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Endocrine Disrupting Compounds and Responses of Longnose Dace in the South Saskatchewan River Basin

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Endocrine Disrupting Compounds and Responses of Longnose Dace in the South Saskatchewan River Basin" submitted by Kenneth Michael Jeffries, in partial fulfillment of the requirements of the degree of Master of Science.

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Abstract

The combined stresses of municipal wastewater and agricultural run-off are common in river basins globally, although they are generally evaluated separately. Endocrine disrupting compounds (EDCs) are introduced to rivers from municipal wastewater and agricultural run-off. Along river gradients, I measured changes in vitellogenin levels in longnose dace to assess a physiological response to exposure to estrogenic compounds, a group of EDCs. Vitellogenin levels increased downstream of municipalities and areas influenced by agriculture. Female-biased sex ratios were detected in the Oldman River, which suggests severe endocrine disruption. I also sampled river water for 28 organic contaminants, which includes many estrogenic compounds, from 23 sites on three rivers. Organic contaminants were detected at every site, which indicates multiple activities within the river basins influence water quality. This thesis demonstrates the presence of organic contaminants spatially and a biological response to the cumulative impacts of municipalities and agriculture on rivers.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
EDC	Endocrine Disrupting Compound
SSRB	South Saskatchewan River Basin
mRNA	Messenger Ribonucleic Acid
QRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
ORBWQI	Oldman River Basin Water Quality Initiative
Hwy	Highway
Nov.	November
BRBC	Bow River Basin Council
SE	Standard Error
Temp.	Temperature
DO	Dissolved Oxygen
S. Cond.	Specific Conductivity
TP	Total Phosphorus
NO_3+NO_2	Nitrate and Nitrite
NH_3	Ammonia
Cl^-	Chloride
YOY	Young of Year
CPUE	Catch Per Unit Effort
cDNA	Complementary Deoxyribonucleic Acid
M-MLV	Moloney Murine Leukemia Virus
ANCOVA	Analysis of Covariance
LS Mean	Least Square Mean
LN	Natural Log
RDWTP	Red Deer Wastewater Treatment Plant
BBWTP	Bonnybrook Wastewater Treatment Plant
FCWTP	Fish Creek Wastewater Treatment Plant
LWTP	Lethbridge Wastewater Treatment Plant

DCM	Dichloromethane
GC-HRMS	Gas Chromatography-High Resolution Mass Spectrometry
HSI	Hepatosomatic Index
GSI	Gonadosomatic Index
ELISA	Enzyme Linked Immunosorbant Assay
PVC	Polyvinyl Chloride

Chapter One: Introduction

Endocrine disrupting compounds (EDCs) are compounds that can potentially interfere with the endocrine systems of humans and animals (Metzler 2002). EDCs pose potential risks to developing organisms. When effects occur, they are typically permanent and irreversible (Colborn et al. 1993). EDCs are suspected to be associated with the recent increases in endocrine specific diseases in humans (Jobling and Tyler 2006), including certain types of cancer. Effects of environmental EDC exposure have been documented in mammals, birds, fish, amphibians and mollusks (Jobling and Tyler 2006), which indicates that EDCs have the potential to impact a variety of organisms.

Concern is increasing over the presence of EDCs in the environment. A large number and variety of EDCs have been introduced into the environment since World War II (Colborn et al. 1993). A major problem is that many EDCs enter the environment simultaneously and there is the potential for synergistic effects of exposure to multiple EDCs. Complex mixtures of EDCs can be introduced to the environment by many sources including municipal and industrial wastewater effluents, agricultural and urban run-off, atmospheric emissions and deposition, and natural sources. Because EDCs enter the environment from numerous sources, EDCs are thought to be globally widespread.

Aquatic systems have been described as "repositories" for EDCs released into the environment (Burki et al. 2006) and may be particularly impacted by EDCs. Many surface waters in the United States and Canada, including Alberta, have detectable levels of EDCs (Kolpin et al. 2002; Metcalfe et al. 2003; Sosiak and Hebben 2005). Few studies have successfully identified the spatial distribution of EDCs in aquatic systems and simultaneously addressed the biological responses to EDC exposure. Widespread incidences of endocrine disruption have been detected in fish populations (Jobling et al. 1998; Aravindakshan et al. 2004). In these cases, the sources of EDCs are often assumed to be only from wastewater effluents. EDCs are likely widely distributed in aquatic systems; however it is still unclear as to what can be considered acceptable levels of EDCs in natural waters and whether biological responses are large enough to induce long-term population level impacts on aquatic organisms.

1.1 Well-known examples of EDC impacts on wildlife

Many of the earliest observations of endocrine disruption in wildlife were discovered accidentally and reported even before it was termed endocrine disruption (Sumpter and Johnson 2005). For example, Blaber (1970) first reported a penis-like structure in female marine snails in Europe. This observation was then discovered in North American mollusks and referred to as an imposex condition (Smith 1971). The masculinization of female snails was eventually linked to exposure to tributyltin, a compound in antifouling paints (Smith 1981). The incidence of imposex has been strongly correlated to the intensity of shipping traffic. Imposex has resulted in reproductive failure of individuals and extirpation of marine snail populations in some areas (Sumpter 2002).

Another well known example of endocrine disruption occurred in Florida alligators in the 1980's. Lake Apopka, Florida, is a lake that receives significant agricultural run-off and received a large pesticide spill in 1980 (Semenza et al. 1997). The alligator population decreased dramatically in Lake Apopka compared to other lakes (Woodward et al. 1993). Juvenile alligators had altered plasma sex steroid levels when compared to reference lakes and males had abnormally small penises (Guillette et al. 1994). Researchers suggested that juvenile alligators were permanently modified during early development, which caused altered steroidogenesis late in life and prevented normal sexual maturation (Guillette et al. 2000). The decrease in the alligator population in Lake Apopka was hypothesized to be due to the inability of males to reproduce. This example shows how EDC exposure early in development can manifest its effects later in the organism's life.

Feminization of male fish is a well-documented effect of endocrine disruption in aquatic systems. Male feminization is caused by exposure to a large class of EDCs that are estrogenic in function. One of the first observations of fish feminization was in the United Kingdom in the 1980's, where there were high incidences of hermaphrodites due to development of ovarian tissue in male testes (Sumpter 2002). This condition was termed intersex, occurred mainly downstream of municipal wastewater treatment plants and was caused by EDCs in effluents (Jobling et al. 1998). Recently, there have been

reports of intersex in fish in the United States in rivers impacted by municipal wastewater (Chambers and Leiker 2006; Woodling et al. 2006). In extreme cases, female-biased sex ratios have been found downstream of wastewater effluent, which demonstrates a population-level effect of estrogenic compound exposure (Woodling et al. 2006).

Many examples of endocrine disruption in wildlife occur in aquatic systems, but recent studies have demonstrated endocrine disruption in terrestrial organisms. For example, polar bears in Greenland have reduced sex organ sizes due to exposure to organohalogen pollutants (Sonne et al. 2006). The primary route of exposure is through dietary consumption of seal blubber, which contains large amounts of organohalogens due to the lipophilic nature of the pollutants (Sonne et al. 2006). The polar bears in Greenland highlight endocrine disruption by bioaccumulation through the food chain. This example shows how EDCs can impact organisms thought to be in relatively pristine environments thousands of kilometers from major anthropogenic activity.

1.2 Modes of action

There are many compounds that have the ability to interfere with the endocrine system by affecting hormone biosynthesis, transport, metabolism or actions (Van Der Kraak et al. 1998). A complication to EDC impacts on wildlife is that it is often unclear what "normal" is for the organism studied, which make decisions as to what is "abnormal" difficult (Sumpter and Johnson 2005). In addition, an organism in the environment is often exposed to many EDCs simultaneously. This is especially true in aquatic systems that receive wastewater. Municipal and industrial wastewaters introduce many EDCs to the environment, which creates chronic exposure to sub-lethal complex chemical mixtures (Sumpter 1997). Generally, organisms tolerate small perturbations to the endocrine pathway; therefore, to determine what is a biologically relevant response to EDC exposure is a challenge (Van Der Kraak et al. 1998). It is important to link biomarkers of endocrine disruption to growth, reproductive impairment and population-level consequences to demonstrate a significant biological response to EDC exposure in wildlife (Mills and Chichester 2005).

1.2.1 Estrogenic and androgenic compounds

Many EDCs that elicit reproductive or developmental responses in organisms are either estrogenic or androgenic in action depending on which hormone pathway they influence. EDCs can be agonistic by mimicking or complementing natural hormones or antagonistic by blocking or preventing natural hormones from eliciting a cellular response (Goksoyr et al. 2003). Interestingly, many estrogenic compounds that have been investigated are agonistic, while many androgenic compounds are antagonistic in function (Van Der Kraak et al. 1998). Therefore, the range of wildlife responses to EDCs is large and to relate effects back to a single compound is difficult.

In fish populations, estrogenic compounds often act on the estrogen receptor in target tissues (Ankley and Giesy 1998) and therefore directly mimic estrogen's effects. However, all estrogenic compounds are not equal in their potency. Natural and synthetic estrogens (e.g. birth control pill metabolites) are the most potent whereas most other estrogenic compounds are considerably less potent and require organisms to be exposed to a higher relative concentration to elicit estrogenic responses (Sumpter and Johnson 2005). This has lead researchers to assess estrogenic compound potency relative to the endogenous estrogen 17β-estradiol in terms of estrogenic equivalents (Coldham et al. 1997). This approach has been used to determine the estrogenicity of wastewater effluents, which are often significant sources of estrogenic compounds to aquatic systems (Fernandez et al. 2007). Because wastewater effluent can make up a considerable proportion of stream flow during certain seasons in some systems, it is important to understand how estrogenic wastewaters can influence aquatic organisms.

One of the most widely accepted biomarkers of estrogenic compound exposure in aquatic systems is production of the female-specific protein vitellogenin by male fish. In natural conditions during ovarian recrudescence in female fish, estradiol is released into the blood by the ovaries and is transported to the liver (Arukwe and Goksoyr 1998; Van Der Kraak et al. 1998). In response to estradiol exposure, estrogen receptors are up-regulated in the liver and bind to estradiol and initiate or enhance transcription of vitellogenin genes. Vitellogenin is then modified and transported to the ovaries for

normal pre-spawning egg development (Arukwe and Goksoyr 1998). In males, vitellogenin synthesis normally remains silent or shows minimal levels. Vitellogenin is readily induced in males exposed to estrogenic compounds, therefore making it an effective indicator of exposure (Rolland 2000). Vitellogenin production in male fish has consequently been used in numerous field studies to examine the overall estrogenic effects of wastewater effluents on receiving waters and the estrogenic potential of many anthropogenic contaminants in laboratory studies.

Androgenic compound effects are not as widely studied in wild fish populations as estrogenic compounds. Androgenic compounds can either mimic natural androgens or can prevent natural androgens from binding to the androgen receptors in target tissues (Goksoyr et al. 2003). Municipal wastewater and cattle feedlot effluents can be sources of androgenic compounds to aquatic systems (Kolodziej et al. 2004; Fernandez et al. 2007). Kraft pulp-mill effluents can induce androgenic responses in fish populations downstream of the effluent input and cause masculinization of females (Bortone and Davis 1994). An interesting example of a biomarker of androgenic compound exposure has been developed in sticklebacks. Male sticklebacks produce a protein to build spawning nests called spiggin, which is stored in the urinary bladder (Goksoyr et al. 2003). Female sticklebacks can produce spiggin when exposed to androgenic compounds (Jakobsson et al. 1999) making this process analogous to vitellogenin production by male fish when exposed to estrogenic compounds (Goksoyr et al. 2003).

1.2.2 Other EDC Effects

Many environmental fish studies have focused on estrogen and androgen receptor mediated responses, however different nuclear receptors can "cross-talk", which can increase the possible modes of gene-regulation (Vasudevan et al. 2002). For example, EDCs can elicit responses in fish via cross-talk between the aryl hydrocarbon receptor and the estrogen receptor (Goksoyr et al. 2003). Aryl hydrocarbon receptor agonists (e.g., dioxins, polyaromatic hydrocarbons and polychlorinated biphenyls) have been demonstrated to be antiestrogenic in fish. The aryl hydrocarbon receptor-ligand complex can suppress estrogenic responses by inhibitory interactions with the estrogen receptor

(Goksoyr et al. 2003). This may reduce vitellogenin production and affect egg development in female fish exposed to aryl hydrocarbon receptor agonists. Complex environmental mixtures of EDCs can bind to multiple nuclear receptors and induce many different mechanisms of endocrine disruption, but because of the potential for nuclear receptors to cross-talk, the potential effects of EDC exposure can be very complicated (Van Der Kraak et al. 1998).

Many chemicals can cause endocrine disruption by interference with hypothalamic-pituitary function. Similar to steroid hormones, thyroid hormones are under hypothalamic-pituitary control and have a crucial role in gonadal development and adult reproductive processes (Arcand-Hoy and Benson 1998). Thyroid hormones may act synergistically with gonadotropins to stimulate steroidogenesis (Cyr and Eales 1996) and thyroid hormones have direct influences on the pituitary and ovary, which could impact steroid levels in fish (Sullivan et al. 1989). Conversely, Cyr and Eales (1996) demonstrated that elevated estradiol levels in fish may depress the conversion of thyroxine to triiodothyronine, the more biologically active thyroid hormone, which controls thyroid hormone levels in fish. Because of the interactions between the steroid hormone and thyroid hormone pathways, EDCs that influence one pathway may elicit effects on the other pathway. Therefore, EDCs can potentially exert disruptive effects on organisms by directly altering the steroid hormone pathway, or indirectly by interacting with other parts of the endocrine pathway, such as the thyroid hormone pathway.

1.2.3 Complex mixture effects

There are multiple EDCs introduced to the environment that cause organisms to be exposed to many EDCs simultaneously. Wastewater effluents often contain complex mixtures of EDCs; therefore, linking a response to a specific compound is virtually impossible (Sumpter 1997). If a suite of EDCs all disrupt the endocrine system the same way (e.g., agonistically via the same receptor) then there is the potential for multiple EDCs to work in combination (Brian et al. 2007). Complex mixtures of estrogenic compounds can induce a greater response in fish than when fish are exposed to compounds individually (Brian et al. 2007). Potentially, many EDCs can have a

synergistic effect on organisms (Smolders et al. 2004), which can make predicting exposure effects on wildlife difficult. Currently, there is no single way to determine exactly how complex EDC mixtures impact wildlife. This has lead to the use of bioindicator species and performance endpoints to determine the cumulative effects of all chemical exposures on growth, reproduction and survival of natural populations (Munkittrick et al. 2000). This approach incorporates all stressors (natural or chemical) that an organism encounters in the environment and consequently is a valuable technique to determine the effects of complex mixtures on wildlife.

1.3 EDCs in the South Saskatchewan River Basin

Much of the research on contaminant impacts on water quality and fish populations in Alberta has focused on pulp mill effluents in Northern Alberta (Cash et al. 2000; McMaster et al. 2004) and the development of the oil sands in Northeast Alberta (van den Heuvel et al. 1999; Tetreault et al. 2003; Nero et al. 2006). Southern Alberta has many different water quality issues. Southern Alberta has the least surface water, but the largest population. There are industrial and intense agricultural activities. All of these factors can influence water quality. Due to over allocation, the provincial government has stopped allocating new water licenses in the South Saskatchewan River Basin (SSRB) to prevent further water removal for industrial and municipal usage until a new water management plan is developed (Alberta Environment, 2005). Despite these concerns over water quantity, Southern Alberta has one of the fastest growing populations in Canada that indicates that water demand will increase. Climate change models and analysis of historical stream flows suggest that river levels in the SSRB will decrease in future decades (Rood et al. 2005; Prairie Adaptation Research Collaborative, 2007, http://www.parc.ca/ssrb). Therefore, water usage will have to become more efficient to provide suitable in-stream flow to maintain river ecosystem integrity.

To address the potential impacts of EDCs in the SSRB, I used longnose dace (*Rhinichthys cataractae*) as a bioindicator species. Longnose dace are an abundant and widely distributed minnow throughout the SSRB. They feed opportunistically on benthic invertebrates (Beers and Culp, 1990), live a maximum 5 years, can reach 13 cm in length

(Nelson and Paetz, 1992), and reside in fast moving water with a cobble substrate (Mullen and Burton, 1998; Scott and Crossman, 1998). Adults typically reach sexual maturity at age two, but some individuals may be able to spawn at age one. Longnose dace have been known to spend over a year within the same reach of a stream (Hill and Grossman, 1987) and are believed to display high site fidelity. Spawning may occur multiple times (Roberts and Grossman, 2001) between mid-May and early-August (Scott and Crossman, 1998), and at least one of the parents defends the spawning territory (McPhail and Lindsay, 1970). Because of their relatively fast life history, site fidelity and wide distribution, longnose dace were chosen to evaluate multiple performance endpoints to examine possible exposures to EDCs along river gradients in the SSRB.

Municipal wastewater effluents are significant point sources of EDCs and many other chemicals to rivers (Kolpin et al. 2002). Agriculture, crop and animal production, are major non-point sources of EDCs and other contaminants to rivers (Kolpin et al. 2002; Kolodziej et al. 2004). Recently, many EDCs, and human and livestock pharmaceuticals have been detected in river water samples from the SSRB (Sosiak and Hebben 2005, Forrest et al. 2006), which indicates that human activities within the SSRB impact river water quality. The cumulative impacts of land-use are affecting longnose dace populations in the Red Deer River, a major river within the SSRB, which demonstrates that impacts on water quality have the ability to affect longnose dace populations (Jeffries 2004). It is very likely that EDCs are introduced to the rivers in the SSRB at concentrations high enough to adversely affect longnose dace populations.

The goals of this thesis is to determine whether EDCs are detectable in SSRB headwaters, and whether estrogenic compounds, a major class of EDCs, are adversely impacting longnose dace populations. This will be accomplished in two ways. First (chapter two), I will sample longnose dace at multiple sites within the SSRB. I will collect liver samples from male and female longnose dace to detect vitellogenin patterns at collection sites to determine how responses to estrogenic compounds change with distance downstream in these rivers. I will then compare the vitellogenin with individual and population level indices of fish health. This chapter will assess how physiological exposure scales up to individual and population levels of organization to demonstrate

biologically relevant consequences of EDC exposure. Very little is known about the "normal" physiology of longnose dace in the SSRB, therefore this thesis will also provide basic information on the environmental physiology of longnose dace. Second (chapter three), I will measure organic contaminants in river water to determine the presence of EDCs and other contaminants in the SSRB. This will address spatially how organic contaminants change with distance downstream at a scale much larger than usually employed in environmental studies. Hopefully, the results will provide SSRB managers sufficient information to incorporate EDCs into long-term water quality management plans by demonstrating the spatial distribution of EDCs and the biological effects on longnose dace in the SSRB.

Chapter Two: Basin-wide impacts of estrogenic compounds on longnose dace (*Rhinichthys cataractae*) populations in two prairie rivers in Southern Alberta, Canada.

2.1 Introduction

Concern is increasing over the presence of a broad range of environmental pollutants that can cause endocrine disruption in humans and wildlife. Endocrine disrupting compounds act on an organism by mimicking or interrupting steroid hormone function, which can affect the natural reproductive process (Mills and Chichester 2005). Many endocrine disrupting compounds, including industrial chemicals, phytoestrogens, synthetic and natural estrogens, have an estrogenic function (Burki et al. 2006). Estrogenic compounds can bind to estrogen receptors and regulate estrogen responsive genes (Jobling et al. 1996). Aquatic ecosystems are particularly affected by estrogenic compounds (Burki et al. 2006) due to a relatively constant input from wastewater effluent and by periodic input from surface run-off. Estrogenic compounds are often detected in rivers that receive wastewater effluent (Kolpin et al. 2002; Servos et al. 2005; Sarmah et al. 2006) or where there are agricultural activities within the basin (Orlando et al. 2004; Kolodziej et al. 2004).

Estrogenic compounds have the potential to affect aquatic life (Mills and Chichester 2005). The impact of estrogenic compounds on riverine fish populations is complicated by the addition of nutrients that can elevate river productivity (Chambers et al. 2000). Many nutrient sources are associated with wastewater effluent and agricultural run-off (Chambers et al. 2000). This can lead to more abundant and bigger fish downstream of nutrient sources (Adams et al. 1996; Gibbons et al. 1998), however the fish may appear healthy morphologically yet suffer physiological stresses from exposure to estrogenic compounds.

To detect the effects of estrogenic compounds, fish are often used because they are vulnerable to chemical pollutants. Chronic exposure to estrogenic compounds can affect fish by causing ovarian tissue development in the testes (Jobling et al. 1998), reduced sperm motility and concentration (Aravindakshan et al. 2004) and in extreme

cases, edema in ovaries, inhibited testicular development and kidney lesions (Palace et al. 2006). In laboratory studies, exposure to estrogenic compounds has resulted in reduced gonad size (Jobling et al. 1996) and female-biased sex ratios (Metcalfe et al. 2001).

A short-term response to estrogenic compound exposure in fish is the production of vitellogenin in male fish or altered vitellogenin production in females. Vitellogenin is a female-specific precursor to egg yolk protein that is produced in the liver. It can also be produced by male fish when they are exposed to estrogen and estrogenic compounds (Jobling et al. 1996). Vitellogenin is a widely used biomarker of estrogenic compound exposure in male fish and has been used to demonstrate the estrogenic effects of wastewater effluents on wild fish populations (Folmer et al. 2001; Burki et al. 2006; Jobling et al. 1998). There is no consensus as to whether low level exposure to estrogenic compounds will have long-term individual or population level consequences on wild fish populations (Hoger et al. 2006).

A sentinel fish approach is an effective technique to assess cumulative impacts on fish populations in rivers (Munkittrick et al. 2000). This approach can be applied at basin-wide scales (Hinck et al. 2006). By sampling multiple fish performance endpoints at morphological and physiological levels of organization, insight into the combined effect of nutrient inputs and estrogenic compound-related stresses on fish populations can be gained. Small-bodied fish are useful sentinel species because they have smaller home ranges, higher abundances and show changes in growth and reproduction faster than larger fish (Gibbons et al. 1998; Gray et al. 2002). Longnose dace (*Rhinichthys cataractae*) are an abundant and widely distributed minnow in Alberta, Canada. Longnose dace respond to changes in land-uses and are a suitable sentinel fish species for studies in Alberta rivers (McMaster et al. 2004; Jeffries 2004).

I used longnose dace to identify areas of estrogenic compound exposure in the Bow and Oldman Rivers, Alberta. I determined estrogenic compound exposure by measuring hepatic vitellogenin mRNA using a quantitative reverse transcription polymerase chain reaction (QRT-PCR) technique. QRT-PCR can detect increased hepatic vitellogenin mRNA levels in fish downstream of wastewater effluents (Burki et al. 2006).

I measured vitellogenin levels plus additional fish performance endpoints to assess how the physiological response of longnose dace to estrogenic compound exposure scales up to individual and population levels of organization. This study examines the cumulative effects of municipal wastewater effluent and agricultural land-uses on wild minnow populations along a prairie river gradient. Wastewater and agriculture can impact water quality and are common stresses in river basins globally.

2.2 Materials and Methods

2.2.1 River descriptions

The Oldman River has a drainage area of 28 200 km^2 and flows 450 km east from the continental divide to its confluence with the Bow River approximately 100 km west of Medicine Hat. The river originates as a fast flowing mountain stream and flows through foothills of the Rocky Mountains, before becoming a meandering prairie river. The city of Lethbridge (population 73 000) is the major municipality along the Oldman River and discharges tertiary treated sewage into the river. The mean annual river discharge at Lethbridge is 81.8 m^3/s (Seneka 2004). Fort Macleod (population 3 000) is located 88 km upstream of Lethbridge, and Taber (population 7 500) is located 85 km downstream of Lethbridge. Fort Macleod and Taber discharge rotating biological contactor treated wastewater (secondary treated effluent) into the Oldman River (C.W. Koning, Alberta Environment, Calgary, personal communication). Flow is regulated by the Oldman Dam located 152 km upstream of Lethbridge. Over 60 percent of the land-use in the Oldman Basin is agriculture. There are intense livestock operations in the basin, especially northeast of Lethbridge, and the region is heavily irrigated making the Oldman Basin one of the most intensively managed agricultural regions in Canada (ORBWQI 2005). Water quality generally deteriorates with distance downstream with no one dominant influence on water quality rather it is the cumulative effects of all activities in the basin that influence water quality (ORBWQI 2005).

I collected longnose dace from eight sites (spanning approximately 300 km) on the Oldman River in April and November 2005 (Fig. 2.1). Of the eight sites, four were

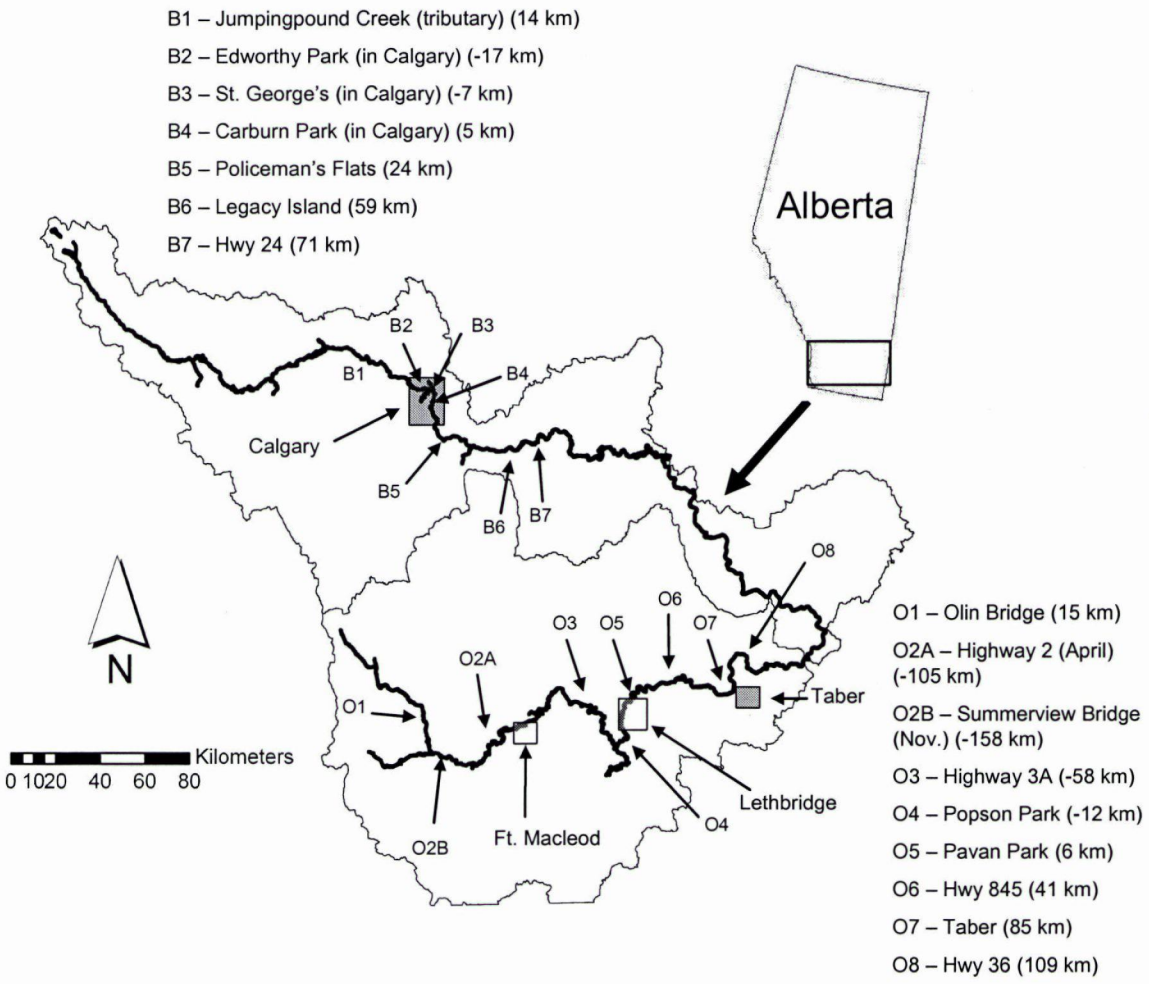


Figure 2.1 Map of the Bow and Oldman River Basins that shows sampling locations. Distances in parentheses are with respect to the Bonnybrook Wastewater Treatment Plant in the Bow River and the Lethbridge Wastewater Treatment Plant in the Oldman River. The Jumpingpound Creek site in the Bow River and Olin Bridge site in the Oldman River are both above reservoirs and therefore distances in parentheses are distances upstream of the reservoirs.

upstream of Lethbridge and four were downstream. The Highway 2 site was sampled in April and was replaced with the Summerview Bridge site in November. Only the Olin Bridge site was above the Oldman Reservoir; the other seven sites were downstream. One year (2005), five year and ten year averages of a suite of water quality parameters taken from three Alberta Environment long-term monitoring stations were used to characterize the Oldman River (Table 2.1). The three Oldman River monitoring stations included two sites where I collected fish and the Highway 3 site, which is located one km downstream of our Highway 3A fish collection site.

The Bow River has a drainage area of 25 132 km^2 and flows east 645 km from the continental divide to its confluence with the Oldman River (BRBC, 2005). Like the Oldman River, the Bow River originates as a fast flowing mountain stream, flows through the foothills of the Rocky Mountains and subsequently into the prairies. The city of Calgary (population 1 000 000) is the major municipality along the Bow River and discharges tertiary treated sewage into the Bow River from the Bonnybrook and Fish Creek Wastewater Treatment Plants. Calgary is the single largest contributor of pollutants and nutrients to the Bow River (BRBC 2005). The mean annual discharge of the Bow River at Calgary is 91.1 m^3/s (Seneka 2004). Flow is heavily regulated by the Bearspaw (immediately upstream of Calgary) and the Ghost Dams (26 km upstream of Calgary).

I sampled longnose dace from seven sites covering approximately 110 km on the Bow River (Fig. 2.1), including three sites upstream of the Bonnybrook Wastewater Treatment Plant and four downstream. Of the four downstream sites, only the Carburn Park site is located upstream of the Fish Creek Wastewater Treatment Plant. Longnose dace were sampled in the Jumpingpound Creek tributary due to the difficulty in collecting longnose dace in the Bow River mainstem upstream of Calgary. Five year and ten year averages of a suite of water quality parameters from two Alberta Environment long-term monitoring stations were used to characterize the Bow River (Carseland Weir, located one km upstream of Highway 24 and Cochrane, located 13 km upstream of Bearspaw Dam) (Table 2.2).

Table 2.1 Water quality parameters for 2005, and five year and ten year averages in the Oldman River, Alberta. Data are presented as means (SE).

River	Site	Average	Temp. (°C)	pH	DO (mg/L)	Turbidity (NTU)	S. Cond. (µS/cm)	TP (mg/L)	NO_3 +NO_2 (mg/L)	Total NH_3 (mg/L)	Cl^-
Oldman	Summerview	1 year	8.4	8.19	10.70	9.9	291.7	0.011	0.089	0.031	0.83
			(1.2)	(0.01)	(0.6)	(2.9)	(5.1)	(0.003)	(0.006)	(0.008)	(0.43)
		5 year	8.3	8.14	11.41	8.7	289.0	0.010	0.100	0.011	1.07
			(0.6)	(0.03)	(0.21)	(1.4)	(4.5)	(0.001)	(0.005)	(0.002)	(0.17)
		10 year	8.6	8.16	11.28	7.8	284.5	0.009	0.090	0.008	1.04
			(0.5)	(0.02)	(0.15)	(1.1)	(3.4)	(0.001)	(0.004)	(0.002)	(0.05)
	Hwy 3	1 year	10.5	8.35	10.80	68.7	368.4	0.070	0.084	0.033	10.67
			(2.2)	(0.07)	(0.44)	(46.5)	(16.5)	(0.050)	(0.025)	(0.008)	(9.07)
		5 year	9.7	8.15	10.77	37.7	342.2	0.069	0.124	0.017	2.46
			(1.0)	(0.03)	(0.22)	(12.8)	(6.1)	(0.039)	(0.019)	(0.004)	(0.54)
		10 year	9.7	8.17	10.66	54.4	330.6	0.078	0.107	0.017	2.19
			(0.7)	(0.02)	(0.15)	(16.2)	(4.5)	(0.027)	(0.015)	(0.003)	(0.30)
	Hwy 36	1 year	10.3	8.36	10.52	70.5	382.3	0.072	0.096	0.045	3.5
			(2.2)	(0.07)	(0.56)	(52.4)	(12.0)	(0.056)	(0.035)	(0.017)	(0.47)
		5 year	9.3	8.31	11.52	37.8	376.3	0.067	0.152	0.038	4.78
			(1.0)	(0.03)	(0.25)	(14.4)	(7.1)	(0.038)	(0.027)	(0.008)	(0.27)
		10 year	9.8	8.31	11.09	36.6	363.3	0.085	0.126	0.039	4.31
			(0.7)	(0.03)	(0.17)	(8.6)	(4.9)	(0.022)	(0.019)	(0.005)	(0.22)

Table 2.2 Water quality parameters for five year and ten year averages in the Bow River, Alberta. Data were not available for 2005 for the Bow River and therefore the one year average is not included. Data are presented as means (SE).

River	Site	Average	Temp. (°C)	pH	DO (mg/L)	Turbidity (NTU)	S. Cond. (µS/cm)	TP (mg/L)	NO_3 +NO_2 (mg/L)	Total NH_3 (mg/L)	Cl^-
Bow	Cochrane	5 year	5.7	8.16	11.73	4.3	287.7	0.006	0.087 (	0.005	1.92
			(0.8)	(0.03)	(0.22)	(2.2)	(4.4)	(0.002)	0.004)	(0.001)	(0.07)
		10 year	6.3	8.20	11.49	3.52	287.2	0.005	0.086	0.004	1.88
			(0.6)	(0.02)	(0.14)	(1.0)	(3.0)	(0.001)	(0.004)	(0.001)	(0.07)
	Hwy 24	5 year	6.7	7.93	11.30	20.8	361.4	0.061	0.921	0.190	11.32
			(1.0)	(0.06)	(0.18)	(8.7)	(7.7)	(0.009)	(0.054)	(0.025)	(0.80)
		10 year	7.0	7.98	11.10	16.1	359.7	0.057	0.841	0.173	10.43
			(0.7)	(0.04)	(0.13)	(4.8)	(4.8)	(0.005)	(0.041)	(0.016)	(0.85)

Temp.=temperature, DO=dissolved oxygen, S. Cond.=specific conductivity TP=total phosphorus, Chl a=chlorophyll a, NO_3 +NO_2=nitrate+nitrite, NH_3=ammonia, Cl^-=dissolved chloride.

2.2.2 Fish collections

Longnose dace were sampled during April 2005 and late October/early November 2005 using a backpack electrofishing unit (Model 12-B POW electrofisher, Smith-Root Inc.). One person operated the electrofisher and two people collected stunned fish with 6 mm mesh dip nets. Young of year (YOY) and adult fish were identified using length-frequency distributions from previous samplings. Length-frequency distributions for longnose dace in Alberta have been verified by otolith analysis and are a reliable technique to estimate age classes (Jeffries, unpublished data). Sites were fished until at least 10 adult females and 10 adult males were caught, if possible. Gender was determined by visual identification. All longnose dace were counted, and all adults were measured for length (forklength, ±1 mm) and weight (wet weight, ±0.01g). Adult longnose dace were dissected for liver and gonad weights (wet weight, ±0.001g). Adults remained alive until dissection, at which point they were euthanized by a cut through the spinal cord. Liver samples were taken for QRT-PCR analysis and were placed into autoclaved individual sterile polypropylene vials containing TRIZOL REAGENT (Invitrogen, Carlsbad, California) and immediately frozen in liquid nitrogen. After returning to the laboratory, samples were kept in a -80°C freezer until analyses were conducted. To maintain consistency among sample sites, only livers from males and females estimated to be age class two and three using length-frequency distributions were sampled for vitellogenin mRNA.

Catch per unit effort (CPUE) was determined at each site using all longnose dace caught. CPUE was used to estimate relative fish abundance and was standardized as the number of fish per 5 000 seconds of electrofishing effort. All adult fish were separated by sex for analysis of length-weight, liver weight-body weight and gonad weight-body weight relationships. Liver and gonad to body weight relationships were used as proxies for energy storage and reproductive effort, respectively (Munkittrick et al., 2000).

2.2.3 Partial cloning of longnose dace vitellogenin and β-actin

Total RNA was mechanically extracted from liver samples using TRIZOL REAGENT (Invitrogen, Carlsbad, California) and converted to cDNA using an oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, California), according to the manufacturer's protocol. Primers were as follows: Vitellogenin: (5'-CAAGAGTCTGATTGAAACTGC -3') and (5'-GCAGGAGATTTCAGAAGAGC-3'), β-actin: (5'-CCTCCATTGTTGGCACC -3') and (5'- CCTCTCTTGCTTTGAGCCTC -3'). PCR was carried out as in Nelson and Habibi (2006) using 3 μl of diluted cDNA, 0.56 μM of forward and reverse primer, 0.2 mM dNTPs, and *Taq* DNA polymerase to a total volume of 50 μl in buffer [1X= 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 1.4 mM $MgCl_2$]. Cycling was 2 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 53.5°C, 1 min at 72°C, followed by 5 min at 72°C. PCR products were run alongside a DNA ladder on 1% agarose gels stained with ethidium bromide. Specific bands were excised, DNA extracted and sequenced (University of Calgary). Details of the sequences can be found in appendix A.

2.2.4 Quantitative PCR

Primers were as follows, and all resulted in one amplicon (as determined by melt curve and gel-elecrophoresis analysis) and had an efficiency greater than 90%; Vitellogenin: (5'-GAAGTGCGCATGGTGGCTTGTATT-3') and (5'-AGCTGCCATATCAGGAGCAGTGAT-3') and β-actin: (5'-CCTCCATTGTTGGCACC -3') and (5'- CCTCTCTTGCTTTGAGCCTC -3'). As an internal control, β-actin was also amplified as described in Nelson et al. (2007). An iCycler iQ Multicolour Real-Time PCR Detection System (Bio-Rad, Hercules, California) was used with the following conditions per well: 0.5 μl of cDNA, 0.26μM of each primer, 0.2 mM dNTPs, Sybr Green (Invitrogen, Carlsbad, California) and *Taq* in buffer [10 mM Tris-HCL (pH 9), 50 mM KCl, 1.4 mM $MgCl_2$, 20 nM fluorescein] to a total volume of 25 μl. Each experimental group was run in triplicate to ensure consistency. Cycling was 3 minutes at 95°C, followed by 50 cycles of 10 seconds at 95°C

and 45 seconds at 55°C. Vitellogenin expression was normalized by converting it to a ratio between vitellogenin and β-actin (a housekeeping gene) expression.

2.2.5 Statistical analyses

I performed all statistical analyses with SAS software v.8.2 (SAS Institute, Cary, North Carolina). Data were natural log transformed, if necessary, to better meet the assumptions of parametric statistics (Sokal and Rohlf, 1995). Fish length-weight relationships were assessed by Analysis of Covariance (ANCOVA), with forklength as the covariate. Site differences in gonad and liver to body weight relationships were also assessed by ANCOVA with adjusted body weight as the covariate. Adjusted body weight for the livers was body weight minus liver weight, while adjusted body weight for the gonads was body weight minus gonad weight. All ANCOVAs were run separately for males and females. Heterogeneity of slopes tests were performed initially, and if the slopes were not statistically different, intercepts were compared. Tukey's pairwise comparisons were used to determine which sites were different. Homogeneity of variances were tested using F-max tests and normality was tested with Kolmogorov-Smirnov tests. All statistics were performed with a critical alpha value of 0.05, however, if the slopes in the ANCOVAs were not homogenous, *a posteriori* contrasts to determine differences in slopes were performed using an adjusted alpha following the Dunn-Šidák procedure. Differences between sites in vitellogenin mRNA levels were analyzed using a nonparametric Kruskal-Wallis test and multiple pairwise comparisons were conducted using the Bonferroni adjustment method (Rao 1998).

2.3 Results

2.3.1 Water Characteristics

During the summer and fall of 2005, there was substantial flooding in Southern Alberta that brought the Oldman River to approximately three and a half times higher than the 95 year mean annual discharge for the month of November (Environment

Canada, 2006, http://www.wsc.ec.gc.ca/hydat/H2O/index_e.cfm). As a result, longnose dace adults were not sampled at the Highway 36 site and insufficient numbers were found at the Highway 2 site in November. Longnose dace were caught 53 km upstream of Highway 2 (Summerview Bridge) and this site was used to replace the Highway 2 site in November 2005. In the Bow River, flooding elevated the river levels to approximately two and a half times the 95 year mean annual discharge for the month of June, but had dropped to approximately one and a half times the 95 year mean annual discharge during October sampling (Environment Canada, 2006, http://www.wsc.ec.gc.ca/hydat/H2O/index_e.cfm). This flooding caused major river bed scouring downstream of Calgary and resulted in adult longnose dace not being found at the Carburn Park and Legacy Island sites during the October sampling, while insufficient numbers of adults were found at Policeman's Flats. Therefore, these sites were removed from the October analyses.

Despite the flooding, water quality parameters for 2005 are similar to the five and ten year averages in the Oldman River except for an increase in mean turbidity. Many water quality parameters are comparable between the Highway 3 and Highway 36 water monitoring sites, which suggests that land-uses in the Oldman Basin between those sites have a similar influence on the water quality with respect to these water quality parameters measured. The one year (2005) average was not available for the Bow River and therefore only five year and ten year averages were determined. For both rivers, specific conductivity, water column chlorophyll a, chloride, phosphorus and nitrogen increase with distance downstream, which can all be indicative of anthropogenic activity within the basins.

2.3.2 Vitellogenin mRNA levels

In all cases, females had greater hepatic vitellogenin mRNA levels than males in the Oldman River (see example, Fig. 2.2a). Only the April samples are presented, however the November results were similar in terms of females produced greater levels of vitellogenin than males. Male longnose dace at the Highway 845 and Taber sites had significantly higher vitellogenin mRNA levels than the most upstream Olin Bridge site

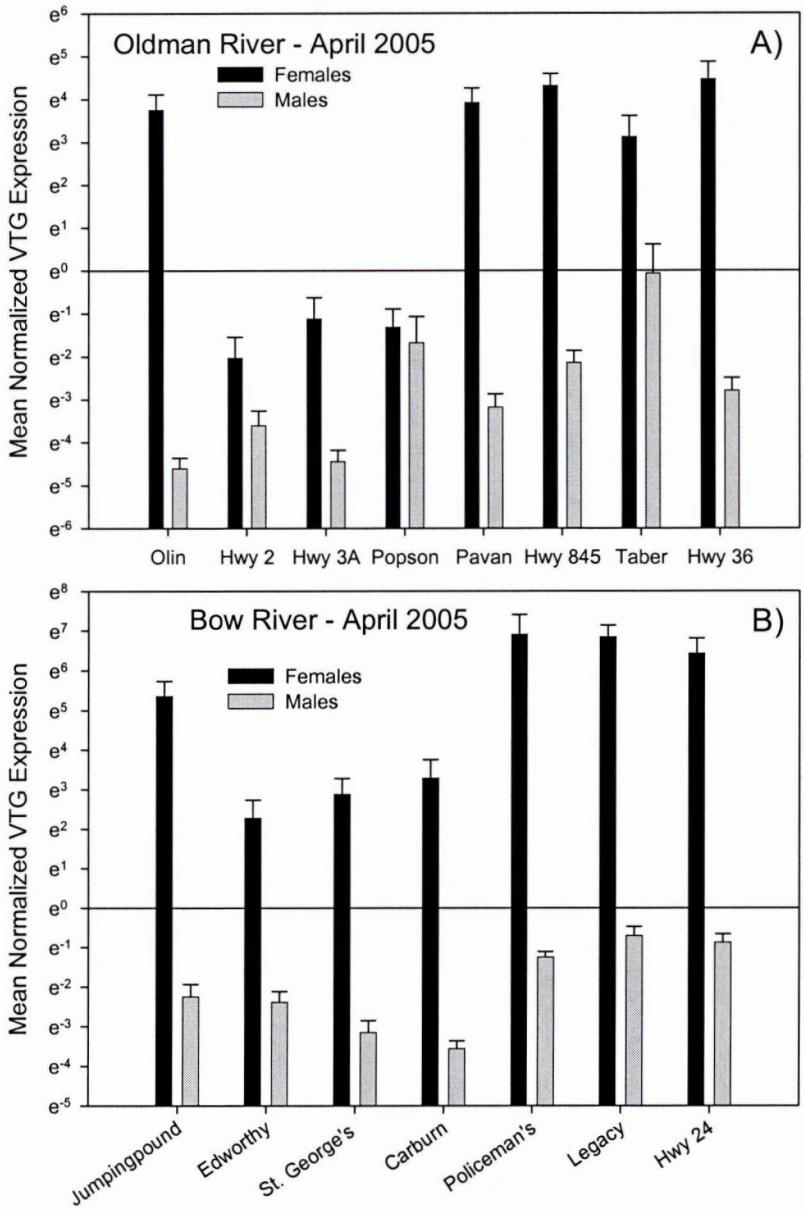


Figure 2.2 Comparison of mean normalized vitellogenin mRNA expression in female and male longnose dace (*Rhinichthys cataractae*) sampled in the A) Oldman River and B) Bow River Alberta, in April 2005.

and the Highway 3A site (χ^2=18.25, p<0.02) in April 2005 (Fig. 2.3). Female longnose dace at the most upstream Olin Bridge site and the four downstream of Lethbridge sites produced significantly more vitellogenin mRNA than females from the remaining three sites (χ^2=42.88, p<0.0001) in April 2005 (Fig. 2.4). In October, males at the three most upstream sites had higher vitellogenin mRNA than the Pavan Park and Highway 845 sites, however the pattern was not significant (χ^2=9.70, p>0.05). In October, females at the Pavan Park and Highway 845 sites produced significantly less vitellogenin mRNA than the three most upstream sites (χ^2=27.00, p<0.0001).

As in the Oldman River, females had greater vitellogenin mRNA levels than males in the Bow River (Fig. 2.2b). Again, only the males and females from April are presented, but the females produced more vitellogenin than males in October. Male longnose dace from the three sites downstream of Calgary had greater vitellogenin levels (χ^2=46.41, p<0.0001) than the upstream sites in April (Fig. 2.5). Females from the three sites downstream of Calgary and the Jumpingpound site had greater vitellogenin mRNA levels than the remaining three sites (χ^2=28.03, p<0.0001) in April (Fig. 2.6). In October, the males at Highway 24 had the highest mean vitellogenin mRNA levels, although this pattern was not statistically significant (χ^2=4.05, p>0.05). Females from the Jumpingpound site had greater vitellogenin levels than the other sites in October (χ^2=17.88, p<0.001).

2.3.3 Relative abundance and sex ratios

Catch per unit effort generally increased downstream of municipalities in the Oldman and Bow Rivers during both sampling occasions. In the Oldman River, CPUE was highest at Taber during April and Highway 845 in November (Fig. 2.7). Sex ratios in the Oldman River were female-biased, and as skewed as much as 90 percent female at the Highway 845 site in April. Only the Taber site in November, which had high incidences of parasitism that made sexual assignment difficult, had more males than females. In the Bow River, the CPUE was highest at the Jumpingpound site on both sampling occasions, but increased at the most downstream of Calgary sites (Fig. 2.8). The sex ratios in the Bow River are also female-biased, but less so than the Oldman River. Only the

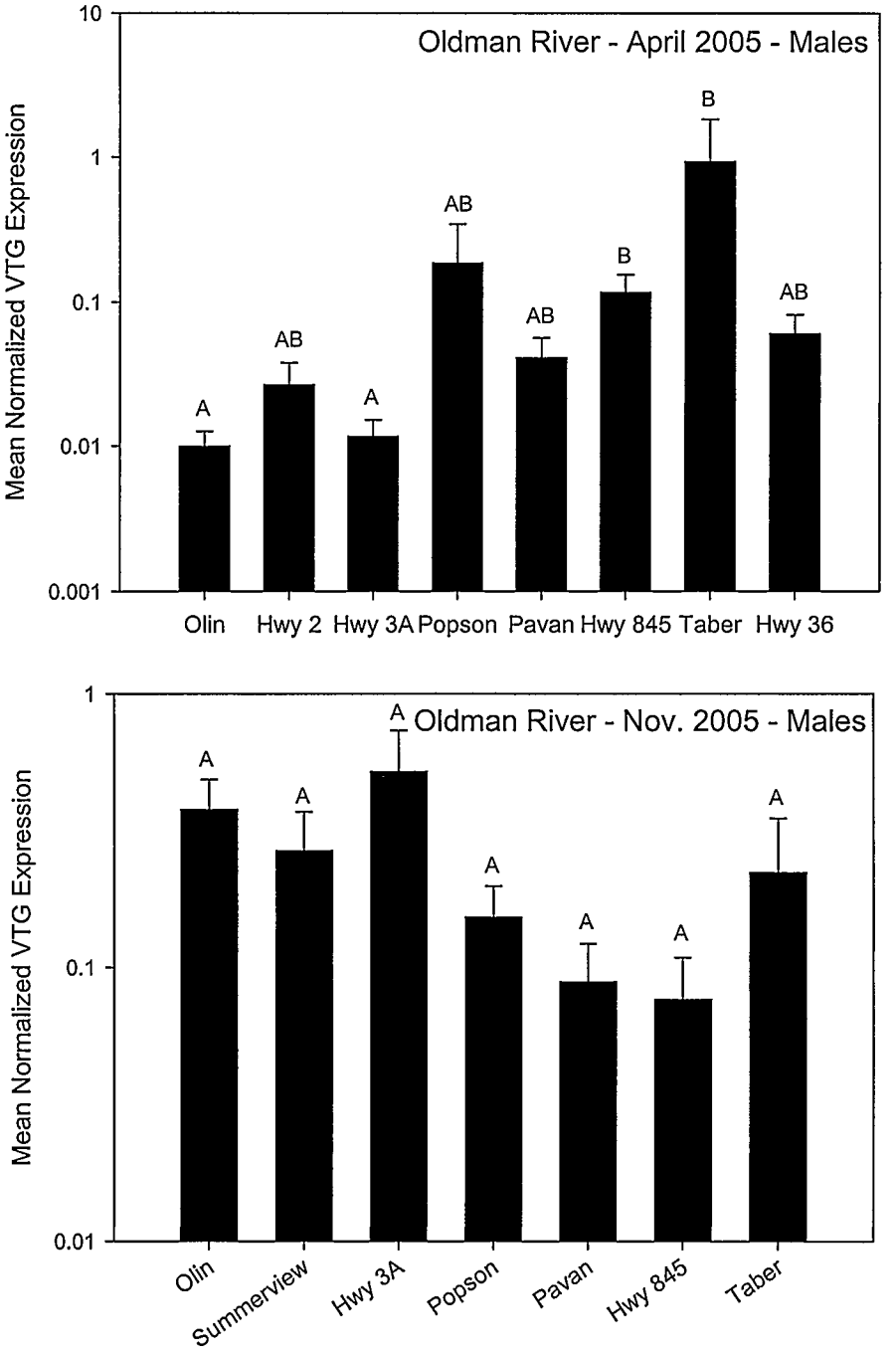


Figure 2.3 Mean normalized vitellogenin mRNA expression in male longnose dace (*Rhinichthys cataractae*) sampled in the Oldman River, Alberta, in April and November 2005. Different letters indicate statistical significance at $p<0.05$.

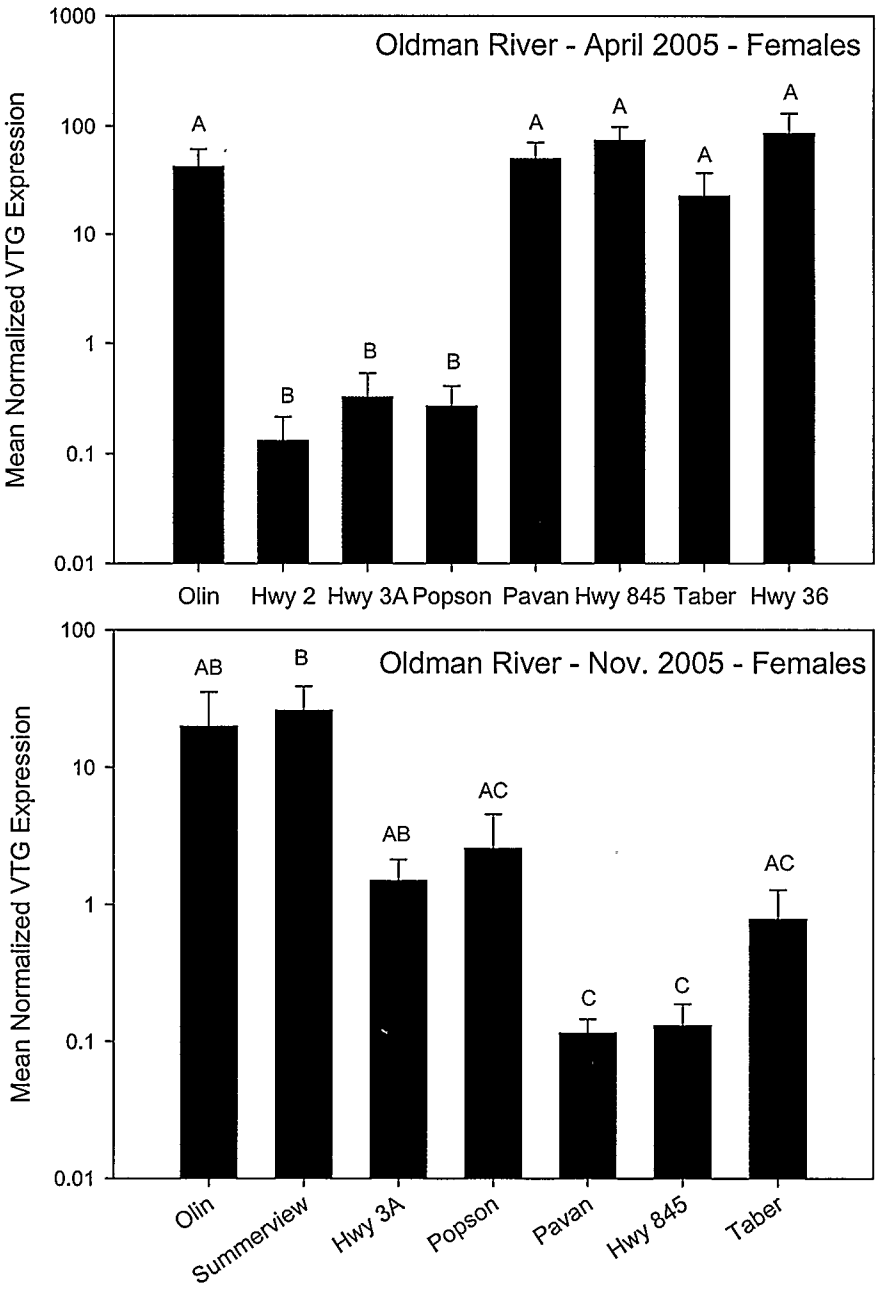


Figure 2.4 Mean normalized vitellogenin mRNA expression in female longnose dace (*Rhinichthys cataractae*) sampled in the Oldman River, Alberta, in April and November 2005. Different letters indicate statistical significance at $p<0.05$.

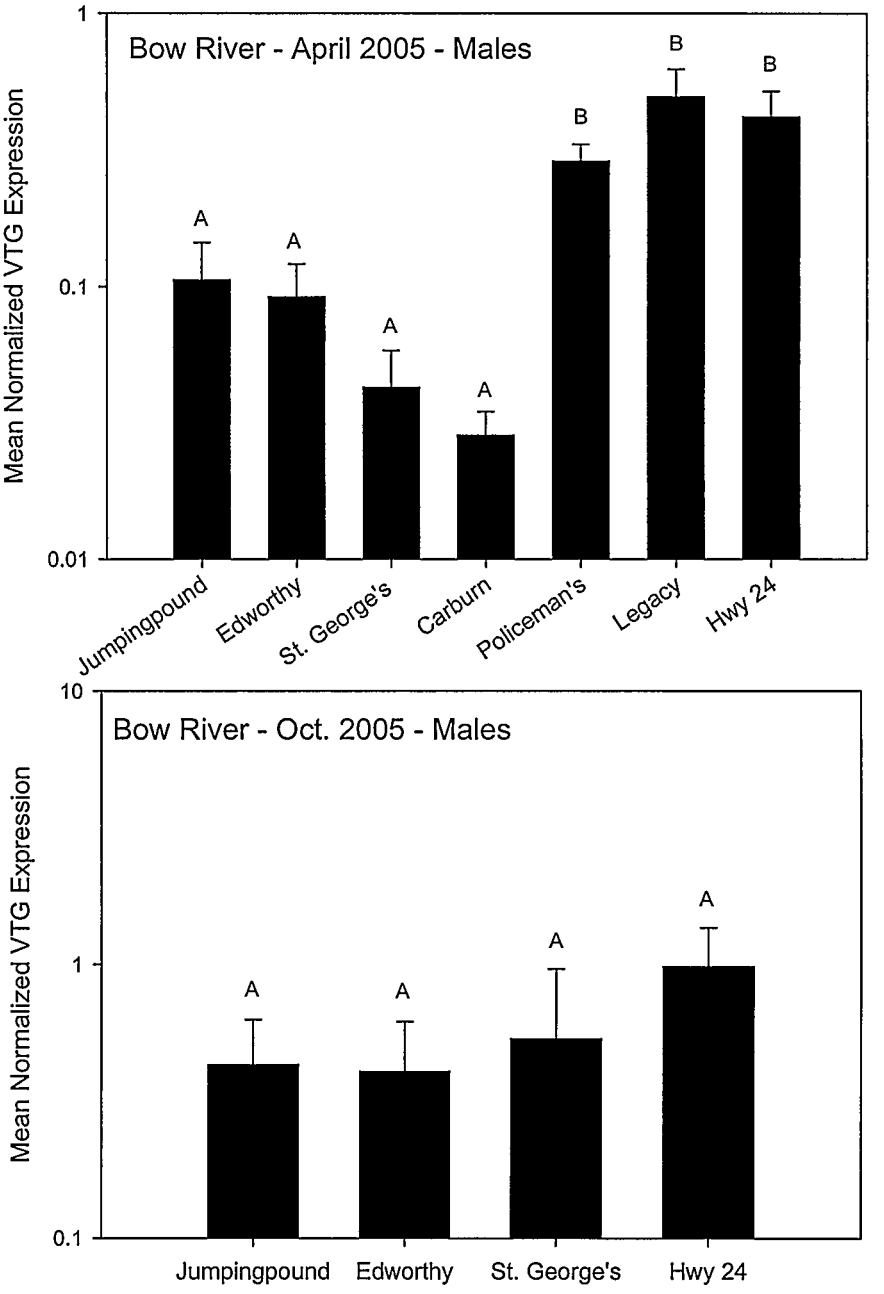


Figure 2.5 Mean normalized vitellogenin mRNA expression in male longnose dace (*Rhinichthys cataractae*) sampled in the Bow River, Alberta, in April and October 2005. Different letters indicate statistical significance at $p<0.05$.

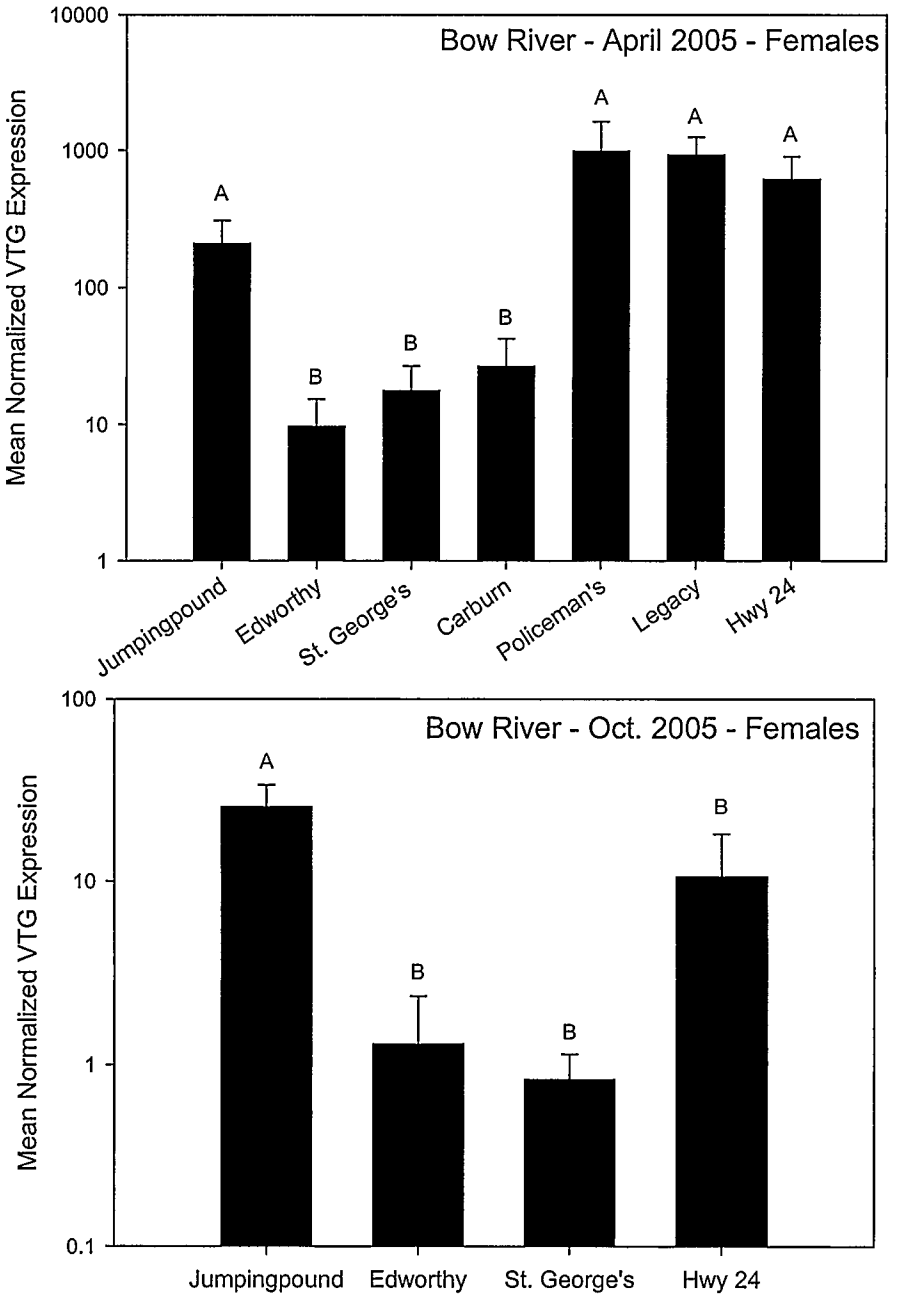


Figure 2.6 Mean normalized vitellogenin mRNA expression in female longnose dace (*Rhinichthys cataractae*) sampled in the Bow River, Alberta, in April and October 2005. Different letters indicate statistical significance at $p<0.05$.

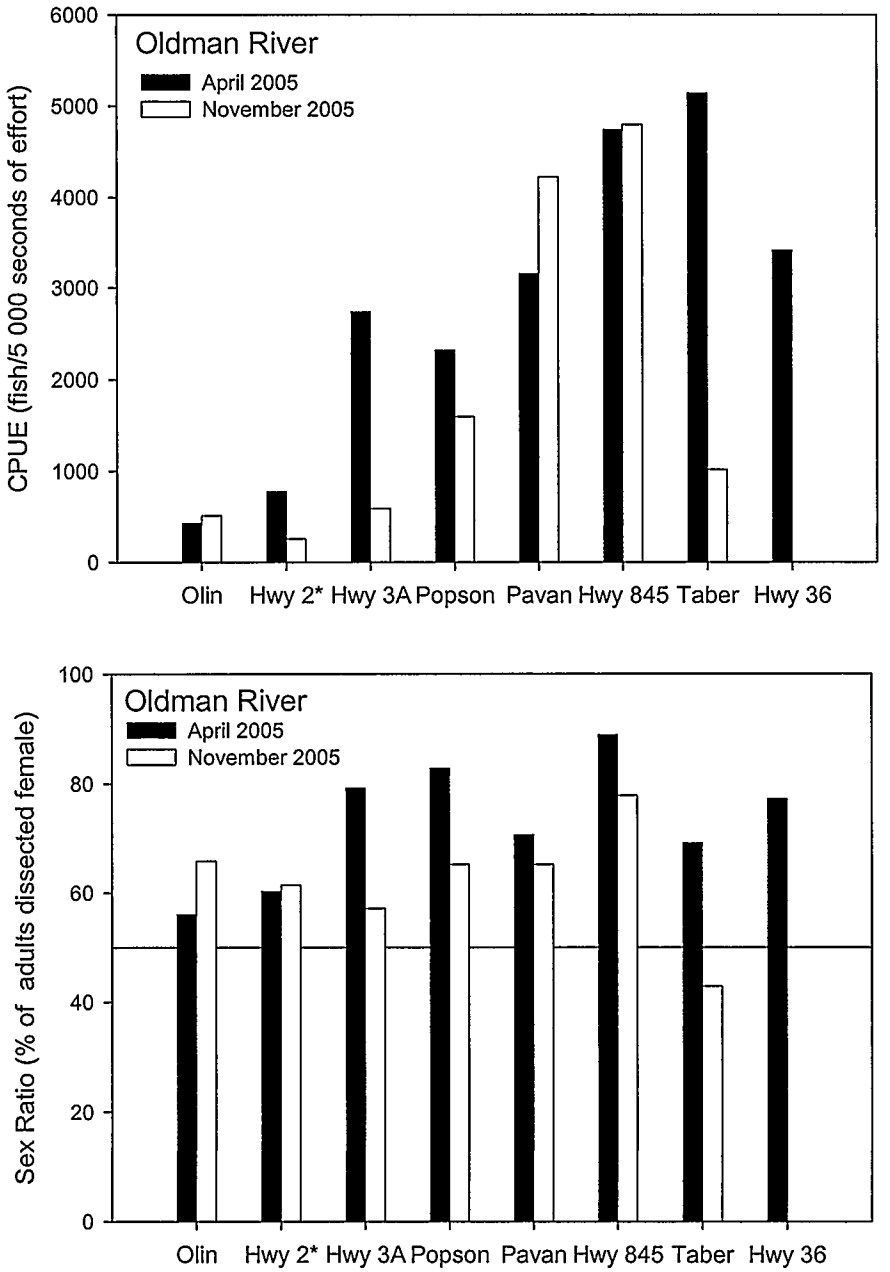


Figure 2.7 Catch per unit effort (CPUE) and sex ratios of longnose dace (*Rhinichthys cataractae*) collected from the Oldman River, Alberta, in April and November 2005. Highway 2 was not sampled in November 2005, therefore the values are for Summerview bridge.

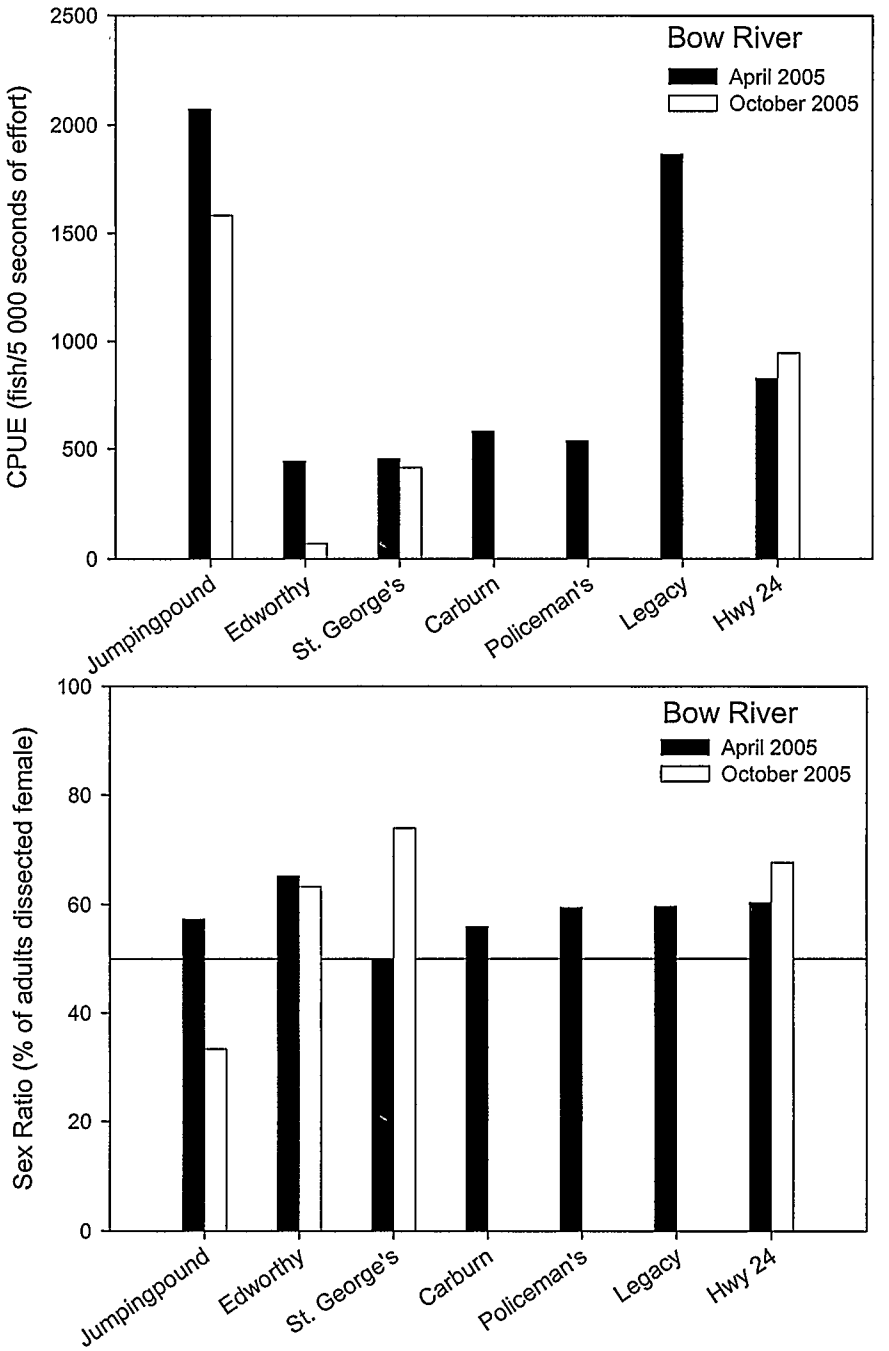


Figure 2.8 Catch per unit effort (CPUE) and sex ratios of longnose dace (*Rhinichthys cataractae*) collected from the Bow River, Alberta, in April and October 2005.

Jumpingpound site in October had more males than females during the two sample periods.

2.3.4 Relative body, liver and gonad weights

Length-weight, liver weight-body weight and gonad weight-body weight relationships were used to estimate relative body weight, liver and gonad weights via ANCOVAs. If the slope of the regression estimate was significantly different, the mean weights were not compared in the ANCOVA because of a violation of the assumptions of the ANCOVA. In these instances, only the slopes of the regression estimates were statistically compared and subsequently any sites with homogeneous slopes were compared by ANCOVA. Longnose dace were heaviest at sites downstream of Lethbridge for males ($F=16.16$, $p<0.0001$) and females ($F=10.43$, $p<0.0001$) in April 2005 (Table 2.3). However, the slopes for the length-weight relationships were not homogeneous ($p<0.0001$), therefore the females at the Highway 2, Highway 845 and Taber sites were excluded from the ANCOVA. Longnose dace had greater relative liver weights downstream of Lethbridge for males ($F=12.21$, $p<0.0001$) and females ($F=15.57$, $p<0.0001$) in April 2005. Males at Taber and Highway 36 had significantly larger gonads than immediately upstream of Lethbridge ($F=3.61$, $p<0.002$). Females at Pavan Park and Highway 36 had significantly larger gonads than at the most upstream (Olin Bridge) site (11.85, $p<0.0001$). The slopes of the gonad weight-body weight relationships were not homogeneous ($p<0.0001$) and the Highway 2 and Highway 845 females were excluded from the ANCOVA.

In November 2005, there was no significant difference in body weight ($F=0.94$, $p>0.05$), liver weight ($F=0.54$, $p>0.05$) or gonad weight ($F=2.17$, $p>0.05$) for male longnose dace in the Oldman River (Table 2.4). For females, there was also no significant difference in body weight between sites ($F=1.15$, $p>0.05$). Females at Highway 3A had significantly larger livers than at Pavan Park and Taber ($F=4.64$, $p<0.001$). Females at Olin Bridge had significantly smaller gonads than those at Summerview ($F=4.33$, $p<0.001$).

Table 2.3 Relative body, liver and gonad weights of longnose dace (*Rhinichthys cataractae*) from the Oldman River, Alberta, in April 2005. Letters within a column indicate statistical differences at $p<0.05$. Standard error of the estimate is in parentheses.

		Body Weight		Liver Weight		Gonad Weight	
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean[a]
Olin Bridge	Male	3.37(0.16)A	0.52(0.03)ABC	0.53(0.20)A	-3.76(0.10)A	1.47(0.27)A	-4.44(0.14)AB
Hwy 2	Male	2.89(0.21)A	0.44(0.02)B	1.34(0.25)A	-3.40(0.06)AB	1.40(0.39)A	-4.35(0.10)AB
Hwy 3A	Male	3.09(0.44)A	0.45(0.02)B	1.76(0.41)A	-3.31(0.08)BC	2.47(0.67)A	-4.41(0.12)AB
Popson Park	Male	2.81(0.30)A	0.47(0.02)AB	0.61(0.35)A	-3.51(0.09)AB	1.91(0.56)A	-4.55(0.13)A
Pavan Park	Male	2.81(0.33)A	0.61(0.02)CD	1.06(0.37)A	-2.99(0.06)C	1.54(0.58)A	-4.11(0.10)AB
Hwy 845	Male	3.18(0.40)A	0.69(0.03)D	0.56(0.40)A	-2.93(0.10)C	2.11(0.64)A	-4.42(0.15)AB
Taber	Male	3.01(0.35)A	0.58(0.02)C	0.69(0.34)A	-3.22(0.06)BC	1.75(0.54)A	-4.05(0.09)B
Hwy 36	Male	2.60(0.32)A	0.56(0.02)AC	1.39(0.40)A	-3.45(0.08)AB	0.87(0.62)A	-3.98(0.11)B
		Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean
Olin Bridge	Female	3.36(0.08)AB	0.70(0.02)AB	1.06(0.09)A	-3.44(0.06)A	0.081(0.002)A	0.098(0.007)A
Hwy 2	Female	3.42(0.08)B	N/A[b]	1.13(0.10)A	-3.15(0.05)BC	0.055(0.004)BC	N/A[b]
Hwy 3A	Female	3.14(0.07)A	0.71(0.01)B	0.99(0.10)A	-2.95(0.05)CDE	0.075(0.004)A	0.123(0.006)AB
Popson Park	Female	3.16(0.10)A	0.72(0.01)AB	0.97(0.13)A	-3.25(0.05)AB	0.073(0.005)A	0.125(0.006)AB
Pavan Park	Female	3.18(0.11)A	0.79(0.01)C	0.93(0.12)A	-2.90(0.05)DE	0.088(0.006)A	0.150(0.006)C
Hwy 845	Female	2.83(0.11)C	N/A[b]	0.93(0.15)A	-2.78(0.05)E	0.038(0.007)C	N/A[b]
Taber	Female	2.72(0.13)C	N/A[b]	1.03(0.20)A	-2.96(0.05)CDE	0.064(0.009)AB	0.142(0.006)BC
Hwy 36	Female	2.99(0.13)AC	0.76(0.01)AC	0.99(0.16)A	-3.09(0.05)BCD	0.083(0.01)A	0.160(0.006)C

[a] LN transformed. [b] was not used in the ANCOVA because the slope was significantly different.

Table 2.4 Relative body, liver and gonad weights of longnose dace (*Rhinichthys cataractae*) from the Oldman River, Alberta, in November 2005. Letters within a column indicate statistical differences at $p<0.05$. Standard error of the estimate is in parentheses.

		Body Weight		Liver Weight		Gonad Weight	
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean[a]
Olin Bridge	Male	2.88(0.17)A	0.73(0.02)A	0.86(0.21)A	-3.21(0.08)A	1.82(0.21)A	-3.99(0.08)AB
Summerview	Male	3.35(0.18)A	0.77(0.03)A	0.96(0.19)A	-3.32(0.09)A	1.42(0.23)A	-3.68(0.11)A
Hwy 3A	Male	3.07(0.39)A	0.73(0.03)A	1.37(0.45)A	-3.09(0.09)A	1.56(0.45)A	-4.09(0.10)AB
Popson Park	Male	2.72(0.51)A	0.78(0.03)A	1.64(0.66)A	-3.15(0.10)A	2.13(0.66)A	-4.28(0.11)B
Pavan Park	Male	2.79(0.41)A	0.76(0.03)A	1.23(0.52)A	-3.19(0.10)A	1.24(0.52)A	-4.15(0.11)AB
Hwy 845	Male	3.20(0.28)A	0.76(0.04)A	1.14(0.32)A	-3.13(0.13)A	0.91(0.32)A	-4.22(0.14)AB
Taber	Male	2.57(0.47)A	0.79(0.02)A	1.57(0.58)A	-3.11(0.08)A	0.10(0.59)B	N/A[b]
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean[a]
Olin Bridge	Female	3.14(0.06)A	0.85(0.01)A	1.23(0.07)A	-3.01(0.05)AB	1.30(0.16)A	-2.18(0.07)A
Summerview	Female	3.05(0.07)A	0.86(0.01)A	0.99(0.07)A	-3.12(0.05)ABC	1.43(0.14)A	-1.86(0.07)B
Hwy 3A	Female	3.26(0.17)A	0.82(0.02)A	1.33(0.18)A	-2.93(0.07)A	1.53(0.56)A	-2.11(0.08)AB
Popson Park	Female	3.38(0.18)A	0.82(0.02)A	1.47(0.18)A	-3.09(0.06)ABC	1.42(0.31)A	-2.16(0.08)AB
Pavan Park	Female	3.24(0.19)A	0.83(0.02)A	0.94(0.20)A	-3.20(0.06)BC	2.09(0.25)A	-2.16(0.06)AB
Hwy 845	Female	3.18(0.25)A	0.87(0.02)A	1.26(0.26)A	-3.15(0.06)ABC	1.70(0.28)A	-1.92(0.07)AB
Taber	Female	2.89(0.39)A	0.85(0.02)A	1.24(0.46)A	-3.38(0.08)C	1.05(0.48)A	-2.00(0.09)AB

[a]LN transformed.

[b] was not used in the ANCOVA because the slope was significantly different.

Table 2.5 Relative body, liver and gonad weights of longnose dace (*Rhinichthys cataractae*) from the Bow River, Alberta, in April 2005. Letters within a column indicate statistical differences at $p<0.05$. Standard error of the estimate is in parentheses.

		Body Weight[a]		Liver Weight[a]		Gonad Weight	
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean
Jumpingpound	Male	3.24(0.13)A	1.06(0.01)A	1.22(0.16)A	N/A[b]	0.031(0.003)A	0.51(0.003)A
Edworthy Park	Male	3.14(0.20)A	0.96(0.02)BC	0.66(0.29)B	-3.13(0.06)A	0.025(0.006)A	0.033(0.003)B
St. George's	Male	2.93(0.11)A	0.92(0.02)B	0.49(0.16)B	-3.32(0.06)A	0.021(0.004)A	0.028(0.004)B
Carburn Park	Male	3.24(0.18)A	0.93(0.02)B	1.68(0.21)C	N/A[b]	0.019(0.004)A	0.039(0.004)AB
Policeman's Flats	Male	2.67(0.34)A	1.08(0.02)A	1.05(0.46)AB	-2.70(0.08)B	0.049(0.007)B	N/A[b]
Legacy Island	Male	3.25(0.18)A	1.02(0.02)AC	1.14(0.20)AB	-2.86(0.06)B	0.028(0.004)A	0.052(0.003)A
Hwy 24	Male	3.19(0.17)A	1.05(0.02)A	0.88(0.20)AB	-2.71(0.06)B	0.030(0.003)A	0.049(0.003)A
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean
Jumpingpound	Female	3.20(0.08)A	1.19(0.01)A	1.16(0.10)A	-2.34(0.04)A	0.10(0.005)A	0.18(0.008)A
Edworthy Park	Female	3.21(0.09)A	1.01(0.01)B	1.02(0.12)A	-2.94(0.05)B	0.074(0.005)B	0.22(0.009)B
St. George's	Female	3.03(0.08)A	1.03(0.02)B	0.95(0.11)A	-2.99(0.06)B	0.078(0.006)B	0.24(0.012)B
Carburn Park	Female	3.25(0.14)A	1.04(0.02)B	1.56(0.15)B	N/A[b]	0.11(0.01)A	0.15(0.012)A
Policeman's Flats	Female	3.26(0.13)A	1.18(0.02)AC	1.27(0.13)AB	-2.55(0.06)AC	0.13(0.007)C	0.10(0.014)C
Legacy Island	Female	3.30(0.11)A	1.12(0.01)C	1.23(0.11)A	-2.58(0.05)C	0.15(0.007)C	0.19(0.01)D
Hwy 24	Female	3.36(0.10)A	1.12(0.01)C	1.11(0.11)A	-2.57(0.05)C	0.11(0.007)A	0.16(0.008)A

[a]LN transformed.

[b] was not used in the ANCOVA because the slope was significantly different.

Male longnose dace at Jumpingpound, Policeman's Flats and Highway 24 were significantly larger ($F=13.65$, $p<0.0001$) than at Edworthy Park, St. George's and Carburn Park (Table 2.5). Males downstream of Calgary had significantly larger liver weights than at Edworthy Park and St. George's ($F=19.00$, $p<0.0001$). This ANCOVA excluded the Jumpingpound and Carburn Park sites as the slopes of the relationships were significantly greater at those two sites ($p<0.001$). Males at Jumpingpound, Legacy Island and Highway 24 had significantly larger gonads than longnose dace at the Edworthy Park and St. George's sites ($F=8.47$, $p<0.0001$). The slope of the gonad weight-body weight relationship for males at Policeman's Flats was significantly greater than the other slopes ($p<0.02$) and was not used in the ANCOVA.

Female longnose dace at Edworthy Park, St. George's and Carburn Park had significantly smaller body weights than the other sites ($F=25.51$, $p<0.0001$) in April 2005. Females at Edworthy Park and St. George's also had significantly smaller livers than the other sites ($F=24.73$, $p<0.0001$); however, the Carburn Park site was not included in the ANCOVA because the slope of the relationship was significantly greater than the other sites ($p<0.02$). The slope of the gonad weight-body weight relationships for females at Edworthy Park and St. George's were significantly lower than at other sites, while at Policeman's Flats and Legacy Island, they were significantly greater ($p<0.0001$). Only sites with parallel slopes were compared with an ANCOVA. There was no difference in relative gonad weight between females at Edworthy Park and St. George's ($F=2.58$, $p>0.05$), and no difference between females at Jumpingpound, Carburn Park and Highway 24 ($F=2.12$, $p>0.05$). Females at Legacy Island had significantly larger gonad weights than at Policeman's Flats ($F=24.47$, $p<0.0001$).

Longnose dace were heavier at the Jumpingpound and Highway 24 sites for males ($F=23.32$, $p<0.0001$) and females ($F=46.61$, $p<0.0001$) in October 2005 in the Bow River (Table 2.6). Relative liver weights were greater at the Jumpingpound and Highway 24 sites for both males ($F=13.05$, $p<0.0001$) and females ($F=18.38$, $p<0.0001$). Males at the Jumpingpound site had significantly greater relative gonads weights than males at the Edworthy Park site ($F=4.42$, $p<0.01$). There was no statistical difference amongst sites for relative gonad weights in October 2005 for females (2.32, $p>0.05$).

Table 2.6 Relative body, liver and gonad weights of longnose dace (*Rhinichthys cataractae*) from the Bow River, Alberta, in October 2005. Letters within a column indicate statistical differences at $p<0.05$. Standard error of the estimate is in parentheses.

		Body Weight		Liver Weight		Gonad Weight	
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean
Jumpingpound	Male	3.11(0.11)A	1.07(0.01)A	1.64(0.24)A	-2.68(0.06)A	0.013(0.002)A	0.034(0.002)A
Edworthy Park	Male	2.63(0.30)A	0.90(0.03)B	1.33(0.52)A	-3.44(0.12)B	0.009(0.007)A	0.021(0.004)B
St. George's	Male	3.12(0.24)A	0.94(0.02)B	1.44(0.36)A	-3.00(0.09)C	0.016(0.004)A	0.029(0.003)AB
Hwy 24	Male	3.01(0.19)A	1.08(0.02)A	1.41(0.29)A	-2.64(0.09)A	0.014(0.003)A	0.028(0.003)AB
Site	Sex	Slope	LS mean[a]	Slope	LS mean[a]	Slope	LS mean
Jumpingpound	Female	2.96(0.07)A	1.16(0.02)A	1.24(0.11)A	-2.75(0.05)A	0.11(0.01)A	0.18(0.01)A
Edworthy Park	Female	3.07(0.14)A	0.99(0.02)B	0.96(0.14)A	-3.20(0.07)B	0.08(0.01)A	0.13(0.02)A
St. George's	Female	3.21(0.09)A	0.99(0.01)B	1.15(0.10)A	-3.09(0.04)B	0.10(0.01)A	0.15(0.01)A
Hwy 24	Female	3.05(0.11)A	1.17(0.01)A	1.05(0.12)A	-2.73(0.05)A	0.09(0.01)A	0.15(0.01)A

[a]LN transformed.

2.4 Discussion

I used longnose dace to detect exposure to estrogenic compounds in the Bow and Oldman Rivers, Alberta, by measuring hepatic vitellogenin mRNA. Longnose dace were used as a sentinel fish species to tell me where they were being exposed to estrogenic compounds. I initially sampled longnose dace populations at multiple sites along natural river gradients and then worked backwards to identify the sources of stresses that may be affecting those populations. The goal of this study was to assess the cumulative impacts of municipal wastewater and agriculture on prairie river fish populations.

2.4.1 Vitellogenin mRNA levels

2.4.1.1 Oldman River

In April 2005 in the Oldman River, male and female longnose dace had greater hepatic vitellogenin mRNA levels downstream of Lethbridge compared to upstream, which indicates estrogenic compound exposure. Estrogenic compounds are commonly detected in rivers downstream of municipal wastewater effluents (Kolpin et al. 2002). In the Oldman River, Sosiak and Hebben (2005) measured pharmaceuticals and endocrine disrupting chemicals, including estrogenic compounds, in river water downstream of Lethbridge, which is consistent with a municipal wastewater source. Male and female longnose dace have elevated vitellogenin levels well downstream of Lethbridge at the Taber and Highway 36 sites. Taber's wastewater effluent likely introduces estrogenic compounds into the river, which may affect longnose dace at the Highway 36 site.

In addition to municipal wastewater inputs, there are numerous cattle feedlots in the Oldman Basin between Lethbridge and Taber which potentially impact the river. Kolodziej et al. (2004) showed that cattle operations can introduce steroid estrogens to rivers and can be a major source of steroid hormones in certain watersheds. Forrest et al. (2006) found various livestock pharmaceuticals in tributaries of the Oldman River, which demonstrates that the cattle operations in the Oldman Basin are having an impact on the river. The vitellogenin response increased with distance downstream, which suggests that

municipal wastewater and cattle operations cause a cumulative impact expressed as the vitellogenin response in longnose dace. In November, longnose dace at the most upstream site in Oldman River also had elevated vitellogenin levels. There are cattle operations in the area, and the Oldman River does not receive any wastewater effluent upstream of that site, which further suggests that cattle operations may be a significant source of estrogenic compounds to the river.

During the summer of 2005, there were large floods in Southern Alberta that caused the Oldman River to exceed normal levels, which was still evident in early November. Floods caused a change in the vitellogenin patterns for males and females. However, the patterns were similar between sexes, which suggests that the patterns are externally driven and not entirely due to natural reproductive processes. Downstream of Lethbridge, females produced significantly *less* vitellogenin than the most upstream sites. Solé et al. (2003) identified a reduction in vitellogenin production in female carp downstream of a wastewater effluent; here estrogen mimics caused a negative feedback of gonadotropin secretion that resulted in reduced vitellogenin synthesis. Estrogenic compounds may potentially inhibit gonadotropin secretion which may result in reduced estradiol released from the ovaries to induce vitellogenin production in the liver. Alternatively, longnose dace at Pavan Park and Highway 845 could be exposed to androgenic or anti-estrogenic compounds, which could enter the river from Lethbridge or from cattle operations. Trenbolone acetate, a synthetic androgenic steroid used in cattle production, is metabolized into its active form trenbolone-β and has a half life of 260 days in manure (Schiffer et al. 2001). Rain in the Oldman Basin prior to sampling, which contributed to the increased flow, may have brought compounds like trenbolone-β and natural androgens into the Oldman River and contributed to the reduced vitellogenin expression. Increased river flow could also cause a dilution effect, which would lower the estrogenic compound concentrations resulting in reduced vitellogenin production. If dilution did occur, then the increase in vitellogenin at Taber would be indicative of new sources of estrogenic compounds upstream of Taber. River water contains complex mixtures of estrogenic and other compounds and thus a combination of many factors may

have caused the reduced vitellogenin production downstream of Lethbridge in November 2005.

2.4.1.2 Bow River

In April 2005, male and female longnose dace had greater vitellogenin production downstream of Calgary. Sosiak and Hebben (2005) found estrogenic compounds (bisphenol A and nonylphenol) and numerous pharmaceuticals downstream of Calgary that demonstrates Calgary's wastewater effluents impact the Bow River. Estrogenic compounds from Calgary's wastewater could cause the vitellogenin response in adult longnose dace downstream of Calgary. In October 2005, male and female vitellogenin levels did not significantly increase downstream of Calgary, consistent with a dilution effect due to flooding in the summer of 2005. For both males and females, the most downstream of Calgary site, Highway 24, had a slight increase in vitellogenin mRNA levels, which indicates there was still an increase in estrogenic compound exposure downstream of Calgary. This may be due to agricultural run-off downstream of Calgary that influences river water quality.

Females at Jumpingpound Creek had elevated vitellogenin expression on both sampling occasions. This site is not on the Bow River mainstem. I hypothesize that dramatic daily changes in water levels caused by flow regulation from the Ghost Dam make the Bow River upstream of Calgary poor habitat for longnose dace. Consequently I used Jumpingpound Creek as our upstream site. Cattle operations near this site may introduce estrogenic compounds that caused increased vitellogenin levels in female longnose dace. This increase did not occur in male fish, which suggests that there are likely natural vitellogenin patterns that contribute to the vitellogenin response in females at this site. Giesy et al. (2000) detected a dose-dependent vitellogenin increase in female fathead minnows (*Pimephales promelas*) exposed to 4-nonylphenol, but not in males. Therefore, there may also be gender-specific responses to specific compounds that cause the vitellogenin patterns at the Jumpingpound site.

2.4.2 CPUE and sex ratios

CPUE in the Oldman River was lowest at the upstream sites and increased with distance downstream. Municipal wastewater effluent and agricultural activities introduce nutrients to rivers that increase their productivity (Chambers et al. 2000). Increased productivity often results in more abundant fish populations due to higher food availability. CPUE was slightly elevated at Highway 3A and Popson Park when compared to the two most upstream sites. There is increased phosphorus and nitrogen upstream of Lethbridge at Highway 3A compared to the Summerview site (ORBWQI 2005), possibly due to Fort Macleod's sewage effluent and agricultural run-off from area farms and feedlots. The highest CPUE was downstream of Lethbridge in April and November suggesting nutrient enrichment downstream of Lethbridge. There is an increase in total phosphorus and total nitrogen levels in the Oldman River downstream of Lethbridge compared to the Summerview site, which suggests nutrient inputs from Lethbridge and non-point source inputs from agriculture. There are also elevated fecal coliform levels at Highway 36 and more agricultural pesticides detected at Highway 845 and Highway 36 when compared to the upstream Summerview site (ORBWQI 2005) which indicate that agricultural activities in the Oldman Basin influence the river. In April 2005, CPUE remained elevated at least ~85-110 km downstream of Lethbridge, a trend that was not found in November. Increased river levels resulted in high turbidity and made fish collections difficult at the most downstream sites in November. Therefore, the CPUE was small.

In the Bow River, CPUE was elevated on both sampling occasions at the Jumpingpound Creek site. This suggests a nutrient enrichment effect at that site. It is likely that the cattle operations in the Jumpingpound Creek Basin introduce nutrients to the creek that allow the longnose dace population to be more abundant. CPUE on the Bow River mainstem increased with distance downstream, however not immediately downstream of Calgary's wastewater effluents. Calgary's wastewater is a major source of nutrients to the Bow River (Hogberg 2004), but the longnose dace population size did not increase until >59 km downstream of Calgary. The effluents from Calgary's two municipal wastewater treatment plants are the largest contributors of pollutants to the

Bow River (BRBC 2005), including large inputs of ammonia, which can be toxic to fish (Rattner and Heath 2003). Hoger et al. (2006) reported rainbow trout (*Oncorhynchus mykiss*) mortality due to ammonia toxicity when fish were exposed to fifteen percent diluted wastewater effluent. It is possible that pollutants or high biological oxygen demand from the wastewater effluents prevent more abundant longnose dace populations in the river immediately downstream of Calgary. CPUE on the Bow River mainstem was highest 59 km and 71 km downstream of Calgary's Bonnybrook Wastewater Treatment Plants. The combined influence of Calgary's wastewater and agricultural run-off likely introduce nutrients to the Bow River which results in more abundant longnose dace populations at the most downstream sites.

The populations of longnose dace in the Oldman River are female-biased, and are as high as 80 to 90 percent female. The skewed sex ratios were mainly at the sites most influenced by municipal wastewater and agriculture. In the Bow River, the sex ratios were also female-biased, but rarely exceeded 60 percent female. Many mechanisms can create altered sex ratios, including environmental conditions (Baroiller et al. 2001), gender-specific mortality and sampling error (Woodling et al. 2006). Our sampling protocol was consistent among rivers and sites. The highly skewed sex ratios were not seen in the Bow River, or previously in the Red Deer River, Alberta (Jeffries 2004). Therefore, sampling error is unlikely the reason for the female-biased sex ratios in the Oldman River. There are no obvious reasons why natural factors would influence sex ratios at certain sites in the Oldman River as river conditions such as temperature, pH and dissolved oxygen were similar among the Oldman River sites considered and I know of no reason why males would be selectively preyed upon.

Estrogenic compounds can cause male fish to develop as females if exposed to the compounds at a critical point in sexual differentiation. Metcalfe et al. (2001) demonstrated female-biased sex ratios in Japanese medaka (*Oryzias latipes*) exposed to estrogenic compounds during development. In Colorado rivers, sex ratios in white suckers (*Catostomus commersoni*) were heavily skewed towards females downstream of municipal wastewater effluent because of estrogenic compound exposure (Woodling et al. 2006). The same estrogenic compounds that caused increased vitellogenin in longnose

dace in the Oldman River could have affected the longnose dace at a critical stage in sexual development, which resulted in female-biased sex ratios.

It is unclear why particular compounds would influence longnose dace in the Oldman River more than in the Bow River. Female-biased sex ratios in the Oldman River may be related to differences in the specific estrogenic compounds found in the two rivers and the exposure period. A major difference between the two basins is the intense agriculture and cattle operations in the Oldman basin, which can introduce many agricultural contaminants, livestock pharmaceuticals and steroid hormones into the river (Kolodziej et al. 2004; ORBWQI 2005; Forrest el al. 2006). Longnose dace spawning may occur multiple times (Roberts and Grossman 2001) between mid-may to early-August (McPhail and Lindsay 1970). This coincides with a time of heavy pesticide activity and manure application to crops. Highway 845 had the most female-biased sex ratio on both sampling occasions. In 2002, various pesticides were detected in the Oldman River from May to August, and the Highway 845 site had the highest number of pesticide detections (ORBWQI 2005). The timing of these activities may be related to the skewed sex ratios in the Oldman River if estrogenic compounds from agricultural activities affect longnose dace at critical stages of sexual development.

2.4.3 Relative body, liver and gonad weights

Increased relative body weight (condition) and relative liver weight are used as estimates of energy storage (Munkittrick et al. 2000). Typically, increased energy storage reflects positive growing conditions for fish and generally occurs in areas with elevated nutrient inputs. Increased liver size can result from many processes, including contaminant metabolism and increased biosynthesis in the liver (Hoger et al. 2006). In the Bow and Oldman Rivers, increased liver size in longnose dace corresponds with increased condition, and suggests that liver size is related to energy storage. Deviations from this pattern may demonstrate an energetic stress response. Elevated nutrients can result in increased relative gonad size, an estimate of reproductive effort (Munkittrick et al. 2000) if fish are able to convert additional energy from increased food availability to reproduction. Estrogenic compounds can reduce gonad size in laboratory fish (Jobling et

al. 1996). Laboratory studies apply estrogenic compounds alone, rather than in conjunction with exposure to increased nutrients. Because nutrients can cause fish to appear healthy morphologically, the impact of estrogenic compounds in field populations is not clear.

In the Oldman River, male and female longnose dace have increased relative body, gonad and liver weights downstream of Lethbridge in April 2005. Nutrient inputs from municipal wastewater effluent and agricultural surface run-off elevate river productivity, which results in positive growth responses in longnose dace. Relative weights follow the same pattern as vitellogenin, which suggests that estrogenic compounds may be introduced to the river from similar sources as nutrients (e.g., wastewater and surface run-off). In November 2005, body, liver and gonad mass did not increase downstream of Lethbridge in males, again similar to the vitellogenin responses, which may be because increased river flows diluted the nutrients and estrogenic compounds in the river. Females actually had significantly smaller livers downstream of Lethbridge, which further indicates that high river flows caused a reduction in the positive growing conditions in the Oldman River. Longnose dace at the upstream sites did not have greater relative weights despite having increased vitellogenin, except at the Summerview site where males and females had larger gonads. These results suggest that estrogenic compounds affect longnose dace physiologically in the Oldman River, but do not influence energy storage and reproductive effort. These measures of fish health appear to be related to nutrients that enter the river and cause an increase in growing conditions in the Oldman River.

Relative body, liver and gonad weights in males were high at Jumpingpound Creek, which suggests nutrient enrichment here. Females had similar patterns, but with no differences in gonad size. Relative weights increased downstream of Calgary in the Bow River, consistent with a nutrient effect from Calgary. As in the Oldman River, the increased relative body, liver and gonad weights occur generally at the same sites with increased vitellogenin levels, and may indicate that estrogenic compounds enter the Bow River from similar sources as the nutrients. Calgary's wastewater is the major contributor to the nutrient pattern downstream of Calgary and is likely the main source of estrogenic

compounds. The patterns in October were similar to April downstream of Calgary, which would be expected if wastewater effluents were a major influence on longnose dace consistently throughout the year. This pattern was not found in the Oldman River.

Longnose dace at Carburn Park, downstream of Calgary's Bonnybrook Wastewater Treatment Plant, did not respond positively to nutrient inputs. Phosphorus concentrations and macrophyte biomass increase downstream of the effluent input that cause an increase in river productivity (Hogberg 2004). The slope of the liver weight-body weight relationship was significantly greater for both males and females at Carburn Park in April 2005, which indicates liver sizes increase at a greater rate with body size. However, longnose dace did not increase in abundance or have heavier relative body weights. These relationships together suggest that longnose dace at this site may be impacted by contaminant inputs from the Bonnybrook Wastewater Treatment Plant, which have increased contaminant metabolism and biosynthesis in the liver. The longnose dace do not have elevated vitellogenin mRNA, which suggests that contaminants other than estrogenic compounds might cause the increased liver size at Carburn Park.

2.5 Conclusions

In the Oldman River, longnose dace show signs of estrogenic compound exposure evinced by increased or altered vitellogenin production. Estrogenic compounds are introduced to the river with nutrients. The nutrients elevate river productivity and result in high abundance and increased body, liver and gonad sizes, primarily at the most downstream sites, which suggests increased food availability although food intake was not measured. Seemingly beneficial effects of increased river productivity cause longnose dace to appear healthy morphologically despite the physiological impacts of exposure to estrogenic compounds. Adult longnose dace populations are heavily female-biased in the most agriculturally intensive areas of the river basin. Skewed sex ratios may be an indicator of severe endocrine disruption that could impact the long-term reproductive health of longnose dace in the river. These results demonstrate the cumulative impacts of

estrogenic compounds introduced to a river from municipal wastewater effluents and agricultural activities on minnow populations in a prairie river.

In the Bow River, there are elevated vitellogenin levels in longnose dace, consistent with estrogenic compound exposure. Calgary's wastewater effluents are likely the major sources of estrogenic compounds as vitellogenin levels increase downstream of the city of Calgary. This pattern agrees with many studies that have demonstrated increased vitellogenin in fish downstream of municipal wastewater effluents. The sex ratios were not as heavily female-biased as in the Oldman River, perhaps due to different estrogenic compound mixtures in the Bow River. The Jumpingpound Creek site, which is not on the mainstem, also had increased vitellogenin levels although there are no municipal wastewater discharges upstream of the site. There are cattle operations along the creek, which further indicates the potential impacts of cattle operations on riverine fish populations.

In this study I used a native and abundant minnow to examine the cumulative impacts of municipalities and agricultural activities as sources of estrogenic compounds in prairie rivers. I used QRT-PCR to detect differences in hepatic vitellogenin mRNA at multiple sites along natural river gradients in male and female longnose dace. Because male and female vitellogenin production patterns were similar, I believe that the female patterns are not due only to natural reproductive processes and also represent evidence of estrogenic compound exposure. I then compared the physiological results to multiple fish performance endpoints to assess the impacts of vitellogenin up-regulation on longnose dace populations. There were indications of estrogenic compound exposure at every site on both rivers demonstrating that to assess the impacts of estrogenic compounds on entire river systems and fish populations; it is more complicated than only considering point-sources. In this study, every site was located in areas with some type of anthropogenic activity. This study demonstrates the importance of assessing rivers at large scales to fully detect the cumulative impacts of land use on water quality.

Chapter Three: Organic contaminants in river water and vitellogenin production in male longnose dace (*Rhinichthys cataractae*) in the South Saskatchewan River Basin, Alberta, Canada.

3.1 Introduction

A broad range of chemical contaminants are introduced to rivers from anthropogenic sources such as municipal wastewater and agricultural run-off (Kolpin et al. 2002). It is often difficult to determine the impact(s) of an individual contaminant on aquatic organisms in the field because multiple chemicals are present and may interact with each other when in complex mixtures (Brian et al. 2007). In nature, estrogenic compounds can disrupt endocrine function and are often present in complex mixtures (Kolpin et al. 2002). Many common organic contaminants, including natural and synthetic steroid hormones, industrial chemicals and phytosterols can be estrogenic (Fernandez et al. 2007) and have the potential to impact aquatic life.

Municipal wastewater is often a source of estrogenic compounds to rivers. Steroid hormones, industrial contaminants and pharmaceuticals are regularly detected in rivers downstream of wastewater effluents (Petrovic et al. 2002; Servos et al. 2005; Sarmah et al. 2006) and have been associated with increased vitellogenin levels, production of ovarian tissue by male testes, and in extreme cases, altered sex ratios in fish populations (Jobling et al. 1998; Burki et al. 2006; Woodling et al. 2006). Biological responses to estrogenic compounds by fish have been documented at least 35 km downstream of a wastewater effluent source (Aravindakshan et al. 2004) and estrogenic compounds have been detected in river water (at μg/L levels) and sediments (at μg/kg levels) almost 30 km downstream of a wastewater effluent at concentrations high enough to induce biological responses (Petrovic et al. 2002). However, the typical distance downstream of wastewater sources where fish are no longer impacted by estrogenic compounds is unknown.

Agricultural run-off and cattle operations can have major impacts on river water quality and introduce estrogenic compounds (Kolpin et al. 2002; Kolodziej et al. 2004). Livestock pharmaceuticals, steroid hormones and pesticides may be detected in rivers

influenced by agriculture and cattle operations. The effect of municipal wastewater effluent is often not evaluated together with agricultural influences along long river gradients, or in multiple rivers within the same basin. The cumulative impacts of municipal wastewater and agricultural inputs have the potential to adversely affect native fish populations (Falconer et al. 2006).

I collected river water at 23 sites on three rivers and measured the concentrations of 28 organic contaminants that can be grouped into major classes of contaminants and linked back to a range of different land-uses. Many of these organic contaminants are estrogenic and commonly occur in wastewater effluents or rivers impacted by humans (Kolpin et al 2002; Sosiak and Hebben 2005). I used contaminant concentration snapshots to estimate what could be found along the rivers throughout the year. I also sampled longnose dace (*Rhinichthys cataractae*), an endemic and abundant minnow in these rivers to assess potential biological responses due to exposure to estrogenic compounds. My goal was to determine the cumulative impacts of municipal wastewater and agricultural activities on organic contaminant concentrations in prairie rivers, then to link these contaminants to fish responses at a large basin-wide scale.

3.2 Methods

3.2.1 River Basins

Water and fish were collected from the Red Deer, Bow and Oldman Rivers, three major tributaries of the South Saskatchewan River (Figure 3.1). The Red Deer River has a mean annual discharge of 47.5 m^3/s and flow is regulated by Dixon Dam, 51 km upstream of Red Deer (population 72 000). Potential sources of organic contaminants to the Red Deer River are Red Deer's tertiary treated wastewater, chemical manufacturing plants downstream of Red Deer, and cattle and crop production in the basin.

The Bow River has a mean annual discharge of 91.1 m^3/s and flow is heavily regulated by multiple dams upstream of Calgary (population 1 000 000). Bearspaw Dam is located immediately upstream of Calgary and is the primary flow regulator in the reach considered in this study. The main sources of organic contaminants are the Bonnybrook

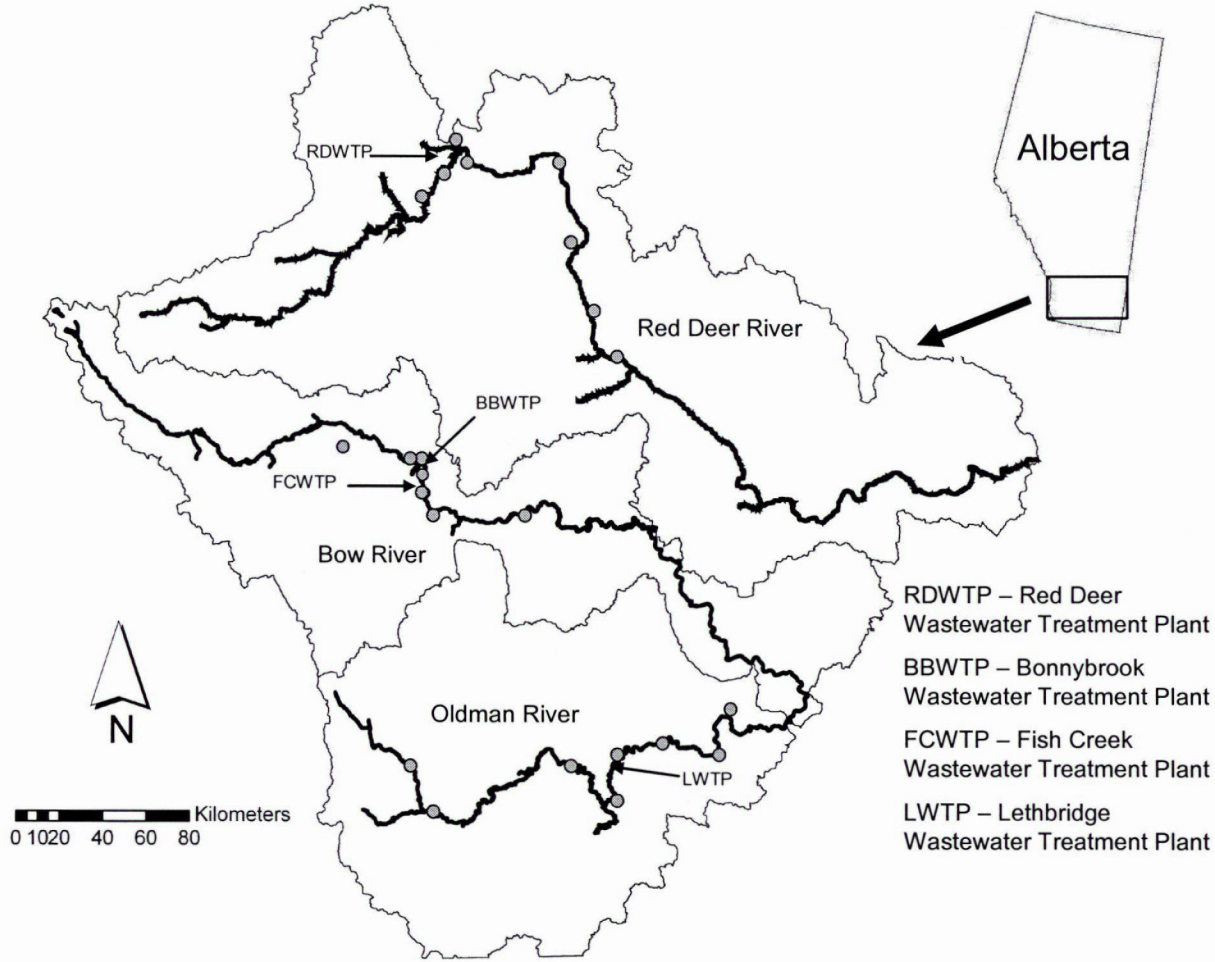


Figure 3.1 Map of the Red Deer, Bow and Oldman River Basins, Alberta, Canada indicating sampling locations and the major municipal wastewater treatment plants.

and Fish Creek Wastewater Plants in Calgary, which both discharge tertiary treated wastewater effluent. There is also agriculture downstream of Calgary.

The Oldman River is a heavily meandering river with a mean annual discharge of 81.8 m^3/s and flow regulated by the Oldman Dam located 152 km upstream of Lethbridge (population 73 000). The Oldman River is mainly impacted by Lethbridge's tertiary wastewater effluent, wastewater effluent from several smaller municipalities, agriculture and intense cattle production in the downstream region of the Oldman Basin.

The sample sites and the distances from major municipal wastewater effluents for the three rivers are presented in Table 3.1. Sites were selected from historical Alberta Environment sampling stations and where longnose dace had been previously collected. The Jumpingpound Creek site was sampled with the Bow River, but is not on the mainstem. Therefore the river distance provided is for where Jumpingpound Creek joins the Bow River, upstream of the Bearspaw Reservoir. The Olin Bridge site was above the Oldman Reservoir and therefore the river distance provided is the distance upstream of the Oldman Reservoir. All sites on the Red Deer River are on the mainstem and within a continuous section of the river.

3.2.2 Water Samples

Twenty-eight organic contaminants were extracted from river water and are listed in Table 3.2 and 3.3 and along with the percentage of water samples with a positive detection for each individual contaminant. These contaminants do not include organic matter associated with increased biological oxygen demand in rivers. They were divided into eight functional groups to identify potential contaminant sources. Water was collected during months of historical low flow for all rivers. The Bow River was sampled in December 2005, while the Red Deer and Oldman Rivers were sampled during April 2006. Twelve liters of water were collected at each site using four litre pre-cleaned and silylated glass amber bottles. The bottles were pre-rinsed with sample water immediately prior to collection following the sample procedure outlined in Sosiak and Hebben (2005). The samples were kept at 4°C until chemical extractions, which were performed within 24 hours of collection.

Table 3.1 Sites where water samples were collected on the Red Deer, Bow and Oldman Rivers, Alberta, with approximate river distances to the major wastewater treatment plants on that particular river and indication whether longnose dace (*Rhinichthys cataractae*) were collected for vitellogenin analysis.

River	Site	River Distance	Fish Collected
Red Deer River	Penhold Bridge	-36 km	Yes
	Ft. Normandeau	-13 km	Yes
	Riverbend	3 km	Yes
	Joffre Bridge	24 km	Yes
	Content Bridge	64 km	Yes
	Tolman Bridge	130 km	Yes
	Morrin Bridge	151 km	Yes
	Drumheller	179 km	Yes
Bow River	Jumpingpound Creek[a]	14 km	Yes
	Edworthy Park	-17 km	Yes
	St. George's Island	-7 km	Yes
	Carburn Park	5 km	No
	Fish Creek Park	15 km	No
	Policeman's Flats	24 km	No
	Highway 24	71 km	Yes
Oldman River	Olin Bridge[b]	15 km	Yes
	Summerview Bridge	-158 km	Yes
	Highway 3A	-58 km	Yes
	Popson Park	-12 km	Yes
	Pavan Park	6 km	Yes
	Highway 845	41 km	Yes
	Taber	85 km	Yes
	Highway 36	109 km	No

[a] The Jumpingpound Creek river distance is where Jumpingpound Creek joins the Bow River upstream of Bearspaw Reservoir.
[b] The Olin Bridge site river distance is the distance upstream of the Oldman Reservoir.

Table 3.2 Organic contaminants targeted for analysis by GC-HRMS in river water from the Red Deer, Bow and Oldman Rivers, Alberta, and the percentage of positive detections. The functional groups used for subsequent figures are in bold.

Target Chemical	Detections (%)[a]	Description
Natural Hormones		
Estrone	52.2	Endogenous female estrogen
17β -Estradiol	47.8	Endogenous female estrogen
17α -Estradiol	4.3	Endogenous female estrogen
Estriol	4.3	Endogenous female estrogen
Testosterone	8.7	Endogenous male androgen
Synthetic Hormones		
Equilin	8.7	Hormone replacement therapy drug
d-Equilenin	17.4	Hormone replacement therapy drug
Mestranol	8.7	Synthetic ovulation inhibitor (birth control pill)
19-Norethindrone	21.7	Synthetic ovulation inhibitor (birth control pill)
17α –Ethynylestradiol	0	Synthetic ovulation inhibitor (birth control pill)
(-)-Norgestrel	26.1	Synthetic ovulation inhibitor (birth control pill)
Veterinary		
α –Zearalanol	17.4	Veterinary drug (growth promoter)
β –Estradiol-3-benzoate	0	Veterinary drug (growth promoter)
Bisphenol A	8.7	Plasticizer, potent EDC from PVC plastics

[a]The percentage of sites with a positive detection (n=23).

Table 3.3 Organic contaminants targeted for analysis by GC-HRMS in river water from the Red Deer, Bow and Oldman Rivers, Alberta, and the percentage of positive detections. The functional groups used for subsequent figures are in bold.

Target Chemical	Detections (%)[a]	Description
Cholesterol and its derivatives		
Cholesterol	100	Animal/plant derived sterol
Desmosterol	82.6	Cholesterol derivative
Cholestanol (coprostanol)	100	Cholesterol derivative
6-Ketocholestanol	34.8	Cholesterol oxidation product
7-Ketocholesterol	91.3	Cholesterol oxidation product
Coprostan-3-one	90.9[b]	Fecal neutral sterol
Ergosterol	69.6	A major sterol produced by fungi
Phytosterols		
β -Sitosterol	100	Phytosterol
Campesterol	100	Phytosterol
Stigmastanol	100	Phytosterol
Stigmasterol	95.7	Phytosterol
Fucosterol	100	Sterol found in seaweed
Totarol	0	Antibacterial diterpenoid
Pinosylvin	4.3	Stilbene found in *Pinus* sp.

[a]The percentage of sites with a positive detection (n=23).
[b]Only 22 sites were able to be tested for coprostan-3-one.

The organic contaminants were extracted by constant stirring for two hours, three litres at a time into 300 ml of dichloromethane (DCM). Samples were spiked with five labelled internal standards (ring-$^{13}C_6$-nonylphenol, propane-d_6-bisphenol A, di-*n*-octylphthalate-d_4, 17β-estradiol-17-acetate, and 2,2,3,4,4,6-d_6-cholesterol). Separatory funnels were used to isolate the DCM, which was then evaporated to approximately twenty mL and sent to the Institute of Ocean Sciences (Department of Fisheries and Oceans, Sydney, British Columbia, Canada) on ice for ultra-trace analytical gas chromatography-high resolution mass spectrometry (GC-HRMS) analysis as outlined in Fernandez *et al.* (2007). Quality control samples for each river consisted of procedural blanks, duplicate blanks and native spiked duplicates. Results are presented as internal standard and blank corrected. Internal standard recoveries were generally greater than 50 percent and were deemed acceptable by the Institute of Ocean Sciences. The concentrations of all contaminants for the three rivers are located in appendix B.

3.2.3 Fish Samples

Male longnose dace were collected during the non-reproductive months of October and early November 2005 from the three rivers using a backpack electrofishing unit (Model 12-B POW electrofisher, Smith-Root Inc.). However, longnose dace collection was not possible at all sites. The longnose dace remained alive until dissection, when they were euthanized by a cut through the spinal cord. Male liver and testes weights were used to calculate hepatosomatic (HSI) and gonadosomatic (GSI) indices (index = organ mass/[body mass – organ mass]*100). Livers were placed in individual polypropylene vials, immediately frozen in liquid nitrogen, and stored at -80°C until analyses were conducted.

Hepatic vitellogenin protein levels were determined using Carp Vitellogenin ELISA Kits (Biosense Laboratories, Bergen, Norway). Longnose dace vitellogenin showed good cross-reactivity to the antibodies used in the Carp Vitellogenin ELISA Kit and therefore the samples were quantifiable (Sven-Inge Kristiansen, Biosense Laboratories, Bergen, Norway, personal communication). Buffer solution (phosphate buffered saline, pH 7.3, with 1% bovine serum albumin) was added to liver samples at a

ratio of 1:50 (weight:volume). The mixture was mechanically homogenized, centrifuged at 14 000 g for 10 minutes and the supernatant was collected and diluted with buffer solution at 1:50 and 1:500 ratios. The sandwich ELISA procedure used in this study is outlined in Nilsen *et al.* (2004). The results are presented as ng of protein/μg of liver tissue.

3.2.4 Statistical Analysis

I performed statistical analyses with SAS software v.8.2 (SAS Institute, Cary, North Carolina). Data were log 10 transformed, if necessary, to better meet the assumptions of parametric statistics (Sokal and Rohlf, 1995). Differences between sites in vitellogenin levels were analyzed using a nonparametric Kruskal-Wallis test. Relationships between HSI or GSI with vitellogenin production were analyzed using Pearson Product Moment correlation analysis. All statistics were performed with a critical alpha value of 0.05.

3.3 Results and Discussion

3.3.1 Natural and synthetic hormones, veterinary pharmaceuticals and bisphenol A

Natural (endogenous human and animal hormones) and synthetic hormones, that are estrogenic to fish, were detected in the Red Deer and Oldman Rivers. Testosterone, the only hormone sampled that is not estrogenic, was detected at Tolman Bridge on the Red Deer River and at Highway 845 on the Oldman River. Natural and synthetic hormones, veterinary pharmaceuticals and bisphenol A were not detected in the Bow River. In the Red Deer River, the total concentration of natural and synthetic hormones increased downstream of Red Deer (Figure 3.2). Sosiak and Hebben (2005) detected estrone and estriol in Red Deer's wastewater effluent at 9.93 ng/L and 2.19 ng/L respectively, but did not detect any natural hormones in the Red Deer River. I was able to detect natural and/or synthetic hormones at every site including two sites upstream of Red Deer. Animal manure can be a source of natural hormones and veterinary pharmaceuticals via defecation and manure application to fields (Burnison et al. 2003).

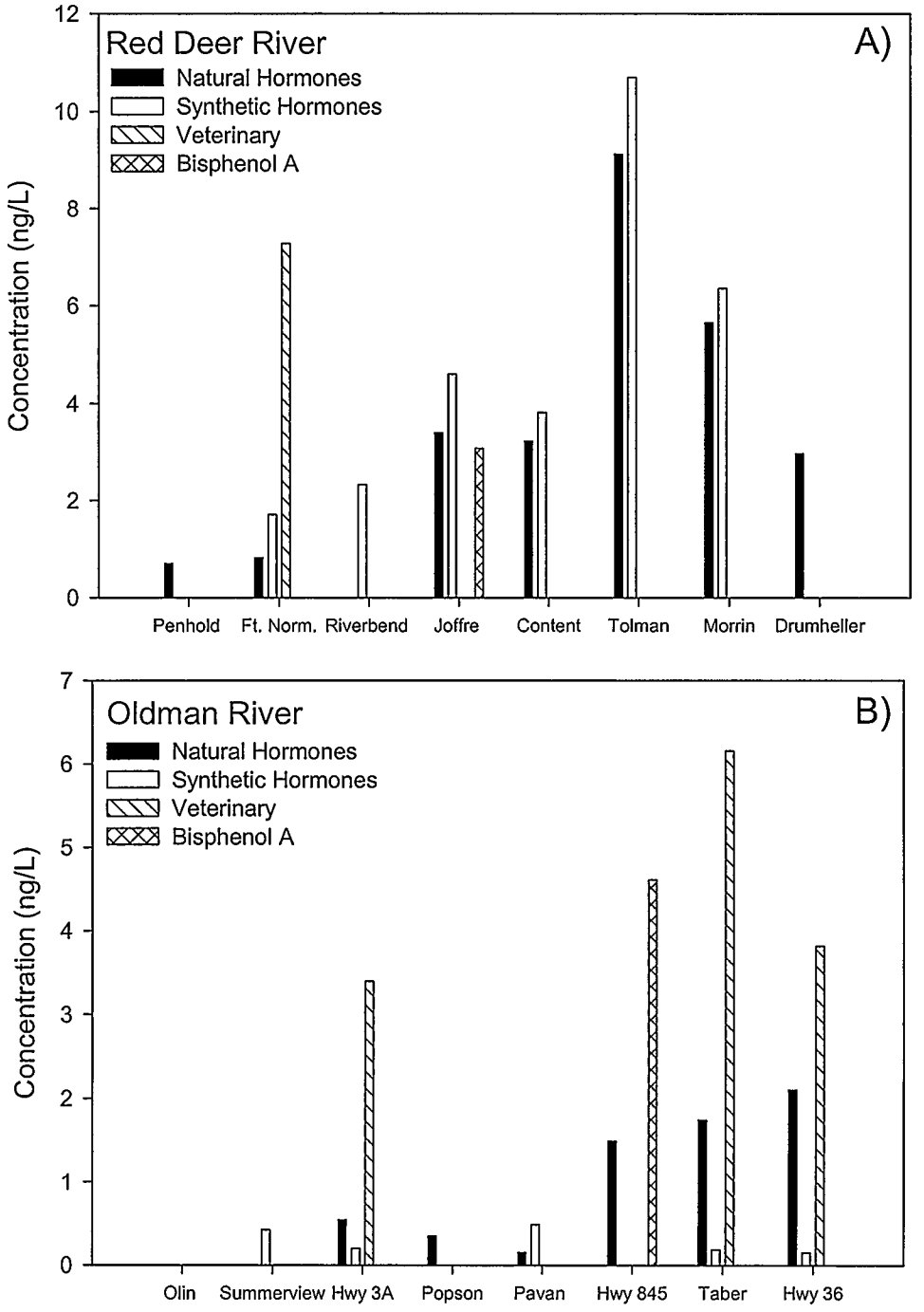


Figure 3.2 Concentrations of natural and synthetic hormones, specific veterinary pharmaceuticals and bisphenol A in river water from the A) Red Deer River and B) Oldman River in Alberta.

Cattle production in the vicinity of the Red Deer area likely contributes to the natural hormone levels in the Red Deer River because veterinary growth promoters were detected upstream of Red Deer at Fort Normandeau.

Synthetic hormones and bisphenol A were detected at sites downstream of Red Deer's wastewater treatment plant, which suggests a municipal source. The synthetic hormones included birth control pill metabolites and hormone therapy pharmaceuticals, which are characteristic of municipal wastewater. Bisphenol A was only detected at Joffre Bridge and not Riverbend, yet it is often detected in municipal wastewater effluents (Fernandez et al. 2007). This may indicate that the wastewater effluent plume was not fully mixed across the river width by Riverbend. Bisphenol A was detected previously downstream of Red Deer (Sosiak and Hebben 2005) which demonstrates consistency in this pattern. The bisphenol A detected at Joffre Bridge may be due to Red Deer wastewater effluent or an alternative unknown source upstream of Joffre Bridge.

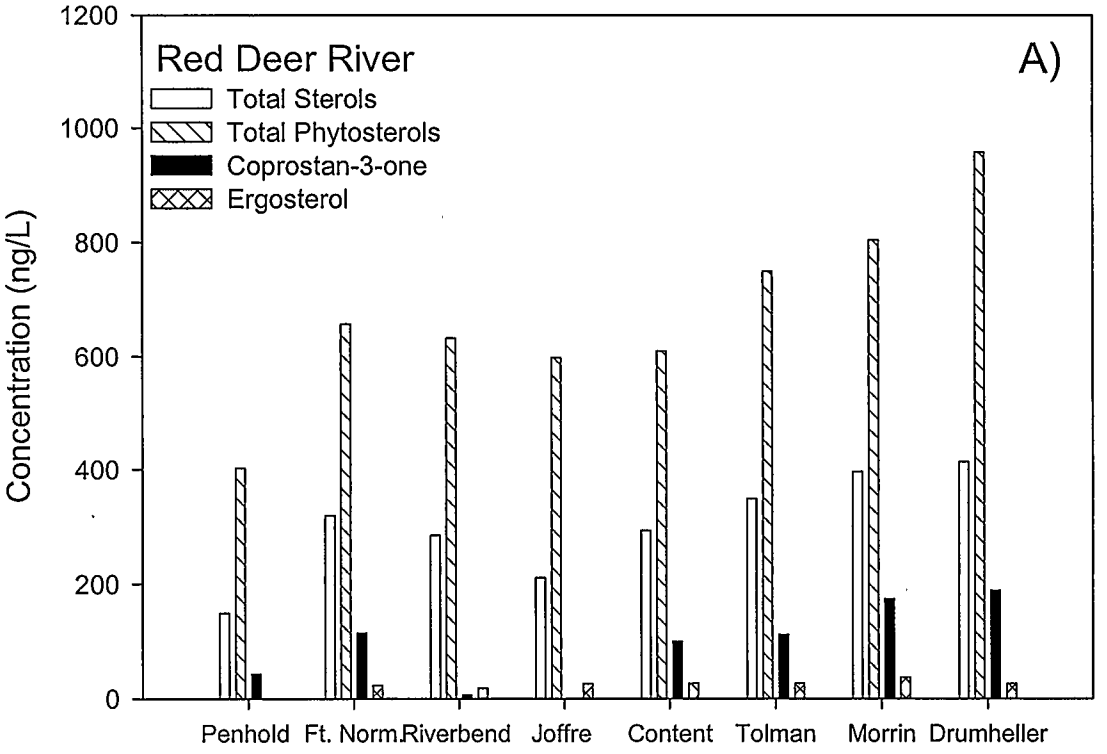
The highest total concentrations of natural and synthetic hormones were found at Tolman Bridge and Morrin Bridge, which are 130 km and 151 km downstream of Red Deer's wastewater effluent, respectively. Red Deer's wastewater footprint can be as large as 120 km depending on the time of year, as determined by stable isotopes (Hogberg 2004). Therefore, Red Deer's wastewater may be responsible for the natural and synthetic hormone concentrations that were detected. There was recent rain before the sampling that increased water levels and river turbidity at the downstream sites. Estrogenic compounds have been detected in sediments downstream of municipal wastewater effluents (Petrovic et al. 2002). The increase in river water levels can mobilize river sediments, which may have the potential to release estrogenic compounds back into the water column and contribute to the high levels of natural and synthetic hormones detected at the Tolman Bridge and Morrin Bridge sites. There are likely additional inputs from other sources that contribute to the hormone concentrations at Tolman Bridge and Morrin Bridge. Wastewater from small municipalities downstream of Red Deer (e.g. Stettler, population 5 200, and Three Hills, population 3 600) may introduce natural and synthetic hormones to the river. Cattle production can also be a source of natural hormones and could contribute to the hormone concentrations in the Red Deer River.

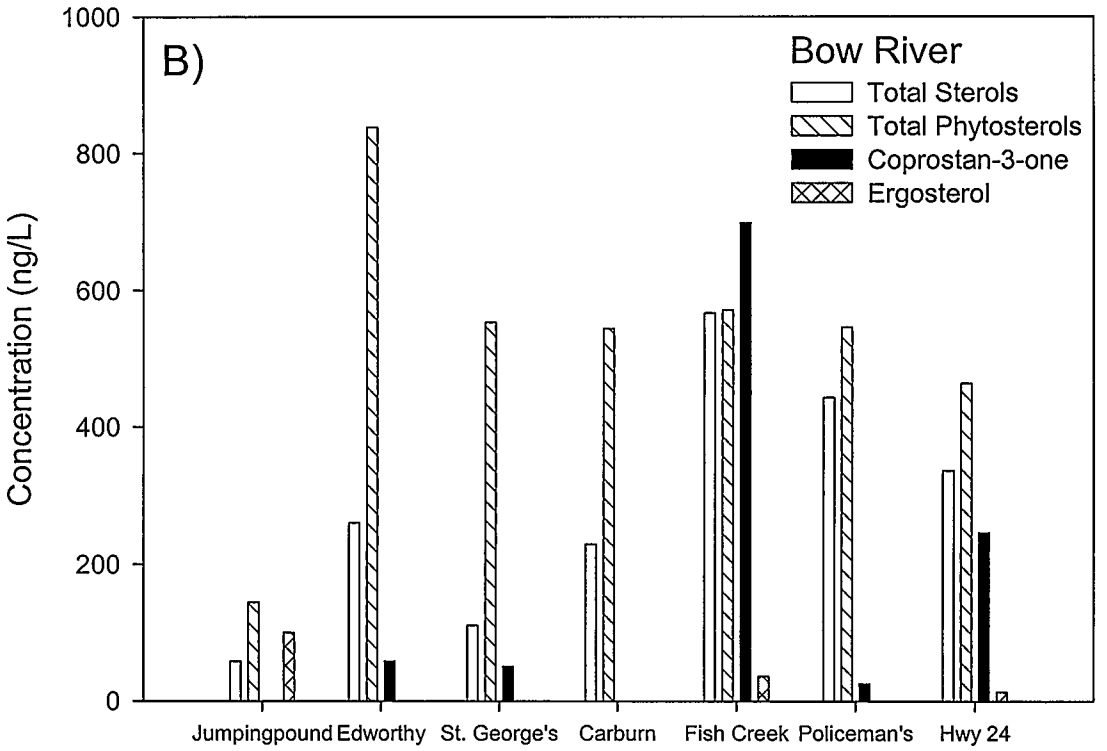
In the Oldman River, concentrations of natural and synthetic hormones increased downstream of smaller municipalities and Lethbridge. As in the Red Deer River, the sites with the highest concentrations of natural hormones are downstream of the major city, Lethbridge. There are intense cattle operations in the Oldman Basin around Lethbridge which may contribute to the levels of natural hormones detected in the Oldman River. Taber and Highway 36, the two sites with the highest concentrations of natural hormones, also have detectable levels of veterinary growth promoters, which indicate cattle operations influence the Oldman River. Water at Highway 3A also had veterinary growth promoters present, which is consistent with cattle operations as a major land use.

Bisphenol A was detected at Highway 845 only. Sosiak and Hebben (2005) also detected bisphenol A at this site, which suggests some consistency to this pattern. Bisphenol A may be introduced to the Oldman River from Lethbridge's wastewater, however it was not detected immediately downstream of the effluent at Pavan Park. The source of bisphenol A is unknown, but this industrial compound has been detected in cattle feedlot lagoons, possibly from polyvinyl chloride (PVC) piping or lagoon liners used at the feedlots (Lee et al. 2004). It is possible that the bisphenol A detected at Highway 845 is associated with the cattle feedlots in the area along with Lethbridge wastewater.

3.3.2 Total sterols, phytosterols, ergosterol and coprostan-3-one

In the Red Deer and Oldman Rivers, the concentrations of total sterols, phytosterols, ergosterol and coprostan-3-one increase downstream of major municipalities, and these compounds are highest at the most downstream sites (Figure 3.3). These compounds can be related to nutrient and fecal waste inputs, which are indicative of increased municipal and agricultural impacts to the Red Deer and Oldman Rivers. Cholesterol and its derivatives (total sterols) are found in human waste and are common in municipal wastewater effluents (Fernandez et al. 2007). Total sterols may also be introduced to rivers via animal waste (Leeming et al. 1996) following manure application. Coprostan-3-one is a fecal neutral sterol that indicates fecal inputs of human


Red Deer River
A)
Total Sterols
Total Phytosterols
Coprostan-3-one
Ergosterol
Concentration (ng/L)
0
200
400
600
800
1000
1200
Penhold
Ft. Norm.
Riverbend
Joffre
Content
Tolman
Morrin
Drumheller


B)
Bow River
Total Sterols
Total Phytosterols
Coprostan-3-one
Ergosterol
Concentration (ng/L)
0
200
400
600
800
1000
Jumpingpound
Edworthy
St. George's
Carburn
Fish Creek
Policeman's
Hwy 24

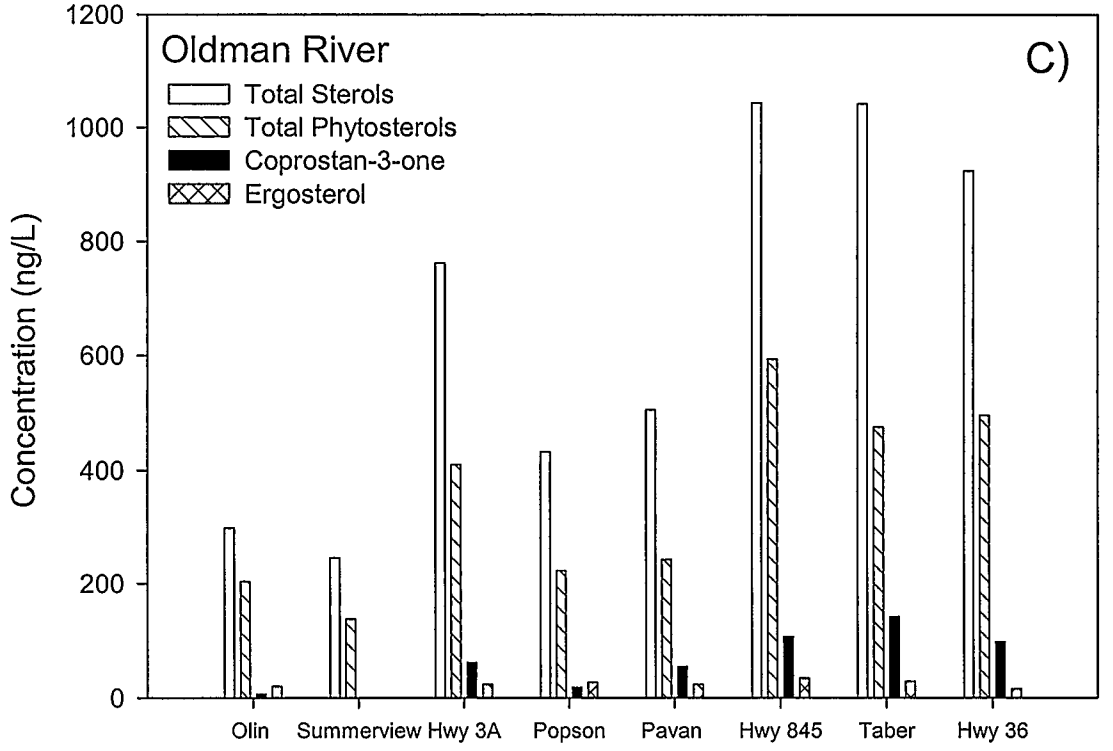


Figure 3.3 Concentrations of sterols, phytosterols, ergosterol and coprostan-3-one in river water from the A) Red Deer River, B) Bow River and C) Oldman River in Alberta.

or animal origin. Coprostan-3-one becomes more abundant with distance downstream, which indicates cumulative impacts of human and animal wastes. In fact, 55 percent of nitrogen inputs to the Oldman River have been estimated to be due to animal manure (Rock and Mayer 2006). Further, levels of fecal coliform bacteria increase with distance downstream in both rivers (ORBWQI 2005; Alberta Environment, 2006, www3.gov.ab.ca/env/water/water_information_centre.cfm), a pattern that would be expected based on the coprostan-3-one findings. However, because fecal coliform bacteria are not simply spatially correlated to large wastewater sources, the increase in fecal coliform bacteria may also be due to wastewater effluent from smaller municipalities (e.g., Fort Macleod and Taber) and non-point source animal manure inputs to the rivers.

Ergosterol is a natural fungal steroid that can be used to estimate fungal biomass (Gardner et al. 1993) and fungal biomass increases in rivers with high concentrations of dissolved organic nutrients (Welch and Lindell 1992). Ergosterol is most abundant at the most downstream sites in the Red Deer and Oldman Rivers, which suggests an increase in organic nutrients with distance downstream. Municipal wastewater effluent and agricultural activities introduce nutrients to rivers that can increase river productivity (Chambers et al. 2000); this could lead to the pattern of ergosterol in these rivers.

Phytosterols are natural plant sterols that can be introduced to rivers by a variety of sources including municipal wastewater effluent (Fernandez et al. 2007), and were found to increase with distance downstream in the Red Deer and Oldman Rivers. The precise sources of phytosterols are difficult to identify because the phytosterol signature of fecal waste is mixed with the phytosterols released by algae, detritus and other plant material in the river (Leeming et al 1996). Aquatic macrophytes and periphyton are more abundant in the Red Deer River downstream of Red Deer (Hogberg 2004) and periphyton biomass increases downstream of Lethbridge in the Oldman River (Alberta Environment, 2006, www3.gov.ab.ca/env/water/water_information_centre.cfm). Phytosterols introduced to the rivers from macrophytes, periphyton and plant decomposition can contribute to the increase in phytosterols in the Oldman and Red Deer Rivers.

In the Bow River, total sterols, ergosterol and coprostan-3-one concentrations generally increase downstream of Calgary's wastewater treatment plants, however not all compounds were detected at every site. Coprostan-3-one and ergosterol were not detected downstream of the Bonnybrook Wastewater Treatment Plant. This may be due to a lack of complete mixing of the wastewater effluent plume across the river width by Carburn Park (Hogberg 2004) as I sampled the left bank (east side) and the wastewater is discharged five kilometres upstream of Carburn Park on the right bank. Total phytosterols increased downstream of the Bearspaw Reservoir, which discharges from the hypolimnion. This may introduce phytosterols to the Bow River at the Edworthy Park site if the Bearspaw Reservoir had macrophyte decomposition in the hypolimnion, which caused phytosterols to be discharged into the Bow River. There are also macrophytes and periphyton in back channels along a municipal golf course upstream of the Edworthy Park site. Decomposition of macrophytes and periphyton upstream of Edworthy Park may have contributed to the total phytosterol concentrations. Phytosterol concentrations did not increase significantly downstream of Calgary, as concentrations did downstream of Red Deer and Lethbridge.

Total sterols and phytosterols were frequently detected the Red Deer, Bow and Oldman Rivers and may generate multiple hormonal responses in fish. In rainbow trout (*Oncorhynchus mykiss*), β-sitosterol is estrogenic and causes significant vitellogenin production at 25 μg/L in laboratory studies (Tremblay and Van Der Kraak 1998). I measured much lower concentrations of β-sitosterol in river water, but β-sitosterol may contribute to the overall estrogenicity of the rivers. In zebrafish (*Danio rerio*), environmentally relevant wood sterol mixtures can elicit estrogenic effects (Nakari and Erkomaa 2003). Pulp-mill effluents however, which can be a significant source of phytosterols to rivers (Mahmood-Khan and Hall 2003), may actually masculinize fish (Mills and Chichester 2005). Fucosterol and cholesterol have an inverse correlation with wastewater estrogenic activity in recombinant yeast assays (Fernandez et al. 2007), but cholestanol, a cholesterol derivative, can induce estrogenic responses in freshwater mussels (Gagne et al. 2001). Therefore, total sterols and phytosterols can potentially

cause undetermined hormonal responses in longnose dace in the Red Deer, Bow and Oldman Rivers.

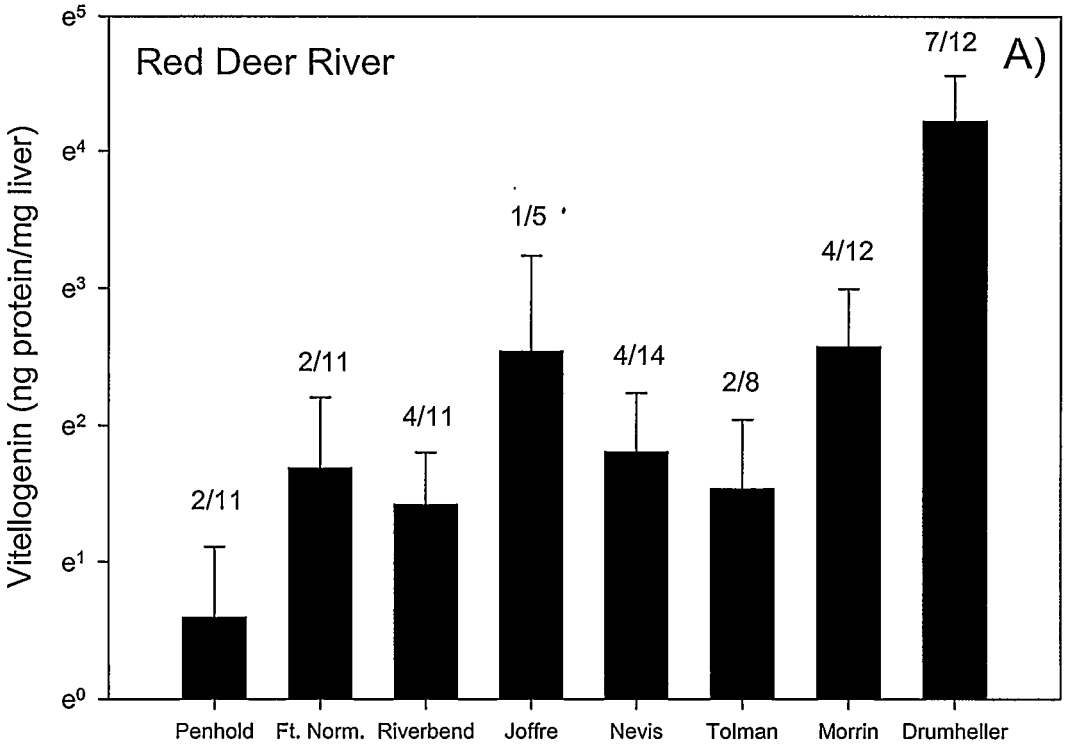
Organic contaminants, including many estrogenic compounds, were detected at every site where water samples were collected in the Red Deer, Bow and Oldman Rivers. This does not mean that organic contaminants are present at these concentrations at all times, but rather that organic contaminants are likely present at detectable levels over large distances and time. There are likely many other organic contaminants that were not measured and could be present in these rivers. This demonstrates however, that estrogenic compounds are widespread throughout the river basins and not limited to downstream of major point sources. Therefore, many activities within these basins may impact longnose dace populations.

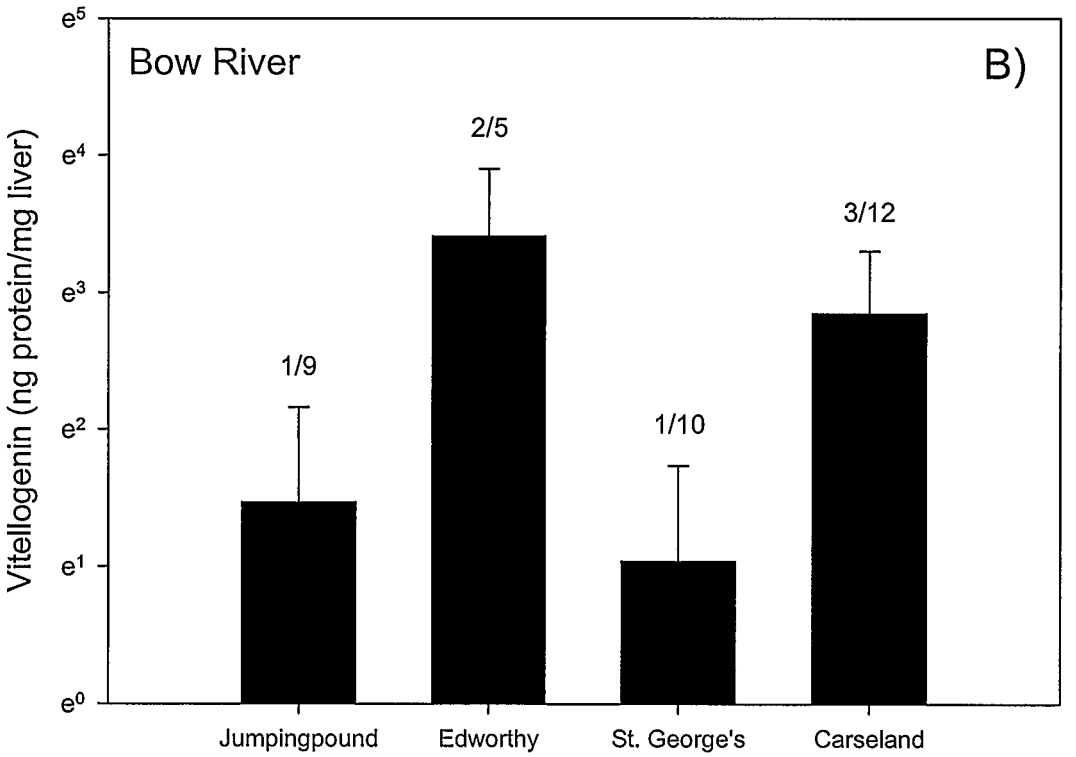
3.3.3 Longnose dace vitellogenin patterns

In the Red Deer, Bow and Oldman Rivers, at least one fish had detectable levels of vitellogenin protein at all sites, except Popson Park. In the Red Deer River, males downstream of Red Deer had higher hepatic vitellogenin protein levels than upstream, although this pattern was not statistically significant (χ^2= 9.6, p>0.05). The males at Drumheller had the highest mean vitellogenin levels and highest proportion of detections. Water at Drumheller had the highest levels of total sterols and total phytosterols. These compounds may contribute to the estrogenicity of the Red Deer River at the Drumheller site.

In the Bow River, Edworthy Park had the highest vitellogenin levels, but this too was not statistically significant (χ^2= 3.1, p>0.05). Edworthy Park also had the highest proportion of vitellogenin detections, yet the sample size was low. The Edworthy Park site had the highest levels of total phytosterols in water samples, which further suggests that natural compounds like phytosterols may contribute to the estrogenic response of longnose dace in the rivers.

In the Oldman River, males immediately downstream of Lethbridge at Pavan Park had the highest vitellogenin levels (Figure 3.4). This pattern was not statistically significant (χ^2= 5.1, p>0.05). Males at Summerview Bridge had the greatest proportion of


Red Deer River
A)
Vitellogenin (ng protein/mg liver)
e5
e4
e3
e2
e1
e0
2/11
2/11
4/11
1/5
4/14
2/8
4/12
7/12
Penhold
Ft. Norm.
Riverbend
Joffre
Nevis
Tolman
Morrin
Drumheller


Bow River
B)
Vitellogenin (ng protein/mg liver)
e5
e4
e3
e2
e1
e0
1/9
2/5
1/10
3/12
Jumpingpound
Edworthy
St. George's
Carseland

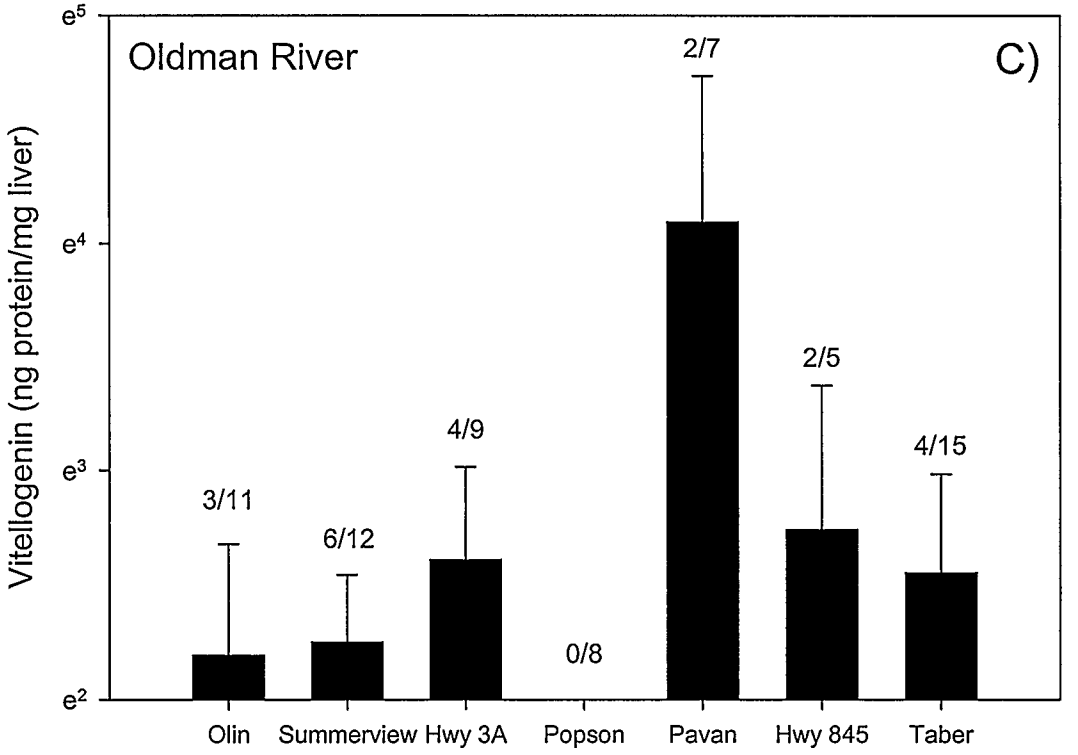


Figure 3.4 Hepatic vitellogenin protein levels in male longnose dace (*Rhinichthys cataractae*) collected from the A) Red Deer River, B) Bow River and C) Oldman River, Alberta. Data are presented as means with SE. Proportion of positive detections at each site determined by ELISAs are above the mean vitellogenin values.

vitellogenin detections. Water at Pavan Park and Summerview Bridge had the highest concentrations of synthetic hormones. This could be expected at sites downstream of municipal wastewater discharges like at Pavan Park. The Summerview Bridge site is downstream of Pincher Creek, a small tributary of the Oldman River which flows through the small town of Pincher Creek (population 3 700). The town of Pincher Creek is not on the Oldman River mainstem. There were detectable levels of 19-norethindrone, a birth control metabolite, at Summerview Bridge, which only has a human source. Synthetic hormones may contribute to the vitellogenin production in longnose dace at Pavan Park and Summerview Bridge.

Hepatic vitellogenin protein levels were measured in male longnose dace at every site where adults were collected as a marker of exposure to estrogenic compounds. The proportion of longnose dace with detectable vitellogenin levels was not very high, which resulted in a lack of statistical significance. There could be differences in exposure histories within sites that cause a variable response among fish. Alternatively, individual fish may vary in their response to similar exposure concentrations. Studies have used whole body homogenates to assess vitellogenin protein levels in small-bodied minnows in the field (Palace et al. 2006). Vitellogenin has a relatively long half-life in male blood plasma because males lack a specific mechanism for removal (Burki et al. 2006). Therefore, there are often higher levels in blood plasma than in the liver (Burki et al. 2006). I might have detected vitellogenin protein in more fish and at greater levels if I had used whole body homogenates rather than liver homogenates. Because vitellogenin protein has a limited half-life and is less abundant in fish livers, longnose dace that did have detectable levels of hepatic vitellogenin protein indicate a considerable physiological response to estrogenic compound exposure in Southern Alberta rivers.

3.3.4 Effects of vitellogenin production

There is a negative relationship between liver size and vitellogenin production in the Red Deer and Oldman Rivers. HSI, a measure of relative liver size, has been used as an estimate of energy storage in wild fish (Munkittrick et al. 2000). Fish that experience beneficial growing conditions often have larger livers because of increased lipid and

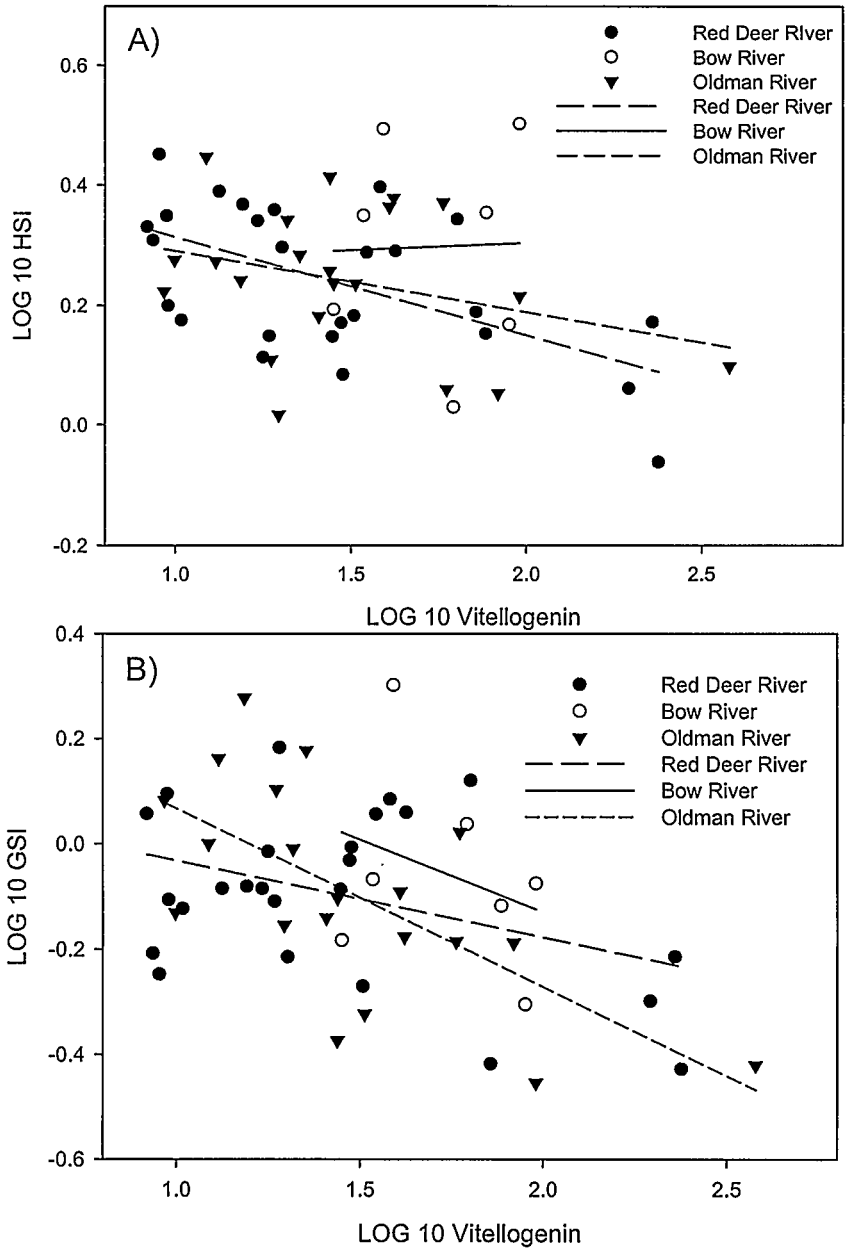


Figure 3.5 A) Hepatosomatic indices (HSI) and B) gonadosomatic indices (GSI) compared with hepatic vitellogenin protein levels in male longnose dace (*Rhinichthys cataractae*) collected from the Red Deer, Bow and Oldman Rivers, Alberta, Canada. Only males with detectable levels of vitellogenin protein were used in these comparisons.

glycogen storage. Production of the egg-yolk precursor protein vitellogenin by males should impose an energetic expense. I found a significant negative correlation in the Red Deer River ($r=-0.57$, $n=26$, $p<0.003$) for males with detectable levels of vitellogenin protein (Figure 3.5). There was a negative correlation in the Oldman River, although it was not significant ($r=-0.32$, $n=21$, $p=0.16$).The individual variation in liver size is high in the Oldman River which probably influenced the statistical outcome of the analysis. When data from the Red Deer and Oldman Rivers are pooled, there is a strong negative correlation between HSI and vitellogenin production ($r=-0.47$, $n=47$, $p<0.001$), which suggests an energetic cost of vitellogenin production in males in the two rivers. There was no relationship between HSI and vitellogenin production in males in the Bow River ($r=0.03$, $n=7$, $p=0.95$). This may result from high variability in liver sizes and a low sample size. There may also be different stresses in the Bow River that are causing different energetic responses when compared to the Red Deer and Oldman Rivers.

There was a negative relationship between gonad size and vitellogenin production in the Red Deer, Bow and Oldman Rivers. There is no consensus as to whether male exposure to estrogenic compounds will have detrimental reproductive effects on fish populations (Mills and Chichester 2005). In the laboratory, Jobling et al. (1996) reported a decrease in gonad growth due to estrogenic compound exposure in rainbow trout and a negative correlation between GSI and vitellogenin production. Gonad size in fish is directly related to reproductive success and GSI is often used as an estimate of reproductive investment in field populations (Munkittrick et al. 2000). I found a significant, negative correlation between GSI and vitellogenin production in the Oldman River ($r=-0.50$, $n=21$, $p<0.02$), and a borderline significant negative correlation in the Red Deer River ($r=-0.39$, $n=25$, $p=0.054$) for males with detectable levels of vitellogenin protein. There was a negative correlation in the Bow River, but this was not significant ($r=-0.31$, $n=7$, $p=0.50$). When all males from the three rivers are pooled, there was a significant negative correlation between GSI and vitellogenin production ($r=-0.39$, $n=53$, $p<0.004$). Because reproductive success is related to gonad size, smaller GSI suggests a reproductive cost of vitellogenin production in male longnose dace. This requires further

investigation to identify the specific mechanisms that cause reduced gonad size in male longnose dace due to estrogenic compound exposure.

3.4 Conclusions

In this study, I detected many organic contaminants from multiple sources at every site sampled in the Red Deer, Bow and Oldman Rivers. Many of these compounds likely contribute to the estrogenic response of longnose dace, an abundant minnow in Alberta rivers. These estrogenic compounds, plus others that were not measured, could cause the vitellogenin responses detected in male longnose dace. There was vitellogenin protein detected in at least one fish at every site sampled except Popson Park. Vitellogenin production in males likely has energetic and reproductive costs evinced by reduced liver and gonad sizes, respectively. I have demonstrated the presence of organic contaminants spatially and a biological response to the cumulative impacts of municipalities and agricultural practices in prairie rivers. As human and animal populations grow in Southern Alberta, which will result in greater impacts on water quality, organic contaminant concentrations may increase in the Red Deer, Bow and Oldman Rivers and potentially affect the long-term viability of longnose dace populations.

Chapter Four: Conclusions

A variety of chemical contaminants are introduced to rivers from anthropogenic sources such as municipal wastewater and agricultural run-off (Kolpin et al. 2002). Many of these compounds have the ability to adversely affect fish populations. These compounds often enter aquatic systems in complex mixtures, which make linking a fish response to an individual compound very difficult (Brian et al. 2007). In the South Saskatchewan River Basin (SSRB), major rivers are impacted by municipal wastewater effluent and agricultural run-off, which can introduce many compounds into the rivers and affect water quality. The goal of this study was to assess the cumulative impacts of municipal wastewater and agriculture on rivers in the South Saskatchewan River Basin.

4.1 Organic contaminants

I detected many organic contaminants from multiple sources at every site sampled in the Red Deer, Bow and Oldman Rivers in the SSRB using GC-HRMS. I detected natural and synthetic hormones primarily downstream of municipal wastewater effluents. I also detected increasing concentrations of total sterols (cholesterol and its derivatives), phytosterols and fecal sterols with distance downstream, which suggests cumulative inputs increase the amounts of organic contaminants in these rivers. Interestingly, estrogenic compounds were detected at every site sampled, which indicates that estrogenic compounds are widespread throughout the rivers and not limited to downstream of major point sources. Few studies have directly assessed the spatial distribution of EDCs in aquatic systems. I used contaminant concentration snapshots to address how prairie rivers change with distance downstream. The numbers of sample sites, the distances covered within each river and the number of contaminants measured at each site, make these results some of the most comprehensive to address the spatial distribution of organic contaminants in rivers.

My samples were taken during months of historical low flow for all rivers. However, river flow fluctuates dramatically throughout the year, which alters the concentrations of organic contaminants in the rivers. Therefore, temporal changes in

contaminant concentrations need to be identified in future studies to fully understand how municipal wastewater and agriculture affect water quality in the SSRB.

Many estrogenic compounds that enter rivers accumulate in river sediments (Petrovic et al. 2002). Contact with contaminated sediments may be an additional exposure route for fish (Mills and Chichester 2005). Longnose dace reside in the benthic substrate and dig into the sediments to find food; therefore they may be particularly sensitive to sediments that are contaminated with estrogenic compounds. To fully understand the total estrogenic compound loading that occurs in rivers and how it affects longnose dace populations; estrogenic compound concentrations in the sediments must be determined. This may also provide estimates of the rate that specific compounds are removed from the water column if concentration gradients are detected in the sediments downstream of point sources.

4.2 Vitellogenin

Bioindicator species are used to determine the cumulative effects of all chemical exposures on growth, reproduction and survival of fish populations. My thesis research used longnose dace as bioindicators of estrogenic compound exposure in major rivers in Southern Alberta. Essentially, I used longnose dace to tell me where they are being exposed to estrogenic compounds along natural river gradients. I used the egg-yolk protein precursor vitellogenin to detect a physiological response to exposure to estrogenic compounds in riverine longnose dace populations. I also measured fish performance endpoints to determine how physiological exposure to estrogenic compounds scales up to individual and population levels of organization.

Longnose dace were useful bioindicators of estrogenic compound exposure because they show changes in hepatic vitellogenin levels at different sites along river gradients. Differences in vitellogenin levels were detected in sites that were relatively close together, but separated by large point sources. For example, longnose dace populations sampled immediately upstream and downstream of Lethbridge had significantly different mean vitellogenin levels. The river distance between the two sampling locations is only 18 km. This indicates that longnose dace and the vitellogenin

response are sensitive to estrogenic compound inputs to the rivers. This also suggests that longnose dace display site fidelity. Therefore, longnose dace are a suitable fish species to determine biological responses to estrogenic compounds in Southern Alberta rivers.

I found that vitellogenin levels were often elevated downstream of municipalities and in areas influenced by agriculture and cattle operations. I used two different techniques to detect vitellogenin levels in longnose dace. I used QRT-PCR to determine vitellogenin mRNA levels in male and female liver samples in April and October/November, 2005. QRT-PCR is a sensitive assay that is able to amplify relatively small amounts of target RNA. This is important for environmental studies using small-bodied fish where tissue samples may be limited. Environmental exposures are often at low concentrations and therefore the fish responses may be small, which would make QRT-PCR an appropriate technique. I was able to quantify vitellogenin mRNA in every longnose dace sampled, even at very low levels. I also used an ELISA technique to detect vitellogenin protein only in male liver samples in October/November, 2005. These were the same liver samples used for the October/November QRT-PCR analysis. The ELISA was less sensitive than the QRT-PCR and therefore I was not able to detect protein levels in every fish sampled. Therefore the ELISA results were not always consistent with the QRT-PCR results. Whole body homogenates may be more appropriate to detect vitellogenin by ELISAs in small-bodied fish rather than liver homogenates. Whole body homogenates may have resulted in a higher detection rate. Because longnose dace are small-bodied minnows and environmental exposures to estrogenic compounds are often at low concentrations, QRT-PCR is a sensitive and valuable technique to detect vitellogenin levels in longnose dace.

Estrogenic compounds can bind to estrogen receptors and regulate estrogen responsive genes (Jobling et al. 1996). Vitellogenin up-regulation due to estrogenic compound exposure may operate via the estrogen receptor pathway. My approach was to assess the endpoint, vitellogenin levels, but this did not provide evidence as to what mechanism caused the vitellogenin up-regulation. It is unknown whether the vitellogenin up-regulation I detected was through the estrogen receptor in longnose dace. I was also

unable to determine if the compounds that caused vitellogenin up-regulation could potentially interact with other processes such as sex hormone secretion, which could affect spawning behaviour. Further research into how estrogenic compounds in the SSRB affects the longnose dace endocrine system could provide insight into how estrogenic compounds generally impact fish populations in the field.

4.3 Implications

This study is one of the first large-scale surveys of longnose dace health in Alberta rivers impacted by municipal wastewater and agriculture. The effect of municipal wastewater effluent is often not evaluated together with agricultural influences along long river gradients, or in multiple rivers within the same basin. Cumulatively, these impacts introduce estrogenic compounds into rivers in the SSRB, which affects longnose dace physiology evinced by vitellogenin production in males. My results suggest that the cumulative impacts of municipal wastewater, agriculture and large cattle operations have a major influence on longnose dace populations in the Oldman River where there was strong evidence of endocrine disruption that included female-biased sex ratios. In the Red Deer and Bow Rivers, municipal wastewater appears to be a major source of estrogenic compounds that affect longnose dace. Surprisingly, vitellogenin production was detected at every site where longnose dace were collected, which indicates widespread exposure to estrogenic compounds in the SSRB. This thesis demonstrates that estrogenic compounds are present in the SSRB at levels high enough to impact fish populations and at large spatial distributions. Therefore, estrogenic compounds should be monitored and incorporated into long-term water quality management plans by SSRB managers because they are already at levels that can adversely affect native biota. If river flow decreases further due to increased future water use, concentrations of EDCs may increase in the SSRB and cause major biological impacts on aquatic organisms.

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APPENDIX A: PARTIAL CLONING OF LONGNOSE DACE VITELLOGENIN AND BETA-ACTIN RESULTS

A.1. Longnose dace partial vitellogenin sequence.

Vitellogenin sequence results as determined by RT-PCR. This sequence has been deposited in Genbank (Accession #: EF202607).

1866 base pair sequence:

TGAAGCTCATGGATCCTCTACTCCACGAGTATGCTGGCATTTGGCCCAAGGAT
CCATTTGTTCCTGCCACTAAGCTCACCTCAGCTCTGGCTGCTCAGCTTCAGAT
TCCCATCAAGTTTGAGTATGCTAATGGTGTGGTTGGAAAGGTATTCGCCCCTG
CAGGAGTCTCCCCTACAGTACTGAACCTGCACAGAGGAATCCTCAACATCAT
TCAGCTCAACCTCAAGAAGACCCAGAACATCTATGAGCTGCAAGAGGATGGA
GTTCAGGGAGTGTGCAGGACCCACTACGTCATCAATGAGGATACAAAGGCCA
ACCACATTATTGTCACCAAGTCTAAGGATCTGAACCACTGTCAGGAGAGAAT
CATGAAGGACGTCGGTTTGGCGTACACTGAGAGGTGTGCTGAATGCACAGAG
AGGGTCAAGAGTCTGATTGAAACTGCATCTTACAACTACATCATGAAACCAT
CTGCCGCCGGTGTACTGATCGCTGAAGCCACAGTTGAGGAAGTGCATCAGTT
TTCACCCTTCAATGAGATCCATGGTGCTGCCCAGATGGAAGCAAAACAAACC
TTGACTTTTGTTGAGATGGAGAAGACGCCTGTTGTTCCAATCAAAGCTGATTA
CTTGGCTCGTGGATCCTTGCACTATGAGTTTTCAACTGAAATTCTTCAGACCC
CCATTCAACTCATGAAGATCAGTGATGCACCCGCTCAGATTATTGAGGTCTTA
AAGCACCTTGTTGAAAACAATGTGGCCATGGTCCATGATGATGCTCCACATA
AGTTTATTGAGCTCATCCAGCTCCTGCGTGCTGCCACTTTGGAGAATATTGAG
GCTATCTGGGCTCAGTTCAAAGACAAACCAGTTTACAGGCGCTGGCTTATGG
ATGCTCTTCCTGCTGTTGGCACACCAGTCATTGTAAAATTCATCAAGGAGAAG
TTTCTGTCTGGTGAACTTACCATTCCTGAGTTCATTCAGGCTCTTGTGGTTGCT
CTGCAAATGGTCACTGCTGATTTGGACACCATCCAGTTGACAGCTAGTTTGGC
TATGCACGAGAAAATCGCCAAAATCCCAGCTCTGCGTGAAGTCGTCATGCTT
GGATATGGTTCCATGATTGCCAAACACTGTTTTGCAGTTCCCACTTGCCCCTC
CGAGCTCCTCAAGCCCATCCATGATATTGCTACAGAGGCCATTTCTAAGAATG
ACATTCCTGAAATCACTTTGGCTCTGAAAGTTATGGGCAATGCTGGTCACCCT
GCTAGTCTTAAAACCATCATGAAGCTCCTACCTGGACTGAGAACTGCAGCTA
CTTCTCTGCCTCTTAAAGTCCAGGTTGATGCCATCTTGGCTCTGAGGAACATT
GCCAAGAAAGAGCACAAACTGGTTCAGCCAGTGGCCCTGCAGCTTGTATTGG
ACAGGGCTCTCCACCCTGAAGTGCGTATGGTTGCTTGTATTGTTTTGTTCGAG
GCCAAGCCCTCAGTGGCTCTCGTCTCCAATCTTGCTGGTGCTTTGAAGACTGA
GACTAACATGCATGTTGCGAGCTTTGCCTATTCCCACATGAAGTCCTtGACCA
GAATCACtGCACCTGATATGGCATCTGTTGCGGGTGCAGCTAATGTTGCCATC
AAGCTCATGAATCGCAAACTGGACAGGCTTAGCTTCCGTTTCAGCAGAGCCA
TTCAGCTGGACTTCTATCATTCTCCTCTTATGATTGGAGCTGCTGGTAGTGCCT

ACATGATCAATGATGCCGCCACCATCCTGCCCAGAGCTGTTGTAGCTAAAG
CACGTGCTTACCTGGCTGGAGCTGCTGCTGATGTTCTTGAGATCGGTGTGAGA
ACTGAAGGAATCCAGGAGGCTCTTC

Corresponding 621 amino acid sequence:

KLMDPLLHEYAGIWPKDPFVPATKLTSALAAQLQIPIKFEYANGVVGKVFAPAG
VSPTVLNLHRGILNIIQLNLKKTQNIYELQEDGVQGVCRTHYVINEDTKANHIIVT
KSKDLNHCQERIMKDVGLAYTERCAECTERVKSLIETASYNYIMKPSAAGVLIAE
ATVEEVHQFSPFNEIHGAAQMEAKQTLTFVEMEKTPVVPIKADYLARGSLHYEF
STEILQTPIQLMKISDAPAQIIEVLKHLVENNVAMVHDDAPHKFIELIQLLRAATLE
NIEAIWAQFKDKPVYRRWLMDALPAVGTPVIVKFIKEKFLSGELTIPEFIQALVVA
LQMVTADLDTIQLTASLAMHEKIAKIPALREVVMLGYGSMIAKHCFAVPTCPSEL
LKPIHDIATEAISKNDIPEITLALKVMGNAGHPASLKTIMKLLPGLRTAATSLPLKV
QVDAILALRNIAKKEHKLVQPVALQLVLDRALHPEVRMVACIVLFEAKPSVALV
SNLAGALKTETNMHVASFAYSHMKSLTRITAPDMASVAGAANVAIKLMNRKLD
RLSFRFSRAIQLDFYHSPLMIGAAGSAYMINDAATILPRAVVAKARAYLAGAAA
DVLEIGVRTEGIQEAL

A.2. Comparison of the partial longnose dace vitellogenin sequence with other organisms.

Organism	Genbank #	% Similarity	# of Gaps
Fathead minnow (*Pimephales promelas*)	AF130354	95.3%	0
Common carp (*Cyprinus carpio*)	AF414432	90.5%	0
White cloud Mountain Minnow (*Tanichthys albonubes*)	EF370398	85.2%	0
Goldfish (*Carassius auratus*)	ABG22139	83.7%	0
Xenopus (*Xennopus laevis*)	CAA68433	42.2%	2
Chicken (*Gallus gallus*)	BAD32701	40.6%	8
Human apolipoprotein B-100	PO4114	24.5%	91

A.3. Longnose dace partial β-actin sequence.

B-actin sequence as determined by QRT-PCR. This sequence has been deposited in Genbank (Accession #: EF202606).

55 base pair sequence:
tggcatgggacagaaggactcatatgtgggagatgaggctcaaagcaagagagga

Corresponding 18 amino acid sequence:

GMGQKDSYVGDEAQSKRG

A.4. Comparison of the partial longnose dace β-actin sequence with other organisms.

Organism	Genbank #	% Similarity	# of Gaps
Common Fat minnow (*Rhynchocypris oxycephalus*)	AAF63689	100%	0
Common carp (*Cyprinus carpio*)	AAR04426	100%	0
Goldfish (*Carassius auratus*)	BAA08756	100%	0
Xenopus (*Xennopus laevis*)	AAX85447	100%	0
Chicken (*Gallus gallus*)	XP_422898	100%	0
Human	AAH12854	100%	0

APPENDIX B: TOTAL CONCENTRATIONS OF ORGANIC CONTAMINANTS.

Twenty-eight organic contaminants were measured at twenty-three sites in the Red Deer, Bow and Oldman Rivers, Alberta. The concentrations are in ng/L and are internal standard and blank corrected. The samples were analyzed using GC-HRMS at the Institute of Ocean Sciences (Department of Fisheries and Oceans, Sydney, British Columbia, Canada). Coprostan-3-one was not measured at Joffre Bridge in the Red Deer River. Compounds that were not detected by the assay are indicated by ND.

B.1. Red Deer River, Alberta.

Compound (ng/L)	Penhold Bridge	Ft. Norm.	Riverbend	Joffre Bridge	Content Bridge	Tolman Bridge	Morrin Bridge	Drum.
Estrone	0.70	0.58	ND	2.55	2.65	5.26	5.15	2.72
17β-Estradiol	ND	0.24	ND	0.64	0.58	0.91	0.51	0.25
17α-Estradiol	ND	ND	ND	0.22	ND	ND	ND	ND
Estriol	ND	ND	ND	ND	ND	ND	ND	ND
Testosterone	ND	ND	ND	ND	ND	2.97	ND	ND
Equilin	ND	ND	ND	1.92	ND	1.67	ND	ND
d-Equilenin	ND	ND	ND	0.72	ND	0.61	0.54	ND
Mestranol	ND	ND	ND	ND	ND	ND	ND	ND
19-Norethindrone	ND	ND	ND	1.02	0.44	0.50	ND	ND
17α-Ethynylestradiol	ND	ND	ND	ND	ND	ND	ND	ND
(-)-Norgestrel	ND	1.71	2.33	0.95	3.38	7.91	5.82	ND
α-Zearalanol	ND	7.28	ND	ND	ND	ND	ND	ND
β-Estradiol-3-benzoate	ND	ND	ND	ND	ND	ND	ND	ND
Bisphenol A	ND	ND	ND	3.08	ND	ND	ND	ND
Cholesterol	110.25	225.44	209.43	176.48	214.97	264.53	281.44	302.56
Desmosterol	17.79	41.26	36.64	ND	40.26	40.95	56.23	54.55
Cholestanol	12.93	36.86	26.97	25.12	27.68	27.78	38.64	42.05
6-Ketocholestanol	0.39	0.65	0.39	ND	ND	0.72	1.28	1.28
7-Ketocholesterol	7.88	15.02	11.03	9.12	10.02	14.35	18.15	12.78
Coprostan-3-one	43.11	114.73	6.34	N/A	100.02	111.93	173.90	188.77
Ergosterol	ND	23.33	18.46	26.09	26.93	26.45	36.62	26.04
β-Sitosterol	72.61	118.17	116.88	117.28	107.46	122.76	137.37	151.26
Campesterol	12.89	21.21	19.72	18.30	18.38	23.23	25.90	28.72
Stigmastanol	22.15	55.80	44.14	48.18	40.99	55.38	64.18	84.61
Stigmasterol	119.57	197.59	194.49	174.26	183.89	247.61	235.51	339.60
Fucosterol	174.67	264.62	256.80	240.02	259.18	299.94	340.77	353.33
Totarol	ND	ND	ND	ND	ND	ND	ND	ND
Pinosylvin	ND	ND	ND	ND	ND	ND	ND	ND

B.2. Bow River, Alberta.

Compound (ng/L)	Jumping. Creek	Edworthy Park	St. George's	Carburn Park	Fish Creek Park	Policeman's Flats	Hwy 24
Estrone	ND	ND	ND	ND	ND	ND	ND
17β-Estradiol	ND	ND	ND	ND	ND	ND	ND
17α-Estradiol	ND	ND	ND	ND	ND	ND	ND
Estriol	ND	ND	ND	ND	ND	ND	ND
Testosterone	ND	ND	ND	ND	ND	ND	ND
Equilin	ND	ND	ND	ND	ND	ND	ND
d-Equilenin	ND	ND	ND	ND	ND	ND	ND
Mestranol	ND	ND	ND	ND	ND	ND	ND
19-Norethindrone	ND	ND	ND	ND	ND	ND	ND
17α-Ethynylestradiol	ND	ND	ND	ND	ND	ND	ND
(-)-Norgestrel	ND	ND	ND	ND	ND	ND	ND
α-Zearalanol	ND	ND	ND	ND	ND	ND	ND
β-Estradiol-3-benzoate	ND	ND	ND	ND	ND	ND	ND
Bisphenol A	ND	ND	ND	ND	ND	ND	ND
Cholesterol	45.86	168.16	101.55	186.22	382.95	294.67	216.06
Desmosterol	ND	67.80	ND	ND	69.61	57.50	45.26
Cholestanol	12.14	17.20	9.22	33.89	77.88	67.37	50.03
6-Ketocholestanol	ND	ND	ND	ND	ND	ND	ND
7-Ketocholesterol	ND	7.61	ND	9.27	36.02	23.03	24.74
Coprostan-3-one	ND	58.48	51.06	ND	698.65	25.44	246.26
Ergosterol	ND	ND	ND	ND	36.17	ND	13.14
β-Sitosterol	43.23	272.18	172.10	199.09	165.22	157.98	152.03
Campesterol	8.21	25.57	17.38	26.20	44.57	32.24	29.18
Stigmastanol	13.20	48.92	ND	5.57	41.71	35.71	24.45
Stigmasterol	16.89	109.74	67.25	9.83	68.78	80.28	71.75
Fucosterol	63.16	381.30	283.23	302.65	250.28	239.62	185.78
Totarol	ND	ND	ND	ND	ND	ND	ND
Pinosylvin	ND	ND	12.72	ND	ND	ND	ND

B.3. Oldman River, Alberta.

Compound (ng/L)	Olin Bridge	Summer. Bridge	Hwy 3A	Popson Park	Pavan Park	Hwy 845	Taber	Hwy 36
Estrone	ND	ND	ND	0.15	0.15	0.72	0.74	0.75
17β-Estradiol	ND	ND	0.54	0.20	ND	0.35	1.01	0.88
17α-Estradiol	ND	ND	ND	ND	ND	ND	ND	ND
Estriol	ND	ND	ND	ND	ND	ND	ND	0.48
Testosterone	ND	ND	ND	ND	ND	0.42	ND	ND
Equilin	ND	ND	ND	ND	ND	ND	ND	ND
d-Equilenin	ND	ND	0.20	ND	ND	ND	ND	ND
Mestranol	ND	ND	ND	ND	ND	ND	0.19	0.15
19-Norethindrone	ND	0.42	ND	ND	0.49	ND	ND	ND
17α-Ethynylestradiol	ND	ND	ND	ND	ND	ND	ND	ND
(-)-Norgestrel	ND	ND	ND	ND	ND	ND	ND	ND
α-Zearalanol	ND	ND	3.40	ND	ND	ND	6.16	3.81
β-Estradiol-3-benzoate	ND	ND	ND	ND	ND	ND	ND	ND
Bisphenol A	ND	ND	ND	ND	ND	4.61	ND	ND
Cholesterol	46.31	74.70	184.37	108.15	124.52	216.10	270.66	217.98
Desmosterol	12.19	28.46	45.58	36.34	35.40	50.57	66.03	51.58
Cholestanol	5.20	3.61	24.08	12.26	15.69	29.77	46.11	36.27
6-Ketocholestanol	ND	ND	0.42	ND	ND	ND	0.70	ND
7-Ketocholesterol	4.79	1.65	13.23	7.27	8.25	12.99	13.44	9.90
Coprostan-3-one	6.77	ND	61.08	18.05	54.29	106.95	141.88	97.58
Ergosterol	20.11	ND	23.54	26.53	23.25	33.97	28.58	15.38
β-Sitosterol	58.26	40.56	99.75	48.62	49.83	152.07	96.93	118.74
Campesterol	6.13	9.43	15.91	10.12	10.73	17.35	18.15	16.75
Stigmastanol	43.96	2.47	32.01	10.64	11.55	39.45	47.09	33.39
Stigmasterol	7.79	19.80	101.17	42.01	44.62	147.47	104.67	118.20
Fucosterol	87.47	65.18	160.82	111.63	126.28	237.39	207.84	208.14
Totarol	ND	ND	ND	ND	ND	ND	ND	ND
Pinosylvin	ND	ND	ND	ND	ND	ND	ND	ND