Does intersex matter? A case study of rainbow darter in the Grand River

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this 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.

Abstract

Endocrine disrupting compounds (EDCs) are present in the environment and can have negative effects on the health of wildlife. Aquatic organisms residing near the outfalls of municipal wastewater effluent (MWWE) are chronically exposed to EDCs, including natural hormones, pharmaceuticals, and industrial chemicals. The vulnerability of aquatic organisms to these compounds is due to the evolutionary conservation of endocrine systems. Although numerous studies have indicated that compounds in MWWE, including estrogenic and anti-androgenic contaminants, feminize male fish, it is still uncertain what the consequences of feminization of male fish are. Research on this topic since the early 1990's has demonstrated that a multitude of compounds in MWWE, are capable of binding to estrogen receptors in fish. Key biomarkers of estrogen exposure are elevation of vitellogenin protein and gene expression levels, as well as the presence of female tissue in male gonads; a condition referred to as intersex. The feminization of male fish and intersex condition has been noted in populations of fish around the world including rainbow darter (*Etheostoma caeruleum*) in the Grand River, Ontario, Canada. Male rainbow darter collected from sites near MWWE outfalls have intersex condition as well as impaired androgen production and sperm development. The aim of the studies in this thesis was to assess the implications of feminized males using rainbow darter as a model. Several questions were addressed through field and laboratory studies.

The first question addressed was whether exposure to MWWE, and intersex in particular, reduced the reproductive success of rainbow darter. To test this, fish were collected in the field during the spawning season, gametes were stripped, and manual fertilizations conducted at each of several sites. Fertilization success and embryo survival were lower at sites downstream of a MWWTP outfall when compared to a rural reference site. Additionally, when grouped into categories based on the severity of intersex (based on number and development of oocytes in testes), it was found that the most severely affected males had the lowest fertilization success. In contrast, no relationship was found between embryo survival and intersex severity, suggesting that egg quality may play a larger role in the survival of embryos. This study concluded that severe intersex condition, which is a marker of MWWE exposure, was an indicator of poor reproductive success in male rainbow darter.

The second question addressed in this thesis was whether reproductive behaviour was altered in fish exposed to MWWE. To test this, rainbow darter were collected from sites in the Grand River along an urban gradient and two separate experiments conducted. The first experiment was a breeding competition experiment where three males, each from a different site, were placed into a spawning tank with a single female from either a reference or MWWE exposed site. Reproductive behaviour of both male and female rainbow darter collected near MWWTP outfalls were impaired. Males spent less time guarding the spawning area and females performed fewer nose digs. Both behaviours are important for reproductive success. The second experiment in this study assessed the response of males to a mirror-competitor. Males with severe intersex condition spent less time performing aggressive acts than males with no intersex condition. From these findings this study concluded that MWWE exposure, as indicated by intersex severity in the second experiment, alters reproductive behaviour of rainbow darter and would likely lead to reduced reproductive fitness in the wild.

The third study in this thesis tested whether observations of impaired reproductive success and behaviour are directly associated with MWWE as opposed to other urban stressors. In this study, male and female rainbow darter were collected from a reference site during the pre-spawning period and placed in breeding groups. Following a 10-day pre-exposure spawning period, rainbow darter breeding groups were exposed to 1 of 5 treatments (control, 17α-ethinylestradiol (EE2, a synthetic estrogen), 1%, 10%, or 20% MWWE) for 21 days. After the 21-day exposure, male behaviour was assessed in a mirror-competitor test. Fecundity (number of eggs) was lower in the highest (20%) dose of MWWE during the first week of the exposure, but was comparable to other groups for the remainder of the experiment. Fertilization success was also lower during the exposure period compared to the pre-exposure period in MWWE exposed and EE2 exposed fish. Males exposed to 20% MWWE were less aggressive and more active than the control group. Although the responses of this experiment were less dynamic than those observed in fish exposed in the wild, they corroborated the findings that rainbow darter exposed to MWWE have reduced reproductive fitness.

The final study in this thesis addressed the issue of repeatability of field studies of feminized males by comparing the variability of biological measures between fall and spring seasons among five years of collections as well as across levels of biological organization. Measures were most consistent in males collected during the fall. Additionally, measures at the tissue level, including

sperm development and intersex incidence and severity, were the most consistent measures among years and seasons. This study concluded that the use of multiple biological measures from several levels of biological organization allowed for the same conclusion to be made about the reproductive health of rainbow darter in all years and both seasons of field collections.

Throughout these studies, intersex was found to be one of the most reliable markers of exposure to endocrine disrupting compounds in municipal wastewater effluent. Additionally, from the sum of these studies it can be concluded that highly prevalent and severe intersex in a population of fish indicates poor reproductive fitness. Whether this results in reduced population size or decreased genetic diversity is not known, and is an important topic for future studies.

Acknowledgements

The journey of this PhD dissertation has been incredible and it gives me great pleasure to acknowledge the people that I have worked with and learned from along the way.

I would like to express deep gratitude to my supervisor Dr. Mark Servos who has provided me with numerous opportunities to learn and advance my career and for his advice and constant support. I am also grateful to my committee, Dr. Brian Dixon, Dr. Mark McMaster, and Dr. Glen Van der Kraak for their encouragement, interest, and valuable input.

The studies conducted throughout this thesis involved the effort of many people. I would like to thank the past and present students, technicians, and research fellows of the Servos lab and Environment and Climate Change Canada for volunteering their time during field seasons and lab exposures. Without their help and positive attitudes, this thesis would be a blank document. A special thank you to Gerald Tetreault who has been an active supporter. I have appreciated you sharing your knowledge, from identifying a rainbow darter to providing feedback on manuscripts. Leslie Bragg has also helped immensely throughout this process. She facilitated all steps of the research and provided expert advice on the sampling and analysis of water chemistry. I would also like to thank the summer students over the years who were assigned the essential, but often mind numbing, tasks of counting eggs, measuring flow rates, cleaning fish tanks, collecting effluent three times a week, and extracting water samples. Additionally, I would also like to acknowledge Jennifer Thomas for copy-editing parts of this dissertation.

The financial support that I received from NSERC pushed me to start this program, and the funding from OGS and the University of Waterloo allowed me to focus on research and worry less about financial stability. Travel funding from SETAC NA allowed me to share my findings with the research community.

Finally, I would like to thank my friends, family, and partner for providing support during the valleys and celebrating with me on the peaks of this ride.

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Preface Statement

This PhD thesis is based on a collection of several manuscripts. At the time of submission one manuscript was published, a second accepted, and a third and fourth submitted for review in refereed journals. These manuscripts, as listed below, are presented in Chapters 2, 3, 4, and 5 respectively. They have been slightly modified for consistency in formatting. In all manuscripts, I am the first author and my supervisor Dr. Mark Servos is the anchor author. The content of each manuscript is dominated by my intellectual effort although there has been a contribution by several other individuals who are recognized as co-authors. The roles of the co-authors are explained in detail below.

1. Fuzzen, M.L., Bennett, C.J., Tetreault, G.R., McMaster, M.E., Servos, M.R., 2015. Severe intersex is predictive of poor fertilization success in populations of rainbow darter (*Etheostoma caeruleum*). Aquat Toxicol 160, 106-116.
2. Fuzzen, M.L.M., Crichton, A., Servos, M.R. Alteration of reproductive behaviours in rainbow darter (*Etheostoma caeruleum*) collected near municipal wastewater treatments plants. In submission.
3. Fuzzen, M.L.M., Dhiyebi, H.A., Bragg, L.M., Servos, M.R. Digging deeper: An assessment of reproductive success and behaviour of a native fish species, rainbow darter, after exposure to municipal wastewater effluent or 17α-ethinylestradiol. In submission.
4. Fuzzen, M. L. M., Bragg, L. M., Tetreault, G. R., Bahamonde, P. A., Tanna, R. N., Bennett, C. J., McMaster, M. E. and Servos, M. R., 2016. An assessment of the spatial and temporal variability of biological responses to municipal wastewater effluent in rainbow darter (*Etheostoma caeruleum*) collected along an urban gradient. PLoS ONE. 11, e0164879.

The ideas in the first manuscript [1] were conceived by me and I carried out the work, including design of the experiment, methodology, analysis, manuscript writing, and revision. Charles Bennett developed the method for the processing of histological samples. Dr. Tetreault, Dr. McMaster and Dr. Servos actively participated in the discussion of the experiment, results, and reviewing of the manuscript.

In manuscript [2], I conceived the key ideas, conducted the experiments, wrote the manuscript and performed the revisions. Alexandra Crichton assisted with conducting the first experiment by analyzing the video. Dr. Servos and Alexandra Crichton participated in discussion of the study design and results, and reviewing of the manuscript.

In manuscript [3], I initiated the research on responses of adult rainbow darter to municipal wastewater effluent exposure, conducted the experiment, sample and data analysis, and wrote and revised the manuscript. Hadi Dhiyebi designed the exposure system and assisted with conducting the experiments. Leslie Bragg advised on water chemistry sampling and methodology and also analyzed water samples for hormones and pharmaceuticals. Dr. Mark Servos actively participated in discussion of the results and reviewing of the manuscript.

In the final manuscript [4], I developed the concept for the paper, conducted three seasons of field work and sample analysis, compiled and analyzed the data and wrote and revised the manuscript. Dr. Tetreault gathered two seasons of field and sample data, Dr. Bahamonde gathered two seasons of field and sample data and developed methods for the analysis of gene expression, and Rajiv Tanna also gathered two seasons of field and sample data and initiated the analysis of intersex severity. Dr. Charles Bennet provided guidance and assistance with histological processing and analysis of all field seasons. All co-authors participated in the discussion of experiment results and reviewing of the manuscript.

Chapter 1
General Introduction

1.1 Introduction

An endocrine disrupting compound (EDC) is defined as any exogenous substance or mixture that adversely affects the synthesis, transport, signaling, or metabolism of an endocrine system (WHO-UNEP, 2012). In the 2012 World Health Organization report "*Status of the Science – Endocrine Disrupting Chemicals*," it was stated that there are close to 800 chemicals that have the potential to be endocrine disruptors (WHO-UNEP, 2012). These endocrine disrupting compounds represent a wide range of chemicals that originate from many different sources. For example, some pesticides (DDT), herbicides (atrazine), and fungicides (vinclozolin) have endocrine active properties. Many industrial or mining byproducts (heavy metals, dioxins, and PCBs), chemicals associated with plastics (phthalates, nonylphenol, and bisphenol A), human hormones (17β-estradiol, estrone), and pharmaceuticals and personal care products (17α-ethinylestradiol, diclofenac, triclosan) are EDCs (Norris and Carr, 2013).

Endocrine functions of organisms are very sensitive systems that are modulated frequently in order to maintain homeostasis (Norris and Carr, 2013). This sensitivity of endocrine systems makes them vulnerable to disruption through several mechanisms, including binding to endocrine receptors (receptor mediated effects), as well as through alterations in the synthesis, metabolism, or excretion of endogenous hormones (Denslow and Sepulveda, 2007). Physiological processes, such as those mediated by endocrine systems, are well conserved evolutionarily. This means that chemicals that mimic natural hormones (e.g. binding to the receptors) or alter hormone synthesis and/or function in humans (i.e., pharmaceuticals) can alter endocrine function in fish when they enter the aquatic environment (Gunnarsson *et al*., 2008). Although the exposure is generally low, EDCs present a potential risk to aquatic organisms because they are capable of causing physiological responses at very low doses (Länge *et al*., 2001; Parrott and Blunt, 2005). The amount of hormone required to modulate endocrine systems is extremely small, in the ng/L range (Norris and Carr, 2013). The outcome of EDC exposure is dependent on sensitivity, concentration, duration, and timing of exposure. Developmental stages of organisms are generally more sensitive than adult forms, and exposure for a short duration during development can have consequences for adult health and reproductive success (WHO-UNEP, 2012). One of the most studied examples of EDC-mediated disruption is the alteration of male reproductive physiology in fishes after exposure to estrogenic compounds. Many cases of feminization of male fish have been observed near the outfalls of

municipal wastewater treatment plants that contain a diversity of potential endocrine disrupting substances.

1.2 Municipal wastewater effluent

Municipal wastewater effluent (MWWE) is a complex mixture of human waste, graywater, industrial effluents, and in the case of combined sewers, urban runoff and storm water. These sources of wastewater contain many contaminants that can act as EDCs including metals, legacy compounds (such as persistent organic pollutants), pesticides, as well as pharmaceuticals and personal care products (PPCPs). Treatment processes of municipal wastewater treatment plants (MWWTPs) vary considerably among plants due to differences in plant design and operation. While MWWTPs are constructed to remove solid waste, reduce biological oxygen demand, and eliminate human pathogens, they are not designed specifically to remove EDCs. Certain processes in MWWTPS, such as nitrification, however, are associated with a higher removal of some PPCPs (Clara *et al*., 2005a). Solids retention time has also been shown to be an important factor for PPCP removal (Clara *et al*., 2005b; Metcalfe *et al*., 2003; Servos *et al*., 2005). Regardless of the treatment processes used, there are some EDCs/pharmaceuticals that are discharged into the receiving environment.

1.3 Feminization of male fish – the initial observations

Initial observations of feminized male fish in the UK were made by fishermen, who caught hermaphroditic (gonads contained both ovarian and testicular tissue) in settlement lagoons of MWWTPs (Sumpter and Johnson, 2008). A fish biologist, Roger Sweeting, working for the water company Thames Water confirmed these casual observations by conducting two surveys. The first survey was conducted downstream of the Rye Mead MWWTP in 1978, the second survey was conducted in two parts of River Lea in 1981. Five of 26 sexually mature roach (*Rutilis rutilis*) captured downstream of Rye Meads MWWTP, and 5 of 100 mature males in the larger survey were reported to have intersex (Sweeting, 1981). Intersex is defined as the simultaneous presence of both male and female gonad tissue in a gonochoristic (non-sex changing) fish species (Tyler and Jobling, 2008). Scientists eventually became aware of the feminization of male fish residing near MWWTPs (Sumpter and Johnson, 2008), sparking an international field of research that has grown exponentially (Figure 1.1).

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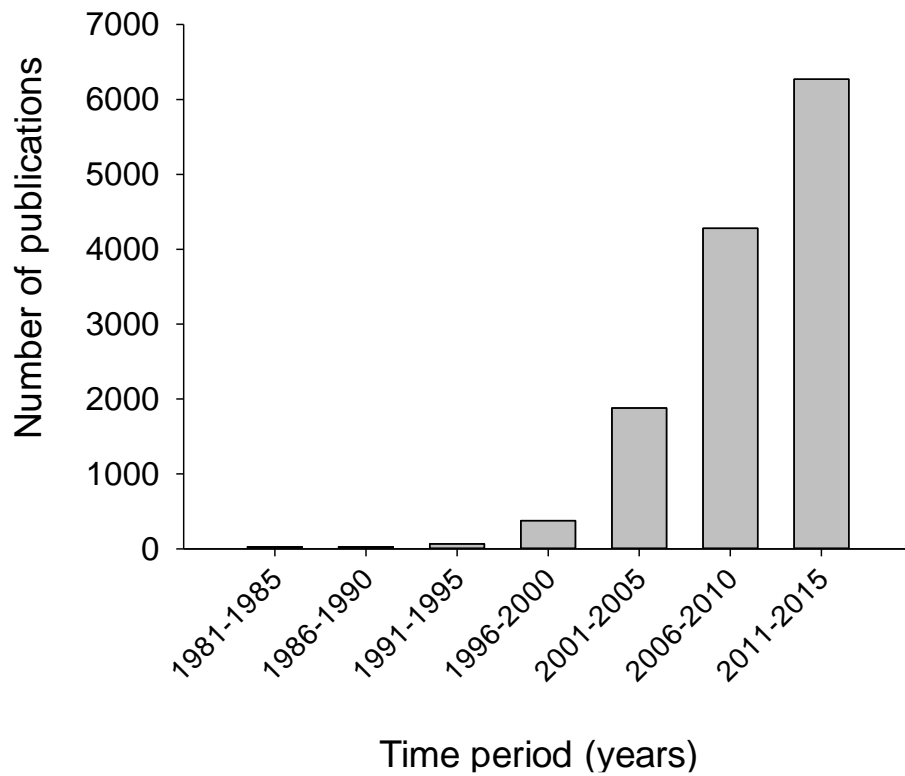
Figure 1.1 Number of articles found when the search terms "wastewater", "estrogenic", and "fish" were entered into the search engine Google Scholar (www.scholar.google.com). Queries were made using five year intervals from 1981-2015.

1.3.1 What constituents of MWWE are responsible for feminization of male fish?

Investigations of causative agents in MWWE identified estrogens as a likely suspect since males collected in the initial surveys had smaller gonad sizes, and females had larger gonad sizes (Sweeting, 1981). Additionally, several papers had been previously published about the potential hazards of estrogens entering the aquatic environment (Aherne *et al*., 1985; Richardson and Bowron, 1985). To test the hypothesis that estrogenic compounds were present in MWWE, Purdom *et al*., (1994) conducted a study using a vitellogenin assay. Vitellogenin (VTG) is an egg yolk precursor protein that is normally found in elevated quantities only in female fish. The presence of elevated VTG in male fish is evidence that estrogenic compounds are acting on estrogen receptors (ER) in the liver (Sumpter and Jobling, 1995). When rainbow trout (*Oncorhynchus mykiss*) were caged downstream of MWWTPs, it was found that male fish had a dramatic elevation of plasma VTG, confirming the presence of estrogenic compounds (Purdom *et al*., 1994).

The next challenge was identifying which compounds in the MWWE were responsible for the estrogenic effects. To assess this, a combination of analytical chemistry and biological screening was conducted. This involved separating the final effluent using solid phase extraction and liquid chromatography and then testing fractions of the elutant using a yeast estrogen screen to isolate and identify specific chemicals (Sumpter and Johnson, 2008). The main estrogenic constituents of MWWE were found to include natural estrogens (estrone (E1), and 17β-estradiol (E2), as well as the synthetic estrogen 17α-ethinylestradiol (EE2) (Desbrow *et al*., 1998, Ternes *et al*., 1999). In addition to these, other chemicals in effluents can bind to the estrogen receptor (e.g. alkyphenols, bisphenol-A). When many xenoestrogens (estrogen receptor binding compounds) are present in one solution, such as in effluent, they can act additively (Thorpe *et al*., 2003). Chemicals may also act through mechanisms other than binding to the estrogen receptor to feminize male fish. Anti-androgenic compounds were found to be a likely contributor to the feminization of wild fish in UK rivers when they were modeled along with estrogenic compounds (Jobling *et al*., 2009). Although the mechanism is not yet understood, the anti-diabetic drug metformin has been shown to feminize male fish at an environmentally relevant dose (Niemuth *et al*., 2015; Niemuth and Klaper, 2015). Thus while the additive activity of steroid hormones and EE2 are likely the causative agents in most cases of MWWE exposure, other compounds contribute to the estrogenicity, and in some cases a high volume of a weakly estrogenic compound (such as alkylphenol) is responsible.

1.3.2 The scope and nature of feminization in fish

In order to assess how widespread the issue of estrogenic compounds in watersheds was, two large surveys were conducted in the early 1990s. The first survey conducted involved the caging of rainbow trout throughout the UK to assess the plasma VTG response to municipal wastewaters (Harries *et al*., 1996). The second was a survey of roach in rivers throughout the United Kingdom (Jobling *et al*., 1998). These studies found widespread estrogenicity and up to 100% incidence of intersex in male roach, both of which were associated with the presence of municipal wastewater treatment plants (MWWTPs). In addition to intersex, studies of roach identified numerous indications of endocrine disruption, including elevation of plasma E2 and testosterone (T), reduced spermiation, milt volume, and sperm density (Jobling *et al*., 2002a) and reduced fertilization success (Jobling *et al*., 2002b).

Since the original investigations into the presence of feminized male fish in the UK, field surveys conducted by government agencies and researchers have found the presence of feminized fish all over the world (Abdel-moneim *et al*., 2015; Bahamonde *et al*., 2013). When a review of intersex condition in fish was conducted by Abdel-moneim *et al*. (2015), they reported that 20 fish families and 54 species in 29 countries had reported cases of gonadal intersex. In the last year an additional six studies have reported the presence of intersex in wild fish, including two new families and four new species (Adeogun *et al*., 2016a; Adeogun *et al*., 2016b; Fritts *et al*., 2016; Guellard *et al*., 2015; Ibor *et al*., 2016; Zheng *et al*., 2015). Several observations can be made from this vast number of studies of intersex condition. First, that the presence of oocytes in male testes was frequently found not only near effluent outfalls but also at reference sites. This has been attributed to the presence of non-point sources, movement of fish, and legacy contaminants. The incidence of intersex at "reference" sites can range from 0 to 55%, as reviewed by Bahamonde *et al*. (2013). Secondly, differences in life history and sensitivity to estrogenic compounds across species leads to differences in their response to exposure. For example, a study by Hinck *et al.,* (2009) found that only four of 16 fish species demonstrated intersex condition in rivers across the US. The third observation that can be made is that the presentation of intersex differs between species and locations. The incidence (proportion of males affected) of intersex has a large range from 0 -100% within a species, with few (~20%) studies reporting an incidence greater than 50% in a sampling area (Abdel-moneim *et al*., 2015). Additionally, presentation of feminized gonads differs among species, with most displaying the

presence of oocytes in testicular tissue and some displaying ovarian cavities, absence of sperm ducts, or altered testicular structure. In order to assess severity of intersex condition, several scoring systems have been developed to take into account the unique presentation of intersex in each species (Anderson *et al*., 2003; Bahamonde *et al*., 2015a; Blazer *et al*., 2007; Faller *et al*., 2003; Jobling *et al*., 1998; Tanna *et al*., 2013; van Aerle *et al*., 2001).

It is important to note that the presence of intersex is just one measure of feminization of male fish. While this is a biomarker that is frequently measured, it is not always present in fish exposed to estrogenic compounds (hence the large range of incidences). Elevated plasma VTG or liver gene expression of *vtg*, reduced androgen production, altered sperm production, and gonad size are also frequently assessed biomarkers of estrogenic compounds in the environment. When assessed for their relationship to intersex, none of the biomarkers were correlated (Bahamonde *et al*., 2013), suggesting variation in the mechanism of intersex development and a need to assess multiple markers of feminization in male fish.

1.3.3 Implications of feminization of male fish

The section above demonstrated that the feminization of males is a widespread issue, which leads to the question "does intersex matter"? Feminized roach have been demonstrated to have reduced fecundity (Jobling *et al*., 2002a), reduced fertilization success (Jobling *et al*., 2002b), and reduced paternity in a competitive breeding scenario (Harris *et al*., 2010). Despite the fact that numerous studies have identified feminization of male fish near MWWE outfalls, reproductive success of wild caught feminized male fish has only been assessed in roach. In order to better understand the implications of feminization, more studies assessing the impacts of MWWE on reproductive fitness of wild fish need to be conducted.

In contrast to the lack of field studies, a vast number of laboratory studies have been conducted which address the issue of pharmaceuticals in the environment and the potential implications for fish populations. These studies have resulted in a greater understanding of many important aspects of exposure and response of fish to pharmaceuticals, including route of exposure, metabolism, mode of action, life-stage dependent sensitivity, and identification of biomarkers (Férard, 2013; Sumpter and Johnson, 2005). Additionally, laboratory studies have demonstrated that the presence of pharmaceuticals can impair reproductive behaviour (Söffker and Tyler, 2012). The use of

behaviour as an endpoint is less common, but the non-invasive and sensitive nature of behaviour make it an important model that should be investigated in further detail. Despite the large amount of knowledge gained through laboratory studies, it remains difficult to apply this knowledge to wild fish. Thus we still do not know what the implications are for populations of fish in which some proportion of males are feminized.

A bold study conducted in northern Ontario was designed to address this gap in our understanding of the link between physiological measures of feminization, and population responses (Kidd *et al*. 2007). Researchers conducted a whole-lake study in an attempt to determine the potential impacts of MWWE in an intact ecosystem. Since MWWE is a complex matrix that varies greatly temporally, seasonally and between treatment plants, a single compound, EE2, was chosen to dose the lake. The biological indicators used to measure reproductive performance suggested impaired reproduction. Multiple species of male fish had elevated levels of plasma VTG, decreased amounts of developed sperm in the testes, presence of intersex and had decreased relative gonad size (Kidd *et al*., 2007; Palace *et al*., 2009; Palace *et al*., 2006). At the population level, a near elimination of a short lived species (fathead minnow; *Pimephales promelas*) from the lake was observed after just one year of EE2 additions and the population of a longer lived species decreased in size (pearl dace; *Margariscus margarita*) (Kidd *et al*., 2007; Palace *et al*., 2009; Palace *et al*., 2006). While this whole-lake experiment demonstrates that individual reproductive measurements were indicative of population impacts, it is uncertain whether fish in open systems would demonstrate similar impacts, or be detectable from natural background variability. Outfalls for MWWE are typically located in rivers and the exposure of fish is dependent on many factors including flow rate of the river and composition of the effluent. Rivers have the added complication of fish movement which could mask the recruitment failure of a single population. Thus in addition to controlled experiments, it is important to study populations of wild fish exposed to effluents containing estrogenic chemicals in order to better understand the implications for fish recruitment and sustainability.

1.4 Environmental monitoring

The vast number of compounds, the low concentrations of EDCs (and PPCPs), and complex matrix make monitoring endocrine disrupting compounds in MWWE a daunting task. The challenges associated with the development of monitoring programs for EDCs in MWWE are the subject of

several recent articles and reviews (Brander, 2013; Jasinska *et al*., 2015; Kilgour *et al*., 2005; Maruya *et al*., 2014; Park and Park, 2015). Despite these challenges, monitoring programs for EDCs/PPCPs or MWWE are being developed by regulators. In July 2015, the European Commission included three estrogens (E1, E2 and EE2), and the non-steroidal anti-inflammatory diclofenac on a "watch list" of the Water Framework Direction (Decision EU 2015/495). These compounds will be monitored across countries in the European Union for up to four years, after which a decision will be made about whether to include them on the priority substances list. There are currently no national regulations in Canada that require sampling and analyses of effluents for environmental estrogens.

The Canadian Ministers of the Environment (CCME) strategy on wastewater called for the establishment of national effluent quality standards. In response, new *Wastewater Systems Effluent Regulations* were developed under subsection 36(5) of the *Fisheries Act* (P.C. 2012-942 June 28, 2012). The goal of the regulation is to set national baselines for effluent quality standards that are achievable through secondary treatment or equivalent to "reduce the threats to fish, fish habitat and human health from fish consumption by decreasing the level of harmful substances deposited to Canadian surface water from wastewater effluent". Unfortunately, the new regulations do not address the emerging issues of endocrine disrupting substances. Interestingly an Environmental Effects Monitoring Program (EEM) was proposed during the *Canada Gazette*, Part 1, but was considered "premature and ahead of the timing in the CCME Strategy" and the EEM requirement was removed from the regulations. However, an "intent to include them at a later date as a regulatory amendment" was stated in the regulation. Research to support the development of effective approaches to assess and monitor municipal effluents in Canada will be needed in the near future. Many jurisdictions are implementing monitoring programs in advance of a national program and therefore research is needed to support the design and interpretation of these efforts.

The objective of environmental monitoring programs is usually to assess environmental change as well as the adequacy of regulations in protecting the environment from adverse effects of pollutants or stressors. Environmental monitoring can consist of chemical, physical, or biological monitoring (or some combination of the three). The EEM program for pulp and paper in Canada has been highlighted internationally as a well-planned environmental monitoring program (Johnson and Sumpter, 2016). The pulp and paper EEM utilizes chemical, physical and biological assessment using a stressor based approach (Kilgour *et al*., 2007). Biological assessment includes sampling of benthic

invertebrate communities and assessment of age structure, growth rate, relative gonad size, and fecundity of fish populations (Walker *et al*., 2002).

In addition to the endpoints in the EEM there are advantages of using multiple levels of biological organization in monitoring (Figure 1.2). The source of contamination can be more readily determined using sub-organismal measures (e.g. gene expression, physiology, and organ levels) that are more directly linked to exposure to specific stressors. Additionally, when mechanistically linked to responses at higher levels of biological organization, predictions can be made about the status of population and community levels measures (Ankley *et al*., 2010). This can provide regulators the information they need to make more effective decisions. A key aspect of biological monitoring programs is the ability to detect deviation in endpoints from "normal", and determine when this is biologically significant. In order to detect change, an understanding of the biology of the organisms used for monitoring, as well as a baseline of data are essential (Arciszewski and Munkittrick, 2015; Munkittrick *et al*., 2009).

In terms of biological monitoring for endocrine disrupting compounds in MWWE, there are limited data available. As described above, a large number of studies have identified the presence of feminized male fish around the world. However, a baseline of this condition in any one population has not been well established. In order for development of biological monitoring programs to be effective for compounds such as E2, EE2, and whole-effluent, we must first gain a better understanding of conditions and mechanisms leading to feminization of male fish in the environment. This can only be gained through a combination of laboratory and intensive field investigations of fish associated with wastewater effluent exposure that includes the evaluation and understanding of the expression of key endpoints.
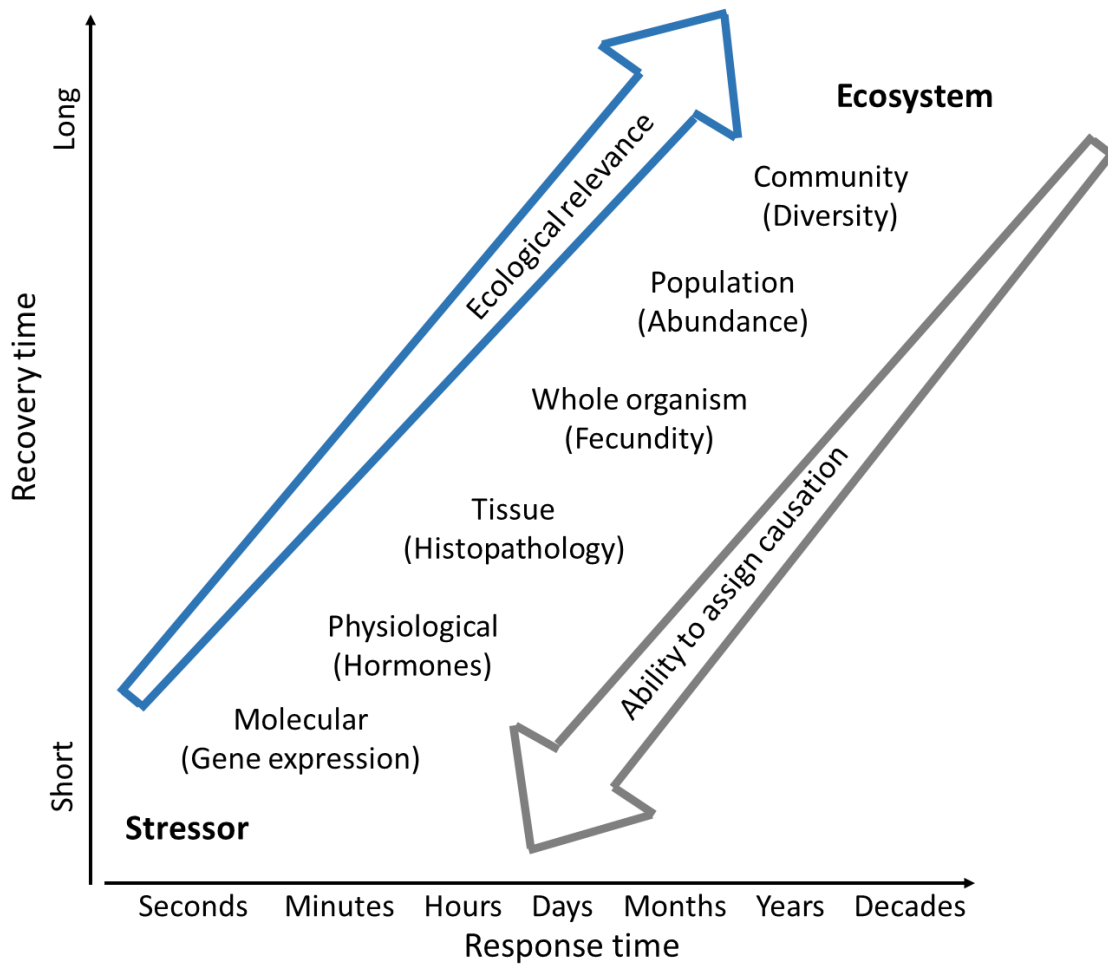
Figure 1.2 Demonstration of the paradox of biomarker selection in biological monitoring. Examples of biological endpoints used for biological monitoring in aquatic environments are given in brackets under the level of biological organization. Adapted from Newman and Unger (2003).

1.5 Rainbow darter in the Grand River watershed

The Grand River watershed in southern Ontario covers 6,800 km^2 and receives effluent from 30 MWWTPs (Figure 1.3). While the flow of the river is managed to maintain summer dilution of MWWE, population growth and extreme weather events put pressure on the river system. This is most obvious in summer months, when dissolved oxygen levels in the central region of the river frequently drop below 4 mg/L (Jamieson *et al*., 2013). Increased nutrients in the central region of the river system are associated with changes in macrophyte density (Hood *et al*., 2014), and stable isotope signatures in food webs suggesting a change in nutrient/energy flow (Loomer *et al*., 2015). Additionally, PPCPs have been detected in surface water (Arlos *et al*., 2015), as well as in tissues of freshwater mussels (de Solla *et al*., 2016) and fish species (Wang *et al*., 2011).

The impacts of PPCPs from MWWE have been studied in two fish species in the central Grand River, greenside darter (*Etheostoma blennoides*), and rainbow darter *(Etheostoma caeruleum)*. In a study that assessed the reproductive health of these fishes in response to exposure to MWWE, it was found that male greenside darter had decreased production of 11-ketotestosterone (11KT), while male rainbow darter also had reduced proportion of spermatozoa (mature sperm) in gonads, and both species had high prevalence of intersex (up to 100%) at field sites downstream of MWWTPs in the Grand River (Tetreault *et al*., 2011). In subsequent studies of rainbow darter, reduced 11KT production, reduced proportion of spermatozoa, and high prevalence of intersex were confirmed and intersex severity assessed (Bahamonde *et al*., 2015a; Tanna *et al*., 2013). Both a high incidence and high severity of intersex were observed in rainbow darter collected downstream of a MWWTP in the central Grand River, making this species an excellent model with which to address questions about feminization of male fish.

Rainbow darter are a small bodied (up to 7 cm), short lived (3-5 years), benthic, and sexually dimorphic percid species that are dispersed throughout northeastern North-America (Kuehne and Barbour, 1983; Page, 1983). Seasonal changes in reproductive endpoints are similar to greenside darter, which has been described to support environmental effects assessment (Tetreault *et al*., 2014). The small home range of rainbow darter along with their wide distribution and abundance at sites throughout the central region of the Grand River make this species an excellent model to study issues surrounding MWWE and feminization of male fish.
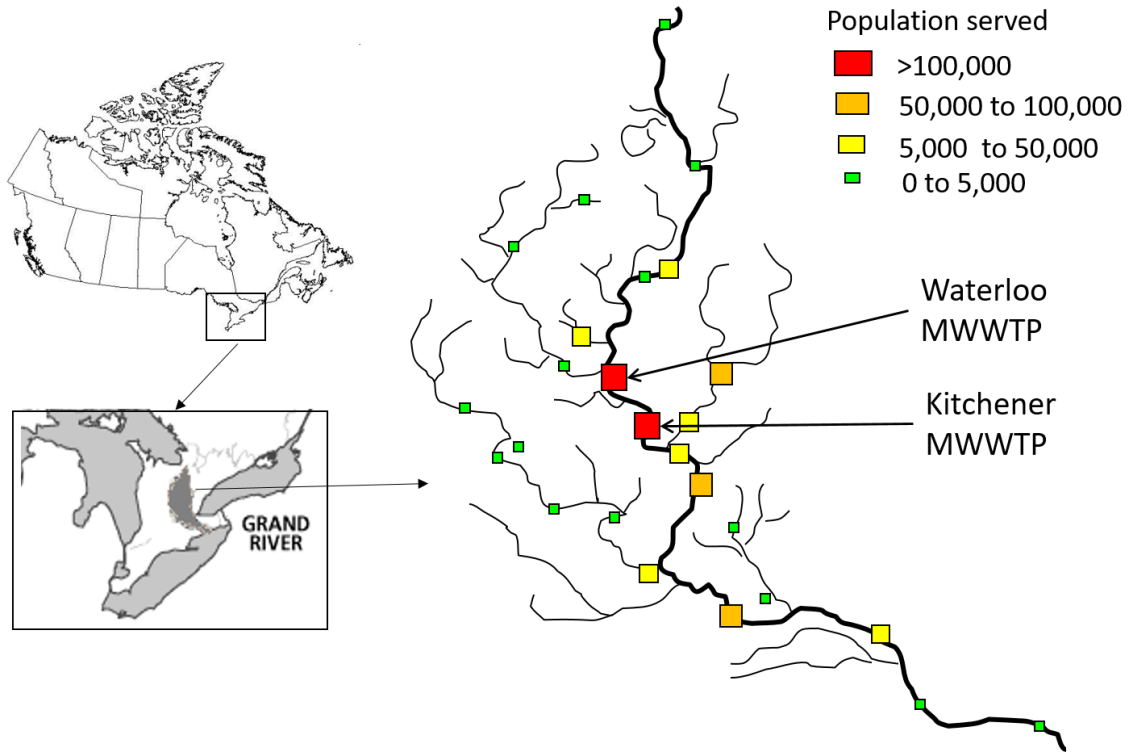
Figure 1.3 The Grand River watershedlocated in southern Ontario, Canada. The location and population size of the 30 municipal wastewater treatment plants were obtained from the Grand River Conservation Authority (GRCA).

1.6 Thesis objectives

The overlying hypothesis was that exposure to MWWE results in reduced reproductive success of rainbow darter in the central Grand River. A series of field and controlled laboratory studies were conducted to test this hypothesis. Specific objectives of each study were to assess:

1. Reproductive success in the form of fertilization success and embryo survival of rainbow darter collected from MWWE exposed sites in the Grand River (Chapter 2);

2. Reproductive behaviour of rainbow darter collected from MWWE exposed sites in the Grand River (Chapter 3);

3. Fecundity, fertilization success, and aggression of rainbow darter exposed to MWWE or EE2 in the laboratory (Chapter 4);

4. Variability of measures of reproductive health in rainbow darter collected through an urban gradient in the Grand River (Chapter 5).

Chapter 2

Severe intersex is predictive of poor fertilization success in populations of rainbow darter (*Etheostoma caeruleum*)

2.1 Overview

Municipal wastewater effluent (MWWE) contains emerging contaminants such as pharmaceuticals and personal care products (PPCPs) that have estrogenic properties. PPCPs are thought to be responsible for feminization of male fish in heavily urbanized areas around the globe. While many observations of feminized male fish have been made, the impact of feminization on reproductive success is not well understood. To address this lack of knowledge of the impacts of feminization, we examined the reproductive fitness of rainbow darter (*Etheostoma caeruleum*), a fish that is also known to have been feminized in some reaches of the Grand River, Kitchener-Waterloo, Ontario, Canada. In order to assess their reproductive health, somatic indices, gonadal steroid production, fecundity, and histological severity of intersex were measured in male rainbow darter collected through an urban gradient. Reproductive fitness was assessed by stripping milt and eggs from wild spawning fish, fertilizing eggs manually, and rearing embryos to hatch. The fertilization success and survival of embryos to hatch were compared among sites. In this study, it was found that rainbow darter collected at sites near a large municipal wastewater treatment plant (MWWTP), had decreased gonad size, increased severity of intersex, and decreased androgen production relative to other sites. Fish collected near the largest MWWTP also had lower fertilization success and survival to hatch. In contrast, fish collected near a second MWWTP farther upstream had comparable fertilization success, but lower survival to hatch compared to the upstream rural reference site. Intersex severity was negatively correlated with fertilization success, but not survival to hatch, suggesting that intersex is a good indicator of a population's fertilization success. Further investigation is required in order to determine if feminization will impact the sustainability of wild populations of fish.

2.2 Introduction

Determining the impact of municipal wastewater effluent (MWWE) on natural fish populations is a research area that has received growing attention over the past 15 years. MWWE is a complex mixture of contaminants that includes nutrients, legacy compounds, and pharmaceuticals and personal care products (PPCPs). While municipal wastewater treatment plants (MWWTPs) are effective at removing pathogens and nutrients, they are not designed to remove PPCPs. Thus many potentially harmful compounds enter the receiving environment via MWWTP effluent. Feminization of male fish is one impact of MWWE exposure, and has been noted in wild fish populations throughout the western world (Jobling *et al.* 1998; Bjerregaard *et al.* 2006; Blazer *et al.* 2011; Tetreault *et al.* 2011). Male fish show various indicators of feminization including smaller testes, lower levels of male sex hormones, reduced secondary sexual characteristics, presence of immature oocytes in testes (intersex) and elevation of plasma vitellogenin (VTG) levels (Desforges *et al.*, 2010; Liney *et al.*, 2005; Tetreault *et al.*, 2011; Vajda *et al.*, 2011). Estrogenic compounds present in the MWWE have been identified as being the major cause of this feminization effect (Harries *et al.*, 1996). The potential impact of feminization on the reproductive success (ability to produce progeny), and sustainability of fish populations remains a major area of uncertainty (Sumpter and Jobling, 2013).

The most thoroughly studied populations of feminized male fish are the roach (*Rutilus rutilus*) in UK rivers, where the degree of feminization correlates with the density of MWWTPs (Jobling *et al.*, 1998). Feminized roach have been characterized as having smaller gonads, intersex condition, and high plasma VTG levels (Jobling *et al.*, 1998). When studies were conducted to determine the reproductive fitness of these fish, feminized males displayed decreased gamete quality (lower sperm density) and diminished fertilization success (Jobling *et al.*, 2002a; Jobling *et al.*, 2002b). Additionally, a competitive breeding trial demonstrated that male roach with intersex condition were less reproductively successful than roach without this intersex condition (Harris *et al.*, 2010). The combination of wide spread feminization of roach in UK rivers, and these findings of decreased fertilization success of feminized males suggests that there might be population level effects of estrogen exposure in these systems. Despite decades of exposure and decreased male reproductive function, roach populations near treatment plants displayed no difference in the amount of genetic diversity compared to populations of roach from a reference area (Hamilton *et al.*, 2014).

This suggests that while estrogen may reduce roach reproductive fitness, this does not impede genetic exchange or seem to endanger the ability of this species to thrive in their environment.

In contrast, the major finding of a controlled whole lake exposure to environmentally relevant levels of 17α-ethinylestradiol (EE2; a component of the birth control pill) over three years was the elimination of a population of fathead minnow, *Pimephales promelas* (Kidd *et al.*, 2007; Kidd *et al.*, 2014). Prior to the crash of the fathead minnow population, an increase in male plasma vitellogenin (VTG), a decrease in relative male gonad size and histological presence of intersex were noted (Kidd *et al.*, 2007; Palace *et al.*, 2002). This group hypothesized that the population crash of fathead minnows was caused by the inability of males to fertilize eggs. Pearl dace (*Margariscus margarita*) that were also present in the EE2 exposed lake demonstrated similar feminization (elevated VTG levels, presence of intersex), but the dace population was found to have only minor decreases in recruitment (Palace *et al.*, 2009; Palace *et al.*, 2006). This study suggests that there is a potential for estrogen exposure to negatively affect the sustainability of a population of fish.

There have been reports of at least 30 species of fish demonstrating feminization of males due to MWWE exposure (Bahamonde *et al.*, 2013). Few studies, however, have investigated whether this feminization leads to impaired reproductive success. This has led to a gap in our understanding of the links between observations of feminization at the individual level, and effects of estrogen exposure at the population level in wild populations of fish. Thus attaining information concerning whether feminized fish in the wild are capable of reproducing is essential in order to assess the actual level of risk of estrogen exposure to fish.

Previous studies have demonstrated feminization of male rainbow darter (*Etheostoma caeruleum*) caught near the outfalls of the larger MWWTP's in the Grand River watershed in southern Ontario (Bahamonde *et al.*, 2014b; Tanna *et al.*, 2013; Tetreault *et al.*, 2011). Decreased testis size, a high incidence of intersex (>80%), decreased androgen production, and elevated *vtg* expression in the liver was found in male rainbow darter (Bahamonde *et al.*, 2014b; Tanna *et al.*, 2013; Tetreault *et al.*, 2011). Despite this feminization, there is still a population of fish present, although at lower densities, downstream of the largest MWWTP (Kitchener) in the Grand River (Tetreault *et al.*, 2013). This population of fish with high incidence of feminized males presents a unique opportunity to study the link between MWWE exposure, feminization, and population level impacts. Therefore, the aim of this study was to assess whether MWWE exposure results in impaired

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reproduction, which in turn may lead to population level effects, by determining the reproductive success of rainbow darter through an urban gradient in the Grand River.

2.3 Methods

The following studies were conducted on rainbow darter in the Grand River watershed in southern Ontario, Canada. Rainbow darter is a small bodied, short lived (up to four years) benthic species of fish. They reach sexual maturity at one year of age and spawn asynchronous clutches in the spring from April to June. They are ubiquitous in riffle habitats across the watershed making them a good sentinel species. Reproductive success was assessed along the urban gradient of Kitchener-Waterloo to determine the effect of feminization in rainbow darter. Gametes (eggs and milt) collected from wild caught rainbow darter were fertilized and monitored for fertilization success and survival to hatch. This experiment was conducted in spring 2012, and repeated in spring 2013 with additional sites in 2013. All procedures adhered to guidelines for animal use set by the Canadian council for animal care and were approved by the University of Waterloo Animal Care Committee under AUPP 10-17.

2.3.1 Treatment plant description and site selection

The Grand River watershed is host to a population of approximately one million people that are serviced by 30 MWWTPs. The majority of the population is in the central reaches of the watershed and served by two wastewater treatment plants close in proximity.   The smaller of the two plants, Waterloo, services ~125,000 people and its outfall is ~21 km upstream of the Kitchener MWWTP outfall which services ~225,000 people. At the time of the study the Waterloo and Kitchener wastewater treatment plants were secondary activated sludge plants with low solids and hydraulic retention times that resulted in non-nitrifying conditions. Although these plants are currently undergoing major upgrades to achieve nitrification, the current study was conducted before the majority of upgrades was completed. Previous studies of the Kitchener MWWTP effluent note that ammonia concentration in the effluent frequently exceeds 20 mg/L (Cooke, 2006). A description of the treatment plant processes and chemical profile (including selected pharmaceuticals) of the surface waters of the Grand River are reported by (Arlos *et al.*, 2014b).

For the initial study, three sites were selected in 2012 (Figure 2.1) in order to determine whether wastewater discharges impact the reproductive success of rainbow darter. A site 1 km downstream of the Kitchener MWWTP (DSK) was chosen to test for near-field downstream impacts. A site 1 km upstream of the Kitchener MWWTP outfall serves as an upstream reference (USK) and a site upstream of the city-limits serves as a reference for urban impacts (R). The study was repeated in 2013 and a second treatment plant (Waterloo), which is located between the rural reference site (R) and the Kitchener MWWTP, was included in the study. The addition of this plant was included in order to determine if any of the impacts observed in 2012 were due to downstream effects of the Waterloo MWWTP. Two sampling sites were added to assess the effects of the Waterloo MWWTP; a site 1 km downstream (DSW) and 1 km upstream (USW) of the outfall. In addition, a second downstream site located 3 km from the Kitchener MWWTP outfall was added in order to observe the far field impacts of wastewater effluent (DSK2).

2.3.2 Fish collection

Rainbow darter were collected from each of the three sites in 2012 three separate times (during the breeding season (April 17-18, May 10-11, May 24-25) in order to obtain a sufficient sample size. In 2013, fish were collected from all six sites over the course of one week in early May (May 6-11). Timing of sampling varied between 2012 and 2013 due to differences in flow and weather conditions, which is variable during this time of year. Fish collected with a backpack electrofisher (Smith Root, LR-20) were held in aerated buckets and sampled on site in a mobile sampling trailer. Length ($\pm$0.1 cm) and weight ($\pm$0.01 g) were recorded prior to the collection of gametes. Milt was collected using a heparinized capillary tube and gentle massaging of the abdomen. Care was taken to avoid collecting urine and feces. After collection, milt volume was measured, and then milt was placed in a labeled 0.5 mL centrifuge tube and kept on ice until used for fertilization and assessment of sperm density. Eggs were collected from females by massaging their abdomen and carefully collecting eggs with forceps. Eggs were placed into petri dishes, photographed with a stereomicroscope (S6, Leica Microsystems, Wetzlar, Germany), and then immediately fertilized (see 2.6). After the gametes were collected, fish were sacrificed and gonads were removed from the fish and weighed ($\pm$0.001 g). In 2012, all testicular and ovarian tissue was kept for histological analysis. In 2013, testes were separated into two lobes and placed in either an excess of Media 199 (Sigma–

Aldrich, St. Louis, MO, USA, pH = 7.6) on ice for the determination of *in vitro* steroid production, or in a histology cassette in Davidsons' solution for histological processing. If total testis weight was less than 20 mg, then both lobes were allocated towards histological analysis.
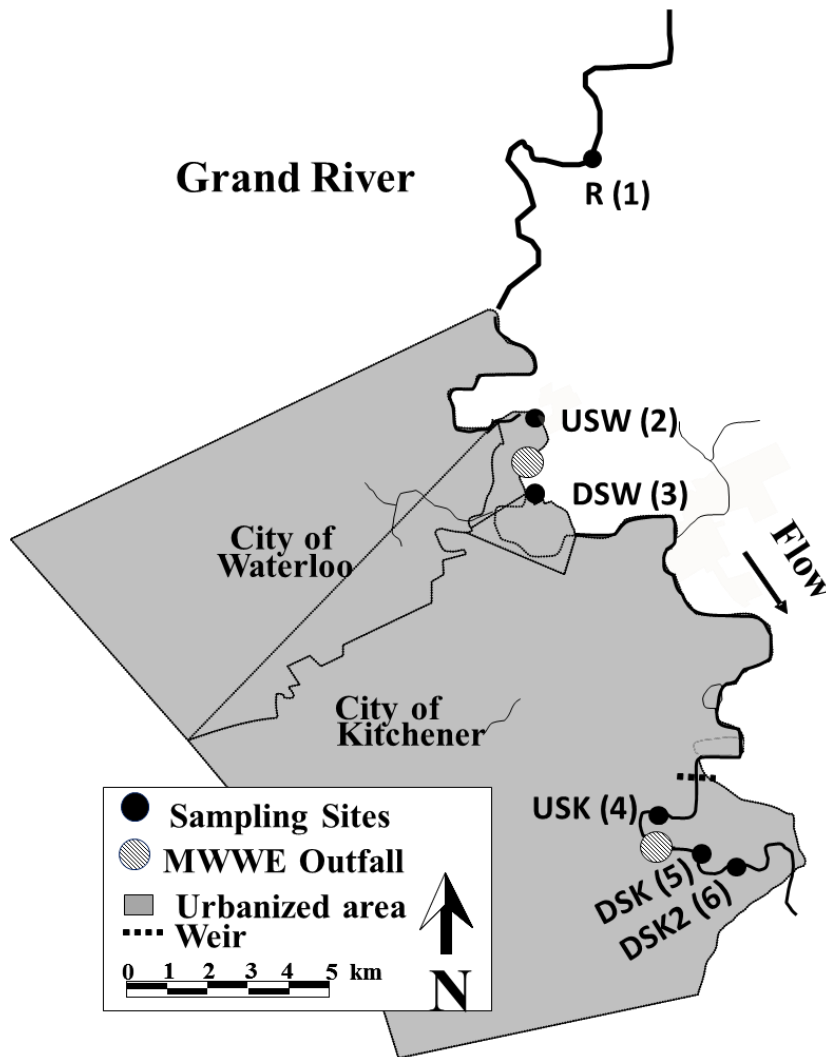
Figure 2.1 Map of sampling area in the Grand River Watershed (southern Ontario, Canada) used for this study. One rural reference (R), two upstream (US) sites, and three downstream (DS) sites were chosen to test if reproductive success of rainbow darter is impacted by municipal wastewater effluent (MWWE).

2.3.3 In vitro steroid production

Single lobes of testes were weighed and tissue placed (to a maximum of 20 mg) into a single well of a 24-well cell culture plate with 1 mL of Media 199. Steroid production was stimulated with 10 IU of human chorionic gonadotropin (Sigma-Aldrich) dissolved in Media 199. Plates were incubated at 16°C for 24 hours. The Media 199 was then removed from the plate and placed into a 1.5 mL centrifuge tube at -80°C for future analysis of testosterone (T) and 11-ketotestosterone (11-KT) by enzyme linked immune-assay (EIA) performed as per kit instructions (Cayman Chemical Company, Ann Arbor, MI, USA).

2.3.4 Histology

After being fixed in Davidson's for 48 h, samples transferred and stored in 70% ethanol until they were dehydrated and embedded in paraffin wax. Forty, 5 µm thick sections were placed onto slides with slide mount, and then stained with hematoxlin and eosin. Males were analyzed for presence and severity of intersex, while the relative proportion of ovarian cell types was determined in female samples.

Intersex condition is classified as the presence of ovarian tissue (oocytes) in predominantly male gonads. Scanning for intersex was conducted by a single trained technician using a Leica DM100 light microscope (at 100 x magnification). The total number of primary oocytes found from 40 sections was tallied. Males were given an intersex severity score from 0 to 7. The scoring system for determining intersex severity in male rainbow darter was based on oocytes number, oocyte development, and proportion of ovarian tissue versus testicular tissue (Table 2-1). Rationale for the scoring system is described in detail by Bahamonde *et al.* (2014a).

For each female, an entire cross section of the ovary was photographed at 5 x magnification and images stitched using Leica Application Suite (v 4.4.0). Oocytes with nuclei were classified as primary, cortical alveolar, early vitellogenic, mid vitellogenic, or late vitellogenic. All classified cells as well as all mature or atretic oocytes and all post-ovulatory follicles (POF) were counted using imaging software (Image J 1.43o8, National Institutes of Health, USA) and the relative proportion of each cell type was determined for all females collected.

Table 2-1 Scoring system for determining severity of intersex condition in rainbow darter (*E. caeruleum*) testes. Scoring system is primarily based on the number of primary oocytes (PO) found in forty sequential H&E stained 5 μM sections (modified from Bahamonde *et al.,* 2015).

Score	# primary oocytes	Other characteristics
0	0	Normal testis
1	1-3	-
2	4-10	-
3	10 +	-
4	-	Cortical alveolar oocytes;
5	-	< 50% ovarian tissue
6	-	Vitellogenic oocytes; > 50% ovarian tissue (Figure 2.6)
7	-	100 % ovarian tissue

2.3.5 Fecundity (eggs and sperm)

The number and size (in 2013 only) of eggs stripped from each female were determined from pictures collected in the field using imaging software (ImageJ). Sperm density (billions of sperm / mL) was assessed using a Cell-Vu sperm count chamber (Millennium Sciences, New York) as per instructions with the following modifications. Milt was diluted 1000x with ddH$_2$O and 5 µL of this diluted solution was placed onto a slide with a cover slip and the number of sperm in each of 10 squares was counted.

2.3.6 Fertilization success and survival of progeny

In order to assess the fertilization success of fish at each site, collected gametes were combined as follows. Eggs collected from each female were divided into 3 clusters of ~20 eggs and placed in a 6 well cell culture plate. Milt was diluted 20 x with fish Ringers solution (NaCl, 111 mM; KCl, 3.35 mM; NaHCO$_3$, 2.38 mM; CaCl$_2$, 2.7 mM), and 20 µL of this solution was immediately pipetted onto each well of eggs, gently mixed, and allowed to rest for 2 minutes. After the two-minute fertilization period, eggs were hardened with a sterile salt based 'egg water' (Westerfield, 1995), transported to the University of Waterloo, and placed in an incubator at 16°C. Successful fertilization was determined as survival to 24 hours post fertilization. Egg water treated with methylene blue (0.03

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M) was changed daily and any dead embryos (identified as white throughout) were removed. Survival was monitored until hatch (11 -14 days post fertilization). Survival to hatch is denoted as the proportion of fertilized embryos that survived until hatch (%).

2.3.7 Statistics

All statistical tests were performed using SigmaPlot 12.3. A one-way analysis of variance (ANOVA) with a Tukey post-hoc test was performed on all normal data to test for differences between sites in reproductive health measures, gamete quality, and reproductive success. Data that was not normal was log transformed to achieve normality, or if this was not successful, a Kruskal-Wallis test was conducted and Dunns' method was employed to test for differences between sampling sites. To determine if there were any correlations between reproductive health measures and fertilization success or survival to hatch, a multivariate regression analysis was performed, where fertilization success or survival to hatch was the dependent variable, and egg size, male GSI, intersex index, or 11-KT production were the independent variables. Outliers (values greater or less than two times the quartile range from the mean) were eliminated from normally distributed data sets (~ 1 in 20).

2.4 Results

The number of fish used in this study varied among sites due to difficulties in finding females with ovulated oocytes as well as difficulty finding mature males with available milt. In 2012, between 35 and 49 fish of each sex were collected from each site in total (three sampling time points). In 2013, between 13 and 25 fish were collected from each site in total (one sampling time point). Despite efforts to normalize the length of fish, the size of fish caught also varied among sites (Table 2-2).

2.4.1 Reproductive health

The relative gonad size of males was found to be smaller downstream of the Kitchener WWTP at DSK compared to the rural reference site (R) in 2012 and 2013 (Table 2-2). LSI of male rainbow darter was not impacted by MWWE exposure. Condition (K) of male fish was higher at DSK compared to the site upstream of the Kitchener WWTP (USK). As for androgen production, *in vitro* 11-KT and T production were lower in males collected surrounding the Kitchener MWWTP in 2013 compared to R (Figure 2.2). Analysis of gonad histology found that a large proportion of males (up

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to 80% of those collected) had presence of oocytes in their testes at USK and DSK in 2012 and at USK, DSK, and DSK2 in 2013 (Figure 2.3). Few males were found to have intersex surrounding the Waterloo MWWTP. The mean intersex severity was found to be higher in males collected from sites in the vicinity of the Kitchener MWWTP in 2012 (USK (1.15±0.25), DSK (1.13±0.24)) and 2013 (USK (0.95±0.26), DSK (2.62±0.46), DSK2 (1.93±0.40) than males from the rural reference site in 2012 (0.10±0.06) or 2013 (0; Figure 2.3).

Female rainbow darter were found to have comparable GSI among sites in 2013, however females caught at DSK in 2012 had larger relative gonad size compared to R (Table 2-2). LSI was elevated in females collected downstream of both the Waterloo and Kitchener sites compared to USK in 2013 and females caught at DSK in 2012 had elevated LSI compared to the rural reference (R) in 2012. K of females at USK was lower than all other sites in 2013, and was lower than DSK in 2012 (Table -2-2). Females collected near the Waterloo MWWTP had similar proportions of ovarian oocyte development in comparison to fish from the rural reference site (R). Fish collected downstream of the Kitchener MWWTP (DSK) had a larger proportion of vitellogenic (2012) or mature oocytes (2013), a larger proportion of atretic oocytes (2013), and a smaller proportion of post ovulatory follicles (2012 and 2013) compared to fish collected from R (Figure 2.4).

Table 2-2 Comparison of mean (± SE) gonad and liver somatic indices (GSI and LSI respectively), as well as condition (K) of male and female rainbow darter collected from the central Grand River, Ontario, Canada in spring 2012 (May 10-11 only; n=15-18) and spring 2013 (n = 13–25). Sites included a rural reference (R), a site upstream and downstream of the Waterloo MWWTP (USW, DSW), as well as one site upstream and two sites downstream of the Kitchener MWWTP (USK, DSK, DSK2). Presence of a common letter between sites indicates that they are not significantly different (p < 0.05) as determined by a one-way ANOVA with a Tukey post-hoc test.

Season	Site	Sex	Length	Weight	GSI	LSI	K
Spring 2012	R	M	6.3 ± 0.1 A	2.95 ± 0.16 A	1.39 ± 0.08 A	1.74 ± 0.12 A	1.16 ± 0.02 A
		F	5.6 ± 0.1 a	2.15 ± 0.17 a	12.80 ± 0.60 a	4.91 ± 0.13 a	1.19 ± 0.02 ab
	USK	M	5.2 ± 0.1 B	1.48 ± 0.16 B	1.33 ± 0.09 AB	1.65 ± 0.13 A	1.00 ± 0.02 B
		F	5.5 ± 0.1 a	2.04 ± 0.15 a	13.52 ± 0.63 ab	4.52 ± 0.1 ab	1.16± 0.02 a
	DSK	M	5.5 ± 0.1 B	1.87 ± 0.18 B	1.14 ± 0.10 B	1.82 ± 0.14 A	1.03 ± 0.03 B
		F	6.2 ± 0.1 b	3.12 ± 0.16 b	15.46 ± 0.57 b	4.25 ± 0.12 b	1.27 ± 0.02 b
Spring 2013	R	M	5.9 ± 0.1 A	2.61 ± 0.16 A	1.33 ± 0.04 A	1.59 ± 0.05 A	1.21 ± 0.02 A
		F	5.3 ± 0.1 ab	1.79 ± 0.15 ab	10.07 ± 0.26 a	4.47 ± 0.11 ab	1.14 ± 0.02 ab
	USW	M	5.4 ± 0.2 A	1.91 ± 0.23 AB	1.21 ± 0.05 AB	1.20 ± 0.07 B	1.16 ± 0.03 A
		F	5.4 ± 0.1 ab	2.01 ± 0.18 ab	9.30 ± 0.34 a	4.49 ± 0.16 ab	1.22 ± 0.03 a
	DSW	M	5.9 ± 0.2 A	2.41 ± 0.23 A	1.21 ± 0.07 AB	1.54 ± 0.75 AB	1.18 ± 0.03 A
		F	5.0 ± 0.1 a	1.51 ± 0.17 a	9.51 ± 0.31 a	4.80 ± 0.11 b	1.15 ± 0.02 ab
	USK	M	5.0 ± 0.2 B	1.29 ± 0.18 B	1.25 ± 0.06 AB	1.36 ± 0.07 AB	1.01 ± 0.02 B
		F	5.0 ± 0.1 a	1.47 ± 0.18 a	9.52 ± 0.41 a	4.23 ± 0.13 a	1.09 ± 0.02 b
	DSK	M	5.9 ± 0.1 A	2.49 ± 0.17 A	1.13 ± 0.05 B	1.46 ± 0.09 AB	1.12 ± 0.02 A
		F	5.3 ± 0.1 b	2.25 ± 0.15 b	9.79 ± 0.47 a	4.88 ± 0.11 b	1.19 ± 0.02 a
	DSK2	M	6.1 ± 0.2 A	2.78 ± 0.23 A	1.11 ± 0.05 B	1.37 ± 0.06 AB	1.16 ± 0.04 A
		F	5.4 ± 0.2 ab	2.07 ± 0.19 ab	9.35 ± 0.39 a	4.37 ± 0.09 ab	1.22 ± 0.03 a
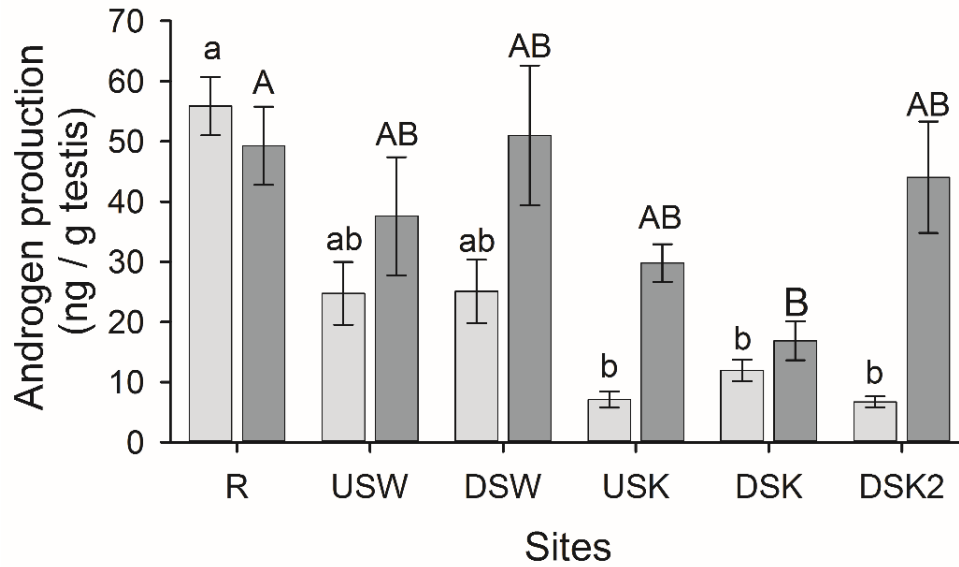
Figure 2.2 Production of 11-ketotestonerone (light grey) and testosterone (dark grey) by rainbow darter testis tissue *in vitro* (n = 8-15) in 2013. Letters not shared between bars are significantly (p <0.05) different from one another as determined by a Kruskal Wallis test with Dunn's method.
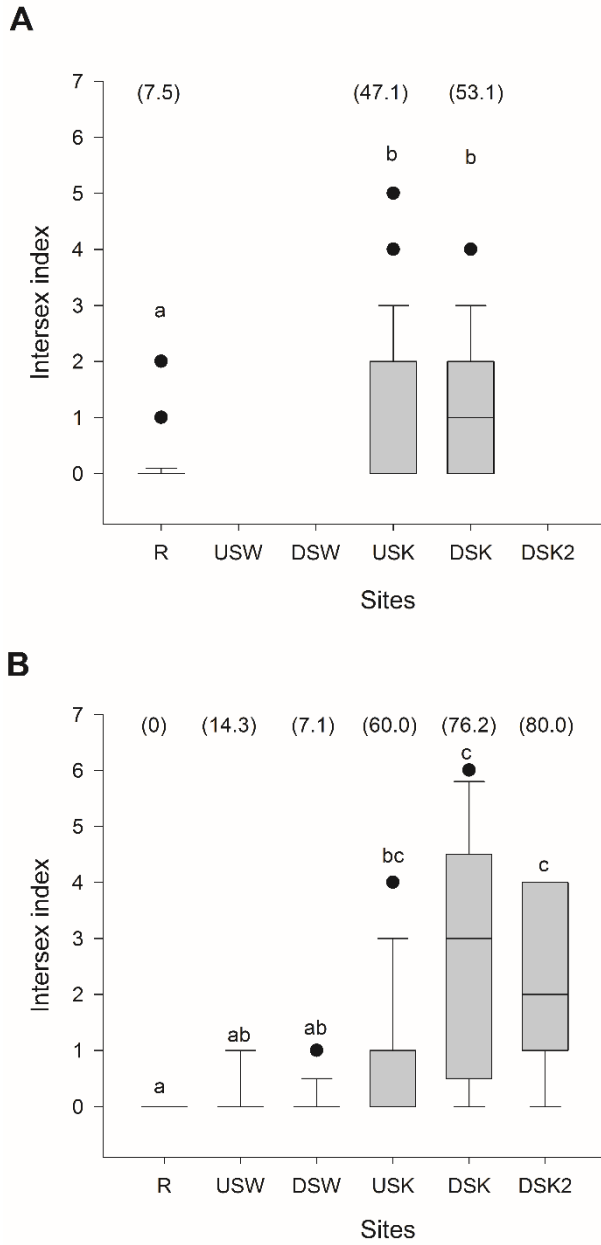
Figure 2.3 Intersex severity (box and whisker plot with outliers (●)) of male rainbow darter populations collected along an urban gradient in (A) spring 2012 (all time points; n = 32-40) and (B) 2013 (n = 14-21). The incidence (% of population) of males displaying intersex is displayed above the bars for each site. Letters not shared between bars are significantly (p <0.05) different from one another as determined by a Kruskal-Wallis one-way ANOVA on Ranks and Dunn's method post-hoc test.
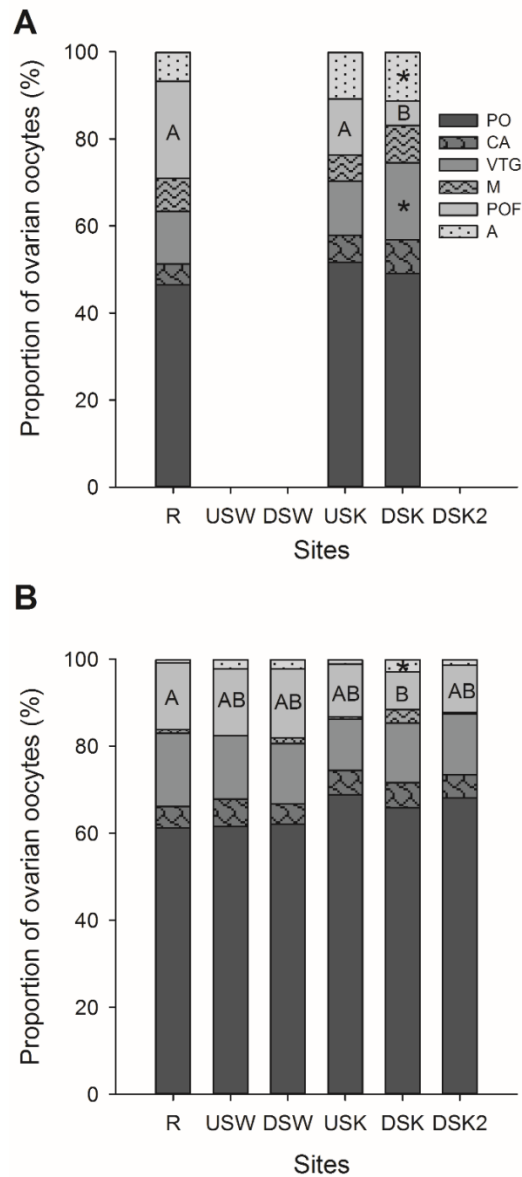
Figure 2.4 Stage of ovarian cell development of female rainbow darter collected from the Grand River in (A) spring 2012 (n =19-23) and (B) 2013 (n = 15-25). Ovaries contained six ovarian cell types including primary oocytes (PO), cortical alveolar oocytes (CA), vitellogenic oocytes (V), mature oocytes (M), post ovulatory follicles (POF) and atretic oocytes (A). Bars that do not share a letter had significantly different proportions of POFs, and * denotes significant (p<0.05) difference in the proportion of cells from the rural reference site (R), as determined by a one-way ANOVA with a Tukey's post-hoc test.

2.4.2 Fecundity

Fecundity was assessed by measuring milt volume and sperm density in males, and egg number and egg diameter in females. Milt volume was found to be greater in males collected downstream of the Kitchener MWWTP compared to the site upstream of the cities (R; Figure 2.5A, B). Milt volume was found to be positively correlated with intersex severity (R^2 =0.275, p<0.001, DF = 93; Figure 2.5C). Only the most severely impacted fish found did not produce any milt (Figure 2.5C, Figure 2.6). Sperm density ranged from 13 – 17 billion sperm / mL milt, and did not differ among (or within) sites in 2012 or 2013 (p>0.05).

A strong positive correlation was found between total length, and the number of eggs collected from individual females (Figure 2.7A; R^2 = 0.260, p<0.001, DF =141). Therefore, when comparing the number of eggs per individual among sites, this data was corrected for length. The number of eggs collected from females did not differ among sites in 2012, however, more eggs were collected from females downstream of the Kitchener MWWTP (DSK) compared to USW and USK in 2013 (Figure 2.7B, D). Eggs collected from sites downstream of the first treatment plant (DSW, USK, DSK and DSK2) were smaller in diameter than eggs collected from R and USW females (Figure 2.7C).
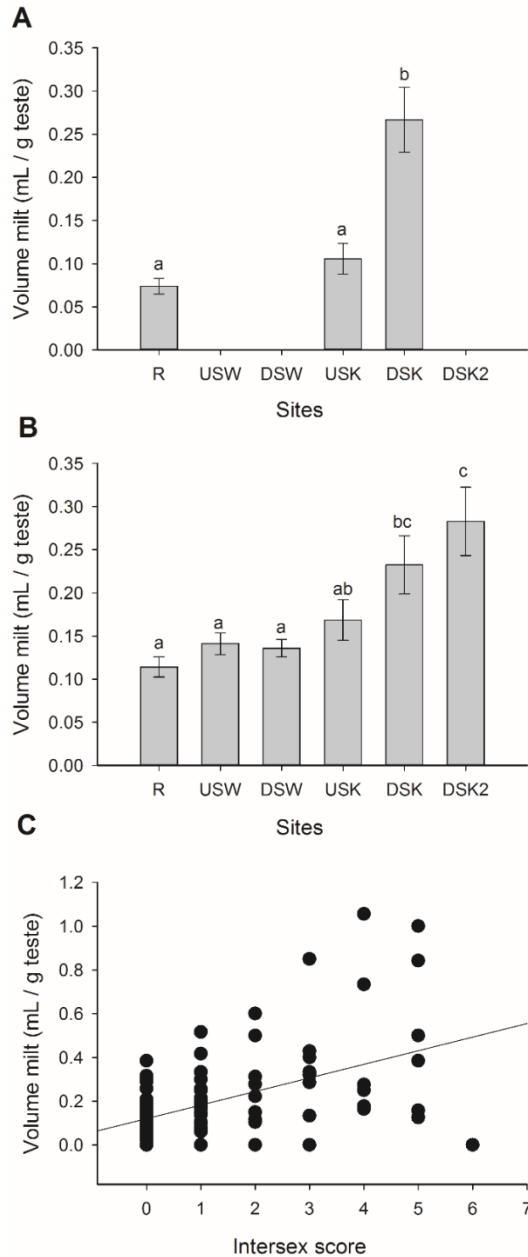
Figure 2.5 Fecundity of male rainbow darter was measured in (A) 2012 and (B) 2013. Milt volume (A, B; n = 27-35, n = 10-22) increased in urbanized areas. A positive correlation (R^2 = 0.275, p <0.001) was found between intersex severity and volume of milt collected from rainbow darter (C; n =111). Letters not shared between bars are significantly (p <0.05) different from one another as determined by a one-way ANOVA and Tukey post-hoc test.
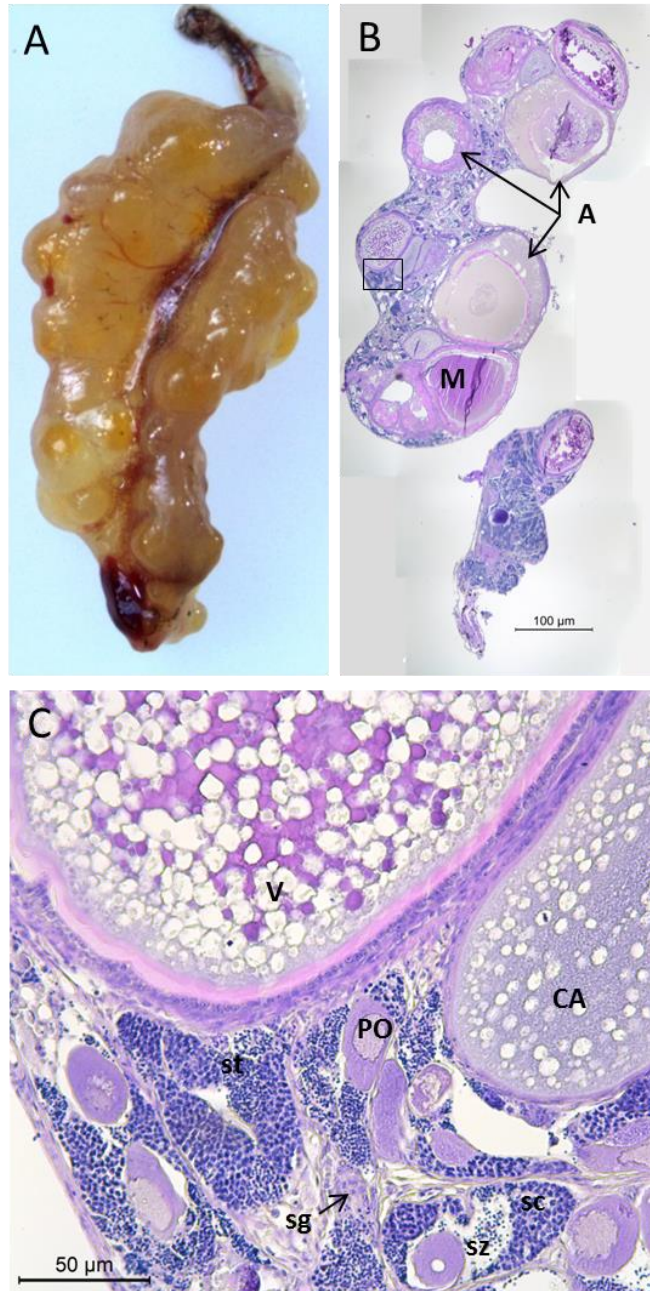
Figure 2.6 Macroscopic (A) and microscopic (B, C) depiction of a gonad displaying severe intersex condition (index score of 6) in rainbow darter. Male cell types such as spermatogonia (sg), spermatocytes (sc), spermatids (st), and spermatozoa (sz), and female cell types such as primary oocytes (PO), cortical alveolar oocytes (CA), vitellogenic oocyte (V), mature oocytes (M), and atretic oocytes (A) were all noted in this specimen.
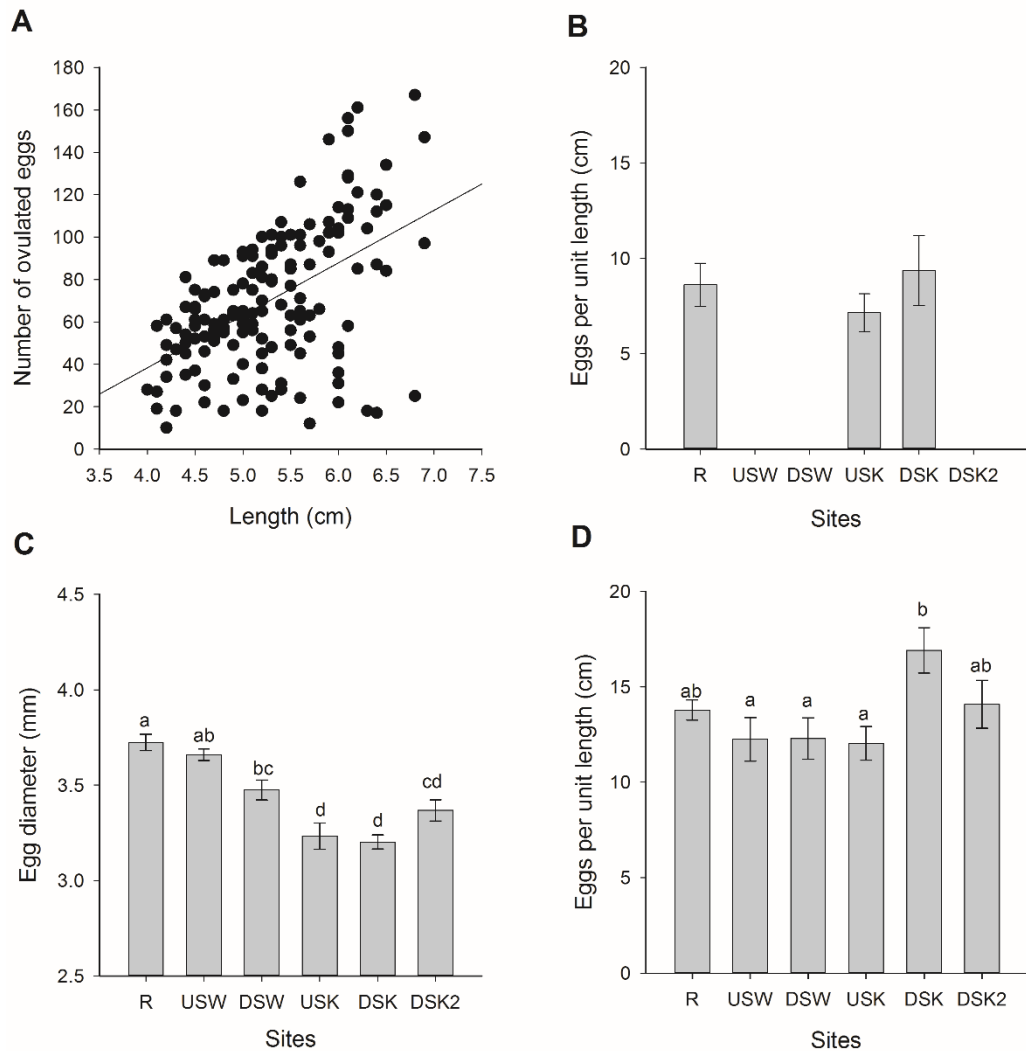
Figure 2.7 Fecundity of female rainbow darter collected from sites in the central Grand River, ON, Canada. A positive relationship between the number of ovulated eggs present in an individual and her length was found (A; R2 = 0.260, p<0.001, n = 143). Egg number corrected for length was compared among sites in 2012 (B; n =18-22) and 2013 (D; n =16-25). Egg diameter was compared among sites in 2013 and the number of eggs per unit length (C, n = 16-25). Bars that do not share a common letter are significantly (p<0.05) different from one another as determined by a one-way ANOVA and Tukey's post-hoc test.

2.4.3 Fertilization success and embryo survival to hatch

Fertilization success was found to be lower for fish collected from DSK (5) in 2012 and 2013 compared to R (1). While fertilization success was lower at USK (4) compared to R (1) in 2012, this trend was not significant in 2013 (Figure 2.8A, C). Survival of fertilized embryos to hatch from fish collected from all sites in the urban area was lower compared to embryos of fish collected at R (1) in both years (Figure 2.8B, D). To address the question of whether intersex severity is indicative of reproductive success, fertilization success was compared among "normal" males (those with zero intersex), moderately feminized males (those with an intersex severity score of 1-3), and severely intersex males (those with an intersex score of 4-6). Severely intersex males had lower fertilization success compared to normal and moderately feminized males, but survival to hatch was comparable among all groups (Figure 2.9 A).

No differences were found in the fertilization success or survival of embryos from the rural reference site (R) between 2012 and 2013, allowing for the comparison of data across years. To test for the presence of relationships between reproductive health measures and reproductive success, data from 2012 and 2013 were pooled because correlational trends observed were similar in both years. A multiple regression analysis was performed to determine whether there were significant relationships between fertilization success or embryo survival to hatch with female or male reproductive health measures. Egg size was not correlated with egg fertilization or with embryo survival to hatch, nor were male reproductive health measures correlated with embryo survival to hatch ($p>0.05$). In contrast, negative correlations were observed between male GSI and fertilization success ($R^2 = 0.050$, $p<0.01$, DF = 110) as well as between intersex severity and fertilization success (Figure 2.9 B; $R^2 = 0.0303$; $p=0.035$, DF = 143).
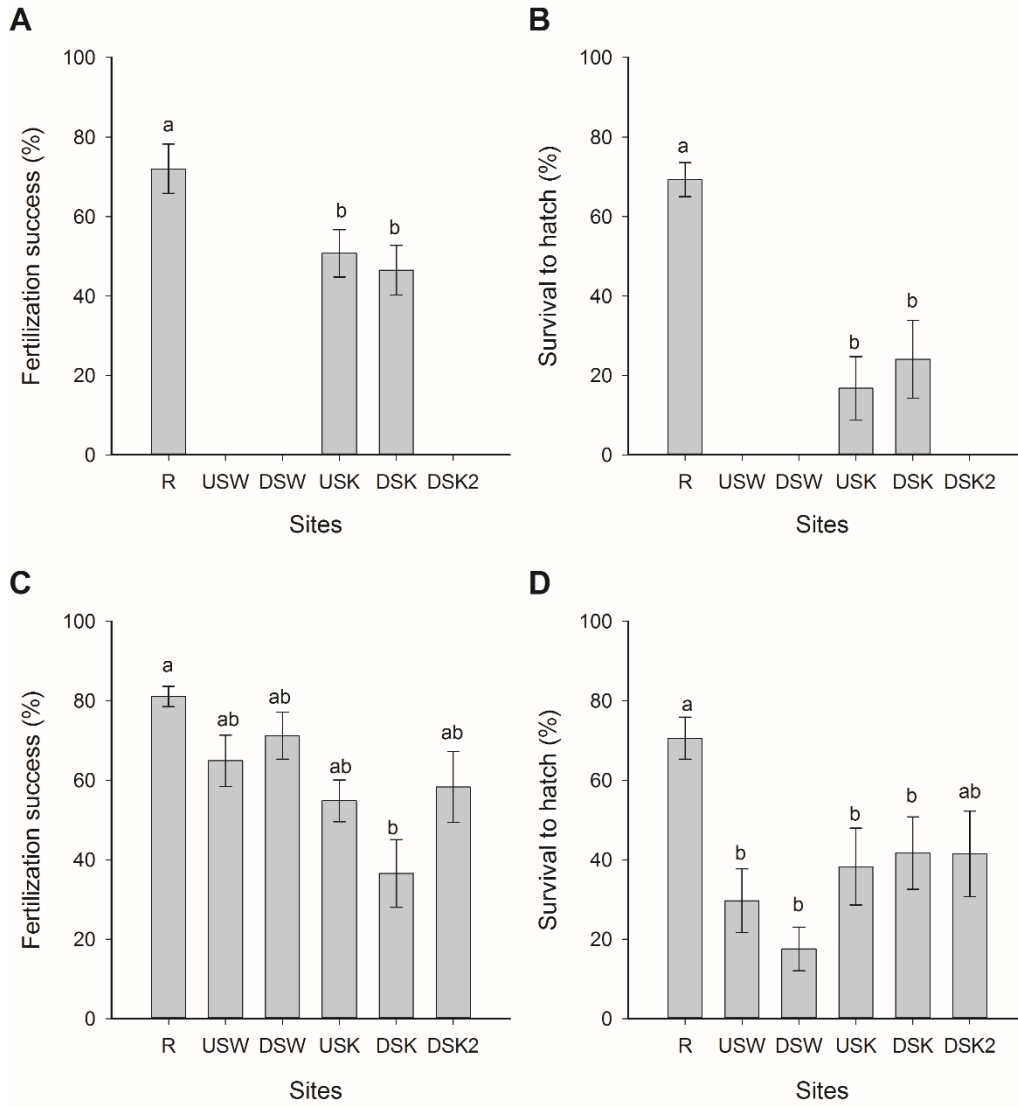
Figure 2.8 Reproductive success of rainbow darters collected near MWWTPs or a reference site (R) was determined in spring 2012 (A, B) and 2013 (C, D). Fertilization success (A, C; n = 17-19, n = 10-17), defined as survival to one day post fertilization, and survival of fertilized eggs to hatch (B, D; n = 15-18, n = 10-15) were the measures chosen to determine reproductive success. Bars that do not share a common letter are significantly ($p<0.05$) different from one another as determined by a one-way ANOVA and Tukey's post-hoc test.
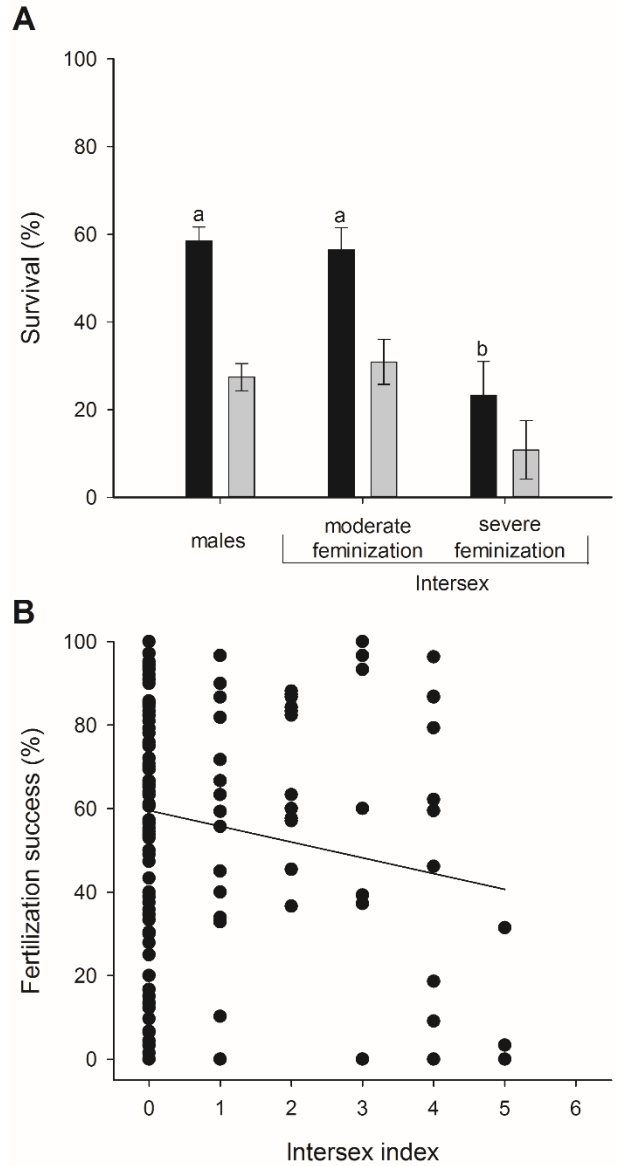
Figure 2.9 Comparison of reproductive success between normal, moderately feminized (intersex score 1-3) and severely feminized (intersex score 4-6) males (A). Fertilization success (black bars) and embryo survival to hatch (grey bars) were the measures used to indicate reproductive success (n=17-91). Regression analysis of pooled data from 2012 and 2013 demonstrated a negative correlation between fertilization success and intersex severity (B; R2= 0.0303; p=0.035, n = 144). Bars that do not share letters are significantly different (p<0.05) as determined by a one-way ANOVA with a Tukey's post-hoc test.

2.5 Discussion

In this study, we found that male rainbow darter collected downstream of a large MWWTP (Kitchener), but not a medium sized MWWTP (Waterloo), had reduced reproductive health measures including decreased gonad size, increased incidence and severity of intersex, and decreased androgen production. Fecundity differed among sites, with fish collected downstream of the large MWWTP having increased milt volume and increased egg number. In contrast, egg size was smaller and fewer post-ovulatory follicles were found in ovaries of females caught downstream of the large MWWTP. Additionally, sperm density did not differ between sites. When gametes of fish caught near the medium sized MWWTP were manually fertilized, the observed fertilization success was unchanged compared to the upstream and reference sites, but there was reduced survival of the progeny to hatch. Fish collected from the areas up- and downstream of the large MWWTP had consistently lower fertilization success and survival to hatch. A negative correlation between intersex severity and fertilization success was found, but no other relationships between reproductive health measures and reproductive success were found.

2.5.1 Reproductive health

The impaired reproductive health measures found in male rainbow darter collected at the sites downstream of the Kitchener MWWTP, DSK (5) and DSK2 (6), sites compared to urban reference, R, agree with what has been found in previous studies of this system (Tetreault *et al* 2011; Tanna *et al.* 2013). While smaller relative gonad size has been found intermittently dependent upon season and year, impaired sex steroid production and the presence of intersex has been consistently found downstream of the Kitchener MWWTP (Bahamonde *et al.*, 2014b; Tanna *et al.*, 2013; Tetreault *et al.*, 2011). Additionally, male rainbow darter collected downstream of the Kitchener MWWTP have elevated levels of liver vitellogenin expression, a yolk protein precursor that is normally highly expressed in females, but not males (Bahamonde *et al.*, 2014b). These male reproductive health measures suggest exposure to estrogenic and/or anti-androgenic compounds. This is supported by chemical analysis of whole effluent and water samples taken from the same sites as fish collection. Previous analysis of the estrogenicity using a Yeast Estrogen Screen (YES) of effluent from the Waterloo and Kitchener MWWTPs found estrogen equivalencies of 4.32 and 16.99 ng/L respectively (Tanna *et al.*, 2013). An effects directed assessment (EDA) using a YES also identified 17β-estradiol, estrone and EE2 as the major estrogens in these effluents. Analysis of effluent taken from these same plants as well as water from the Grand River for pharmaceutics has identified a variety of pharmaceuticals including several anti-androgens such as triclosan in the Grand River (Arlos *et al.*, 2014b).

In this study, the incidence of intersex in the male fish collected was up to 80%. A high intersex incidence (between 80 -100% of male fish) has also been found in some areas of the United Kingdom and

United States. Studies of wild roach (Bjerregaard *et al.*, 2006; Jobling *et al.*, 2009; Jobling *et al.*, 1998)), and largemouth bass (*Micropterus salmoides)* (Blazer *et al.*, 2012; Blazer *et al.*, 2014; Blazer *et al.*, 2007) found that intersex severity was dependent upon the density of human and animal populations in the watershed. We observed the same trend in this study, where the most densely populated area (Kitchener) was correlated with the highest score of intersex severity.

The benthic habit of rainbow darter might make them more susceptible to the effects of MWWTP compounds. Blazer *et al.* (2014) found that a higher number and concentration of estrogenic compounds were found in sediments compared to the water column in Pennsylvanian watersheds. In addition to adults interacting with sediment, increased contact with sediment occurs during early development, since eggs are deposited in the gravel of riffle areas. This exposure may explain the female dominated sex ratios and examples of extreme intersex condition (Figure 2.6) occasionally observed at this site. While measurements of pharmaceuticals have been made in the water column (Arlos *et al.*, 2014a), no measurements have been made in the sediments at this site. This is an interesting area for future work.

Somatic indices are reported for females at the time of collection. Although some differences were noted in these measures, they are hard to interpret since the gonad and liver sizes are changing rapidly during spawning. In general, the GSI suggest that all fish collected were spawning since they were lower than 20% (observed GSI just prior to spawning; Fuzzen, unpublished data). Additionally, post-ovulatory follicles were present in most females, also indicating that they were caught during spawning. It is important to note that many female rainbow darter were caught at each site, however only females with eggs that could be easily stripped were included in this study. Anecdotally, fewer females (~ 1 in 10) collected downstream of the Kitchener MWWTP (DSK) had eggs that could be easily stripped compared to females collected from the reference site (~ 1 in 3). We did not expect to see differences in ovulation among sites since previous studies have not found differences in ovarian maturation (Bahamonde *et al.*, 2014a; Tetreault *et al.*, 2011). Thus this study was not designed to compare proportion of females with available eggs.

2.5.2 Fecundity

While a larger number of smaller eggs were collected from females at DSK (5) than USK (4) in 2013, we also found a lower proportion of post-ovulatory follicles in females collected from DSK. Additionally, a larger proportion of atretic follicles were present in female ovaries collected from DSK to females from R. Together these data, along with the anecdotal observation that fewer females collected from DSK had eggs available for collection, suggest that ovulation in females downstream of the Kitchener MWWTP is being impaired. A somewhat similar effect was also observed in English sole (*Parophrys vetulus*), where evidence suggested that fish collected from highly urbanized areas had

delayed ovulation and spawning (Johnson *et al.*, 2008). Studies of killifish (*Fundulus heteroclitus*) from Newark Bay, NJ, found that females from a contaminated site (legacy compounds and MWWE) had reduced ovarian maturation and egg production (Bugel *et al.*, 2010, 2011). Similarly, a study of yellow perch (*Perca flavescens*) in the Chesapeake Bay area of Eastern USA observed impaired oocyte maturation in fish from highly populated areas (Blazer *et al.*, 2013). Laboratory studies of zebrafish (*Danio rerio*) and fathead minnow have shown that MWWE decreases the number of eggs laid, and that ammonia specifically impairs ovulation (Filby *et al.*, 2010; Lister *et al.*, 2009; Thorpe *et al.*, 2009). While these studies suggest that there are some possible effects of MWWE on egg production, relatively few studies have focused on the effects of MWWE exposure on female reproductive function in the wild.

When fecundity was measured in male rainbow darter, we found no differences in sperm density from fish collected near either of the MWWTPs. While this is similar to what was found in largescale suckers (*Catostomus macrocheilus*) in the Columbia River, USA (Jenkins *et al.*, 2014), it had been hypothesized that milt density would be reduced in fish exposed to effluent in this study. Decreased androgen production and delayed spermatogenesis have been noted in rainbow darter at this site (Bahamonde *et al.*, 2014a; Tetreault *et al.*, 2011), which was suspected to lead to lower concentrations of mature spermatozoa in milt. Additionally, roach collected from areas near MWWTPs in UK rivers, as well as smallmouth bass (*M. dolomieu*) collected from the south branch of the Potomac River (VA, USA) had lower sperm density than fish from reference areas with low incidence of intersex (Blazer *et al.*, 2012; Jobling *et al.*, 2002a). While sperm density was unchanged, an increase in milt volume was observed through the urban gradient. When compared to intersex severity, a positive correlation was found with milt volume ($R^2 = 0.275$, $p < 0.001$). Since fish were collected in the middle of their spawning season, we cannot be certain whether this positive correlation suggests an increase in milt production, or a decrease in spawning activity due to MWWE exposure.

2.5.3 Fertilization success and survival to hatch

Fertilization success and survival of fertilized eggs to hatch was found to be lower in gametes collected from sites near the Kitchener MWWTP (USK (2012), DSK1, DSK2 (2013)). These were the same sites in which indicators of feminization of males had been observed. Additionally, the sites in which the feminization was more severe (higher median intersex index score, lower 11-KT production) were the sites that had the least reproductive success. While no indicators of feminization (gonad size, 11-KT production, intersex index) were observed near the Waterloo treatment plant, reduced embryo survival (but not fertilization success) was observed.

The decreases in fertilization success and embryo survival of fish from feminized males in this study are similar to what has been found by Jobling *et al.*, (2002b). Roach collected from rivers in the UK

were found to have a 20% decrease in fertilization success between reference fish and fish collected from a heavily urbanized river (Jobling *et al.*, 2002a). When separated by the severity of intersex, it was found that only severe intersex fish (>33% ovarian tissue in gonad, many with secondary oocytes) had reduced fertilization success. A direct negative correlation was found between intersex severity and fertilization success ($R^2 = 0.363$, $p < 0.001$). In addition, when a competitive breeding trial was conducted with wild roach collected downstream of MWWTPs, it was found that males with higher severity of intersex (index ≥ 4) had reduced reproductive success (proportion of hatched progeny sired by them). While we also observed a negative correlation between intersex severity and fertilization success, this correlation was not as strong ($R^2 = 0.170$; $p<0.001$). When fertilization success was compared based on intersex severity, we found that the most severely intersex fish (index ≥ 4) had significantly reduced fertilization success compared to normal (index of 0) and moderate intersex (index of 1-3) males. However, this comparison can be misleading, since the severe intersex fish were all collected from downstream of the MWWTP. When the fertilization success of normal males was compared with severely intersex males from the site downstream of the Kitchener MWWTP (DSK), no differences were found. While this study supports previous studies that males with severe intersex condition will have reduced fertility and reproductive success, our study also suggests that severe intersex condition is only an indicator of chronic exposure. Since males exposed to MWWE that did not display severe intersex also had reduced fertilization success, it is likely that the mechanism of decreased fertility either does not relate to intersex itself, or that there are multiple mechanisms being affected.

Embryo survival to hatch was not correlated with any of the other reproductive health or gamete quality measures in this study. Additionally, reduced embryo survival was noted at all of the urban sites tested. It is possible that the reduced embryo survival is mediated by a different mechanism than reduced fertilization success. Urban runoff contains a large number of potentially harmful compounds, including legacy compounds such as polycyclic aromatic hydrocarbons (Hoffman *et al.*, 1984). Any of these lipophilic compounds which may have been present in the female, would have been deposited into the collected eggs (Niimi, 1983), and could explain the decreased survival of rainbow darter embryos surrounding the MWWTPs (West *et al.*, 2014). Further studies would be required to determine the cause of the observed decreased embryo survival in this study.

2.6 Conclusion

This study confirmed the finding that fish reproduction is impaired in densely populated urban areas. We found that a decrease in fertilization success was found in the same areas as endocrine disruption of males. This suggests that these measures of reproductive health are good predictors of reproductive fitness within a population of fish. In the future, it will be important to determine the long

term impact of this reproductive impairment on the population structure and genetic diversity. It is our hope that this unique opportunity to study a population with severe intersex condition will aid efforts to create a monitoring program for municipal wastewater treatment plants.

Chapter 3

Alteration of reproductive behaviours in rainbow darter (*Etheostoma caeruleum*) collected near municipal wastewater treatment plants

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In submission.

3.1 Overview

Municipal wastewater effluent (MWWE) is a complex matrix of contaminants that is released into aquatic environments. Many constituents of MWWE are endocrine disrupting compounds (EDCs) and have been shown to disrupt the reproductive physiology of fishes. Reproductive behaviour is also susceptible to disruption by EDCs, but few studies have investigated the effects of MWWE on the reproductive behaviour of wild fish. In this study, rainbow darter (*Etheostoma caeruleum*) were collected from sites in the Grand River and reproductive behaviour and physiology were assessed in non-exposed and MWWE-exposed fish. Two behaviour experiments were conducted. First, a competitive breeding experiment was conducted, where one male from each of three field sites and a single female were placed into a breeding tank. Interactions among the males, as well as the behaviours of the female, were assessed in each of two trials (one with a female from a reference site, and the second with a female from an exposed site). The second behaviour experiment was a mirror-competitor test, where males collected from six different field sites through an urban gradient were placed into individual tanks and their interactions with a mirror were recorded and analyzed. Indications of impaired male and female reproductive behaviour were observed in rainbow darter collected from a site downstream of a MWWTP. Exposed females performed fewer nose digs and spawned fewer eggs than reference females. Similarly, exposed males were less colourful, produced less 11-ketotestosterone, spent less time defending the spawning ground, and when assessed by level of intersex, spent less time interacting with their mirror image than unexposed males. These findings suggest that exposure to MWWE impairs reproductive behaviour in rainbow darter. This study demonstrates the importance of studying behavioural endpoints in addition to physiological measures.

3.2 Introduction

Municipal wastewater effluent (MWWE) represents one of the largest sources of pharmaceuticals entering aquatic ecosystems and it contains a diversity of stressors for aquatic vertebrates (aus der Beek *et al.*, 2016). In addition to pharmaceuticals and personal care products (PPCPs), stressors include metals and legacy compounds (Luo *et al.*, 2014), which have been demonstrated to negatively affect the endocrine function of aquatic vertebrates (WHO-UNEP, 2012). Endocrine disrupting chemicals (EDCs) in MWWE are associated with impairment of reproductive physiology including the feminization of males and the disruption of egg production and oviposition in females (Jobling and Tyler, 2003; Overturf *et al.*, 2015).

In addition to reproductive physiological processes, EDCs in MWWE have the potential to disrupt reproductive behaviour. Reproductive behaviours in teleosts are modulated by hormonal processes including gonadal steroids, neuropeptides, and prostaglandins (Gonçalves and Oliveira, 2010). For example, oviposition (release of ovulated eggs) in females is induced by prostaglandins, and androgens are required for aggressive reproductive behaviours in males (Munakata and Kobayashi, 2010). Alteration of endocrine function could lead to dysfunction through subtle changes in behaviour. Some studies have suggested that behavioural endpoints are more sensitive to disruption than physiological endpoints (Melvin and Wilson, 2013). A review of this subject by Söffker and Tyler (2012), however, suggests that they are equally sensitive. There have been relatively few studies conducted on the impacts of MWWE on fish reproductive behaviour compared with the large number of studies on the impacts of MWWE on reproductive physiology. The studies on the impacts of MWWE on reproductive behaviour of fish have been focused on male reproductive behaviour and have had equivocal findings. Exposure of goldfish (*Carassius auratus*) to MWWE for ten weeks resulted in no change in reproductive behaviour (Schoenfuss *et al.*, 2002). Male fathead minnows (*Pimephales promelas*) exposed to estrogenic MWWE for three weeks failed to compete for nest sites (Martinović *et al.*, 2007). Similarly, three-spined stickleback (*Gasterosteus aculeatus*) exposed to MWWE with anti-androgenic properties built fewer nests and spent less time courting females (Sebire *et al.*, 2011). In contrast, male eastern mosquitofish (*Gambusia holdbooki*) collected from a MWWE-exposed site were found to spend more time associating with females than males from a reference site (Saaristo *et al.*, 2014). The fact that relatively few studies have been conducted on critical aspects of the reproductive behaviour of fish exposed to MWWE makes assessment of the potential risk to fish populations highly uncertain.

Studies that assess the effects of EDCs on female reproductive behaviour are sparse, and to our knowledge none have investigated the effects of MWWE on female reproductive behaviour directly. Exposure of fathead minnows to the progestin gestodene at 10 ng/L for 8 days induced aggressive behaviours (chases and nips) in females (Frankel *et al.*, 2016). Similarly, exposure to the androgen 17β-

trenbolone at 6 ng/L resulted in female mosquitofish spending less time associating with males than control females (Saaristo *et al.*, 2013). Female zebrafish (*Danio rerio*) exposed to 17α-ethinylestradiol (EE2) at 9.86 ng/L showed diminished courting responses towards males and had lower reproductive success than control females (Lavelle and Sorensen, 2011). Another study tested the effects of bisphenol A (BPA) exposure on sexual isolation between native and invasive species. While females under control conditions spent more time with conspecifics, BPA-exposed females spent equal time with conspecific and heterospecific males (Ward and Blum, 2012). These studies suggest that there is potential for MWWE to affect female reproductive behaviour, and this should be assessed in greater depth.

Rainbow darter (*Etheostoma caeruleum*) in the Grand River (Ontario, Canada) collected near MWWE outfalls have disrupted reproductive physiology, with males having reduced androgen production, reduced spermatogenesis, and intersex gonads (presence of ovarian tissue in testis) (Bahamonde *et al.*, 2015; Bahamonde *et al.*, 2014; Fuzzen *et al.*, 2015; Tanna *et al.*, 2013; Tetreault *et al.*, 2011). While the reproductive behaviour of this species has been studied under natural conditions (Fuller, 1998b, 1999; Fuller and Montgomery, 2003), no studies have assessed the sensitivity of rainbow darter reproductive behaviour to chemical perturbation. Rainbow darter are a small, benthic, insectivorous species of fish and geographically are one of the most common and abundant species of darter (Kuehne and Barbour, 1983). They reside in creeks and small to mid-sized streams and rivers throughout the Great Lakes and Ohio River valley regions of North America (Ray *et al.*, 2006). Rainbow darter are sexually dimorphic, with males having bright blue and orange colouration and females being a mottled sandy colour (Zhou *et al.*, 2014). The spawning season of rainbow darter is latitude dependent (Hubbs, 1985); it takes place from late April to early June in Southern Ontario (personal observation). Rainbow darter are gonochoristic and are asynchronous spawners, with females developing multiple clutches of eggs over the course of the spawning season (Fuller, 1998a; Heins *et al.*, 1996). During the breeding season, males will guard small moving territories in the fast-moving waters at the head of a riffle (Reeves, 1907; Winn, 1958a, b). Competition between males occurs frequently, since breeding grounds are often crowded. Males use displays (extension of the first dorsal fin and darkening of colouration) to intimidate opponents and will attack other males from the area (Reeves, 1907). Females reside at the bottom of the riffle and they move to the top of the riffle to breed. When females enter a spawning area, they are often followed by several males. The dominant male (typically the largest) will defend the female, attacking other males that approach (Reeves, 1907). Females perform nose digs and bury their bodies in the gravel in preparation for spawning, which signals their readiness to males (Reeves, 1907). Males will mount a buried female and then they both quiver simultaneously to release gametes. During spawning, sneaker males are commonly present and will release milt nearby. Larger males tend to be guarders, while smaller males tend to be sneakers (Fuller, 1998b, 1999). While guarding males do not achieve 100% paternity

when multiple males are present in the spawning area, they are more likely to have single spawning events with females than non-guarding males, which ensures paternity and reproductive advantage (Fuller, 1999). Additionally, a study of the competitive breeding behaviour of rainbow darter revealed that male actions, such as guarding, played a larger role in mating success than female choice (Fuller and Montgomery, 2003). Thus guarding of a spawning area is important to the reproductive success of rainbow darter.

The purpose of this study was to determine whether rainbow darter exposed to MWWE have altered reproductive behaviour. Two experiments were conducted with rainbow darter collected from sites through an urban gradient, which included municipal wastewater treatment plants (MWWTPs). Competitive breeding trials were conducted to assess the reproductive behaviours of males and females, and comparisons were made between individuals collected from exposed and non-exposed sites. Reproductive behaviour of males was also assessed in a mirror-opponent aggression test.

3.3 Methods

All procedures were in accordance with guidelines provided by the Canadian Council of Animal Care (CCAC) and were approved by the University of Waterloo Animal Care Committee under AUPP 10-17. In both years, rainbow darter were collected during the early reproductive period in the Grand River.

3.3.1 Behaviour experiment 1: Competitive breeding trials

The purpose of the competitive breeding experiment was to test whether reproductive behaviour differed among rainbow darter collected from a reference site and two sites near a MWWTP outfall. One male from each of the three sites was placed in a spawning tank with a single female and a small breeding arena.

3.3.1.1 Fish collection

Ten male and ten female rainbow darter were collected from each of three field sites by backpack electrofisher in April 2012. Sites included a rural reference site (R), an urban reference site upstream of the Kitchener MWWTP (USK), and an exposure site downstream of the Kitchener MWWTP (DSK) (Figure 3.1). Rainbow darter were transported to the University of Waterloo and held in well water in 40 L aquaria separated by site and sex for a three-week acclimation period. Light cycle was 12 h light: 12 h dark and water temperature was maintained at 15 (±1) °C. Fish were fed frozen blood worms and frozen brine shrimp twice daily after the first week of acclimation.

49
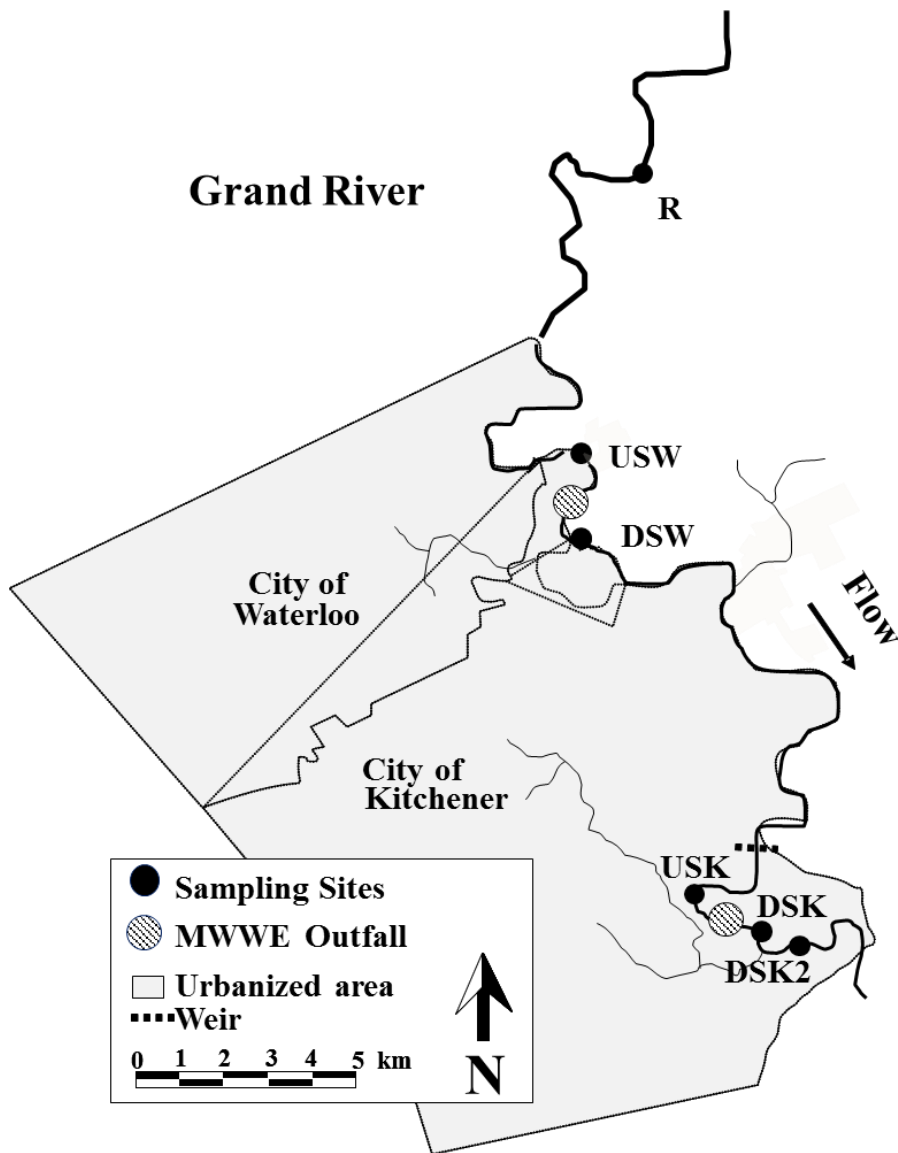
Figure 3.1 Depiction of sites from which rainbow darter were collected for assessment of reproductive behaviour. Fish were collected from a rural reference (R) site, as well as sites upstream of municipal wastewater effluent (MWWE) outfalls (USW, USK), and sites downstream of MWWE outfalls (DSW, DSK, DSK2).

3.3.1.2 Competitive breeding trials

The length and weight of each fish was measured and milt was collected before the fish were placed into one of ten experimental tanks. To identify individuals in the video footage of the competitive breeding trial, males were fin clipped. Fish were anesthetized in MS-222 and a small piece of tissue was removed from an area of the second dorsal fin. Fish were randomly assigned to receive a clip on the anterior region of the second dorsal fin, a clip on the posterior region of the second dorsal, or no clip. Each experimental tank contained one male from each of the three field sites (R, USK, DSK), which were size matched (± 0.1 cm), and one female. Two trials were conducted with the same males; however, the origin of the female was changed in each trial. The first trial was conducted with females from the R site and the second trial was conducted with DSK females. For each female, the competitive breeding trials lasted for three days. Each competitive breeding trial began 24 h after the addition of a female by placing a breeding tray into the experimental tanks. Breeding trays consisted of two stacked saucers. The top saucer had a mesh bottom and was filled with gravel, allowing spawned eggs to fall into the second collection tray underneath. Trays were placed into tanks for three hours in the morning of each of the three days of the trial and the interactions of the rainbow darter were recorded using GoPro Hero 2 video cameras. Breeding trays were removed after each three-hour trial to maximize the chance that spawning and guarding behaviours would be captured and to assess the number of eggs spawned during each trial. Females were removed from the tanks that contained males after the completion of the third trial and sacrificed with a lethal dose of MS-222. The length (±0.1 cm), weight (±0.001 g), gonad weight, and liver weight of these females were recorded. The second set of females was placed in the tanks after a two-day resting period. After the completion of all competitive breeding trials, males were sampled in a manner similar to females, except that their testes were placed in Davidson's solution for histological analysis.

3.3.1.3 Sperm density and motility

Sperm density (billions of sperm / mL) and motility (% motile) were assessed using a Cell-Vu sperm count chamber (Millennium Sciences, New York) according to the manufacturer's protocol, with the following modifications. For each male, 1 µL of milt was diluted with 99 µL of ddH2O, and 5 µL of diluted milt was placed onto a slide with a gridded cover slip. The number of motile and immotile sperm in each of 10 squares of the grid was counted.

3.3.1.4 Histological analysis of male gonads

Male testes were fixed in Davidson's solution for 24 h then transferred to 70% ethanol until they were processed and embedded in paraffin wax. To assess each individual for the presence of intersex, 40 sections that were 5 µm in thickness were mounted onto slides for each individual and stained with

hematoxylin and eosin. Each section was viewed with a light microscope using the 40x objective lens and scanned for the presence of oocytes. The presence of oocytes, the number of oocytes, and the stage of oocytes in all sections were recorded, and then each fish was assigned a severity score from 0 to 7 as detailed in Table 3-1.

Table 3-1 Intersex severity scores assigned to individuals after 40, 5-µm sections of testis tissue mounted on slides were scanned. Score (from 0 to 7) is dependent on the number of primary oocytes (po), the presence of advanced oocyte types (cortical alveolar (ca), vitellogenic (vtg)), and the proportion of ovarian tissue present in the gonad.

Severity score	0	1	2	3	4	5	6	7
Number of po's	0	1-3	4-9	10+	10+	NA	NA	NA
Ooocyte stages present	none	po	po	po	clustering of po's; presence of ca	vtg	vtg	NA
Proportion ovarian tissue	0	<50%	<50%	<50%	<50%	<50%	>50%	100%

3.3.1.5 Video analysis

Videos were analyzed by a single viewer who was blind to the collection site of the fish in the trial. Females were assessed for three behaviour types: proportion of time spent in the spawning area, number of nose digs, and number of spawning events. Male reproductive behaviour was analyzed in three ways: individuals were assessed for the proportion of time they spent on the breeding tray, the number of chases initiated by each male, and the number of spawning acts each male conducted with females. All behaviours were totaled over three days.

3.3.1.6 Statistics

Statistical testing was conducted with SigmaPlot (version 12, Systat software, San Jose, CA). Length, gonad somatic index (GSI), liver somatic index (LSI), and condition factor (K) were compared among sites using a one-way analysis of variance (ANOVA) in males, with a student's t-test between sites in females. Milt volume, sperm density, and sperm motility were compared among sites using a one-way ANOVA, with a Tukey's post-hoc test. Intersex severity was compared among sites using a Kruskal-Wallis one-way ANOVA with a Mann-Whitney U post-hoc test. For the analysis of male behaviour, the proportion of time spent in the spawning area, the number of chases and the number of spawning events were compared among sites and according to whether males were in the presence of an R or DSK female with a two-way ANOVA with repeated measures and a Tukey's post-hoc test. For the analysis of female

behaviour, the proportion of time in the spawning area, the number of nose digs, and the number of spawning events were compared between females from the R and DSK sites using a student's t-test. For statistical tests of reproductive health measures, $\alpha=0.05$. For statistical tests of behavioural measures, $\alpha=0.1$. This reduced stringency for type I error was used in this study because field collection and behaviour data are highly variable, and the combination of the two made the possibility of committing a type II error more likely.

3.3.2 Behavioural experiment 2: Mirror-opponent trial

The purpose of the mirror-opponent trial was to test whether exposure to MWWE alters the response of males to an identical competitor. To test this, rainbow darter males were collected through an urban gradient. After acclimation, males were placed individually into experimental tanks and a mirror was placed into the tank with them. The response of rainbow darter males to the mirrors were video recorded.

3.3.2.1 Field collection

Fifteen male rainbow darter were collected from each of six different sites by backpack electrofisher between May 6 and 10, 2013. Three of the sites were the same as in experiment 1 (R, USK, DSK). The three additional sites included a site upstream and a site downstream of the Waterloo MWWTP (USW, DSW) as well as a second site further downstream of the Kitchener MWWTP (DSK2; Figure 3.1). Rainbow darter were transported to the University of Waterloo, where they were sorted by site (five fish per tank) into 10 L tanks in an aquatic habitat system. Fish were acclimated for three weeks before experimentation to a 14 h light: 10 h dark light cycle at 13±1 °C and were fed a diet of frozen brine shrimp and blood worms twice daily.

3.3.2.2 Interaction with mirror

The experimental tanks were 20 L aquaria held at 14±2 °C with a white cloth placed on the outside of each tank to reduce visual stimuli from neighboring tanks. Males were transferred into individual tanks and held for 24 h before the start of the experiment. GoPro Hero 2 cameras were mounted with a side view of the experimental tanks. Mirrors were placed into the end of each tank and behaviours of males were recorded for 30 min. After the 30 min experiment, males were sacrificed with a lethal dose of MS-222 and were photographed on a standardized background. Milt was then collected in a capillary tube for analysis of milt volume and sperm density, and length (±0.1 cm), weight (±0.001 g), gonad weight, and liver weight were recorded. Gonads were divided into two lobes if they were heavier than 20 mg and either were placed in 5 mL of Media 199 and set on ice for in vitro steroid hormone production or were placed in Davidson's solution for histological analysis.

3.3.2.3 Sperm density

Sperm density was assessed as described in section 3.3.1.3.

3.3.2.4 *In vitro* production of androgens

Gonads collected from males during Experiment 2 were assessed for in vitro gonad steroid production. Testes were reweighed (up to 20 mg) and placed into a single well of a 24-well tissue culture plate with 1 mL of Media199 and 10 IU of human chorionic gonadotropin (Sigma-Aldrich, Oakville, ON). Plates were incubated for 24 h at 16 °C after which media was removed from each well, placed into centrifuge tubes, and stored at -80 °C for future steroid analysis. Steroids analysis consisted of measurement of 11-ketotestosterone (11KT) in media by enzyme-linked immunosorbent assay (ELISA) as described by manufacturer's protocols (Cayman Chemical, Ann Arbor, MI, USA).

3.3.2.5 Histological analysis of male gonads

Males were assessed for intersex severity as described in section 3.3.1.4.

3.3.2.6 Colour score

Photographs of males were assessed for colour intensity by assigning a score of 1 to 4 (as depicted in Figure 3.2), with the option of assigning half scores (i.e., 2.5) for individuals that did not fit into distinct groups.

3.3.2.7 Video analysis

Videos were analyzed by a single viewer who was blind to the identity of the site from which the fish was collected. Individuals were assessed for fifteen minutes after the time at which they identified themselves in the mirror. During the fifteen minutes, the proportion of time the fish spent displaying each of five behaviour types (lateral display, frontal display, attack, swimming, and resting on bottom of tank) was assessed. Additionally, the proportion of time spent occupying areas of the tank in relation to the mirror (near mirror and interacting, near mirror and not interacting, away from mirror) was recorded. To assess differences in behaviour, the proportion of time spent displaying aggressive behaviour (displays or attacks) was compared among treatment groups.
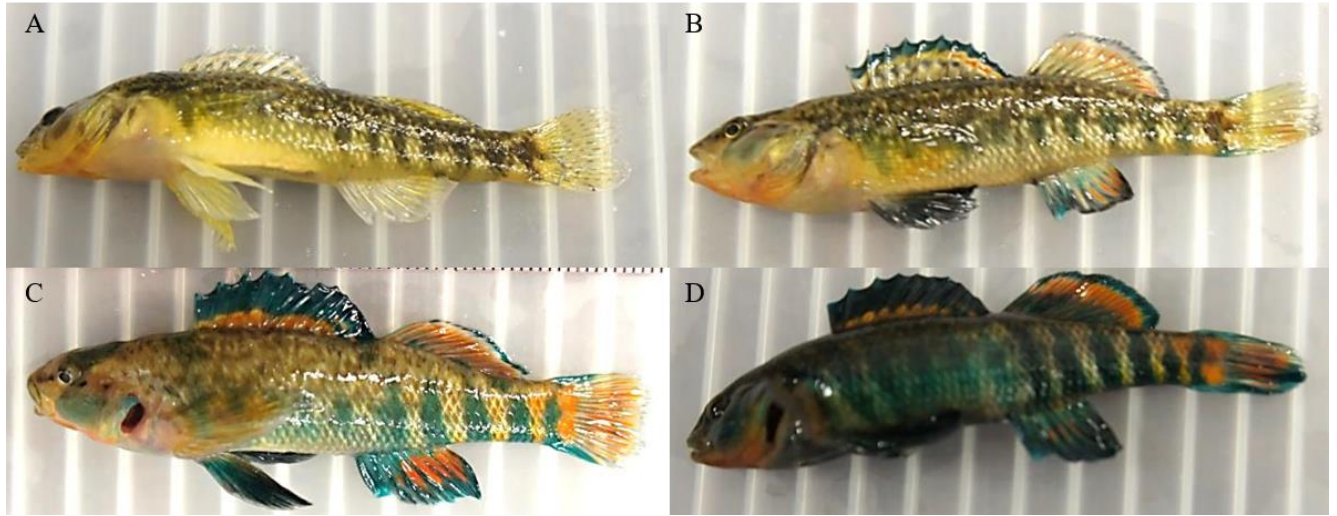
Figure 3.2 Colour scale for assessment of secondary sexual characteristics of rainbow darter. Photographs of males on a standard background were assigned a score of (A) 1, (B) 2, (C) 3, or (D) 4.

3.3.2.8 Statistics

Length, gonad somatic index (GSI), liver somatic index (LSI), condition factor (K), milt volume, 11KT production, and intersex severity were compared among sites using a one-way ANOVA with a Tukey's post-hoc test. Colour score of males was compared among sites using a Kruskal-Wallis one-way ANOVA with a Mann-Whitney U post-hoc test. The proportion of time spent displaying aggressive behaviours (sum of attacks, lateral displays, and frontal displays) was compared among sites using a one-way ANOVA. For statistical tests of reproductive health measures, α=0.05 was used. For statistical tests of behavioural measures, α=0.1 was used.

3.1 Results

3.1.1 Behaviour experiment 1: Competitive breeding trials

Two mortalities occurred in this experiment. Both were DSK females who died on the third day of the competitive breeding trial. The cause of death was unknown, and video analysis revealed that one of the two females had been behaving normally. The second female remained inactive through the experiment and was thus eliminated from the analysis. Additionally, analysis of video revealed that interactions did not occur in all competitive breeding tanks. Only tanks in which male-male interactions or spawning events occurred were included in the analysis (8 of 10 tanks with R females, and 9 of 10 tanks with DSK females).

3.1.1.1 Reproductive health

Somatic indices of male rainbow darter did not differ among sites (Table 3-2). Somatic indices and K of females did differ between sites, however, with females collected from downstream of the Kitchener MWWTP (DSK) having higher GSI, lower LSI, and higher K than R females (Table 3-2). When the milt volume, density, and motility of males were assessed, no differences were found among sites. The severity of the intersex condition was greater in males collected from DSK than in R males (Figure 3.3).

Table 3-2 Length, gonad somatic index (GSI), liver somatic index (LSI), and condition factor (K) of male (M) and female (F) rainbow darter sampled after the completion of a competitive breeding behavioural experiment. * indicates significant (p <0.05) difference between R and DS females as determined by a student's t-test.

Site	Sex	Length (cm)	GSI	LSI	K
R	M	6.1 ± 0.1	0.59 ± 0.13	1.32 ± 0.07	1.18 ± 0.03
USK	M	6.1 ± 0.1	0.40 ± 0.09	1.23 ± 0.08	1.13 ± 0.02
DSK	M	6.1 ± 0.1	0.38 ± 0.22	1.44 ± 0.10	1.13 ± 0.02
R	F	5.8 ± 0.1	5.92 ± 0.82	1.95 ± 0.08	1.05 ± 0.04
DSK	F	6.1 ± 0.1	16.34 ± 1.67 *	1.27 ± 0.10 *	1.15 ± 0.02*

3.1.1.2 Behavioural responses during competitive breeding trials

When the reproductive behaviours of females in the competitive breeding trials were assessed, we found no difference in the proportion of time females spent occupying the breeding area (Figure 3.4A). DSK females performed fewer nose digs than R females (Figure 3.4B); however, the number of spawning events did not differ (Figure 3.4C). The number of eggs spawned did differ: R females spawned a mean of 23 (±7) eggs during the trial whereas DSK females did not spawn any eggs.

During the first competitive breeding trial with the R female, the relative amount of time spent guarding the breeding tray was lower in males collected from DSK and USK than in R males (Figure 3.5A). The relative proportion of chases conducted by males also differed among sites, with DSK and USK males conducting fewer chases than R males (Figure 3.5B). However, no differences in the number of spawning events were found among males (Figure 3.5C). During the second trial with the DSK female, no difference in male guarding time was found among sites. DSK males, however, were found to spend more time guarding the spawning area in the presence of the DSK female than the R female (Figure 3.5A). The proportion of chases performed by males did not differ among sites in the presence of the DSK female, and it also did not differ between breeding trials with R and DSK females (Figure 3.5B). While the number of spawning events did not differ among sites, the number of spawning events by R males was lower in the presence of DSK females than in the presence of R females (Figure 3.5C).
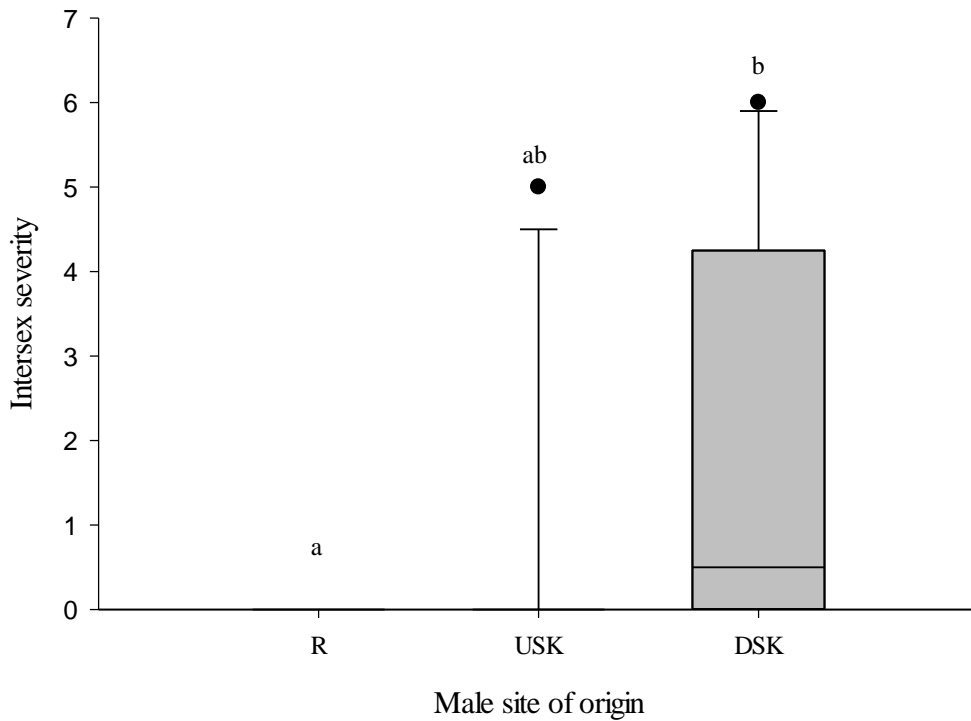
Figure 3.3 Intersex severity of male rainbow darter collected from sites in the Grand River including a reference site (R), a site upstream (USK) and a site downstream (DSK) of the Kitchener MWWTP. Box plots represent median of data (line in middle of box), the 25th and 75th percentile (lower and upper limits of the box), the 10th and 90th percentile of the data (whiskers), as well as outliers (black circles). Boxes that do not share letters are significantly different as determined by a Kruskal-Wallis one-way analysis of variance with a Mann-Whitney U post-hoc test (p<0.05).
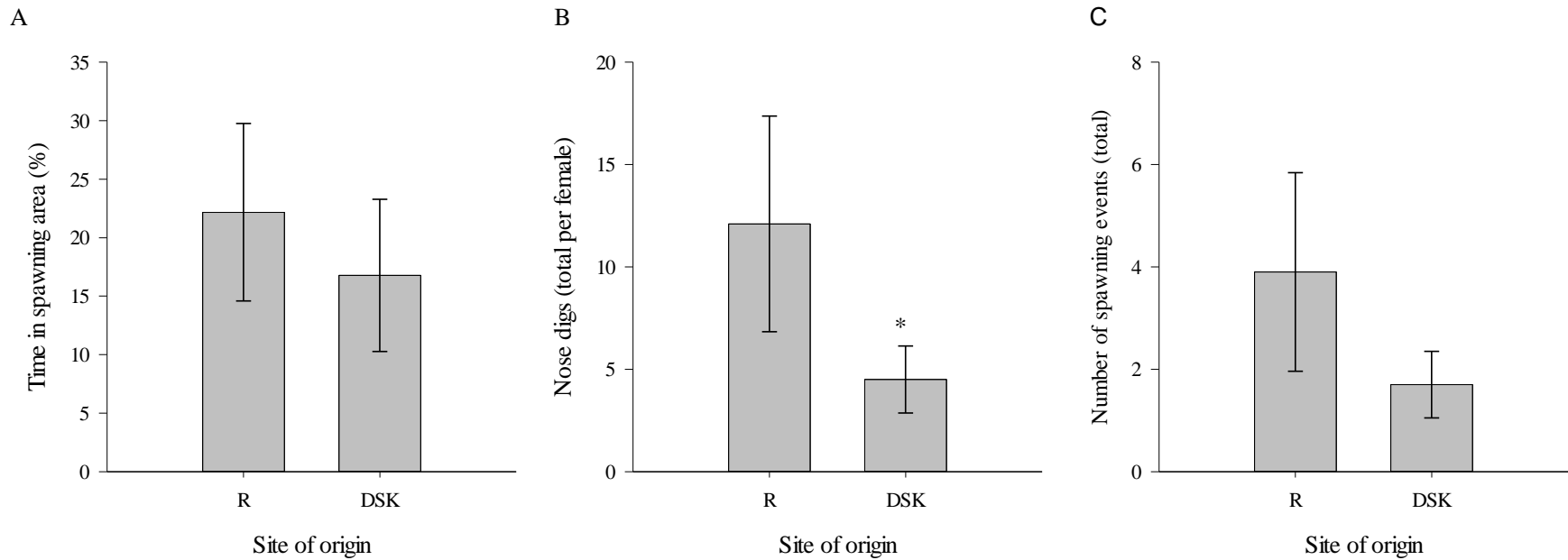
Figure 3.4 Behaviours of female rainbow darter placed in breeding tanks with three males and a spawning area. Assessment of mean (±SE) (A) proportion of time spent in the spawning area, as well as (B) number of nose digs performed in spawning area, and (C) number of spawning events was conducted for females collected from a rural reference site (R) or a site downstream of the Kitchener municipal wastewater treatment plant (DSK). * indicates significant difference (p<0.1) from R site as determined by a student's t-test.
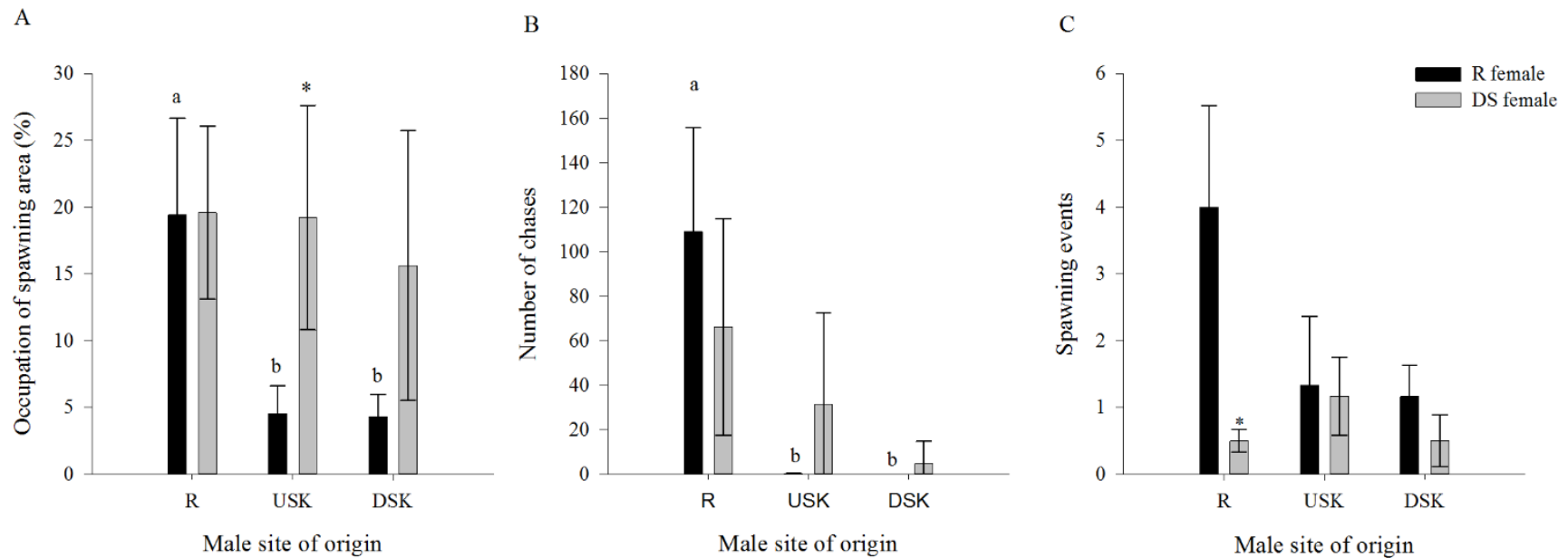
Figure 3.5 Assessment of reproductive behaviour in male rainbow darter collected from three field sites (reference (R), upstream of the Kitchener municipal wastewater treatment plant (MWWTP; USK), downstream of the Kitchener MWWTP (DSK)) in the Grand River and placed into a competitive breeding trial with a single female from either the R site (black bars), or the DSK site (grey bars). Behaviours assessed included mean ($\pm$ SE) of the (A) proportion of time spent in the spawning ground, (B) number of chases, and (C) number of spawning events. Bars that do not share letters are significantly (p<0.1) different as determined by a two-way analysis of variance with repeated measures and a Tukey post-hoc test. * indicates significant difference of behaviour in a group (site of origin) of males in the presence of R versus DSK females.

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3.1.2 Behaviour experiment 2: Mirror opponent trials

Videos in which males did not recognize their mirror image were eliminated from the study. The number of videos included in the assessment ranged from 9 to 13 per site.

3.1.2.1 Male reproductive health

Male GSI and LSI were comparable across sample sites. Condition factor was lower in DSK and DSK2 males than in R males (Table 3-3). Milt volume of males collected at DSW was higher than that of males collected at USW but not higher than that of males collected from the R site (Figure 3.6A). Milt volume of males collected downstream of the Kitchener MWWTP (DSK, DSK2) was also elevated when compared with that of males collected at USW and R (but not when compared with that of males collected at USK) (Figure 3.6A). Sperm density was similar among fish collected from all sites. No differences in production of 11KT were found between sites when a one-way ANOVA was conducted (Figure 3.6C). We found that males collected downstream of the Kitchener MWWTP had higher intersex severity than fish collected from the R site or from USK. Fish collected near the Waterloo MWWTP had intersex severity similar to that of fish collected at the R and USW sites (Figure 3.6D). Colour intensity of males collected downstream of the Kitchener MWWTP (at DSK and DSK2) was lower than that of fish collected upstream of the Waterloo MWWTP (R and USW) (Figure 3.7).

3.1.2.2 Behavioural responses to mirror

There were few differences in the behaviour of male fish from the various sites in terms of their response to the presence of a mirror opponent. Total aggression of DSK males was lower than that of DSK2 males, but not lower than that of R, USW, or USK males (Figure 3.8A). When males were classified as being unexposed, mildly exposed, or heavily exposed to MWWE based on intersex severity score (0, 1-3, 4-6 respectively), it was found that heavily exposed males spent less time displaying aggressive acts (Figure 3.8B). When a multiple regression analysis was performed, no relationship was found between colour score and aggressive behaviours ($p>0.1$), and a weak positive correlation was found between 11KT and aggressive behaviours ($R=0.27$, $DF=53$, $p=0.049$).

Table 3-3 Length and somatic indices including gonad somatic index (GSI), liver somatic index (LSI), and condition factor (K) of male rainbow darter sampled after the completion of the mirror-opponent behavioural experiment. Males were collected from six sites in the Grand River including a reference site (R), sites upstream (USW, USK), and sites downstream (DSW, DSK, DSK2) of municipal wastewater treatment plants. Measures that do not share letters are significantly (p <0.05) different as determined by a one-way ANOVA with a Tukey's post-hoc test.

Site	Length (cm)	GSI	LSI	K
R	6.1 ± 0.2	1.08 ± 0.10	1.34 ± 0.08	1.02 ± 0.02 a
USW	5.9 ± 0.1	0.92 ± 0.13	1.49 ± 0.09	1.02 ± 0.03 ab
DSW	5.5 ± 0.1	0.84 ± 0.13	1.35 ± 0.11	1.00 ± 0.02 abc
USK	5.6 ± 0.1	0.90 ± 0.10	1.23 ± 0.09	0.96 ± 0.03 abc
DSK	5.8 ± 0.2	0.84 ± 0.12	1.20 ± 0.07	0.93 ± 0.03 bc
DSK2	5.4 ± 0.2	0.66 ± 0.11	1.18 ± 0.06	0.93 ± 0.03 c

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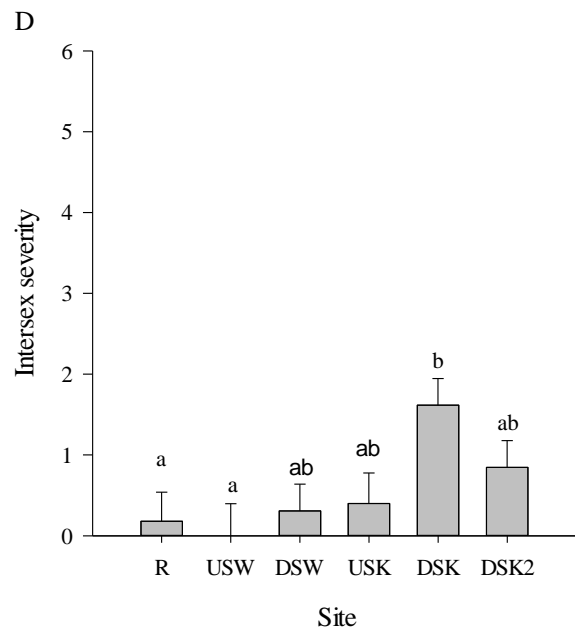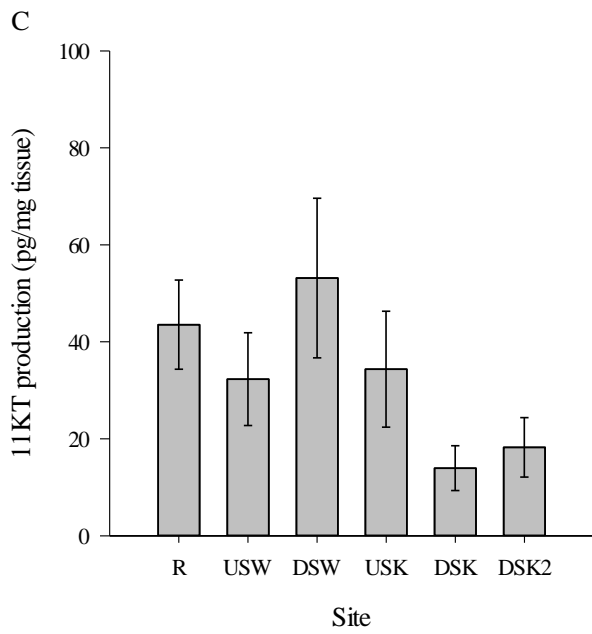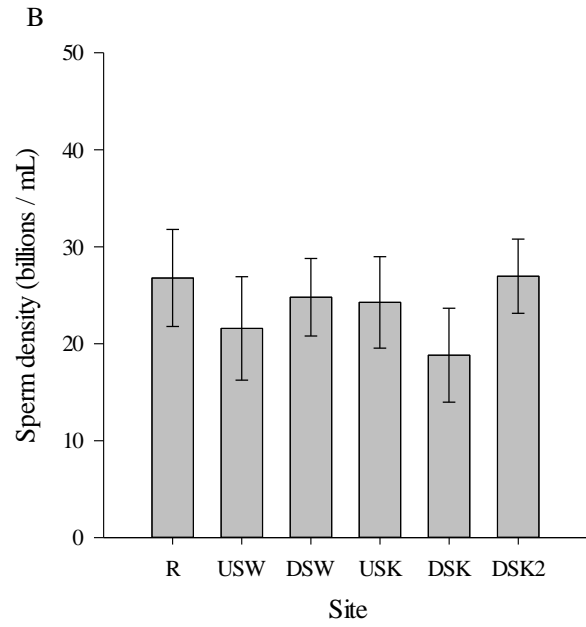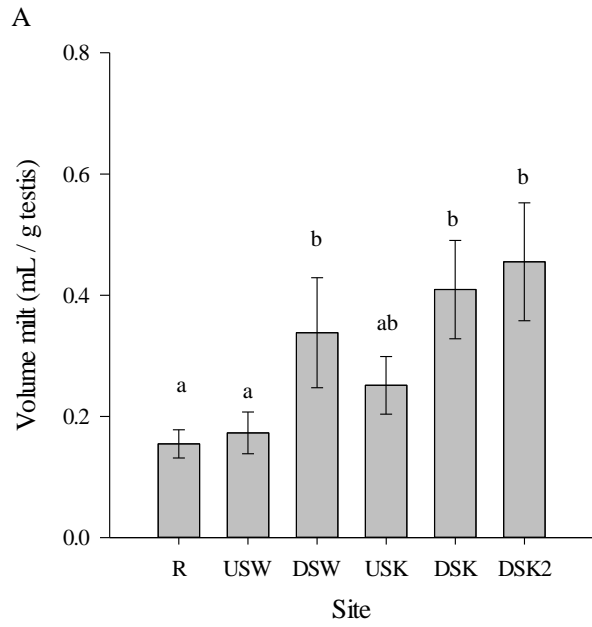
Figure 3.6 Reproductive health measures of male rainbow darter after completion of the mirror-opponent behaviour test. Fish were collected from a rural reference site (R), a sites upstream of the Waterloo (USW) and Kitchener (USK) municipal wastewater treatment plants (MWWTP), as well as sites downstream of the Waterloo (DSW) and Kitchener (DSK, DSK2) MWWTPs. Measures included mean (± SE) (A) milt volume, (B) sperm density, (C) *in vitro* **11-ketotestosterone (11KT) production, and (D) intersex severity. Bars that do not share a common letter are significantly (p<0.05) different as determined by a one-way analysis of variance with a Tukey's post-hoc test.**
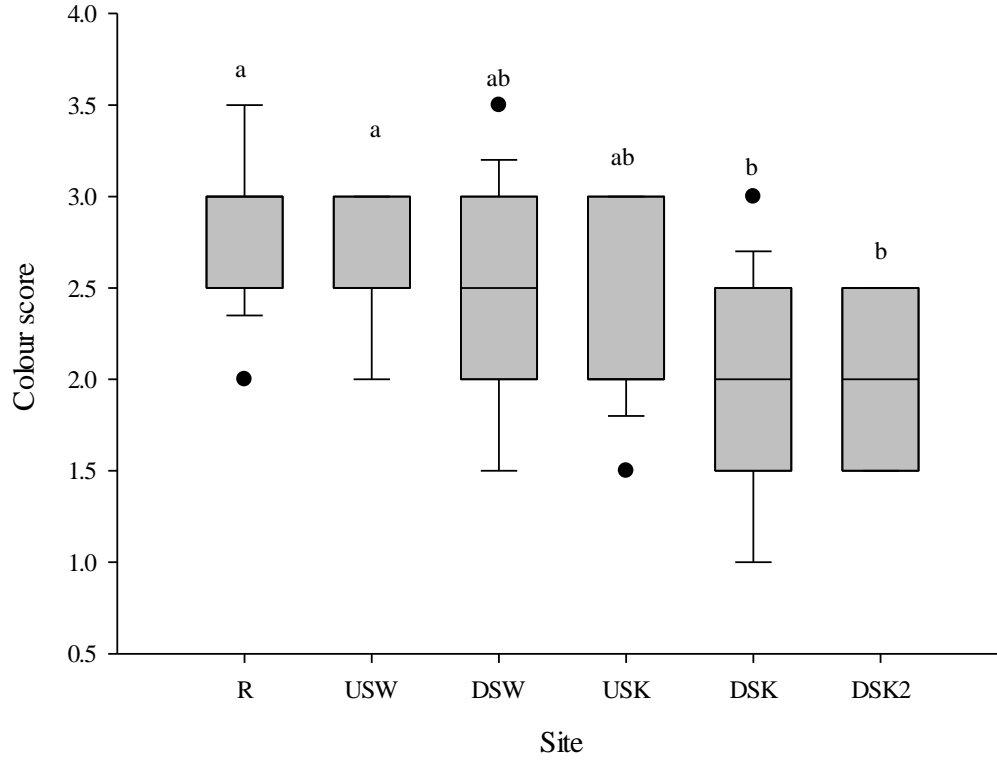
Figure 3.7 Assessment of male secondary sexual characteristics through colour. Photographs of males were assigned a score from 1 to 4. Box plot represents median of data (line in middle of box), the 25th and 75th percentile (lower and upper limits of the box), the 10th and 90th percentiles of the data (whiskers), as well as outliers (black circles). Boxes that do not share letters are significantly different as determined by a Kruskal-Wallis one-way analysis of variance with a Mann-Whitney U post-hoc test (p<0.05).

A
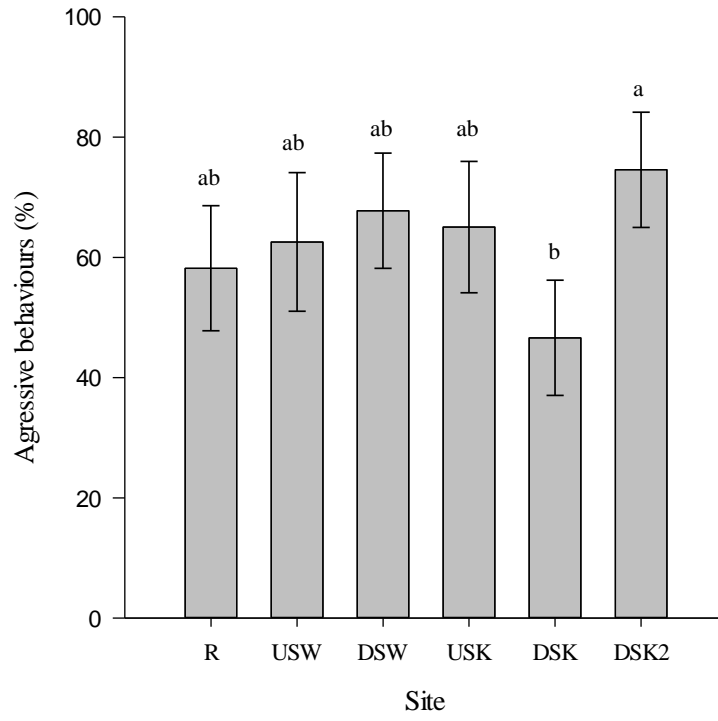
B
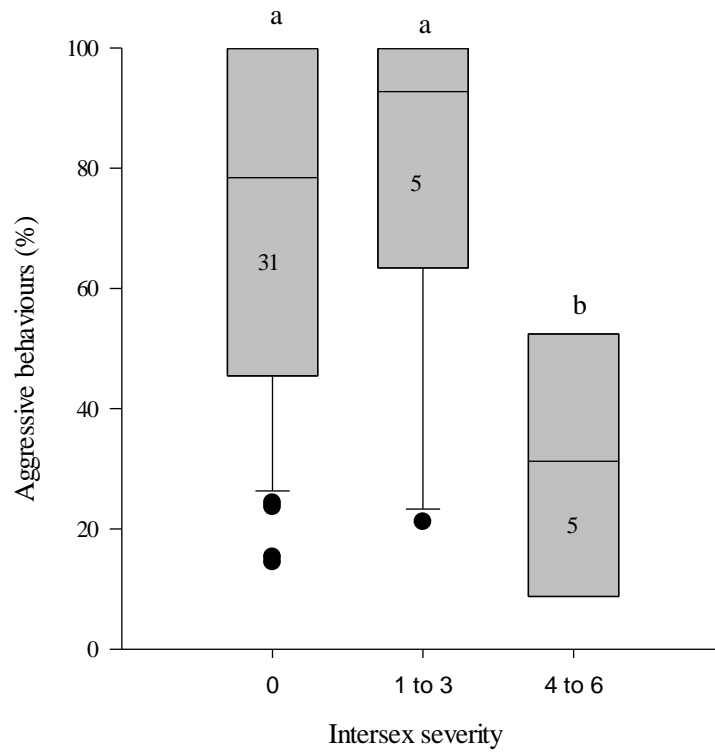
Figure 3.8 Male rainbow darter collected from field sites through an urban gradient were presented with a mirror-competitor. Mean (±SE) aggression was compared (A) among sites. Additionally, aggressive behaviours were compared among rainbow darter based on (B) intersex severity. Box plot represents median of data (line in middle of box), the 25th and 75th percentile (lower and upper limits of the box), the 10th and 90th percentile of the data (whiskers), as well as outliers (black circles). Numbers in boxes represent sample size. Bars that do not share a common letter are significantly different (p<0.1) as determined by a one-way analysis of variance, with a Tukey's post-hoc test (A). Boxes that do not share letters are significantly different as determined by a Kruskal-Wallis one-way analysis of variance with a Mann-Whitney U post-hoc test (p<0.1) (B).

3.2 Discussion

In this study, rainbow darter collected near MWWTPs during the spawning season were placed into competitive scenarios. The responses of fish collected from sites downstream of MWWTPs were compared with those of fish collected from a rural reference site (R). Females collected downstream of a MWWTP performed fewer nose digs and deposited fewer eggs than R females but did not differ in terms of the amount of time they spent in the spawning area or their number of spawning events. Compared with males collected from a R site, males collected from downstream of one MWWTP (Kitchener) spent less time guarding a spawning area and less time chasing other males in the presence of a female from a R site. Additionally, males presenting with high intersex scores had reduced aggression towards a mirror competitor compared with males with no intersex. The results suggest that although the endpoints are highly variable, subtle effects on reproductive behaviour in rainbow darter are associated with exposure to the urban environment and wastewater effluents. This is one of few studies to assess reproductive behaviours of native fish exposed in the wild and has implications for future watershed management.

3.2.1 Female reproductive behaviour

The reproductive behaviour of female rainbow darter consists of selecting a mate, performing nose digs in gravel to partially bury their body, and then releasing eggs in the presence of a spawning male (Reeves, 1907; Winn, 1958a). In this study we found no difference in the amount of time females spent in the spawning area. The number of nose digs performed by females did differ by collection site, with females collected from the site downstream of the Kitchener MWWTP (DSK) performing fewer nose digs than females from the rural reference (R) site. While the number of spawning events was similar between R and DSK females, the number of eggs collected differed, with (23±7) spawned by R females and none by DSK females. Additionally, the GSI of females after the experiment was completed was larger in DSK females than in R females, suggesting the lack of egg deposition. It is possible that since DSK females were tested after R females, that males had less interest in spawning, resulting in no egg deposition. This is unlikely, however, since the spawning season in the wild lasts several months and males spawn with multiple partners. Additionally, females have been shown to deposit eggs in substrate even in the absence of males (Winn, 1958a). The observations of reduced nose digs and lack of egg deposition suggest that females from DSK either were not ovulating eggs, or that they did not perform adequate behaviours to oviposit their ovulated eggs.

The reduced number of nose digs and the absence of eggs spawned may be related to disruption of the female endocrine system. Prostaglandins (PGs), which are produced in the mature ovary, have three main functions in female reproduction. They act to stimulate follicular rupture, stimulate female reproductive behaviour and sexual receptivity, and act as a pheromone, stimulating male sexual behaviour

(Sorensen and Goetz, 1992). A disruption of PG production would explain the lack of egg deposition as well as the alteration of male-male dynamics between the two experimental trials. In the first trial of the competitive breeding experiment, males collected near the Kitchener MWWTP were less likely to defend the spawning area, with fewer chasing events in the presence of a female from the R site. In contrast, during the second competitive breeding trial with DSK females, no differences in defence of the spawning ground or the number of chases was observed. Additionally, males from the R site performed fewer spawning acts with DS females than R females. These changes in male-male interactions could be due to a difference in pheromone production between R and DS females and is an area for future study.

3.2.2 Male reproductive behaviour

We did not observe any instances in which more than one male was present during spawning, despite this being a common occurrence (in 5 of 11 replicates) in a study by Fuller and Montgomery (2003). The lack of sneaker males in this study may be due to the similarity of size of males contained in each tank. Observations of spawning events with multiple males found that large males frequently acted as the guarder, with smaller males acting as sneakers (Fuller, 1999). Thus in future studies it may be important to perform experiments with larger breeding groups that include a range of sizes to more accurately simulate breeding events in wild.

As previously mentioned, males collected from sites near the Kitchener MWWTP were less aggressive than males collected from the R site in the presence of R females. Few other studies have attempted to assess the sexual behaviour of fish collected from sites near MWWE in the wild. Saaristo *et al*., (2014) collected mosquitofish from pristine and MWWE-exposed sites late in the breeding season. In contrast to this study, the authors found that MWWE-exposed male mosquitofish spent more time associating with and chasing females than fish collected from the pristine site. The differences in responses between these studies could be due to many reasons, including differences in reproductive strategies, environmental conditions, or the composition of effluent.

When the number of spawning events was compared among males from the R site and sites near the MWWTPs, no differences were found. While this seems to contradict the fact than DSK males spent less time in the spawning area and performed fewer chases, the comparable number of spawning events across groups may be related to the nature of the reproductive behaviour of female rainbow darter. Previous studies have noted that female fish spawn with multiple males, even in the presence of a dominant, guarding male (Winn, 1958a). To increase their chances of reproductive success, females may have pursued multiple males, encouraging spawning events with less desirable males due to a lack of choice. It is uncertain whether the choice of R females is related to the dominance of males displayed through aggressive behaviour or some other physical trait. In a study by Fuller *et al.*, (2003) rainbow

darter females collected from two different field sites displayed a preference for males from a singular site. This preference was not due to size, and the authors concluded that this must have been related to visual cues. Male rainbow darter have bright colour displays, with an iridescent blue colouration in their cheeks, dorsal fins, pectoral fins, and lines on their body that darkens during competition and mating events (Ciccotto and Mendelson, 2016; Zhou *et al.*, 2014). While not assessed in the competitive breeding study, male colouration was found to be less brilliant in males from DSK collected for the mirror-opponent aggression test. It is possible that MWWE-exposed males are less attractive due to the lack of male colouration and darkening response in interactions with conspecifics. Interpreting mate choice can be difficult and confounded by interactions between fish and individual reproductive status. This is an area of research that could be tested more accurately in future studies with the use of fish models or robotic fish (Ciccotto and Mendelson, 2016; Phamduy *et al.*, 2014).

The second experiment of this study assessed male aggression towards a mirror competitor. The time spent displaying aggressive behaviours differed little among fish collected from the different sites. When males were grouped by intersex severity, however, a clear decrease in aggressive behaviours was noted in males with high-severity intersex condition. Since intersex is associated with MWWE exposure, males with higher intersex severity probably had either more direct or a longer duration of exposure to MWWE. Intersex scoring was conducted in the competitive breeding studies as well; however, fewer individuals had intersex present compared with in the mirror-competitor experiment. The small proportion of males with moderate or severe intersex (2 and 4 respectively) in the competitive breeding trial made assessment of behaviours according to intersex severity statistically unsound. A weak positive correlation was found between gonad production of 11KT and total aggression. No relationship was found between colour score or size and aggression. Previous studies have indicated that larger, more colourful males are more aggressive, a trait that allows them to achieve dominance (Winn, 1958b). In contrast, this study suggests that male aggression varies by individual and is dependent on perceived advantage. When faced with a male of equal size and colour, male response was similar across size and colour classes. In exposed males, it is likely that the reduced production of 11KT resulted in lower aggression. Aggressiveness of male fish is promoted by 11KT and testosterone (Oliveira and Gonçalves, 2008). Rainbow darter males use aggressive acts to defend prime spawning area, as well as to guard a choice female when she enters a male's territory. Decreased aggression would probably lead to a reduced number of sole spawning events and an increase in the number of group spawning events, both of which would result in decreased fitness for the individual.

Our understanding of the effects of chronic exposure to MWWE on the reproductive behaviours of native fish species is limited because only a small number of studies have been conducted. In this study, we found that there is a potential for MWWE to negatively affect reproductive behaviours of fish

in the wild. Decreased reproductive behaviour led to reduced reproductive success in this study (no eggs were deposited by DSK females). When these findings are considered along with those of previous studies (Fuzzen *et al.*, 2015) in which rainbow darter collected from the same site had reduced fertilization success and embryo survival, it is likely that rainbow darter fitness is reduced in the population sampled downstream of the Kitchener MWWTP. Whether this reduced fitness leads to changes in population size is not known and should be investigated in future studies.

Chapter 4

Digging deeper: An assessment of reproductive success and behaviour of a native fish species, rainbow darter, after exposure to municipal wastewater effluent or 17α-ethinylestradiol

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4.1 Overview

Municipal wastewater effluent (MWWE) is a contributor of multiple contaminants, including endocrine disrupting compounds, into the aquatic environment. In a previous study, reduced reproductive success, including reduced fertilization success and survival to hatch, were found in a sentinel fish species, rainbow darter (*Etheostoma caeruleum*) exposed to municipal wastewaters in the Grand River, Ontario. In order to further test effects of wastewater on aspects of reproductive fitness, such as fecundity and behaviour, a controlled laboratory study was necessary. In this study, rainbow darter collected from a reference site were placed in breeding groups and held in dechlorinated tap-water for a 10-day pre-exposure period. Following the pre-exposure spawning period, breeding groups were exposed for 21 days to control, 10 ng/L 17α-ethinylestradiol (EE2) or diluted MWWE at 1%, 10%, or 20%. Reproductive health (gonad somatic index, testicular steroid production), reproductive success (fecundity, fertilization success) and behaviour (aggression, activity) were assessed. A small reduction in the number of eggs spawned was observed after 7 days of exposure to 20% MWWE, but no differences were present after 14 or 21 days and no changes were observed with EE2 exposure. Fertilization success was lower in breeding groups exposed to 20% MWWE or EE2, and after the exposure, a larger volume of milt was collected from males exposed to 20% MWWE and EE2. Similarly, vitellogenin (*vtg*) expression in livers was elevated in 20% MWWE and EE2 exposed males, while cytochrome P4501A (*cyp1a*) expression was elevated in 20% MWWE exposed males, but not in EE2 exposed males. When a mirror test was conducted to assess levels of aggression and activity in males after the 21-day exposure period, it was found that exposure to 20% MWWE decreased aggression and increased activity, while EE2 exposure had no impact on either behaviour. Overall these findings suggest that treated MWWE exposure had minor effects on reproductive success of rainbow darter at the highest concentration, and that while these effects were partially mimicked by EE2, not all effects could be attributed to estrogenic compounds.

4.2 Introduction

Municipal wastewater effluent (MWWE) is a ubiquitous toxicant in the environment. Since MWWE is a complex matrix, assessing the potential risk of MWWE on the health of the receiving environment is a challenge. In addition to nutrients, metals, and legacy compounds, MWWE contains pharmaceuticals and personal care products (PPCPs). Whole MWWE can be acutely toxic, however, it is usually diluted in the receiving environment reducing the toxicity. Exposure to low concentrations of MWWE can still cause adverse effects on aquatic wildlife by disrupting endocrine pathways (WHO-UNEP, 2012). The most thoroughly studied cases of endocrine disruption due to MWWE exposure is disruption of reproduction in fish, resulting in feminization of males and reduced reproductive fitness (Tyler and Jobling, 2008). These effects have been attributed to estrogenic compounds, such as alkyl-phenols, and hormones including natural and synthetic estrogens as well as anti-androgenic compounds (Sumpter, 2005).

A key series of studies that brought the issue of feminization of male fish to light was conducted on populations of roach (*Rutilus rutilus*) collected from rivers throughout the United Kingdom. In this instance, an observation of widespread sexual disruption was made including the presence of intersex (oocytes in testes) (Jobling *et al*., 1998). This observation caused the researchers to investigate the cause and consequences of intersex condition in fish populations. To address these questions, both field, and laboratory experiments were conducted. The studies concluded that intersex was induced partly by estrogenic compounds (Routledge *et al*., 1998), that reproductive success was negatively impacted in feminized males (Tyler and Jobling, 2008), but that no differences were found in the genetic diversity of roach populations living in urbanized areas versus rural areas. However, the high level of variability in the allelic markers at all sites, may mask a small change in genetic diversity if present (Hamilton *et al*., 2014). While this series of studies provided a wealth of information about the presence of feminized male fish, the risk of reduced fertilization success, and the potential for effects at the population level, it is uncertain if the conclusions drawn from roach, a large, long lived, mobile fish species, can be extrapolated to other fish species and sites around the world. An example of such a contrast is found in a study in which a whole lake was dosed with the synthetic estrogen 17α-ethinylestradiol (EE2) at approximately 5 ng/L. Male fathead minnow (*Pimephales promelas*) were feminized in a manner similar to roach (decreased androgens, increased vitellogenin (*vtg*) protein and presence of intersex). In contrast to roach, fathead minnow males ceased reproducing, and the population collapsed after two years of exposure (Kidd *et al*., 2007). This demonstrates the importance of investigating multiple species and aquatic systems to gain greater understanding of potential implications of endocrine disrupting chemicals (EDCs), such as estrogens, in the environment.

In the central Grand River (Ontario, Canada) several species, including rainbow darter (*Etheostoma caeruleum*) have been found to be sexually disrupted (Tanna *et al*., 2013; Tetreault *et al*., 2011). Rainbow darter (a small, short lived, benthic, percid species) collected from sites near MWWE outfalls were found to consistently produce less androgen, have delayed spermatogenesis, and higher incidence and severity of intersex when compared to a rural reference site (Bahamonde *et al*., 2015a; Fuzzen *et al*., 2015; Tetreault *et al*., 2011). Similar to the researchers in the UK, the question was posed as to whether these observations would lead to reduced reproductive success. In order to determine the potential impact of this feminization on reproductive success, a field study was conducted. Males and females were collected from a reference site, as well as upstream and downstream of a municipal wastewater treatment plant (MWWTP) during their spawning season. In a manual fertilization study, it was found that fertilization success and survival of progeny to hatch was lower in fish collected near MWWE outfalls (Fuzzen *et al*., 2015). Additionally, when stripped, males collected near the MWWTP had a higher volume of milt and females had a larger number of ripe but unspawned eggs (in one of two years of the study), suggesting a possible reduction in spawning frequency. While this suggests that MWWE has a negative impact on rainbow darter reproductive success, several observations could not be attributed directly to MWWE due to the complex nature of the natural environment. It was difficult to exclude the potential for cumulative effects since males both directly upstream and downstream of the treatment plant had lower fertilization success and progeny survival (which may be due to movement or upstream effects). Fecundity of females could not be accurately assessed from ovarian histology due to the fact that females develop multiple clutches in their ovary, but do not ripen and spawn all of their immature eggs (Fuller, 1998). Also, while the feminization of males suggests estrogen exposure, and estrogenicity has been detected in the MWWE, expression of *vtg* was not highly expressed in liver tissue of males collected near the outfall (Bahamonde *et al*., 2014). This could be due to acclimation, or could suggest that estrogenic compounds are not the main constituent causing these effects.

Thus the purpose of this study was to further address the question of whether MWWE exposure affects reproductive success by conducting a controlled laboratory experiment. By using controlled conditions, questions that were raised from field observations can be addressed more directly. Namely, whether MWWE impairs fecundity, or alters spawning behaviour of breeding rainbow darter, and whether changes observed can be attributed to estrogenic compounds. To address these questions, rainbow darter were collected from a reference area, were transported to the laboratory, and were exposed to three concentrations of MWWE (1%, 10%, or 20%), or 10 ng/L 17α-ethinylestradiol for three weeks during spawning. Reproductive health (somatic indices, androgen production), reproductive success (fecundity, fertilization success), and behaviour (aggression, activity) were assessed.

4.3 Methods

All procedures are in accordance with guidelines provided by the Canadian Council of Animal Care (CCAC), and were approved by the University of Waterloo Animal Care Committee under AUPP 10-17.

4.3.1 Fish collections

Rainbow darter spawn in late April to early June annually and are asynchronous clutch spawners. In this study, rainbow darters were collected at the beginning of the spawning season from a rural reference on April 24, 2014. While rainbow darter spawn within the first year of life, the minimum length of fish collected was 4.5 cm to ensure sexual maturation. Male and females were separated by sex and transported back to the University of Waterloo. Fish were acclimated for two weeks at a density of 10 fish per 10 L tank. Water temperature was maintained at 12 °C; light cycle was 12 h light: 12 h dark. Fish were fed a diet of frozen blood worms and frozen brine shrimp twice daily.

4.3.2 Exposure

After the acclimation period, fish were sorted into 20 L breeding tanks with two males and two females per tank based on size. Males and females were size matched to within 0.1 cm length (Appendix A, Table S4-1) and randomly assigned to breeding tanks. All tanks had a flow-through design with a minimum of two tank renewals per day (3.0 ± 0.4). Each tank received control water (dechlorinated tap water) for ten days during a pre-exposure breeding period, then received one of five specific treatments for 21 days during the exposure period. The treatment groups consisted of control (dechlorinated tap water), a positive control of 10 ng/L 17α-ethinylestradiol (EE2), 1% municipal wastewater effluent (MWWE), 10% MWWE or 20% MWWE (replicates of six tanks for each treatment group). Dilutions of MWWE were selected to mimic realistic concentrations in the river during low flow (20%), high flow (10%), and after further dilution and mixing (1%). MWWE was collected from the Kitchener MWWTP after UV disinfection three times per week, transported to the University of Waterloo, and placed into a chilled (4 °C) reservoir. MWWE was pumped from the reservoir into mixing cells for the three different dilutions of exposure using FMI pumps (models QG50 MB, QG150MB; Fluid Metering Inc., USA). EE2 was obtained from Sigma Aldrich (Oakville, ON, Canada). A stock solution was used to spike a 20 L reservoir, which was pumped into a 1 L mixing cell, which was released into a header tank that flowed into fish tanks. During the exposure the mean number of tank changes per day was 2.9 ± 0.3. Temperature of breeding tanks was controlled using a circulating water bath and a Delta star water chilled cooler (DS-10-WC). Temperature was modified to mimic diurnal fluctuations of temperature in the wild, and were

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matched to river temperature taken from the Grand River Conservation Authority real-time water quality monitoring website (https://apps.grandriver.ca/waterdata/kiwischarts/wq_temp.aspx).

4.3.3 Exposure chemistry

Water quality parameters (dissolved oxygen, temperature, ammonia, and nitrate) were monitored three times per week using a YSI proplus multimeter (YSI Incorporated, OH, USA). Additionally, grab samples of tank water were collected in 500 mL amber glass bottles to assess concentration of EE2 and to characterize estrone (E1) and 17β-estradiol (E2). Wastewater was collected in 125mL bottles each time MWWE was collected for the exposure to assess the concentration of a subset of pharmaceuticals (ibuprofen, naproxen, diclofenac, triclosan, fluoxetine, atorvastatin, and venlafaxine). Tank water samples were extracted with a method specific for hormones (Arlos *et al*., 2015), and MWWE was extracted with a method that captures a broad range of pharmaceuticals (Tanna *et al*., 2013). After solid phase extraction, samples were reconstituted in 500 µL of methanol, and analyzed using LC-MS/MS as described previously (Wang *et al*., 2011).

4.3.4 Assessment of reproductive success

Fecundity, fertilization success, and embryo survival to hatch were determined for each pair as follows. Breeding tray systems consisting of two plant saucers (20 cm in diameter), one with a mesh bottom filled with gravel and a second collection tray underneath, were placed in each tank with the breeding group (Figure 4.1). In the wild, female rainbow darter perform nose-digs in the gravel and deposit eggs during spawning. In the breeding tray system the mesh bottom of the top tray allowed eggs to drop into the collection tray, thus preventing them from being consumed by adults. Breeding trays were checked daily for the deposition of eggs. Any eggs present were gently removed from the breeding trays, rinsed with control water and checked for fertilization using a dissecting microscope (S6D; Leica microsystems, ON, Canada). At the end of the 21-day exposure period, females were removed from the breeding tanks and sacrificed with a lethal dose of MS-222, the total length (± 0.1 cm), body, liver and gonads weight (± 0.001 g) were recorded.

Figure 4.1 Picture of breeding tray used to assess reproductive success of rainbow darter under control conditions, or exposed to 10 ng/L 17α-ethinylestradiol (EE2), 1, 10, or 20% municipal wastewater effluent (MWWE).

4.3.5 Behaviour experiment and analysis

Following the 21 days of exposure, males were separated in each 20 L tank using an opaque divider for 24 hours. At the start of the behaviour experiment, a mirror was placed inside each half of the aquaria and mounted to the glass using Velcro. Interactions between the rainbow darter males and the mirror were recorded using GoPro Hero 2 cameras mounted with a side view of each tank (so that two males could be observed in each video) for 30 minutes. Males were sacrificed after the 30-minute recording and sampled in the same manner as described previously for females, with the following addition. After determining the weight, testicular tissue was placed in 5 mL of Media 199 and placed on ice for assessment of stimulated *in vitro* steroid production.

Videos were analyzed by a single viewer who was blind to the treatment identity. Individuals were assessed for fifteen minutes after the time at which they identified themselves in the mirror. During the fifteen minutes, the proportion of time spent displaying one of five behaviour types (lateral display, frontal display, attack, swimming or resting on bottom of tank) was assessed. Additionally, the proportion of time spent occupying areas of the tank in relation to the mirror (near mirror and interacting, near mirror and not interacting, away from mirror) and the proportion of time being active or inactive were assessed. Since rainbow darter are a benthic species, any time spent in the water column was considered as being active (or swimming). To assess differences in behaviour, the proportion of time displaying aggressive behaviour (displays or attacks) were compared between treatment groups. Additionally, the activity of fish (resting versus swimming) was compared among treatment groups.

4.3.6 In vitro steroid production and steroid measurement

Testicular tissue was weighed (up to 20 mg) and placed into a well of a 24-well cell culture plate with 1 mL of Media 199 and stimulated with 10 IU of human chorionic gonadotropin. Tissue was incubated in stimulated solution for 24 hours at 16 °C, after which the solution was removed and stored at -80 °C for future analysis of testosterone (T) and 11-ketotestosterone (11KT) generated by the testicular tissue. Steroids were measured by enzyme linked immune-assay (EIA) following manufacturers guidelines (Cayman Chemical, MI, USA).

4.3.7 RNA isolation and integrity

Total RNA was isolated from liver tissue of using RNeasy kits (Qiagen, ON, Canada) according to manufacturer instructions. Tissue was homogenized using a tissue homogenizer (Power Gen 125; Fisher Scientific, ON, Canada) after the addition of 900 mL of QIAzol. After purification, samples were eluted from columns using 60 µL of RNase free water. RNA integrity was evaluated using the Tape Station (2200, Agilent Technologies, ON, Canada) with the RNA Screen Tape according to the

manufacturer's protocol. RNA integrity values (RIN scores) averaged 9.3 ± 0.46. RNA concentration was measured using a plate reader (Spectra Max M3, Molecular Devices LLC, CA, USA) with an adapter for small volume samples (µMax low volume 24-well microplate). RNA was stored at -80 $^\circ$C until cDNA synthesis.

4.3.8 cDNA synthesis and gene expression profiling using real-time PCR

cDNA was prepared from 500 ng total RNA according to the Bio-Rad iScript cDNA Synthesis Kit (CA, USA), in a total volume of 20 µL. The reaction was conducted in a thermal cycler (BioRad), according to manufacturer's protocol. cDNA was diluted 20 fold in DNase-RNase free water and stored at -20 $^\circ$C until analysis by real-time PCR.

Real-time PCR reactions were performed using SsoFast™ EvaGreen Supermix (Bio-Rad), 100 nmol of each primer, and 2.5 µL of diluted cDNA. Primers for reference genes and *vtg* were designed by a previous study (Bahamonde *et al.*, 2014), while primers for *cyp1a* were designed using Primer3 (http://biotools.umassmed.edu/bioapps/primer3 www.cgi) (Table 1). All primers were synthesized by Sigma-Aldrich. Reactions were assayed using the CFX96™ real-time PCR Detection System (Bio-Rad). The cycling parameters for real-time PCR were as follows: initial 1-cycle Taq polymerase activation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s, and primer annealing at optimal temperature (58 °C or 60 °C) for 5 s. After 40 cycles, a dissociation curve was generated starting at 65.0 °C and ending at 95.0 °C with incremental increases of 0.5 °C every 5 s. Plate design included controls to test for genomic DNA contamination including no reverse transcriptase (NRT) and no cDNA template (NTC). Negative controls indicated successful DNase treatment, and melt curves for all genes indicated a single product being formed. Each sample and standard were measured in duplicate. Biological replicates were as follows: control (n=10), EE2 10 ng/L (n=7), 1% MWWE (n=6), 10% MWWE (n=7), 20% MWWE (n=7). Efficiency and R^2 for each primer were calculated from a 7-point stand curve (Table 4-1).

Two reference genes *18s* and *ef1a*, were used to normalize the expression of target genes. No significant differences were found between control and treatment groups for either of the reference genes tested. For the reference genes, a combined M-value of 0.48 and CV of 0.17 were determined by the target stability function in CFX96 software, based on geNORM (Hellemans *et al.*, 2007). Normalized gene expression values were extracted using CFX Manager 2.1 software (Bio-Rad laboratories) using a relative ΔΔCq method.

Table 4-1 Real-time PCR primers used to measure mRNA levels in rainbow darter livers. The fit (R^2) and efficiency (E) of each pair of primars was determined using an eight-point standard curve.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	R^2	E (%)
18s	CGGTTCTATTTTGTGGGTTTTC	ACCTCCGACTTTCGTTCTTG	172	0.99	105.5
ef1α	ATCGGCGGTATTGGAACTG	CGGATTTCTTTGACGGACAC	197	0.997	102.9
vtg	CTACGCTCATTCCTGGGTTC	CAGTGGCAGTCGTTGTCCT	188	0.996	99.0
cyp1a	CTGTTTGGAGCGGGTTTTGA	ATGGTGAAGGGCAGGAATGA	213	1.0	94.8

4.3.9 Statistics

Chemistry of tank water was compared across the three weeks of exposure and tested for differences over time, and among treatment groups using a two-way analysis of variance (ANOVA). Fecundity and fertilization success were compared within each treatment group before and after the start of exposure using a student's t-test. Length, gonad somatic index (GSI), liver somatic index (LSI), condition factor (K), milt volume, gene expression, aggression, and activity were compared between exposure groups for male and female rainbow darter using a one-way ANOVA with a Tukey's post-hoc test. Gonad androgen production was not normally distributed, and thus was tested using a Kruskal-Wallis one-way ANOVA.

4.4 Results

4.4.1 Exposure chemistry

The concentration of MWWE in the exposure tanks varied slightly from the nominal concentrations. Proportion of effluent in exposure tanks was determined from conductivity measures and were 1.8 ± 0.1, 13.1 ± 0.5, and 21.0 ± 0.6 for the 1, 10, and 20% MWWE exposure tanks, respectively. Concentration of EE2 in tank water was 9.4 ± 0.7 ng/L.

Ammonia was elevated in 10% and 20% MWWE tanks compared to controls during week one (but not EE2, or 1%). This trend varied slightly over the course of the exposure and was related to changes in concentrations in the whole effluent. While conductivity increased, ammonia concentrations decreased over the course of the study (Table 4-2). Water temperature increased throughout the experiment in a manner that paralleled the water temperature in the original site of field collection (Table 4-2, Appendix A, Figure S4.1). Additionally, dissolved oxygen content in tanks decreased during the third week of the exposure (Table 4-2).

Concentrations of ibuprofen, naproxen, diclofenac, triclosan, fluoxetine, atorvastatin, and venlafaxine in MWWE collected from the Kitchener MWTP varied little over the course of the exposure

(Figure 4.2A). In fish tanks the concentration of E1 did vary among treatments, with 10% and 20% MWWE tanks having higher concentrations compared to control tanks in week one (Figure 4.2B). Concentration of estrone did not differ among treatment groups in week two, or week three. The concentration of E2 varied among weeks, but was not significantly different among treatments.

4.4.2 Fish health measures and reproductive success

No differences in GSI, LSI, or K in female or male fish were found after 21 days of exposure to MWWE or EE2 (Appendix A, Table S4-1, Table S4-2). A higher volume of milt (collected manually at the end of the experiment) was observed in both the males exposed to EE2 and to 20% MWWE when compared to control males (Figure 4.3A). The synthesis of 11KT and T from hCG stimulated male testicular tissue was found to be unaffected by any of the exposure treatments (Figure 4.3 B, C).

The rate of egg laying in females was variable throughout the exposure period (Figure 4A). The reason for this variability in egg production is not known, but could be related to the fact that they are asynchronous clutch spawners. Exposure to 20% MWWE resulted in a reduction in female fecundity after 7 days of exposure compared to control females (Figure 4.4B). This decrease in egg laying, however, was no longer present at later points in the exposure (day 14 and day 21) (Figure 4.4B). Fertilization success of fish was generally lower during the exposure period compared to the pre-exposure, however a significant difference in fertilization success was only observed in fish exposed to EE2 or 20% MWWE (Figure 4.5).

4.4.3 Male behavioural endpoints

When the aggressive behaviour of male fish was assessed, it was noted that frontal and lateral displays were the most common type of behaviour, with attacks occurring infrequently (Appendix A, Figure S4.2). The portion of time spent displaying aggressive behaviour was lower in males exposed to 20% MWWE (Figure 4.6A) when compared to the control group. Exposure to EE2 had no effect on the proportion of time spent displaying aggressive behaviour (Figure 4.6A). Additionally, male rainbow darter exposed to 20% MWWE spent more time in the behaviour experiment being active than fish in the control or EE2 treatments (Figure 4.6B). When a multiple regression analysis was conducted, no relationships were noted between aggressive or active behaviour and sex steroid synthesis (T or 11KT) by testicular tissue. A weak positive correlation was found between length of fish and aggressive behaviour (R^2=0.1; $p<0.05$), but not between length and activity of fish.

Table 4-2 Mean ± SE of conductivity, ammonia, and temperature measures taken in individual exposure tanks (n=6) of rainbow darter throughout the pre-exposure (five measurements) and exposure (three measurements per week). Treatments that do not share a common lower case letter, and time points that do not share a common upper case letter, are significantly different as determined by a two-way analysis of variance (p<0.05).

	Conductivity (µSiemens)			Ammonia (mg/L)			Temperature (°C)			Dissolved oxygen (mg/L)		
Pre-exposure	642 ± 12			0.24 ± 0.03			14.6 ± 0.3			8.9 ± 0.4		
Exposure week	1	2	3	1	2	3	1	2	3	1	2	3
Control	618 ± 18 a, A	646 ± 20 a, B	623 ± 1 a, AB	0.15 ± 0.05 a, A	0.12 ± 0.04 a, B	0.10 ± 0.03 a, B	17.3 ± 0.7 a, A	17.7 ± 1.9 a, B	20.9 ± 0.3 a, C	8.3 ± 0.3 a, A	8.3 ± 0.4 a, A	7.4 ± 0.1 a, B
EE2	612 ± 19 a, A	640 ± 15 a, B	620 ± 14 a, AB	0.15 ± 0.04 a, A	0.17 ± 0.08 b, A	0.10 ± 0.03 a, B	17.4 ± 0.7 a, A	18.0 ± 1.7 b, B	21.0 ± 0.3 a, C	8.4 ± 0.2 a, A	8.2 ± 0.4 a, A	7.4 ± 0.2 a, B
1%	642 ± 20 b, A	674 ± 22 b, B	655 ± 3 b, AB	0.17 ± 0.06 a, A	0.18 ± 0.04 b, A	0.10 ± 0.03 a, B	17.4 ± 0.6 b, A	17.9 ± 1.8 b, B	21.0 ± 0.3 a, C	8.2 ± 0.2 a, A	8.4 ± 0.4 a, A	7.0 ± 0.2 b, B
10%	789 ± 19 c, A	879 ± 23 c, B	878 ± 9 c, B	0.70 ± 0.20 b, A	0.42 ± 0.11 c, B	0.20 ± 0.07 b, C	17.4 ± 0.6 ab, A	17.8 ± 1.8 ab, B	21.0 ± 0.3 a, C	8.4 ± 0.2 a, A	8.1 ± 0.4 a, A	7.1 ± 0.3 ab, B
20%	923 ± 35 d, A	1011 ± 17 d, B	1027 ± 42 d, B	1.30 ± 0.41 c, A	0.70 ± 0.17 d, B	0.29 ± 0.11 c, C	17.3 ± 0.6 ab, A	18.0 ± 1.7 b, B	21.0 ± 0.3 a, C	8.4 ± 0.2 a, A	8.1 ± 0.5 a, A	7.4 ± 0.1 ab, B
Whole-effluent	2113 ± 9	2367 ± 26	2412 ± 48	11.05 ± 3.27	7.34 ± 1.37	4.07 ± 1.27						
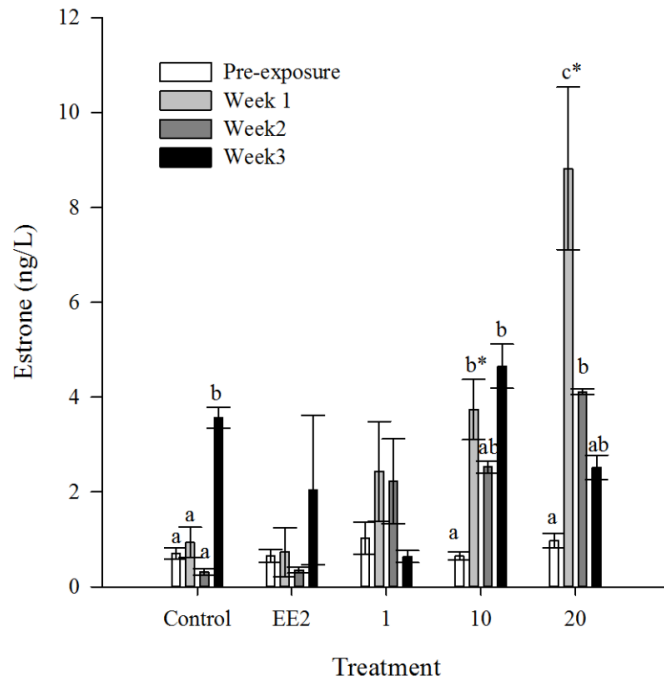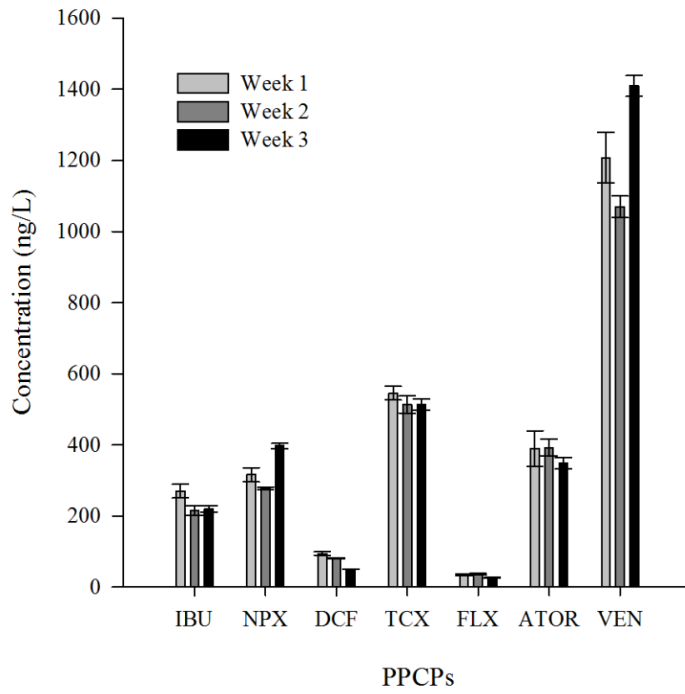
Figure 4.2 Mean (± SE) concentration of (A) pharmaceuticals and personal care products (PPCPs) in final municipal wastewater effluent (MWWE) and (B) estrone during each of three weeks of the exposure. PPCPs measured included ibuprofen (IBU), naproxen (NPX), diclofenac (DCF), triclosan (TCX), fluoxetine (FLX), atorvastatin (ASTOR), and venlafaxine (VEN). Estrone was measured in tanks of fish exposed to dechlorinated tap water (control), 10 n/gL 17α-ethinylestradiol (EE2), or MWWE diluted to 1, 10, or 20%. Bars within an exposure treatment that have different letters indicates a significantly difference ($p<0.05$) among exposure weeks. Additionally, bars with an asterisk are significantly different from the control treatment at that time point (week). Differences were determined using a two-way analysis of variance with a Tukey's post-hoc test.
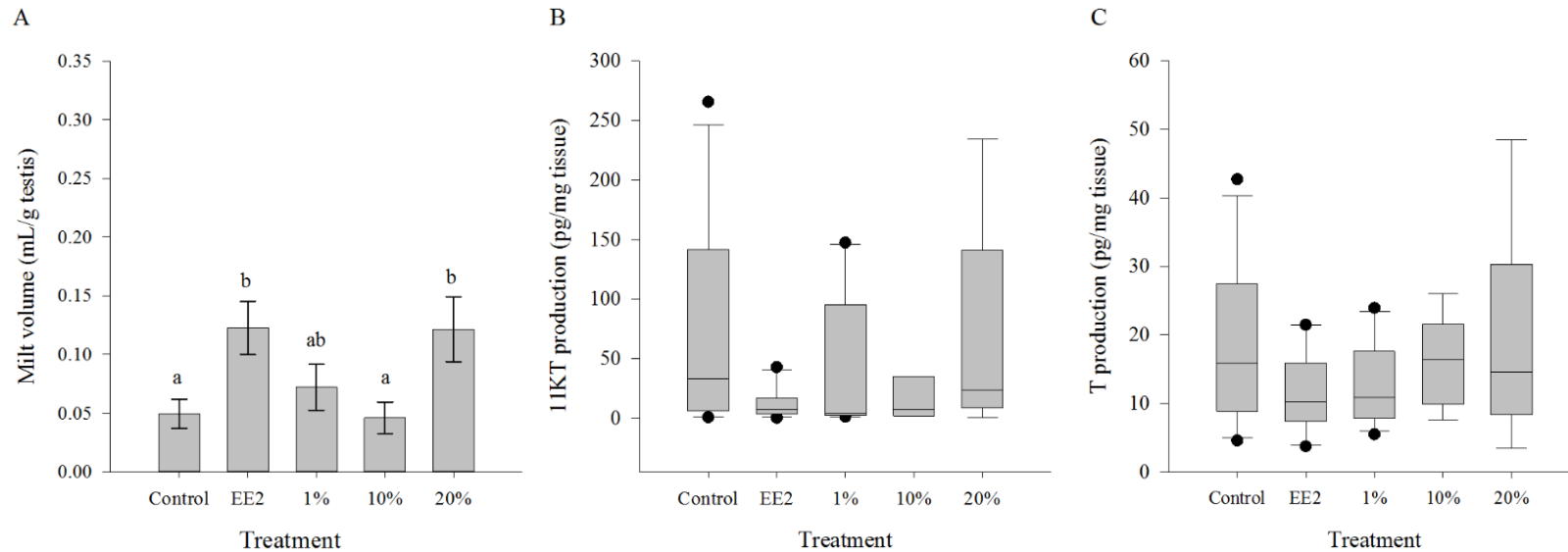
Figure 4.3 Measures of reproductive fitness collected from male rainbow darter exposed to dechlorinated tap water (control), 10 ng/L 17α-ethinylestradiol (EE2), or diluted municipal wastewater effluent (MWWE; 1, 10, 20%) including (A) milt production (mean ± SE), and *in vitro* synthesis of (B) 11-ketotestosterone (11KT) and (C) testosterone (T) synthesis in testis tissue (box plot representation where the horizontal line in box plots represents the median, the boundaries of the box represent the 25th and 75th percentiles, the 10th and 90th percentiles are represented by the whiskers, and the black dots represent outliers). Bars that do not share a common letter are significantly different as determined by a one-way analysis of variance with a Tukey's post-hoc test (p <0.05).

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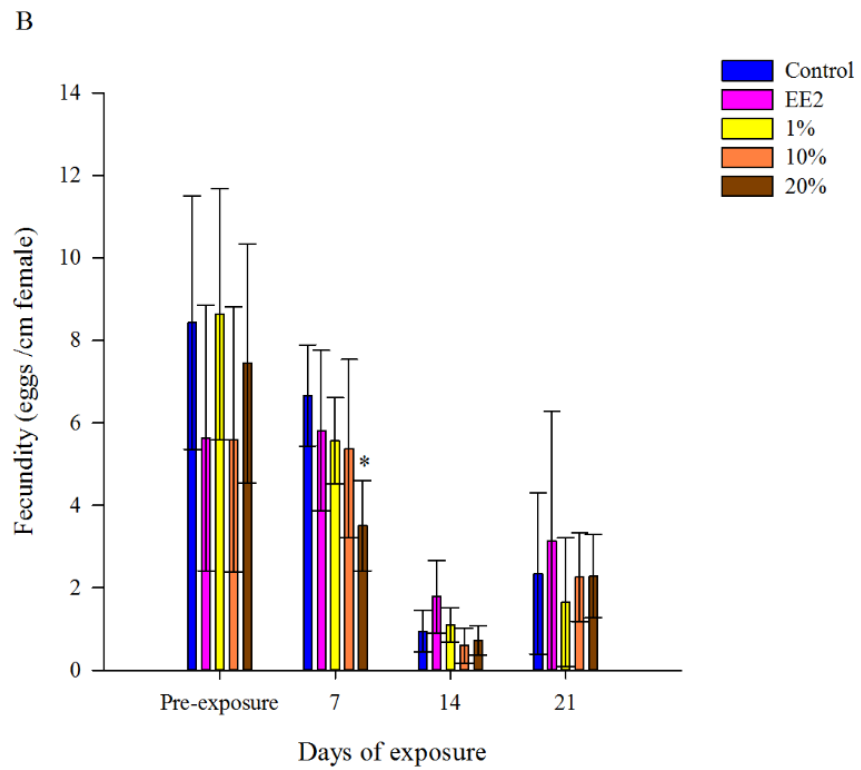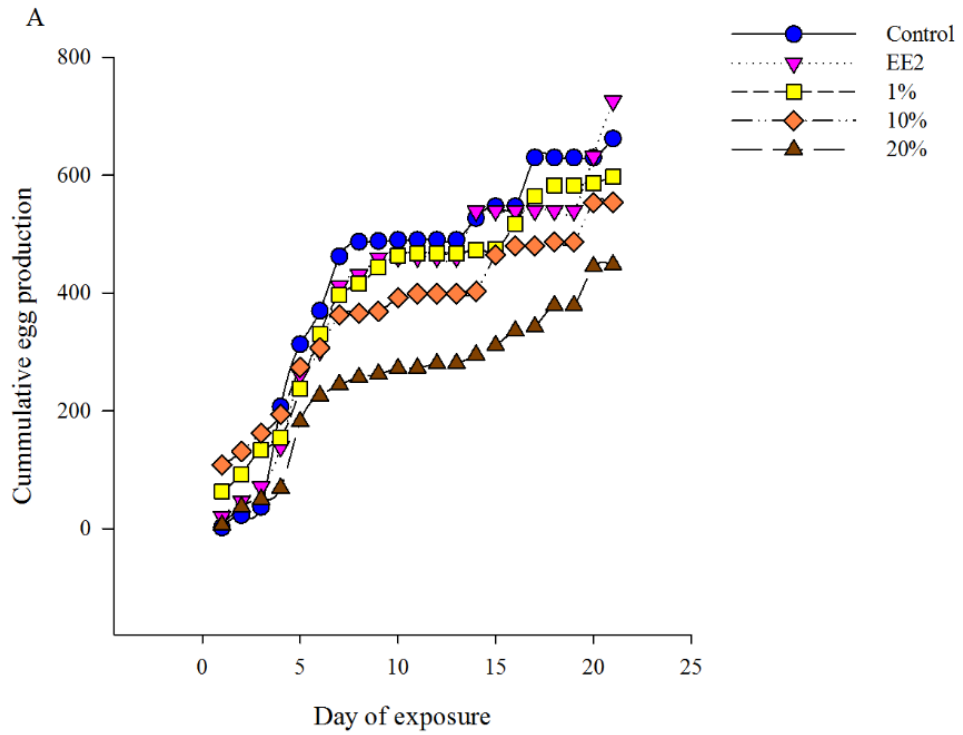
Figure 4.4 Egg production by groups of rainbow darter exposed to dechlorinated tap water (control), 10 ng/L 17α-ethinylestradiol (EE2), or diluted municipal wastewater effluent (MWWE; 1, 10, 20%). (A) Cumulative egg production over the 21-day period and (B) mean (± SE) fecundity (number of eggs deposited per cm length of females) of rainbow darter through the pre-exposure period and for each week (mean across 7 days) of the exposure. * indicates significant difference (p < 0.05) from control at that time point as determined by a student's t-test.
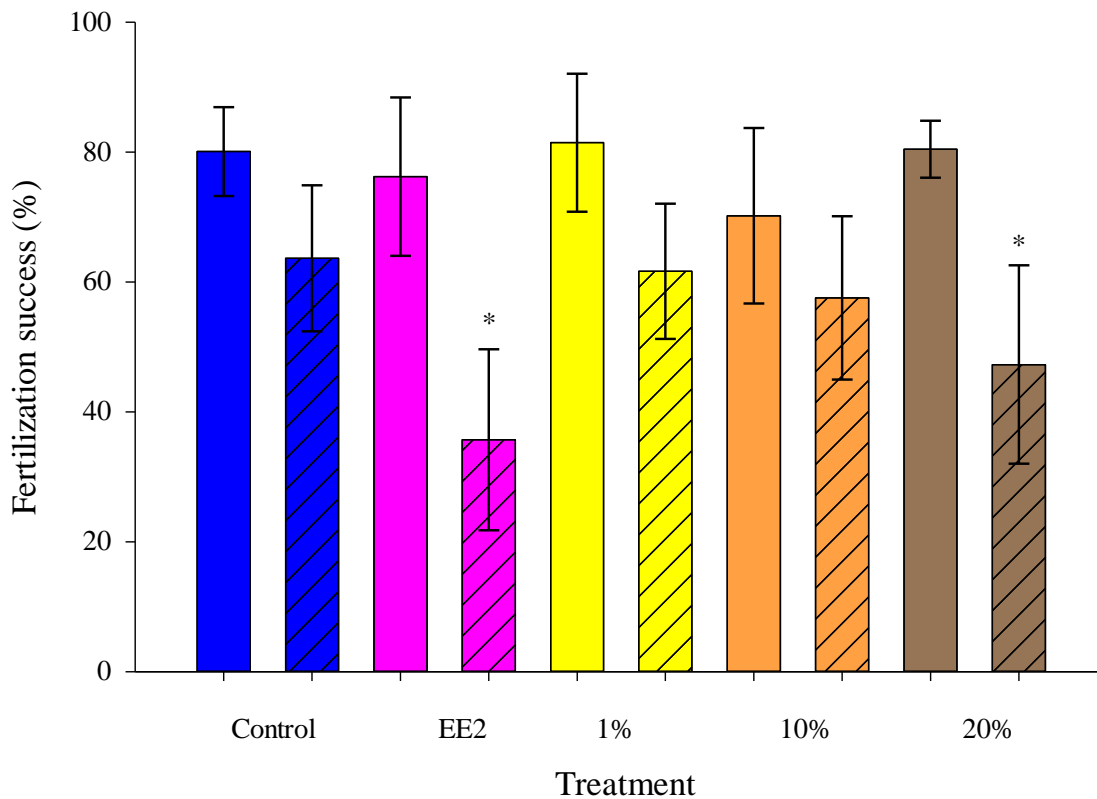
Figure 4.5 Fertilization success (mean ± SE) of rainbow darter through the pre-exposure (open bars) and exposure period (diagonal lines) to dechlorinated tap water (control), 10 ng/L 17α-ethinylestradiol (EE2), or diluted municipal wastewater effluent (1, 10, 20%). * indicates significant difference (p < 0.05) in fertilization success between pre-exposure and exposure within a treatment and was determined using a student's t-test.

A
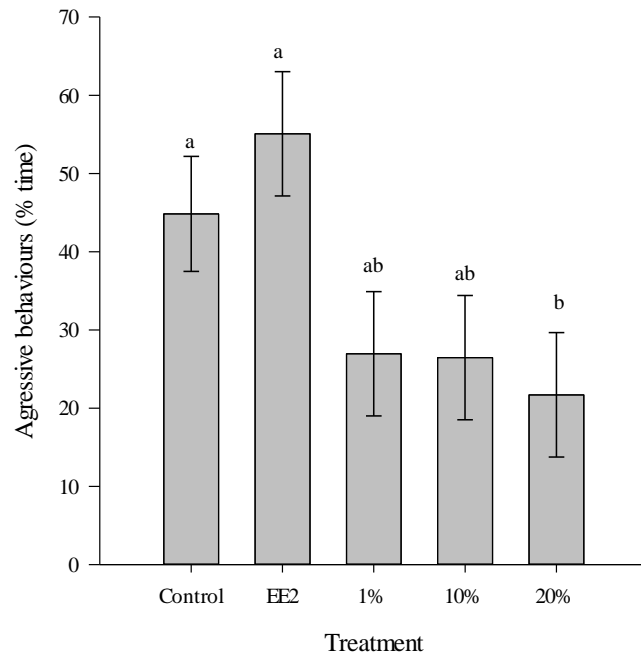
B
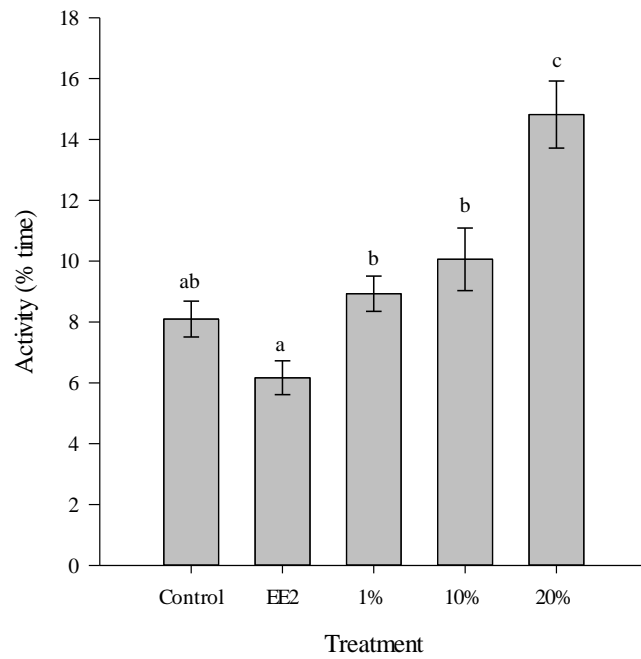
Figure 4.6 Comparison of mean (± SE) proportion of time male rainbow darter spent (A) displaying aggressive behaviours and (B) swimming actively in a mirror test after 21 days of exposure to dechlorinated tap water (Control), 10 ng/L 17α-ethinylestradiol (EE2), or diluted municipal wastewater effluent (1%, 10% or 20%). Bars that do not share a common letter are significantly different as determined by a one-way analysis of variance with a Tukey's post-hoc test (p <0.05).

4.4.4 Gene expression

The expression of male *cyp1a* was reduced in males exposed to EE2 for 21 days when compared to control males. While no difference in *cyp1a* expression was found after 21 days of exposure to 1 or 10% MWWE, males exposed to 20% effluent had increased *cyp1a* expression (Figure 4.7 A). Expression of *vtg* in males was induced over five-fold by EE2 exposure, and a small (1.5 fold) induction in *vtg* expression was also observed after exposure to 20% MWWE (Figure 4.7 B).
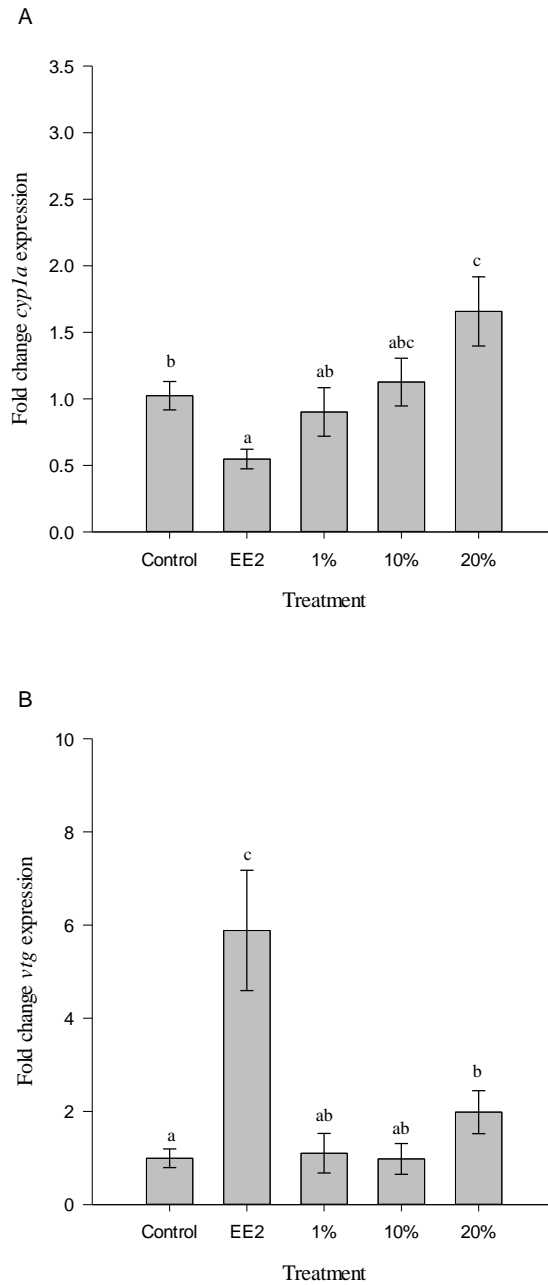
Figure 4.7 Relative expression (mean ± SE) of *cyp1a* (A) and *vtg* (B) in male liver tissue collected after being exposed to dechlorinated tap water (Control), 10 ng/L 17α-ethinylestradiol (EE2), or diluted municipal wastewater effluent (MWWE; 1, 10, 20%). Bars that do not share a common letter are significantly different as determined by a one-way analysis of variance with a Tukey's post-hoc test ($p < 0.05$).

4.5 Discussion

In this study a native fish species, rainbow darter, was exposed to environmentally relevant doses of municipal wastewater effluent (MWWE) for 21 days during which reproductive success and male reproductive behaviours were assessed. Minor effects on reproductive health and success were observed after exposure to the highest concentration of MWWE (20%). Milt volume and gene expression of *cyp1a* and *vtg* were elevated in 20% MWWE exposed fish compared to controls, while egg laying, fertilization success, and aggression were reduced. While the positive control (EE2) also demonstrated minor effects on fertilization success and a larger induction of *vtg*, no change in egg laying or male reproductive behaviour were noted. This study is unique due to the use of the native fish species and enhances our understanding of what may have occurred in previously studied populations of rainbow darter exposed to MWWE in the wild.

4.5.1 Exposure chemistry and molecular response

Analysis of the chemical composition of the effluent demonstrated that it contained a wide variety of pharmaceuticals. Venlafaxine (ananti-depressant) was the most concentrated with over 1 µg/L present in final effluent. Triclosan (an anti-microbial with anti-androgenic activity) was the second most concentrated compound. Although pharmaceuticals were not assessed in tank water, estrogenic compounds were. The concentration of EE2 was below the method detection limit in all tanks except for the EE2 treatment group, in which the measured concentration (9.4 ± 0.7) was close to the nominal concentration (10 ng/L). The concentration of E2 did not differ among treatment groups. The concentration of E1, however, was higher in the 10% and 20% MWWE tanks during the first week of the exposure. Estrone is commonly detected in MWWE and contributes a large portion to the overall estrogenicity of a sample (Atkinson *et al*., 2012). A small increase in *vtg* expression was observed in males exposed to 20% MWWE compared to controls. While E1 likely contributed in part to the estrogenicity, there are numerous other compounds in MWWE that were not measured that can act additively on the estrogen receptor (Thorpe *et al*., 2003). A larger (five-fold) increase in *vtg* expression was observed in males exposed to 10 ng/L EE2 than males exposed to 20% MWWE. This suggests that the diluted effluent has lower estrogenicity than the EE2 exposure (~20ng 17β-estradiol equivalency).

Expression of *cyp1a* was higher in males exposed to 20% MWWE, but lower in males exposed to 10 ng/L EE2 compared to controls. Increased *cyp1a* expression after exposure to MWWE

has been observed in other studies (Chen *et al*., 2016; Ings *et al*., 2011) and is associated with exposure to contaminants such as poly-aromatic hydrocarbons (Billiard *et al*., 2004), and polychlorinated biphenyl (Roy *et al*., 2003). It is unclear whether PPCPs in MWWE can impact *cyp1a* expression since some studies have observed increases in *cyp1a* with exposure to PPCPs such as diclofenac (Mehinto *et al*., 2010), while others have observed decreases after exposure to PPCPs including EE2 (Laville *et al*., 2004; Thibaut *et al*., 2006).

4.5.2 Reproductive health and success

This study was designed to mimic a fathead minnow 21-day reproductive test. While we observed a steady baseline of egg laying with rainbow darter during the 10-day pre-exposure and the first 7 days of exposure, the rate of egg laying during the later portion of the exposure period (day 14 to 21) was lower in all groups. The decrease in spawning activity in the later portion of the experiment may be a natural lull in the breeding period of rainbow darter, but this would require further investigation. The mean fecundity of control females in this study was 148 ± 32, which was lower than the mean annual fecundity of 309 ± 44 found by Fuller (1998). In addition to potential difference in egg laying potential due to site specific differences, lower egg laying in this study may be due to difference in the number of females held per tank (two in our experiment vs one female per tank with two males), or the duration of the experiment (31 days vs. 54 days) between our study and the study conducted by Fuller (1998). While this study was important in order to answer key questions from the field, this species may not be an ideal laboratory model for long-term reproductive studies.

Minor indications that reproductive success of rainbow darter was reduced were observed when fish were exposed to the highest dose (20%) of MWWE. Females spawned fewer eggs during the first portion (7 days) of MWWE exposure, but this effect was no longer present after 14, and 21 days of exposure. This may be due to the change in effluent quality. As seen in Table 2 and Figure 2, the concentration of ammonia and pharmaceuticals present in the effluent decreased throughout the course of the exposure. Previous studies have demonstrated that exposure to MWWE during the reproductive period reduces egg production in fish, but this typically occurs at concentrations > 25% (Cavallin *et al*., 2016; Filby *et al*., 2010; Galus *et al*., 2013; Lister *et al*., 2009; Ma *et al*., 2005; Schilling, 2015; Smolders *et al*., 2002; Thorpe *et al*., 2009). Another possible explanation for why an effect was observed at 7 days, but not at 14 or 21 days, is adaptation to MWWE exposure after the

first week. A recent study clearly demonstrates the recovery of fathead minnow egg production throughout a 21-day exposure to 100% MWWE (Cavallin *et al*., 2016). A study by Ankley and Villeneuve (2015) demonstrated that the reproductive axis (hypothalamic pituitary gonad axis) of fathead minnows compensate for suppression of plasma estrogen production after a 7 to 10-day period of exposure to compounds such as fadrazole, prochloraz, trilostane and trenbolone.

       We observed no effect of exposure to 10 ng/L EE2 on egg laying in this study. Whether exposure to estrogenic compounds (E2, EE2) during spawning has an effect on egg laying seems to depend on dose (Brion *et al*., 2004; Filby *et al*., 2010; Jobling *et al*., 2004; Lister *et al*., 2009; Santos *et al*., 2007; Schilling, 2015; Seki *et al*., 2002; Thorpe *et al*., 2009) and species/strain sensitivity (Bosker *et al*., 2016; Söffker *et al*., 2012). Similar to this study, the majority of studies have found no effect of exposure on spawning fish to 10 ng/L of EE2 on egg production (Bosker *et al*., 2016; Filby *et al*., 2010; Jobling *et al*., 2004; Lister *et al*., 2009; Thorpe *et al*., 2009). It is thus likely that the slight reduction of egg laying observed in the 20% MWWE group is not due to estrogenic compounds, but some other constituent of MWWE.

       In addition to reduced egg production, we observed increased total milt volume in males exposed to 20% MWWE or to 10 ng/L EE2. Increased milt volume was also observed in a previous study of male rainbow darter collected from sites downstream of MWWTPs during the spawning season (Fuzzen *et al*., 2015). We interpreted this increased milt volume as a reduction in the release of milt for spawning events. This hypothesis is supported by the fact that fertilization success was lower in rainbow darter exposed to 20% MWWE and EE2 (when compared to fertilization success of these groups during the acclimation period). The literature demonstrates conflicting reports about the effects of MWWE exposure during spawning on fertilization success, with one study showing reduced fertilization success (Ma *et al*., 2005), another showing no effect (Cavallin *et al*., 2016), and a third demonstrating a positive effect (Chen *et al*., 2016). Similarly, the literature examining the effect of EE2 exposure during spawning on fertilization success is conflicted. In some studies, similar to this one, fertilization success is shown to be reduced after exposure to doses $\leq$ 10 ng/L EE2 (Hill and Janz, 2003; Söffker *et al*., 2012), while others demonstrated no effect at doses $\geq$ 10 ng/L (Brion *et al*., 2004; Hashimoto *et al*., 2009; Martinović *et al*., 2008). Additionally, Peters *et al*. (2007) found a decrease in fertilization success of mummichog (*Fundulus heteroclitus*) at 1 and 100 ng/L EE2, but not at 10 ng/L. This variability seems to suggest that this response is quite variable between individuals, and is likely dependent on the timing of exposure in relation to sperm development.

Overall it is possible that estrogenic compounds in MWWE are at least partially responsible for reduced fertilization success observed in this study.

4.5.3 Male behaviour

In this study, males exposed to 20% MWWE were less aggressive than control males. A decline in spawning behaviour/success has also been reported in other studies in which fish were exposed to MWWE (Garcia-Reyero *et al*., 2011; Martinović *et al*., 2007; Schoenfuss *et al*., 2002; Sebire *et al*., 2011). Additionally, altered reproductive behaviour has been observed in studies of fluoxetine exposure (Dzieweczynski and Hebert, 2012; Perreault *et al.*, 2003). While concentrations of fluoxetine in this experiment were low, venlafaxine was the most concentrated of all the pharmaceuticals measured and may be contributing to the alteration of male behaviour in the 20% MWWE exposed fish. Similar to other endpoints, other studies have observed contradicting results concerning the effects of MWWE on male reproductive behaviour. Some studies demonstrate an increase (Chen *et al*., 2016; Garcia-Reyero *et al*., 2009; Saaristo *et al*., 2014) and other demonstrate no change (Barber *et al*., 2007) in sexual or aggressive behaviour in fish exposed to MWWE. No changes in male aggression were noted after exposure of rainbow darter to 10 ng/L EE2 in this study. In contrast, many studies have observed decreased nest holding or spawning behaviours after exposure to EE2 (Coe *et al*., 2008; Martinović *et al*., 2008; Salierno and Kane, 2009).

In addition to reduced aggression, we observed an increase in activity of male fish exposed to 20% MWWE. Activity, or locomotion has been demonstrated to be increased after exposure to the pesticide atrazine (Rohr and McCoy, 2010), the anti-depressant sertraline (Xie *et al*., 2015), and psychiatric drugs such as diazepam (Brandão *et al*., 2013; Oggier *et al*., 2010). Similar to aggression, no changes in activity were noted after exposure to EE2, suggesting that the alterations of behaviour observed in the 20% MWWE exposed males were not related to estrogenic compounds in this study.

4.5.4 Conclusions

In this study we observed minor reproductive disruption after exposure to an environmentally relevant dose of MWWE to a local fish species. While the acute duration of this exposure cannot account for the long-term exposure and accumulation of contaminants over the life-span of a fish, it did allow us to ask specific questions about fecundity and behaviour that could not be addressed from field collections. We found that fecundity was affected when effluent contained higher concentrations

of ammonia, that behaviour of male fish was also altered by exposure to the highest concentration of MWWE, and that these two responses were not influenced by an estrogenic compound (EE2) at a concentration higher than expected in the effluent. In addition to physiological alterations previously observed in wild fish, these alterations of fecundity and reproductive behaviour would reduce reproductive fitness of affected fish. This could potentially lead to a reduced populations of rainbow darter in areas with high ($\geq$20%) concentrations of MWWE from outfalls.

Chapter 5

An assessment of the spatial and temporal variability of biological responses to municipal wastewater effluent in rainbow darter (*Etheostoma caeruleum*) collected along an urban gradient

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5.1 Overview

Municipal wastewater effluent (MWWE) and its constituents, such as chemicals of emerging concern, pose a potential threat to the sustainability of fish populations by disrupting key endocrine functions in aquatic organisms. While studies have demonstrated changes in biological markers of exposure of aquatic organisms to groups of chemicals of emerging concern, the variability of these markers over time has not been sufficiently described in wild fish species. The aim of this study was to assess the spatial and temporal variability of biological markers in response to MWWE exposure and to test the consistency of these responses between seasons and among years. Rainbow darter (*Etheostoma caeruleum*) were collected in spring and fall seasons over a 5-year period in the Grand River, Ontario, Canada. In addition to surface water chemistry (nutrients and selected pharmaceuticals), measures were taken across levels of biological organization in rainbow darter. The measurements of hormone production, gonad development, and intersex severity were temporally consistent and suggested impaired reproduction in male fish collected downstream of MWWE outfalls. In contrast, ovarian development and hormone production in females appeared to be influenced more by urbanization than MWWE. Measures of gene expression and somatic indices were highly variable between sites and years, respectively, and were inconclusive in terms of the impacts of MWWE overall. Robust biomonitoring programs must consider these factors in both the design and interpretation of results, especially when spatial and temporal sampling of biological endpoints is limited. Assessing the effects of contaminants and other stressors on fish in watersheds would be greatly enhanced by an approach that considers natural variability in the endpoints being measured.

5.2 Introduction

Municipal wastewater effluent (MWWE) is the largest (by volume) source of pollution in the aquatic environment in Canada (Holeton *et al*., 2011). Over the past century, municipal wastewater treatment plants (MWWTPs) have enhanced their processes to effectively remove pathogens, organic matter, and some portion of the nitrogenous wastes from the effluents. The use of these treatment technologies has vastly improved public and environmental health (Cutler and Miller, 2005; Naik and Stenstrom, 2012). While progress in treatment processes has greatly reduced their environmental impacts, new challenges have arisen. Chemicals of emerging concern (CECs), such as human steroid hormones, and pharmaceuticals and personal care products, are passed through the body or washed down sink drains and enter municipal wastewater systems (Halling-Sørensen *et al*., 1998; Heberer, 2002). MWWTPs are not currently designed to remove the diversity of CECs that enter the wastewater streams (Daughton and Ternes, 1999). Although some compounds are fully degraded through existing wastewater treatment processes, others are either transformed into other compounds or remain intact and released into the receiving environment (Kasprzyk-Hordern *et al*., 2009; Luo *et al*., 2014; Miege *et al*., 2009). Thus, numerous CECs have been detected in surface waters around the world (Ternes *et al*., 1999).

The presence of CECs in aquatic environments is of concern because of their wide distribution and their potential to have adverse effects on aquatic organisms (Corcoran *et al*., 2010; Sumpter, 2005). The concentrations of these compounds in surface waters are typically very low, often below the detection limits of current analytical methods (Prasse *et al*., 2015). However, it has been demonstrated that CECs can impair the endocrine function of aquatic organisms at environmentally relevant concentrations (Overturf and Huggett, 2015). This high activity at low concentrations is due to the similarity of the structure of these chemicals to endocrine hormones, allowing them to bind to endocrine receptors (Blair *et al*., 2000). Estrogenic compounds such as 17α-ethinylestradiol (EE2), which are ubiquitous in MWWE (Ternes *et al*., 1999), can impair reproduction in fish populations at concentrations of a few ng/L (Lange *et al*., 2008; Lange *et al*., 2009; Parrott and Blunt, 2005). A study in which a whole-lake was dosed with an average of 5 ng/L of EE2 for three consecutive summers found that fathead minnows (*Pimephales promelas*) exhibited disrupted reproductive health followed by a collapse of the population (Kidd *et al*., 2007; Kidd *et al*., 2014). Despite the strong relationship between exposure to an estrogenic compound and the collapse of a population of fish in the whole-lake study, it remains difficult to make direct associations between

CECs in effluents and adverse effects in aquatic organisms. Laboratory (Filby *et al*., 2007; Liney *et al*., 2005a; Liney *et al*., 2005b) and caging (Jasinska *et al*., 2015; Kolok *et al*., 2007; Writer *et al*., 2010) exposures of aquatic organisms to MWWE have improved our understanding of the changes in biomarkers that occur as a result of exposure to CECs (Sumpter and Jobling, 2013). Adverse outcome pathways (AOPs) have synthesized the knowledge gained in laboratory and caging studies by linking stressors to biological responses across levels of biological organization. AOPs identify molecular initiating events, key biological events, and adverse outcomes (Ankley *et al*., 2010). Although many AOPs are able to predict adverse outcomes to groups of stressors under controlled laboratory conditions, they have not yet expanded to include the complexities of exposure in aquatic environments.

There are many additional variables present in the field that complicate the assessment of the effects of CECs in MWWE. Wastewaters are complex mixtures of chemicals and nutrients that can vary temporally, and their composition is also dependent on the degree and type of treatment processes (Baynes *et al*., 2012; Luo *et al*., 2014; Prasse *et al*., 2015; Verlicchi *et al*., 2012). Additionally, natural systems contain many other anthropogenic and natural stressors that can vary spatially and temporally across watersheds, creating confounding or cumulative effects (Pal *et al*., 2010; Park and Park, 2015). As a result, measures of fish health in receiving waters vary widely over time. The main challenge we face in assessing the potential adverse effects of CECs in MWWE is separating changes in biological indicators due to exposure from changes due to natural variability. The variability of biological measures associated with endocrine disruption has not been well explored in wild fish in the literature.

A number of studies have examined wild fish in areas influenced by MWWE outfalls to assess the effects associated with CECs (Bizarro *et al*., 2014; Bjerregaard *et al*., 2006; Blazer *et al*., 2007; Brander *et al*., 2013; Huang *et al*., 2016; Jobling *et al*., 1998; Prado *et al*., 2011; Vajda *et al*., 2008; Vethaak *et al*., 2005). While many studies have examined key biological responses associated with CECs in MWWE, few have examined the variability in these responses. The variability that has been examined includes variability due to species differences (van Aerle *et al*., 2001), spatial variation (Blazer *et al*., 2007), and seasonal variation (Allen *et al*., 1999; Blazer *et al*., 2012). To date, only one study has examined the annual variability of reproductive disruption (Blazer *et al*., 2012). Although consistent responses were found between two years of study in that report, there is still a great need for additional information concerning the amount of variability in the response of fish

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populations to MWWE and the consistency of the response over time. To be predictive, biological monitoring programs need to be able to confidently detect a change in a biological measure that can be associated with a source or stressor. Once a change is confidently detected and placed within the context of an adverse outcome pathway, more appropriate and effective management decisions can be made.

The aim of this study is to better understand the spatial and temporal variability of key biological measures associated with CEC exposures. A large data set was assembled by compiling previous research data and gathering additional samples from the Grand River, Ontario, which is an area with known MWWE impacts (Bahamonde *et al*., 2015a; Fuzzen *et al*., 2015; Tetreault *et al*., 2011; Tanna *et al*, 2013). The intense study of this unique system presents an opportunity in which to address questions about the variability of biological responses exposed to MWWE.

5.3 Materials and Methods

Rainbow darter (*Etheostoma caeruleum*) are a small-bodied, benthic-dwelling, sexually dimorphic percid species. Reproductive maturity is reached at one year of age and adults spawn annually (asynchronously in clutches) from mid-April to late May. This species was chosen as a model because it is highly abundant in the watershed and has low mobility. To assess reproductive health, fish were collected after gonad recrudescence in the fall (late October/early November) and just before the spawning season in the spring (late March/early April).

Surface water chemistry and biological measures were collected in fall and spring seasons for up to 5 years from 7 sites. These sites were dispersed through an urban gradient that encompasses the outfalls of two MWWTPs in the Grand River, Ontario, Canada (Figure 5.1). In addition to novel data collected in fall of 2011, spring of 2012, and fall of 2012, data were gathered from studies conducted in the Grand River watershed from fall 2007 to spring 2011. These studies used similar methodology to collect rainbow darter and measure indicators of their health. The data from fall 2007 to spring 2011 were originally used to address independent research questions related to the endocrine-disrupting effects of wastewater (Bahamonde *et al*., 2015a; Bahamonde *et al*., 2015b; Bahamonde *et al*., 2014; Fuzzen *et al*., 2015; Tanna *et al*., 2013; Tetreault *et al*., 2011).
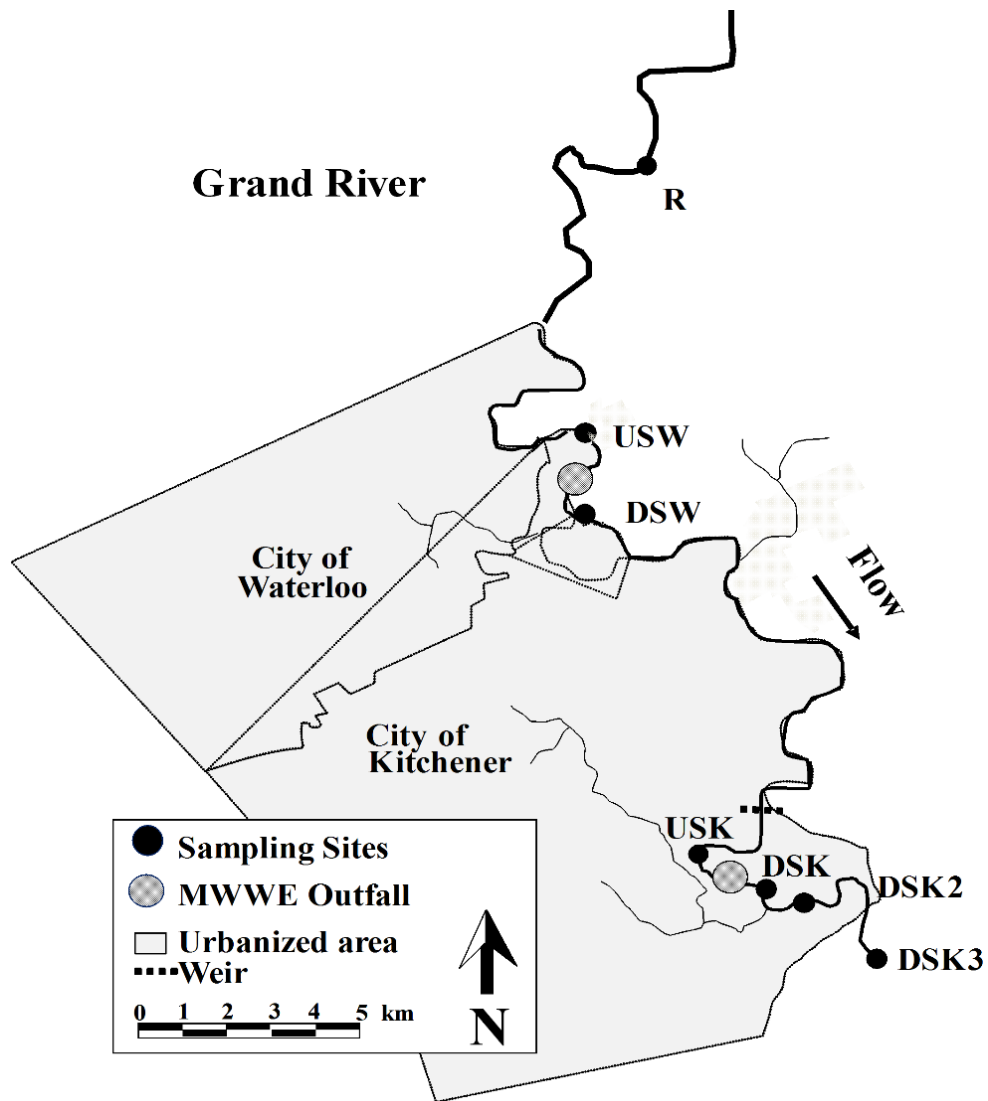
Figure 5.1 Map of sampling area in the Grand River watershed (southern Ontario, Canada). Fish and surface water were collected from one reference (R) site, two sites upstream (US), and four sites downstream (DS) of municipal wastewater effluent (MWWE) outfalls.

The Grand River watershed supports close to one million people spread across several population centers. There are 30 MWWTPs in the watershed, which vary greatly in terms of the size of population served and process type. The two largest treatment plants (Waterloo and Kitchener) are located close to one another in the most urbanized central reaches of the watershed. At the time of the studies, both treatment plants were operated with an activated sludge process with minimal or partial nitrification, as described in Table 5-1.

Table 5-1 Properties of Waterloo and Kitchener municipal wastewater treatment plants from 2007 to 2011 (Waterloo, 2012).

Municipal wastewater treatment plant	Population served	Treatment process	Capacity rating (m^3/day)	Volume (m^3/day)
Waterloo	120,265 - 127,688	Conventional activated sludge with chemical phosphorus removal, anaerobic sludge digestion, and chlorine disinfection	54,600	41,358 – 47,562
Kitchener	215,247 – 227,761	Conventional activated sludge with chemical phosphorus removal, anaerobic sludge digestion, sodium hypochlorite disinfection, and sodium bisulphite dechlorination	122,700	64,329 – 74,935

5.3.1 Site description

Fish were collected across an urban gradient that included the outfalls of the two largest wastewater treatment plants (Figure 5.1). Although the number of sites varied among seasons and years because of changes in the objectives of the original collections, access, and weather conditions, a subset of sites were selected for analysis in this study. Up to seven sites from each sampling period were included. A rural reference (R) site, an upstream and downstream site at the Waterloo MWWTP (USW, DSW), and an upstream site and three downstream sites at the Kitchener MWWTP (USK, DSK, DSK2, DSK3) were selected.

5.3.2 Surface water and effluent sampling, preparation, and analysis

Surface water was collected from the fish collection sites during fall and spring of 2010, 2011, and 2012 and analyzed for selected pharmaceuticals and personal care products as well as

nutrients (ammonia). For pharmaceutical analysis, grab samples of surface water were collected in triplicate (one sample each collected from the near, center, and far bank of the river, or within the plume of effluent for sites downstream of MWWTPs) using pre-cleaned 500 mL amber glass bottles. Samples were preserved on site with sodium azide (1 g/L) and ascorbic acid (50 mg/L) and stored at 4°C until extraction. The preparation, extraction and analyses of water samples are described in detail by Tanna *et al.,* (2014). Briefly, compounds were analyzed using solid phase extraction followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 HPLC (Mississauga, ON, Canada) coupled to an Applied Biosystems 3200 QTRAP mass spectrometer (ABSciex, Concord, ON, Canada). Additional grab samples of surface water were collected using 500 mL plastic bottles and were submitted to Maxxam Analytics (Mississauga, ON, Canada) for analysis of ammonia.

5.3.3 Field fish collections

In this study, all animals were handled in strict accordance with the principles of the Canadian Council on Animal Care, and the protocol was approved by the Animal Care Committee of the University of Waterloo (Permit Numbers 04-24, 08-08, and 10-17). Fish were stunned using a backpack electrofisher (Smith-Root LR-24), collected with a net, and placed into aerated buckets. Rainbow darter were brought to an on-site sampling trailer where the length (to 0.1 cm), weight (to 0.001 g), gonad weight (to 0.001 g), and liver weight (to 0.001 g) were measured. Livers were placed into cryo-vials and flash frozen in liquid nitrogen for gene expression analysis. Gonads were divided and alternately allocated to gene expression, steroid, or histological analysis. The number of fish collected and the endpoints studied varied in some years, depending on the research questions being addressed. A table showing the sample size for each endpoint in all seasons/years is provided in the supplemental materials (Appendix B, Table S5-1).

5.3.4 Quantification of mRNA by real-time reverse transcriptase-polymerase chain reaction

RNA extraction, cDNA synthesis, and real-time reverse transcriptase-polymerase chain reaction (qPCR) were performed as described by Bahamonde *et al.,* (2014). The RNA integrity of fall 2010 samples was reported by Bahamonde *et al.,* (2014). The RNA integrity of fall 2011 and 2012 samples was evaluated using A260/A280 and the Tape Station (2200, Agilent Technologies)

with the RNA Screen Tape according to the manufacturer's protocol. RNA Integrity Number equivalent (RIN^e) values averaged 8.93 ± 0.07 (mean $\pm$ SE). Briefly, 1 µg total RNA was reverse transcribed to cDNA using iScript cDNA reverse transcriptase (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's protocols. Gene-specific primer sequences for rainbow darter, vitellogenin (*vtg*), 18S ribosomal RNA (*18s*), elongation factor 1-α (*ef1α*), and *β-actin* were obtained from Bahamonde *et al.,* (2014) and are listed in Table 5-2. Reference genes were combined using geNORM (Hellemans *et al*., 2007), and normalized expression levels for target genes (*vtg*) were extracted using CFX Manager 2.1 software (Bio-Rad Laboratories) using a relative ΔΔCq method. Expression data are reported as fold change $\pm$ SE from reference (R) male mRNA levels.

Table 5-2 Real-time PCR primers used to measure mRNA levels in rainbow darter livers. R^2 and efficiency (E) were calculated from fall 2011 and fall 2012 samples only.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	R^2	E (%)
18s	CGGTTCTATTTTGTGG GTTTTC	ACCTCCGACTTTCGTTC TTG	172	0.992	105.5
ef1α	ATCGGCGGTATTGGA ACTG	CGGATTTCTTTGACGG ACAC	197	0.997	102.9
β-actin	CCAACAGGGAGAAGA TGACAC	GAGGATGGCGTGAGGT AGAG	188	0.993	112.5
vtg	CTACGCTCATTCCTGG GTTC	CAGTGGCAGTCGTTGT CCT	188	0.996	99.0

5.3.5 In vitro steroid production

Gonad *in vitro* sex steroid production was determined as described in detail by Fuzzen *et al*. (2015). Briefly, gonads collected in the field were placed in media 199 and transported on ice to the laboratory, where up to 20 mg of tissue was placed into a 24-well cell culture plate with 1 mL of media 199 (Sigma-Aldrich) and stimulant and incubated at 16°C for 24 h. Stimulation of sex steroid production differed between years. From fall 2007 to spring 2011, Forskolin was used (as described by Tetreault *et al.* (2011)). From fall 2011 onward, steroid production was stimulated with 10 IU of human chorionic gonadotropin (hCG) solubilized in media 199. At the end of the incubation period, media was removed from the wells, placed into centrifuge tubes, and stored at -80°C for analysis of sex steroid hormones. Testosterone (T) and 11-ketotestosterone (11KT) from testis incubations and

17β-estradiol (E2) and T from ovary incubations were measured by radioimmunoassay (RIA) as described by McMaster *et al*. (1995) from fall 2007 to fall 2011 and by enzyme immunoassay (EIA) kit (Cayman Chemicals, Ann Arbor, MI, USA) according to manufacturer's directions in spring and fall 2012. Briefly, 200 µL (RIA) or 50 µL (EIA) aliquots of each sample were measured in duplicate.

5.3.6 Histological processing and analysis

One lobe of gonad tissue was placed into an individual histocassette in Davidson's solution for histological analysis and fixed for 48 h. Samples were processed and slides made as described by Fuzzen *et al.* (2015). Males were analyzed for the presence and severity of intersex. Intersex condition is classified as the presence of ovarian tissue (oocytes) in predominantly male gonads. Males were given an intersex severity score from 0 to 7. The scoring system for determining intersex severity in male rainbow darter was based on number of oocytes, oocyte development, and proportion of ovarian tissue versus testicular tissue. The rationale for the scoring system is described in detail by Bahamonde *et al.* (2015a) and Fuzzen *et al.* (2015). In addition to intersex, males were assessed for the relative proportion of spermatogonia, spermatocytes, spermatids, and spermatozoa as described by Tetreault *et al.* (2011). For each female, the relative proportion of perinuclear, cortical alveolar, pre-vitellogenic, and mature (mid-vitellogenic, late-vitellogenic, and vitellogenic) oocytes was determined for all females collected, as described previously (Bahamonde *et al*., 2015a; Fuzzen *et al*., 2015; Tetreault *et al*., 2011).

5.3.7 Somatic indices

Gonad somatic index (GSI; gonad weight / total body weight *100), liver somatic index (LSI; liver weight / total body weight *100), and condition factor (K; weight/ (length^3) *100) were determined for all individuals collected and the mean was determined for males and females at each site in each season and year.

5.3.8 Statistical analyses

Data from the three new field seasons and from the earlier studies conducted from fall 2007 to spring 2011were compiled into a single database. The mean, standard deviation, and standard error were determined for each endpoint within each season, year, site, and sex (for biological data). For chemistry data, a two-way analysis of variance (ANOVA) was conducted to test for the effects of site, year, and interactions between site and year. A Tukey's post-hoc test was conducted to test for

differences between individual sites. This test was performed on data for ammonia as well as for pooled CECs (consisting of naproxen, ibuprofen, venlafaxine, carbamazepine, and triclosan). For chemical profiles of select pharmaceuticals a metric to test for statistical differences was placed on each graph. For all biological data, the same metric to test for statistical differences and a second metric to test for biological differences were placed onto the graphs. Both of these metrics were calculated from a pooled data set from the R site in all years for each season and sex. The first metric calculated was the 95% confidence interval of the pooled data from the R site determined using SPSS (Version 23; IBM, New York, USA). Values that exceeded the 95% confidence interval of the mean R site value were considered to be statistically different. The second metric calculated was the critical effect size (CES), which was based on thresholds set in the environmental effects monitoring program for pulp and paper from Environment Canada and recommendations from Munkittrick *et al*., (2009). The CES was set to ± 10% of the mean for the condition factor and ± 25% of the mean for all other measures. Values that exceeded the CES were considered to be biologically different.

5.3.9 Cluster analyses

To further explore the data, we conducted three ordination tests to assess the influence of MWWE on rainbow darter biology. The first test was a principal coordinates analysis (PCO) ordination test, which was used to explore site clustering and test for seasonal effects. To determine which variables were responsible for the separation along the axis, a canonical analysis of principal coordinates (CAP) ordination was conducted. The third test, distance-based redundancy analysis (DISTLM), was used to explore what amount of the variation in biological variables changing at downstream sites was attributable to MWWE constituents. Ordination analyses were conducted on the mean of biological variables. Analyses were conducted on biological and chemical data normalized within the Primer+PERMANOVA software package (PRIMER-E Ltd) (Clarke and Gorley, 2006).

5.3.10 Variability assessment of biological variables

The variability of several measures in the study was determined by normalizing each data point to the mean of its site. To test the variability of measures across levels of biological organization, four key endpoints in male rainbow darter (*vtg* expression, testicular testosterone production, spermatozoa proportion, and gonad somatic index) were compared at the R site in the fall

season. Additionally, site-specific variability was assessed by comparing these same endpoints between the R site and the DSK site.

5.4 Results

5.4.1 Chemistry

Surface water concentration of nutrients (ammonia in this study) and CECs (pharmaceuticals) increased downstream of both the Waterloo and Kitchener MWWTPs in all years and all seasons (Figure 5.2). The absolute concentration of nutrients and pharmaceuticals varied between seasons and years but the profiles remained consistent. The patterns of ammonia and pharmaceutical concentrations resembled one another, and when a regression analysis was conducted they were found to be directly correlated ($R^2 = 0.644$, $p < 0.05$). The profiles of 4 of the 5 CECs investigated in this study (ibuprofen, venlafaxine, carbamazepine, and triclosan) that were found at high concentrations in surface water are presented in Figure 5.3. The detection of elevated levels of these compounds at sites downstream of MWWTPs demonstrates that MWWE is a source of measurable concentrations of pharmaceuticals in surface river water. These profiles also demonstrate that CECs persist in the watershed even after dilution due to mixing, because concentrations were higher at the DS3 site than at the R site (Figure 5.3).

5.4.2 Gene expression

Quantitative measurement of female liver *vtg* in the fall and spring revealed no impact of MWWTP outfall on expression (Figure 5.4A, C). Although some increases in *vtg* were observed within the urban area in fall 2011, no additional increases were observed directly downstream of the MWWTP (Figure 5.4A). Male liver *vtg* was found to increase downstream of both MWWTP outfalls in some fall seasons and one spring season (Figure 5.4B, D).

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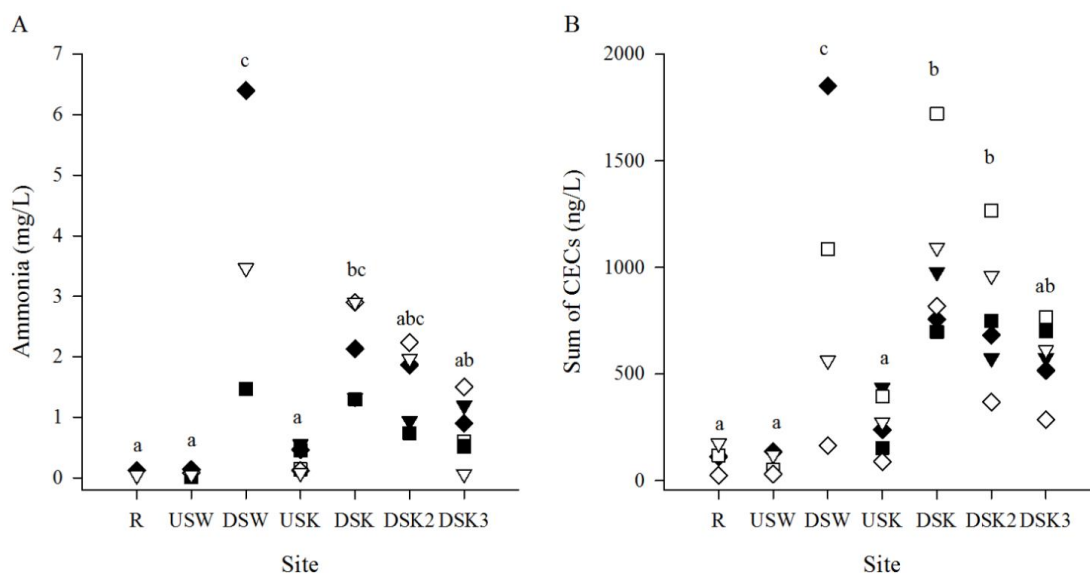
Figure 5.2 Concentration of nutrients and pharmaceuticals increased downstream of MWWTPs. (A) Mean concentration of ammonia (mg/L) and (B) the sum of dominant chemicals of emerging concern (naproxen, ibuprofen, venlafaxine, carbamazepine, and triclosan) in river surface water. Samples were collected in the fall (dark symbols) and in the spring (open symbols) of 2010 (squares), 2011 (diamonds), and 2012 (down-facing triangles). Sites that share a common letter are not significantly different ($p > 0.05$) as determined by a two-way ANOVA with a Tukey's post-hoc test (no interactions were found for season and site).

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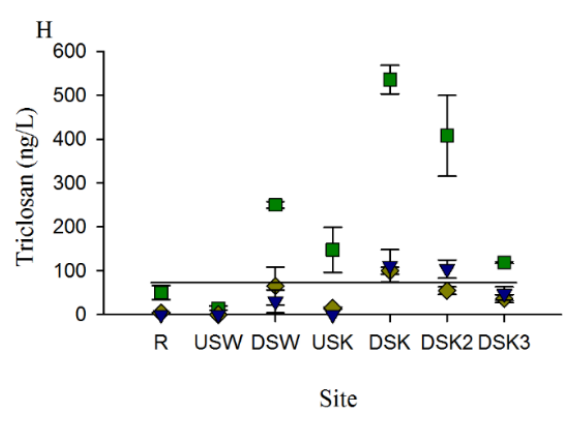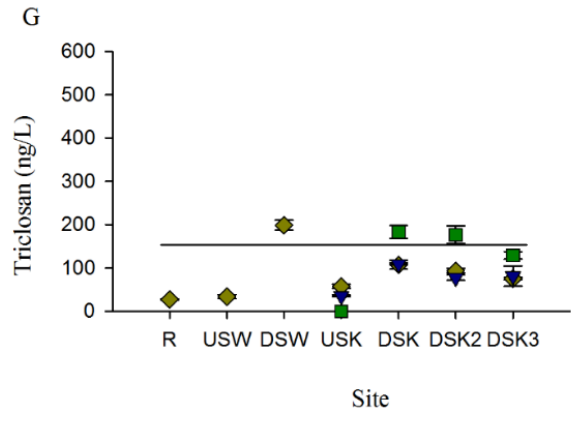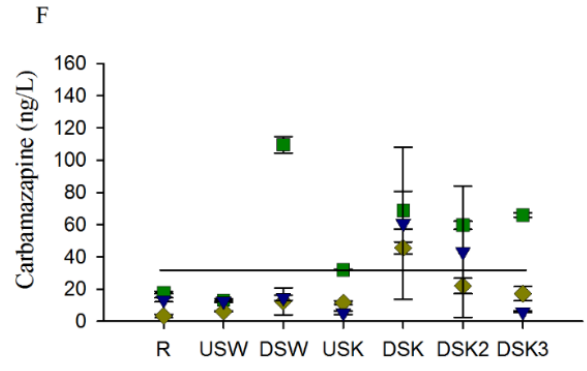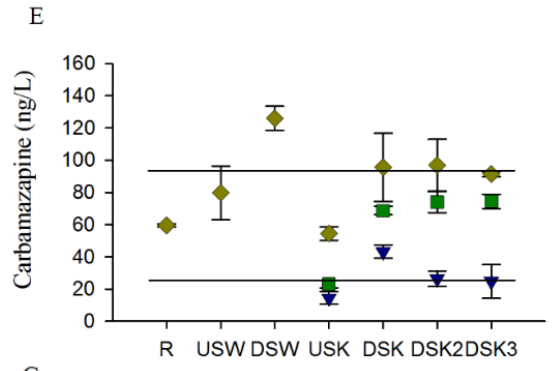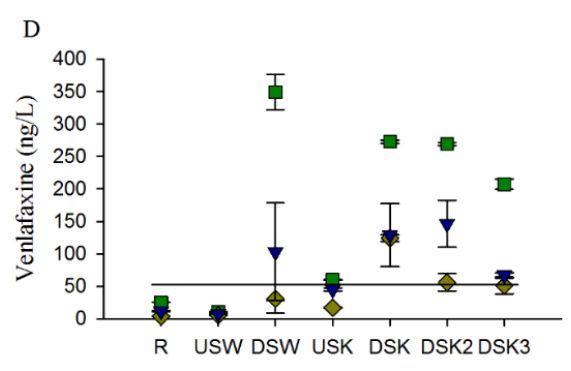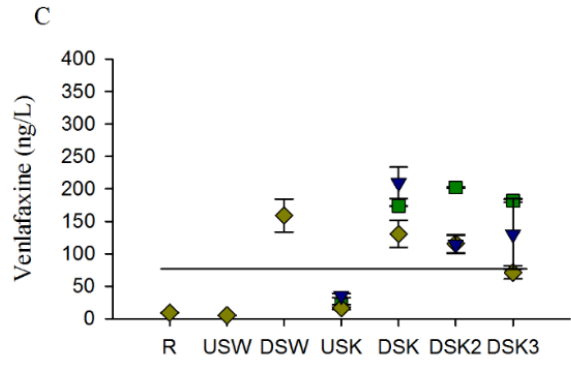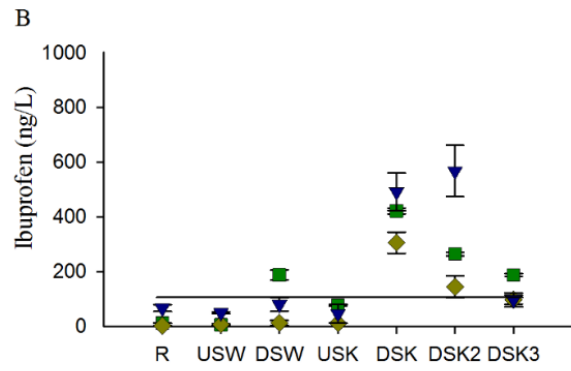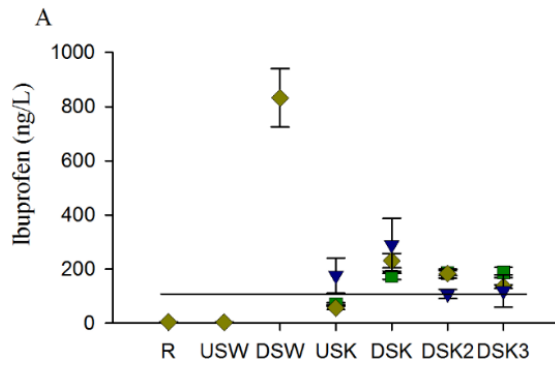
Figure 5.3 Profile of pharmaceuticals and personal care products in surface water. Mean concentration (± SE) of select pharmaceuticals and personal care products in river surface water through an urban gradient. Samples were collected in the (A, C, E, G) fall and in the (B, D, F, H) spring 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles). The solid lines indicate the 95% confidence interval calculated from the mean of the data from the rural reference site (R).

Figure 5.4 Vitellogenin (vtg) expression is higher in males collected downstream of MWWTP outfalls. Relative change in vitellogenin (*vtg*) expression in liver tissues of (A, C) female and (B, D) male rainbow darter collected from sites through an urban gradient, compared with that in R males. Expression was measured in the (A, B) fall of 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles), as well as in the (C, D) spring of 2011. The dashed line indicates the upper 25% critical effect size calculated from the mean of the data from the rural reference site (R) over all three years, and the solid line indicates the upper 95% confidence interval from the pooled R. In the spring (panels C, D), * indicates significant (*p* < 0.05) difference between R and DSK sites as determined by a student's t-test.

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5.4.3 Steroids

In vitro gonad steroid production was measured in male and female rainbow darter in three fall field seasons and two spring field seasons. To compare the response among years, data were normalized to the reference site (R) within each field season. While trends appeared similar across years, absolute values of hormone measurement among years varied greatly (Appendix B, Table S5-2). Differences in absolute measures have also been noted in an inter-laboratory study of hormone measurements by radioimmunoassay and enzyme immunoassay (Feswick *et al*., 2014). Although steroids were measured using different techniques in different field seasons, the relative values demonstrated consistent trends. Stimulated ovarian T production was reduced through the urban area in the fall (Figure 5.5B). Exposure to MWWE was not found to impact stimulated ovarian steroid production of T in the spring (Figure 5.5D). E2 production was found to be reduced downstream of the Waterloo and Kitchener MWWTPs in 2 of 3 years in the fall (Figure 5.5A) and was lower downstream of the Kitchener plant in 1 of 2 years in the spring compared with E2 production at the reference site (Figure 5.5C). Stimulated testicular production of 11KT and T was found to be lower at sites downstream of the Kitchener and Waterloo MWWTPs in fall and spring in most years (Figure 5.6). While the size of the impact varied between treatment plants and years, there was a consistent trend of suppression of androgen production downstream of the MWWTPs compared with the reference (R) site.
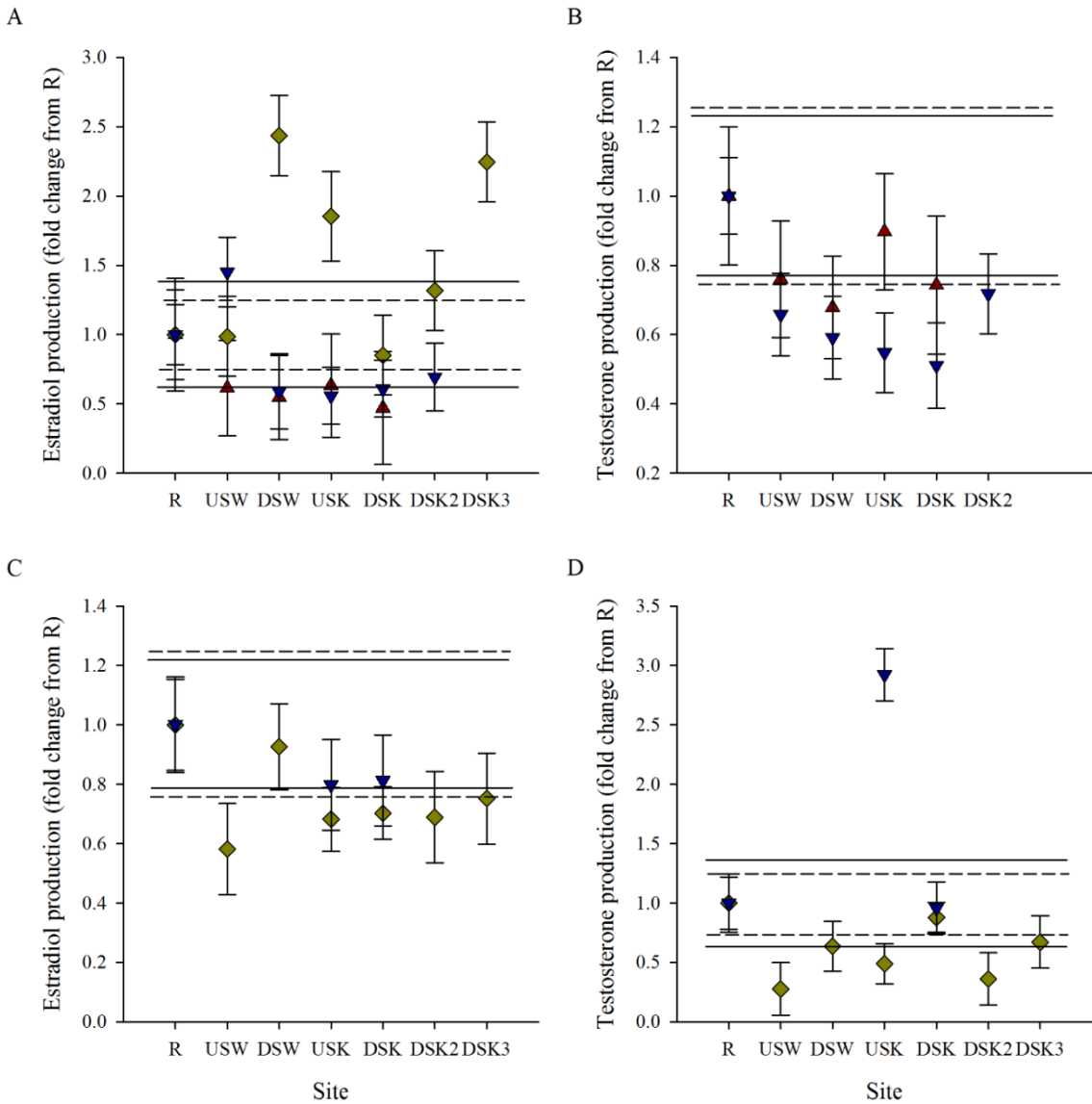
Figure 5.5 Hormone production of ovaries varies through the urban gradient and between seasons. Normalized (to mean R site) *in vitro* **steroid production of (A, C) estradiol and (B, D) testosterone in stimulated ovarian tissues collected from rainbow darter in the wild through an urban gradient. Measurements were taken in the (A, B) fall of 2007 (red triangles), 2011 (yellow diamonds), and 2012 (blue down-facing triangles), and well as in the (C, D) spring of 2011 (yellow diamonds) and 2012 (blue down-facing triangles). The dashed line indicates the upper 25% critical effect size calculated from the mean of the data from the rural reference site (R), and the solid line indicates the upper 95% confidence interval from the pooled R.**
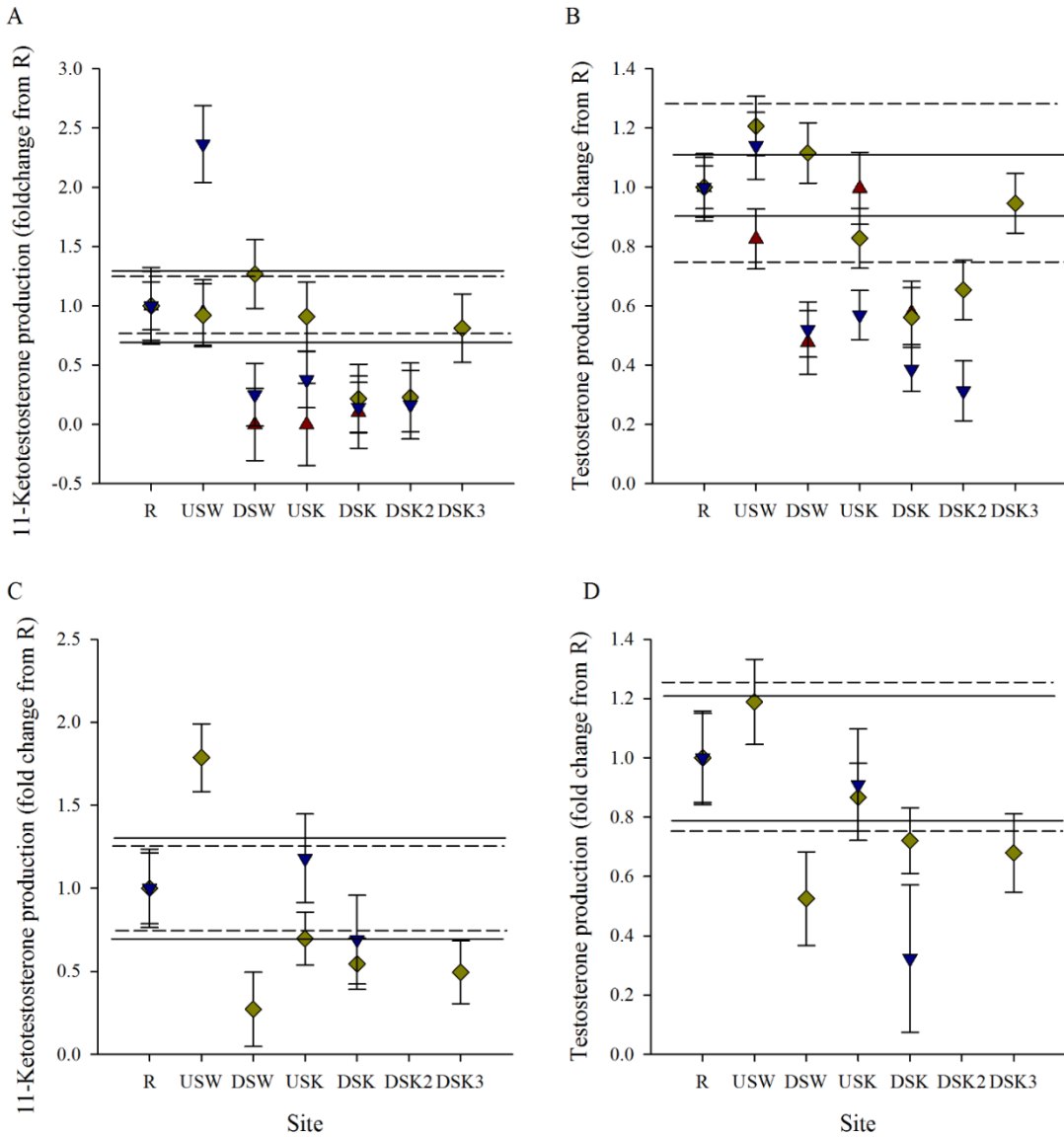
Figure 5.6 Hormone production of testes is reduced in males exposed to MWWE. Normalized (to mean R site) *in vitro* **steroid production of (A, C) 11-ketotestosterone and (B, D) testosterone in stimulated testis tissue collected from rainbow darter in the wild through an urban gradient. Measurements were taken in the (A, B) fall of 2007 (red triangles), 2011 (yellow diamonds), and 2012 (blue down-facing triangles), as well as in the (C, D) spring of 2011 (yellow diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the upper 25% critical effect size calculated from the mean of the data from the rural reference site (R), and the solid line indicates the upper 95% confidence interval from the pooled R.**

5.4.4 Histology

While multiple categories of stages of gonad development were assessed in this study, data for only the most advanced stages are presented in this section. The relative proportion of other stages is presented in the supplemental information (Appendix B, Figure S5.1-S5.4). Ovarian development was found to be moderately impacted by urbanization (Figure 5.7A, C). An increase in the development of ovarian follicles was observed through the urban area in the fall with an increase in the proportion of pre-vitellogenic oocytes (Figure 5.7A). Additionally, there was a moderate decrease in the proportion of vitellogenic oocytes in the urban area in the spring (Figure 5.7C); however, no clear impact of MWWE was observed. During the fall field collections, sperm development was consistently delayed in males collected from sites downstream of MWWTPs, with a lower proportion of spermatozoa (Figure 5.7 B). The proportion of spermatozoa in spring 2009 was unchanged through the gradient. In spring 2011, the proportion of spermatozoa was found to be lower at the site upstream of the MWWTPs but not downstream when compared with the R site (Figure 5.7 D).

Intersex incidence and severity were consistently higher at sites downstream of the Waterloo and Kitchener MWWTPs than at upstream sites and the R site in the fall field seasons (Figure 5.8A, B). The spring field collections demonstrated an increase in intersex incidence and severity through the urban region, with additional increases downstream of MWWTPs (Figure 5.8C, D).
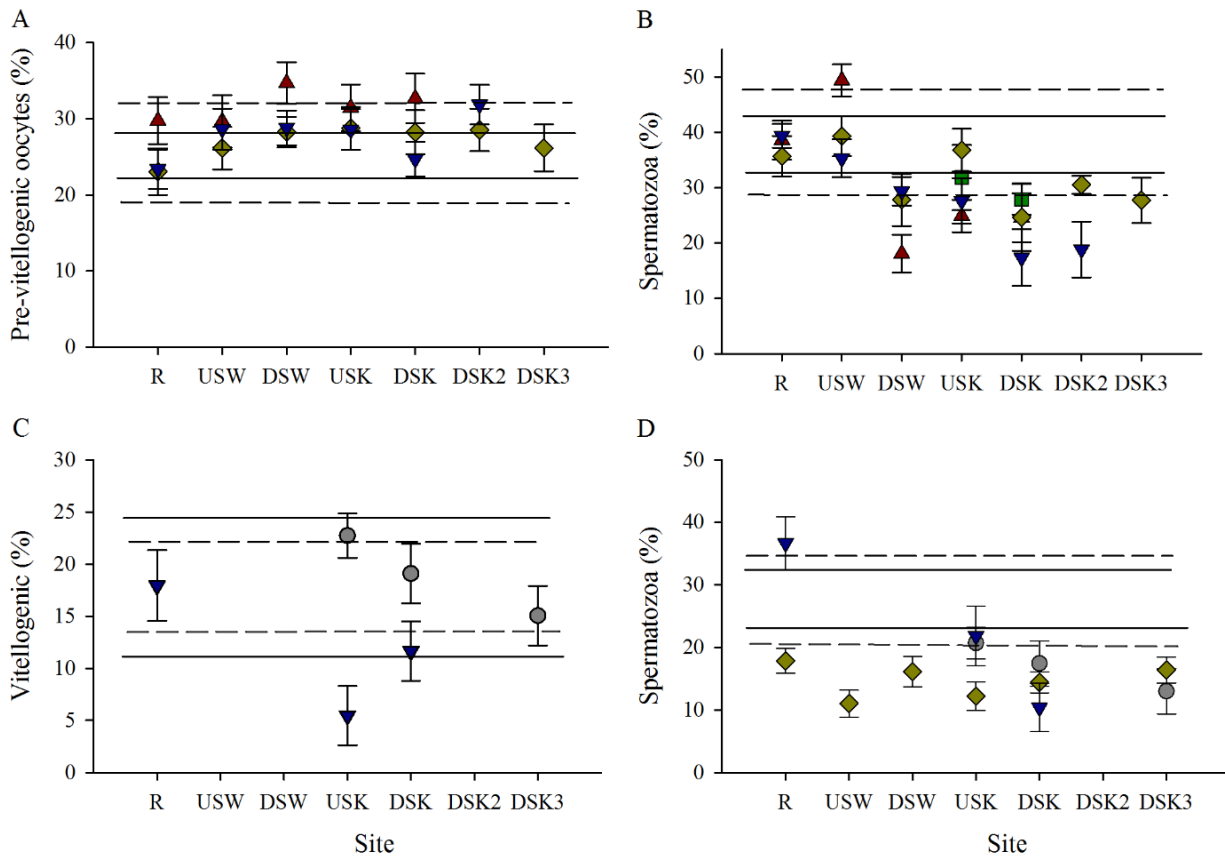
Figure 5.7 Impact of MWWE on gonad development is clear in males, but not females.
Proportion of advanced cell types in (A, C) female and (B, D) male gonads of rainbow darter
collected through an urban gradient. The proportion of all cell types was assessed through
histological analysis of female and male gonads collected in (A, B) fall and (C, D) spring field
seasons in 2007 (red triangles), 2009 (grey circles), 2010 (green squares), 2011 (yellow
diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the upper 25%
critical effect size calculated from the mean of the data from the rural reference site (R), and
the solid line indicates the upper 95% confidence interval from the pooled R.
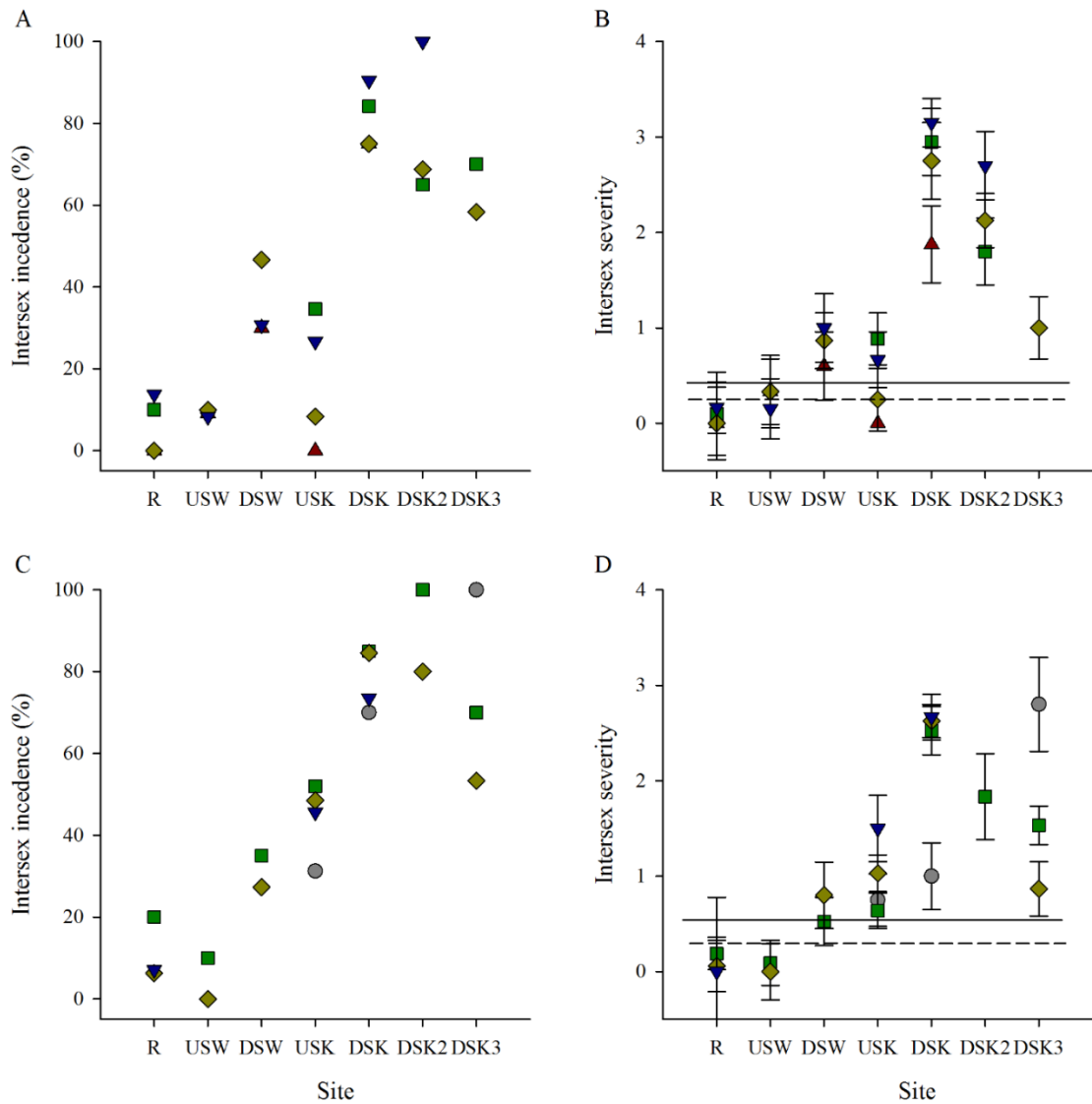
Figure 5.8 Intersex incidence and severity are consistently higher at sites downstream of MWWTP outfalls. Intersex (A, C) incidence and (B, D) severity of male rainbow darter collected through an urban gradient in the (A, B) fall of 2007 (red triangles), 2009 (grey circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles) and in the (C, D) spring of 2009 (grey circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the upper 25% critical effect size calculated from the mean of the data from the rural reference site (R), and the solid line indicates the upper 95% confidence interval from the pooled R.

5.4.5 Somatic indices

No direct impacts of MWWE on relative gonad size were observed in female rainbow darter. While GSI was higher at some sites in fall 2010, this occurred throughout the urban area and seemed to be driven by annual differences, not site differences (Figure 5.9A). Female GSI was higher at sites upstream of MWWTPs in some years during spring collections; this effect persisted downstream in some subsequent years but dissipated downstream in other years (Figure 5.9C). In the fall, decreases in male GSI were found downstream of the Waterloo MWWTP (DSW) in all years, and inconsistent decreases were observed at the site downstream of the Kitchener MWWTP (DSK). These decreases approached biological significance in fall 2007 and fall 2009 (Figure 5.9B). In contrast, GSI was statistically but not biologically higher at the second and third sites downstream of Kitchener MWWTP (DSK2 and DSK3) than at the R site in fall (Figure 5.9B). No consistent changes in male GSI were noted in the spring field collections (Figure 5.9D). Annual variability in GSI was greater at the R site during the spring than the fall for both males and females (Figure 5.9).

In the fall field collections, the relative size of female livers increased modestly downstream of the Waterloo MWWTP (DSW) in fall 2011 and 2012 (but not 2007) and increased at the second and third sites downstream of the Kitchener MWWTP (DSK2 and DSK3) in the fall of all years (Figure 5.10A). No consistent trends in female LSI were observed in the spring (Figure 5.10C). Similar to the finding in females, male LSI increased at DSW in the fall of 2011 and 2012. The increases in liver size downstream at the DSK2 and DSK3 sites were less consistent in males than females, with biologically significant changes observed in fall 2010 and 2011, but not 2012 (Figure 5.10B). No trends were observed in male LSI during the spring field collections (Figure 5.10D). Similar to GSI, the spring LSI at the R site was found to be highly annually variable in both sexes.

Condition factor was higher in females collected downstream of both MWWTPs in the fall in all years. This increase was only biologically significant in females collected at DSK and DSK2 in fall 2009, 2010, and 2012 (but not 2011; Figure 5.11A). In males, condition factor was also elevated at sites downstream of MWWTPs in the fall, but the response was less consistent. A statistically and biologically significant increase was found at DSW in fall 2007 (but not 2011 or 2012), at DSK in fall 2012 (but not 2009, 2011, or 2012), and at DSK2 in fall 2010 and 2012 (but not 2011) (Figure 5.11B). No clear changes in condition factor were observed in females or males between the reference (R) sites and sites downstream of MWWTPs during the spring field collection (Figure 5.11C, D). The variability of condition at the R site was larger in the spring than the fall (Figure 5.11).

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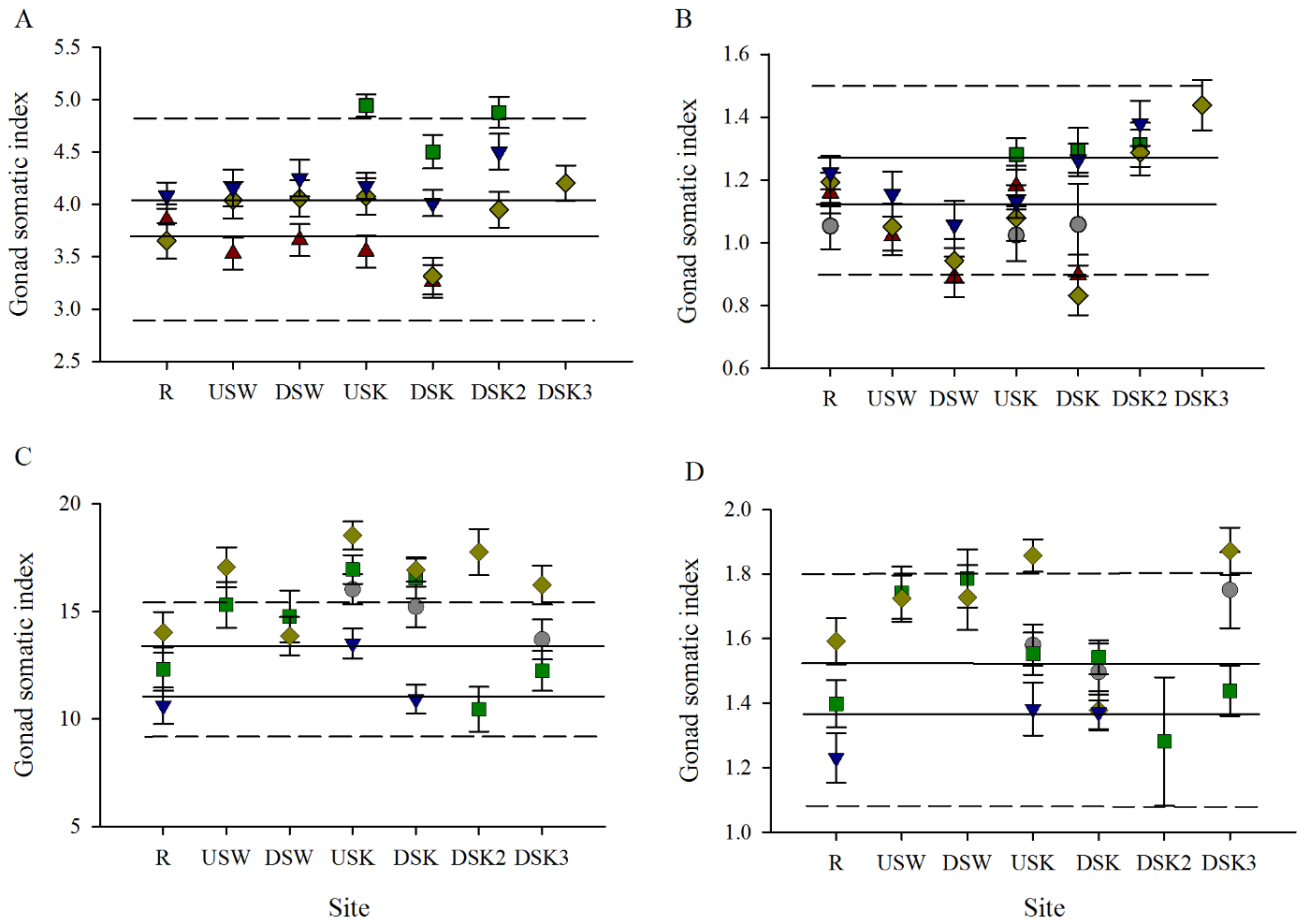
Figure 5.9 Changes in gonad somatic index through the urban gradient are variable and difficult to interpret. Gonad somatic index of (A, C) female and (B, D) male rainbow darter collected through an urban gradient in the (A, B) fall of 2007 (red triangles), 2009 (grey circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles) and in the (C, D) spring of 2009 (grey circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the 25% critical effect size calculated from the mean of the data from the rural reference site (R), and the solid line indicates the 95% confidence interval from the pooled R.
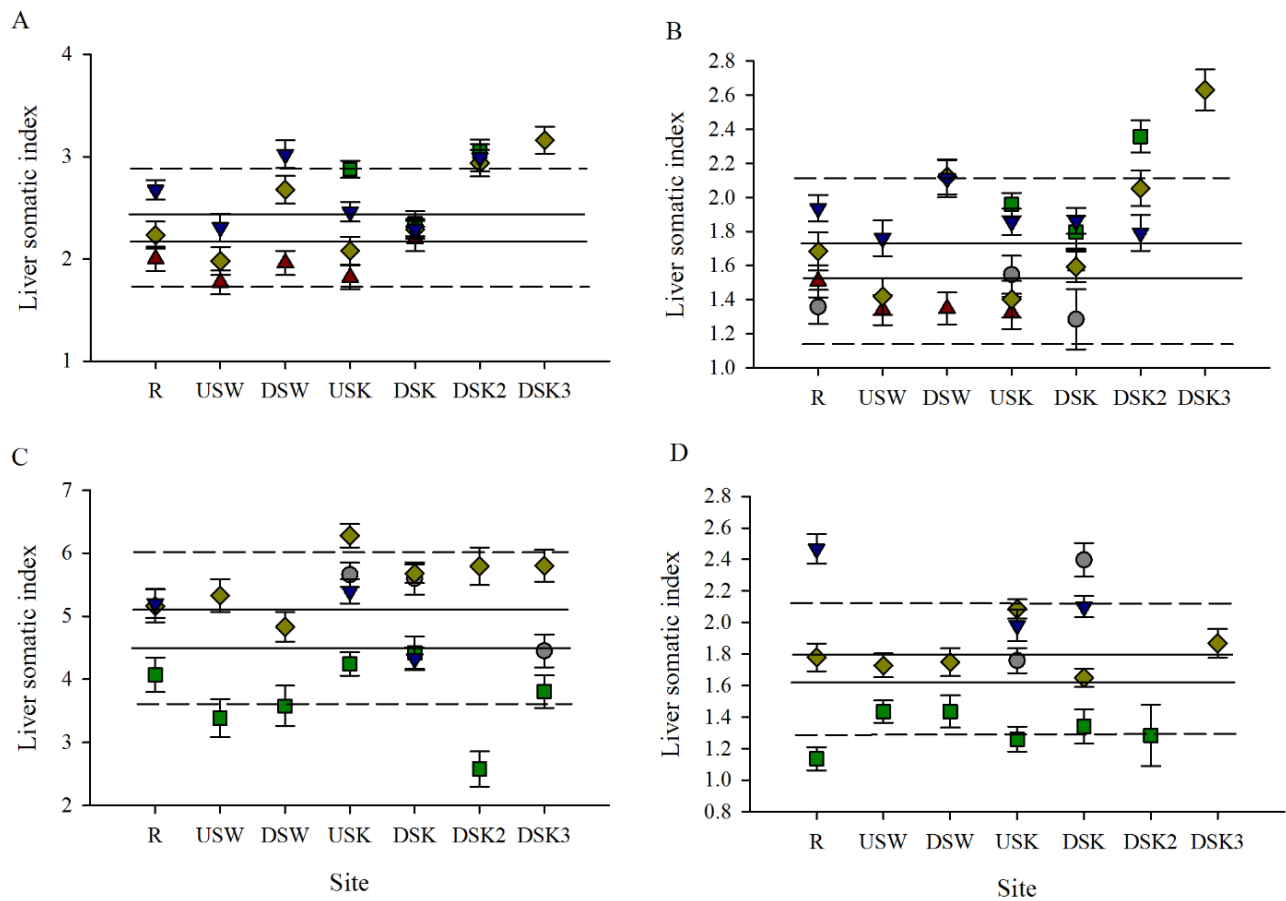
Figure 5.10 Liver somatic index is variable through the urban watershed. Liver somatic index of (A, C) female and (B, D) male rainbow darter collected through an urban gradient in the (A, B) fall of 2007 (red triangles), 2009 (grey circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles) and in the (C, D) spring of 2009 (grey circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the 25% critical effect size calculated from the m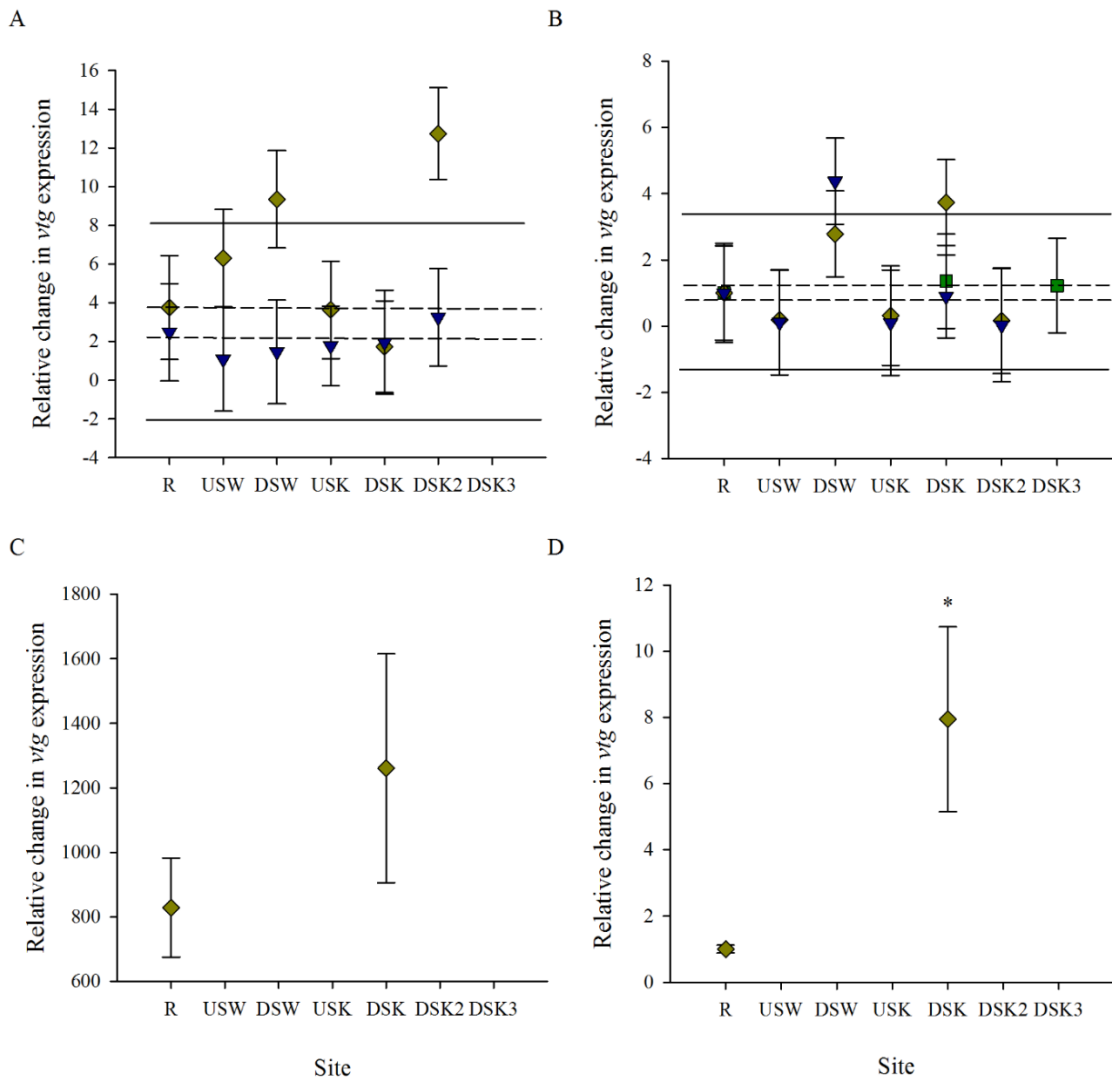ean of the data from the rural reference site (R), and the solid line indicates the 95% confidence interval from the pooled R.
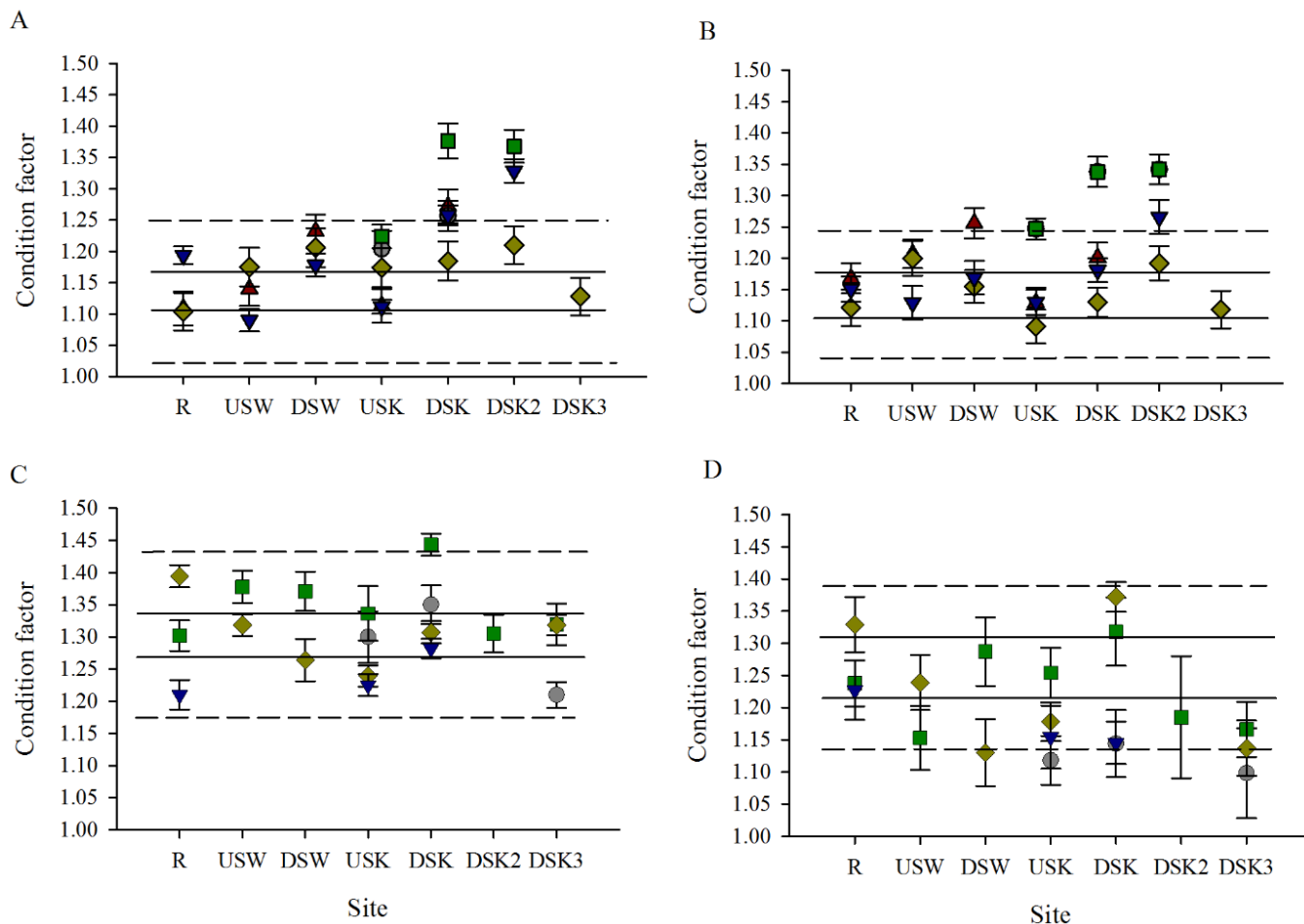
Figure 5.11 Condition factor increases downstream of MWWTP outfalls in fall, but not spring.
Condition factor of (A, C) female and (B, D) male rainbow darter collected through an urban
gradient in the (A, B) fall of 2007 (red triangles), 2009 (grey circles), 2010 (green squares), 2011
(yellow diamonds), and 2012 (blue down-facing triangles) and in the (C, D) spring of 2009 (grey
circles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue down-facing triangles).
The dashed line indicates the 10% critical effect size calculated from the mean of the data from
the rural reference site (R), and the solid line indicates the 95% confidence interval from the
pooled R.

5.4.6 Cluster analyses

Principal coordinates (PCO) analysis of female data demonstrated that there was distinct separation of biological variables between seasons. This separation occurred mainly by PCO1. Some separation of sites could be observed in fall data by PCO2, with downstream sites clustering more closely together and with some overlap between the reference site and upstream sites (Figure 5.12A). When a canonical analysis of principal components (CAP) test was performed on female data, it was found that there were no significant variables that explained a majority of the separation (Figure 13A). PCO analysis of male biological endpoints exhibited less distinction between seasons than that of the female endpoints (which separated along PCO1) (Figure 5.12B). A separation of the sites along PCO2 was noted with exposed (downstream) sites grouping together (Figure 12B). The CAP revealed that site separation in males was correlated with intersex severity ($r = 0.786$) along CAP1, and some slight separation along CAP2 was noted as well, which was correlated with somatic indices ($r = 0.55$, 0.59 and -0.84 for GSI, LSI, and K respectively) (Figure 5.13B).

Somatic indices were excluded from the distance-based redundancy (DISTLM) analyses due to their variable nature. All other biological measures were included. Female biological measures were associated with carbamazepine, but no other pharmaceutical (Appendix B, Table S5-3). In contrast, male biological measures were associated with all pharmaceuticals except for carbamazepine (Appendix B, Table S5-3).
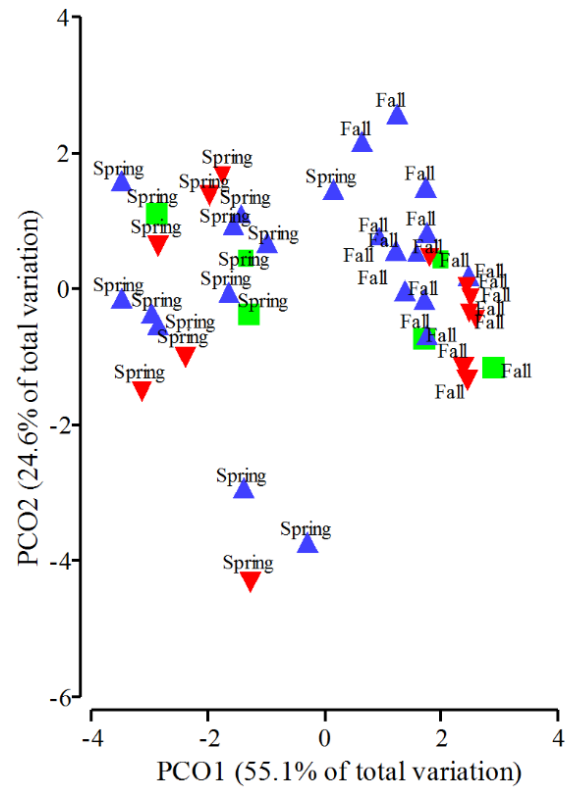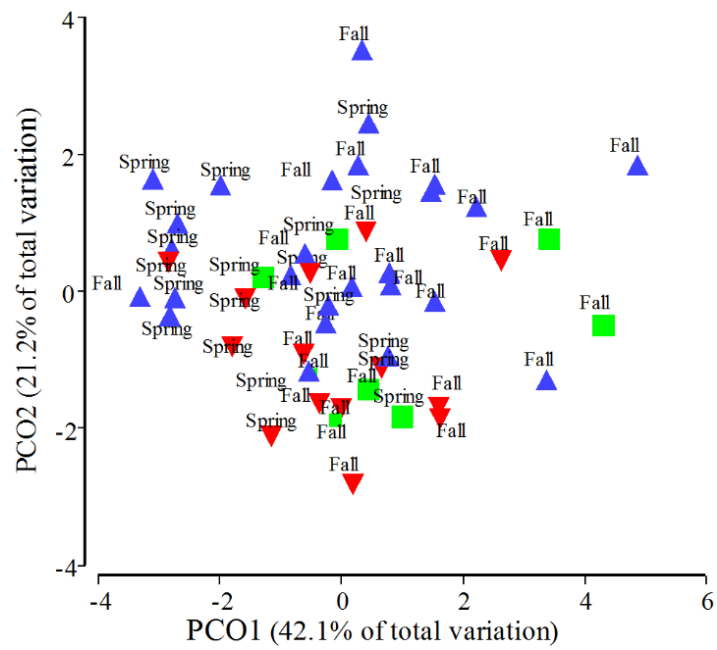
Figure 5.12 Principal coordinates (PCO) analysis of biological measures. (A) Female and (B) male biological measures collected from a reference site (green squares), sites upstream of municipal wastewater treatment plant (MWWTP) outfalls (red downward triangles), or downstream of MWWTP outfalls (blue triangles). Each sampling site is labeled with the season (spring or fall) that data were collected. PCO is based on a Euclidean distance resemblance matrix.
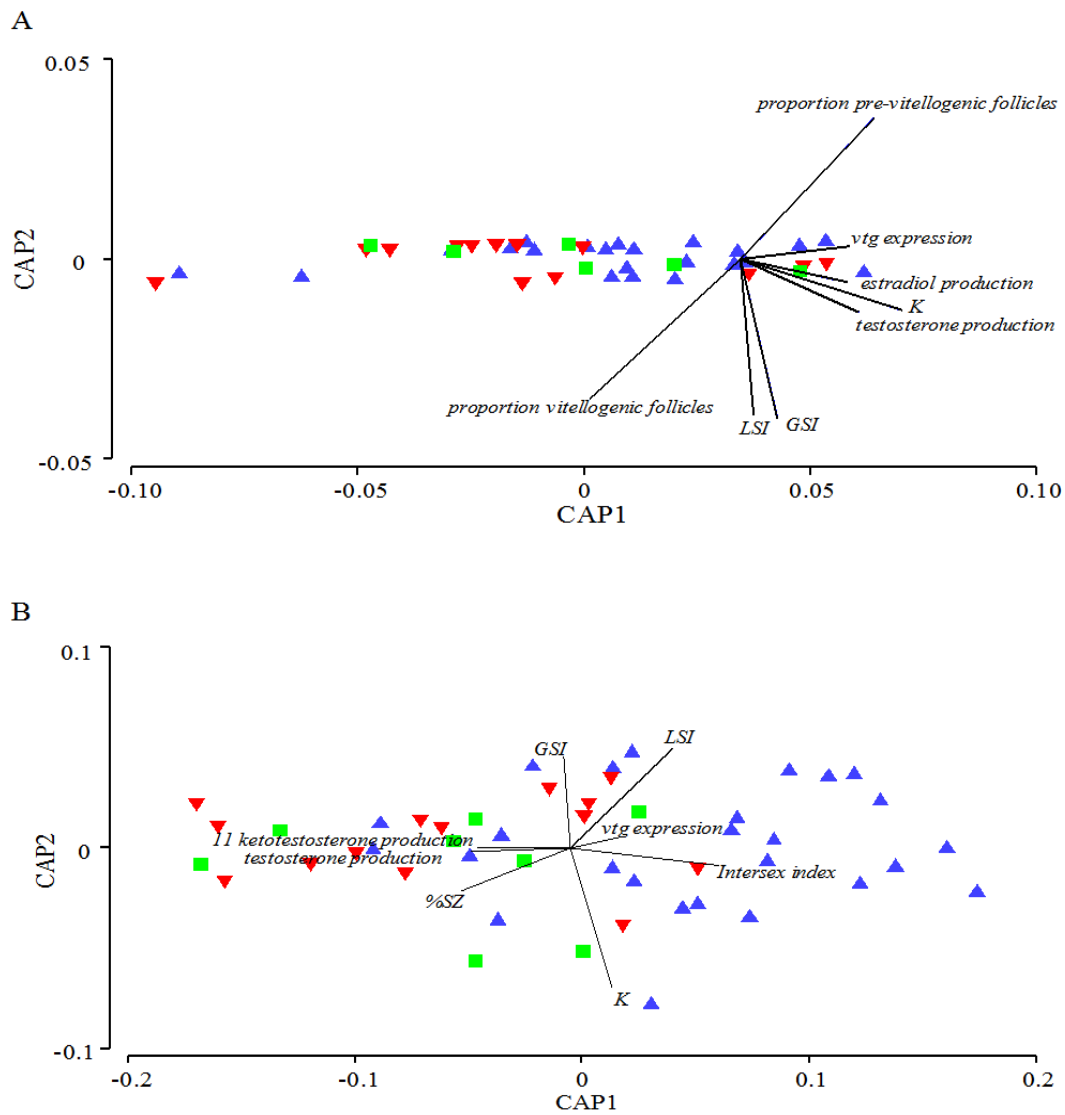
Figure 5.13 Canonical analysis of principal coordinates (CAP) ordination with biological variables as vectors. (A) Female and (B) male biological measures collected from a reference site (green squares), sites upstream of municipal wastewater treatment plant (MWWTP) outfalls (red downward triangles), or downstream of MWWTP outfalls (blue triangles).

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5.4.7 Consistency / variability

In females, the interpretation of whether an impact of MWWE was observed on certain endpoints was dependent on the reference site chosen. Changes in measures at lower levels of biological organization were present when compared with the rural reference site (R) but not as clearly present when compared with the site directly upstream of the MWWTPs (USW, USK; Table 5-3). The consistency between years of the presence or absence of a response when compared with the R site was greater in the fall than in the spring, and more consistent downstream of the Waterloo MWWTP than the Kitchener MWWTP (Table 5-3). In males, there was some inconsistency in interpretation depending on which reference site was used for the comparison (rural reference (R) versus direct upstream reference (USW, USK)), but not to the same extent as in females (Table 5-4). The consistency of male responses to MWWE between years was greater in the fall than the spring (Table 5-3). While responses of males downstream of the Waterloo MWWTP were more consistent than the responses of males downstream of the Kitchener MWWTP in fall, this relationship reversed in the spring field collections.

To determine the amount of variability within a site/season within each endpoint, data were normalized so that the mean equaled 1 within the site. The standard deviation then represented the amount of variability in each measure within a year/season/site. Variability of endpoints was found to decrease in measures as they increased in complexity in terms of biological organization (Figure 5.14A). This was true at the R site as well as at the DSK site (Figure 5.14). While the variability of gene expression was comparable between sites, the variability of other measures was greater at the DSK site than at the R site (Figure 5.14).

Table 5-3 Comparison of significant changes observed in female rainbow darter downstream of the Waterloo (DSW) and Kitchener (DSK) MWWTPs in relation to either the rural reference site (R) or the immediate upstream (USW and USK, respectively) reference site. Comparisons are made for fall and spring field collections.

Female endpoints	# years in which differences were observed in fall				# years in which differences were observed in spring			
Downstream site	DSW		DSK		DSW		DSK	
Reference site	R	USW	R	USK	R	USW	R	USK
Gene expression	1/2	0/2	0/2	0/2	NA	N/A	0/1	N/A
Testosterone production	2/2	0/2	3/3	0/2	1/1	0/1	2/3	2/2
Estradiol production	3/3	1/3	2/2	1/3	0/1	1/1	1/2	0/2
Ovarian development	1/3	0/3	1/3	0/3	NA	NA	0/2	0/2
GSI	0/3	0/3	0/4	2/4	0/2	1/2	2/4	1/4
LSI	2/3	2/3	0/4	2/4	0/2	0/2	0/4	2/4
K	0/3	2/3	4/5	3/5	0/2	0/2	1/4	3/4

Table 5-4 Comparison of significant changes observed in male rainbow darter downstream of the Waterloo (DSW) and Kitchener (DSK) MWWTPs in relation to either the rural reference site (R) or the immediate upstream (USW, USK respectively) reference site. Comparisons are made for fall and spring field collections.

Male endpoints	# years in which differences were observed in fall				# years in which differences were observed in spring			
Downstream site	DSW		DSK		DSW		DSK	
Reference site	R	USW	R	USK	R	USW	R	USK
Gene expression	1/2	2/2	1/3	1/2	N/A	N/A	1/1	N/A
Testosterone production	2/3	2/3	3/3	2/3	1/1	1/1	2/3	1/2
11-ketotestosterone production	2/3	2/3	3/3	1/3	1/1	1/1	1/2	0/2
Testis development	3/3	2/3	4/4	2/4	0/1	0/1	3/3	1/3
Intersex severity	3/3	1/3	4/4	4/4	1/2	1/2	4/4	3/4
GSI	1/3	0/3	2/5	3/5	0/2	0/2	0/4	1/4
LSI	2/3	2/3	0/5	1/5	0/2	0/2	1/4	2/4
K	1/3	0/3	1/4	2/4	1/2	2/2	0/4	1/4
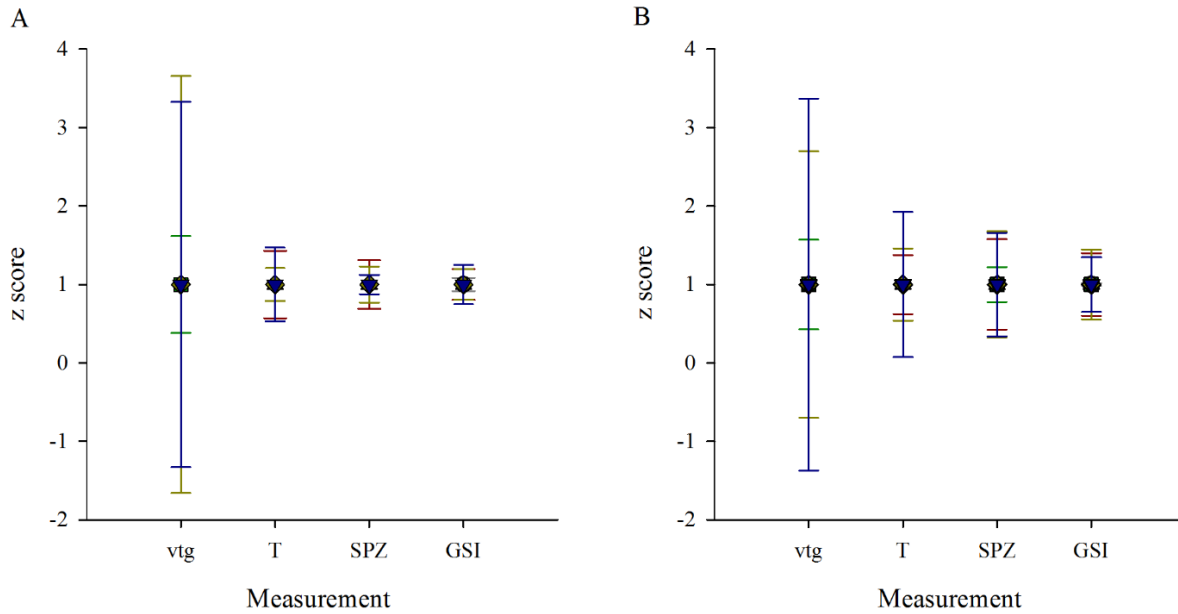
Figure 5.14 Variability of biological measures decreases with increasing biological complexity.
A comparison of variability in measures of male rainbow darter reproduction across levels of
biological organization at a (A) reference site and a (B) MWWE-exposed site from collections in
the fall of 2007 (red triangles), 2010 (green squares), 2011 (yellow diamonds), and 2012 (blue
down-facing triangles). Measures compared include gene expression of *vtg* (VTG), *in vitro*
production of testosterone (T), gonad development and relative proportion of spermatozoa
(SPZ), and gonad somatic index (GSI).

5.5 Discussion

This study is among the few to examine the variability in the response of wild fish to watershed stressors, including municipal wastewater effluents, across levels of biological organization in multiple seasons and years. While field collections can often be difficult to interpret due to the many confounding factors and the presence of multiple stressors, we found that the use of endpoints across multiple levels of biological organization allowed us to more clearly identify stressors and assess their impact. Through the analysis of multiple endpoints across levels of biological organization and over multiple seasons and years, we concluded that MWWE has impacts on reproduction in male rainbow darter. The degree of the reproductive disruption and the number of biological measures affected did vary between sexes, seasons, and years. The largest differences in the responses were found to be associated with season. Additionally, the consistency of the reproductive disruption and variability differed for each of the selected measures across the levels of biological organization. Measures at the tissue level, such as gonad development and presence of intersex, were the most consistent and were also the most useful endpoints in discerning the effects of point sources such as wastewater outfalls. In the following sections the general patterns in the measures are first described and then the seasonal variability and consistency/variability of biological measures are explored.

5.5.1 Impacts associated with the urban gradient and wastewater outfalls (spatial changes)

A clear change in surface water chemistry in response to urbanization, especially associated with wastewater outfalls, was observed in this study. The central Grand River receives high levels of nitrogenous waste. While some of the nitrate in the river originates from upstream (probably associated with intensive agriculture), there was a clear increase in ammonia and nitrate downstream of the two MWWTP outfalls. The pharmaceuticals analyzed in this study followed a trend similar to that of the nitrogenous compounds (i.e., ammonia, nitrate), with large increases directly downstream of the MWWTP outfalls. This is to be expected, as MWWE is well known to be a source of CECs. While some of the chemicals we measured disappeared quickly downstream (e.g., triclosan), others appear to persist for many kilometers downstream (i.e., carbamazepine, venlafaxine). Through the use of models, Arlos *et al.,* (2015) demonstrated that while some chemicals (like triclosan) are probably removed through photolysis, the concentrations of other chemicals are decreased primarily through dilution or attenuation. Since the volume of effluent is relatively consistent, the river flow plays an

important role in modifying exposure temporally. The data presented in this study consist of grab samples and are highly dependent on the weather and resulting flows immediately before sampling and thus may not accurately reflect the exposure of fish during critical life stages.

The impact of MWWE on female reproductive health was not clear in this study. Overall, some subtle differences between sites were observed in the fall field collections but not in the spring (as demonstrated in Figure 5.12A). When we examined the individual biological markers across levels of biological organization, we observed few consistent and clear changes associated with MWWE. For instance, although synthesis of ovarian estradiol and testosterone was usually lower near the MWWTPs in spring and fall than at the rural reference site (R), few differences were noted between directly upstream and downstream sites in either season. Similarly, increases in the proportion of pre-vitellogenic cells were observed in the fall downstream of the two MWWTPs when compared with the R site but not the USW or USK sites. Additionally, the proportion of vitellogenic oocytes was lower downstream of the Kitchener MWWTP than at the R site during one spring season. While these observations were inconsistent, the direction of the changes is suggestive of long-term exposure to estrogenic compounds. The lower proportion of vitellogenic oocytes in the spring, however, could also be explained by differences in the timing of spawning. Although we did not observe ovulated eggs in the ovarian cavity, it is possible that fish spawned earlier at sites near the MWWE. Other field studies of MWWE impacts have reported changes in ovarian development and noted delayed ovarian maturation (Woodling *et al*., 2006), increased oocyte atresia (Jobling *et al*., 2002a), interruption of spawning (Tetreault *et al*., 2012), as well as decreased plasma estradiol and testosterone concentrations (Blazer *et al*., 2012). In the laboratory, exposure to MWWE has been shown to induce atresia of ovarian follicles (Lange *et al*., 2011), alteration of ovarian development (Lange *et al*., 2011), and reduction of egg laying in fishes (Filby *et al*., 2010; Jobling *et al*., 2003; Lister *et al*., 2009; Thorpe *et al*., 2009). Although these studies suggest that there are potential negative impacts of MWWE on females, we found these hard to separate from the effects of other stressors in the wild. The changes observed in females in this study tended to occur both upstream and downstream of the MWWTPs, suggesting that any impacts present may have been due to cumulative urban effects rather than just MWWE.

Male reproductive health measures responded to MWWE exposure in both seasons and at most levels of biological organization. The PCOS clearly demonstrated a separation of sites based on the biological endpoints (Figure 5.12B), which were indicative of MWWE exposure. Male *vtg*

expression increased directly downstream of the treatment plants in some years. Increases in *vtg* expression have been associated with the presence of estrogenic compounds (Palace *et al*., 2002; Rose *et al*., 2002; Sumpter and Jobling, 1995) and are frequently found downstream of MWWTPs (Adeogun *et al*., 2016b; Harries *et al*., 1996; Sanchez *et al*., 2011). Male gonad sex steroid production and gonad development were found to be decreased and delayed, respectively. Additionally, a consistent increase in the occurrence and severity of intersex was found downstream of MWWE outfalls in the fall. These measures clearly demonstrate exposure to MWWE, resulting in endocrine disruption and impaired sexual development. Similar to the increased expression of *vtg*, these endpoints are all suggestive of the presence of estrogenic compounds. The estrogenicity of the two MWWTPs in this study was assessed using the yeast estrogen screen (YES) in a previous study. The concentrations of estradiol equivalents in treated effluent in that study were found to be 4.3 ± 0.1 and 17.0 ± 0.4 ng/L in the Waterloo and Kitchener wastewater, respectively (Tanna *et al*., 2013).

5.5.2 Consistency of interpretation between seasons

While there were fewer data available for spring field collection than fall, we generally found greater consistency in the interpretation of a response in the fall than in the spring. This is partly due to the high annual variability in most measures in the spring field season compared with the fall. The somatic indices demonstrate this phenomenon most clearly, with large variation between years at the R site. There are several explanations for the increased variation in somatic indices during the spring. First, the variability between years in spring LSI could be due to differences in habitat (e.g., temperature, flow, food availability). Rainbow darter rapidly increase the size of their gonad and liver during the period just before spawning, a process that is dependent on food availability (Galloway and Munkittrick, 2006) and thus could be altered by variations in weather patterns. Second, the timing of sampling (during this biologically dynamic period) in the spring can be variable because access to sample sites is dependent on river flow, which is elevated by snow melt. Third, the interpretation of the impacts of MWWE may differ between seasons because of the increased fish movement in the spring, as rainbow darter move larger distances in the early spring than in the fall or summer. The increase in movement during the spring is probably related to locating ideal spawning sites (Winn, 1958a). Increased movement during spring and summer would explain the higher levels of intersex incidence and severity above the Kitchener MWWTP in spring than in fall.

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In terms of developing a biological monitoring program for CECs, this study demonstrates how an understanding of the indicator species is essential to developing an effective program. In a northern climate, small-bodied species such as darters that spawn in early spring are difficult to consistently sample, due to the aforementioned influences of variable weather conditions and movement associated with spawning. It is therefore strongly recommended that sampling occur in late fall when gonad development and physiology are more consistent. Barrett and Munkittrick (2010) reached a similar conclusion based on an assessment of the extensive Environmental Effects Monitoring program results in Canada.

5.5.3 Consistency/variability of measurements (across levels of biological organization)

When examining the impacts of MWWE, we found that measures at the lowest end of the biological scale (i.e., gene expression, Figure 5.4) were less consistent in their responses between years and were the most variable endpoint in terms of the range of data collected within a field season/site. Measures in the middle of the biological scale (i.e., intersex, Figure 5.8) were more consistent between seasons and years and also varied less within a year. While measures at the highest biological level measured (i.e., somatic indices; Figure 5.9-5.11) were the least consistent among years, they also varied the least within a season (Figure 5.12, Table 5-3 and 5-4). We observed variation in the data sets not only across levels of biological organization but also across space. Measures in the middle and at the upper end of the biological scale were more variable at the exposed site than the R site (Figure 5.12).

The high variability in gene expression within a site/year was not unexpected. Gene expression is a dynamic process that changes hourly in response to the ever-changing physiological demands of an organism. While an increase in variability within a site can provide us with information about the nature of the response (exposure, movement, etc.), it can be problematic for interpretation within a biological monitoring program. A clear increase in the expression of *vtg* in the liver of male rainbow darter downstream of the MWWTPs was present in some years; however, in other years this response was blunted or absent. Although this could be due to fluctuations in the composition of MWWE, or the amount of dilution of MWWE, not enough is known about the variability in the *vtg* response of a chronically exposed fish to draw any conclusions about this observation.

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Somatic indices were the least variable endpoint we measured in terms of the standard deviation at a site. They were also the least consistent endpoint between years of collections. It is difficult to know if the annual changes in the site-specific patterns of somatic indices are associated with abiotic fluctuations (exposure, flow, effluent quality, oxygen, etc.) or natural variability. In some years, fish may be exposed during a critical life stage to specific contaminants, or there may be extreme conditions (i.e., drought) that result in changes in somatic indices. Because somatic indices are more likely to be associated with general stressors or food availability, they may respond differently than endpoints that are more mechanistically linked to exposure to CECs in MWWE (e.g., intersex). High annual variability in select biomarkers has been observed in other studies as well (Hanson *et al*., 2009). While it has been suggested that the use of multiple reference sites may alleviate this variability (Kilgour *et al*., 2007), another study has found that this variability is reduced only when more than four reference sites, or four years of data collected from one site, are assessed (Hanson *et al*., 2010). The results from our study demonstrate how dynamic somatic indices can be between years in a small-bodied fish like darters and show the risk of over-interpreting them when only one year of sampling is completed or taken out of context of other endpoints. Many studies in the literature rely on this type of minimal sampling design and do not fully consider annual variability. For example, our own studies previously reported differences in somatic indices at these same sites and attributed them to wastewater exposure (Tanna *et al*., 2013).

In contrast to the high variability of gene expression and the low consistency of somatic indices, the variability of histological measures was low and the consistency of these measures was high. Gonad development in males was delayed in most fall field collections (but not spring). Additionally, the intersex incidence and severity were the most consistent measure in this study across years (in all the fall field seasons and most spring field seasons). Similarly, a study of male smallmouth bass (*Micropterus dolomieu*) found that the incidence of intersex was similar in two years of collection (Blazer *et al*., 2012). While smallmouth bass intersex incidence and severity were comparable between spring and fall, the measures were both lower at sites in the summer (Blazer *et al*., 2012; Blazer *et al*., 2007).

This examination of variability and consistency in endpoints across levels of biological organization provides contrasting messages about the appropriate design of a biological monitoring program. While it seems that it is necessary to collect multiple years of data to assess the impacts of MWWE on somatic indices, individual measures, such as intersex, are fairly consistent among years

and would not require multiple years of biological monitoring. Similarly, while measures at the low end of the biological scale (gene expression) are highly variable, measures at higher levels of biological organization (somatic indices) are less variable. This must be considered in the design and when applying statistics to the data sets generated. For endpoints with large variability the sample size must be increased to be able to detect change. Much larger sample sizes will be needed for measures of gene expression than for measures of somatic indices. However, this is seldom considered in monitoring designs or studies and often, because of the cost or other consideration (e.g., sample availability), sample size is not optimized for each endpoint. Additionally, a larger sample size may not be practical for a few reasons: because of the amount of fishing effort required, because removing additional fish from an affected area could have negative impacts on the population, and because it is difficult to produce an ethical justification for using larger numbers of fish to detect minimal change.

5.5.4 The population-level conundrum

The largest gap in this study is the inability to link the observation that male rainbow darter have impaired reproductive health to a higher level endpoint, namely implications for population sustainability. While measures of sex ratio were taken in some years, it was determined that the habitat preferences of males and females differed and changed across seasons, thus making these data inconclusive. The lack of population-level endpoints is a major weakness in most studies that evaluate the impacts of CECs from MWWE. This is a significant issue because measures at higher levels of biological organization provide regulators with the information necessary to make decisions. For example, a recently proposed framework for screening sites at risk from CECs suggested identifying sites where population and community effects are observed (Diamond *et al*., 2015).

Despite the importance of these measures, the assessment of population-level endpoints in response to CECs proves to be problematic for a couple of reasons. Firstly, there are few standardized methods that test for population-level responses to CECs. This was recently addressed in a review by Hamilton *et al*. (2016). The authors suggested several manners in which population-level effects could be assessed with modern technology. A second reason why assessing population-level endpoints are problematic is the amount of variability. We were unable to associate changes in somatic indices with a specific stressor in our multi-stressor system, despite there being strong indications of an effect related to endocrine disruption. It is likely that it will be even more difficult to

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detect change in measures at the population, community or ecosystem levels and directly associate them with CECs.

5.6 Conclusion

This study supports the use of the hierarchical biological system in biological monitoring programs. Making linkages to specific stressors (e.g., effluents) is strengthened by this approach, but natural variability and complexity of stressors (e.g., effluents) and the environment (e.g., flow, habitat) make it difficult to establish cause and effect (e.g., predictive) relationships. This study also cautions against the over-interpretation of monitoring data that do not consider the lifecycle of the sentinel species and the implications of natural variability (spatial and temporal). By placing our results in the context of an adverse outcome pathway (AOP) we can better understand the associations between individual- and population-level impacts of MWWE and the thresholds of these impacts.

Chapter 6

General Conclusion

In the title of this thesis the question "does intersex matter" was posed. This question has been addressed in various manners throughout the thesis. As described in the introduction, intersex condition is found in male fish around the world and is heavily associated with municipal wastewater effluent (MWWE). Thus, it is important to better understand the implications of intersex condition for reproductive success and population dynamics. Additionally, it is important to determine the thresholds (if present) of intersex incidence and/or severity for a healthy and sustainable population of fish. Our understanding of these issues was advanced in by the studies presented in this thesis.

1. In the second Chapter the relationship among intersex severity and fertilization success and embryo survival were investigated. Males with severe intersex (those with scores between 4 and 6) had reduced fertilization success but survival of embryos was not correlated with intersex severity. The conclusion drawn from this study was that severe intersex in males was associated with exposure to MWWE leading to reduced fertilization success.

2. In the third Chapter of this thesis reproductive behaviours of fish from reference sites and MWWE exposed sites were compared. While relationships weren't found directly with intersex, males collected downstream of the Kitchener municipal wastewater treatment plants (MWWTP) were found to spend less time guarding a spawning area. Additionally, aggressive behaviour was found to be lower in males with severe intersex in a mirror-opponent test.

3. When fish were collected from the reference site and exposed to MWWE in the fourth Chapter, a reduction in aggressive behaviour was observed in the same mirror-opponent test. Together with Chapter three, these studies suggest that male reproductive behaviour is reduced by MWWE exposure.

4. When an assessment of the variability of biological measures of MWWE exposure was conducted in Chapter five, it was demonstrated that intersex is one of the least variable measures of MWWE exposure in rainbow darter.

These studies advance the science of this field by contributing to the understanding of the implications and thresholds of intersex severity. From the studies in this thesis, it can be concluded that presence of severely intersex males in a population of fish is indicative of a reduction in reproductive function. This can be clearly seen when the evidence gathered from this thesis is presented in the format of an adverse outcome pathway (AOP) for the feminization of male fish

(Figure 6.1). The alteration of measures of reproductive health, such as increased intersex severity and reduced steroid production, result in the occurrence of adverse outcomes, such as reduced recruitment.

This thesis advanced the understanding of the implications of feminization of male fish. There is an opportunity, however, for this topic to be explored even further using rainbow darter in the Grand River as a model system. It is uncertain whether decreases in reproductive success would result in reduced genetic diversity or eventual population collapse. The lack of evidence of population level effects in wild exposed fish has led to uncertainty of the relevance of endocrine disruption. Populations of roach in UK rivers with intersex males were not altered in their genetic diversity and were deemed to be self-sustaining (Hamilton *et al*., 2014). Despite reduced recruitment and low abundance of rainbow darter downstream of the Kitchener MWWTP (Tetreault *et al*. 2013) a population remains present after many generations of exposure. From our studies, it can't be determined if this is continued presence of rainbow darter is due to migration from nearby populations or if this population is also self-sustaining. To better understand the implication of intersex in male fish, it is important to test the genetic diversity of rainbow darter. This could be accomplished with the use of microsatellite markers. One could examine the heterozygosity of individuals from heavily exposed areas and compare to unexposed sites, as well as across time. Since microsatellite markers have been developed previously for rainbow darter and other darter species, this experiment is feasible and could be conducted using tissues of previously collected specimens (Beneteau *et al*., 2009; Haponski *et al*., 2009).
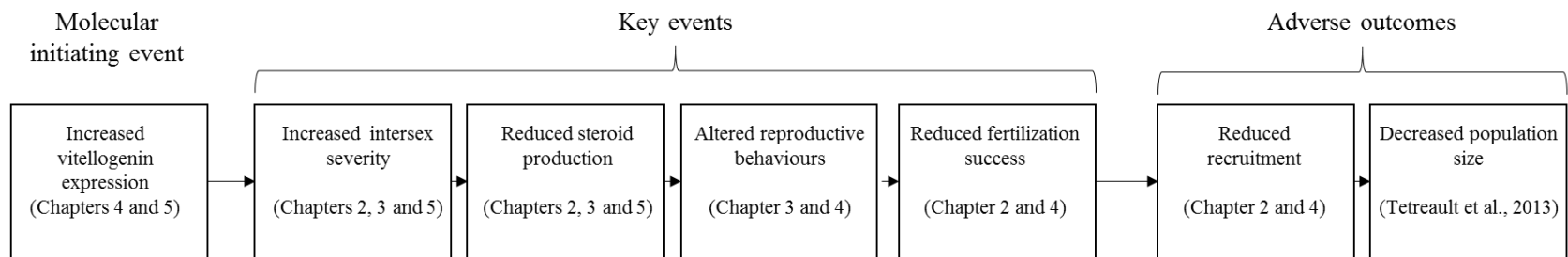
Figure 6.1 Proposed adverse outcome pathway for the exposure of rainbow darter to municipal wastewater effluent based on observations of rainbow darter from studies in this thesis and Tetreault *et al*. 2013 (decreased population size).

In addition to providing a better understanding of the implications of intersex condition in male fish, this thesis has contributed to a better understanding of the threshold of when these adverse effects occur. Comparisons of biological measures were made among categories of intersex severity throughout this thesis. A trend was present where fish with moderate intersex severity were not altered in their biological function, but fish with severe intersex condition had reduced reproductive functions. This was true for fertilization success and reproductive behaviours. Since severe intersex rainbow darter were less reproductively successful, the sustainability populations of rainbow darter is likely dependent on the proportion of individuals with severe intersex. The mean proportion of males presenting with severe intersex at the site downstream of the Kitchener MWWTP from all studies conducted was 34%, which is presumable below a critical threshold of population collapse. To determine a threshold of intersex, future studies could use data from this thesis to determine what proportion of severe intersex male rainbow darter would result in a lack of recruitment in the Grand River.

Does intersex matter? Yes. The evidence in this thesis suggests that populations in which there are individuals with severe intersex, reduced recruitment is likely occurring and leading to low population abundance. There are situaltions in which intersex is more likely to be a critical indicator of a population in disstress. For instance, populations in which a large proportion of severely intersex individuals would decrease in abundance more rapidly than those with a lower proportion of severely intersex individuals. Additionally, species of fish that are already under pressure from other environmental stressors will be more sensitive to the presence of intersex individuals. This second situation is especially relevant to fish populations in North America where of the 140 species of darter (Kuehne and Barbour, 1983; Page, 1983), 74 species are listed as either vulnerable, near endangered, critically endangered, or extinct (e.g. the Maryland darter; *Etheostoma sellare*) (IUCN, www.iucnredlist.org). When examined based on threats to populations, 31 species were identified as being threatened by domestic and urban wastewater. Since rainbow darter are widespread throughout northeastern North America, they could be used as sentinels to identify stressors in areas of concern for threatened species. Thus, in addition to adding to the knowledge base of the implications of intersex, these studies have identified variables in rainbow darter that could be used to protect more sensitive species.

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Appendix A

Supplemental information for Chapter 4
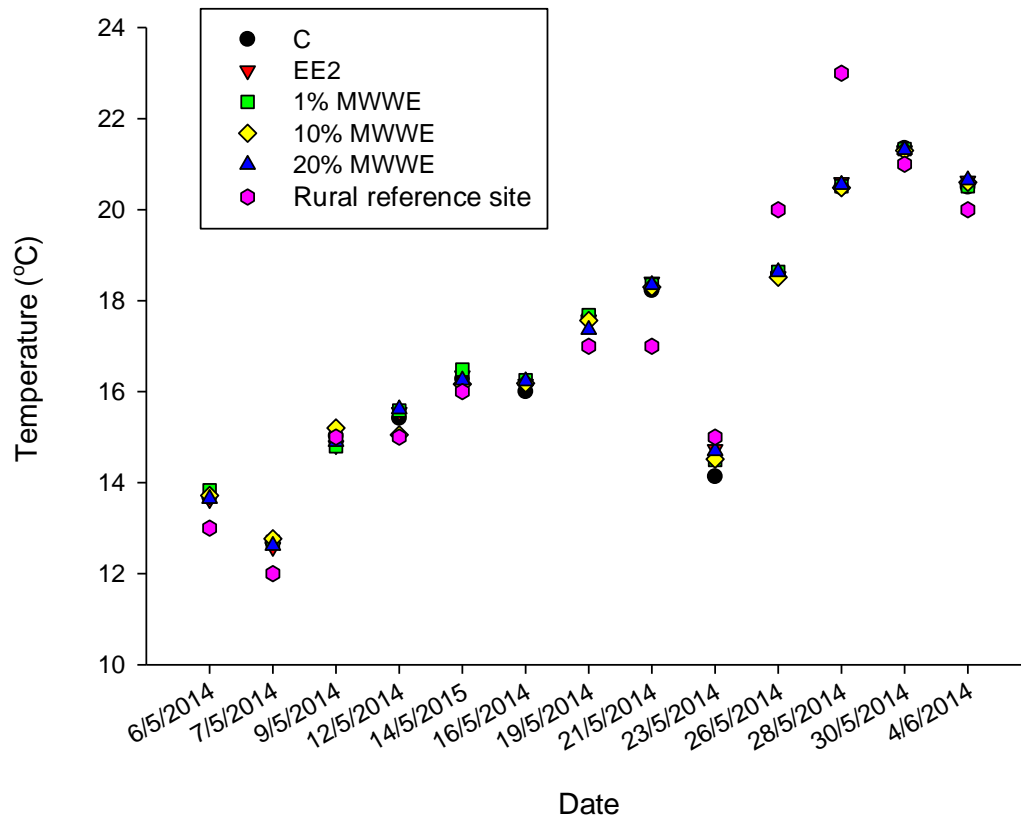
Figure S4.1 Water temperature (°C) in exposure tanks during a 21 day of exposure of rainbow darter to dechlorinated tap water (C), 10 ng/L 17α-ethinylestradiol (EE2), or municipal wastewater effluent (MWWE) diluted to 1, 10, or 20%. This figure also depicts the water temperature of the field site from which fish were collected (Rural reference site at West Montrose, ON, Canada). River temperature was taken from the Grand River Conservation Authority real-time water quality monitoring website (https://apps.grandriver.ca/waterdata/kiwischarts/wq_temp.aspx).
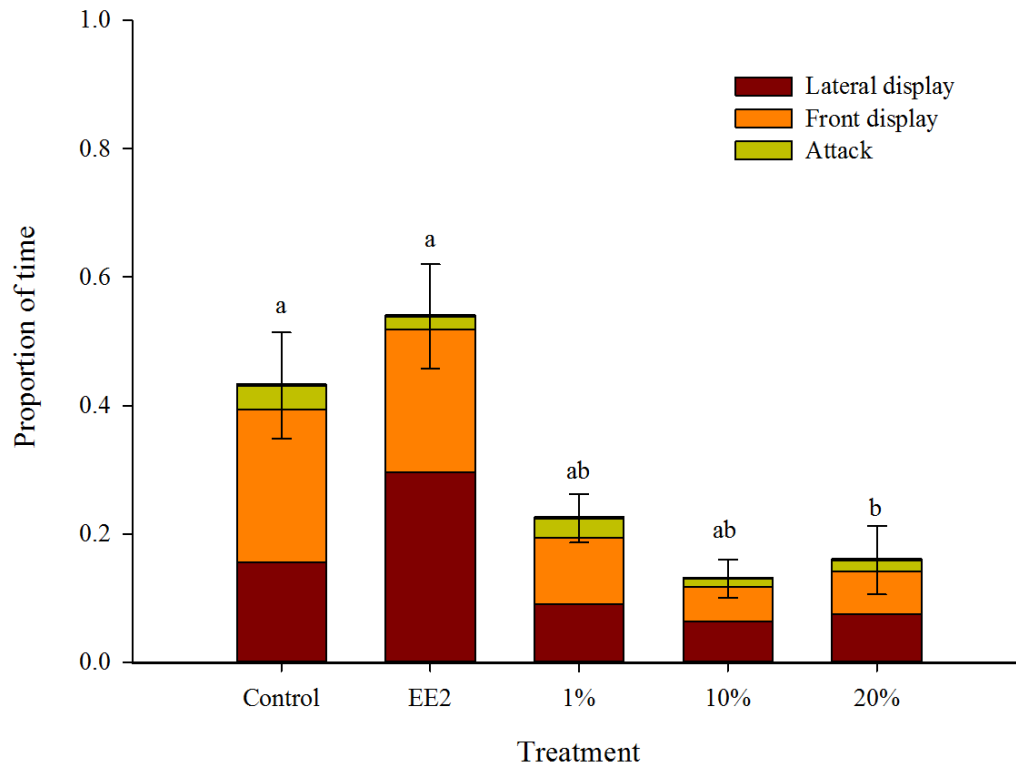
Figure S4.2 Comparison of mean (± SE) proportion of time male rainbow darter spent displaying three types of aggressive behaviours in a mirror test after 21 days of exposure to dechlorinated tap water (Control), 10 ng/L 17α-ethinylestradiol (EE2), or diluted municipal wastewater effluent (1%, 10%, 20%). Bars that do not share a common letter are significantly different as determined by a one-way analysis of variance with a Tukey's post-hoc test ($p < 0.05$).

Table S4-1 Pre-exposure sample size (N), length (cm ± se), and condition factor (K; ± se) of male (M) and female (F) rainbow darter randomly assigned to control, 10 ng/L 17α-ethinylestradiol (EE2), 1% municipal wastewater effluent (MWWE), 10% MWWE, or 20% MWWE treatments. * Indicates significant difference from control as determined by a one-way analysis of variance with a Tukey's post-hoc test (p < 0.05).

Treatment	Sex	N	Length	Weight (g)	K
Control	M	24	5.7 ± 0.1	2.02 ± 0.14	1.05 ± 0.02
	F	24	5.6 ± 0.1	1.93 ± 0.14	1.06 ± 0.02
EE2	M	12	6.0 ± 0.1	2.41 ± 0.19	1.08 ± 0.03
	F	12	5.9 ± 0.1	2.37 ± 0.19	1.12 ± 0.03
1% MWWE	M	12	5.9 ± 0.1	2.39 ± 0.19	1.10 ± 0.03
	F	12	5.9 ± 0.1	2.31 ± 0.19	1.10 ± 0.03
10% MWWE	M	12	5.7 ± 0.1	1.93 ± 0.19	1.03 ± 0.03
	F	12	5.6 ± 0.1	1.93 ± 0.19	1.08 ± 0.03
20% MWWE	M	12	5.6 ± 0.1	1.92 ± 0.19	1.09 ± 0.03
	F	12	5.6 ± 0.1	2.04 ± 0.19	1.17 ± 0.03 [*]

Table S4-2 Post-exposure sample size (N), length (cm ± se), gonad somatic index (GSI), liver somatic index (LSI), and condition factor (K; ± se) of male (M) and female (F) rainbow darter control, 10 ng/L 17α-ethinylestradiol (EE2), 1% municipal wastewater effluent (MWWE), 10% MWWE, or 20% MWWE treatments. No significant differences from control were found when tested with a one-way analysis of variance with a Tukey's post-hoc test (p < 0.05).

Treatment	Sex	N	Length	Weight (g)	GSI	LSI	K
Control	M	13	5.5 ± 0.1	1.80 ± 0.11	0.95 ± 0.16	1.22 ± 0.10	1.08 ± 0.02
	F	16	5.4 ± 0.1	1.93 ± 0.14	5.99 ± 1.12	1.73 ± 0.17	1.07 ± 0.02
EE2	M	12	5.7 ± 0.1	1.74 ± 0.11	1.13 ± 0.17	1.42 ± 0.10	1.08 ± 0.03
	F	12	5.6 ± 0.1	1.95 ± 0.16	6.25 ± 1.29	1.40 ± 0.20	1.07 ± 0.03
1% MWWE	M	12	5.8 ± 0.1	2.05 ± 0.16	0.94 ± 0.17	1.30 ± 0.10	1.08 ± 0.03
	F	12	5.7 ± 0.1	2.08 ± 0.16	7.10 ± 1.29	1.57 ± 0.20	1.09 ± 0.03
10% MWWE	M	12	5.4 ± 0.1	1.75 ± 0.16	0.98 ± 0.17	1.33 ± 0.11	1.04 ± 0.03
	F	12	5.4 ± 0.1	1.66 ± 0.16	4.61 ± 1.35	1.80 ± 0.21	1.05 ± 0.03
20% MWWE	M	12	5.4 ± 0.1	1.70 ± 0.16	0.94 ± 0.17	1.37 ± 0.10	1.06 ± 0.03
	F	12	5.3 ± 0.1	1.67 ± 0.16	7.25 ± 1.29	1.78 ± 0.20	1.05 ± 0.03

Appendix B

Supplemental information for Chapter 5

Table S5-1. Sample sizes of biological endpoints including analysis of gene expression of vitellogenin (*vtg*), stimulated in vitro steroid production of testosterone (T), 11-ketotestosterone (11KT), or 17β-estradiol (E2), histological analysis of gonad development (GD), incidence and severity of intersex (IS), relative proportion of gonad (GSI), relative proportion of liver (LSI), and condition factor (K).

| Year / Season | Site | Male | | | | | | | Female | | | | | |
		vtg	*T*	*11KT*	*GD*	*IS*	*GSI LSI*	*K*	*vtg*	*E2*	*T*	*GD*	*GSI LSI*	*K*
2007/ Fall	R	NA	8	8	5	10	20	20	NA	5	5	10	20	20
	USW	NA	10	11	5	11	22	23	NA	7	7	8	20	20
	DSW	NA	9	9	5	10	20	20	NA	9	9	10	20	20
	USK	NA	7	7	5	10	20	20	NA	6	7	10	20	20
	DSK	NA	9	9	5	8	20	20	NA	5	5	9	19	19
2009/ Fall	R	NA	NA	NA	NA	NA	19	NA	NA	NA	NA	NA	NA	NA
	USK	NA	NA	NA	NA	NA	15	NA	NA	NA	NA	NA	NA	NA
	DSK	NA	NA	NA	NA	NA	6	NA	NA	NA	NA	NA	NA	NA
	DSK2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	DSK3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2009/ Spring	USK	NA	17	17	10	16	37	37	NA	11	18	18	35	35
	DSK	NA	9	9	5	10	20	20	NA	6	9	10	20	20
	DSK3	NA	6	6	5	5	11	11	NA	7	10	10	20	20
2010/ Spring	R	NA	NA	NA	NA	42	42	42	NA	NA	NA	NA	18	18
	USW	NA	NA	NA	NA	22	22	22	NA	NA	NA	NA	15	71
	DSW	NA	NA	NA	NA	19	19	19	NA	NA	NA	NA	13	31
	USK	NA	NA	NA	NA	36	36	36	NA	NA	NA	NA	39	80
	DSK	NA	NA	NA	NA	19	19	19	NA	NA	NA	NA	20	169
	DSK2	NA	NA	NA	NA	6	6	6	NA	NA	NA	NA	17	15
	DSK3	NA	NA	NA	NA	30	30	30	NA	NA	NA	NA	20	62
2010/ Fall	R	10	NA	NA	NA	10	NA	NA	NA	NA	NA	NA	NA	NA
	USK	NA	NA	NA	5	26	40	40	NA	NA	NA	NA	40	40
	DSK	10	NA	NA	5	20	20	30	NA	NA	NA	NA	18	18
	DSK2	NA	NA	NA	5	20	21	21	NA	NA	NA	NA	21	21
	DSK3	10	NA	NA	NA	11	NA	NA	NA	NA	NA	NA	NA	NA

2011/ Spring	R	8	11	11	17	17	30	30	8	10	10	NA	20	63
	USW	NA	12	12	14	14	30	30	NA	10	10	NA	20	71
	DSW	NA	10	9	11	10	15	20	NA	11	11	NA	25	31
	USK	NA	19	20	13	33	62	62	NA	20	17	NA	40	80
	DSK	8	20	21	23	40	66	105	8	30	30	NA	63	169
	DSK2	NA	NA	NA	NA	NA	NA	NA	NA	10	10	NA	15	15
	DSK3	NA	14	14	15	15	29	29	NA	10	10	NA	21	62
2011/ Fall	R	9	10	10	5	9	14	14	7	8	NA	10	16	16
	USW	9	10	10	5	9	15	15	8	10	NA	12	15	15
	DSW	12	10	12	5	15	17	15	8	10	NA	13	15	15
	USK	9	10	10	5	12	16	17	8	8	NA	12	15	15
	DSK	12	10	10	5	8	22	22	9	10	NA	9	15	15
	DSK2	8	10	10	5	16	16	16	9	10	NA	12	16	16
	DSK3	NA	10	10	5	12	13	13	NA	10	NA	10	16	16
2012/ Spring	R	NA	10	9	5	16	26	26	NA	9	8	7	25	67
	USK	NA	6	9	5	15	23	23	NA	10	10	10	36	120
	DSK	NA	4	7	5	23	49	49	NA	10	10	10	41	167
2012/ Fall	R	9	20	21	5	18	30	30	8	19	16	14	30	69
	USW	8	8	8	5	13	16	16	7	14	14	13	15	46
	DSW	12	12	12	5	10	15	15	7	12	14	14	15	44
	USK	8	15	15	5	15	30	30	12	21	15	13	30	120
	DSK	13	20	20	5	20	31	31	7	21	13	18	29	55
	DSK2	7	10	10	5	10	16	16	8	15	15	14	16	40

Table S5-2. Concentration of steroid hormones (ng/g tissue) produced by gonad tissue stimulated with forskolin (F), or hCG (H) and measured by RIA (R) or EIA (E), as described in the methods section for *in vitro* steroid production.

Year / Season	Site	Male		Female	
		T	11KT	E2	T
2007 / Fall	R	3.84 ± 0.58	ND	2.80 ± 0.74	0.79 ± 0.33
(F, R)	USW	2.66 ± 0.38	ND	2.97 ± 0.45	0.88 ± 0.24
	DSW	1.96 ± 0.17	ND	2.59 ± 0.41	0.89± 0.22
	USK	2.64± 0.28	ND	2.39 ± 0.63	1.06 ± 0.30
	DSK	1.0 ± 0.21	ND	1.72 ± 0.31	0.90 ± 0.19
2009 / Spring	USK	0.75 ± 0.09	0.82 ± 0.25	0.81 ± 0.13	0.46 ± 0.14
(F, R)	DSK	0.77 ± 0.10	0.76 ± 0.12	0.98 ± 0.21	0.71 ± 0.16
	DSK3	0.51 ± 0.04	0.70 ± 0.12	0.82 ± 0.24	0.38 ± 0.01
2011 / Spring	R	35.5 ± 3.9	49.1 ± 7.5	43.6 ± 5.4	29.7 ± 5.9
(F, R)	USW	42.2 ± 3.8	87.7 ± 7.2	25.4 ± 5.4	8.3 ± 5.9
	DSW	18.6 ± 4.1	14.8 ± 8.3	41.3 ± 5.2	18.9 ± 5.6
	USK	30.8 ± 3.0	24.2 ± 5.5	29.8 ± 3.9	14.6 ± 4.5
	DSK	25.6 ± 2.9	26.8 ± 5.4	30.7 ± 3.1	26.1 ± 3.4
	DSK2	NA	NA	30.1 ± 5.4	10.7 ± 5.9
	DSK3	24.1 ± 3.5	24.3 ± 6.6	32.8 ± 5.4	20.0 ± 5.9
2011/ Fall	R	49.6 ± 3.3	50.2 ± 6.2	8.3 ± 4.2	NA
(H, R)	USW	59.8 ± 3.3	46.7 ± 5.6	8.2 ± 3.8	NA
	DSW	55.3 ± 3.3	63.7 ± 6.2	20.3 ± 3.8	NA
	USK	41.0 ± 3.3	45.7 ± 6.2	15.4 ± 4.2	NA
	DSK	27.8 ± 3.3	10.9 ± 6.2	7.1 ± 3.8	NA
	DSK2	32.4 ± 3.3	11.5 ± 6.2	11.0 ± 3.8	NA
	DSK3	46.9 ± 3.3	40.8 ± 6.2	18.7 ± 3.8	NA
2012 / Spring	R	21.4 ± 4.1	43.2 ± 8.3	48.6 ± 5.7	25.7 ± 6.6
(H, E)	USK	22.7 ± 5.3	34.2 ± 5.5	38.8 ± 5.4	75.0 ± 5.9
	DSK	6.9 ± 6.5	26.8 ± 5.4	39.5 ± 5.4	24.6 ± 5.9
2012/ Fall	R	20.8 ± 2.3	17.1 ± 4.3	23.1 ± 2.7	1.22 ± 0.15
(H, E)	USW	23.6 ± 3.7	27.7 ± 6.9	33.6 ± 3.2	0.81 ± 0.16
	DSW	10.8 ± 3.0	4.3 ± 5.6	13.7 ± 3.4	0.72 ± 0.16
	USK	11.9 ± 2.7	6.5 ± 5.0	12.9 ± 2.6	0.67 ± 0.15
	DSK	8.1 ± 2.3	2.4 ± 4.4	14.1 ± 2.6	0.62 ± 0.16
	DSK2	6.5 ± 3.3	2.9 ± 6.2	16.0 ± 3.1	0.88 ± 0.15

Table S5-3. Distance-based redundancy analysis reveals that there is no association between select pharmaceuticals and female biological endpoints, but there is an association between some pharmaceuticals and male biological endpoints. Significance is indicated by * when p<0.05.

Sex	Pharmaceutical	SS	Pseudo-F	P	Proportion
Female	Naproxen	5.30	1.36	0.321	0.061
	Ibuprofen	1.39	0.341	0.688	0.016
	Venlafaxine	-0.953	-0.227	0.879	0.011
	Carbamazepine	16.8	5.01	0.020 *	0.193
	Triclosan	-2.78	-0.650	0.960	-0.032
Male	Naproxen	13.9	4.10	0.040 *	0.124
	Ibuprofen	19.9	6.29	0.009 *	0.178
	Venlafaxine	22.7	7.40	0.007 *	0.203
	Carbamazepine	10.6	3.05	0.086	0.095
	Triclosan	20.7	6.60	0.007 *	0.185

A

B

C
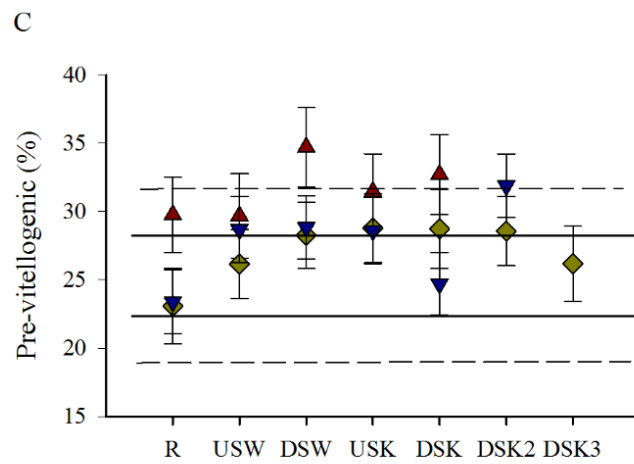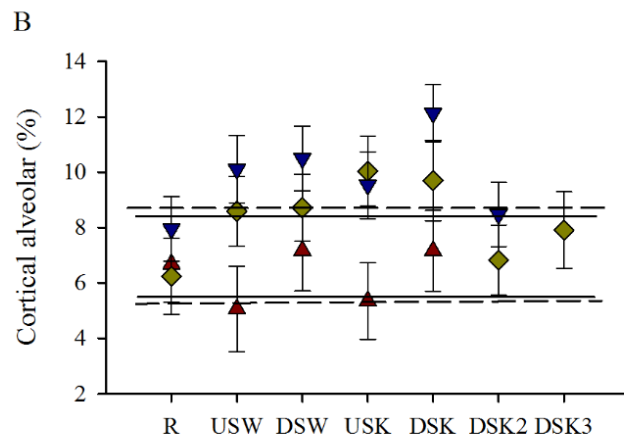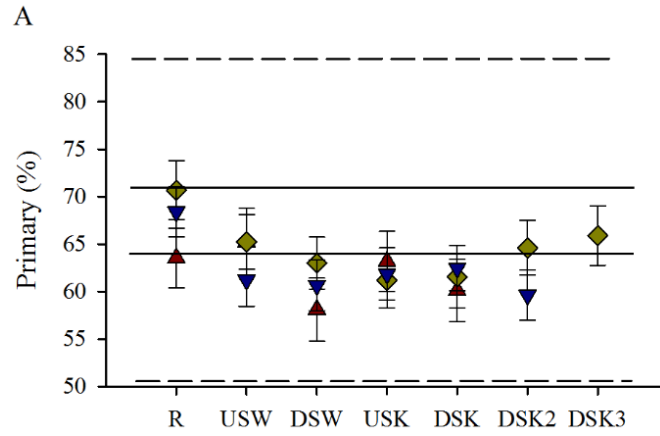
Site

Figure S5.1 Changes in ovarian development through an urban gradient in fall collections. Relative proportion of primary (A), cortical alveolar (B) and pre-vitellogenic oocytes represented by the mean (± SE). Assessment was determined through histological analysis of female gonads collected in fall field season of 2007 (red triangles), 2011 (yellow diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the upper 25% critical effect size calculated from the mean of the data from the rural reference site (R), and the solid line indicates the upper 95% confidence interval from the pooled R.
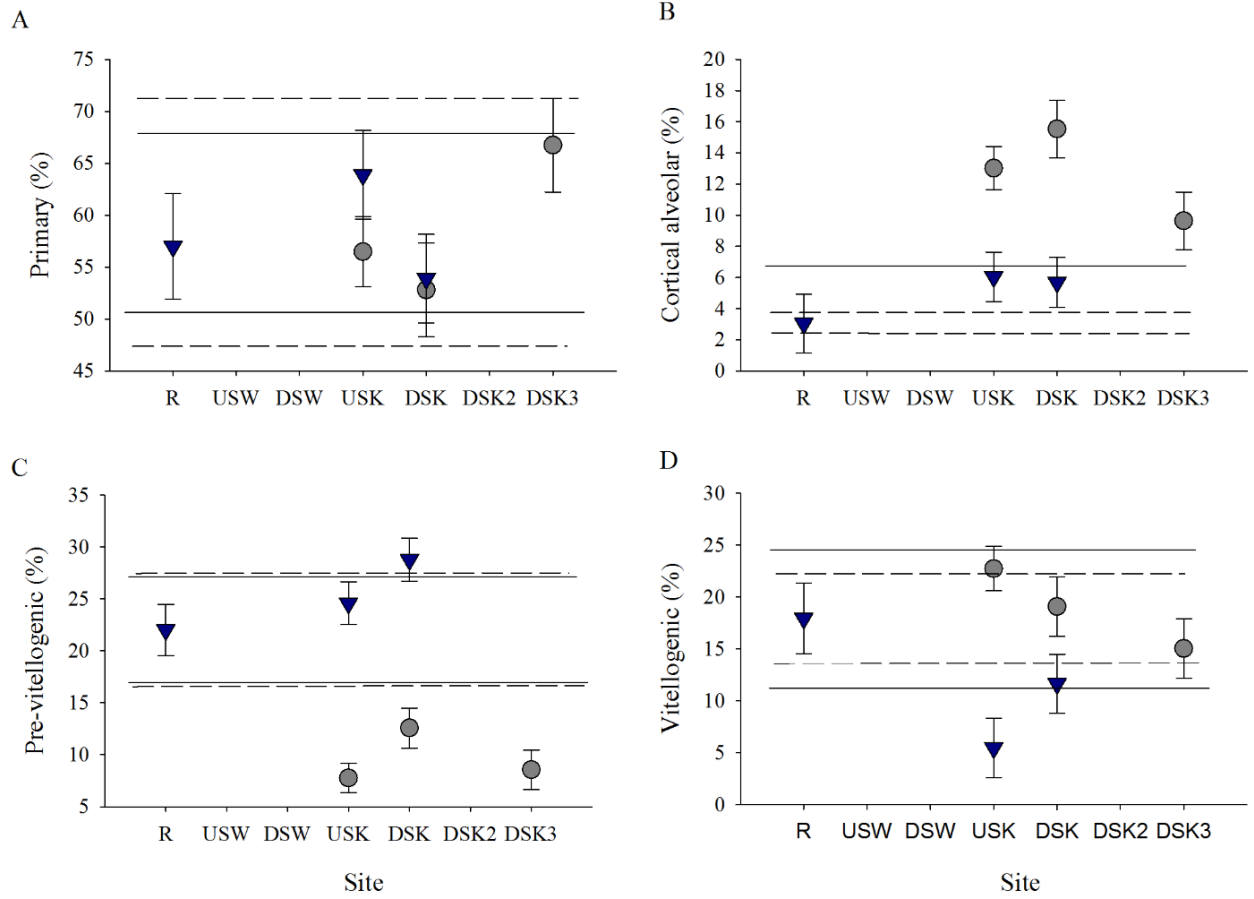
Figure S5.2 Changes in ovarian development through an urban gradient in spring collections. Relative proportion of primary (A), cortical alveolar (B), pre-vitellogenic (C), and vitellogenic (D) oocytes represented by the mean (± SE). Assessment was determined through histological analysis of female gonads collected in spring field season of 2009 (grey circles) and 2012 (blue down-facing triangles). The dashed line indicates the upper 25% critical effect size calculated from the mean of the data from the rural reference site (R), and the solid line indicates the upper 95% confidence interval from the pooled R.
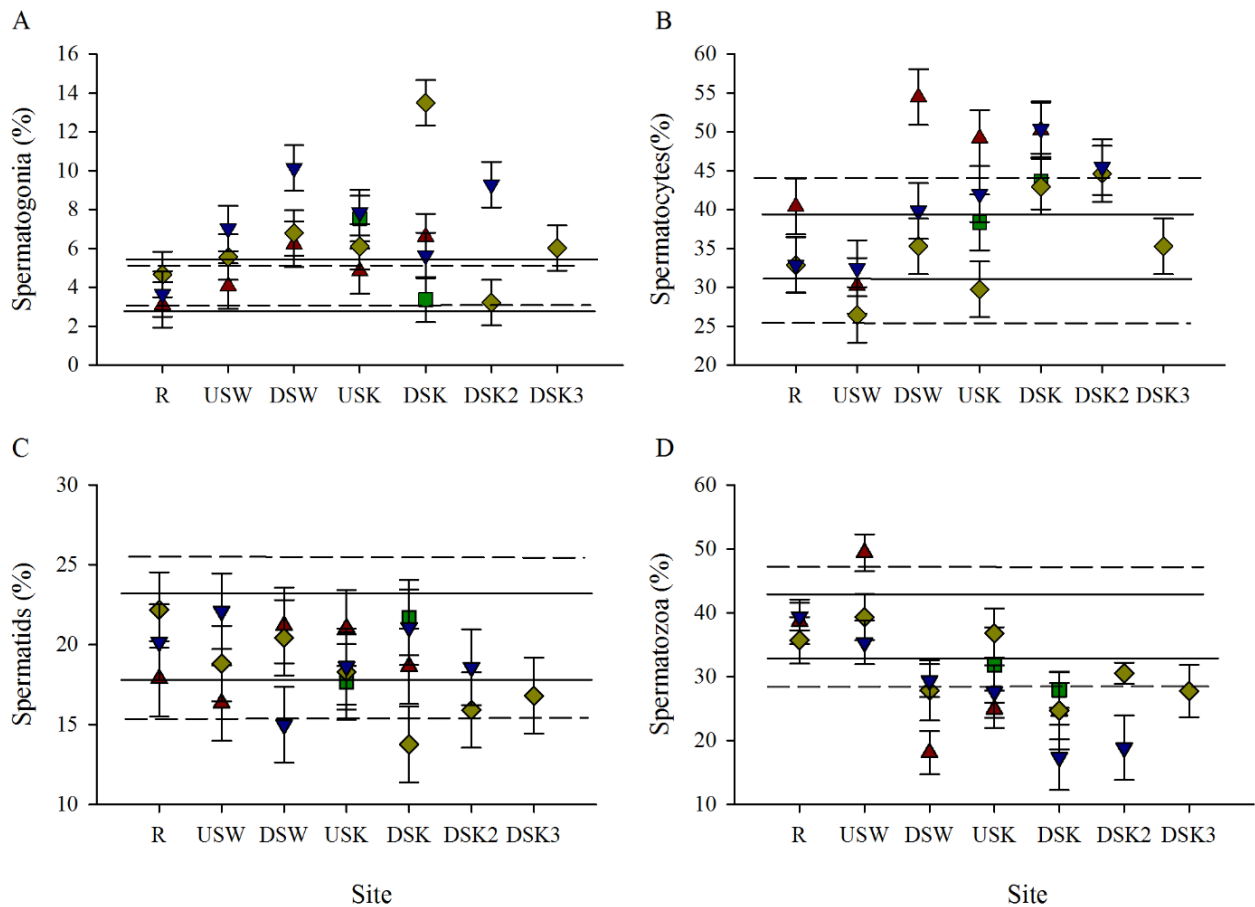
Figure S5.3 Changes in testis development through an urban gradient in fall collections.
Relative proportion of spermatogonia (A), spermatocytes (B), spermatids (C), and spermatozoa
(D) represented by the mean (± SE). Assessment was determined through histological analysis
of male gonads collected in fall field season of 2007 (red triangles), 2010 (green squares), 2011
(yellow diamonds), and 2012 (blue down-facing triangles). The dashed line indicates the upper
25% critical effect size calculated from the mean of the data from the rural reference site (R),
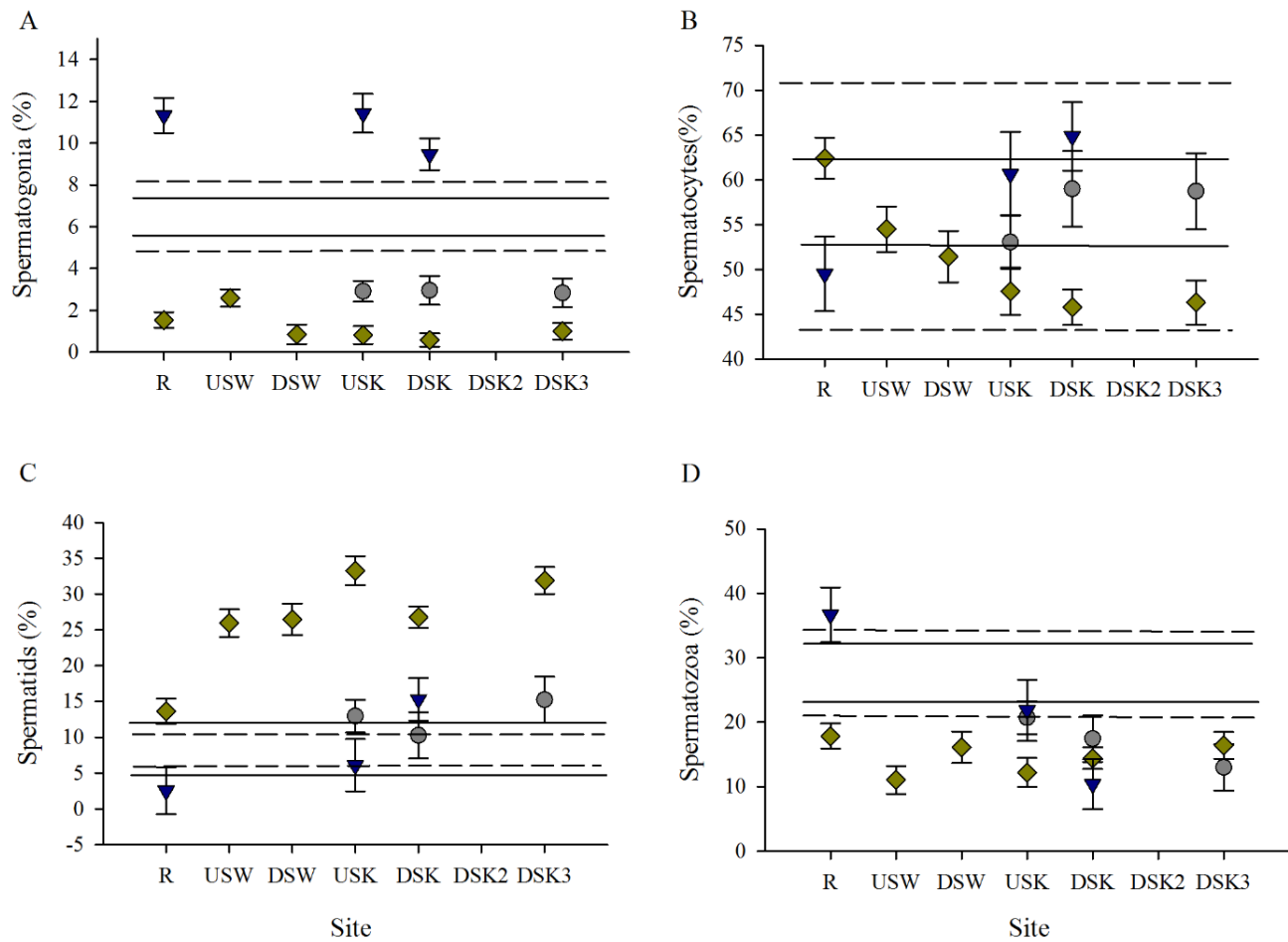and the solid line indicates the upper 95% confidence interval from the pooled R.

Figure S5.4 Changes in testis development through an urban gradient in spring collections.
Relative proportion of spermatogonia (A), spermatocytes (B), spermatids (C), and spermatozoa
(D) represented by the mean (± SE). Assessment was determined through histological analysis
of male gonads collected in spring field season of 2009 (grey circles), 2011 (yellow diamonds),
and 2012 (blue down-facing triangles). The dashed line indicates the upper 25% critical effect
size calculated from the mean of the data from the rural reference site (R), and the solid line
indicates the upper 95% confidence interval from the pooled R.