

Association of Intersex in Wild Fish with Wastewater Effluent in the Grand River, Ontario

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

The Grand River watershed is the largest watershed in southern Ontario, and is expected to see major development and urban densification over the next 20 years. An expected 57% increase in population over the next two decades in urban centers such as Kitchener – Waterloo, Cambridge, Guelph and Brantford means an added load on the existing 30 wastewater treatment facilities serving the watershed. A subsequent increase in the amount of average flow and effluent released into the Grand River via the Waterloo and Kitchener wastewater treatment plants is also expected. The Waterloo and Kitchener wastewater plants are both secondary treatment plants, although neither plant currently nitrifies the wastewater prior to release. As a result, increased concentrations of ammonia and nitrate are found downstream of the treatment plant outfalls. Compounds introduced into the Grand River via the discharge of wastewater effluent can have impacts on resident biota such as fish. Disruption of the normal function of endocrine systems in fish has been associated with municipal effluents as well as chemicals that have been detected in these effluents. One of the major responses has been the presence of intersex (oocytes in testes) in fish downstream of the wastewater plant outfalls at sites around the globe. The research in this thesis examined resident fish for the variability and extent of intersex condition by adapting a new fragmented testis technique. Biomarkers of response such as relative gonad weight (GSI), relative liver weight (LSI) and condition (K) were also measured. The study focused on the Rainbow Darter (*Etheostoma caeruleum*) a dominant benthivorous fish species in the riffle habitats in the Grand River. An increased proportion of male Rainbow Darters sampled immediately downstream of the Waterloo outfall had a gonad lobe containing at least one testis-oocyte. The number of Rainbow Darter with more severe intersex (10-99 oocytes/lobe) also increased immediately downstream of the Waterloo outfall. A much more dramatic expression of intersex proportion and severity (>100 oocytes per testis lobe) was observed further downstream, below the Kitchener outfall. These patterns in intersex presence and severity were also observed in two other species collected at a subset of the original sites. Although there were minimal changes in GSI, LSI and K below the Waterloo outfall, differences were observed downstream of the Kitchener wastewater effluent outfall. Female Rainbow Darter downstream of the Kitchener outfall showed significant decreases in GSI and LSI, and increases in K. Male Rainbow Darter GSI and LSI data across sites did not express changes that coincided with MWWWE outfalls, although small increases in condition were observed. These patterns of intersex and organism level responses suggest that the assimilation of wastewater effluent into natural receiving environments may have impacts on endocrine function and energy use and allocation in wild fish.

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Having been exposed to the content of a number of upper level graduate courses in Toxicology and Environmental Risk Assessment set the stage for one of my last undergraduate classes with Dr. Mark Servos. In this class, Mark integrated concepts across disciplines enthusiastically and in a manner which instantly piqued my interest. His energy is such that he has drawn in and collaborated with a number of talented people that I consider myself extremely lucky to have worked under and in collaboration with. I have learned something valuable and/or benefitted from the efforts of each and every individual that has worked in the Mark Servos lab at the University of Waterloo and the Mark McMaster lab at Environment Canada, Burlington.

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Through the last two years I have been surrounded by individuals who continually question and discuss various topics that fall under the very interdisciplinary field that is Ecotoxicology. It is this dialogue, and the encouraging nature of these individuals that has made this experience one I will always draw upon. I wouldn't trade this for the world.

Thank you all.

Dedication

I dedicate this thesis to the natural environment, and to its continued health with which we are inextricably linked.

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Chapter 1:

Introduction

Rapid urban intensification and unprecedented population growth experienced in southern Ontario has strained both existing water and wastewater infrastructure, and potentially, the assimilative capacities of aquatic receiving environments. With accelerated urban growth comes an inflexible demand for potable water and removal of wastewater, resulting in defined infrastructure costs, but varied environmental costs. If left unchecked, urban growth may outstrip both finite infrastructure resources and environmental carrying capacities, placing both ecological and human health at risk.

The Grand River drains its 6,965 km² watershed from its headwater origins near Dundalk, ON, 280 km through to its discharge into Lake Erie. The majority of the land mass surrounding the Grand River and its tributaries are either under intensive agricultural uses or urban areas, with limited undisturbed or protected areas. As the Grand River watershed is west of Ontario's protected Greenbelt region, urban development occurring west of Toronto under Ontario's "Places to Grow" act is ultimately targeted for the existing urban centers of Kitchener, Waterloo, Cambridge, Guelph and Brantford, which translate into population increases of 57% between 2001 and 2031 (Grand River Conservation Authority 2005; Ministry of Public Infrastructure Renewal 2006). The >900,000 individuals currently residing in the watershed are served by 30 sewage treatment plants which release treated effluent into the Grand River and its tributaries. To mitigate the impacts of population growth and increases in effluent released into aquatic receiving environments, a series of municipal wastewater treatment plant (MWWTP) upgrades are being implemented to increase the effluent quality (Region of Waterloo 2007).

MWWTPs receive a mixture of liquid waste comprising residential wastewater, industrial wastewater, and in some cases, storm water. Upon reaching a wastewater treatment plant, raw sewage is typically subject to multiple stages of treatment: preliminary treatment, primary treatment, secondary treatment. In some cases, tertiary treatment processes are employed to remove specific components.

Preliminary treatment physically removes larger objects and materials from wastewater to facilitate the adsorption and deposition of lighter suspended solids during primary treatment. Secondary treatment employs aerobic processes to enable bacteria to convert the remaining organic compounds into carbon dioxide and water. Efficient secondary treatment thus reduces the biological oxygen demand (BOD) of effluent. While the effectiveness of the treatment processes vary with hydraulic and solids retention times at each phase, the resulting secondary-treated wastewater effluent (depending on the individual MWWTP) is either subject to tertiary treatment such as sand filtration, and or released after disinfection directly into a nearby waterway. Disinfection can be accomplished through many processes but is usually accomplished by chlorination or application of UV light. Despite extensive treatment the resulting effluent can still contain a wide variety of contaminants.

Municipal wastewater effluents (MWWEs) represent the largest point source discharges of pollutants to Canadian surface waters (Environment Canada 2001b), and often contain a number of “emerging” contaminants. Recent advances in environmental analytical chemistry allow for the quantification of these compounds at trace levels. Many of the emerging contaminants detected in MWWEs are biologically active, and can interfere with normal organism function by mimicking or interfering with endogenous hormones (Sonnenschein and Soto 1998). EDCs, including pharmaceutical and personal care products (PPCPs) can also be introduced into natural environments via wastewater discharges (Daughton and Ternes 1999).

A number of recent studies have attempted to characterize the ecological effects of the continual exposure of resident fish such as Rainbow Darter (*Etheostoma caeruleum*) and Greenside Darter (*Etheostoma blennioides*) to MWWEs within the Grand River watershed. These species are suitable sentinel species for detecting impacts as they are abundant, sensitive, and widely distributed throughout the watershed (Brown et al. 2011; Tetreault et al. 2011). Tetreault et al. (2011) demonstrated one of the first instances of widespread intersex condition (the coexistence of male and female gonadal tissue) in

wild fish in a Canadian freshwater ecosystem. The discovery of primary oocytes in male Rainbow and Greenside Darter testis tissue downstream of the Waterloo and Kitchener secondary-treated MWWWE outfalls is strongly suggestive of the presence of EDCs in these receiving environments. At MWWWE-exposed sites with higher rates of intersex, male Rainbow Darter and Greenside Darter gonads also demonstrated a reduced ratio of mature to immature testis tissue cells (Tetreault et al. 2011). Increased concentrations of PPCPs have been characterized near the Waterloo and Kitchener MWWWE outfalls in both water and in fish (Wang et al. 2011a). Bioconcentration of these compounds in fish, while modest, confirms their availability in tissues and their potential for interactions with endocrine mechanisms. In addition to impacts on reproductive systems, municipal wastewater effluents have recently been shown to induce stress responses and impair the ability of fish to respond to secondary stressors (Ings et al. 2011). These biochemical and organ level responses to MWWWE exposure necessitates further exploration and characterization of the extent and variability of these responses in fish throughout the watershed to develop a baseline of responses. By understanding the distribution of the impacts of MWWWE in the watershed the impacts of current and future activities can be better assessed.

While MWWTPs partially remove some PPCPs and EDCs from wastewater, others pass through largely unmodified and enter aquatic environments with effluents released from these facilities (Metcalf et al. 2003; Servos et al. 2005). Although concentrations of PPCPs are very low, they are designed to exert biological activity at a minimal therapeutic dose (Daughton and Ternes 1999; Fent et al. 2006). Consequently, the widespread detection of numerous PPCPs and EDCs in MWWEs and their receiving environments at trace concentrations raises the possibility of adverse effects to even very low-level exposure to fish.

Environmental exposure to exogenous chemicals can interfere with endocrine signaling pathways within organisms (Cheek et al. 1998), resulting in altered hormone synthesis, metabolism or modified receptor levels (Sonnenchein and Soto 1998). The ability for EDCs to impair or alter function of

endocrine signalling pathways by diverse mechanisms, together with the promiscuity of some receptors to bind a variety of structurally similar and diverse ligands (Cooper and Kavlok 1997), can result in various non-specific physiological impairments and disruptions. There have been many recent cases of EDCs having an effect on freshwater, estuarine, and marine exposed organisms (Mills and Chichester 2005). A wide variety of EDCs including (organometals, pesticides, polycyclic aromatic hydrocarbons, surfactants) have been reported in the environment (Kümmerer 2001). Among the most bioactive environmental contaminants in municipal wastewater effluents due to their intended function are natural and synthetic estrogens such as 17 β -estradiol (E2), estrone, estriol and the synthetic superanalogue 17 α -ethynylestradiol (EE2) (Solé et al. 2000; Mills and Chichester 2005). Although usually found as complex mixtures at low ng/L concentrations in MWW they represent a major contribution to the overall estrogenicity (Desbrow et al. 1998; Snyder et al. 1999; Baronti et al. 2000). In male fish, the activation of signaling pathways by estrogens and estrogen mimics such as nonylphenol, 4-tert-octylphenol and bisphenol-A can also result in feminization (Brian et al. 2009). Mixtures of these ligands can act in an additive manner increasing the magnitude of the potential response in exposed fish. As MWW are complex and dynamic mixtures containing EDCs, our knowledge of the cause and effect linkages in exposed organisms in the natural environment is limited (Sumpter 2009).

Estrogenic compounds are well known for their ability to induce production of vitellogenin and intersex condition in male fish (Jobling et al. 1998; Denslow 1999; Aerni et al. 2004). Estrogens and xenoestrogens entering the environment can elicit biochemical responses outside of an organism's normal physiology. In females the effects of additional estrogens are difficult to discern while in males the resulting feminization is more readily observed. The production of the egg yolk precursor protein vitellogenin (Vg), while a normal event in the pre-spawning cycle of female fish, can be elicited by estrogens in males. The elevated concentration of Vg in males is thus a useful endpoint indicative of

exposure to estrogenic chemicals. Commensurate with Vg production, intersex is a strong indication of the presence of estrogenic contaminants in a waterbody (Jobling et al. 1998).

Intersex condition attributed to MWW entering receiving environments was observed in early studies on Roach (*Rutilus rutilus*) in rivers in the United Kingdom. The detection of feminization and the occurrence of intersex in fish associated with municipal wastewaters is a discovery that has become the focus of considerable research globally (Sumpter and Andrew 2008). Intersex has not been widely investigated in Canada and only a few studies have reported this condition in fish in Canada (e.g. Mikaelian et al. 2002; Kavanagh et al. 2004). Very recently, Tetreault et al. (2011) described the high incidence of intersex in an urbanized river in southern Ontario. A summary of studies investigating intersex condition are summarized in Table 1.

As outlined by Ankley et al. (2010), numerous studies are available for natural (e.g. E2) and synthetic (e.g. EE2) estrogens that have generated large amounts of toxicity data relevant to the assessment of the impacts on aquatic species such as fish. The adverse outcome pathway presented by Ankley et al. (2010) involves the interaction of these chemicals with estrogen receptors resulting in a variety of changes in protein synthesis and ultimately changes in reproductive performance. For estrogen receptor activation, the adverse outcomes predicted (based on empirical linkages) include Vg induction, intersex, impaired spawning behaviour, altered sex ratios, and reduced fecundity. The outcome pathway proposes that intersex is induced independently and in parallel with Vg induction and morphological effects. Other early and recent studies supporting these predicted outcomes demonstrated reductions in 11-ketotestosterone in males, the primary androgen responsible for the development and maturation of male gametes (Schultz et al. 2003; Tetreault et al. 2011). In the gonads the interaction between an estrogenic ligand and the estrogen receptor results in the stimulation of germ cells which develop into gametes and somatic cells. Differentiation of germ cells into oocytes within testes tissue (testis-ova) thereby constitutes intersex condition.

Table 1: Review of studies by country where intersex condition (female oocytes in male testicular tissue) has been observed in resident wild fish collected downstream of MWWWE discharges (adapted from Tetreault et al. 2011).

Country	Species	Common name	Reported intersex	Study
Belgium	<i>Gobio gobio</i>	Gudgeon	5-20%	Douxflis et al. (2007)
	<i>Barbatula barbatula</i>	Stoneloach	11%	Douxflis et al. (2007)
Canada	<i>Coregonus clupeaformis</i>	Lake Whitefish	1.2-11.7%	Mikaelian et al. (2002)
	<i>Morone Americana</i>	White Perch	22-83%	Kavanagh et al. (2004)
	<i>Etheostoma blennioides</i>	Greenside Darter	0-60%	Tetreault et al. (2011)
	<i>Etheostoma caeruleum</i>	Rainbow Darter	0-75%	Tetreault et al. (2011)
Denmark	<i>Rutilus rutilus</i>	Roach	4.5-6.5%	Bjerregaard et al. (2006)
France	<i>Platichthys flesus</i>	Flounder	8%	Minier et al. (2000)
Germany	<i>Abramis brama L</i>	Bream	0.5-6%	Hecker et al. (2002)
Italy	<i>Barbus plebejus</i>	Barbell	50%	Viganò et al. (2001)
Netherlands	<i>Abramis brama</i>	Bream	37%	Vethaak et al. (2002)
	<i>Platichthys flesus</i>	Flounder	0%	Vethaak et al. (2002)
Spain	<i>Cyprinus carpio</i>	Carp	18-50%	Sole et al. (2002, 2003)
United Kingdom	<i>Rutilus rutilus</i>	Roach	16-100%	Jobling et al. (1998, 2002a)
	<i>Platichthys flesus</i>	Flounder	20%	Allen et al. (1999)
	<i>Gobio gobio</i>	Gudgeon	6-15%	van Aerle et al. (2001)
	<i>Esox Lucius</i>	Pike	14-26%	Vine et al. (2005)
	<i>Catostomus commersoni</i>	White Sucker	4 fish; 18-22%	Woodling et al. (2006); Vajda et al. (2008)
	<i>Micropterus spp.</i>	Black Bass	9%	Hinck et al. (2006)
USA	<i>Micropterus dolomieu</i>	Smallmouth Bass	0-100%; 44%; 82-100%	Blazer et al. (2007); Hinck et al. (2009); Iwanowicz et al. (2009)
	<i>Micropterus salmoides</i>	Largemouth Bass	44%; 23%	Hinck et al. (2009); Iwanowicz et al. (2009)
	<i>Ictalurus punctatus</i>	Channel Catfish	50%	Hinck et al. (2009)

In order to characterize the presence and severity of intersex condition, traditional histology methods have been employed to process male gonadal tissue samples. Immediately after evisceration, the gonadal tissues (testes) are placed into Davidson's solution (a buffered fixative). Following a minimum period of 24 h in fixative, the sample is dehydrated using a series of water and alcohol mixtures. Finally a solvent is used to dissolve remaining alcohol and enable the hot paraffin embedding agent to fully infiltrate. Once set in a solid mould of paraffin, the sample is sectioned to a 5 μm thickness using a microtome, and set onto microscope slides. The slides are stained with hematoxylin and eosin to provide definition and enhance contrast of cell nuclei, acidic structures, cytoplasmic proteins and other extracellular structures. This allows for clear analysis of the developmental stage of the testes tissue, as well as an opportunity to detect the presence of any interspersed oocytes. This method however, lacks the capacity to accurately quantify gonadal disruption manifested as testis-ova within a single sample without preparation of multiple sections over multiple slides. Further, the traditional approach can introduce error since oocytes are larger than 5 μm in diameter, and thereby may appear in multiple 5 μm slices of tissue, potentially overestimating intersex due to a single oocyte being counted multiple times. Consequently, studies presenting data that specify the severity of intersex in individual fish are rare (Harris et al. 2011). Rather, the proportions of intersex fish at a given site sampled or within a river are usually presented. The ability to easily quantify the severity of intersex within a sample obtained from a single fish would be of great advantage in evaluations linking intersex and other fish health parameters.

As a result of continuous exposure to MWWE, resident fish populations are exposed to a complex mixture of EDCs, simple nutrients, and physicochemical stressors impacting development and disrupting metabolism (Gibbons and Munkittrick 1994). The manifestation of these cumulative stressors can be seen as changes in fish health indices such as relative gonad size (GSI), liver size (LSI) and condition (K). Studies evaluating the impacts of MWWE on these fish health parameters have yielded varied patterns of response, perhaps reflecting the simultaneous nutrient enrichment and heightened food availability

coincident with exposure to EDCs. The nutrient enrichment can impact energy allocation manifested as changes in GSI, LSI and K (Allen et al. 1999; McMaster et al. 2005). However, other studies found fish exposed to MWWWE experienced no significant changes in energy allocation (Jobling et al. 1998; van Aerle et al. 2001; Hinck et al. 2009; Iwanowicz et al. 2009). Effects of nutrient-laden MWWWE additions to nutrient poor environments are more likely to be observed as changes in nutrient uptake, energy assimilation, and somatic distribution relative to those already saturated with nutrients (McMaster et al. 2005). Thus, the quality and degree of MWWWE dilution and nutrient status of each receiving environment are factors potentially masking influences of MWWWE discharges on fish health. Non-specific responses to multiple stressors, including MWWWEs, often involve diminished reproductive success and impacts on food utilization (Gibbons and Munkittrick 1994). In some MWWWEs, such as that released from the Kitchener MWWTP, nutrients in the form of very high levels of ammonia (up to 30 mg/L during “supernating” activities), are a further stressor on fish populations. Sludge lagoons at the Kitchener MWWTP collect municipal and septic tank sludge from adjacent municipalities not served by a MWWTP, as well as wastewater collected by private septic contractors. This additional wastewater contains high concentrations of ammonia that result in effluent ammonia concentrations as high as 27.68 mg/L (Grand River Conservation Authority, personal communication). Fish exposed to high concentrations of ammonia can experience chronic and acute toxicity via hypoxic anaemia as most freshwater fish species lack strategies to avoid ammonia toxicity (Randall and Tsui 2002). The low tolerances for ammonia in resident freshwater fish species, coupled with continual exposure to EDCs from two MWWTPs in the Grand River, presents a scenario allowing for the examination of cumulative stressors on energy allocation and fish reproductive physiology.

Although intersex has been documented in two species of forage fish in the central portion of the Grand River, the study evaluated a limited number of sites and a small sample size due to the labour intensive histological techniques. A recent method described by researchers in Japan (Lin et al. 2009) was

adapted in this study to allow rapid and sensitive assessment of both the extent (proportion) and severity (number of testis-ova) of intersex in fish. The objective of this thesis was to validate and apply this technique to better quantify the distribution of intersex in fish across the watershed, especially in association with several major municipal wastewater effluent outfalls. The specific objectives include:

- 1) Development and validation of a differential staining procedure for rapid quantification of intersex severity in small forage fish.
- 2) Evaluation of the extent and severity of intersex in forage fish species of Grand River in relation to an urban gradient, including municipal wastewater treatment plant outfalls (e.g. Kitchener, Waterloo).
- 3) Evaluation of changes in somatic indices (relative gonad size, liver size and condition) across the urban gradient and determine the potential relationship to severity of intersex.

Chapter 2:

Occurrence and severity of intersex (testis-ova) across an urban gradient in the Grand River, Ontario, Canada

It is anticipated that this chapter will be submitted as a manuscript to a leading environmental science journal.

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- Mark Servos: Co-supervisor to Rajiv Tanna and assisted with field work, ideas, research direction, editing, and general advice

Introduction

There have been many advances in the treatment and management of wastewater in our cities over the past few decades. By increasing the quality of treated municipal wastewater effluent (MWW), we have created an environment that can support healthy ecosystems, but one that remains exposed to a wide variety of contaminants. Many of these contaminants that can act as endocrine disruptors (EDCs) have recently been detected in treated sewage effluent, and their respective receiving environments throughout the world (Desbrow et al. 1998; Ternes et al. 1999b; Lee and Peart 2000; Servos et al. 2001a). Due to their high affinity for biological receptors, a variety of natural hormones, pharmaceuticals and industrial contaminants found in effluents can induce responses in aquatic organisms at very low concentrations (low nanogram per litre range) (Brian et al. 2005; Tyler et al. 1998).

The discovery of intersex condition in male Roach (*Rutilus rutilus*) associated with MWWs in the United Kingdom in the mid 1990s prompted studies which explored the extent and variability of the condition as well as potential implications on wild fish populations (Purdom et al. 1994; Jobling et al. 2002a). Although the prevalence of intersex in Roach has been found to be as high as 100% at some sites in the UK (Jobling et al. 1998) a smaller portion of these affected fish displayed severe intersex. Recent investigations of the impacts of MWW on fish in other parts of the world have generated further evidence of the widespread occurrence of this phenomenon. Notable examples indicating high rates of intersex (50% or greater) associated with MWW include Smallmouth Bass (*Micropterus dolomieu*) and Channel Catfish (*Ictalurus punctatus*) in the United States (Blazer et al. 2007; Hinck et al. 2009; Iwanowicz et al. 2009), Carp (*Cyprinus carpio*) in Spain (Sole et al. 2002; 2003), Barbell (*Barbus plebejus*) in Italy (Vigano et al. 2001), and White Perch (*Morone americana*) in Canada (Kavanagh et al. 2004).

Despite growing concern about the increasing rates of intersex in environments exposed to estrogenic chemicals via MWW, only minimal evidence regarding reproductive impairment has been

generated. Severely intersex fish show delayed maturation of reproductive tissue, decreased sperm density, as well as lower quality of sperm (impacted motility) (Jobling et al. 2002a; Vajda et al. 2008; Tetreault et al. 2011). While these fish do show impacted fertilization success, and correlations with other fish response parameters, the proportion of intersex fish displaying severe intersex is low (Jobling et al. 1998; Minier et al. 2000; Bjerregaard et al. 2006; Blazer et al. 2011). As a result, the likelihood of intersex condition directly leading to population level effects through recruitment failure is presumed to be low. However, this condition represents a useful tool to detect the exposure of fish to endocrine disrupting chemicals in the environment. Intersex may be an early indicator for other biological responses which may go otherwise unnoticed until dramatic population level changes occur. Experimental exposure of a whole lake ecosystem to environmentally relevant levels of 17 α -ethinylestradiol (EE2) by Kidd et al. (2007) sustained a Fathead Minnow (*Pimephales promelas*) population which displayed high levels of intersex (and vitellogenin induction) for three years prior to recruitment failure. Similar levels of EE2 exposure have been observed to adversely affect and even inhibit fish reproduction in laboratory settings (Länge et al. 2001; Nash et al. 2004).

The discovery of intersex condition in the Grand River by Tetreault et al. (2011) marked the first report of a high occurrence of intersex in a Canadian river system associated with MWWWE inputs. The Grand River watershed is influenced by both intensive agriculture and 30 MWWWE outfalls. Two of the major municipal wastewater treatment plants (MWWTPs) in the central reach of the watershed (Waterloo and Kitchener) serve a combined population of 352,135 individuals (Region of Waterloo 2011), and release 110,323 m³/day of treated effluent that is known to contain a variety of biologically active contaminants (Metcalf et al. 2003; Servos et al. 2005; Servos et al. 2007; Wang et al. 2011). Tetreault et al. (2011) showed increasing proportions of intersex in both Rainbow Darter (*Etheostoma caeruleum*) and Greenside Darter (*Etheostoma blennioides*) in the Grand River moving downstream with the proportion of testes with detectable oocytes being >65% below the Kitchener outfall. Unfortunately these studies

examined a limited number of sites and fish and did not quantify the severity of the intersex condition (e.g. number of oocytes per testes). A recent method proposed by Lin et al. (2009) using a simple testes fragmentation approach was modified and validated to allow rapid quantification of intersex incidence in Rainbow Darter and small forage fish. The primary objective of the present study was to determine the severity of intersex in a common sentinel species across the urbanized reach of the Grand River by applying a rapid testes fragmentation method.

Methods

Study Area

The Grand River watershed is the largest in southern Ontario (6,965 km²) draining southward into eastern Lake Erie (Cooke 2006). In addition to intensive crop and animal agriculture, there are a series of small treatment plants and septic systems that serve residents across the upper Grand River watershed. A series of dams and weirs are operated in the headwaters for flood control and to sustain flows throughout dryer summer months. With a number of tributaries draining into the main stem of the Grand River, the river transitions from a 6th order to 7th order stream over the study area. Two major secondary MWWTPs that are the focus of this study release effluent into the Grand River in this reach. The Waterloo MWWTP serves a population of more than 120,055 and the Kitchener MWWTP serves more than 190,000 individuals (Table 2). At the time of the study the Kitchener WWTP also received wastewater sludge from other local wastewater plants which is stored in supernating lagoons which contribute to high loads of ammonia in the final effluent. Both treatment plants are currently undergoing multi-million dollar upgrades to increase effluent quality.

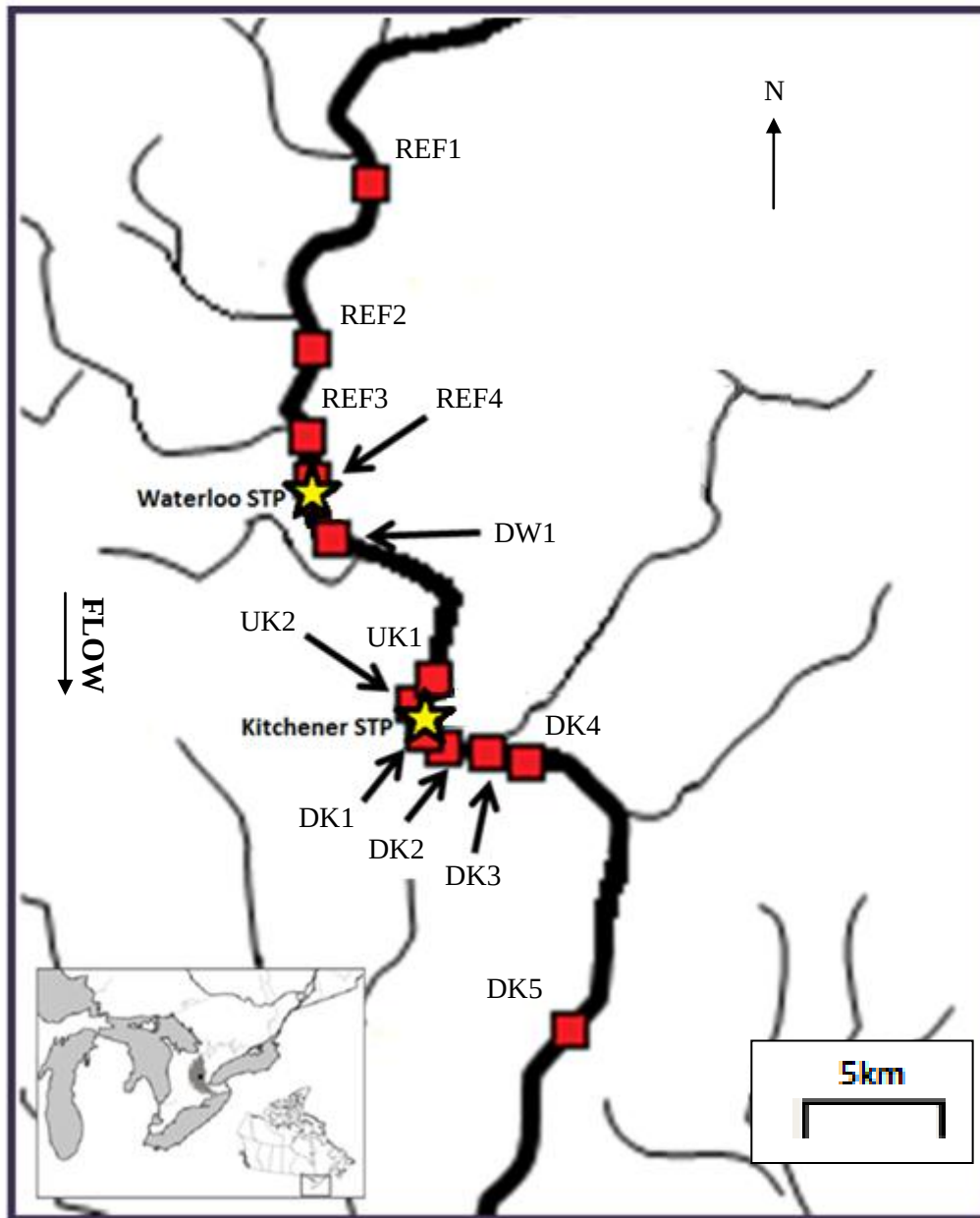


Figure 1: Map of the Grand River through the City of Waterloo and City of Kitchener, Ontario, Canada with sampling sites used in spring 2010 indicated.

Table 2: Description of the City of Kitchener and the City of Waterloo wastewater treatment plants in 2008 (Region of Waterloo, 2011)

Parameters	Kitchener	Waterloo
Population Served	190,000	120,055
Rated capacity m ³ /day	122,745	56,050
Discharge m ³ /day	77,768	23,802
Secondary Treatment	Conventional Activated Sludge	Conventional Activated Sludge
Combined Sewers	Some Foundation Drains	No
Disinfectant	Sodium Hyperchlorite *	Sodium Hyperchlorite
Other	Receives aerobic sludges in winter from other Regional plants, stored in lagoons	-

* High ammonia from supernating lagoons interferes with disinfection

Water Sampling

In order to characterize the level of exposure of MWWE on the areas sampled, a series of pharmaceuticals were quantified in surface water at each site using a method described by Wang et al. (2011a; 2011b). Four 500 mL amber glass bottles of surface water were collected at each sampling site at the time of fish collections. Each water sample was preserved with sodium azide, stabilized with ascorbic acid and spiked with deuterated standards. Samples were extracted with 500 mg Oasis HLB solid phase extraction (SPE) cartridges (Waters Corporation, Milford, MA, USA). Cartridges were preconditioned with 5 mL each of MTBE methanol and water. Elution was completed with 5 mL of methanol followed by 5 mL of 90:10 ratio of MTBE to methanol. Samples were evaporated to dryness and reconstituted in methanol with internal standards. Separation was completed using an Agilent 1200 liquid chromatograph (LC) with a gradient of methanol/water at a flow rate of 0.8 mL/min. Two solvents were used for the mobile phase. Mobile Phase A (water + 5 mM ammonium acetate) and Mobile Phase B (methanol). Detection was completed on an AB Sciex3200 Qtrap mass spectrometer (MS/MS) using electrospray ionization in both positive and negative modes.

Fish Sampling

Rainbow Darter was selected for this study as the species is common in streams and rivers of southern Ontario. Preliminary field studies identified Rainbow Darters as abundant and spatially distributed in riffle habitats across selected sites in the Grand River watershed. Rainbow Darter is a benthivorous fish with strong site fidelity (Loomer 2008; Tetreault et al. 2011) making it a good sentinel species. Fifteen sites sampled in early spring, April-May, 2010 (Table 3) were chosen based on distance relative to MWWE outfall, wadability and comparability with respect to habitat (Tetreault et al. 2012; Brown 2010).

Starting at the downstream reach of each site, the individual operating the backpack electroshocker (Smith-Root Model 12) and two netters moved to each side covering all accessible areas in transects gradually moving upstream. All fish that could be caught were removed using dip nets (approx 0.5 cm mesh size). A minimum of 15 female and 20 male fish was targeted (although this was not achieved at all sites) for sampling of gonad and liver tissue. Fish were taken to an adjacent on-site laboratory and remained in aerated buckets until they were sampled. Fish were sacrificed by severance of the spinal cord according to a protocol approved by the University of Waterloo's Animal Care Committee (AUP 08-08). Fish fork length was measured to the nearest millimeter and body weight was measured to the nearest tenth of a gram. Gonad and liver samples were weighed using an electronic balance (Sartorius TE153S) to the nearest milligram and preserved in Davidson's solution and liquid nitrogen respectively. Weights less than 5 mg were excluded from analysis as the balance produced inconsistent values over repeated measurements of the same sample below this value. Condition [k = (body weight/length³)*100], gonadosomatic index [GSI = (gonad weight/body weight)*100], and liversomatic index [LSI = (liver weight/body weight)*100] were calculated to measure fish responses across sites.

Intersex

In order to determine the severity of intersex of individual fish accurately and efficiently, a histological protocol was developed based on Lin et al. (2009) to allow quantification of the total number of primary oocytes within each testis. One lobe of the testes from each male fish was subject to this process following fixation in Davidson's solution. Testis samples were placed into 1.5 mL graduated microcentrifuge tubes, to which a Methylene Blue based differential stain solution was added to submerge the sample. The stain solution was produced by dissolving 5.5 mg of Methylene Blue into 1 mL 70% ethanol and 7.5 mL of glycerine, then adding 15 mL of deionized water. The sample was homogenized with a Teflon drill bit to allow for consistent and even exposure to the stain. The microcentrifuge tube was then spun in a pulse centrifuge (VWR Scientific Products GALAXY MINI C1213) for 30 seconds to

Table 3: Co-ordinates for spring 2010 sampling sites on the Grand River.

Site	Symbol	Latitude (N)	Longitude (W)
Reference 1	REF1	43° 38' 9"	80° 26' 24"
Reference 2	REF2	43° 30' 19"	80° 28' 28"
Reference 3	REF3	43° 32' 3"	80° 28' 50"
Reference 4	REF4	43° 30' 19"	80° 28' 26"
Waterloo MWWWE outfall	WMWWWE	43° 28' 47"	80° 28' 55"
Downstream Waterloo 1	DW1	43° 28' 28"	80° 28' 28"
Upstream Kitchener 1	UK1	43° 24' 29"	80° 25' 30"
Upstream Kitchener 2	UK2	43° 24' 8"	80° 25' 50"
Kitchener MWWWE outfall	KMWWWE	43° 24' 5"	80° 25' 18"
Downstream Kitchener 1	DK1	43° 23' 51"	80° 24' 55"
Downstream Kitchener 2	DK2	43° 23' 38"	80° 24' 47"
Downstream Kitchener 3	DK3	43° 23' 14"	80° 23' 13"
Downstream Kitchener 4	DK4	43° 23' 7"	80° 21' 46"
Downstream Kitchener 5	DK5	43° 16' 35"	80° 20' 51"

draw down any sample spread over the inner surface of the tube. After 5 minutes of incubation at room temperature, a volume of glycerol equal to the amount of stain solution was added. The resulting solution was then mounted onto a glass microscope slide using a Pasteur pipette, and sealed using a glass cover slip and mounting medium. The development of the differential stain and processing procedure allows for the rapid enumeration of testis-ova in a consistent and cost effective manner. The resulting slides carrying tissue “smears” were scanned under a microscope in serial transects to enumerate the number of primary oocytes in each sample thereby quantifying the severity of gonadal disruption in individual fish (per testis lobe) (Figure 2).

In order to validate the results produced using the modified fragmented testes method (smear method), gonad halves (lobes) from Rainbow Darters at a selected site were processed using both traditional histology and the developed smear method. Samples selected for traditional histological analysis were sectioned using a microtome at a 5 μ m thickness, stained using hematoxylin and eosin (Luna 1992), and cover slipped using mounting medium (Figure 3). Mounting multiple serial sections per slide produced between 8 and 44 slides for an entire sample (single testis lobe). Since a primary oocyte is larger in diameter than the section widths produced from microtoming, a single oocyte may appear in multiple sections. In order to produce reliable values for total oocyte counts for comparison across methods, each identifiable oocyte was counted in each section produced. As a result, a single oocyte may have reappeared across multiple sections using traditional histology methods. Thus, count data generated from the traditional method were log transformed prior to comparison with the smear method to account for overestimation through multiple recounting of a single oocyte.

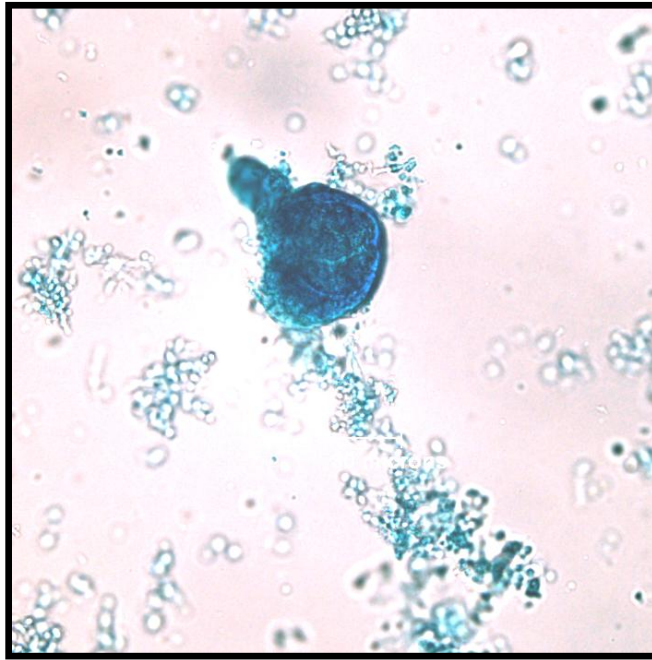


Figure 2: Sample prepared using the smear protocol, demonstrating differential staining of testis-ova found in male Rainbow Darter.

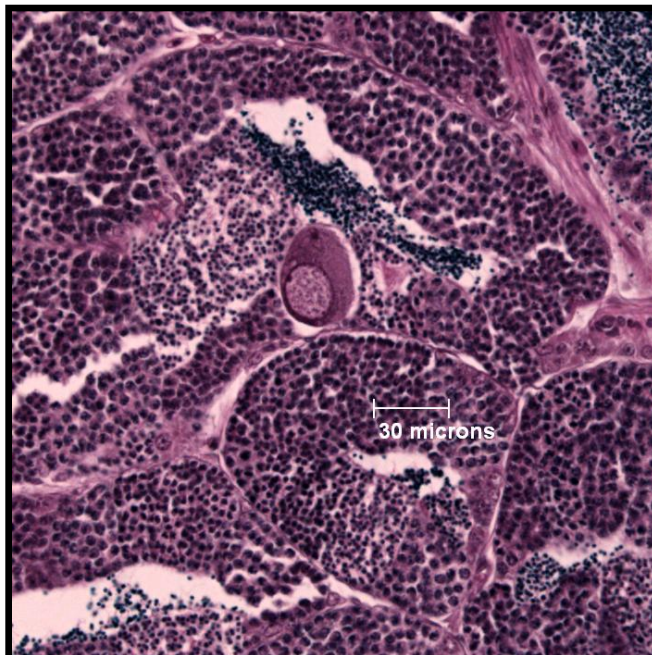


Figure 3: Sample prepared using standard histology procedures showing testis-ova found in male Rainbow Darter.

Analysis

Statistical comparisons were made among sites between sexes. Regression analysis was performed on the log length and log weight of each data set (e.g. species) to identify outliers and transcription errors. Liversomatic index was tested using an ANOVA and Tukey's post hoc test to identify individual site differences. Differences in condition factor, gonadosomatic index, and intersex severity were tested using nonparametric Kruskal-Wallis tests, as the datasets failed the assumption of equal variance. Intersex incidence rates were compared between sites using Fisher's Exact probability. Relationships between severe intersex and other fish diagnostics were tested using Pearson's product-moment correlation. Due to the low number of severe intersex fish (>100 oocytes per testis lobe), moderate intersex fish (>10 oocytes per testis lobe) were included in this relational analysis. Differences in all data analyses were determined using SPSS 19.0 statistical software (IBM SPSS 2010).

Results

Water Quality

Several contaminants representative of those associated with MWWEs impacted basins were dramatically elevated in river water below the MWWWE discharges, while they were at very low levels (or below detection limits) at the upstream sites. All analytes examined, with the exception of atrazine and fluoxetine, were quantified at elevated concentrations downstream of the Waterloo MWWWE outfall relative to upstream locations (Figure 4). These spikes in analyte concentrations associated with the Waterloo MWWWE were rapidly attenuated with downstream distance (UK1, UK2), before again increasing (except atrazine) with the discharge from the Kitchener MWWWE outfall.

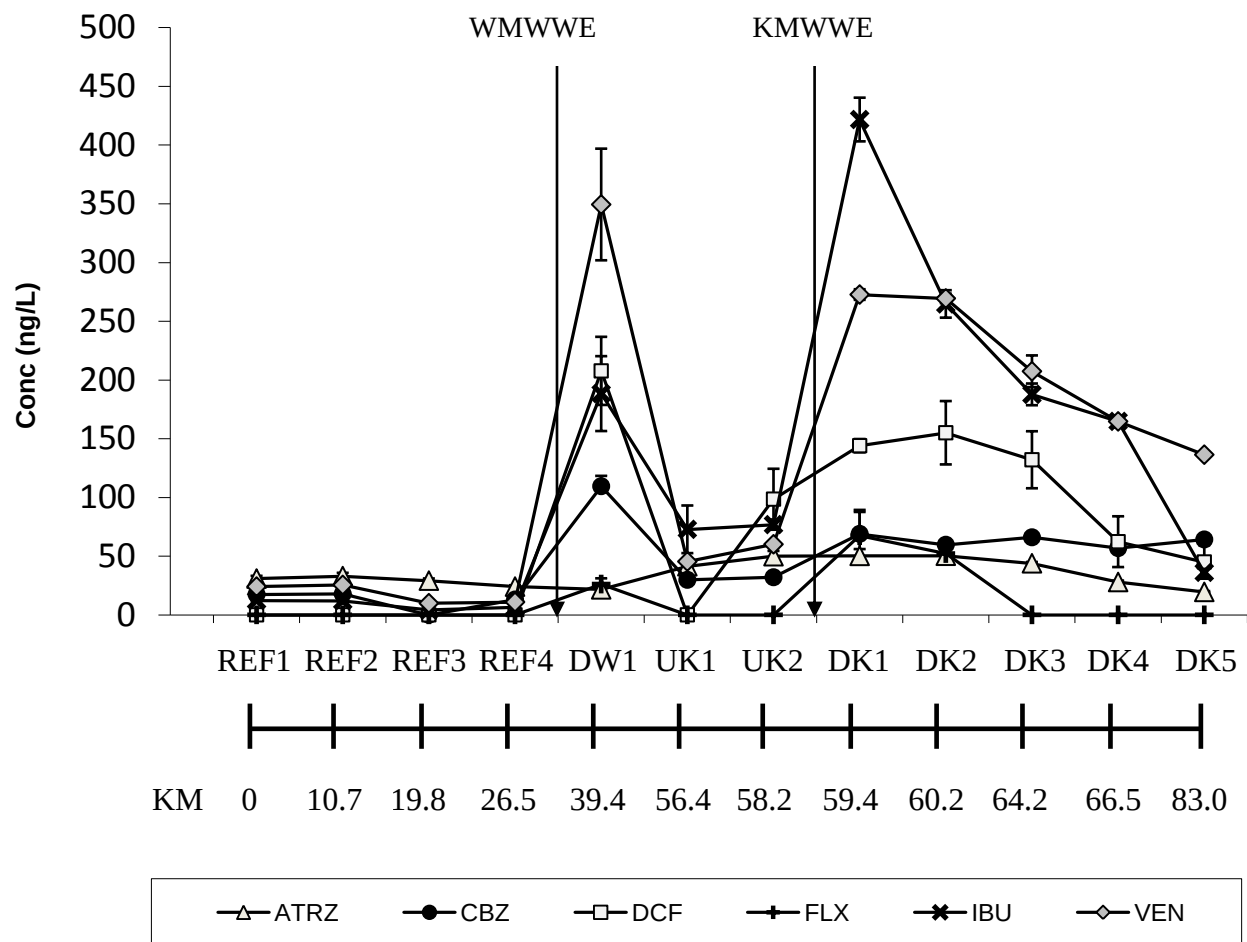


Figure 4: Concentration profile of selected contaminants in water samples collected in spring 2010 on the Grand River. ATRZ = Atrazine, CBZ = Carbamazepine, DCF = Diclofenac, FLX = Fluoxetine, IBU = Ibuprofen, and VEN = Venlafaxine. Values are means $\pm$ SE (n=4).

Intersex

Smear Method Validation

Primary oocyte counts generated from paired gonad lobes separated for analysis using both traditional histology and the smear method were compared (Figure 5), yielding a statistically significant correlation of $R^2 = 0.911$ between both methods ($P < 0.001$). This relationship was not 1:1, with traditional histology sectioning giving slightly higher values most likely as a result of counting eggs repeatedly that were larger than the sectioning width of 5 μm (Figure 5).

Intersex Severity

Male Rainbow Darter testis lobes from fish collected upstream and downstream of the MWWTP outfalls were processed for the presence and severity of oocytes (Figure 6). Intersex severity was very low (< 1 oocyte/lobe) and did not differ among reference sites (REF1, REF2, REF3, REF4) ($P \geq 0.310$). Rainbow Darter near the Waterloo MWW outfall (DW1) did not differ from the collective upstream reference sites in intersex severity ($P \geq 0.287$), nor did stations further downstream (UK1 and UK2; $P \geq 0.287$). However, there were some sites below the Waterloo outfall which differed in the degree of intersex from some reference sites, including UK1, which displayed higher intersex severity than REF4 ($P = 0.018$), while site UK2 was greater than all reference sites except REF3 ($P \leq 0.036$). Fish collected below the Kitchener MWW outfall (DK1) expressed the highest severity of intersex relative to all upstream sites ($P \leq 0.001$), and other downstream sites ($P \leq 0.040$). Sites further downstream of the Kitchener MWW showed a gradual decrease in intersex severity, with site DK4 showing no statistical differences from all reference sites ($P \geq 0.186$).

Intersex Presence

Intersex presence expressed as a proportion of male Rainbow Darter testis lobes carrying oocytes to those without was determined for each site (Figure 6). No differences in intersex presence were found in fish among reference sites (REF1, REF2, REF3, REF4) ($P \geq 0.423$) while fish from sites DK1, DK2 and DK3 at and below the Kitchener MWWWE outfall exhibited higher proportions of intersex than all reference sites ($P \leq 0.001$). Proportions of intersex closer to the MWWWE outfall (DK1 and DK2) were higher than those sites (DK4 and DK5) further downstream ($P \leq 0.0237$). Site DK4 was not different from all reference sites ($P \geq 0.240$), and site DK5 was not different from REF1, REF2 and REF3 ($P \geq 0.094$). While the proportion of male Rainbow Darter with intersex was higher below the Waterloo MWWWE outfall (DW1) relative to fish from reference sites, DW1 was not statistically different from any reference site ($P \geq 0.057$). Downstream of DW1, site UK1 was statistically similar to all reference sites except REF4 ($P = 0.026$), and UK2 was significantly greater than all reference sites except for REF2 ($P \leq 0.047$).

Somatic Indices

Female Rainbow Darter collected from one site downstream of the Kitchener MWWWE outfall (DK2), had statistically smaller ovaries (Figure 7) compared to fish collected from all reference and upstream sites ($P \leq 0.035$) with the exception of site REF2 ($P = 0.096$). Relative gonad sizes in females directly exposed at DK1 were relatively larger than those collected at REF1 and REF2 ($P \leq 0.010$), but were not different from REF3 and REF4 ($P \geq 0.191$). Relative gonad sizes in females directly exposed to Waterloo MWWWE (DW1) were not different from samples collected at reference sites ($P \geq 0.102$). Female Rainbow Darter from site UK1 (downstream of the Waterloo MWWWE outfall, but upstream of the Kitchener discharge) showed increased relative ovary size compared to directly exposed fish ($P \leq 0.015$) and reference sites REF1 and REF2 ($P \geq 0.001$). Female fish from site UK2 also had relatively larger gonads, but their size did not differ from those collected from DW1 ($P = 0.111$) nor any reference sites ($P \geq 0.052$) except for REF2 ($P = 0.013$). Fish collected from the far downstream sites DK4 and DK5 below

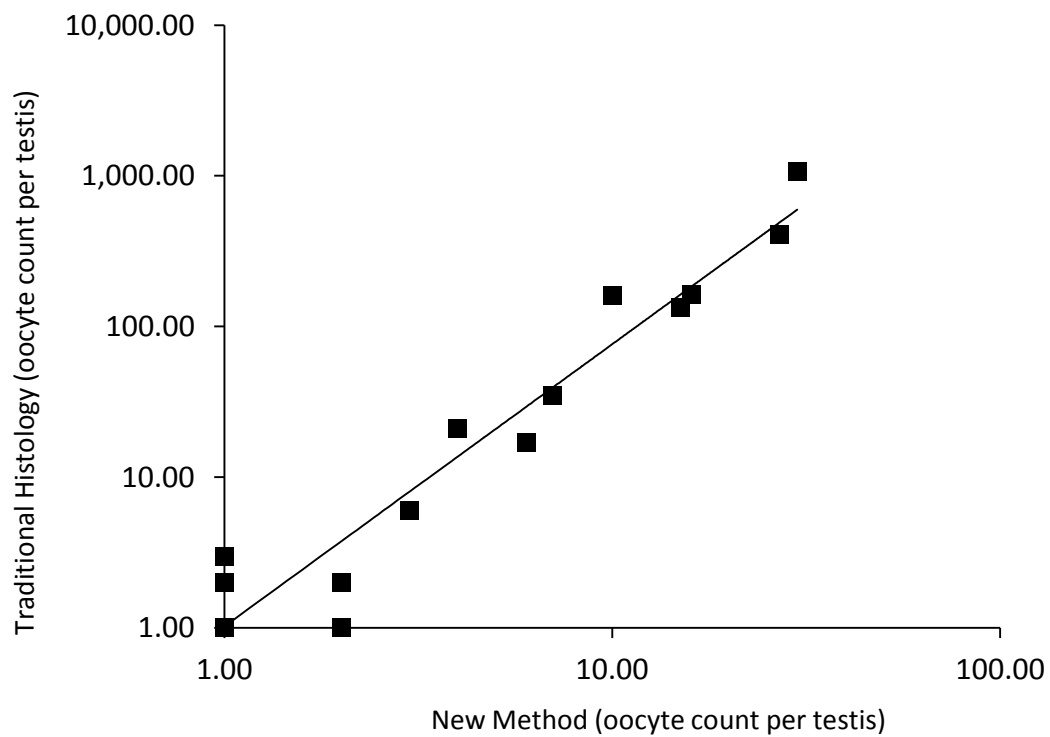


Figure 5: Paired samples correlation comparison of oocyte count values generated from traditional histology and smear method in paired gonad samples (lobes) at site DK3 ($n=19$, Pearson's correlation coefficient $R^2= 0.911$, $P<0.001$).

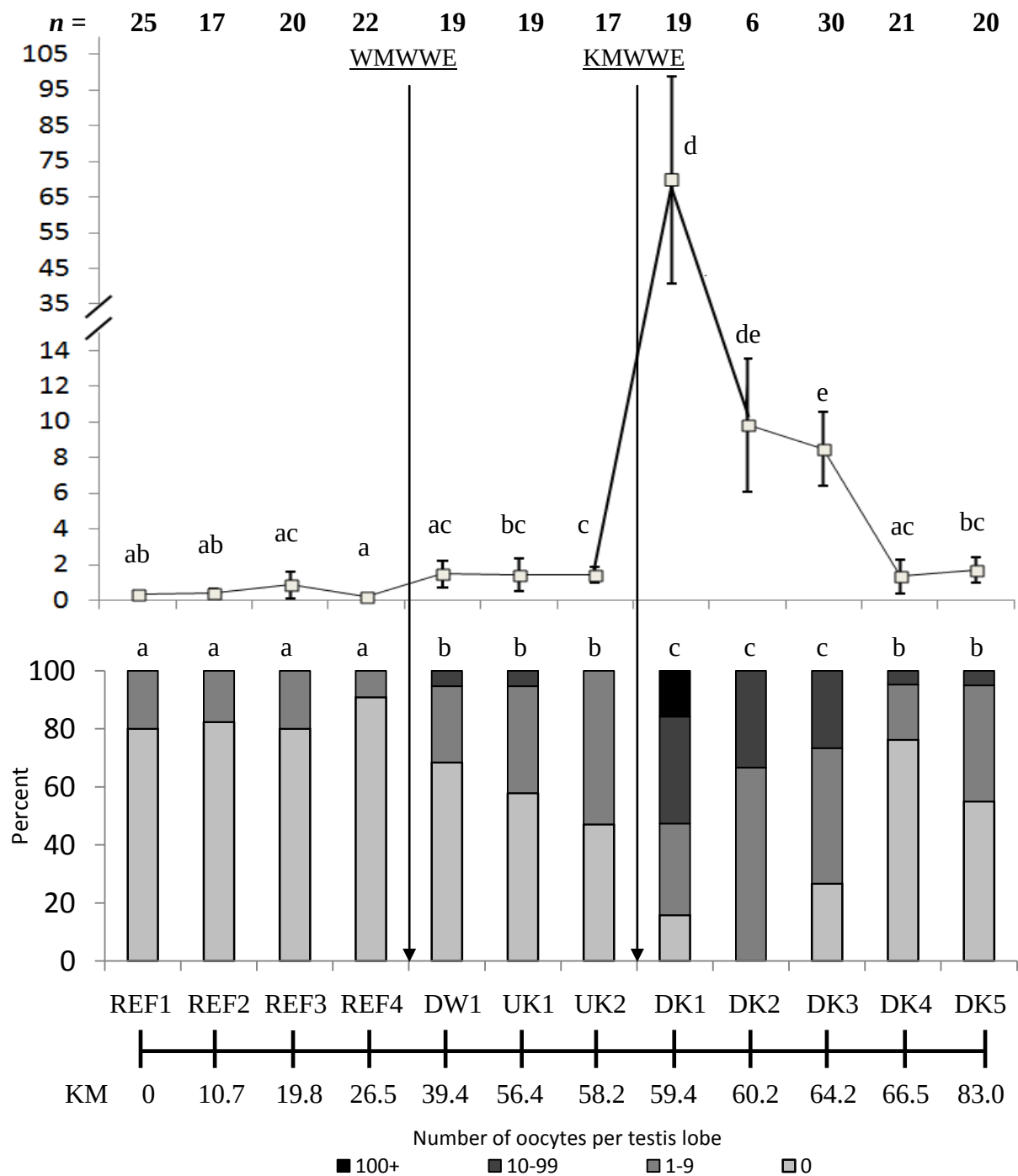


Figure 6: Oocyte occurrence (mean±SE) and proportional intersex severity in male Rainbow Darter collected from the Grand River in spring 2010 adjacent the Waterloo and Kitchener MWWWE outfalls (indicated by arrows). Lettering indicates significant differences determined using Mann-Whitney-U test. Distance from farthest upstream site indicated below sites.

the Kitchener wastewater outfall demonstrated increased ovary sizes relative to fish from DK2, with values not different from those of the reference sites ($P \geq 0.051$).

Relative liver sizes in female Rainbow Darter were lowest at site DK2 (downstream of the site directly exposed to Kitchener MWW, DK1), and significantly lower than those in fish collected from REF1 and REF2 ($P \leq 0.002$), upstream sites UK1, UK2 and directly exposed DK1 ($P \leq 0.001$). There were no statistical differences in liver size between fish directly exposed to Waterloo MWW and any other site sampled ($P \geq 0.303$), nor were there any differences between reference sites ($P \geq 0.510$). Relative liver size at far downstream sites DK3, DK4 and DK5 gradually increased and returned to values not statistically different from any sites upstream of and including DK1 ($P \geq 0.582$).

Female Rainbow Darter directly exposed to Kitchener MWW (DK1) had the highest condition (heaviest relative to length) of all sites sampled, and were statistically greater than those of fish collected from REF2 and REF4 ($P \leq 0.046$). No significant differences were observed in condition between females exposed to Waterloo MWW (DW1) and all reference sites ($P \geq 0.312$). Downstream of Kitchener MWW outfall at DK2, condition decreases significantly relative to DK1 ($P = 0.003$), and shows a gradual increase over far field sites (DK2, DK3, DK4, DK5) whereas large fluctuations are observed at upstream sites.

Comparisons of relative gonad size in male Rainbow Darter at exposed sites to reference sites (Figure 8) showed a statistical increase at the Waterloo MWW outfall (DW1) relative to only 1 reference site (REF3) ($P = 0.008$). Testes from fish at reference site REF3 were lower than downstream REF4 ($P = 0.019$), but REF4 and exposed site DW1 did not differ ($P = 1.000$). Rainbow darter testes from the far downstream site (DK4) were smaller than those from the exposed site DW1 ($P = 0.025$). No other statistically significant between site differences in relative gonad size among male Rainbow Darters were observed.

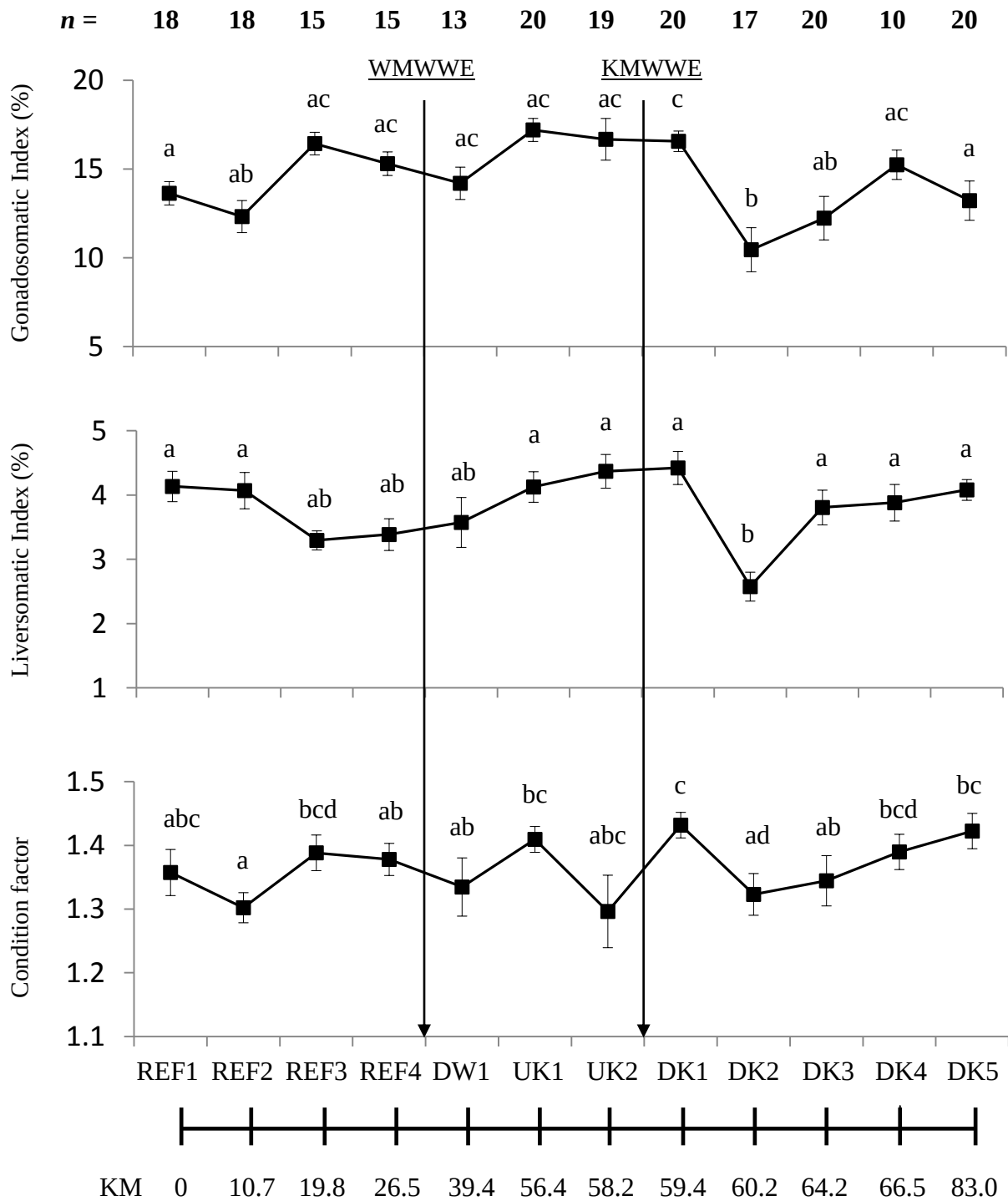


Figure 7: Gonadosomatic index (GSI), livensomatic index (LSI) and condition factor (mean $\pm$ SE) for female Rainbow Darter collected from the Grand River in Spring 2010. Arrows indicate the Waterloo and Kitchener MWWWE outfalls, respectively. Lettering indicates significant differences.

Relative liver sizes in male Rainbow Darter gradually increased with downstream conditions, with larger livers at the Waterloo MWWWE outfall (DW1) relative to those from fish at REF1, REF2 and REF3 ($P \leq 0.032$). Immediately downstream of DW1, liver size decreased significantly at UK1 ($P = 0.005$). No significant differences were found between remaining downstream sites except for between DK1 and DK4 ($P = 0.033$) as well as DK3 and DK4 ($P = 0.003$). Site DK2 showed the greatest variability in both relative liver size and relative gonad size in male Rainbow Darter compared to other sampled sites, however this can be attributed to the small sample size. Condition in male Rainbow Darter was highest at sites immediately downstream of Waterloo (DW1) and Kitchener (DK1) MWWWE outfalls. Both DW1 and DK1 had significantly greater condition than reference sites REF2, REF3 and REF4 ($P \leq 0.001$), as well as far downstream sites DK2, DK3, and DK4 ($P \leq 0.001$). Condition at DK1 was also significantly greater than upstream UK2. Reference sites REF2, REF3 and REF4, upstream site UK2, and far downstream sites DK2, DK3 and DK4 showed no statistical differences in condition. First reference site REF1 and furthest downstream site DK5 were also statistically similar.

Intersex vs. Fish Indices of Somatic Health

Relationships between increased intersex severity and fish morphometrics and indices of somatic and gonadal health were tested in male Rainbow Darter at the Kitchener MWWWE outfall (DK1). A weak but significant positive correlation ($R^2 = 0.634$) was found in male Rainbow Darter between intersex severity and fish length (Figure 9; $P = 0.049$). A significant negative correlation ($R^2 = -0.815$) was found in male Rainbow Darter between intersex severity and gonadosomatic index (Figure 10; $P = 0.004$).

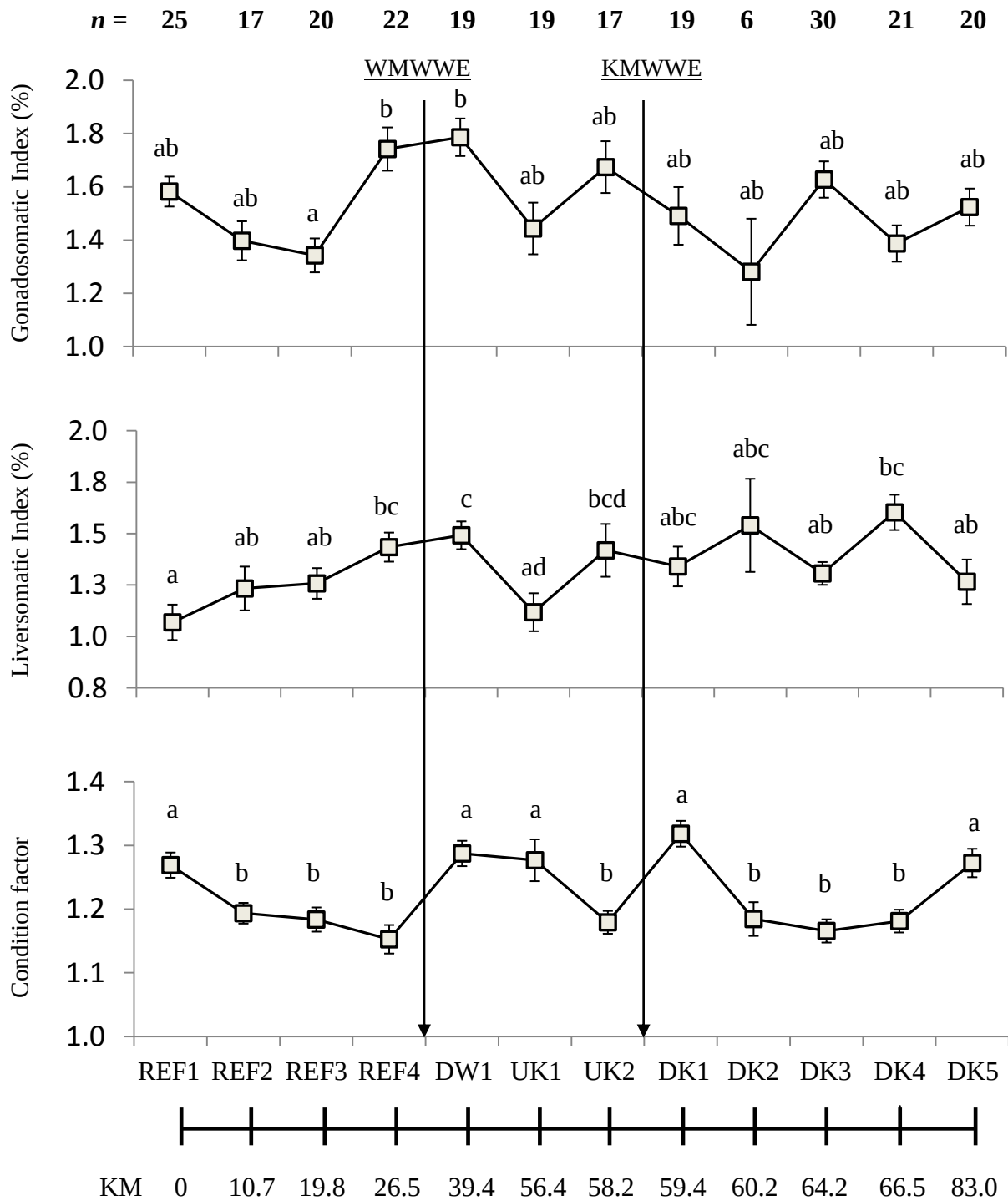


Figure 8: GSI, LSI and condition factor (mean±SE) for male Rainbow Darter on the Grand River in spring 2010. Arrows indicate the Waterloo and Kitchener MWWWE outfalls, respectively. Lettering indicates significant differences.

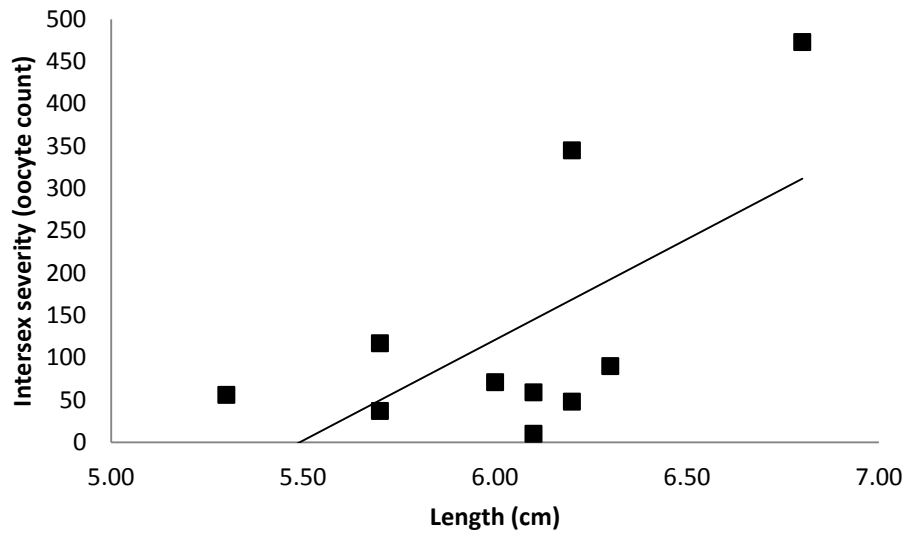


Figure 9: Comparison of intersex severity and male Rainbow Darter length in fish expressing moderate to severe intersex at a station (DK1) immediately downstream of the Kitchener MWWWE outfall ($n=10$, $R^2=0.634$, $P=0.049$).

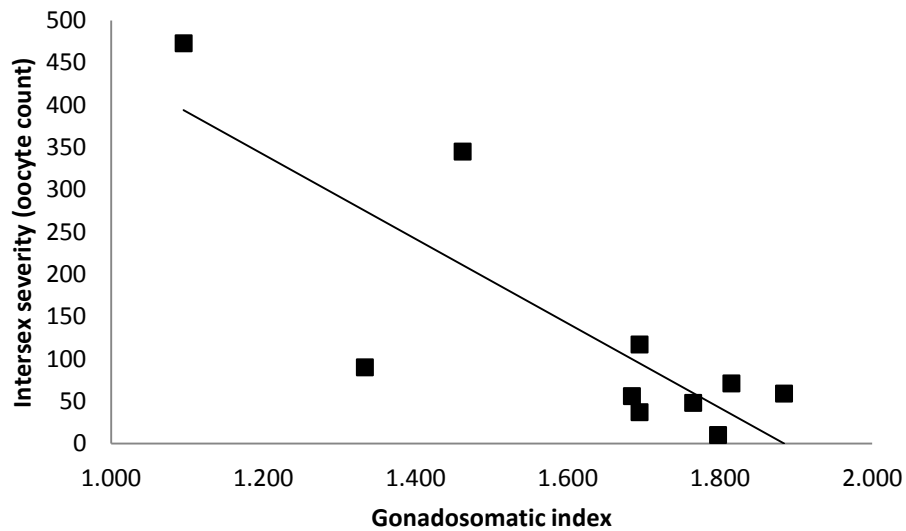


Figure 10: Intersex severity and gonadosomatic index in male Rainbow Darter expressing moderate to severe intersex at a station (DK1) immediately downstream of the Kitchener MWWWE outfall ($n=10$, $R^2 = -0.815$, $P=0.004$).

Discussion

This study is the first to demonstrate both the presence and severity of intersex in fish associated with Canadian municipal wastewater effluents across an urbanized watershed. A new “fragmented testis” quantitative evaluation technique for testis-ova described by Lin et al. (2009) was adapted and validated in these studies to allow for larger sample sizes and rapid assessment. This resulting “smear” method compared well with traditional histological techniques. The fish collected upstream in reference sites showed very low incidence of intersex, despite being influenced by the assimilation of several smaller MWWTPs and high agricultural density. A slight increase in the presence and severity of intersex was observed in male Rainbow Darters downstream of the Waterloo treatment plant outfall, which persisted at the sites (up to 18 km) further downstream of the outfall. Rainbow Darters collected downstream of a second wastewater outfall (Kitchener STP; DK1) showed a significant increase in proportion of males exhibiting intersex as well as the severity relative to background levels and the sites immediately upstream. Intersex severity as quantified by the smear method, was 70 times greater in male Rainbow Darter immediately below (DK1) the Kitchener MWWWE outfall relative to the fish immediately upstream (UK2). Proportion of intersex in these exposed Rainbow Darter populations reached 85% (16 of 19) immediately downstream of the outfall (DK1) and increased to 100% (6 of 6) at a site further downstream (DK2). However, intersex occurrence returned to background levels observed at reference sites (REF1-4) further downstream (DK4,5). The lower sample size at DK2 reflects the difficulty in collecting male fish at this site which suggests a shift in sex ratio due to exposure to MWWWE. A shift in sex ratio has been observed in fish exposed to wastewater effluents in the UK (Filby et al. 2007).

While intersex gonads may be a natural occurrence in gonadal development in some species of fish, the presence of testis-ova is not considered normal for a gonochoristic fish species like the Rainbow Darter (Yamazaki 1983). Because intersex gonads are not a natural feature in gonochoristic fish species, their presence in sexually mature darters indicates the evidence of bipotential germ cells in the gonad, as

well as exposure to EDCs (Vine et al. 2005; Metcalfe et al. 2010). As a differentiated gonochoristic species (Atz 1964) the gonads in Rainbow Darter develop from indifferent gonad tissue directly into ovaries or testes. Locally high prevalence of intersex associated with municipal wastewater outfalls have been reported in many fish species such as Roach (*Rutilus rutilus*, 100%, Jobling et al. 1998; 2002a), Smallmouth Bass (*Micropterus dolomieu*, 100%, Hinck et al. 2009), Largemouth Bass (*Micropterus salmoides*, 91%, Hinck et al. 2009), and White Perch (*Morone Americana*, 83%, Kavanagh et al. 2004). A study completed by Tetreault et al. (2011) on the Grand River showed prevalence of intersex in Rainbow Darter as high as 75% immediately downstream of the Kitchener MWWWE outfall in the fall of 2007 while the prevalence was 100% in spring collections.

Changes in gonadosomatic index (GSI) and liversomatic index (LSI) indicate that changes at the whole organism level were also associated with exposure to municipal wastewater outfalls in this watershed. The Waterloo outfall has minimal effect on either GSI or LSI in Rainbow Darter but there were distinct differences below the Kitchener outfall. Female Rainbow Darter downstream of the Kitchener outfall (DK2) showed significant decreases in both GSI and LSI and recovered to upstream values further downstream (i.e. 4 km). Male Rainbow Darter GSI and LSI data did not show significant change coinciding with MWWWE outfalls. While condition did increase downstream of each treatment plant relative to unexposed sites in male Rainbow Darter, there is considerable variability among sites. Female Rainbow Darter condition was also greatest downstream of the Kitchener MWWWE outfall. Similar changes have been noted in this population of fish in previous years (Tetreault et al. 2011) with a reduction in GSI and increased condition in both male and female Rainbow Darter exposed to Kitchener MWWWE. However the study of Tetreault et al. (2011) also noted increases in LSI likely reflecting the nutrient enrichment afforded by MWWWE, which may manifest as increased glycogen production due to surplus energy and larger liver size (Kilgour et al. 2005). The reductions in liver size (LSI) observed in the current study suggests that there were differences among years in energy allocation or toxicity

associated with the Kitchener effluent. Exposure to multiple stressors impacting energy storage and limiting available energy can be manifested as reduced LSI and reduced GSI respectively (Gibbons and Munkittrick 1994). Ings et al. (2011) also observed a stress response and increased energy demand in Rainbow Trout exposed to tertiary treated wastewater effluents in the Grand River watershed (i.e. Guelph). Differences in effluent quality, dilution or habitat may also influence annual responses in exposed fish populations.

Differences in responses downstream of the Waterloo and Kitchener MWWTP outfall can be attributed to differences in removal efficiency of EDCs in influent entering the MWWTP, as well as dilution of the resulting effluent. The Waterloo MWWTP serves a population of 120,055 individuals, has a rated capacity of 56,050 m³/day, and an average daily flow of 45 994 m³/day. In comparison, the Kitchener MWWTP serves a population of 190,000 individuals (58% greater than Waterloo), and has a rated capacity of 122,745 m³/day and sees an average daily flow of 64,329 m³/day (Region of Waterloo 2011). Neither the Waterloo nor Kitchener MWWTP nitrify the effluent in its treatment process which results in high ammonia concentrations in the final effluents. The PPCPs quantified as markers of exposure in this study downstream of the Waterloo MWWTP outfall did show either greater or comparable values to those downstream of the Kitchener MWWTP outfall despite an average discharge from the Waterloo MWWTP that is 70% lower than Kitchener's MWWTP (23,802 and 77,768 m³/day respectively). Samples obtained for the present study below the Waterloo outfall were collected on the same side as the effluent discharge pipe, which is located on the left side of the river facing upstream. Minimal mixing of the effluent below the Waterloo outfall across the river immediately downstream of the outfall (DW1), could have contributed to greater values in PPCPs despite greater assumed dilution. In comparison, the effluent discharge pipe at Kitchener is submerged at the middle of the river and allows for greater mixing of the plume across the river at the site immediately downstream of the outfall (DK1). However, the effluent below the Kitchener treatment plant has also been shown to not fully mix for

several km downstream (Loomer 2008). The dilution capacity of the Grand River downstream of both treatment plants was greatly diminished in the months prior and during sampling conducted in spring 2010, due to an unusually low snow melt that typically occurs in March (Region of Waterloo 2011). Because of decreased snow fall over the preceding winter of 2009-2010, relatively higher proportions of effluent in the Grand River can potentially be attributed to the increased proportion of intersex and greater biological responses in fish relative to the spring of 2009 (Tetreault et al. 2011). Despite lower dilution afforded by the environment during the spring through diminished snow melt, this short period may or may not have been sufficient to generate an increase in a latent response such as intersex. Increases seen in PPCPs downstream of the Waterloo and Kitchener MWWWE outfalls show gradual decreases in concentration further downstream. These decreases could have resulted from further dilution, as well as the degradation of these pharmaceuticals. In comparison, the concentration profile for atrazine (a herbicide used in agriculture) remains relatively stable across sites sampled. Atrazine, having a half-life in water of several months (Comber 1999) serves as a “reference” contaminant in this study as it isn’t introduced into the environment via MWWWE discharges.

While a reduction in dilution capacity afforded by the flow of the Grand River may be a contributing factor in the responses observed, the Speed River, a tributary of the Grand River, is further limited in its dilution capacity. The Guelph MWWTP serving the City of Guelph is a tertiary treatment plant that discharges an average of 49,124 m³/day into the relatively small Speed River. Many PPCPs quantified downstream of the Guelph MWWWE outfall were found at levels comparable to unexposed sites in the Grand River, and no significant changes in biological responses were observed in both male and female Rainbow Darter. Although some intersex was observed in males directly exposed to the effluent outfall at Guelph (Appendix A), proportion (30%) and severity (0.68 ± 0.31 oocytes/lobe) were limited (Appendix B). The higher solids retention time (extended activated sludge) and tertiary treatment at Guelph are possible factors in the increased removal efficiency of PPCPs and potentially EDCs causing

the impacts on Rainbow Darters in the Grand River (Servos et al. 2005). Where the hydraulic retention time for the Waterloo and Kitchener MWWTPs are a few days, the retention time at the Guelph MWWTP is anywhere from 15 to 28 days (City of Guelph Wastewater Treatment Plant 2007). In addition, denitrification and phosphorous removal through sand filtration (City of Guelph Wastewater Treatment Plant 2007) further contribute to higher effluent quality by reducing effluent ammonia and phosphorous concentrations. The optimization of the treatment processes at the Guelph MWWTP (Wheeler et al. 2010) is largely responsible for the increased quality of effluent and minimal impact on fish communities in the Speed River (Brown et al. 2011).

An additional factor potentially impacting fish health downstream of the Kitchener MWWTE is high concentrations of ammonia. The Kitchener wastewater plant does not nitrify or denitrify and ammonia concentration in the effluent routinely exceeds 20 mg/L (Grand River Conservation Authority, personal communication). Fluctuations of ammonia can result in metabolic stress in fish as well as chronic and acute toxicity (Randall and Tsui 2002). In addition, nitrification and high primary productivity in the river result in regular oxygen depletion downstream at Blair Landing (DK3), resulting in oxygen levels often being well below 4 mg/L during the early morning throughout summer months (Cooke 2006). The combination of complex MWWTE containing many potential endocrine disrupting substances, high ammonia or nitrate (further downstream) as well as low oxygen presents a scenario with multiple stressors acting through a variety of mechanisms. Changing flow conditions between years can result in differing exposure scenarios during critical life-stages, altering biochemical and/or whole organism responses.

While other studies have shown an increase in intersex proportion and have quantified severity through testis-ova size and average count data across a sample size of tissue sections (Jobling et al. 1998; Minier et al. 2000; Bjerregaard et al. 2006; Blazer et al. 2007), this study utilizes a method which allows rapid quantification of testis-ova in an entire tissue sample of a small fish. By utilizing an entire tissue

sample (lobe), the likelihood of missing an occurrence of testis-ova by processing a small subset of cross sections is reduced/eliminated, increasing accuracy. This is especially true and likely where testis-ova occur in clusters and are not evenly distributed throughout tissue, and could be missed. Clustering of oocytes is acknowledged and accounted for in some studies using an incremental increase in the scale of severity (Jobling et al. 1998; Minier et al. 2000; Blazer et al. 2011). Other studies have found it suitable to use a severity scale based on average oocytes counts across tissue sections alone (Bjerregaard et al. 2006). Comparison of the smear method to traditional histology techniques within sampled fish showed highly comparable results, and is thus a reliable method for quantifying severity in a rapid cost effective manner. Studies quantifying intersex severity through development of a severity index have shown a significant association between MWWWE exposure and severity (Blazer et al. 2011) as well as with increasing agricultural density (Blazer et al. 2011). A significant increase in intersex severity using the smear method was seen downstream of each the Waterloo (DW1) and Kitchener MWWWE outfall, with much more severe intersex seen downstream of the Kitchener MWWWE (DK1). Corresponding to decreasing values for PPCPs selected as markers of exposure, intersex severity and proportions decreased at far downstream sites indicating possible attenuated response and or recovery.

The use of actual count data of testis-ova in quantifying disruption is particularly useful in developing relationships between intersex severity and other biomarkers of response. Decreases in gonadosomatic index downstream of MWWWE outfalls noted in the present study have been associated with intersex severity and other impacts on maturation in other studies. In a study by Jobling et al. (2002a), an inverse relationship between more severe intersex and relative gonad size was observed in Roach. Blazer et al. (2011) showed a similar inverse relationship between intersex severity and sperm motility in Smallmouth Bass. Increased intersex severity and GSI in male Rainbow Darter directly exposed at the Kitchener MWWWE outfall (DK1) in the present study also showed a statistically significant inverse relationship. Tetreault et al. (2011) showed decreased GSI as well as a delay in gonad

development at the same site (DK1) in the fall of 2007. Reduction of GSI noted in Bream (*Abramis brama* L.) exposed to MWW in the Elbe River, Germany by Hecker et al. (2002), was also believed to be likely due to an inhibition of maturation. Other studies on gudgeon (*Gobio gobio*) by Dourfils et al. (2007) also showed delayed spermatogenesis downstream of MWW outfall in the Vesdre River in Belgium. These correlations between intersex severity and maturation or sperm quality in wastewater influenced streams indicate the importance of evaluating intersex severity as a biomarker of exposure in conjunction with other parameters.

Continual assimilation of MWW into receiving environments containing wild fish through critical life stages and onward throughout development provides an opportunity to evaluate the adaptability and or possible resilience to exposure. Germ cells present in undifferentiated gonad tissue have the ability to become oogonia or spermatogonia. While exposure of undifferentiated gonad tissue to EDCs via MWW presents a possible increased likelihood of inducing intersex condition, testis-ova can also be induced in older fish (Kang et al. 2002). As noted by Jobling and Tyler (2003), severe intersex fish are often observed in older individuals that have undergone prolonged exposure. Thus the potential for induction of testis-ova in sexually mature and older fish may be due to bipotentiality or the plasticity of testicular germ cells (Okutsu et al. 2006). In this study, despite a relatively small sample size, a statistically significant positive correlation was observed between increased intersex severity and length (as a surrogate for age) in male Rainbow Darter directly exposed to MWW from the Kitchener plant (DK1). Since hormone receptors themselves can also be up-regulated or down-regulated via exposure to EDCs, it is possible that Rainbow Darter exposure to MWW at critical life stages can induce an increased sensitivity beyond sexual maturity (Liney et al. 2005).

A wide diversity of PPCPs have been reported in Canadian waters receiving MWW (Metcalf et al. 2003) including the Grand River (Metcalf et al. 2003; Servos et al. 2005; Servos et al. 2007; Wang et al. 2011b). Although these compounds are not likely responsible for the effects described here they are

useful to characterize the effluent exposure. Other researchers have used a similar approach (Jobling et al. 2002a). The quantification of a series of PPCPs throughout the sites sampled in the spring of 2010 in the Grand River serves as a marker of exposure to MWW and produces patterns that coincide with biomarkers of response measured in darters. While these corresponding patterns between exposure and response were clear downstream of the Kitchener MWW outfall and through to far downstream sites, response was minimal downstream of the Waterloo MWW outfall despite similar significant increases in exposure.

Reproductive dysfunction and intersex has been associated with a variety of EDC that are found in the environment. Laboratory studies investigating the impacts of estrogenic compounds introduced into receiving environments via MWW such as 17 α -ethynylestradiol (EE₂) (Pawlowski et al. 2004), 17 β -estradiol (E₂) (Panter et al. 1998), nonylphenol (Harries et al. 2000; Pickford et al. 2003) octylphenol (Brian et al. 2009) bisphenol-A (BPA) (Sohoni et al. 2001) on fish have shown varying degrees of response both individually and in mixtures (Thorpe et al. 2003; Brian et al. 2005). While these studies and others (Routledge et al. 1998; Desbrow et al. 1998) have linked estrogenic compounds found in municipal wastewater to responses in fish, additional studies (Blazer et al. 2007; Hinck et al. 2009) have linked agriculture to estrogenic responses in fish as well. Specific to the Canadian environment, numerous EDCs have been detected in Canadian effluents or receiving environments exposed to MWW (Lishman et al. 2006) including E₂ and EE₂ (Ternes et al. 1999b; Servos et al. 2007), alkylphenols (Bennie 1999; Lee and Peart 1998b; Servos et al. 2001a) and BPA (Lee and Peart, 2000). These compounds are often found in very low concentrations making them difficult to detect or quantify. However, these compounds can cause biological changes in fish and other organisms at very low concentrations (Parrott and Beverley 2005) and may act in an additive fashion (Brian et al. 2005) in the environment. Recently researchers have suggested that in addition to estrogens a number of chemicals acting as androgen antagonists may also be involved in reproductive responses observed in fish exposed to wastewater effluents (Gibson et al.

2005; Ankley et al. 2010; Jobling et al. 2009). The mechanisms by which these compounds cause reproductive changes in fish requires further research to understand how different species respond to these complex mixtures of environmental contaminants (Leet et al. 2011; Jobling et al. 2009). While estrogenic compounds have long been thought to be the main cause for the feminization of male fish in environments exposed to MWW, it is more likely that mixtures of compounds ranging from pesticides and herbicides (Metcalf et al. 2000 - DDT) to pharmaceuticals, acting as both estrogens and/or anti-androgens are possible causes (Blazer et al. 2007; Jobling et al. 2009).

Effects on biomarkers of exposure and reproduction in the Grand River appear to be impacted by the assimilation of MWW from the Kitchener and Waterloo MWWTPs. While the induction of intersex condition to varying degrees is reflective of possible changes in reproductive capacity, it is likely only the most severe cases which lower the actual reproductive success of resident fish (Jobling et al. 2002b). Further studies characterizing the actual concentrations of estrogens, anti-estrogens and anti-androgens that resident fish populations are exposed to in the Grand River, as well as continued biological monitoring are needed before drawing conclusions on potential fish population and ecosystem level impacts.

Chapter 3:

Conclusion

The results from this study provide strong evidence of EDC based response via exposure to municipal wastewater in the form of a high level of intersex severity. While differences in other fish somatic indices were most apparent in the female, which are classically more sensitive at spawning, minimal changes in males were observed that coincided with MWWE outfalls. The successful execution of this study was largely dependent upon the rapid and reliable ability to measure intersex presence and absence as well as severity using an adaptation of a new method. Beyond showing a very strong correlation with traditional histology methods, at a fraction of the time and cost, this method enabled further relational analyses between intersex and GSI as well as intersex and age observed in previous studies.

While intersex condition remains a strong indicator of the presence and availability of EDCs in the environment, a clearly defined mechanism for its induction remains unknown. While in the present study, biologically active compounds were quantified as a valid marker of exposure, the quantification of estrogenic compounds such as EE2 could have produced further evidence regarding specific compounds of interest. With evidence in current literature suggesting a synergistic effect promoting intersex via the presence of anti-androgens in wastewater effluent, it is critical that future studies include the quantification of hormonally active compounds. The establishment of an endocrine pathway activated by specific ligands quantified in the environment would further define other expected whole organism effects on fish exposed to these compounds. Since the effects observed in this study are rarely seen in the absence of other physiological changes, it is also valuable to further investigate the relationship between severe intersex and the observed patterns between GSI and age. Targeted sampling of Rainbow Darter immediately downstream of the effluent outfall at Kitchener to produce additional severe intersex samples could further solidify this relationship, and allow measurements of steroid production and other

experiments to strengthen conclusions regarding mechanisms and potential population level implications of severe intersex.

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Appendix A

Speed River Sampling Sites

Table 4: Description of the City of Guelph wastewater treatment plant for 2007 (City of Guelph Wastewater Treatment Plant 2007)

Parameter	Description
Population Served	126,000
Capacity m ³ /day	64,000
Discharge m ³ /day	54,834
Secondary Treatment	Conventional and Extended Activated Sludge
Tertiary Treatment	Rotating Biological Contactors and Sand Filtration
Combined Sewers	No
Disinfectant	Sodium Hyperchlorite
Dechlorination	Sodium Bisulphite
Current Upgrades	Various to reach effluent criteria for capacity of 73,330 m ³ /day

Table 5: Coordinates for spring 2010 sampling sites on the Speed River.

Site	Symbol	Latitude (N)	Longitude (W)
Upstream Guelph STP	GUS	43° 38' 9"	80° 26' 24"
Downstream Guelph STP	GDS	43° 30' 19"	80° 28' 28"

Appendix B

Speed River Sampling Results

Table 6: Summary of fish health parameters and intersex severity and presence at sites sampled on the Speed River in spring 2010.

Species	Site	n	Sex	GSI	LSI	k	Intersex Severity (oocytes per lobe)	Percent Intersex
Rainbow Darter	GUS	20	Male	1.78 ± 0.06	1.14 ± 0.09	1.35 ± 0.04	0.00 ± 0	0
	GDS	20		1.70 ± 0.08	1.36 ± 0.12	1.29 ± 0.02	0.68 ± 0.31	31.5
	GUS	20	Female	13.56 ± 0.88	3.54 ± 0.17	1.39 ± 0.04	-	-
	GDS	20		15.85 ± 0.76	3.83 ± 0.21	1.51 ± 0.06	-	-

Appendix C

Profile of Selected Contaminants in the Speed River

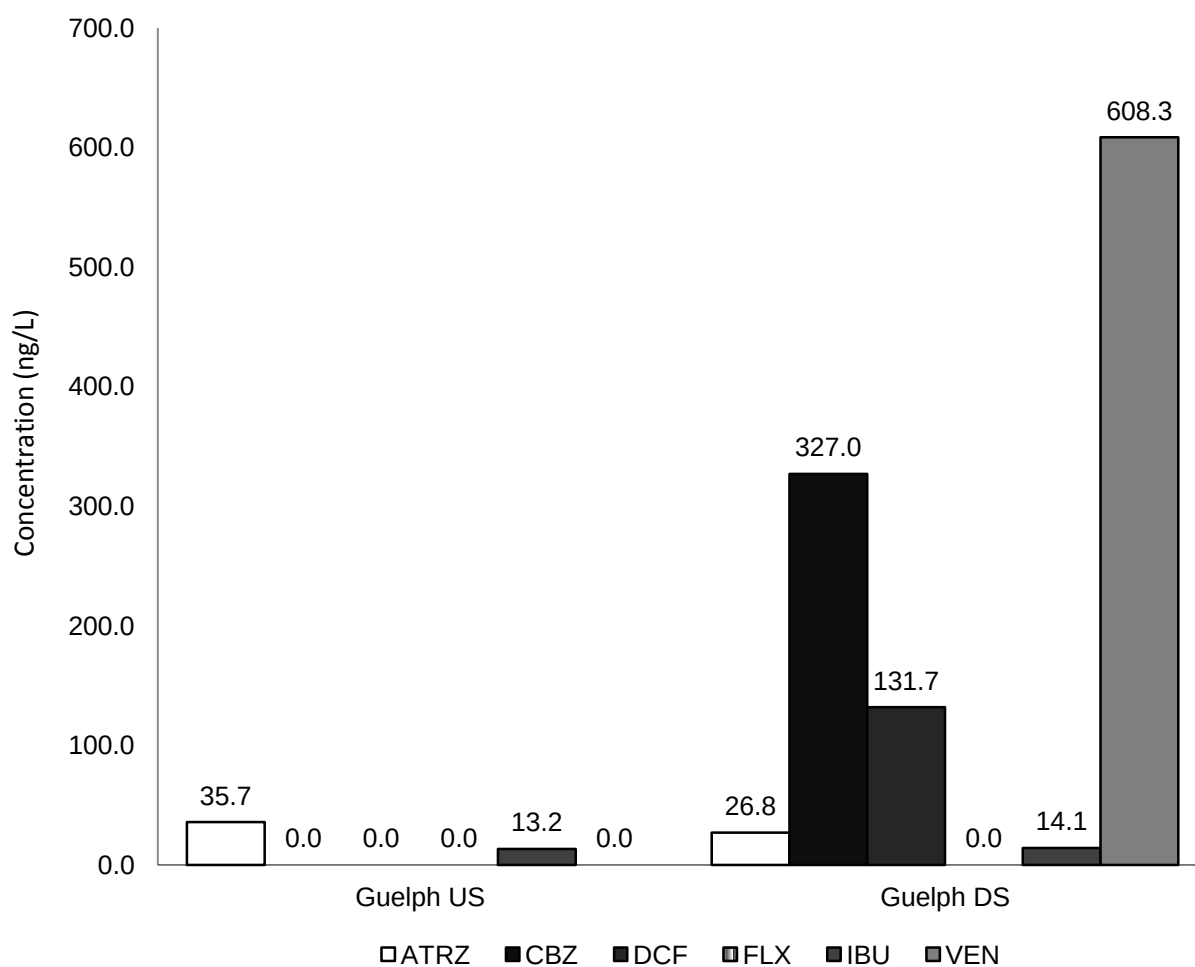


Figure 11: Concentration profile of selected contaminants in water samples collected in spring 2010 on the Speed River. ATRZ = Atrazine, CBZ = Carbamazepine, DCF = Diclofenac, FLX = Fluoxetine, IBU = Ibuprofen, and VEN = Venlafaxine. Values are means $\pm$ SE (n=4).

Appendix D

Intersex Condition in Longnose Dace

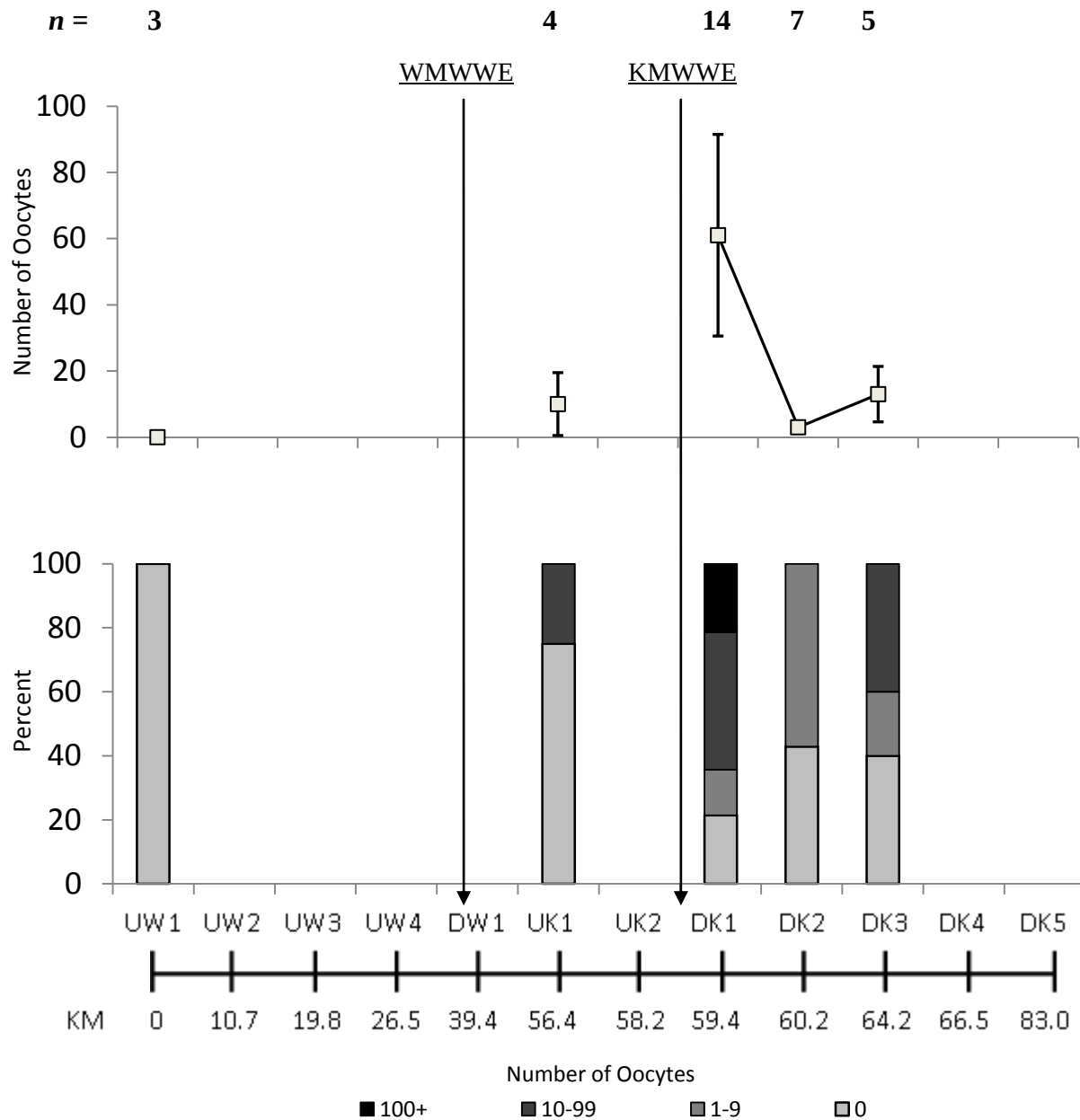


Figure 12: Mean values for intersex severity and proportion of levels of intersex severity in male Longnose Dace on the Grand River in Spring 2010. Arrows indicate the Waterloo and Kitchener MWWE outfalls, respectively. Distance from farthest upstream site indicated below sites.

Appendix E

Intersex Condition in Johnny Darter

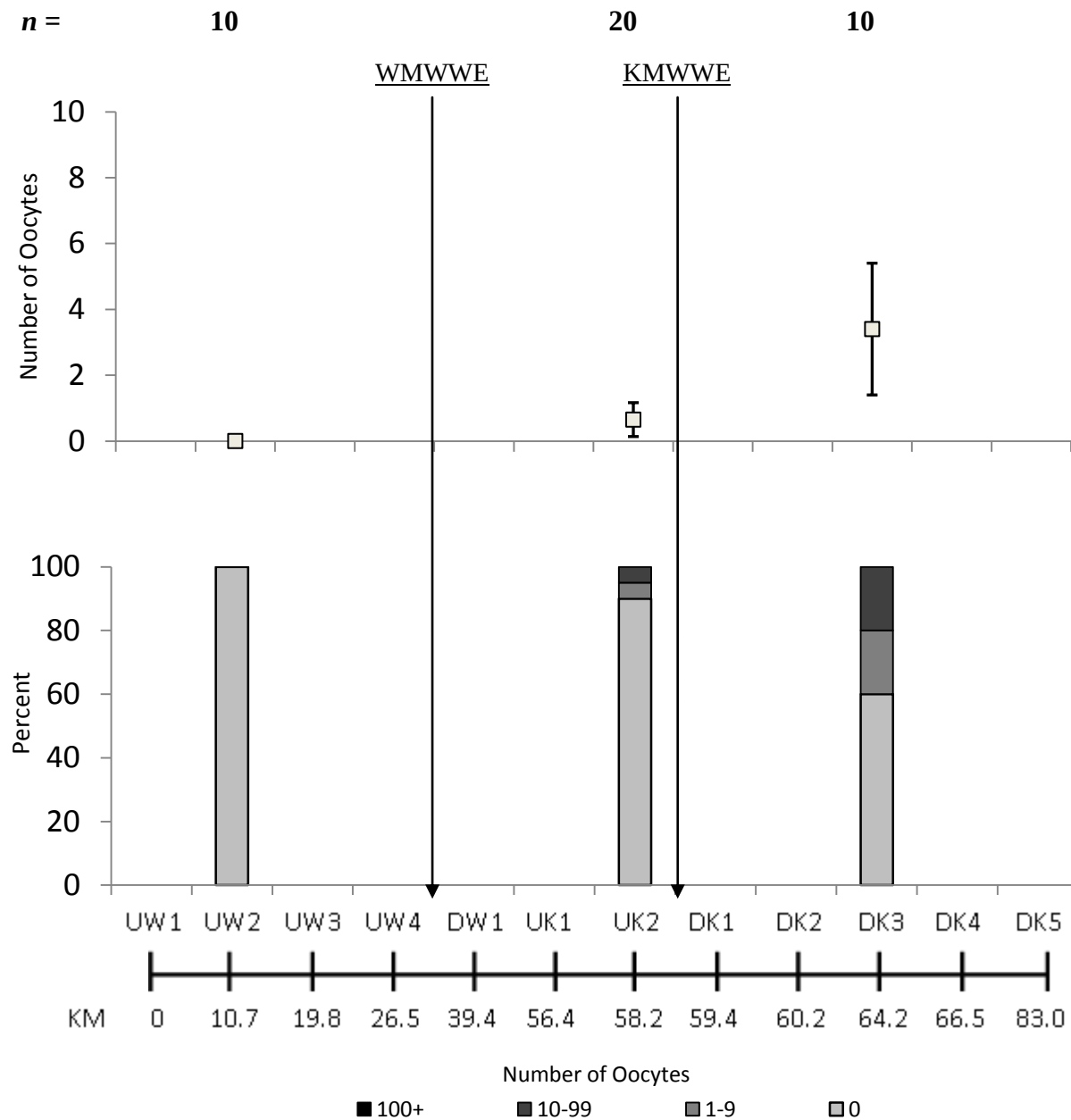


Figure 13: Mean values for intersex severity and proportion of levels of intersex severity in male Johnny Darter on the Grand River in Spring 2010. Arrows indicate the Waterloo and Kitchener MWWWE outfalls, respectively. Distance from farthest upstream site indicated below sites.

Appendix F

Physicochemical Parameters

Table 7: Values for physicochemical parameters measured during sampling in spring 2010.

Site	Site Name	Date Sampled	pH	Temp (°C)	Conductivity (µS)
1	Inverhaugh	April 16/2010	8.45	11.74	477
2	West Montrose	April 15/2010	8.18	8.97	450
2	West Montrose	April 15/2010	8.18	8.97	450
3	Conestogo Confluence	April 29/2010	9	16.5	462
4	Kiwanis Park	April 21/2010	8.32	12.5	524
5	E.I.Trailway	April 19/2010	7.9	12.3	1071
6	Manheim	April 14/2010	8.52	13.47	613
7	Horseranch	April 13/2010	7.99	10.77	644
8	Pioneer Tower 1	April 12/2010	8.26	11.5	N/A*
9	Pioneer Tower 2	April 12/2010	8.36	12.5	N/A*
10	Blair	April 30/2010	8.3	16.63	822
10	Blair	April 14/2010	7.84	10.2	736
11	Speed Confluence	April 28/2010	8.6	11.53	814
12	Glenn Morris	April 22/2010	8.58	14.79	852
13	Guelph Upstream	April 18/2010	8.28	7.88	588
14	Guelph Downstream	April 18/2010	7.96	10.5	1312

*Conductivity reading exceeding measureable levels due to calibration