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Urban-Derived Contaminants Cause Reproductive Disruption in an Aquatic Sentinel Species, Longnose Dace

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Urban-Derived Contaminants Cause Reproductive Disruption in an Aquatic Sentinel Species, Longnose Dace

by

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Abstract

We investigated potential adverse impacts of urban-derived environmental contaminants, such as pharmaceuticals, steroids, surfactants and plasticizers, on Longnose Dace (*Rhinichthys cataractae*) along two rivers, the Bow and Elbow Rivers, in the City of Calgary. Fish were sampled to evaluate physiological and morphological endpoints associated with reproduction and development, including adult sex ratios, changes in body and organ weight, and gonad malformation. Significant male bias was observed downstream of three wastewater treatment plants (WWTPs) on the Bow River, and significant female bias was observed on the Elbow River, suggesting the presence of environmental contaminants with hormone-like activity, dependent on location. To investigate the mechanisms of adverse fish health effects we quantified the expression of liver vitellogenin, estrogen receptor alpha, cytochrome P450, and insulin-like growth factor-1. Decreased IGF1 and ERα expression levels were observed downstream of WWTP effluent in the Bow River, while increased vitellogenin and ERα expression levels were noted in the Elbow River within Calgary. Results support the hypothesis that waterborne environmental contaminants may be responsible for the adverse health effects, such as biased sex ratios, of Longnose Dace within the City of Calgary.

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Dedication

This thesis is dedicated to my brother.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
EDC	Endocrine Disrupting Compound
WWTP	Waste Water Treatment Plant
DEHP	Di-(2-ethylhexyl)-phthalate
DBP	Di(n-butyl) Phthalate
DEP	Dithyl Phthalate
DNP	Dinonyl Phthalate
DIBP	Diisobutyl Phthalate
BBP	Butylbenzyl Phthalate
DnOP	Di-n-octyl Phthalate
DMP	Dimethyl Phthalate
PAH	Polyaromatic Hydrocarbons
PCB	Polycyclic Chlorinated Biphenyls
DDT	1,1,1 –trichloro-2,2-bis(*p*-chlorophenyl)ethane
BPA	Bisphenol A
E2	Estradiol 17β
EE2	17alpha-ethinylestradiol
SSRB	South Saskatchewan River Basin
IGF1	Insulin-like Growth Factor I
ERα	Estrogen Receptor Alpha
CYP1A	Cytochrome P450 1A
CPUE	Catch Per Unit Effort
MS222	Tricaine Methanesulfonate
QRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
H&E	Hemotoxylin and Eosin
cDNA	Complementary Deoxyribonucleic Acid
M-MLV	Moloney Murine Leukemia
ANOVA	Analysis of Variance
mRNA	Messenger Ribonucleic Acid
HSI	Hepatosomatic Index
GSI	Gonadosomatic Index
EROD	Ethoxyresorufin-*O*-deethylase
AHR	Aryl Hydrocarbon Receptors
GH	Growth Hormone
EPA	Environmental Protection Agency

Chapter One: Introduction

Endocrine disrupting compounds (EDCs) are a class of environmental contaminants that have the capacity to alter the function of the endocrine system, potentially altering homeostasis, behaviour, reproduction, and growth (Mills and Chichester 2005). Data show that EDCs impact the endocrine system on numerous levels of biological organization. Reproductive abnormalities in wildlife resulting from EDCs have been widely documented, and range from the feminization of alligators due to exposure to dichlorophenyltrichloroethane (*o,p*'-DDT) in Lake Apopka (Guillette et al. 1996), to deformities in birds in the great lakes (Giesy et al. 1994). Dramatic effects are not only seen in wildlife, but in humans as well. Links between estrogens and cancers have been established (McLachlan and Arnold 1996), and lower sperm concentrations and motility have been associated with environmental exposure to EDCs (Oliva et al. 2001). These widespread impacts that EDCs have on ecosystems demonstrate that EDCs are a pervasive problem.

In addition to an exploding global population, there is an increasing migration of people to urban areas. As of 2008 over half of the world's population lives in cities, and while intense urban growth comes with many benefits, there are often widespread environmental and watershed impacts (The Commission on Growth and Development 2008). Growth and development not only disturbs land, but also puts stress on local water resources as surface waters continually receive discharge and runoff from anthropogenic sources such as wastewater treatment plants (WWTPs), pulp and paper mills, storm water, agricultural practices, and livestock operations. Of concern to researchers are chemicals such as EDCs that are not removed or effectively treated from wastewater effluent or stormwater before they are discharged into aquatic ecosystems. Aquatic organisms are especially susceptible to EDCs as their surrounding

environment is the ultimate sink through discharge and runoff. Although concentrations of EDCs measured in riverine systems are for the most part relatively low, they still have the ability to cause detrimental effects on aquatic life because such contaminants are biologically active at low concentrations.

Traditionally, toxicological testing for most anthropogenic chemicals has focused on the acute impacts of high doses of single compounds in model organisms. These tests do not consider synergistic effects of numerous compounds and often do not identify chronic effects. Chronic effects can often be difficult to detect, as they tend to manifest themselves over long periods of time. To further complicate the issues, multiple EDCs are often present at relatively low concentrations in aquatic environments (Sosiak and Hebben 2005, Jeffries et al. 2010, Chang et al. 2011), making dose-response tests irrelevant for determining long-term effects. Studies that identify chronic effects and population-level consequences are needed to fully understand the impacts of EDCs on fish populations.

Biomarkers are commonly employed to better understand the ways in which contaminants affect individuals or populations. Biomarkers are defined as changes in biological responses that can be related to the presence and magnitude of toxicants (van der Oost et al. 2003), and common biomarkers include behavioural, genetic, physiological, or reproductive responses to exogenous compounds. Endpoints such as alterations in body size (condition factor), organ size (gonadosomatic and hepatosomatic indices) and histopathology (intersexuality) are commonly used to assess the impact of contaminants on individuals. The challenge with morphological measurements is that they do not tell us anything about the physiological mechanisms by which changes occur. Molecular biomarkers using genomics are also used to imply reproductive impairment, and can be useful tools to measure exposure of very

small concentrations of chemicals. For example, the induction of vitellogenin (a precursor to the egg yolk protein) in male fish is commonly used as a biomarker for exposure to estrogen or estrogen-like compounds (Jeffries et al. 2008b, Zhang et al. 2009). However, linkages between gene expression and population level consequences are difficult to establish and interpret, and are not well understood. Morphological and physiological indicators cannot effectively connect cause-effect relationships thus putting an increased importance on considering multiple indicators to determine consistent messages and to better understand mechanisms of EDC impacts.

1.1 Urban sources of environmental contaminants

A broad range of chemical compounds are regularly detected in surface waters of industrialized nations (Kolpin et al. 2002). Water quality in relation to urban centres is largely a function of wastewater and stormwater inputs. While most WWTPs target the reduction of contaminants such as pathogens, heavy metals, nitrogen, and phosphate, they are unequipped to reduce or remove all EDCs in the treatment process. Consequently, EDCs are discharged into receiving surface waters. Numerous studies have demonstrated that sewage effluent and surface waters receiving discharge from WWTPs have detectable levels of endocrine disruptors, including natural and synthetic estrogens, androgens and other biologically active compounds (Kolpin et al. 2002, Sosiak and Hebben 2005, Jeffries et al. 2010, Chang et al. 2011)

Stormwater that results from urban surface water runoff and sewer overflow is a second major source of urban discharge. Xenobiotic organic compounds, including polycyclic aromatic hydrocarbons (PAH), pesticides, phthalates and polycyclic chlorinated biphenyls (PCB), are known contaminants in stormwater (Makepeace et al. 1995). And, while cities continuously

work on ways to reduce stormwater runoff and sediment loads, such as creating stormwater retention ponds, treatment facilities are largely not present and stormwater from urbanized areas is discharged directly into receiving water bodies. Understanding the impacts of stormwater discharges into rivers is needed to adequately protect aquatic ecosystems.

1.2 EDC impacts on aquatic ecosystems

EDCs effects on aquatic ecosystems have been noted since the early 1980's when sport fishermen noticed intersex gonads in Wild Roach (*Rutilus rutilus*) downstream of a wastewater treatment plant (Shears et al. 2005, Tyler and Jobling 2008). Subsequent investigations have shown substantial evidence of a wide range of hormone-associated effects in fish due to EDCs in the environment, with the most widespread being feminization (Lange et al. 2008). The effects of EDCs on reproduction can be manifested by abnormalities in development (Jobling and Tyler 2003), reduced gonad size (Jobling et al. 1996, Jeffries et al. 2008a), biased sex ratios (Jeffries et al. 2008b, Vajda et al. 2008), alterations of gene expression (Filby et al. 2007, Garcia-Reyero et al. 2009a, Garcia-Reyero et al. 2009b), presence of intersex (both ovarian and testicular tissue in the gonads (Woodling et al. 2006), and in extreme cases, population collapse (Kidd et al. 2007).

1.2.1 Endocrine disruption of sexual differentiation

Generally, sex steroids regulate sexual differentiation in teleost fish by affecting the development of germ cells and organs involved in sexual differentiation (Sandra and Norma 2009). Therefore, sexual differentiation of teleost fish has proven responsive to exogenous estrogenic and androgenic steroid hormones (Edmunds et al. 2000). Exposure to EDCs at the time of sexual determination can impact the course of gonadal differentiation and lead to

complete sex reversal (Devlin and Nagahama 2002). In extreme cases male fish may develop phenotypically as females or females may develop phenotypically as males.

Linkages between biased sex ratios and EDCs have been widely noted. For example, permanent male-to-female sex reversal has been demonstrated in Japanese Medaka (*Oryzias latipes*) following exposure to the weakly estrogenic pesticide *o,p'* –DDT (Edmunds et al. 2000). White Sucker (*Catostomus commersoni*) at a site downstream of a sewage effluent site in Boulder Creek, CO were reported to have a significantly female biased population (Vajda et al. 2008), whereas upstream sites displayed a higher male frequency (2x). These examples suggest that a shift to female-biased sex ratios due to environmental EDCs disrupt the reproductive potential of native fish and cause adverse population-level consequences.

1.3 Modes of action

Alterations to the endocrine system due to environmental contaminants are inherently complex as their modes of biological action are highly variable. Where endogenous hormones exert their effect by activating gene transcription through specific receptors, the EDCs can bind to these receptors and interfere with the natural gene regulation process by mimicking or antagonizing endogenous steroids. For example, metabolites of *o,p*'-DDT inhibit androgen binding to the androgen receptor (Kelce et al. 1995), and bisphenol A (BPA) has been shown to directly compete with estradiol for estrogen receptors (Bonefeld-Jorgensen et al. 2001). Moreover, some endocrine disruptors have the ability to modify the synthesis and metabolism of endogenous hormones and hormone receptors (Sonnenschein and Soto 1998). Additionally, the same chemicals have the potential to bind to several different receptors in different tissues, subsequently affecting several hormone pathways (Lemaire et al. 2006). For example, some

environmental chemicals, such as DDE (a metabolite of DDT), have been reported to have *both* estrogen agonist and androgen antagonist properties (Gray et al. 1995). To further complicate an understanding of EDC mechanisms of action, exposure to multiple compounds can have additive or synergistic effects (Mills and Chichester 2005). Mixtures of estradiol 17β (E2) and 17α-ethinylestradiol (EE2), for example, were shown to induce vitellogenin, a common biomarker of endocrine disruption, in an additive manner at low effect levels (Thorpe et al. 2003). Furthermore, the chemical mixtures detected in aquatic environments vary spatially and temporally, making it difficult to determine dose response relationships of any specific contaminants.

1.4 Previous studies from southern Alberta

Previous studies conducted in Southern Alberta have focused on the South Saskatchewan River Basin (SSRB), including the Red Deer, Bow and Oldman Rivers. Jefferies et al. (2008a, 2008b) observed endocrine disruption occurring along these rivers at a basin-wide scale. Vitellogenin induction in male Longnose Dace (*Rhinichthys cataractae*) was reported at every site along the Oldman and Bow Rivers indicating widespread EDC exposure. These findings are in conjunction with the detection of many compounds with endocrine activity in sewage effluent and their receiving waters throughout Alberta (Sosiak and Hebben 2005, Jeffries et al. 2010, Evans et al. 2012). Female-biased sex ratios, skewed up to 90%, were observed in the Oldman River; however, sex ratios in the Bow River were not significantly female-biased. Contrasting patterns may be related to different resources and contaminants in the different regions. The Oldman River is located in a region of agriculture and intensive livestock operations while the

Bow River is subject to multiple urban inputs, including multiple WWPTs from Calgary, Canmore, Banff and Lake Louise.

Alberta has one of the fastest growing populations in Canada (Statistics Canada 2012), and the majority of future growth is projected to take place in Calgary and its surrounding area. While studies such as those of Jefferies et al. (2008a, 2008b) are important in understanding basin-wide patterns of endocrine disruption, they do not consider localized impacts of urban development. To better understand the effects of fish exposed to urban contaminants, patterns and processes need to be examined at spatial scales that reflect localized changes in contaminant concentrations.

1.5 Model species

For this study I used Longnose Dace as a small-bodied sentinel fish species. Longnose Dace is a native species with the widest distribution of any minnow occurring in Alberta (Nelson and Paetz 1992). Spawning occurs from May to August, and fish may spawn more than once each reproductive season (Roberts and Grossman 2001). Longnose Dace were ideal for this study, in part because they are very abundant in the Bow River. Secondly, the species of choice had to be small-bodied. Because small fish species such as Longnose Dace have smaller home ranges (Gibbons et al. 1998, Gray et al. 2002), observed changes in these fish are indicative of their environment. Small-bodied fish also tend to be more abundant, have shorter life spans, and show alterations in growth sooner than larger fish species (Gibbons et al. 1998), making small species suitable for contaminant exposure studies. Lastly, Longnose Dace are a native fish species in the Bow and Elbow river systems. Native fish are already disturbed by numerous anthropogenic stressors including habitat degradation, introduction of invasive species, and

altered flow regimes (Scott and Helfman 2001), and these native fish populations may be the most susceptible to EDCs.

The overall goal of my research was to provide insight into the population level consequences of urban-derived endocrine disrupting compounds on the development and reproductive function of Longnose Dace, a native teleost, sampled in two rivers within the urban footprint of Calgary. Site-wise comparisons were made of phenotypic variables along each river gradient, including sex ratios, condition factor, somatic indices, and gonadal histology. To complement the morphological measurements and attempt to identify any unusual patterns I examined several molecular biomarkers of contaminant exposure, including vitellogenin, insulin-like growth factor 1 (IGF1), estrogen receptor alpha (ERα), and cytochrome P450 1A (CYP1A).

Chapter Two: Urban-derived environmental contaminants linked to the masculinization of wild fish populations in the Bow River, Alberta

2.1 Abstract

The present study was conducted to investigate the adverse impacts of urban-derived environmental contaminants on Longnose Dace in the Bow River, Calgary (Alberta, Canada). Fish were sampled at ten sites to evaluate physiological and morphological endpoints associated with reproduction and development, including adult sex ratios, changes in body and relative organ weight, and gonadal malformation. Significant male bias was observed downstream of three wastewater treatment plants (WWTP), suggesting the presence of environmental contaminants with hormone-like activity. To investigate the mechanisms of adverse effects on fish health, the expression of liver vitellogenin (an estrogen-sensitive egg yolk protein), estrogen receptor alpha (ERα) that mediates estrogen response, cytochrome P450 (CYP1A) that is responsible for the biotransformation of organic pollutants such as polycyclic aromatic hydrocarbons, and insulin-like growth factor-1 (IGF-I), that normally mediates growth induction by growth hormone, were quantified. Results support the hypothesis that urban-derived environmental contaminants may be responsible for the observed adverse health effects, such as masculinization, of Longnose Dace in the Bow River.

2.2 Introduction

A wide range of biologically active organic pollutants with hormone-like activity, which mimic or antagonize estrogens (Kolpin et al. 2002, Sosiak and Hebben 2005) and/or androgens, (Thomas et al. 2002) are regularly measured in effluents and surface waters of many urban areas. Aquatic ecosystems are especially susceptible to contaminant loadings as they receive

continuous inputs through discharge and runoff. Major sources of aquatic contaminants include sewage effluent from WWTPs, pulp and paper mill discharge, storm water, and runoff from agricultural landscapes and intensive livestock operations. Although concentrations of endocrine disrupting compounds (EDCs) in riverine systems are relatively low, previous studies demonstrate that these environmental contaminants, even at low concentrations, are biologically active and can disrupt normal biological processes leading to detrimental physiological and morphological health effects. The adverse effects of EDCs on fish have been documented since the early 1980s when sport fishermen noticed intersex gonads in Wild Roach (*Rutilus rutilus*) downstream of a wastewater treatment plant in England (Tyler and Jobling 2008). Subsequent investigations have demonstrated that exposure to environmental contaminants can result in hormonal imbalance and feminization in fish. Evidence that environmental contaminants cause endocrine disruption includes the presence of intersex (Woodling et al. 2006, Tyler and Jobling 2008, Sanchez et al. 2011), reduced fertility (Jobling et al. 2002), female-biased sex ratios (Jeffries et al. 2008a), alterations of gene expression (Jeffries et al. 2008b, Diniz et al. 2009), changes in organ and body weights (Jeffries et al. 2008a) and in extreme cases, population collapse (Kidd et al. 2007).

Previous research conducted in southern Alberta on the Bow and Oldman Rivers reported EDC impacts at a basin-wide scale (Jeffries et al. 2008b). The induction of vitellogenin in males, an egg yolk protein normally synthesized by females, is a widely used biomarker for estrogenic exposure and was observed in male Longnose Dace at a number of sites along the Bow and Oldman Rivers. In the Oldman River significant female-biased sex ratios were observed (over 90% at some sites), suggesting feminization due to the presence of estrogen-like contaminants (Jeffries et al. 2008b). In fish, the presence of contaminants with estrogen-like activity at the

early stages of gonadal development can cause sex change and the development of male fish into phenotypic females (Devlin and Nagahama 2002).

Studies with a large spatial scale such as that of Jefferies et al. (2008b) have identified basin-wide patterns; however, they typically do not consider the localized impacts of urban development. To better understand the effects on fish exposed to municipal contaminants, patterns and processes should be examined at smaller spatial scales that reflect changes in contaminant concentrations and associated biological responses. The City of Calgary has three WWTPs within city limits, and numerous organic contaminants have been regularly documented in Bonnybrook WWTP effluent and receiving waters (Table 2.1). By sampling fish upstream and downstream of wastewater effluent inputs possible adverse effects of urban-derived contaminants on fish health can be identified.

Small-bodied fish are widely used in contaminant exposure studies as they typically have small home ranges and show changes in reproduction and growth over a short period of time (Gibbons et al. 1998, Gray et al. 2002, Jeffries et al. 2008b). Longnose Dace are a common native minnow in Alberta (Nelson and Paetz 1992), and their abundance, small size, 4-5 year life span and small home range make them an ideal sentinel species.

Our objective was to determine population-level consequences of urban-derived environmental contaminants on Longnose Dace in the Bow River (Alberta, Canada) by testing the hypothesis that environmental contaminants from urban sources cause adverse health effects in Longnose Dace. The expression patterns of a number of genes involved in growth and reproduction were correlated with adult sex ratios and morphological parameters, identifying potential mechanisms of endocrine disruption in Longnose Dace in an aquatic ecosystem with high urban impacts.

Table 2.1 Concentrations of organic wastewater contaminants detected in the Bonnybrook WWTP effluent and the Bow River (AB, Canada) receiving waters in 2002/2003 (Sosiak and Hebben 2005).

Compound	WWTP effluent concentration (ng/L)	Receiving river concentration (ng/L)	Description
Pharmaceuticals			
Benzafibrate	0.144	0.010	Lipid regulator
Diclofenac	0.359	0.021	Analgesic/anti-inflammatory
Gemfibrozil	0.799	0.023	Lipid regulator
Ibuprofen	0.383	0.023	Analgesic/anti-inflammatory
Indomethacin	0.105	0.020	Analgesic/anti-inflammatory
Naproxen	1.785	0.059	Analgesic/anti-inflammatory
Trimethoprim	0.907	0.018	Antibiotic
Pentoxifylline	0.099	0.015	Vasodilator
Carbamazepine	0.925	0.094	Anti-epileptic
Caffeine	0.405	0.064	Stimulant
Cotinine	0.165	0.007	Metabolite of nicotine
Phthalate esters			
DEHP	390.3	684.1	Plasticizer in a broad range of consumer products
DBP	101.2	169.4	PVC production and nitrocellulose lacquers
DEP	22.9	48.5	Plasticizer and in cosmetics, insecticides and aspirin
DNP	3.6	20.0	Film and sheeting, extruded and molded automotive applications
DIBP	10.7	9.0	Solvent, PVC production, synthetic rubber manufacture
BBP	8.0	21.7	PFC and nitrocellulose resin. Used to coat electrical wires
DnOP	1.8	4.0	Plasticizer, pesticide
DMP	1.4	3.2	Rubber softener, also used in wood stains and varnishes

Compound	WWTP effluent concentration (nag/L)	Receiving river concentration (ng/L)	Description
Cholesterol	413.65	285.10	Animal/plant derived sterol
Desmosterol	ND	165.23	Cholesterol derivative
Cholestanol	132.3	10.01	Cholesterol derivative
7-Ketocholesterol	14.82	105.98	Cholesterol oxidation product
6-Ketocholestanol	ND	7.39	Cholesterol oxidation product
Coprostan-3-one	154.49	17.65	Fecal neutral sterol
β-Sitosterol	202.85	73.28	Phytosterol
Campesterol	153.24	19.98	Phytosterol
Stigmastanol	29.81	8.73	Phytosterol
Stigmasterol	177.14	ND	Phytosterol
Fucosterol	577.54	135.87	Sterol found in seaweed
Nonylphenol	1733.51	292.27	Potent EDC from surfactants, formulants in pesticides, lubricating oil additive, curing of epoxy resins
Bisphenol A	83.57	1.80	Potent EDC from PVC plastics
Kaempferol	ND	2.84	Flavonoid found in woody plants
Estrone	2.56	ND	Endogenous female estrogen
Estriol	3.99	ND	Endogenous female estrogen
17β-Estradiol	2.08	ND	Endogenous female estrogen

2.3 Materials and methods

2.3.1 River descriptions

The Bow River flows approximately 645 km from the continental divide of the Rocky Mountains southeast to its confluence with the Oldman River. Its drainage basin is 25,123 km^2 and it is the largest tributary of the South Saskatchewan River Basin (Bow River Basin Council 2005). Approximately 1.12 million people reside in the Bow River watershed, with over 80% of the total population in the City of Calgary. Forty percent of the Bow River basin's total annual natural flow is altered, making it the most regulated river in Alberta Basin (Bow River Basin Council 2005). Calgary discharges water into the river through sanitary and storm water sewers. WWTPs are the largest municipal input in the Bow River basin: Bonnybrook WWTP has a capacity of 500,000 m^3/day, Fish Creek WWTP has a capacity of 500,000 m^3/day and Pine Creek WWTP a capacity of 100,000 m^3/day Basin (Bow River Basin Council 2005). Bonnybrook WWTP effluent is discharged at an approximate rate of 400 000 m^3/day (Bathory et al. 2005). Storm sewers carry surface runoff directly into the river, 90% of which receives little to no treatment. Treatment, when it does occur, consists primarily of settling of particulates in storm water retention ponds and constructed wetlands.

Longnose Dace were collected from eight sites along the Bow River in both September 2009 and May 2010 (Fig. 2.1). Sites were located along 45 km of river between Edworthy Park and Cottonwood Golf Course (Fig. 2.1). In addition to the initial eight sites, Kensington and Glenmore Trail sites were added in 2010. The order in which sites were sampled was randomly determined, and each sampling event took place over 20 days. One site was visited per day. Two sampling times were included to encompass non-reproductive (September 2009) and reproductive (May 2010) stages of Longnose Dace. Sites were primarily selected based on

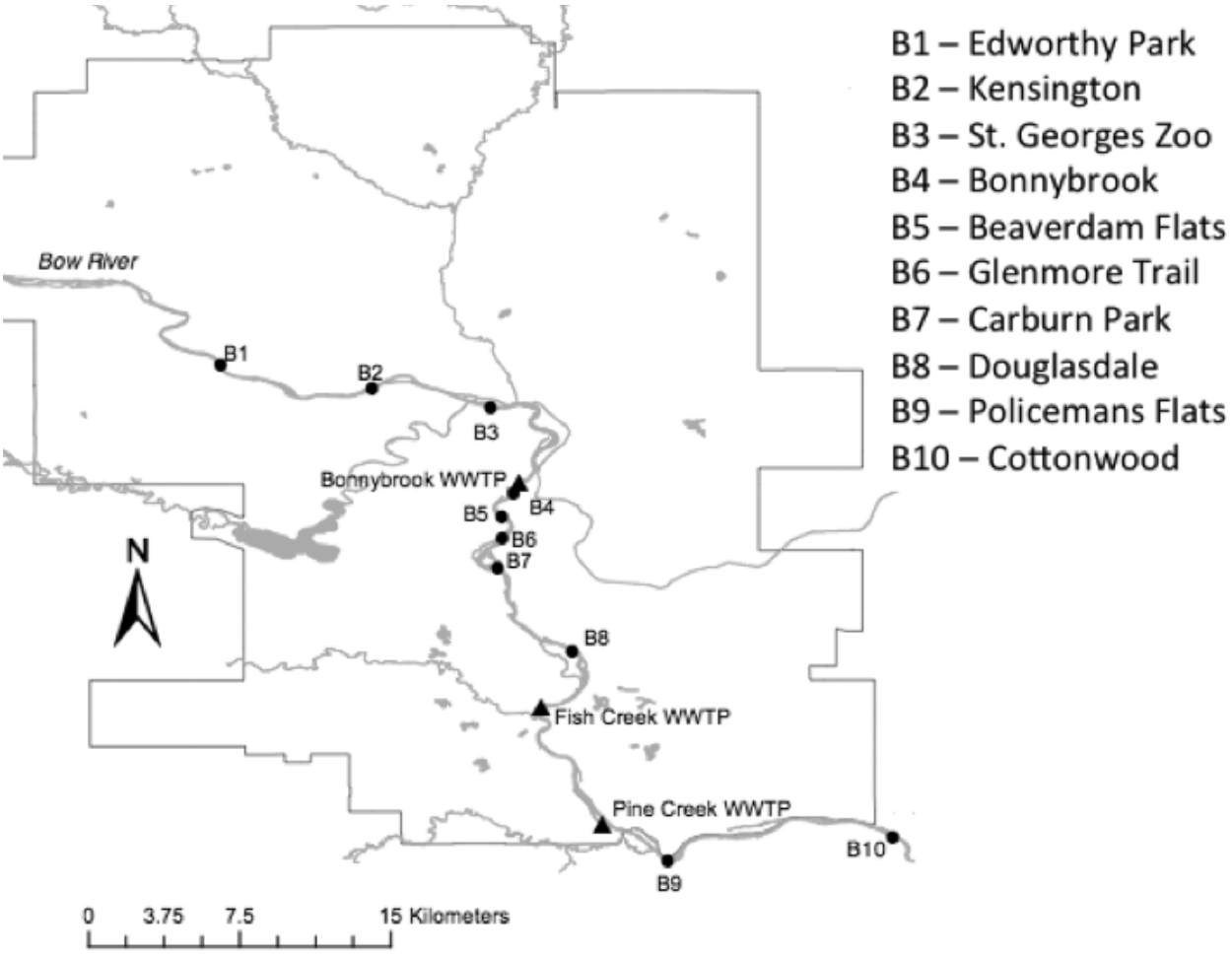

Figure 2.1 Sampling locations along the Bow River within the City of Calgary, Alberta.

potential areas of urban discharge such as storm water inputs, proximity to golf course runoff and wastewater treatment plant discharge. Of the ten sites, three were upstream of any Calgary WWTP, seven were located downstream of the Bonnybrook WWTP and two were downstream of the Fish Creek and Pine Creek WWTPs. Annual averages of water quality characteristics were reported in 2009 and 2010 for sites upstream and downstream of Bonnybrook and Fish Creek WWTPs by The City of Calgary and used to characterize the Bow River (Table 2.2).

2.3.2 Fish collections

Thirty to 40 Longnose Dace were collected from each site using a backpack electrofishing unit (model 12-B POW electrofisher; Smith-Root, Vancouver, WA, USA). One person operated the backpack electrofisher while one or two people collected stunned fish with 6 mm mesh size dip nets. Fishing areas were selected based on typical Longnose Dace habitat consisting of moderate gradient and riffle-run-pool development (Roberts and Grossman 2001) and the accessibility of the area to the backpack electrofishing unit. Catch per unit effort (CPUE) and length-weight relationships of the fish were determined in the field.

One gonad from eight males and eight females at each site was collected for histological analyses. The gonad was placed in glass vial containing 1 mL of 10% buffered formalin solution. Following a minimum of 24 hours in the formalin, samples were transferred to 70% ethanol and stored at 4 °C until preparation of histological sections.

Table 2.2 Water quality variables for the Bow River (AB, Canada) up and down stream of Bonnybrook and Fish Creek WWTPs in 2009 and 2010. Data are presented as means (SE) (City of Calgary, unpublished data).

Sample Location	Year	Dissolved Oxygen (mg/L)	TP (mg/L)	Temp (°C)	TSS (mg/L)	TKN (mg/L)
Upstream of Bonnybrook WWTP	2009	11.15 (0.40)	0.022 (0.008	6.1 (2.0)	6.25 (1.1)	0.194 (0.045)
	2010	10.62 (0.43)	0.018 (0.004)	3.2 (1.9)[a]	6.5 (1.7)	0.162 (0.021)
Downstream of Bonnybrook WWTP	2009	11.24 (0.30)	0.085 (0.013)	6.7 (1.6)	8.0 (1.2)	0.557 (0.065)
	2010	10.63 (0.33)	0.087 (0.010)	3.5 (2.2)[a]	7.2 (1.5)	0.630 (0.078)
Upstream of Fish Creek WWTP	2009	11.51 (0.33)	0.043 (0.007)	6.5 (1.9)	8.2 (2.2)	0.358 (0.041)
	2010	11.23 (0.52)	0.040 (0.005)	3.7 (2.3)[a]	6.9 (2.0)	0.357 (0.044)
Downstream of Fish Creek WWTP	2009	11.10 (0.41)	0.044 (0.007)	6.8 (1.9)	9.2 (2.4)	0.856 (0.112)
	2010	10.39 (0.49)	0.050 (0.006)	3.0 (1.7)[a]	7.7 (2.4)	0.796 (0.083)

[a] - Temperature data obtained from 2010 only available from January to May
TP – Total Phosphorus
Temp – Temperature
TSS – Total Suspended Solids
TKN – Total Kjeldhal Nitrogen

2.3.3 Gonad histology

Gonads were dehydrated through a series of graded ethanol (30-99.9%), NEO-CLEAR® Xylene Substitute and embedded in paraffin. The central portion of each gonad was sectioned at 6 μm using a microtome and then mounted to give a total of 5 sections per fish. Sections were mounted and stained with hemotoxylin and eosin (H&E). The sections were examined by light microscopy for the presence of oocytes and ovarian cavities in the testes at 40x and 100x magnification.

2.3.4 Quantitative PCR

Total RNA was extracted from the liver using TRIZOL reagent (Invitrogen, CA, USA), treated with DNAse (Ambion) according to the manufacturer's protocol, and quantified using spectrophotometric readings at 260 and 280 nm. Four micrograms of total RNA was reverse transcribed into cDNA using oligo(dT) primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's protocol. The cDNA was then diluted and stored at -20 °C for use in QRT-PCR.

Primers for QRT-PCR were designed as follows: GAPDH: (5'-TGATGCTGGTGCCCTGTATGTAGT-3') and (5'-TGTCCTGGTTGACTCCCATCACA-3'), vitellogenin: (5'-GAAGTGCGCATGGTGGCTTGTATT-3') and (5'-AGCTGCCATATC AGGAGCAGTGAT-3'), ERα (5'-TATGTACCCTAAGGAGGAGC-3') and (5'-TGAGTCTCCACACACTCTTCAG-3'), CYP1A: (5'-CGTCGTCGTGGCTGTAGCG-3') and (5'-TGCCCTTGAGGAGCACATCAGC-3') and IGF-1: (5'-CAACGGCACACGGACATC-3') and (5'-CCTCGGCTTGAGTTCTTCTG-3'). A melt curve and gel-electrophoresis determined that all primers resulted in one amplicon and had an efficiency greater than 90%. A Bio-Rad

iCycler iQ Multicolor Real-Time PCR Detection System was used with the following conditions per well: 1 μL of cDNA, 0.3 μL of each primer, 0.2 μL deoxyribonucleotide triphosphates, 2.5 μL SYBR® Green pCR Master Mix, 0.6 μL Taq DNA polymerase in buffer (10 mM Tris-HCl [pH 9], 50 mM KCl, 1.4 mM $MgCl_2$, and 20 nM fluorescein), and water to a total volume of 25 μl. Each sample was run three times to ensure consistency. Cycling was 3 minutes at 95 °C, followed by 48 cycles of 10 seconds at 95 °C and 40 seconds at 57 °C for GAPDH (the internal control), 3 minutes at 95 °C, followed by 45 cycles of 10 seconds at 95 °C and 45 seconds at 55 °C for vitellogenin, 3 minutes at 95 °C, followed by 50 cycles of 10 seconds at 95 °C and 40 seconds at 55 °C for ERα, and 3 minutes at 95 °C, followed by 45 cycles of 10 seconds at 95 °C and 40 seconds at 57 °C for CYP1a. All data are expressed as the change with respect to corresponding GAPDH Ct levels.

2.3.5 Statistics

Statistical analyses were performed with Prism 5 (Version 5.0a). All analyses were run separately for males and females. If necessary the data were log transformed to better meet the assumptions of parametric statistics, including normality and homogeneity of variance. Length-weight relationships and organ-body weight ratios were analyzed using analysis of variance (ANOVA). Tukey's pair-wise comparisons were used to identify which sites were significantly different from one another. If data were normally distributed, differences in vitellogenin, ERα, CYP1A and IGF1 mRNA levels between sites were analyzed using ANOVA followed by Tukey's pair-wise comparisons to identify which pairs of sites differed. In cases where data were non-normal, nonparametric Kruskal-Wallis tests were performed, and multiple pair wise

comparisons were conducted using Dunn's Multiple Comparison Test. Homogeneity of variances were tested using Bartlett's test for equal variances and normality was tested using Shapiro Wilks normality tests. Differences from an expected 50:50 sex ratio at each site were evaluated with Chi-squared tests.

2.4 Results

2.4.1 Sex ratios

Morphological examination of Longnose Dace caught in the Bow River revealed significant (p<0.05) male-bias phenotypes downstream of WWTPs in spring (2010) and fall (2009) samplings (Fig. 2.2). In May 2010 (breeding season) four sites downstream of Bonnybrook WWTP (Glenmore, Carburn Park, Policemans Flats and Cottonwood) displayed significant (p<0.05) male-biased sex ratios, approaching 83% males at Cottonwood, which is downstream of three WWTPs (Fig. 2.2a). Sampling in August 2010 (non-breeding season) revealed some variations in sex ratios, presumably due to changes in seasonal discharge and water flow volume. A trend toward male bias was observed at the same locations; however, only one site downstream (Cottonwood) had a male-biased sex ratio significantly different from 50-:50 (p<0.05, Fig. 2.2). During both seasons a tendency for increased female/male ratios at the Kensington and Beaverdam Flats locations was also observed. A significant female-bias sex ratio (80%; Fig. 2.2a) was observed at Kensington, which is upstream of the WWTPs, during May 2010, and a 77% female-biased ratio was observed at Beaverdam Flats, which is approximately 2km downstream of Bonnybrook WWTP, in August 2009 (Fig. 2.2b). Histological examination of gonads (n=288) did not indicate intersex characteristics in any fish examined, regardless of sex, season or site.

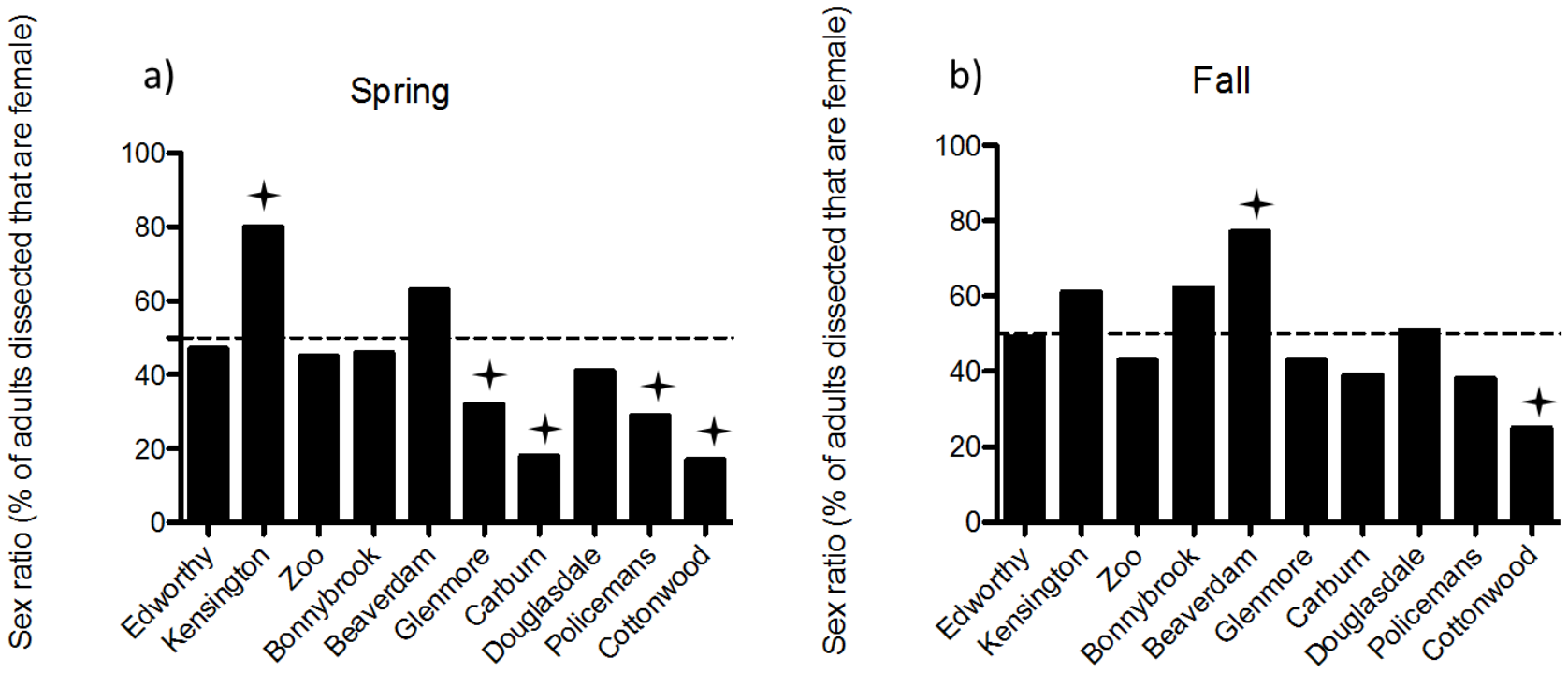

Figure 2.2 Sex ratios of Longnose Dace (*Rhinichthys cataractae*) collected in the Bow River in August (a) and May (b) of 2010. Stars indicate that the sex ratio was significantly different from 50:50 ($p<0.05$) as determined by a chi-square analysis.

2.4.2 Length-weight, liver and gonad relationships

The condition factor of female Longnose Dace was significantly larger during the spring than fall (Table 2.3, $p<0.05$), but no differences were observed for male condition factors between different seasons ($p>0.05$). There was a general increase in condition factor of fish sampled at locations downstream of Edworthy, which increased downstream of WWTP in both males and females during both samplings (Table 2.3).

The hepatosomatic index (HSI) of males and females was significantly larger during spring than during the fall ($p<0.05$). There was a general tendency for increased HSI in males and females sampled along the river gradient downstream of Bonnybrook WWTP. The trend for increased HSI was more pronounced among fish sampled in the fall.

There was a tendency for increased gonadosomatic index (GSI) in males and females sampled along the river gradient, and as expected the GSI of males and females were significantly larger during the pre-reproductive spring sampling ($p<0.05$). Comparison of specific sites revealed an increase in GSI downstream of Bonnybrook WWTP in May 2010 for males and females. Furthermore, male and female Longnose Dace had significantly smaller gonads at Douglasdale during spring of 2010.

2.4.3 Cytochrome P4501A mRNA levels

Generally, Longnose Dace CYP1A mRNA levels were higher in males than females (Fig. 2.3). While the differences were not statistically significant, the results suggest a sex-related bias in the CYP1A mRNA levels, as male Longnose Dace show a general increase in CYP1A along the river gradient into areas of wastewater effluent mixing (Fig. 2.3a). A similar trend was not observed in female fish.

Table 2.3 Abundance, condition and relative liver and gonad weights (mean ± standard error of the mean [SEM]) of male and female Longnose Dace (*Rhinichthys cataractae*) collected in the Bow River in August 2009 and May 2010. Different letters indicate statistical differences ($p<0.05$).

Males - August 2009

Site	CPUE[a]	n	Condition Factor		HSI		GSI[bc]	
Edworthy Park	351.28	11	8.77 ± 0.72	A	0.97 ± 0.56	A	0.95 ± 0.26	A
St. George's Zoo	252.45	13	8.83 ± 0.78	A	1.78 ± 0.55	ABC	0.63 ± 0.05	A
Bonnybrook	377.47	10	8.89 ± 0.65	A	1.43 ± 0.76	AB	0.68 ± 0.08	A
Beaverdam Flats	585.94	10	9.21 ± 0.69	A	1.66 ± 0.71	ABC	0.71 ± 0.10	A
Carburn Park	436.97	16	9.18 ± 0.8	A	2.28 ± 0.86	C	0.84 ± 0.14	A
Douglasdale	561.80	12	9.80 ± 0.54	A	2.00 ± 0.40	BC	0.66 ± 0.07	A
Policeman's Flats	570.43	27	9.35 ± 0.68	A	2.06 ± 0.62	BC	0.77 ± 0.06	A
Cottonwood	372.42	12	9.58 ± 1.00	A	2.22 ± 0.76	BC	1.01 ± 0.18	A

Females - August 2009

Site	n	Condition Factor		HSI[b]		GSI[b]	
Edworthy Park	30	9.14 ± 1.01	AB	1.56 ± 0.61	AB	2.66 ± 0.44	A
St. George's Zoo	27	8.75 ± 0.48	A	1.98 ± 0.76	AB	1.17 ± 0.18	BC
Bonnybrook	27	8.88 ± 0.83	AB	1.61 ± 0.43	AB	2.10 ± 0.28	AB
Beaverdam Flats	33	9.31 ± 0.65	AB	1.71 ± 0.40	A	2.96 ± 0.27	A
Carburn Park	21	9.27 ± 0.85	B	2.41 ± 1.10	B	2.29 ± 0.35	AB
Douglasdale	31	9.76 ± 0.91	AB	1.96 ± 0.66	AB	2.83 ± 0.30	A
Policeman's Flats	22	9.00 ± 0.74	AB	2.13 ± 0.80	AB	0.79 ± 0.06	C
Cottonwood	21	9.73 ± 0.62	AB	2.13 ± 0.55	AB	3.40 ± 0.52	A

Males - May 2010

Site	CPUE[a]	n	Condition Factor		HSI		GSI[b]	
Edworthy Park	112.44	17	8.82 ± 0.21	A	1.57 ± 0.13	A	1.27 ± 0.19	AB
Kensington	230.26	6	9.57 ± 0.38	ABC	2.20 ± 0.15	AB	1.34 ± 0.44	AB
St. George's Zoo	159.63	17	9.15 ± 0.21	AB	1.88 ± 0.20	AC	1.29 ± 0.14	AB
Bonnybrook	249.11	19	8.76 ± 0.22	A	1.67 ± 0.12	A	1.78 ± 0.21	AB
Beaverdam Flats	180.22	13	9.39 ± 0.22	ABC	1.69 ± 0.14	AD	2.21 ± 0.18	AB
Glenmore	120.64	23	10.00 ± 0.23	BC	2.61 ± 0.12	B	2.21 ± 0.24	AB
Carburn Park	120.64	28	9.42 ± 0.20	AB	2.03 ± 0.15	AB	2.44 ± 0.85	AB
Douglasdale	120.64	19	9.35 ± 0.21	AB	2.50 ± 0.19	BC	1.35 ± 0.24	A
Policeman's Flats	103.52	20	9.53 ± 0.26	ABC	2.39 ± 0.18	BCD	1.44 ± 0.17	AB
Cottonwood	227.27	29	10.33 ± 0.19	C	2.55 ± 0.12	BC	2.43 ± 0.24	B

Females - May 2010

Site	n	Condition Factor[b]		HSI[b]		GSI	
Edworthy Park	15	8.75 ± 0.31	A	2.04 ± 0.16	AD	6.06 ± 0.72	A
Kensington	30	9.27 ± 0.49	A	2.46 ± 0.14	ACD	7.07 ± 0.60	A
St. George's Zoo	24	9.91 ± 0.27	A	1.95 ± 0.19	A	6.20 ± 1.03	A
Bonnybrook	26	9.28 ± 0.18	A	2.26 ± 0.08	ACD	8.23 ± 0.80	AB
Beaverdam Flats	22	9.77 ± 0.22	A	2.51 ± 0.16	ABCD	9.10 ± 0.73	AB
Glenmore	11	10.51 ± 0.26	A	3.64 ± 0.20	BD	12.14 ± 1.95	B
Carburn Park	6	10.51 ± 0.51	A	2.33 ± 0.46	ABCD	10.27 ± 3.82	AB
Douglasdale	13	10.13 ± 0.28	A	3.38 ± 0.25	BCD	6.16 ± 0.96	A
Policeman's Flats	8	11.43 ± 0.21	A	3.76 ± 0.91	D	11.23 ± 1.32	AB
Cottonwood	6	10.72 ± 0.30	A	3.33 ± 0.52	D	9.88 ± 2.11	AB

Note: Data are presented as the mean ± standard error. Sexes were analyzed separately by ANOVA. Capital letters that are different within columns represent statistical differences at $p<0.05$. HSI = leptosomatic index. GSI = gonadosomatic index

[a] - Catch per unit effort data (CPUE) measured as fish/5 000 seconds of effort. Data are for sexes combined

[b] - data were log transformed to meet the assumptions of ANOVA

[c] - a Kruskal Wallis was performed as data did not meet the assumptions of normality

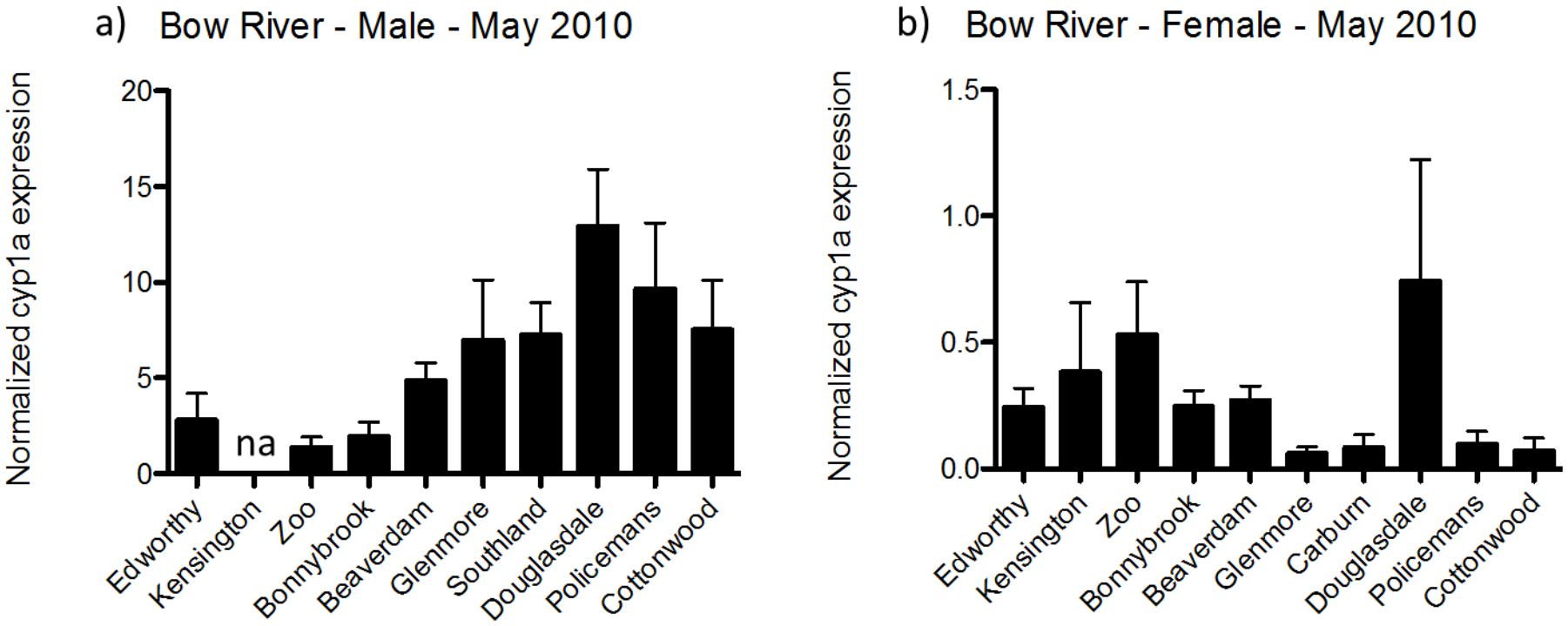

Figure 2.3 Mean cytochrome P4510A mRNA expression in male (a) and female (b) Longnose Dace (*Rhinichthys cataractae*) collected in the Bow River in May 2010. No males were found at Kensington. Bars represent SEM.

2.4.4 Insulin-like growth factor 1 mRNA levels

During spring and fall, hepatic IGF1 mRNA levels displayed comparable levels of expression in male and female Longnose Dace (Fig. 2.4). No site-specific differences were noted in males or females for May samples ($p<0.05$). No seasonal differences were noted in female ($p<0.05$); however, males showed significantly higher hepatic IGF1 mRNA expression in May ($p<0.05$). An overall decrease in male and female IGF1 expression was observed downstream of wastewater effluents during August (Fig. 2.4a, b). Male Longnose Dace sampled from the Zoo site in August showed significantly higher IGF1 expression than fish sampled from the Bonnybrook site (Fig. 2.4a, $p<0.05$). Female fish sampled at the Edworthy site in August contained significantly higher IGF1 mRNA levels than those sampled at the Beaverdam site ($p<0.05$).

2.4.5 Vitellogenin mRNA levels

Longnose Dace hepatic vitellogenin mRNA levels were generally higher in females than in males (Fig. 2.5). Spring females produced significantly higher vitellogenin mRNA than fall females ($p<0.05$) at all sites with the exception of Carburn park ($p<0.05$). Vitellogenin mRNA was also detected in male Longnose Dace at all sites in August 2009 and May 2010 (Fig. 2.5a, c). While there was some variation in the male liver vitellogenin mRNA levels from site to site, there were no consistent patterns, and vitellogenin levels in males did not increase to the levels seen in females (Fig. 2.5).

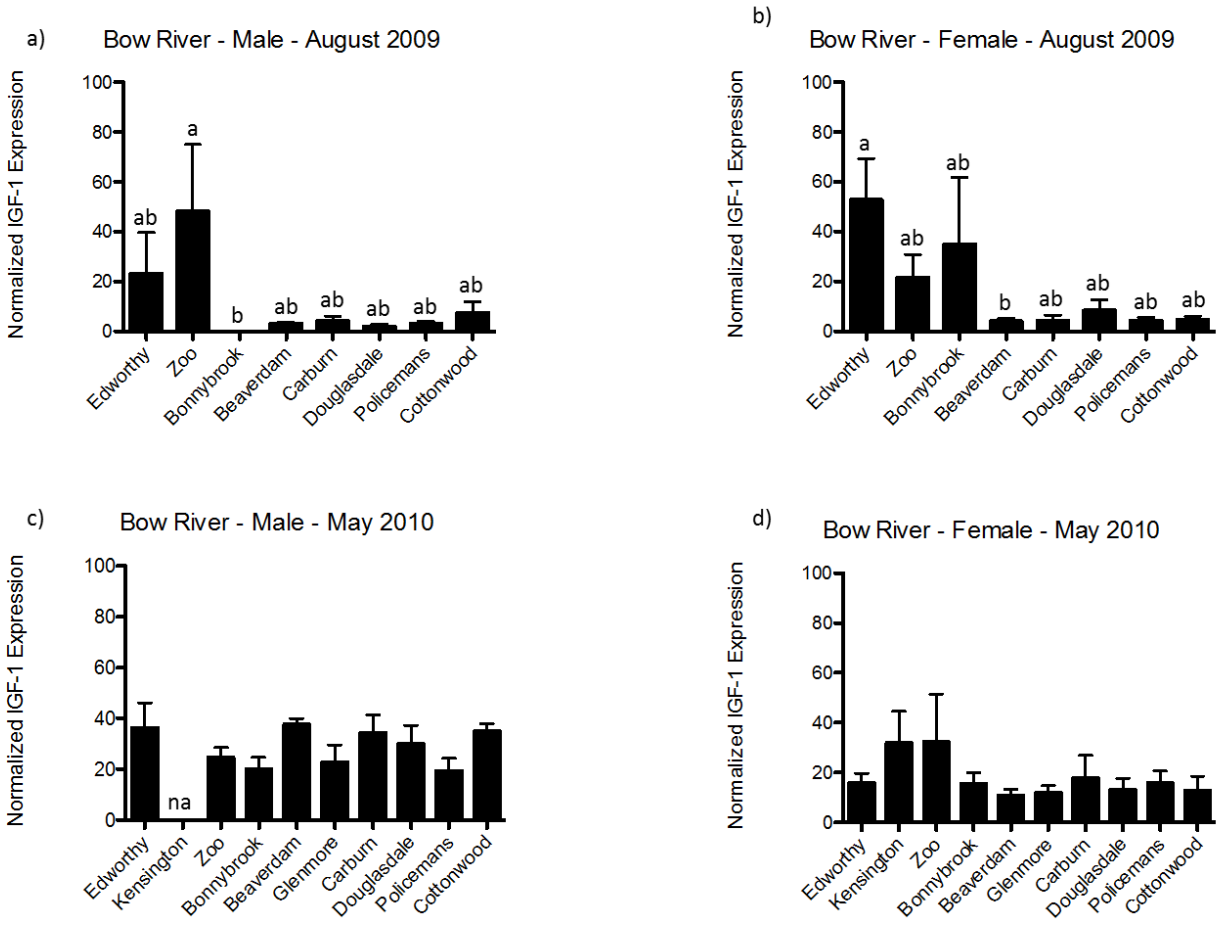

Figure 2.4 Mean IGF1 mRNA expression in female and male Longnose Dace (*Rhinichthys cataractae*) collected in the Bow River in August 2009 and May 2010. No males were found at Kensington. Different letters indicate statistical significance ($p<0.05$). Bars represent SEM.

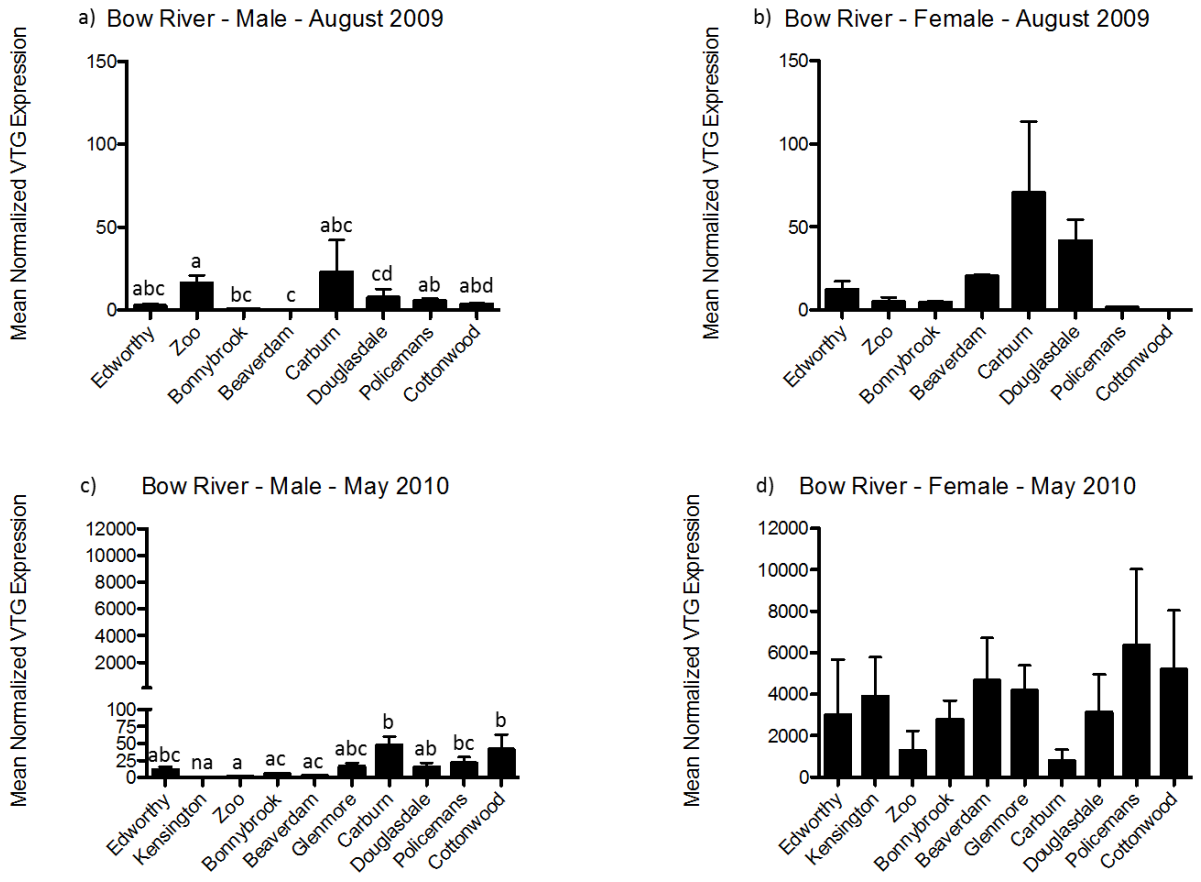

Figure 2.5 Mean vitellogenin mRNA expression in female and male Longnose Dace (*Rhinichthys cataractae*) collected in the Bow River in August 2009 and May 2010. No males were sampled at Kensington. Different letters indicate statistical significance ($p<0.05$). No significant differences were observed in females. Bars represent SEM.

2.4.6 Estrogen receptor alpha mRNA levels

During spring and fall Longnose Dace hepatic ERα mRNA levels were higher in females than in males (Fig. 2.6). During spring female ERα mRNA levels were higher upstream of WWTP effluent compared to the very low levels observed downstream, with the Zoo being significantly higher than Carburn and Douglasdale ($p<0.05$). During fall female ERα mRNA levels were higher upstream of WWTP effluent; however, these differences were not statistically significant ($p>0.05$). Male hepatic ERα mRNA levels remained low and displayed no statistical differences spatially or temporally, which is consistent with the observed vitellogenin levels and male bias along the river gradient ($p>0.05$).

2.5 Discussion

An effects-based approach was used to evaluate potential effects of urban-derived environmental contaminants on fish health. High adult male bias was reported at a number of sites downstream of three existing WWTPs, within Calgary's urban footprint. The observed expression patterns of vitellogenin and ERα mRNA were consistent with the increase in male sex bias, which suggests the presence of contaminants with hormone-like activity along the Bow River in Calgary. This is supported by previous studies that have detected endocrine disrupting compounds in Calgary WWTP effluents and receiving waters (Sosiak and Hebben 2005, Jeffries et al. 2010). Current results provide new insights regarding the impacts of a large urban area on riverine fish populations and demonstrate a link between urban-derived environmental contaminants and masculinization of Longnose Dace.

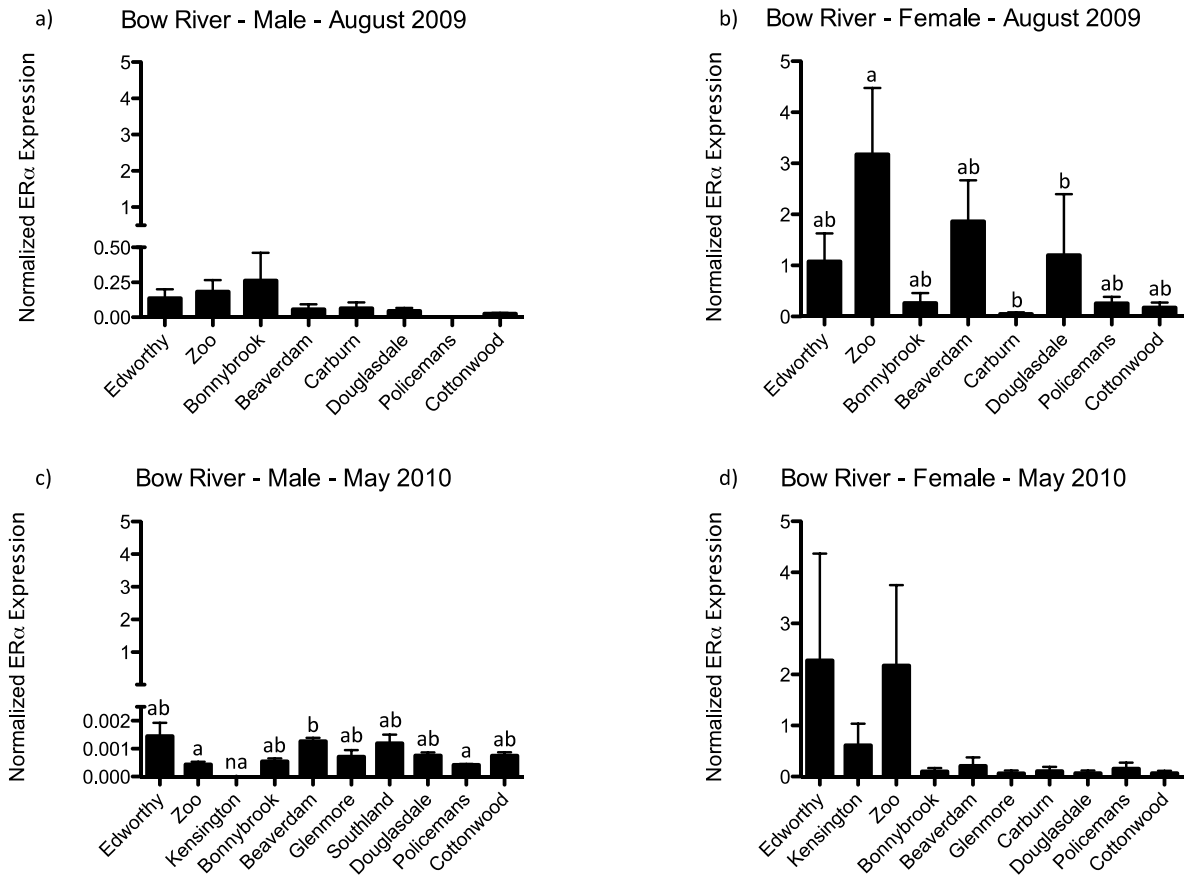

Figure 2.6 Mean estrogen receptor alpha mRNA expression in female and male Longnose Dace (*Rhinichthys cataractae*) collected in the Bow River in August 2009 and May 2010. No males were sampled at Kensington. Different letters indicate statistical significance ($p<0.05$). Bars represent SEM.

2.5.1 Sex ratios

Our results demonstrate a significant male bias along the Bow River at sites downstream of WWTPs, consistent with phenotypic changes in gonadal development following exposure to compounds with hormone-like activity. Fish are among the most diverse group of vertebrates with respect to their gonadal development and reproductive biology. While the sex determinant gene in Longnose Dace has not been characterized, there is evidence that gonadal development into males or females is a function of internal hormone balance during the labile period (Devlin and Nagahama 2002). Higher levels of estrogens and estrogen receptors favour development into a female, and higher levels of androgens can induce the development of the undifferentiated gonad into a testis. Under normal circumstances molecular and cellular events during early development lead to the formation of ovaries or testes, which results in higher production of estrogens or androgens. In the presence of external contaminants with pro-, or anti-, estrogen or androgen like activity, the normal reproductive process can be disrupted. The degree of disruption depends on the concentration of contaminants and duration of exposure, which can result in a range of responses from small development changes in the ovaries to total sex reversal. There are also cases of intersex that result from development of ovarian follicles in the testis under the influence of compounds with estrogen-like activity. It is hypothesized that the observed increase in male: female sex ratios in fish downstream of WWTPs is from exposure to 1) compounds with androgen-like activity, 2) compounds that block estrogens, 3) compounds that inhibit aromatase activity which is responsible for estrogen formation, 4) down regulation of estrogen receptors, or, 5) a combination of some or all of these factors. Based on the present results we cannot distinguish between these mechanisms; however, we have strong evidence that

downstream from large urban WWTPs a complex mixture of organic contaminants and pharmaceuticals, such as those identified in Bonnybrook WWTP effluent, affect different aspects of gonadal development in fish. The low level of vitellogenin in male fish downstream from WWTPs suggests the presence of anti-estrogens that block the effect of EDCs with estrogen-like activity (often detected in urban wastewater effluents) and/or the presence of compounds with androgen like activity. Morthorse et al. (2010) demonstrated permanent masculinization of zebrafish exposed to environmental concentrations of the androgenic steroid trenbolone, and male bias has also been noted in fish exposed to pulp mill effluent known to contain androgenic compounds (Orn et al. 2006). Bellet et al. (2012) reported extremely high levels of androgen agonists and numerous steroid receptor antagonists in raw sewage, thus it is very possible that compounds with androgenic or anti-estrogenic activity are present in Calgary wastewater effluent, and that this mixture causes masculinisation of Longnose Dace, leading the population to develop predominantly as males.

In the absence of a sex determinant genetic marker, the use of adult fish is the only effective way to study gonadal abnormalities. Gonads in immature young fish, whether male or female, are small and undifferentiated, making it difficult to assess sex ratios in a field based study. Transcript measurements of genes related to sexual development during early development can indicate developmental disruption, but do not provide evidence for sex reversal in the absence of a sex determining gene marker. Changes in sex ratios of adult fish, however, are a better indicator of potential sex reversal if other physical and chemical conditions are kept constant. There were observed differences in sex ratios in fish sampled from different locations along the Bow River, while other non-contaminant factors, such as water depth, temperature, flow rates and other physical conditions were relatively constant. This suggests that the observed

sex bias was caused by chemical exposure, leading to sex change. This phenomenon is not unique to the Bow River. In fact, in the Oldman River, which is immediately adjacent to the Bow River, strong female-biased Longnose Dace populations were present downstream of major urban and agricultural influences (Jeffries et al. 2008b). The Oldman River is bordered by agriculture and intensive livestock operations, whereas the Bow River is subject to multiple urban point source inputs, including three WWTPs in Calgary.

2.5.2 Length-weight, liver and gonad relationships

Male and female Longnose Dace are heavier and have larger relative liver size beginning downstream of the Bonnybrook WWTP, the first major point source of nutrient inputs in the Bow River. Our findings are consistent with increased nutrient enrichment and productivity downstream from the wastewater effluent point source. Many studies have shown increased body and liver size downstream of point source inputs such as effluent plumes (Ma et al. 2005, Jeffries et al. 2008a), and although fish appear morphologically healthy, they may suffer physiologically from chronic exposure to contaminants. A contributing factor to the observed elevated HSI could also be the induction of liver detoxification enzymes, which has been seen in conjunction with elevated EROD activity in areas with high concentrations of environmental contaminants such as aromatic hydrocarbons (Arcand-Hoy and Metcalfe 1999, Jeffries et al. 2008a). This is consistent with the increasing, but not statistically significant, male CYP1A expression. Further work is needed to determine the mechanisms behind spatial changes in relative liver and body weights and to separate contaminant-related and nutrient-related processes.

Exposure to estrogens during sexual maturation has been shown to disrupt gonadal growth and development (Jobling et al. 1998) making GSI one indicator of estrogen exposure.

Longnose Dace did not show a reduction in GSI downstream of wastewater effluent inputs, with the exception of Douglasdale. Jobling et al. (2002) reported up to 50% reductions in GSI in fish downstream of wastewater effluents, which is different from our results. This difference is possibly due to variations in different municipal environments, city size, and the types of wastewater treatment processes employed. Variations in GSI can also be attributed to non-contaminant factors such as nutrition and temperature (Kestemont 1989). Due to the complexity of these indices it is difficult to pinpoint the nature of the disruption; therefore, these indices alone are not diagnostic of environmental contaminants.

2.5.3 Patterns of gene expression

CYP1A expression was analyzed to investigate possible exposure of fish to environmental contaminants such as organic pollutants. CYP1A can be induced by exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans and dibenzodioxins (Bucheli and Fent 1995, dos Anjos et al. 2011). There is evidence that CYP1A induction is related to the presence of environmental contaminants in 93% of field studies, making CYP1A a useful biomarker of exposure to these groups of environmental contaminants (Bucheli and Fent 1995). Upregulation of CYP1A was measured in male Longnose Dace predominantly downstream of WWTPs, suggesting the presence of compounds that interact with aryl hydrocarbon receptors (AHR), which can induce CYP1A. The present results are consistent with previous studies that have documented CYP1A induction in fish following exposure to sewage effluent (Ma et al. 2005). Our results also demonstrate gender specific CYP1A expression with hepatic CYP1A mRNA levels over 20-fold lower in spawning female Longnose Dace than in adult males. Gender differences in CYP1A expression have been

observed in other teleost species, with CYP1A levels over 40-fold lower in spawning females (Gray et al. 1991). Dose-related suppression of CYP1A by estradiol treatment has also been documented in post-spawn fish (Gray et al. 1991) and it is possible that reproduction-related hormonal levels rather than exogenous estrogens supress CYP1A expression in females.

IGF1 mRNA expression in the liver of Longnose Dace decreased along the river gradient. IGF1 is an important mediator of growth hormone (GH), which is a key regulator of growth (Wood et al. 2005). Nutritional status is a principal regulator of circulating IGF1 levels, and IGF1 expression is positively correlated with food consumption (Reinecke 2010). Fasting or food restrictions significantly decreased hepatic IGF1 levels in several fish species (Pierce et al. 2005, Montserrat et al. 2007). In accordance with this an increase in liver IGF1 mRNA was seen following increases in amounts fed (Pierce et al. 2005). It is therefore expected that in areas of high productivity such as downstream of wastewater effluents, higher IGF1 levels would be seen, which was not the case in this study. It is possible that nutritional status is not causing the decrease in hepatic IGF1 mRNA levels downstream of WWTPs. Natural and xeno-oestrogens have been demonstrated to interact with IGF1 in fish. Estradiol 17β has a dramatic inhibitory effect on IGF1 expression (Carnevali et al. 2005) and administration of the synthetic ovulation inhibitor EE2 during early development exerts a long-term suppressive effect on hepatic IGF1 expression and synthesis. Exposure to environmental contamination with EE2 results in decreased hepatic IGF1 mRNA expression (Filby et al. 2007). Pesticides have also been demonstrated to affect IGF1 expression. Exposures to environmentally relevant concentrations of pesticides such as deltamethrin have resulted in decreased IGF1 expression (Aksakal et al. 2010). Our results suggest that exposure to environmental contaminants from Calgary's wastewater

impairs the GH-IGF1 axis, as demonstrated by decreased IGF1 expression downstream of wastewater effluent inputs.

As expected, vitellogenin levels in females were significantly higher in spring compared to August, as spring fish are going through vitellogenesis. Longnose Dace spawn between March and July (Roberts and Grossman 2001) and vitellogenin concentrations rise during female sexual maturation. Male vitellogenin was very low in both seasons. There was no clear pattern in vitellogenin in the fish sampled at different sites, indicating that high estrogenicity is not a predominant characteristic of Bow River water. This supports the significant male bias observed downstream from WWTPs. An increase in male liver vitellogenin levels is considered to be a reliable biomarker for estrogen-like compounds (Lange et al. 2008). Our results, however, do not indicate a total lack of estrogen-like substances in the Bow River. Compounds with estrogenic activity have been documented in Bonnybrook wastewater effluent (Sosiak and Hebben 2005, Jeffries et al. 2010), and in a number of samples there was a small increase in male mRNA vitellogenin expression. A more likely cause for the increased phenotypic development of Longnose Dace to males, is the presence of anti-estrogens, or increased levels of compounds with androgen-like activity downstream from WWTPs that counteract endogenous estrogens and the environmental contaminants with estrogen-like activity. Similar findings of male-biased sex ratios and vitellogenin induction have been documented in zebrafish exposed to effluent water from a Swedish pulp mill that contained confirmed androgens (Orn et al. 2006).

A small increase in vitellogenin was observed in males upstream of wastewater effluent inputs and was most apparent at the Zoo site in August 2009. This is likely due to other urban sources, such as surface and storm water runoff that may contain estrogenic compounds like veterinary pharmaceuticals from zoo animals, pesticides and/or herbicides (e.g., 2,4-D), that have

been shown to induce vitellogenin synthesis (Okoumassoun et al. 2002). Our vitellogenin results are consistent with previous findings in southern Alberta (Jeffries et al. 2008b).

A number of studies have reported induction of ERα, an ER subtype, in male fish following exposure to environmental concentrations of EE2 (Filby et al. 2007, Nelson et al. 2007, Lange et al. 2008). Increased expression of ERα, following exposure to EE2, has also been demonstrated to accompany other responses such as vitellogenin induction (Filby et al. 2007, Lange et al. 2008). Our results demonstrate a decrease in ERα in female Longnose Dace downstream of wastewater effluent, coincident with observed male bias downstream of WWTPs. Often environment contaminants with demonstrated estrogen-like activity are not purely estrogenic and could interact with other receptor types as an agonist or antagonist. Antagonistic activity of various pesticides towards ERα has previously been demonstrated (Lemaire et al. 2006). Induced AHR can inhibit E2 responsive genes such as ERα, and they can also induce CYP1A (Geoghegan et al. 2008). The precise mechanisms acting on ERα in the Bow River are unclear at the present time. Changes in ERα expression downstream of wastewater effluent could be explained by: 1) alterations in circulating steroid levels, 2) a potential reduction in estrogen responsiveness and steroid signalling in females, or, 3) the blocking of ERα expression in females by estrogen antagonists. Male Longnose Dace showed extremely low levels of ERα in the Bow River. Gender differences in ERα have been noted in previous studies that showed small levels of ERα expression in male Zebrafish (Meng et al. 2010), which is consistent with the present results.

Physiological responses in fish can be influenced by diet, age, sex and reproductive status (Arcand-Hoy and Metcalfe 1999). Biomarker responses are also influenced by: 1) the mobility of

riverine fish resulting in exposure to variations of EDC concentrations (Winter et al. 2005), 2) genotypic adaptation following chronic exposure to contaminant and, 3) greater genetic diversity of wild fish (Coe et al. 2009) leading to increased tolerance to pollutants. In addition, the precise exposure histories of wild fish populations are often unknown, making linkages between contaminant exposures and biomarker responses difficult. However, transcript measurements can be valuable to identify potential endocrine disruption mechanisms. In studies conducted on Longnose Dace on the Oldman River, correlations were observed between vitellogenin expression in males and female-biased sex ratios (Jeffries et al. 2008b, Evans et al. 2012), supporting the hypothesis that fish in the Oldman River were exposed to compounds with estrogen-like activity. No significant changes in vitellogenin expression were observed in the fish sampled along the Bow River, which is consistent with the observed male bias, and it is possible that fish may have been exposed to compounds with androgenic or antiestrogenic properties. Despite these limitations, toxicological tests on wild fish are a useful indicator of EDC exposure and effects in an ecological context.

Overall, clear patterns of male bias downstream of WWTPs in the Bow River suggest that masculinization is likely caused by contaminants derived from sewage wastewater effluent. Although general water quality in the Bow River is similar to the adjacent Red Deer and Oldman River in Alberta (Jeffries et al. 2008a, Jeffries et al. 2008b), the fish have different morphological and physiological patterns. Gagne and Blaise (1998) observed anti-estrogenic effects in wastewater from large populated areas, while smaller populated areas more often had estrogenic effects. It is possible that industrial processes related to large cities such as Calgary, contribute to the anti-estrogenic effects seen. The majority of studies to date have focused on the estrogenic properties of wastewater effluent and their feminizing effects (Filby et al. 2007, Tyler

and Jobling 2008), and the effects of non-estrogenic steroids are still relatively unclear. And while numerous organic contaminants with endocrine disrupting activity have been detected in Calgary's wastewater effluent, complete characterization of the EDC composition is unknown and thus the presence and concentration of all androgens or estrogen-inhibitors is also unknown. Additional water chemistry analyses are needed to further understand the variation in the presence and concentration of EDCs. The present findings provide strong support for the hypothesis that environmental contaminants, resulting from urban discharge, are causing masculinization of fish populations in the Bow River.

Chapter Three: Urban-derived environmental contaminants linked to the feminization of wild fish populations in the Elbow River, Alberta.

3.1 Abstract

The objective of this study was to determine the population level consequences of urban-derived environmental contaminants on Longnose Dace in the Elbow River. Fish were collected from sites within the City of Calgary in addition to an upstream reference site. Impacts of environmental contaminants on fish were evaluated using phenotypic and morphological endpoints associated with reproduction and development, including sex ratios, changes in body, liver and gonad weight, and the occurrence of intersexuality. Significant female bias and changes in condition and relative liver size were observed within the city limits, suggesting the presence environmental contaminants with hormone-like activity in the Elbow River. In an attempt to explain the observed physical changes we quantified a number of genes related to gonadal development, including vitellogenin (an estrogen-sensitive egg yolk protein), estrogen receptor alpha (ERα; which mediates estrogen response), and cytochrome P450 (CYP1A; involved in the detoxification of organic pollutants). The results support the hypothesis that urban-derived environmental contaminants with possible estrogenic-like activity, resulting from urban sources may be responsible for the health impacts observed in Longnose Dace in the Elbow River.

3.2 Introduction

The City of Calgary is one of the fastest growing cities in North America, the largest city in Alberta, and the fourth largest metropolitan area in Canada (Statistics Canada 2012). One of the many problems associated with increased population growth, such as that in Calgary, is the

impact it has on the surrounding watershed, and while Calgary continues to expand, the loss and degradation of aquatic habitats and water quality in Calgary rivers is of major concern.

Urbanization, the conversion of natural or agricultural lands to urban environments, drastically increases the rate and flow of stormwater (City of Calgary 2009). Contaminants resulting from various urban activities such as construction, transportation, pesticide application, de-icing, washing of cars, air deposition etc. all can be detected in surface water and storm water discharge (Eriksson et al. 2007). In fact, the Environmental Protection Agency (EPA; 2003) has identified stormwater as a form of non-point-source pollution due to its high volume and effects on water quality. As freshwater systems are important for both drinking water and the preservation of aquatic life, there is a growing need to assess the environmental impacts of urban inputs, such as stormwater, on receiving water bodies.

There are currently over 300 storm water outfalls within Calgary, and 75% to 80% of this stormwater effluent does not receive treatment, instead, it discharges directly into local streams and rivers (City of Calgary 2009). Storm water discharge contributes approximately ten times more total suspended solids into Calgary's waterways than all of its wastewater treatment plants combined (City of Calgary 2009). Not only does this stormwater carry sediments and debris into the rivers, but metals and xenobiotic compounds (i.e. polycyclic aromatic hydrocarbons [PAH], pesticides, polycyclic chlorinated biphenyls [PCB], dioxins, furans) are well documented pollutants in stormwater (Eriksson et al. 2007). Moreover, urban stormwater has been proven to be a major source of xenobiotic compounds reported in surface water (Makepeace et al. 1995). Although the presence of contaminants in stormwater well documented, there is little understanding of how urbanization and stormwater impact aquatic communities and fish health.

The objective of this study was to identify the impacts of urban-derived environmental contaminants on the health and reproductive function of a sentinel fish species, and test the hypothesis that environmental contaminants from urban sources cause widespread sexual disruption. For the purpose of this study Longnose Dace were collected along the Elbow River (Alberta, Canada) upstream and within the City of Calgary limits. Longnose Dace are small-bodied fish that have a short life span, a small home range (Roberts and Grossman 2001) and are abundant in the lower reaches of the Elbow River, making them an ideal fish to investigate localized impacts of contaminant exposure. The Elbow River was investigated, as it currently does not receive any discharge from wastewater treatment plants; therefore, the primary source of urban discharge from Calgary into the Elbow River is stormwater effluent. By sampling fish upstream and downstream of areas that receive storm water effluent we can examine potentially adverse effects of urban-derived contaminants on fish health. Sex ratios, gonad malformation, and several morphological indices were correlated with expression patterns of a number of genes in an attempt to better understand mechanisms behind observed phenotypic changes in Longnose Dace in the Elbow River.

3.3 Materials and Methods

3.3.1 River description

The Elbow River flows approximately 124 km from the continental divide of the Rocky Mountains east to the City of Calgary where it joins the Bow River Basin (Bow River Basin Council 2005). The Elbow River has a drainage basin of 1,235 square kilometers and discharges an average of 246,920,355 m^3 into the Bow River annually Basin (Bow River Basin Council

2005). Sixty four percent of the watershed lies within Kananaskis Country, a provincial reserve. It then flows through the Hamlet of Bragg Creek, the municipal district of Rockyview #44, the Tsuu T'ina nation, and the City of Calgary (Elbow River Watershed Partnership 2008). The Glenmore reservoir is a drinking water supply for Calgary and is located directly downstream of the Tsuu T'ina nation. There are no authorized direct discharges of sewage wastewater to the Elbow River upstream of the Glenmore reservoir; however, Bragg Creek is continually under an active boil water advisory due to confirmed aquifer contamination likely due to septic effluent plumes (Elbow River Watershed Partnership 2008). Storm sewers downstream of the Glenmore Reservoir carry surface runoff to the Elbow River with limited treatment.

Longnose Dace were collected from four sites along the Elbow River in May and August 2010 (Figure 3.1). Sites were located along a 26 km stretch of river, and were primarily selected based on potential areas of urban discharge such as storm water inputs and areas of runoff from golf courses. One site (Highway 8) was upstream of the City of Calgary, and was used as a reference site with no urban influences. No sampling sites were located further up the river gradient, as Longnose Dace were not abundant upstream of the Highway 8 site. Three sites were located within the urban footprint of the City of Calgary between the Glenmore Reservoir and the Elbow River's confluence with the Bow River.

3.3.2 Fish Collections

Thirty to 40 Longnose Dace were collected from each site. Fish were sampled using a backpack electrofishing unit (model 12-B POW electrofisher; Smith-Root, Vancouver, WA,

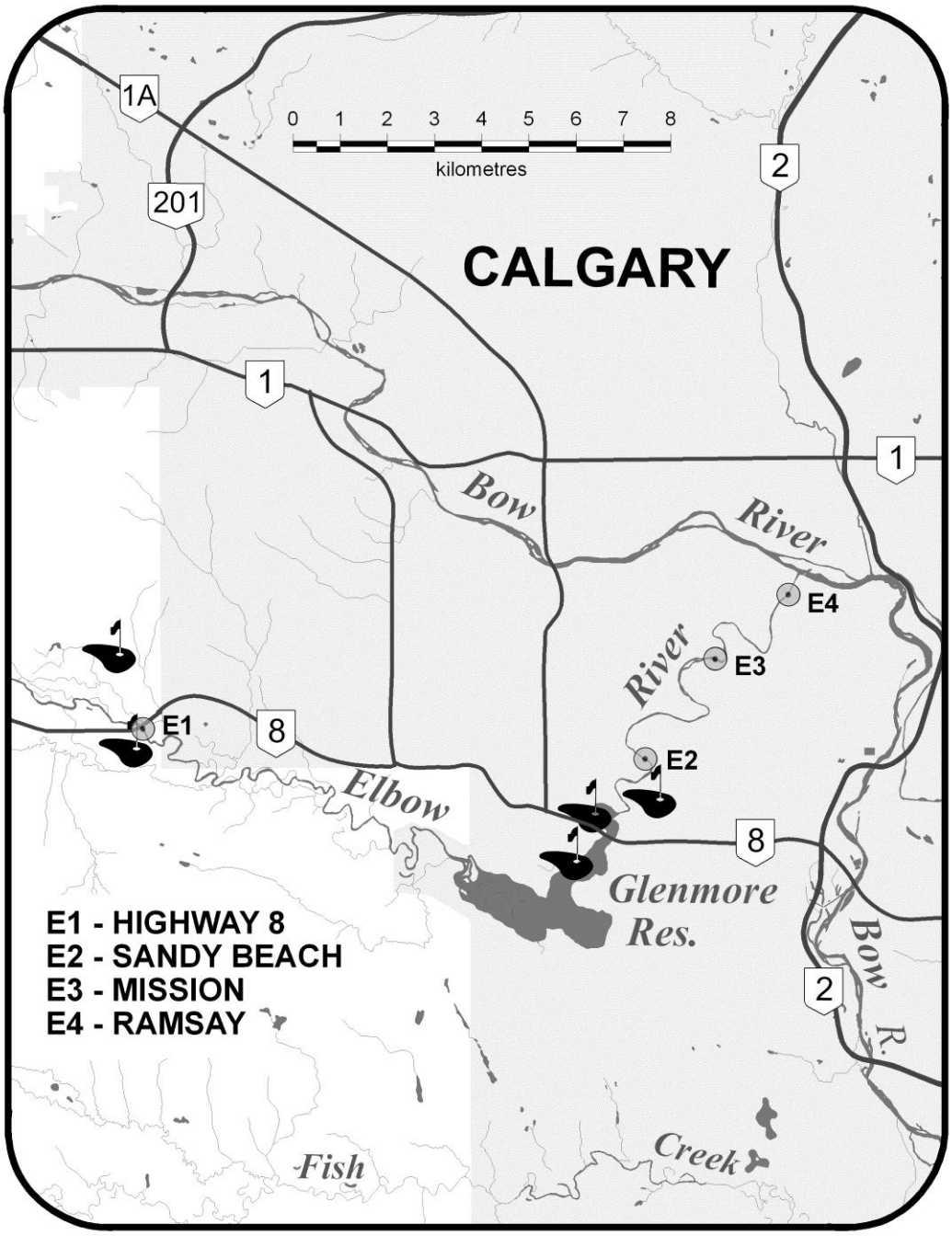

Figure 3.1 Sampling locations along the Elbow River within and above the City of Calgary, Alberta. Calgary's area is shaded grey and golf course locations are indicated in black. Highway 8 was used as a reference site.

USA). One person operated the backpack electrofisher while two people collected the stunned fish with 6 mm mesh size dip nets. Areas to fish were selected based on typical Longnose Dace habitat consisting of moderate gradient and riffle-run-pool development (Roberts and Grossman 2001) and on accessibility of the backpack electrofishing unit. Catch per unit effort (CPUE) and length-weight relationships were determined in the field.

Fish were brought to the laboratory and anesthetised using Trichina Methanesulfonate (MS222), then euthanized by a cut through the spinal cord at the level of the brain stem. Fish were dissected for sex determination, and liver and gonad weights ($\pm$ 0.01 g). A cut-off of a 50 mm fork length was used as fish smaller than this often had undiscernable sex. Hepatosomatic and gonadosomatic indices were calculated as [organ wt/(body wt – organ wt)] x 100], and condition factor was measured (total wt/fork length^3). Liver and gonad tissues were removed for reverse transcriptase quantitative polymerase chain reaction (QRT-PCR) analyses. Samples were placed into 1.5 mL RNase free polypropylene tubes and immediately snap frozen in liquid nitrogen. Samples were stored in a -80°C freezer until analyzed.

One gonad from eight males and eight females at each site were collected for histology. The gonad was placed in a glass vial containing 1 mL of 10% buffered formalin solution. Following a minimum of 24 hours in the formalin, samples were transferred to 70% ethanol and stored at 4°C until preparation of histological sections.

3.3.3 Gonad Histology

Histological analyses were conducted on samples collected in May and August. Gonads were dehydrated through a series of graded ethanol (30-99.9%) NEO-CLEAR® Xylene Substitute and embedded in paraffin. The center of each gonad was sliced into 6 μm sections

using a microtome, and 5 sections per fish were mounted on a slide. The sections were stained with hemotoxylin and eosin (H&E) and examined using 40x light microscopy to determine the presence of intersex.

3.3.4 Quantitative PCR

Gene expression levels of the May 2012 samples were determined using quantitative PCR. Total RNA was extracted from the liver using TRIZOL reagent (Invitrogen, CA, USA) and quantified using spectrophotometric readings at 260 and 280 nm. Four micrograms of total RNA was reverse transcribed into cDNA using oligo(dT) primer and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's protocol. The cDNA was then diluted and stored at -20°C for use in RT-PCR.

Primers were designed and cycling was conducted as described in Chapter 2. All data are expressed as the change with respect to corresponding GAPDH Ct levels.

3.3.5 Statistical analyses

Statistical analyses were performed with Prism 5 (Version 5.0a). Analyses were run separately for males and females. If necessary the data were log transformed to meet the assumptions of parametric statistics, including normality and homogeneity of variance. Homogeneity of variances was tested with Bartlett's tests, and normality was tested with Shapiro Wilks normality tests. May 2010 length-weight relationships and organ-body weight ratios were analyzed using an analysis of variance (ANOVA). Tukey's pair-wise comparisons were used to determine significant differences between sites. Site differences in vitellogenin, ERα and CYP1a mRNA levels were analyzed using either ANOVA or a nonparametric Kruskal-Wallis test, and

multiple pair wise comparisons were conducted using Tukey's pair-wise comparisons or Dunn's Multiple Comparison Test. The number of male and female Longnose Dace from each site in May and August were combined, and differences from an expected 50:50 sex ratio at each site were determined with chi-squared tests.

3.4 Results

3.4.1 Sex ratios

Morphological examination of Longnose Dace gonads from the Elbow River demonstrated significant ($p<0.05$) female-biased sex phenotypes downstream of the Glenmore Reservoir (Fig. 3.2). Two sites downstream of the reservoir (Sandy Beach and Mission) displayed significant female-biased sex ratios, approaching 77% females at Sandy Beach ($\chi^2=18.85$, $p<0.05$). Highway 8, upstream of Calgary, had a statistically significantly male-biased sex ratio compared to 50:50 ($\chi^2=4.08$, $p<0.05$). Histological examination of gonads (n = 128) did not show intersex in any fish examined, regardless of sex, season or site.

3.4.2 Relative abundance and relative body, liver and gonad weights

Catch per unit effort increased within Calgary city limits (Table 3.1), and Longnose Dace were most abundant at the Sandy Beach site in May 2010. Longnose Dace were heaviest at the Ramsay (E4) site and lightest Sandy Beach (E2) for males ($F=3.34$, $p<0.05$) and females ($F=14.28$, $p<0.05$). Males ($F=3.20$, $p<0.05$) and females ($F=7.10$, $p<0.05$) had HSIs at the Highway 8 site upstream of Calgary. The HSIs were significantly lower at Sandy Beach for

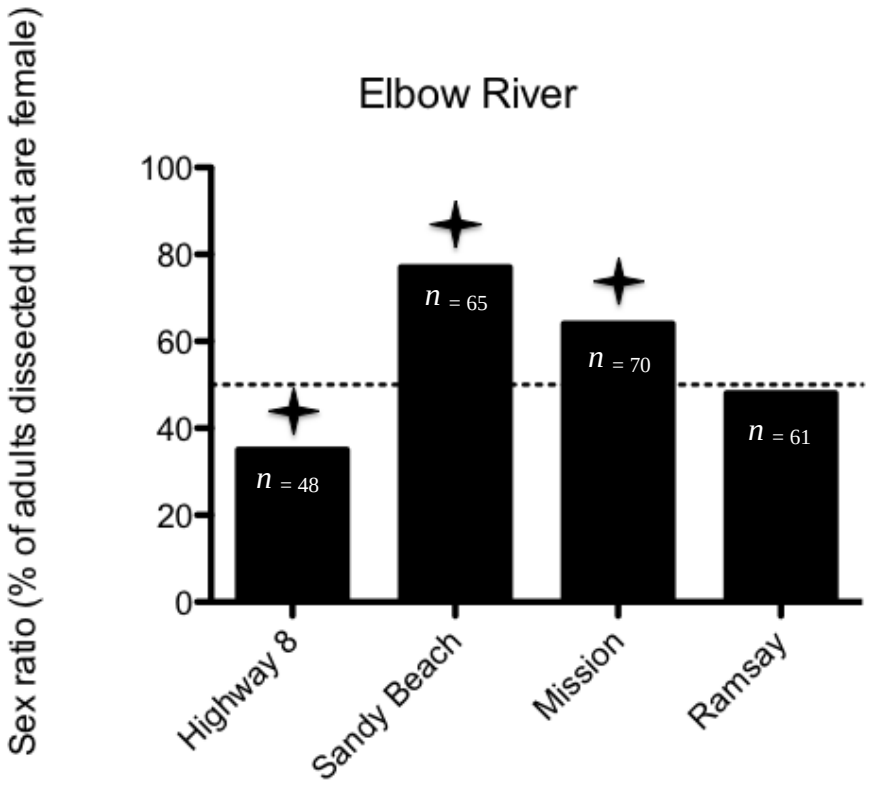

Figure 3.2 Sex ratios of Longnose Dace (*Rhinichthys cataractae*) collected in the Elbow River, Alberta in May and August of 2010. Stars indicate that the sex ratio was significantly different from 50:50 ($p<0.05$) as determined by a chi-square analysis.

Table 3.1 Abundance, condition and relative liver (HSI) and gonad (GSI) weights (mean ± SEM) of male and female Longnose Dace (*Rhinichthys cataractae*) collected in the Elbow River (AB, Canada) in May 2010. Different letters indicate statistical differences ($p<0.05$).

Males - May 2010								
Site	**CPUE[a]**	**n**	**Condition Factor**		**HSI**		**GSI[b]**	
Highway 8	49.50	20	9.07 ± 0.18	AB	2.21 ± 0.20	A	1.60 ± 0.26	A
Sandy Beach	107.49	8	8.27 ± 0.16	A	1.30 ± 0.17	B	1.85 ± 0.48	AB
Mission	97.60	11	9.08 ± 0.21	AB	1.78 ± 0.16	AB	2.27 ± 0.24	AB
Ramsay	41.05	13	9.33 ± 0.25	B	1.75 ± 0.17	AB	2.36 ± 0.25	B
Females - May 2010								
Site		**n**	**Condition Factor[c]**		**HSI**		**GSI[b]**	
Highway 8		6	9.34 ± 0.18	AB	2.60 ± 0.33	A	6.44 ± 1.38	A
Sandy Beach		27	8.61 ± 0.14	A	1.49 ± 0.10	B	7.87 ± 0.39	A
Mission		24	9.34 ± 0.15	B	1.86 ± 0.10	B	8.77 ± 0.28	A
Ramsay		16	10.43 ± 1.01	B	1.97 ± 0.17	AB	8.50 ± 1.16	A

Note: Data are presented as the mean ± standard error. Sexes were analyzed separately by ANOVA. Capital letters that are different within columns represent statistical differences at $p<0.05$. HSI = hepatosomatic index. GSI = gonadosomatic index

[a] - Catch per unit effort data (CPUE) measured as fish/5 000 seconds of effort. Data are for sexes combined

[b] - data were log transformed to meet the assumptions of ANOVA

[c] - a Kruskal Wallis was performed as data did not meet the assumptions of normality

males (F=3.20, $p<0.05$) and females (F=7.10, $p<0.05$). Females at Highway 8 had significantly smaller gonads than those at Ramsay (F=1.41, $p<0.05$). There was a tendency for increased gonad weight in males and females sampled along the river gradient downstream of Glenmore Reservoir; however, this pattern was not significant in males (F=3.42, $p>0.05$).

3.4.3 Cytochrome P4510A mRNA levels

Male and female Longnose Dace displayed comparable CYP1A expression (Fig. 3.3). No significant between-site differences were observed in males (F=2.22, $p>0.05$) or females (F=2.32, $p>0.05$); however, an overall increase in CYP1A expression was observed in fish sampled downstream of the Glenmore Reservoir, with highest expression levels at the Mission site in males and females (Fig. 3.3), but differences were not statistically significant.

3.4.4 Vitellogenin mRNA levels

Longnose Dace hepatic vitellogenin mRNA levels were generally over 1000 fold higher in females than in males (Fig. 3.4). A greater level of variability was observed in females versus males (Fig. 3.4b). Vitellogenin mRNA was also detected in male Longnose Dace at all sites (Fig. 3.4a); however, male expression was relatively low relative to females. No significant differences in male vitellogenin expression were observed between sites (F=0.13, $p>0.05$); however, an overall increase in male vitellogenin expression was seen downstream of the Glenmore Reservoir at the Sandy Beach site (Fig. 3.4a). Females did not display any significant differences between sites (F=2.53, $p>0.05$); however, a general decrease in mean hepatic vitellogenin was observed downstream of the Glenmore Reservoir within the city limits.

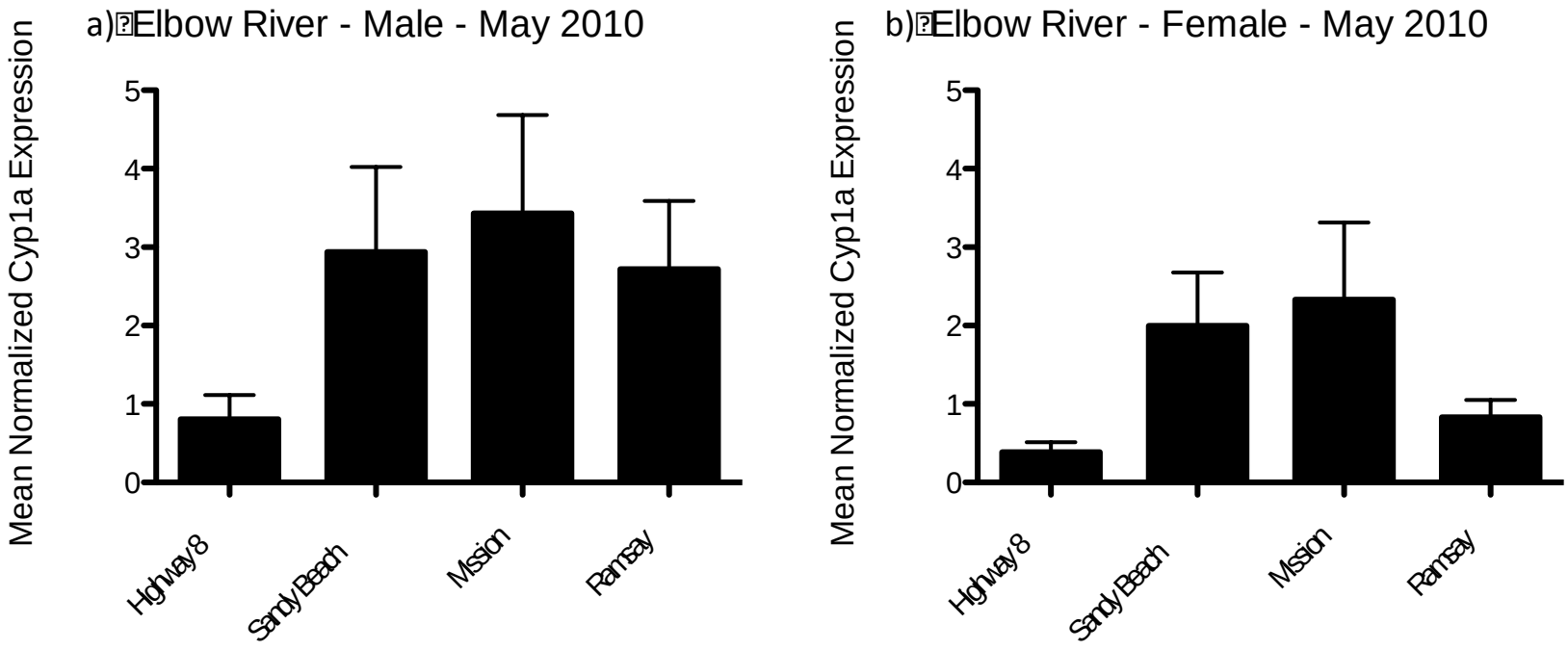

Figure 3.3 Comparison of mean CYP1A mRNA expression in male (a) and female (b) Longnose Dace (*Rhinichthys cataractae*) collected in the Elbow River, Alberta in May 2010. Ten fish of each sex were analyzed for each site. There were no significant differences between sites ($p>0.05$). Bars represent SEM.

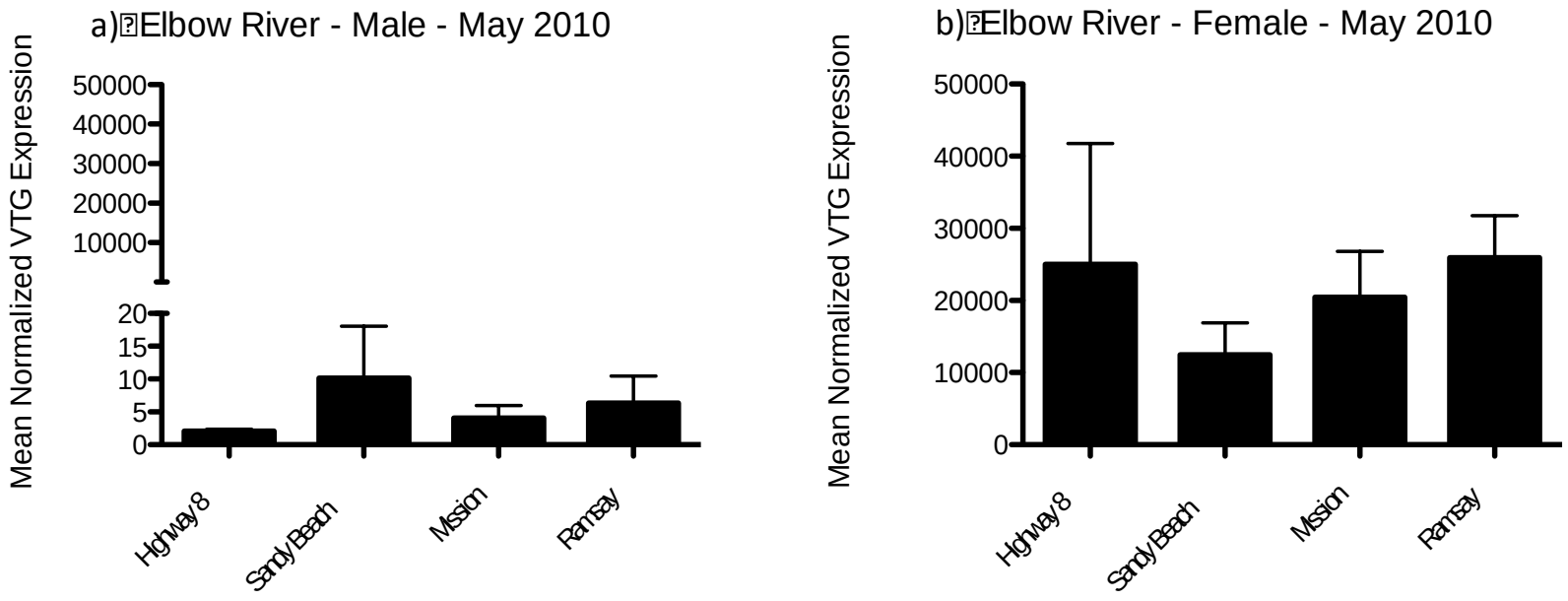

Figure 3.4 Comparison of mean vitellogenin mRNA expression in male (a) and female (b) Longnose Dace (*Rhinichthys cataractae*) collected in the Elbow River, Alberta in May 2010. Ten fish of each sex were analyzed for each site. There were no significant differences between sites (p>0.05). Bars represent SEM.

3.4.5 Estrogen receptor alpha mRNA levels

Hepatic ERα levels in Male and Female Longnose Dace were low. Males at Sandy Beach (E2) had significantly higher ERα mRNA levels than the most upstream Highway 8 site (Fig 3.5a; $F=2.80$, $p<0.05$). Males at the two most downstream sites (Mission [E3] and Ramsay [E4]) had higher ERα mRNA than the most upstream Highway 8 site; however, the difference was not significant ($F=2.80$, $p>0.05$). Females at the three sites within Calgary (Sandy Beach, Mission and Ramsay) had greater ERα mRNA levels than the Highway 8 site (Fig 3.5b); however, the differences between sites were not significant ($F=1.21$, $p>0.05$).

3.5 Discussion

In the present study we investigated the impacts of an urban footprint on fish health along the Elbow River, a relatively pristine river with no treated wastewater inputs. Changes in several morphological and physiological parameters associated with reproduction and development were measured in Longnose Dace to determine the potential effects of urban-derived contaminants. The observed high female-bias sex ratio immediately upon entering the city limits suggests that Longnose Dace were, at some point during early development, exposed to environmental contaminants with estrogen-like activity. The observed expression patterns of CYP1A and ERα mRNA levels are consistent with an increase in female bias. The feminizing changes were observed downstream or upstream of the Glenmore Reservoir, which is not affected by municipal wastewater effluent. A likely possibility is that endocrine disrupting compounds may have been discharged into the Elbow River downstream of the Glenmore Reservoir by means of storm water and surface runoff. The high female-bias in the Elbow River within Calgary shows long-term permanent reproductive effects of xenobiotic exposure, and provides support for the

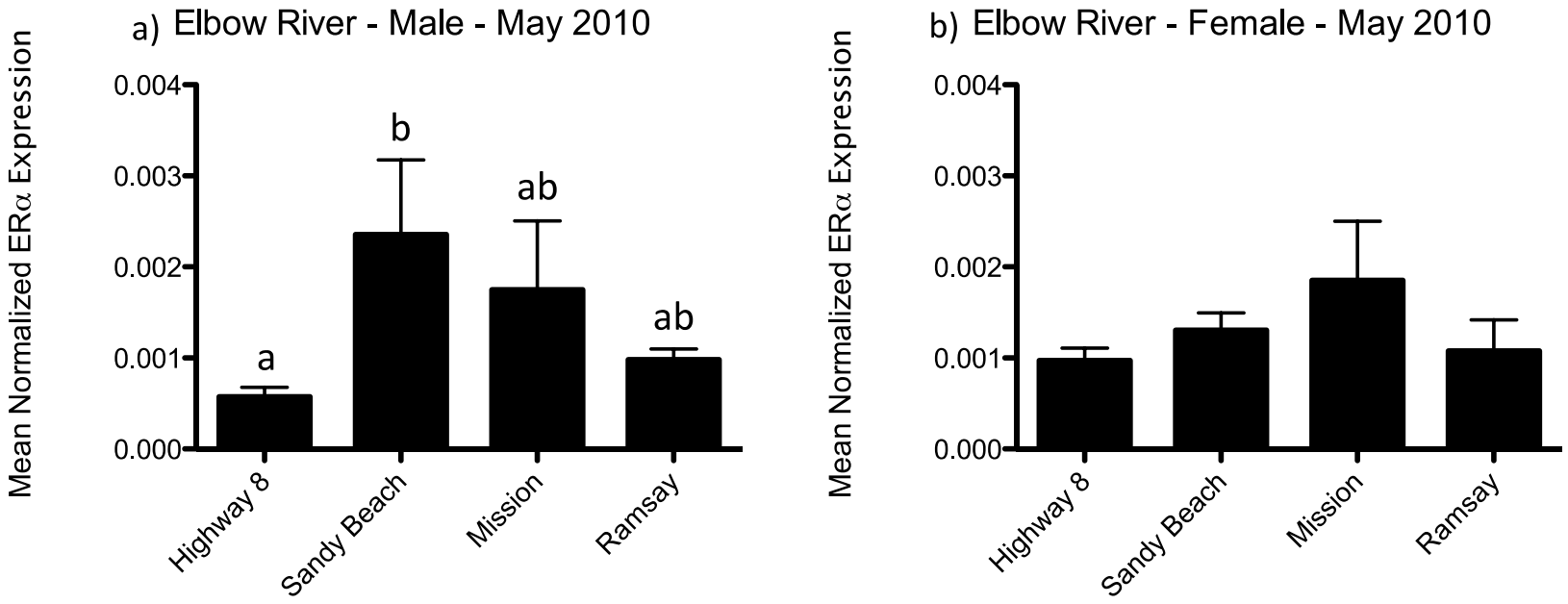

Figure 3.5 Comparison of mean estrogen receptor α mRNA expression in male (a) and female (b) Longnose Dace (*Rhinichthys cataractae*) collected in the Elbow River, Alberta in May 2010. Ten fish of each sex were analyzed for each site. Different letters indicate statistical significance ($p<0.05$). There were no significant differences between sites for females ($p>0.05$). Bars represent SEM.

hypothesis that urban-derived environmental contaminants appear to be causing adverse impacts on fish health in the Elbow River.

3.5.1 Sex ratios

The results demonstrate significant female bias along the Elbow River within Calgary. Female bias is consistent with phenotypic changes in gonadal development resulting from exposure to compounds with estrogen-like activity. While a sex determinant gene has not been characterized in Longnose Dace, evidence suggests that gonadal development into males or females may be a function of hormone imbalance (Devlin and Nagahama 2002), where increased estrogen and estrogen receptors develop the undifferentiated gonads into ovaries, or high levels of androgens result in the development of testes. This sex determination process can be partially reversed or overridden by exposure to endocrine disrupting compounds (Rempel and Schlenk 2008). Formation of intersex gonads has been linked to exposure to environmental estrogens and incomplete sex reversal (Woodling et al. 2006, Tyler and Jobling 2008). Complete sex reversal can occur if exposure to compounds with estrogen-like activity is at a high enough concentration and during a sufficient period of time at the critical time of sexual differentiation (Rinchard et al. 1999). Studies have demonstrated that treatment of many teleost fish with estrogens such as estradiol-17β can result in the feminization of genetic males (Devlin and Nagahama 2002). In this context, no intersex gonads were observed in any Longnose Dace, suggesting that complete sexual differentiation at the time of development is the likely cause of female-bias on the Elbow River. The most common source of exogenous estrogens from large cities is sewage wastewater

effluent (Sumpter 2005), and female-biased sex ratios have been noted downstream of many wastewater treatment plants (Woodling et al. 2006, Vajda et al. 2008).

It is interesting that female-bias has been observed in Elbow River, which is considered relatively pristine and has no wastewater treatment plants along its reach. This demonstrates that estrogen-like compounds are coming from multiple sources in urban environments. Environmental contaminants with estrogen-like activity such as pesticides can impact the sexual differentiation of fish. Exposure of Medaka (*Oryzias latipes*) to *o,p*'-DDT, an estrogenic insecticide, resulted in male-to-female sex reversal (Edmunds et al. 2000). Surface water sampling conducted by Alberta Environment has previously detected the presence of pesticides in the Elbow River (Protection 1998) within Calgary. This suggests that pesticides are a potential inducer of the female bias reported in the Elbow River. A second possible source of estrogen-like contaminants is leaky septic fields from Bragg Creek. Water quality data are needed to identify sources of EDCs in the Elbow River, and to determine if these sources are releasing estrogenic compounds.

Other factors such as environmental conditions can also influence sexual determination in teleost fish (Sandra and Norma 2009). Due to the small distance between sites environmental factors such as temperature and pH are unlikely responsible for the female-biased sex ratios; however, this needs to be confirmed with water quality data. There is also no documented evidence of sex-specific predation on Longnose Dace. Male-bias was observed in the Bow River (Chapter 2), which indicates that sex selectivity of sampling equipment was also not the cause. Sampling protocol was consistent between sites, and sex ratios displayed similar patterns for

multiple sampling events; therefore, sampling error is not likely responsible for the unbalanced sex ratios within Calgary. The female bias is likely the result of phenotypic changes in gonadal development due to exposure to compounds with estrogen-like activity during a critical time in sexual development.

A sixty-five percent male bias was noted at the Highway 8 site upstream of the City of Calgary, which was meant to sere as the reference site. It must be noted that at this site the sample size was relatively low as Longnose Dace are at the edge of their distribution on the Elbow River. In addition, the river was experiencing high flows at the time of sampling due to spring runoff, making fish collection difficult. Further analysis is needed to confirm if male bias is truly present at this site, or if the high number of males was due to the small sample size.

3.5.2 Patterns in gene expression

No significant changes in the expression of male hepatic vitellogenin and CYP1A gene expression were observed between sample sites. This suggests that exposure to estrogen-like activity potentially occurred prior to sampling, but not too distant in the past. This is supported by significant increases in ERα expression within Calgary, which is consistent with exposure to estrogenic contaminants. CYP1A is a detoxification enzyme, and can be induced in fish liver when exposed to organic chemical pollutants such as polycyclic aromatic hydrocarbons (PAHs), commonly derived from gasoline and gasoline by-products (Bucheli and Fent 1995, Chen et al. 2001). For this reason it is commonly used as a biomarker for exposure to contaminants such as hydrocarbons (dos Anjos et al. 2011). Many PAHs have previously been detected in stormwater

(Makepeace et al. 1995). Characterization of stormwater and Elbow River water was not conducted in this study; however, the trend towards increased expression of CYP1A in male and female Longnose Dace suggests that fish were exposed to environmental contaminants such as PAHs.

Longnose Dace spawn between March and July (Roberts and Grossman 2001), and the high vitellogenin levels detected in females were expected as concentrations rise during female sexual maturation. Vitellogenin induction in males is generally a good indicator of exposure to compounds with estrogen-like activity (Sumpter and Jobling 1995) and can reach levels seen in female fish when exposed to estrogen-like compounds. Vitellogenin expression in male and female Longnose Dace within the city limits was not significantly different compared to the site upstream of Calgary, indicating that concentrations of contaminants with estrogen-like activity change periodically in the Elbow River. This view is supported by the observed female bias and significant increase in male ERα mRNA expression. During exposure, vitellogenin transcriptional response is directly proportional to the amount of synthesized estrogen receptor (Flouriot et al. 1997), and estrogen receptors can be specifically induced or repressed by E2 and other estrogenic compounds (Flouriot et al. 1997, Lange et al. 2008).

3.5.3 Relative abundance and relative body, liver and gonad weights

Relative body and liver size are frequently used to assess response to aquatic stressors. Decreased body and liver size in fish has been documented following exposure to contaminants such as sewage effluents and bleached kraft mill effluents (McMaster et al. 1991, Ma et al.

2005), and alterations in body size and growth have been noted in Rainbow Trout exposed to stormwater runoff (Milukaitė et al. 2010). Longnose Dace taken from Sandy Beach were reported to be smaller and have smaller livers than fish upstream and downstream, which suggests fish at this site are suffering from exposure to contaminants. A slight increase in male vitellogenesis was also observed at sites with decreased body and liver weights, and it is also possible that energy allocation towards vitellogenin production may be contributing to the loss in body and liver weight. Similar negative correlations between liver weight and hepatic vitellogenin production have been observed in Longnose Dace in the Oldman River (Jeffries et al. 2010), and weight loss coinciding with vitellogenin production has also been observed in Japanese Medaka (Ma et al. 2005).

The City of Calgary maintains an Integrated Pest Management Plan in which pesticides are applied to neighbourhood parks to manage pests (weeds, insects, diseases, etc.) when pest levels are above pre-determined threshold levels (Alberta Environmental Protection 1998). Golf courses, eight of which are located in the Elbow River watershed, are also heavy users of fertilizers and pesticides to maintain turfs. While guidelines to protect human health and aquatic life are in place, the endocrine disrupting properties of pesticides are not generally taken into consideration. Many pesticides have proven to be potent inducers of vitellogenin (Okoumassoun et al. 2002), and at least 127 pesticides have been noted to have endocrine disrupting properties (McKinlay et al. 2008). A study by Lemaire et al. reported fifteen pesticides to stimulate ERα mediated transcription in a dose dependant manner (Lemaire et al. 2006). It is quite likely that storm water is carrying pesticides and other environmental contaminants from urban sources

such as golf courses, residential properties and city parks, and discharges them into the Elbow River, contributing to the increase in male vitellogenin and ERα expression.

The feminizing effects seen in the Elbow River are in contrast to observations on the Bow River, which was sampled at the same time. While Longnose Dace in the Elbow River downstream of the Glenmore Reservoir in Calgary had a clear female-bias, populations in the Bow River downstream of Bonnybrook wastewater treatment plant are significantly male-biased. One major difference between the two rivers is the wastewater effluent in the Bow River. Reproductive dysfunction of fish in urban settings is most commonly associated with wastewater effluent (Woodling et al. 2006, Lange et al. 2008, Jobling et al. 2009). Our data from the Elbow River suggest that wastewater effluents are not the only source of estrogen-like compounds prevalent in urban settings. The Elbow River flows through highly residential areas where industrial and commercial development surrounds the Bow River. Storm water and surface water runoff have different chemical compositions that contribute to the differences in reproductive effects that we observed. To better understand the processes causing changes in urban settings, further studies on the nature of environmental contaminants in both surface and storm water is needed.

Chapter Four: Conclusion

Industry produces hundreds of new compounds annually, many which have hormone-like activity, and there has been little to no testing of the consequences of the release of these compounds into the environment. Although many contaminants at environmental concentrations have been reported to impair reproductive success of wildlife, only estrogen-like compounds have received much attention. Indeed, effects of organic contaminants with hormone-like activity are rarely noted unless they cause morphological abnormalities. The overall goal of this study was to examine the toxicological effects of urban-derived contaminants present within the City of Calgary on wild Longnose Dace (*Rhinichthys cataractae*).

To characterize the effects of urban-derived organic contaminants we measured spatial and temporal changes in biomarkers related to the development and reproduction of a sentinel species (Longnose Dace) in the Bow River watershed. Longnose Dace were collected during spring and fall along the length of the Bow and Elbow Rivers within the City of Calgary. Fish were analyzed for morphological endpoints including sex ratios, presence of intersex, HSI, GSI, and condition factor. To further understand changes in morphological endpoints several physiological endpoints were also measured, including hepatic vitellogenin, IGF1, CYP1a and ERα mRNA levels.

Morphological endpoints observed in Longnose Dace displayed significant changes in relation to point source inputs, including WWTP effluents and stormwater discharges, along the Bow and Elbow rivers. Changes observed in the Bow River occurred primarily in relation to

Bonnybrook WWTP. The most prominent change was the prevalence of male dominated populations downstream of WWTP effluents. Male bias occurred in conjunction with increases in HSI in addition to decreased IGF1 and ERα expression levels. No intersex was observed. These results, in conjunction with contaminant data that has previously been measured in the Bow River, provide evidence that EDCs present in WWTP effluent are disrupting the normal reproductive process of Longnose Dace. Observed effects are masculinizing in nature, leading Longnose Dace to develop predominantly as males.

Hepatic vitellogenin levels did not change downstream of Bonnybrook WWTP, which signifies that estrogenicity is not a predominant characteristic of Calgary's WWTPs. Results of this study suggest the presence of antiestrogens that block the effect of EDCs with estrogen-like activity and/or the presence of compounds with androgen-like activity. This is supported by the male bias.

Significant female bias was seen upstream of WWTPs in the Bow River which suggests that alternate urban discharge sources such as storm water and surface water runoff, may contain contaminants with estrogen-like activity. This view is supported by the increases in hepatic ERα mRNA levels occurring in conjunction with elevated vitellogenin levels at the same locations. Similarly, significant female-bias sex ratios in conjunction with elevated ERα were detected in the Elbow River, downstream of the Glenmore Reservoir, where no WWTPs are present.

Results of this study suggest a unique chemical footprint within the City of Calgary. Although numerous EDCs have been detected in the Bow River (Sosiak and Hebben 2005, Jeffries et al. 2010), most water chemistry has focused on xenoestrogenic compounds in

wastewater treatment effluent. The nature of the effects on fish in the Bow and Elbow rivers suggests that multiple EDC contributors are present throughout these river systems and that contaminants are not purely estrogenic in nature. Further investigations are required to identify the contributors of EDCs in the Bow and Elbow rivers and to characterize anti-estrogenic and androgenic compounds present.

While this study highlights several impacts of organic contaminant on the endocrine system of wild fishes, precise mechanisms by which EDCs manifest themselves in wild fish populations is unclear. Furthermore, the long-term implications that the morphological and physiological changes will have on the reproductive potential and sustainability of fish populations are unknown. Future research is needed to determine the mechanisms behind the changes, and to address the population-level impacts of urban-derived contaminants as they have implications to long-term fish populations, and potentially to human health.

In conclusion, this study presents novel findings on the impacts that urban effluents have on wild fishes. Spatial changes in Longnose Dace biomarkers along the river gradient indicate that organic contaminants are impacting riverine fish populations. Moreover, our results demonstrate that the effects of environmental contaminants can be seen throughout an urban landscape such as the City of Calgary, not only downstream of wastewater treatment plants. In addition, the expression patterns of IGF1, CYP1a, and ERα that occur spatially along both rivers identify them as useful biomarkers for contaminant exposure.

References

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