2.5 Unique cell pathways in rainbow darter from DSW, DSK1 and DSK4 sites identified with subnetwork enrichment. Presented are the median change of the pathway, $\alpha < 0.05$, measured neighbours (representing the number of measured genes involved in a cell pathway), total number of neighbours that represents all genes that are known to define a specific pathway, and the percentage of measured versus total number of neighbours (simply the percentage of genes that were detected in a cell process in the rainbow darter in contrast to the total number of genes of the pathway).

SNEA cell pathways	Median change	p-value	Measured neighbors	Total # of neighbors	% of measured vs total neighbors
DSW					
mRNA processing	1.17	0.011	34	396	8.6
Poly(A)+ mRNA-nucleus export	1.43	0.023	14	84	16.7
rRNA processing	1.27	0.006	34	396	8.6
Ribosome biogenesis and assembly	1.21	0.034	34	142	23.9
Cation transport	-1.65	0.009	5	31	16.1
Nucleocytoplasmic transport	1.17	0.036	20	122	16.4
Cytoplasmic transport	1.15	0.042	15	45	33.3
Mitochondrial protein transport	1.17	0.017	8	26	30.8
Lipid peroxidation	-1.25	0.019	41	220	18.6
LDL oxidation	-1.43	0.024	15	84	17.9
Glycerol metabolism	-1.60	0.006	6	12	50.0
Cell proliferation	-1.05	0.020	466	4932	9.4
Apoptosis	0.00	0.038	516	4624	11.2
Xenobiotic clearance	-1.28	0.001	42	276	15.2

Tables S2.5 continues

Growth rate	0.00	0.009	111	709	15.7
Sperm cell adhesion	0.00	0.048	7	45	15.6
DSK1					
Leucocyte cell adhesion	1.09	0.016	23	214	10.7
Monocyte recruitment	-1.04	0.016	18	140	12.9
Establishment of T-cell polarity	-1.28	0.020	6	65	9.2
T-cell tolerance	-1.35	0.021	7	71	9.9
Eosinophil chemotaxis	-1.29	0.029	5	67	7.5
Leukocyte migration	-1.02	0.041	43	408	10.5
Transcytosis	1.17	0.015	15	94	16.0
Cellular extravasation	-1.09	0.019	10	76	13.2
Synaptic vesicle exocytosis	-1.15	0.039	5	49	10.2
Migrating myoelectric complexe	1.17	0.026	5	29	17.2
Artery contraction	-1.06	0.027	8	51	15.7
Vein blood flow	1.17	0.046	5	31	16.1
Protein-protein cross-linking via L-cystine	1.12	0.035	16	75	21.3
Glycolysis	1.21	0.038	36	255	14.1
Arachidonic acid metabolism	1.12	0.043	9	70	12.9
Strand invasion	1.15	0.025	7	32	21.9
Genetic instability	1.05	0.048	17	113	15.0
Startle response	1.34	0.039	6	48	12.5

Table S2.5 continues

Response to temperature	-1.14	0.009	32	144	22.2
Epidermal proliferation	1.17	0.038	7	72	9.7
DSK4					
Response to osmotic stress	1.26	0.009	11	68	16.2
Regulated secretory pathway	-1.15	0.013	5	24	20.8
Triacylglycerols biosynthesis	1.06	0.026	10	47	21.3
Alveolus formation	1.28	0.026	6	75	8.0
Pyruvate oxidation	1.38	0.028	5	15	33.3
Nutrient uptake	1.28	0.028	6	25	24.0
Vitellogenesis	1.04	0.039	5	28	17.9
K+ import/homeostasis	1.25	0.004	8	67	11.9
Hatching	1.12	0.044	21	95	22.1
Cell fusion	1.09	0.044	22	200	11.0
Granulocyte differentiation	-1.04	0.045	10	57	17.5
B lymphocyte proliferation	-1.09	0.046	30	227	10.8
Diastolic function	-1.11	0.047	9	58	15.5

Appendix B

Supplemental information for Chapter 3

Table S3.1 Unique subnetwork enrichment analysis (SNEA) cell processes in rainbow darter from the Waterloo MWWTP exposed site across years, from 2011 to 2014. Presented are cell process ID, median change of the process, and its significance.

SNEA cell process	Median change	p-value
DSW 2011		
Cardiomyocyte death	-1.33	0.001
Systolic function	-1.59	0.003
Endothelialization	-1.68	0.007
Membrane polarization	-1.8	0.01
Cell aging	-1.19	0.012
Epithelial cell adhesion	-1.51	0.013
Protein kinase cascade	-1.8	0.018
Recombinational repair	-1.46	0.019
Kidney function	1.1	0.03
Necrotic cell death	1.06	0.031
Chloride ion homeostasis	1.15	0.032
Neuron apoptoses	1.06	0.032
Centrosome separation	-1.39	0.034
Gastric acid secretion	-1.34	0.034
Intrinsic pathway of apoptosis	-1.1	0.035
Response to heat shock	-1.38	0.038
Cell-matrix adhesion	-1.2	0.039
Mitochondrion organization and biogenesis	-1.25	0.039
Platelet activation	-1.22	0.04
Error-prone post-replication DNA repair	-1.46	0.041
Microglial activation	-1.08	0.042
Blood-testicular barrier	-1.38	0.044
DNA double-strand break formation	-1.35	0.046
Synapsis	-1.43	0.048
Site selection	-1.47	0.049

Table S3.1 continuous

Centriole duplication	-1.29	0.049
DNA metabolism	-1.25	0.049
DSW 2012		
Establishment of T-cell polarity	1.21	0.004
Eye movement	1.23	0.004
Heart contraction	1.09	0.007
Lymphocyte adhesion	-1.7	0.01
Non-selective vesicle targeting	-1.35	0.011
Hemostasis	-1.26	0.016
Exit from mitosis	1.59	0.018
Fatty acids import	-1.14	0.021
Response to ethanol	-1.4	0.022
Action potential duration	-1.34	0.022
Migrating myoelectric complexe	1.57	0.022
Triacylglycerols biosynthesis	-1.32	0.025
Ketone biosynthesis	-1.34	0.025
Vesicle docking	-1.24	0.028
Cholesterol export	-1.16	0.028
Glycogen degradation	-1.17	0.032
Natural killer cell proliferation	1.4	0.033
Thermogenesis	-1.17	0.033
Contraction	-1.09	0.034
Muscle strength	1.29	0.034
Cholesterol metabolism	-1.04	0.034
Epidermal proliferation	-1.14	0.035
Granulocyte function	-1.28	0.035
Degranulation	1.06	0.036
Th1 immune response	1.17	0.036
Bile acid secretion	1.15	0.039
Adherens junction assembly	-1.14	0.042
Blood vessel permeability	-1.07	0.044

Table S3.1

Intestinal absorption	1.04	0.044
Oral tolerance	-1.73	0.047
Contractile activity	-1.11	0.047
DSW 2013		
mRNA degradation	1.38	0.002
mRNA processing	1.3	0.008
Pigment biosynthesis	-1.72	0.013
Heat-shock response	1.48	0.013
mRNA metabolism	1.56	0.013
Neurotransmitter uptake	-1.1	0.016
Lipid peroxidation	-1.17	0.018
Kidney filtration	1.21	0.019
Sperm cell adhesion	0	0.022
RNA elongation	1.39	0.022
Nucleocytoplasmic transport	1.32	0.026
mRNA stabilization	1.3	0.028
Cation transport	-1.35	0.028
Heart relaxation	-1.49	0.035
Poly(A) + mRNA -nucleus export	1.71	0.035
Protein folding	1.25	0.039
Glia proliferation	-1.52	0.041
Translation initiation	1.27	0.047
DSW 2014		
T-cell tolerance	1.36	0.002
Neutrophil function	1.26	0.004
Autolysis	1.53	0.006
Myoblast proliferation	1.24	0.021
Meiosis	-1.16	0.025
Tolerance induction	1.36	0.025
Genetic instability	-1.27	0.028
Hatching	1.3	0.031

Table S3.1 continuous

Cellular extravasation	-1.2	0.036
Protein-protein cross-linking via L-cystine	1.21	0.032
Capillary permeability	1.19	0.037
RBC maturation	1.41	0.041
Leukocyte accumulation	1.06	0.041
Ovary function	1.14	0.046
Microsatellite instability	-1.2	0.046
Growth pattern	1.36	0.049
T-cell suppression	1.28	0.049

Table S3.2 Unique subnetwork enrichment analysis (SNEA) cell processes in rainbow darter from the Kitchener MWWTP exposed site across years, from 2011 to 2014. Presented are cell process ID, median change of the process, and its significance.

SNEA cell process	Median change	p-value
DSK1 2011		
Centrosome separation	1.36	0.004
Adherens junction assembly	-1.24	0.005
DNA replication checkpoint	1.53	0.006
Cell aging	-1.15	0.01
Vasodilation	-1.17	0.01
S-G2 transition	-1.05	0.011
G2/M transition	1.02	0.011
Genetic instability	1.2	0.015
Platelet adhesion	-1.17	0.016
Muscle relaxation	-1.02	0.016
Mitosis	1	0.024
Interphase	1.15	0.024
Lung development	1.08	0.025
Mitochondrial damage	-1.05	0.027
Engraftment	-1.17	0.029
DNA metabolism	-1.17	0.029
Fatty acid metabolism	-1.07	0.03
Intracellular signaling cascade	-1.17	0.03
Glial cell adhesion	-1.24	0.033
Meiosis	1.08	0.034
Calcium ion homeostasis	-1.09	0.035
Respiratory gaseous exchange	-1.22	0.036
Epithelial cell adhesion	-1.21	0.037
Chromosome condensation	1.1	0.041
Genome stability	1.17	0.043
M phase	1.13	0.045
Heart relaxation	-1.17	0.046

Table S3.2 continuous

Cilium biogenesis	-1.16	0.046
Contractile activity	-1.16	0.048
Vein blood flow	-1.19	0.049
Mitotic checkpoint	-1.27	0.049
DSK1 2012		
Spermatid development	1.3	0.003
Mast cell apoptoses	-1.38	0.005
Eye movement	1.2	0.009
Cross priming	-1.28	0.01
Germinal center formation	-1.6	0.013
T-cell function	-1.1	0.018
Chemosensitivity	1.15	0.027
T-cell homeostases	1.2	0.031
Lymphocyte activation	1.15	0.036
Necrotic cell death	1.2	0.037
Spindle assembly	-1.1	0.037
Triacylglycerols biosynthesis	1.17	0.038
Glia proliferation	1.35	0.042
B cell function	-1.3	0.042
Strand invasion	-1.39	0.044
Th1 immune response	-1.3	0.044
Platelet activation	1.04	0.049
Formation of immunological synapse	-1.48	0.049
Nuclear division	1.32	0.049
DSK1 2013		
Keratinocyte differentiation	1.23	0.001
Establishment of cell polarity	1.11	0.002
Kidney function	1.1	0.003
Complement activation	-1.34	0.005
Cell recognition	-1.34	0.007

Table S3.2 continuous

Cytolysis	-1.1	0.009
Cell motility	-1.04	0.008
Growth pattern	1.25	0.01
Lamellipodium formation	-175	0.011
Muscle metabolism	-1.3	0.012
Diastolic function	1.62	0.013
Actin filament depolymerization	-1.08	0.013
Osteoclast differentiation	1.09	0.015
Fibrinolysis	-1.07	0.017
Anagen	1.18	0.017
Blastocyst development	-1.33	0.018
Glial cell differentiation	1.26	0.018
Myocyte proliferation	-1.32	0.02
Mitochondrial Ca2+ transport	-1.34	0.02
Myogenesis	-1.02	0.021
Host invasion	-1.49	0.022
Muscle function	-1.24	0.024
Lymphocyte chemotaxis	-1.49	0.025
Dormancy	-1.48	0.03
Monocyte response	-1.42	0.035
Tissue invasion	-1.24	0.036
Cytoskeleton organization and biogenesis	-1.1	0.036
T-cell tolerance	-1.29	0.037
Eosinophil degranulation	-1.02	0.037
Endothelial cell proliferation	-1.1	0.041
Neurulation	-1.19	0.045
Invasive growth	1.19	0.048
ECM degradation	-1.07	0.05
DSK1 2014		

Table S3.2 continuous

Macrophage chemotaxis	1.19	0.005
Receptor clustering	1.66	0.002
Transmembrane signaling	1.66	0.004
Nutrient uptake	1.55	0.003
Membrane ruffling	1.38	0.006
Granulocyte function	-1.27	0.011
Myoblast proliferation	1.35	0.011
Apoptosis of neutrophils	1.08	0.015
Macroautophagy	1.78	0.018
Cholesterol metabolism	-1.21	0.019
mRNA metabolism	-1.5	0.021
Mitochondrial translocation	-1.28	0.029
K+ import/homeostasis	1.52	0.03
Acid secretion	-1.04	0.031
Sex maturation	1.55	0.032
Immune system activation	1.2	0.032
Tropism	-1.27	0.033
Intestinal absorption	-1.18	0.034
Nucleocytoplasmic transport	1.45	0.036
Cation transport	1.52	0.036
Artery blood flow	-1.04	0.037
Fatty acids import	-1.22	0.043
Phosphate import	-125	0.044
Nervous system physiology	1.55	0.046
Bile acid secretion	-1.22	0.047
Osteoclast function	1.26	0.049

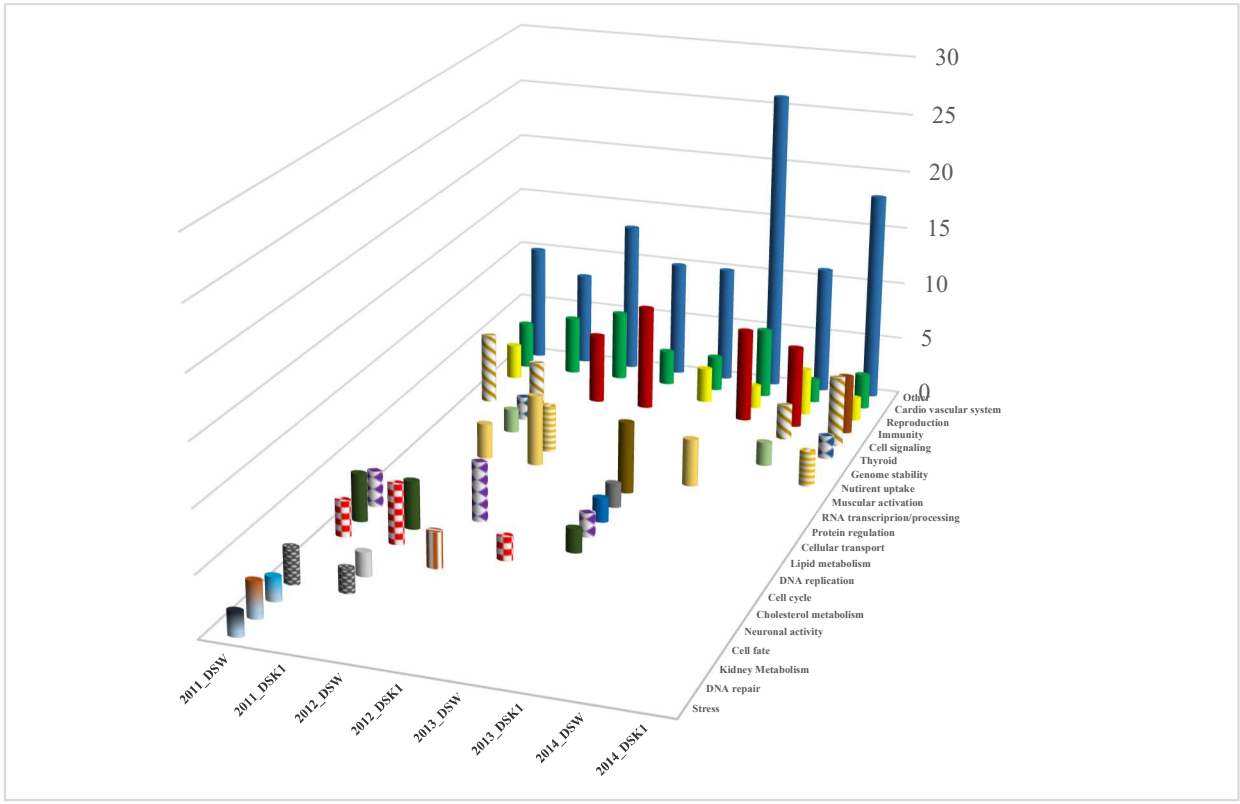


Figure S3.1 Subnetwork enrichment analysis (SNEA) cell process pathways organized in 20 categories in rainbow darter males collected downstream of the Waterloo MWWTP (DSW) and the Kitchener MWWTP (DSK1) in 2011, 2012, 2013 and 2014.

Table S3.3 Entities involved in pathway analysis of differently expressed cell processes presented in Figure 3.6 in rainbow darter males collected below the Kitchener MWWTP in 2011.

Name	Type	Description	Connect-ivity	Probe Value	Local Connect-ivity	In-degree	Out-degree
MAPK14	Protein	mitogen-activated protein kinase 14	3054	1.1143	1	0	1
AGT	Protein	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	2928	-1.168	1	0	1
GH1	Protein	growth hormone 1	2138	-1.2635	1	0	1
INS	Protein	insulin	5379	1.3982	1	0	1
FAS	Protein	Fas (TNF receptor superfamily, member 6)	2047	-1.4641	3	0	3
SOD1	Protein	superoxide dismutase 1, soluble	822	-1.3073	2	0	2
RB1	Protein	retinoblastoma 1	1319	3.3197	3	0	3
CASP3	Protein	caspase 3, apoptosis-related cysteine peptidase	2558	-1.1512	1	0	1
Genome stability	Cell Process		334		37	37	0
CDC42	Protein	cell division cycle 42 (GTP binding protein)	1319	-1.4956	1	0	1
MMP2	Protein	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1700	-1.169	2	0	2
CDK1	Protein	cell division cycle 2, G1 to S and G2 to M	1224	2.1241	3	0	3
HSPB1	Protein	heat shock 27kDa protein 1	789	-1.2237	1	0	1
CDK5	Protein	cyclin-dependent kinase 5	651	-1.2078	1	0	1
SERPINE1	Protein	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1256	-1.2868	1	0	1
GAPDH	Protein	glyceraldehyde-3-phosphate dehydrogenase	838	-1.5998	1	0	1
EIF4E	Protein	eukaryotic translation initiation factor 4E	546	-1.2097	1	0	1

Table S3.3 continuous

HMGB1	Protein	high mobility group box 1	872	-1.1676	2	0	2
TFAM	Protein	transcription factor A, mitochondrial	189	-1.2052	1	0	1
ID1	Protein	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	431	1.8042	2	0	2
PTTG1	Protein	pituitary tumor-transforming 1	300	1.272	2	0	2
Cell aging	Cell Process		315		40	40	0
TIMP1	Protein	TIMP metallopeptidase inhibitor 1	892	-1.5741	1	0	1
G6PD	Protein	glucose-6-phosphate dehydrogenase	431	-1.238	1	0	1
F2RL1	Protein	coagulation factor II (thrombin) receptor-like 1	759	2.8552	1	0	1
NPM1	Protein	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	434	-1.247	2	0	2
ING4	Protein	inhibitor of growth family, member 4	96	1.276	1	0	1
CEBPD	Protein	CCAAT/enhancer binding protein (C/EBP), delta	413	1.2302	1	0	1
NCL	Protein	nucleolin	388	-1.2115	1	0	1
PRSS1	Protein	protease, serine, 1 (trypsin 1)	68	2.6223	1	0	1
BIRC5	Protein	baculoviral IAP repeat containing 5	754	1.8422	3	0	3
KRAS	Protein	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	715	-1.5665	2	0	2
PIN1	Protein	peptidylprolyl cis/trans isomerase, NIMA-interacting 1	431	1.3172	3	0	3
Genetic instability	Cell Process		146		17	17	0
PCNA	Protein	proliferating cell nuclear antigen	870	1.2974	4	0	4
OSGEP	Protein	O-sialoglycoprotein endopeptidase	71	1.6112	3	0	3
HDAC1	Protein	histone deacetylase 1	890	1.1374	1	0	1
MBL2	Protein	mannose-binding lectin (protein C) 2, soluble	387	1.0199	1	0	1

Table S3.3 continuous

CDKN2B	Protein	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	205	1.519	2	0	2
SPARC	Protein	secreted protein, acidic, cysteine-rich (osteonectin)	510	1.89	1	0	1
DNMT1	Protein	DNA (cytosine-5-)-methyltransferase 1	446	1.2951	1	0	1
TERF1	Protein	telomeric repeat binding factor (NIMA-interacting) 1	147	-1.0529	2	0	2
Autolysis	Cell Process		92		11	11	0
PRDX1	Protein	peroxiredoxin 1	187	1.1343	1	0	1
HMMR	Protein	hyaluronan-mediated motility receptor (RHAMM)	122	1.5243	1	0	1
GPI	Protein	glucose-6-phosphate isomerase	238	1.1102	1	0	1
FTH1	Protein	ferritin, heavy polypeptide 1	168	-1.0421	1	0	1
RAD51	Protein	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	460	-1.2479	4	0	4
PRDX5	Protein	peroxiredoxin 5	96	1.2003	1	0	1
H2AFX	Protein	H2A histone family, member X	406	1.0597	1	0	1
HMGA1	Protein	high mobility group AT-hook 1	346	2.1269	1	0	1
RAP1A	Protein	RAP1A, member of RAS oncogene family	627	-1.2006	1	0	1
PHB	Protein	prohibitin	211	-1.2457	1	0	1
VRK1	Protein	vaccinia related kinase 1	65	-1.4248	1	0	1
SUMO2	Protein	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae)	156	-1.1505	1	0	1
GSTT1	Protein	glutathione S-transferase theta 1	100	1.1422	1	0	1
POLB	Protein	polymerase (DNA directed), beta	206	1.0774	2	0	2
FANCA	Protein	Fanconi anemia, complementation group A	99	1.2	1	0	1

Table S3.3 continuous

GMNN	Protein	geminin, DNA replication inhibitor	198	-1.245	1	0	1
SBDS	Protein	Shwachman-Bodian-Diamond syndrome	54	-1.5704	1	0	1
ING1	Protein	inhibitor of growth family, member 1	137	-1.0675	1	0	1
FHIT	Protein	fragile histidine triad gene	190	1.1859	1	0	1
SUMO3	Protein	SMT3 suppressor of mif two 3 homolog 3 (S. cerevisiae)	135	1.1005	1	0	1
RAD18	Protein	RAD18 homolog (S. cerevisiae)	88	1.4498	1	0	1
HMGA2	Protein	high mobility group AT-hook 2	227	-1.3398	1	0	1
RHEB	Protein	Ras homolog enriched in brain	145	-1.4681	1	0	1
TSG101	Protein	tumor susceptibility gene 101	155	2.0883	1	0	1
DNA replication checkpoint	Cell Process		65		6	6	0
DDB1	Protein	damage-specific DNA binding protein 1, 127kDa	147	-1.6698	2	0	2
MORF4	Protein	mortality factor 4	16	1.3234	1	0	1
FEN1	Protein	flap structure-specific endonuclease 1	168	1.2326	3	0	3
RAD51L1	Protein	RAD51-like 1 (S. cerevisiae)	42	-1.2914	1	0	1
RPL23A	Protein	ribosomal protein L23a	164	1.1471	1	0	1
XRCC4	Protein	X-ray repair complementing defective repair in Chinese hamster cells 4	80	-1.0515	1	0	1
RUVBL2	Protein	RuvB-like 2 (E. coli)	105	1.4123	1	0	1
DNA metabolism	Cell Process		95		11	11	0
XRCC2	Protein	X-ray repair complementing defective repair in Chinese hamster cells 2	50	-1.2479	1	0	1
DUT	Protein	deoxyuridine triphosphatase	83	1.3292	1	0	1

Table S3.3 continuous

NEK1	Protein	NIMA (never in mitosis gene a)-related kinase 1	40	1.6973	1	0	1
FIS1	Protein	fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)	54	-1.243	1	0	1
BCCIP	Protein	BRCA2 and CDKN1A interacting protein	32	1.1639	1	0	1
SHFM1	Protein	split hand/foot malformation (ectrodactyly) type 1	40	-1.1287	1	0	1
YEATS4	Protein	YEATS domain containing 4	48	1.3081	1	0	1
CCBL1	Protein	cysteine conjugate-beta lyase, cytoplasmic	55	1.6973	1	0	1
PDXK	Protein	pyridoxal (pyridoxine, vitamin B6) kinase	27	1.1741	1	0	1
SNF8	Protein	SNF8, ESCRT-II complex subunit, homolog (S. cerevisiae)	17	1.2271	1	0	1
MORF4L1	Protein	mortality factor 4 like 1	47	1.2469	1	0	1
UFD1L	Protein	ubiquitin fusion degradation 1 like (yeast)	53	-1.0552	1	0	1
PGD	Protein	phosphogluconate dehydrogenase	76	1.5002	1	0	1
MTERF	Protein	mitochondrial transcription termination factor	17	1.4317	1	0	1
SPIN1	Protein	spindlin 1	15	-1.3536	1	0	1
RNF4	Protein	ring finger protein 4	41	1.2521	1	0	1
UCK2	Protein	uridine-cytidine kinase 2	29	-1.2581	1	0	1
MORC3	Protein	MORC family CW-type zinc finger 3	24	-1.3146	1	0	1
SPC24	Protein	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	22	1.527	1	0	1
KATNB1	Protein	katanin p80 (WD repeat containing) subunit B 1	16	1.6973	1	0	1

Table S3.3 continuous

MCAT	Protein	malonyl CoA:ACP acyltransferase (mitochondrial)	5	1.1987	1	0	1

Table S3.4 Entities involved in pathway analysis of differently expressed cell processes presented in Figure 3.7 in rainbow darter males collected below the Kitchener MWWTP in 2013.

Name	Type	Description	Connect-ivity	Probe Value	Local Connect-ivity	In-degree	Out-degree
MAPK14	Protein	mitogen-activated protein kinase 14	3054	1.1143	3	0	3
AGT	Protein	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	2928	-1.168	3	0	3
GH1	Protein	growth hormone 1	2138	-1.2635	4	0	4
MAPK1	Protein	mitogen-activated protein kinase 1	4849	1.0688	2	0	2
INS	Protein	insulin	5379	1.3982	5	0	5
EPO	Protein	erythropoietin	1590	-1.5741	2	0	2
TNFRSF1B	Protein	tumor necrosis factor receptor superfamily, member 1B	416	-1.218	2	0	2
FAS	Protein	Fas (TNF receptor superfamily, member 6)	2047	-1.4641	7	0	7
NPY	Protein	neuropeptide Y	1092	-1.418	1	0	1
ANXA5	Protein	annexin A5	503	1.3541	1	0	1
CASP3	Protein	caspase 3, apoptosis-related cysteine peptidase	2558	-1.1512	3	0	3
NEU1	Protein	sialidase 1 (lysosomal sialidase)	630	1.0621	3	0	3
RHOA	Protein	ras homolog gene family, member A	1809	-1.5042	3	0	3
CDC42	Protein	cell division cycle 42 (GTP binding protein)	1319	-1.4956	3	0	3
MMP2	Protein	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	1700	-1.169	1	0	1
Th1 immune response	Cell Process		89		6	6	0
HSPB1	Protein	heat shock 27kDa protein 1	789	-1.2237	1	0	1
LEP	Protein	leptin	2189	1.2168	5	0	5
CDK5	Protein	cyclin-dependent kinase 5	651	-1.2078	1	0	1
KCNE1	Protein	potassium voltage-gated channel, Isk-related family, member 1	135	-1.0602	1	0	1
SERPINE1	Protein	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1256	-1.2868	1	0	1
GAPDH	Protein	glyceraldehyde-3-phosphate dehydrogenase	838	-1.5998	1	0	1

Table S3.4 continuous

GNAQ	Protein	guanine nucleotide binding protein (G protein), q polypeptide	830	1.2605	1	0	1
NOTCH1	Protein	notch 1	1644	1.081	1	0	1
GNAI2	Protein	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2	312	1.2905	1	0	1
LEPR	Protein	leptin receptor	379	-1.0901	1	0	1
CCL4	Protein	chemokine (C-C motif) ligand 4	555	-1.1903	2	0	2
HSP90B1	Protein	heat shock protein 90kDa beta (Grp94), member 1	408	-1.0803	1	0	1
F2RL1	Protein	coagulation factor II (thrombin) receptor-like 1	759	2.8552	1	0	1
BNIP3	Protein	BCL2/adenovirus E1B 19kDa interacting protein 3	207	1.2027	1	0	1
AIF1	Protein	allograft inflammatory factor 1	155	1.0308	1	0	1
Lymphocyte activation	Cell Process		382		23	23	0
Platelet activation	Cell Process		388		32	32	0
PPID	Protein	peptidylprolyl isomerase D	160	1.2461	1	0	1
ID2	Protein	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	252	-1.1497	1	0	1
TNNI3	Protein	troponin I type 3 (cardiac)	351	-2.2777	1	0	1
MCL1	Protein	myeloid cell leukemia sequence 1 (BCL2-related)	586	1.0969	2	0	2
PPARA	Protein	peroxisome proliferator-activated receptor alpha	1361	-1.0326	2	0	2
MIF	Protein	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	894	1.2328	2	0	2
SCD	Protein	stearoyl-CoA desaturase (delta-9-desaturase)	364	-1.1708	1	0	1
SPI1	Protein	spleen focus forming virus (SFFV) proviral integration oncogene spi1	672	-1.1377	1	0	1
KIT	Protein	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	710	-1.1763	1	0	1
HPRT1	Protein	hypoxanthine phosphoribosyltransferase 1	113	2.2819	1	0	1
SAA1	Protein	serum amyloid A1	394	-1.335	1	0	1

Table S3.4 continuous

IL10	Protein	interleukin 10	2485	-1.0668	7	0	7
STIM1	Protein	stromal interaction molecule 1	164	-1.247	3	0	3
T-cell function	Cell Process		546		35	35	0
Formation of immunological synapses	Cell Process		72		5	5	0
CNR1	Protein	cannabinoid receptor 1 (brain)	758	1.0013	1	0	1
SLC3A2	Protein	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	209	-1.2052	1	0	1
Neutrophil function	Cell Process		158		14	14	0
MYD88	Protein	myeloid differentiation primary response gene (88)	673	-1.2401	3	0	3
T-cell homeostases	Cell Process		123		12	12	0
F11R	Protein	F11 receptor	254	1.6973	1	0	1
FADD	Protein	Fas (TNFRSF6)-associated via death domain	397	-1.0743	1	0	1
S100B	Protein	S100 calcium binding protein B	408	-1.6301	1	0	1
OSGEP	Protein	O-sialoglycoprotein endopeptidase	71	1.6112	1	0	1
LGALS3	Protein	lectin, galactoside-binding, soluble, 3	658	1.2217	6	0	6
MBL2	Protein	mannose-binding lectin (protein C) 2, soluble	387	1.0199	1	0	1
USF2	Protein	upstream transcription factor 2, c-fos interacting	202	-1.1347	1	0	1
SNAP23	Protein	synaptosomal-associated protein, 23kDa	167	1.2039	1	0	1
APOH	Protein	apolipoprotein H (beta-2-glycoprotein I)	261	-1.4444	1	0	1
SPARC	Protein	secreted protein, acidic, cysteine-rich (osteonectin)	510	1.89	2	0	2
SLPI	Protein	secretory leukocyte peptidase inhibitor	302	1.2908	3	0	3
CD59	Protein	CD59 molecule, complement regulatory protein	255	-1.1615	1	0	1
B cell function	Cell Process		167		11	11	0
BTK	Protein	Bruton agammaglobulinemia tyrosine kinase	415	-1.1121	3	0	3

Table S3.4 continuous

CFH	Protein	complement factor H	237	1.1539	1	0	1
CD9	Protein	CD9 molecule	392	1.5057	1	0	1
HMGA1	Protein	high mobility group AT-hook 1	346	2.1269	1	0	1
RAP1A	Protein	RAP1A, member of RAS oncogene family	627	-1.2006	4	0	4
B2M	Protein	beta-2-microglobulin	501	-1.2093	1	0	1
TPT1	Protein	tumor protein, translationally-controlled 1	199	1.8478	2	0	2
MBP	Protein	myelin basic protein	740	1.0199	1	0	1
CD48	Protein	CD48 molecule	131	-1.3325	2	0	2
MAL	Protein	mal, T-cell differentiation protein	102	-1.1915	1	0	1
HSP90AB1	Protein	heat shock protein 90kDa alpha (cytosolic), class B member 1	151	-1.1557	1	0	1
DAPP1	Protein	dual adaptor of phosphotyrosine and 3-phosphoinositides	65	-1.161	1	0	1
MALT1	Protein	mucosa associated lymphoid tissue lymphoma translocation gene 1	141	1.1938	1	0	1
C1QBP	Protein	complement component 1, q subcomponent binding protein	139	-1.1045	1	0	1
RAP1B	Protein	RAP1B, member of RAS oncogene family	209	-1.1504	1	0	1
Mast cell apoptoses	Cell Process		39		6	6	0
SLA	Protein	Src-like-adaptor	75	1.0971	2	0	2
PIP5K1B	Protein	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	30	-1.1613	1	0	1
HAX1	Protein	HCLS1 associated protein X-1	104	1.1533	1	0	1
FABP7	Protein	fatty acid binding protein 7, brain	78	1.877	1	0	1
ST3GAL1	Protein	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	46	1.1308	2	0	2
UNC119	Protein	unc-119 homolog (C. elegans)	54	-1.0687	1	0	1
RGS18	Protein	regulator of G-protein signaling 18	28	1.0582	1	0	1
LGALS2	Protein	lectin, galactoside-binding, soluble, 2	32	1.2217	1	0	1

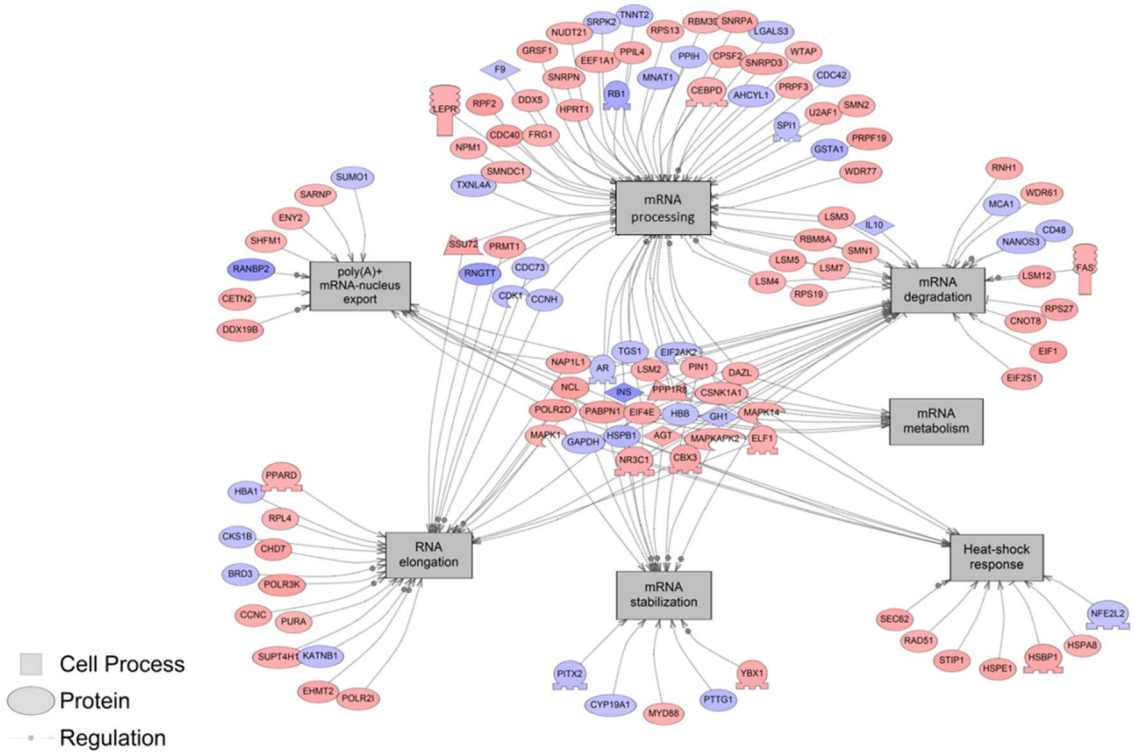


Figure S3.2 Pathway analysis of cell processes differently regulated by the Waterloo MWWTP effluent in 2013. The majority of transcripts were associated with RNA and mRNA processing, and were up-regulated. Heat-shock response was also increased at the transcriptome level in fish from this particular site in 2013. Transcripts colored in red are upregulated whereas those in violet are downregulated. Details on specific entities involved in the presented pathways can be found in the table below (Table S3.3).

Table S3.5 Entities involved in pathway analysis of differently expressed cell processes displayed in the diagram above (Figure S3.2) in rainbow darter males collected below the Waterloo MWWTP in 2013.

Name	Type	Description	Connectivity	Probe Value	Local Connect-ivity	In-degree	Out-degree
EIF2S1	Protein	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	386	-1.1873	1	0	1
MAPK14	Protein	mitogen-activated protein kinase 14	3054	-1.1115	2	0	2
Heat-shock response	Cell Process		108		14	14	0
AGT	Protein	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	2928	1.2405	2	0	2
GH1	Protein	growth hormone 1	2138	-1.299	2	0	2
MAPK1	Protein	mitogen-activated protein kinase 1	4849	1.0391	2	0	2
INS	Protein	insulin	5379	2.1587	4	0	4
FAS	Protein	Fas (TNF receptor superfamily, member 6)	2047	-1.6701	1	0	1
RB1	Protein	retinoblastoma 1	1319	1.1097	1	0	1
CDC42	Protein	cell division cycle 42 (GTP binding protein)	1319	-1.4779	1	0	1
mRNA stabilization	Cell Process		122		15	15	0
CDK1	Protein	cell division cycle 2, G1 to S and G2 to M	1224	-1.4688	2	0	2
HSPB1	Protein	heat shock 27kDa protein 1	789	1.5235	3	0	3
TNNT2	Protein	troponin T type 2 (cardiac)	275	1.6155	1	0	1
GAPDH	Protein	glyceraldehyde-3-phosphate dehydrogenase	838	1.4033	2	0	2
HSPA8	Protein	heat shock 70kDa protein 8	597	-1.0763	1	0	1
EIF4E	Protein	eukaryotic translation initiation factor 4E	546	1.2983	5	0	5
mRNA degradation	Cell Process		255		34	34	0
PTTG1	Protein	pituitary tumor-transforming 1	300	1.6316	1	0	1
LEPR	Protein	leptin receptor	379	1.2963	1	0	1
NFE2L2	Protein	nuclear factor (erythroid-derived 2)-like 2	828	-1.1417	1	0	1
NPM1	Protein	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	434	-1.1065	1	0	1

Table S3.5 continuous

PPARD	Protein	peroxisome proliferator-activated receptor delta	761	1.1098	1	0	1
SUMO1	Protein	SMT suppressor of mif two 3 homolog 1 (S. cerevisiae)	471	1.3003	1	0	1
SPI1	Protein	spleen focus forming virus (SFFV) proviral integration oncogene spi1	672	1.0901	1	0	1
NR3C1	Protein	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	1569	1.1626	2	0	2
HSPE1	Protein	heat shock 10kDa protein 1 (chaperonin 10)	183	1.2284	1	0	1
EIF2AK2	Protein	eukaryotic translation initiation factor 2-alpha kinase 2	545	1.3743	3	0	3
AR	Protein	androgen receptor	1742	1.0554	3	0	3
mRNA processing	Cell Process		516	-2.5164	63	63	0
HPRT1	Protein	hypoxanthine phosphoribosyltransferase 1	113	-3.6773	1	0	1
IL10	Protein	interleukin 10	2485	-1.9054	2	0	2
CEBPD	Protein	CCAAT/enhancer binding protein (C/EBP), delta	413	1.1134	1	0	1
NCL	Protein	nucleolin	388	-1.735	4	0	4
CYP19A1	Protein	cytochrome P450, family 19, subfamily A, polypeptide 1	675	1.0554	1	0	1
MYD88	Protein	myeloid differentiation primary response gene (88)	673	-1.8009	1	0	1
MAPKAPK 2	Protein	mitogen-activated protein kinase-activated protein kinase 2	386	1.042	2	0	2
HBB	Protein	hemoglobin, beta	373	-1.6098	3	0	3
PIN1	Protein	peptidylprolyl cis/trans isomerase, NIMA-interacting 1	431	1.2767	3	0	3
mRNA metabolism	Cell Process		56		7	7	0
LGALS3	Protein	lectin, galactoside-binding, soluble, 3	658	1.9294	1	0	1
STIP1	Protein	stress-induced-phosphoprotein 1	130	-1.1019	1	0	1
GSTA1	Protein	glutathione S-transferase alpha 1	118	-1.209	1	0	1
F9	Protein	coagulation factor IX	229	1.1656	1	0	1

Table S3.5 continuous

poly(A)+ mRNA-nucleus export	Cell Process		116		14	14	0
RAD51	Protein	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	460	-1.8862	1	0	1
RNA elongation	Cell Process		239		28	28	0
EHMT2	Protein	euchromatic histone-lysine N-methyltransferase 2	161	-1.9493	1	0	1
EEF1A1	Protein	eukaryotic translation elongation factor 1 alpha 1	373	1.2795	1	0	1
YBX1	Protein	Y box binding protein 1	234	1.2795	1	0	1
NANOS3	Protein	nanos homolog 3 (Drosophila)	12	1.2639	1	0	1
CCNH	Protein	cyclin H	85	-1.7118	2	0	2
PRPF19	Protein	PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae)	55	1.2886	1	0	1
CD48	Protein	CD48 molecule	131	1.4979	1	0	1
DAZL	Protein	deleted in azoospermia-like	57	1.5372	2	0	2
PRMT1	Protein	protein arginine methyltransferase 1	238	-1.1982	2	0	2
CKS1B	Protein	CDC28 protein kinase regulatory subunit 1B	109	1.5728	1	0	1
SNRPN	Protein	small nuclear ribonucleoprotein polypeptide N	41	-1.76	1	0	1
CHD7	Protein	chromodomain helicase DNA binding protein 7	59	-1.7534	1	0	1
RPS19	Protein	ribosomal protein S19	72	-1.017	2	0	2
NAP1L1	Protein	nucleosome assembly protein 1-like 1	89	1.2757	2	0	2
PITX2	Protein	paired-like homeodomain 2	260	1.1586	1	0	1
ELF1	Protein	E74-like factor 1 (ets domain transcription factor)	113	1.0365	2	0	2
HBA1	Protein	hemoglobin, alpha 1	167	-1.333	1	0	1
CCNC	Protein	cyclin C	80	1.7858	1	0	1
WTAP	Protein	Wilms tumor 1 associated protein	29	1.5702	1	0	1
CETN2	Protein	centrin, EF-hand protein, 2	36	1.4671	1	0	1

Table S3.5 continuous

CBX3	Protein	chromobox homolog 3	198	-1.1896	2	0	2
PPP1R8	Protein	protein phosphatase 1, regulatory (inhibitor) subunit 8	118	-1.1401	3	0	3
SRPK2	Protein	SRSF protein kinase 2	43	-1.7426	1	0	1
LSM7	Protein	LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)	21	-1.3274	2	0	2
AHCYL1	Protein	adenosylhomocysteinase-like 1	40	1.3703	1	0	1
MNAT1	Protein	menage a trois homolog 1, cyclin H assembly factor (Xenopus laevis)	78	1.4446	1	0	1
DDX5	Protein	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	66	-1.0572	1	0	1
CSNK1A1	Protein	casein kinase 1, alpha 1	123	1.038	2	0	2
SHFM1	Protein	split hand/foot malformation (ectrodactyly) type 1	40	1.0914	1	0	1
ENY2	Protein	enhancer of yellow 2 homolog (Drosophila)	10	-1.1356	1	0	1
POLR2D	Protein	polymerase (RNA) II (DNA directed) polypeptide D	42	1.0671	3	0	3
U2AF1	Protein	U2 small nuclear RNA auxiliary factor 1	44	1.059	1	0	1
PRPF3	Protein	PRP3 pre-mRNA processing factor 3 homolog (S. cerevisiae)	33	-1.1975	1	0	1
RPF2	Protein	ribosome production factor 2 homolog (S. cerevisiae)	11	-2.1396	1	0	1
WDR77	Protein	WD repeat domain 77	13	-1.5351	1	0	1
LSM2	Protein	LSM2 homolog, U6 small nuclear RNA associated (S. cerevisiae)	28	1.1393	3	0	3
SMN1	Protein	survival of motor neuron 1, telomeric	233	-1.76	2	0	2
SSU72	Protein	SSU72 RNA polymerase II CTD phosphatase homolog (S. cerevisiae)	33	-1.2749	2	0	2
SMN2	Protein	survival of motor neuron 2, centromeric	68	-1.76	1	0	1
DDX19B	Protein	DEAD (Asp-Glu-Ala-As) box polypeptide 19B	41	-1.4776	1	0	1
POLR2I	Protein	polymerase (RNA) II (DNA directed) polypeptide I, 14.5kDa	33	-1.4207	1	0	1
RNH1	Protein	ribonuclease/angiogenin inhibitor 1	13	-1.3092	1	0	1
EIF1	Protein	eukaryotic translation initiation factor 1	69	-1.2938	1	0	1

Table S3.5 continuous

RBM39	Protein	RNA binding motif protein 39	29	-1.1223	1	0	1
MCA1	Protein	DNA segment, MCA1, multiple CA repeat 1	3	-1.1284	1	0	1
GRSF1	Protein	G-rich RNA sequence binding factor 1	17	-1.816	1	0	1
SUPT4H1	Protein	suppressor of Ty 4 homolog 1 (S. cerevisiae)	16	1.6417	1	0	1
RNGTT	Protein	RNA guanylyltransferase and 5'-phosphatase	66	-1.2146	2	0	2
RANBP2	Protein	RAN binding protein 2	116	-1.2194	1	0	1
LSM5	Protein	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)	20	-1.1043	2	0	2
PURA	Protein	purine-rich element binding protein A	111	1.0253	1	0	1
LSM4	Protein	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)	21	1.3326	2	0	2
LSM3	Protein	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)	21	1.048	2	0	2
CDC73	Protein	cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	93	1.1283	2	0	2
HSBP1	Protein	heat shock factor binding protein 1	12	1.3021	1	0	1
PPIH	Protein	peptidylprolyl isomerase H (cyclophilin H)	21	-1.6997	1	0	1
SEC62	Protein	SEC62 homolog (S. cerevisiae)	24	1.3308	1	0	1
POLR3K	Protein	polymerase (RNA) III (DNA directed) polypeptide K, 12.3 kDa	8	-1.6417	1	0	1
TGS1	Protein	trimethylguanosine synthase 1	61	-1.3116	2	0	2
CDC40	Protein	cell division cycle 40 homolog (S. cerevisiae)	25	1.3834	1	0	1
PABPN1	Protein	poly(A) binding protein, nuclear 1	85	-2.5164	4	0	4
SNRPA	Protein	small nuclear ribonucleoprotein polypeptide A	42	-1.1862	1	0	1
TXNL4A	Protein	thioredoxin-like 4A	8	-1.7906	1	0	1
FRG1	Protein	FSHD region gene 1	17	-1.7661	1	0	1
SARNP	Protein	SAP domain containing ribonucleoprotein	13	-1.1223	1	0	1
CNOT8	Protein	CCR4-NOT transcription complex, subunit 8	28	1.0406	1	0	1

Table S3.5 continuous

RPS13	Protein	ribosomal protein S13	20	1.1948	1	0	1
SMNDC1	Protein	survival motor neuron domain containing 1	16	1.1351	1	0	1
RPL4	Protein	ribosomal protein L4	29	1.0882	1	0	1
RBM8A	Protein	RNA binding motif protein 8A	22	-1.3197	2	0	2
RPS27	Protein	ribosomal protein S27	14	-1.3489	1	0	1
KATNB1	Protein	katanin p80 (WD repeat containing) subunit B 1	16	1.5924	1	0	1
NUDT21	Protein	nudix (nucleoside diphosphate linked moiety X)-type motif 21	15	-1.7423	1	0	1
BRD3	Protein	bromodomain containing 3	13	-1.3886	1	0	1
CPSF2	Protein	cleavage and polyadenylation specific factor 2, 100kDa	15	-1.4083	1	0	1
WDR61	Protein	WD repeat domain 61	5	-1.5659	1	0	1
PPIL4	Protein	peptidylprolyl isomerase (cyclophilin)-like 4	2	-1.1032	1	0	1
SNRPD3	Protein	small nuclear ribonucleoprotein D3 polypeptide 18kDa	16	-1.3148	1	0	1
LSM12	Protein	LSM12 homolog (S. cerevisiae)	7	-1.0806	1	0	1

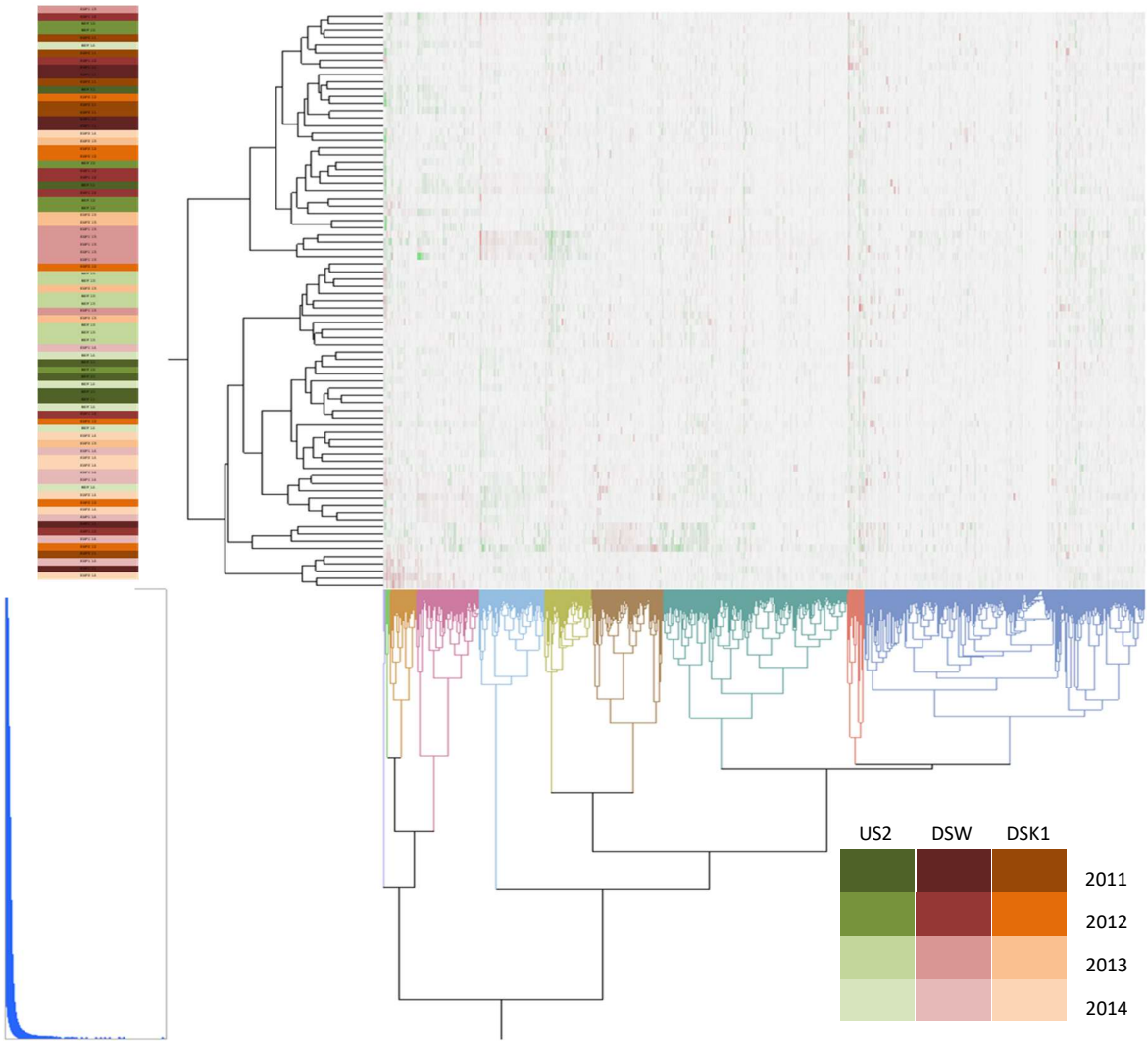


Figure S3.3 Hierarchical clustering of rainbow darter transcriptome data across years and sites. Samples did not clearly segregate over time (2011 - 2014), neither across sites (US2, DSW, DSK1). A tendency for reference sites and/or polluted sites to cluster more often, regardless of year, was noticed.

Appendix C
Supplemental information for Chapter 4

1. Rainbow darter morphometric characteristics

Figure S4.0 and Tables S4.1 to S4.3 are summarizing information regarding morphometric characteristics [length, weight, gonadal somatic index (GSI), liver somatic index (LSI), condition factor (k)] of rainbow darter used in the main study and the supporting studies assessing *in vitro* steroid production in the central Grand River. Data on GSI, LSI, and k were statistically analysed using one-way analysis of variance (ANOVA) with multiple comparison Holm-Sidak post-hoc test. There were no consistent patterns in any of the morphometric parameters measured that could have been associated with the urbanization effects in the central Grand River or patterns suggesting recovery following the upgrades. In addition, rainbow darter males from US2 collected from 2011 to 2016 were used to determine if there was a correlation between the GSI and steroid production (11-ketotestosterone). The correlation found was relatively week ($R^2 = 0.09$) (Figure S4.0).

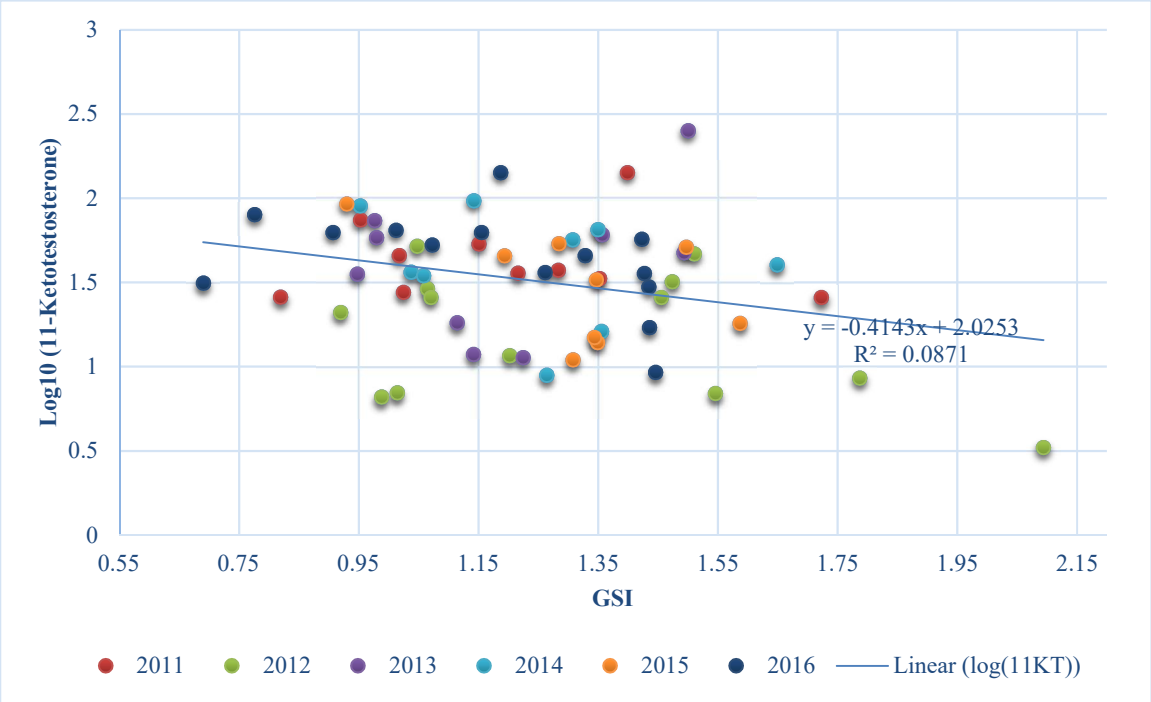


Figure S4.0 Linear regression between the gonadal somatic index (GSI) and 11-ketotestosterone concentration calculated using rainbow darter collections from US2 (reference site) over six years (2011 - 2016). The low value of the coefficient of determination suggests poor correlation between these two endpoints in rainbow darter males in the present study**.**

Table S4.1: Summary table for rainbow darter analysed to assess seasonal variability in *in vitro* androgen production at two reference sites (US2, US3) and a site below the Waterloo MWWTP outfall over approximately one year. Included is information regarding sample size analysed for 11-ketotestosterone (11KT) and testosterone (T) production, fish length (cm), weight (g), gonadal somatic index (GSI), liver somatic index (LSI) and condition factor (k). One-way analysis of variance with Holm Sidak post-hoc test was used to determine significant differences in GSI, LSI and k within each month. Presented are means ± standard deviation.

Time	Site	# of 11KT samples	# of T samples	Length (cm)	Weight (g)	GSI	LSI	k
October	US2	10	9	5.99 ± 0.62	2.78 ± 1.01	1.38 ± 0.25	2.18 ± 0.22[a]	1.24 ± 0.09
2015	US3	10	10	6.04 ± 0.41	2.86 ± 0.83	1.17 ± 0.19	1.72 ± 0.23[b]	1.23 ± 0.11
	DSW	10	10	6.26 ± 0.57	3.14 ± 0.98	1.26 ± 0.21	1.72 ± 0.24[b]	1.26 ± 0.08
November	US2	25	23	5.82 ± 0.52	2.55 ± 0.85	1.19 ± 0.24	1.80 ± 0.36	1.25 ± 0.11[a]
2015	US3	11	12	5.79 ± 0.36	2.59 ± 0.59	1.20 ± 0.18	1.97 ± 0.31	1.31 ± 0.09[ab]
	DSW	26	20	6.12 ± 0.48	3.06 ± 0.74	1.17 ± 0.24	1.87 ± 0.24	1.32 ± 0.06[b]
December	US2	14	13	5.66 ± 0.45	2.26 ± 0.74	1.17 ± 0.28	2.04 ±0.61	1.20 ± 0.09[a]
2015	US3	13	12	5.71 ± 0.38	2.53 ± 0.64	1.02 ± 0.27	2.18 ± 0.34	1.25 ± 0.10[ab]
	DSW	14	12	5.85 ± 0.53	2.58 ± 0.84	1.25 ± 0.24	2.06 ± 0.28	1.33 ± 0.09[b]
April	US2	12	16	5.42 ± 0.54	2.13 ± 1.06	1.65 ± 0.30[ab]	1.88 ± 0.29	1.29 ± 0.33
2016	US3	14	14	5.78 ± 0.42	2.64 ± 0.71	1.53 ± 0.25[a]	1.80 ± 0.30	1.33 ± 0.09
	DSW	15	14	5.87 ± 0.56	2.63 ± 0.77	1.84 ± 0.31[b]	2.03 ± 0.36	1.25 ± 0.10
May 2016	US2	18	13	5.85 ± 0.60	2.41 ± 0.88	1.07 ± 0.24	1.49 ± 0.24	1.15 ± 0.08
	US3	12	11	5.93 ± 0.61	2.40 ± 0.85	1.18 ± 0.26	1.49 ± 0.32	1.10 ± 0.11
	DSW	15	12	6.19 ± 0.65	2.86 ± 1.08	1.11 ± 0.17	1.63 ± 0.24	1.17 ± 0.06
Septemb.	US2	15	15	5.98 ± 0.31	2.50 ± 0.44	0.43 ± 0.22[a]	1.35 ± 0.17[b]	1.16 ± 0.08[ab]
2016	US3	11	11	5.61 ± 0.39	1.95 ± 0.47	0.31 ± 0.07[ab]	1.02 ± 0.29[a]	1.09 ± 0.06[a]
	DSW	15	12	6.26 ± 0.67	3.18 ± 0.95	0.23 ± 0.12[b]	1.26 ± 0.16[b]	1.25 ± 0.15[b]
October	US2	14	16	5.67 ± 0.61	2.34 ± 0.80	1.10 ± 0.33	1.56 ± 0.44	1.25 ± 0.08
2016	US3	15	13	5.62 ± 0.48	2.34 ± 0.59	1.05 ± 0.20	1.58 ± 0.20	1.30 ± 0.12
	DSW	13	15	5.52 ± 0.71	2.19 ± 1.15	1.08 ± 0.20	1.72 ± 0.16	1.21 ± 0.12

[a] and [b] indicate significant differences

Table S4.2 Summary table for rainbow darter analysed to assess the impact of sample handling on *in vitro* androgen production at a reference site (US2) and a polluted site below the Waterloo MWWTP outfall (DSW). Included is information regarding the time of sampling of fish subsets (1 h, 6 h and 24 h), number of samples analysed for each steroid (11-keto testosterone; 11KT, and testosterone; T), fish length (cm), weight (g), gonadal somatic index (GSI), liver somatic index (LSI), and condition factor (k). Two-way analysis of variance determined significant differences in k between 1 h and 6 h, and between US2 and DSW rainbow darter. Presented are means ± standard deviation.

Time	Site	# of 11KT samples	# of T samples	Length (cm)	Weight (g)	GSI	LSI	k
1 h	US2	25	26	5.82 ± 0.52	2.55 ± 0.85	1.19 ± 0.24	1.80 ± 0.36	1.25 ± 0.11
	DSW	26	23	6.12 ± 0.48	3.06 ± 0.74	1.17 ± 0.24	1.87 ± 0.18	1.32 ± 0.06
6 h	US2	24	23	5.53 ± 0.45	2.00 ± 0.52	1.18 ± 0.23	1.90 ± 0.25	1.16 ± 0.05
	DSW	20	20	6.09 ± 0.43	2.91 ± 0.75	1.21 ± 0.22	1.72 ± 0.38	1.26 ± 0.13
24 h	US2	23	23	5.72 ± 0.61	2.32 ± 0.89	1.17 ± 0.27	1.41 ± 0.31	1.19 ± 0.07
	DSW	23	23	6.30 ± 0.49	3.27 ± 0.95	1.12 ± 0.26	1.73 ± 0.35	1.27 ± 0.10

Table S4.3 Summary table for rainbow darter used to assess *in vitro* androgen production across an urban environment in the central Grand River over six years (2011 - 2016). Included are information regarding sample size analysed for 11-ketotestosterone (11KT) and testosterone (T) production, fish length (cm), weight (g), gonadal somatic index (GSI), liver somatic index (LSI), and condition factor (k). One-way analysis of variance with Holm Sidak post-hoc test was used to determine significant differences between fish from various sites within individual years. Presented are means ± standard deviation.

Site	# of 11KT samples	# of T samples	Length (cm)	Weight (g)	GSI	LSI	k
2011							
US2	11	10	5.56 ± 0.68	2.11 ± 0.91	1.19 ± 0.26^{bc}	1.74 ± 0.25^{b}	1.13 ± 0.08^{ab}
US3	13	9	6.43 ± 0.64	3.52 ± 1.32	1.05 ± 0.26^{bb}	1.38 ± 0.49^{c}	1.20 ± 0.07^{ab}
DSW	11	10	5.67 ± 0.92	2.48 ± 1.36	0.98 ± 0.27^{b}	2.19 ± 0.72^{ab}	1.22 ± 0.11^{a}
INT2	11	10	5.65 ± 0.81	2.14 ± 1.10	1.11 ± 0.13^{b}	1.44 ± 0.36^{c}	1.06 ± 0.11^{b}
DSK1	11	10	5.40 ± 0.74	1.96 ± 0.85	1.05 ± 0.21^{b}	1.71 ± 0.41^{b}	1.13 ± 0.11^{ab}
DSK2	11	10	5.83 ± 0.91	2.57 ± 1.55	1.47 ± 0.12^{ac}	2.15 ± 0.36^{ab}	1.13 ± 0.10^{ab}
DSK3	11	10	5.00 ± 0.84	1.64 ± 0.97	1.50 ± 0.25^{a}	2.64 ± 0.41^{a}	1.12 ± 0.11^{ab}
2012							
US1	7	9	6.12 ± 0.74	2.89 ± 1.11	1.15 ± 0.19^{ab}	1.78 ± 0.28	1.15 ± 0.14
US2	13	12	5.46 ± 0.77	2.12 ± 1.14	1.32 ± 0.36^{ab}	1.94 ± 0.27	1.16 ± 0.10
US3	8	8	5.24 ± 0.18	1.71 ± 0.29	1.10 ± 0.18^{ab}	1.79 ± 0.32	1.14 ± 0.12
DSW	12	12	6.23 ± 0.63	3.08 ± 1.03	1.16 ± 0.33^{ab}	2.08 ± 0.27	1.19 ± 0.07
INT2	6	5	5.00 ± 0.73	1.59 ± 0.72	1.21 ± 0.18^{ab}	1.81 ± 0.38	1.17 ± 0.06
DSK1	9	9	5.84 ± 0.85	2.64 ± 1.34	1.53 ± 0.32^{a}	1.74 ± 0.37	1.19 ± 0.07

Table S4.3 continuous

DSK2	10	10	5.90 ± 1.03	2.89 ±1.41	1.34 ± 0.19^{ab}	1.76 ± 0.31	1.27 ± 0.08
DSK4	7	7	5.56 ± 0.78	2.26 ±1.17	1.05 ± 0.35^{b}	1.98 ± 0.18	1.19 ± 0.08
2013							
US1	9	9	6.28 ± 0.42	3.39 ± 0.75	1.11 ± 0.17	2.12 ± 0.18	1.37 ± 0.07^{ab}
US2	9	10	6.05 ± 0.53	2.89 ± 0.80	1.19 ± 0.22	2.32 ± 0.27	1.24 ± 0.06^{bd}
US3	10	12	5.98 ± 0.53	2.77 ± 0.98	1.22 ± 0.20	1.91 ± 0.28	1.25 ±0.08^{bd}
DSW	9	9	5.91 ± 0.36	2.49 ± 0.48	1.38 ± 0.23	2.04 ± 0.25	1.19 ± 0.10^{d}
INT1	8	8	5.54 ± 0.80	2.35 ± 1.12	1.18 ± 0.30	2.10 ± 0.27	1.25 ± 0.13^{bd}
INT2	7	7	5.51 ± 0.49	2.14 ± 0.63	0.98 ± 0.22	2.19 ± 0.40	1.25 ± 0.09^{bd}
DSK1	9	9	5.50 ± 0.69	2.14 ± 0.93	1.26 ± 0.15	2.01 ± 0.25	1.23 ± 0.08^{bd}
DSK2	10	12	5.86 ± 0.82	2.79 ± 1.30	1.22 ± 0.33	2.07 ± 0.35	1.29 ± 0.08^{bcd}
DSK3	10	11	5.43 ± 0.83	2.58 ± 1.43	1.26 ± 0.26	2.23 ± 0.43	1.49 ± 0.14^{a}
DSK4	9	10	6.16 ± 0.80	3.55 ± 1.40	1.15 ± 0.34	1.97 ± 0.34	1.42 ± 0.10^{ac}
2014							
US1	11	11	6.18 ± 0.44	3.29 ± 0.75	1.14 ± 0.27^{b}	2.13 ± 0.41^{ab}	1.35 ± 0.09^{a}
US2	9	9	5.67 ± 0.53	2.49 ± 0.68	1.21 ± 0.17^{b}	2.27 ± 0.34^{ab}	1.27 ± 0.06^{ab}
US3	9	11	5.96 ± 0.56	2.83 ± 0.93	1.40 ± 0.20^{ab}	1.87 ± 0.22^{b}	1.30 ± 0.07^{ab}
DSW	10	10	5.98 ± 0.54	2.81 ± 0.90	1.32 ± 0.23^{ab}	1.89 ± 0.21^{abc}	1.27 ± 0.09^{ab}
INT1	7	9	6.25 ± 0.51	3.33 ± 1.04	1.13 ± 0.35^{b}	1.91 ± 0.30a^{bc}	1.35 ± 0.11^{ab}
INT2	9	10	6.03 ± 0.64	2.86 ± 1.02	1.23 ± 0.22^{ab}	1.54 ± 0.27^{c}	1.32 ± 0.05^{ab}
DSK1	9	9	5.94 ± 0.83	2.71 ± 1.11	1.40 ± 0.17^{ab}	2.06 ± 0.42^{abc}	1.22 ± 0.14^{ab}

Table S4.3 continues

DSK2	10	9	6.07 ± 0.73	2.90 ± 1.16	1.16 ± 0.28^{b}	1.62 ± 0.50^{c}	1.28 ± 0.09^{ab}
DSK3	9	9	5.79 ± 0.69	2.62 ± 1.22	1.19 ±0.26^{b}	2.40 ± 0.35^{a}	1.33 ± 0.13^{ab}
DSK4	11	11	5.40 ± 0.68	2.05 ± 1.14	1.58 ± 0.26^{a}	2.23 ± 0.25^{a}	1.21 ± 0.11^{b}
2015							
US1	10	11	5.79 ± 0.52	2.50 ± 0.86	1.35 ± 0.20	3.54 ± 0.20^{a}	1.24 ± 0.07^{ab}
US2	10	9	5.99 ± 0.62	2.78 ± 1.01	1.38 ± 0.25	2.18 ± 0.22^{ab}	1.24 ± 0.09^{ab}
US3	10	10	6.04 ± 0.41	2.86 ± 0.83	1.17 ± 0.19	1.72 ± 0.23^{b}	1.23 ± 0.11^{ab}
DSW	10	10	6.26 ± 0.57	3.14 ± 0.98	1.26 ± 0.21	1.71 ± 0.24^{b}	1.26 ± 0.08^{ab}
INT1	10	10	6.20 ± 0.42	3.00 ± 0.69	1.27 ± 0.30	1.82 ± 0.24^{ab}	1.24 ± 0.06^{ab}
INT2	10	11	5.97 ± 0.69	2.66 ± 1/09	1.08 ± 0.30	1.89 ± 0.38^{ab}	1.27 ± 0.10^{ab}
DSK1	10	11	6.28 ± 0.56	3.30 ± 1.23	1.18 ± 0.17	1.93 ± 0.23^{ab}	1.18 ± 0.13^{a}
DSK2	9	10	5.75 ± 0.69	2.45 ± 0.91	1.20 ± 0.18	1.99 ± 0.41^{ab}	1.23 ± 0.06^{ab}
DSK3	9	9	6.18 ± 0.59	3.16 ± 1.24	1.19 ± 0.21	1.82 ± 0.30^{ab}	1.28 ± 0.10^{ab}
DSK4	10	10	5.98 ± 0.68	3.04 ± 1.14	1.12 ± 0.23	2.04 ± 0.31^{ab}	1.35 ± 0.12^{b}
2016							
US1	15	16	5.83 ± 0.59	2.59 ± 0.90	1.13 ± 0.22	1.53 ± 0.26^{ab}	1.26 ± 0.09
US2	16	16	5.67 ± 0.61	2.34 ± 0.80	1.10 ± 0.33	1.56 ± 0.44^{ab}	1.25 ± 0.08
US3	15	13	5.62 ± 0.48	2.34 ± 0.59	1.05 ± 0.20	1.58 ± 0.20^{ab}	1.30 ± 0.12
DSW	14	15	5.52 ± 0.71	2.19 ± 1.15	1.08 ± 0.20	1.72 ± 0.16^{ab}	1.21 ± 0.12
INT1	14	15	5.47 ± 0.46	2.15 ± 0.65	1.26 ± 0.18	1.79 ± 0.35^{ab}	1.28 ± 0.11
INT2	16	15	5.54 ± 0.64	2.22 ± 0.89	1.17 ± 0.22	1.66 ± 0.23^{ab}	1.28 ± 0.11

Table S4.3 continues

DSK1	16	14	5.25 ± 0.70	2.11 ± 1.07	1.16 ± 0.20	1.87 ± 0.38[a]	1.34 ± 0.13
DSK2	15	15	5.62 ± 0.69	2.3 ± 1.07	1.11 ± 0.27	1.75 ± 0.30[ab]	1.25 ± 0.12
DSK3	15	14	5.36 ± 0.63	2.12 ± 1.01	1.08 ± 0.20	1.54 ± 0.39[ab]	1.31 ± 0.08
DSK4	17	18	5.56 ± 0.72	2.36 ± 1.32	1.05 ± 0.34	1.50 ± 0.22[b]	1.26 ± 0.15

[a], [b], and [c] indicate significant differences

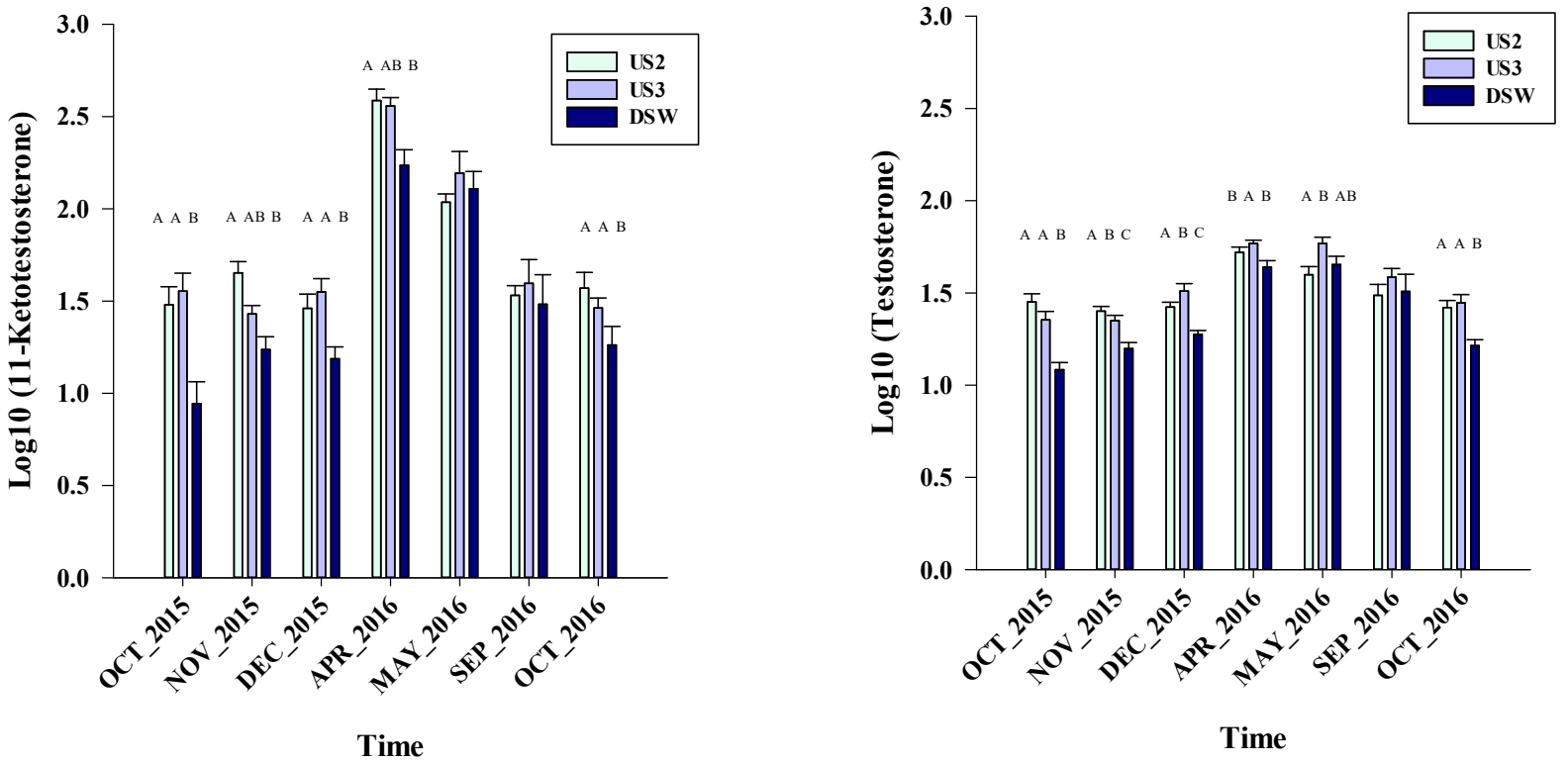


Figure S4.1 Annual cycle (October 2015 to October 2016) of human chorionic gonadotropin stimulated production of 11-ketotestosterone (A) and testosterone (B) by testes from male rainbow darter collected downstream of the Waterloo MWWTP (DSW) and 2 upstream reference sites (US2 and US3). One-way analysis of variance was conducted on log10 transformed data within each month. Statistical differences are indicated by capital letters above vertical bars (mean ± standard error of the mean).

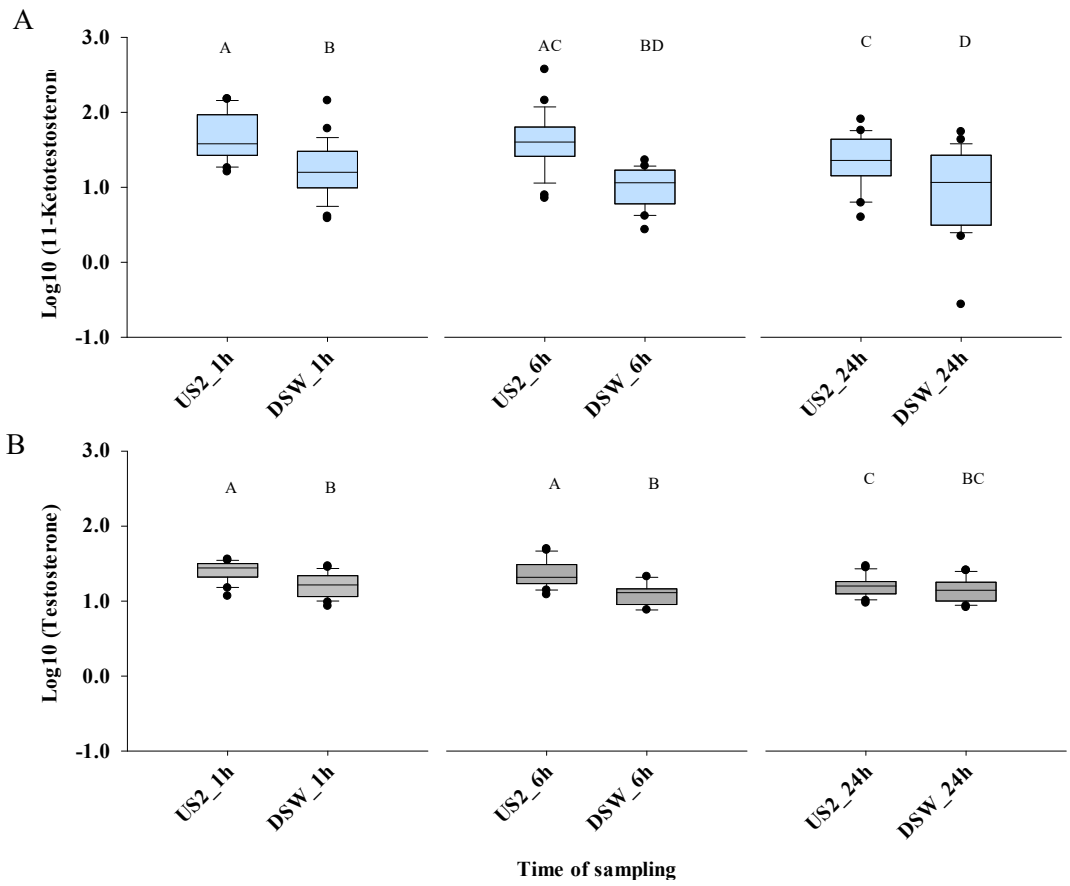


Figure S4.2 Impact of post-capture time of sampling (1 h, 6 h and 24 h) on human chorionic gonadotropin stimulated production of 11-ketotestosterone (A) and testosterone (B) by the testes from male rainbow darter collected downstream of the Waterloo MWWTP (DSW) and from the upstream reference site (US2). Two-way analysis of variance was conducted on log10 transformed data. Statistical differences are indicated by capital letters above box plots (mean ± standard error of the mean).

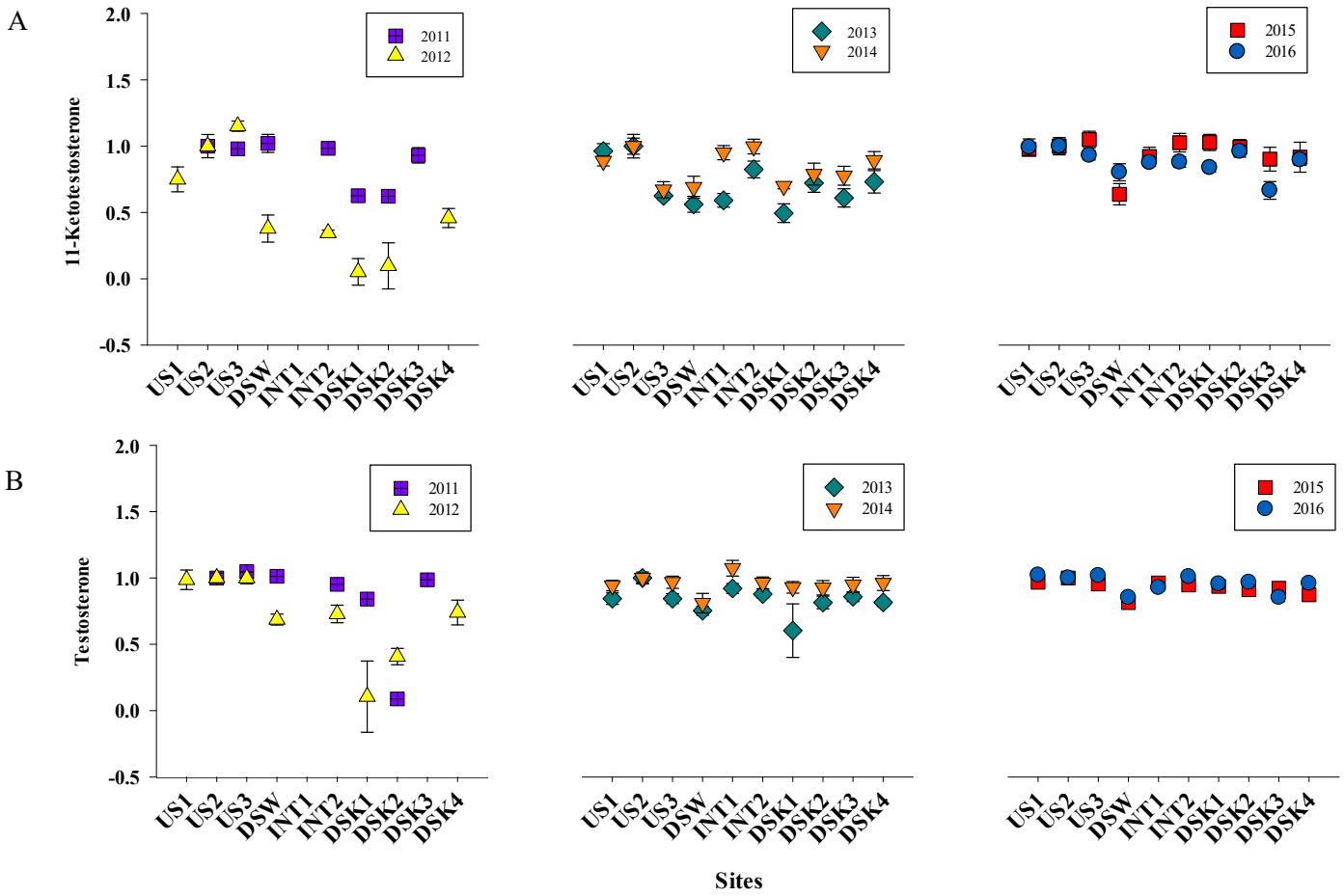


Figure S4.3 Time dependant changes in human chorionic gonadotropin stimulated production of 11-ketotestosterone and testosterone by the testes from male rainbow darter collected across the urbanized environment in the central Grand River during the pre-upgrade period (2011 - 2012), transition period (2013 - 2014), and the post-upgrade period (2015 - 2016). The data presented are log10 transformed steroid concentrations (pg/mg of gonad tissue), normalized to the levels produced by fish from the US2 site. Statistical differences are indicated by capital letters above scatter plots (mean ± standard error of the mean).

2. Yeast cell preparation for yeast estrogen screen (YES)

Refer to Appendix A.

3. Conestogo River as a source of anthropogenic impacts affecting steroid production in US3 fish

In the study that measures steroid production in rainbow darter males across the urban environment in the central Grand River over six years (2011 – 2016), there were three reference sites located above the urbanized area at different distances upstream of the Waterloo MWWTP outfall. The closest refrence site (US3) to the urbanized area was located 6 km above the Waterloo MWWTP outfall. The second reference site (US2) was added as it is well upstream of the major urbanized area; 22 km upstream of the Waterloo outfall. The third reference site (US1) was 11 km further upstream and was an additional control to the two reference sites (i.e., 33 km upstream of the Waterloo outfall). Although US1 and US2 are located in a relatively rural area there are several small wastewater treatment plants that discharge into the river further upstream. There is a greater degree of urbanization around US3 hence the increased concentration of selected pharmaceuticals and nitrougenous products that were reported in the surface water collected from this site than US1 and US2 (Marjan et al., 2017). In addition, there is a tributary upstream of this site, Conestogo River, that carries agricultural run-off as it flows through a rural area dominated by farming and agricultural acivities. In the first two years of the study (2011, 2012), steroid production in rainbow darter from US3 was comparable to the production measured in fish from the further upstream reference sites. However, in 2013 and 2014 significant reduction in 11-keto testosterone production was detected in rainbow darter from US3 compared to upstream reference sites. In contrast, testosterone production was not affected and was comparable to its production in US2 and US3 rainbow darter. 11-Keto testosterone is reported to be the main androgen in teleost fish (Evans et al., 2013; Norris and Lopez, 2011) and detecting significant reduction in its production in two consecutive years at the urban reference sites raised a concern that there may be some confounding factors or contaminants enetering the watershed upstream. To test this hypothesis it was decided to further isolate US3 rainbow darter collection and to investigate if there are upstream sources of contamination supressing normal production of 11-keto testosterone (and testosterone). In November 2015 rainbow darter were collected from US3, US2 and three sites in between primamrely targeting the main stream in the Conestogo River (CR), and two sites in the Grand River stream one below the confuluence (CDS) and the other above the confluence (CUS) (Figure S4.4). Approximately ten rainbow darter males were sampled from each site and a portion of their gonad tissue was collected and used to measure *in vitro* production of 11-keto testosterone (and testosterone) according to the methods described in the main manuscript. Although, rainbow darter collected in the Conestogo River showed lower production of 11-keto testoserone compared to fish collected in the Grand River,

this reduction in its production was not statisitically significant (Figure S4.5). This supporting study suggested that there were no potential impacts upstream of US3 at least not in 2015. Moreover, in this year 11-keto testosterone in fish from US3 recovered and were no longer significantly lower compared to US2 and US1 rainbow darter. It remained unclear whether 11-keto testosterone reduction in rainbow darter in 2013 and 2014 was affected by confounding factors at the actual site or there were contaminants entering through the Conestogo River that subsequently affected 11-keto testosterone production

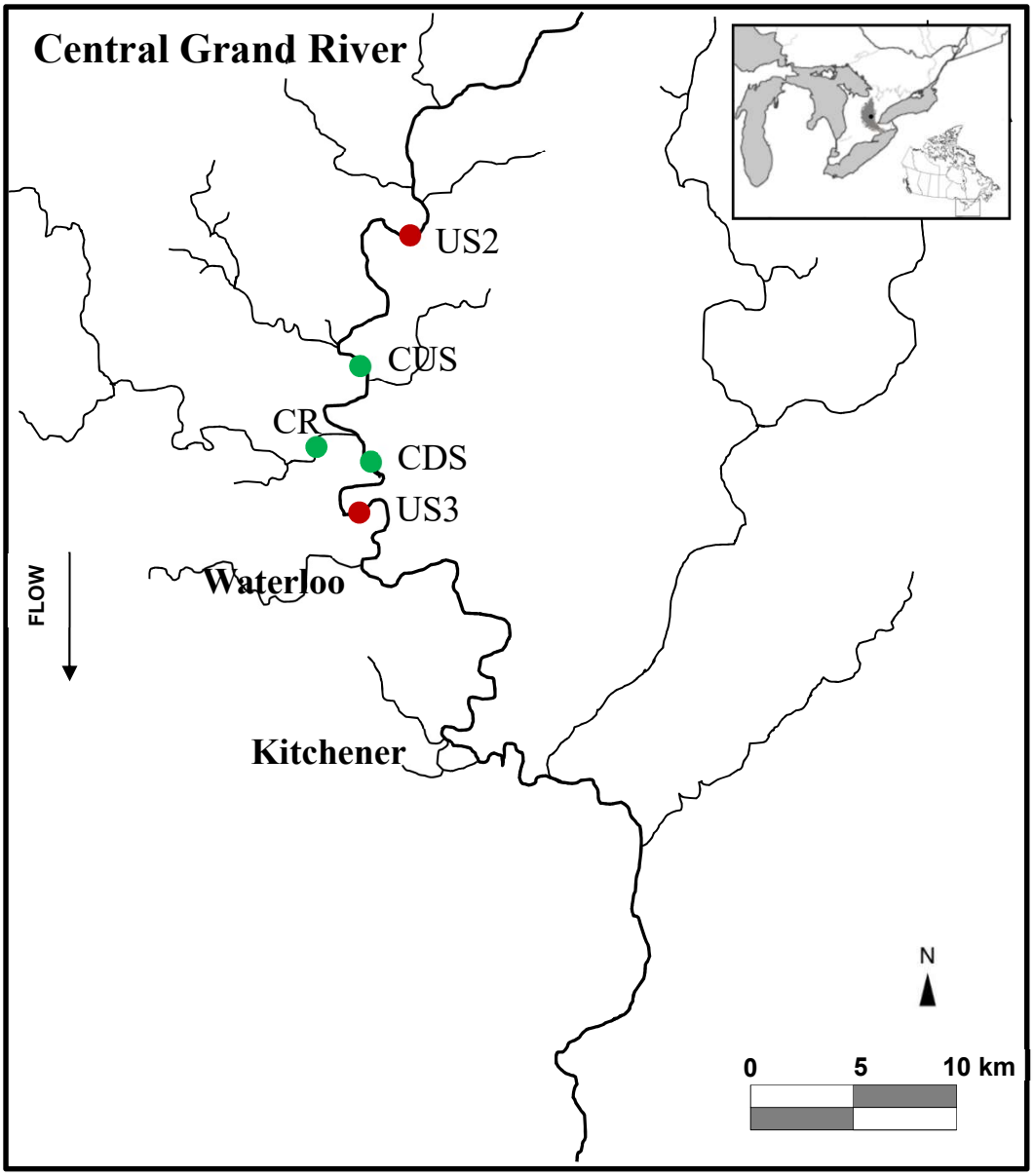


Figure S4.4 Location of sampling sites. Rainbow darter males were collected from the two upstream reference sites (US2 and US3) in 2015 and three additional sites: one located in the main stream of the Conestogo River (CR) and two located in the Grand River: upstream (CUS) and downstream (CDS) of the confluence.

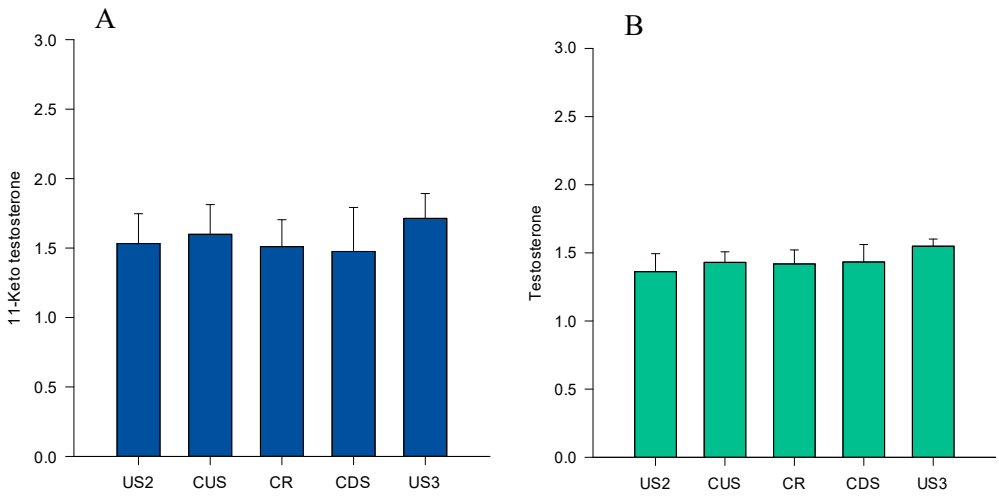


Figure S4.5 Concentration of 11-ketotestosterone (A) and testosterone (B) measured in the rainbow darter in 2015 showed that Conestogo River had no major effects on steroid production in fish at the urban reference site (US3) in the central Grand River. Presented are log10 transformed androgen steroid concentrations (pg/mg of gonad tissue; mean + standard deviation).