THE EFFECTS OF WASTEWATER TREATMENT PLANT EFFLUENT AND AGRICULTURAL RUNOFF ON THE REPRODUCTIVE SYSTEMS OF FATHEAD MINNOW, *PIMEPHALES PROMELAS*.

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ABSTRACT

Endocrine disrupting compounds and pesticides have been detected in rivers and irrigation canals of Southern Alberta, a semiarid region with irrigation-dependent crop production, intensive livestock operations, and a growing human population. However, little is known about the effects of agricultural runoff or wastewater treatment plant (WWTP) effluent in Southern Alberta on fish. Reproductive effects of WWTP effluents from the cities of Lethbridge and Medicine Hat, as well as agricultural runoff in the Lethbridge Northern Irrigation District canals, were investigated in a field study with wild fathead minnows (FHMN) in the Oldman and the South Saskatchewan rivers, in Alberta, Canada, and in a laboratory study with laboratory reared FHMN exposed *in vivo* to the city of Lethbridge WWTP effluent for 21 days. Biochemical and morphological endpoints were measured to characterize reproductive status. Liver vitellogenin, a biomarker of exposure to estrogen mimics, was analyzed using quantitative RT-PCR, and gonadal histology was used to determine sex, gonadal maturity, and intersex. Adverse reproductive effects were detected in FHMN exposed for 21 days to 10 and 25% of Lethbridge WWTP effluent. In the field, effluents from both Lethbridge and Medicine Hat had an effect on the reproductive systems of FHMN. In canals, reproductive effects were detected in wild fathead minnows in years when water quality in irrigation drain canals decreased. Exposure to pesticides was estimated using acetylcholinesterase (AChE) inhibition. Exposure to Lethbridge WWTP effluent did not inhibit AChE, whereas results from the field study were inconclusive. In conclusion, reproductive systems of fathead minnows in Southern Alberta were impacted by anthropogenic chemicals.

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AChE - acetylcholinesterase
AENV - Alberta Environment
ANOVA – analysis of variance
AOPA - Agricultural Operations Practices Act
BD - Battersea Drain
BPA - bisphenol-A
CF - condition factor
CFO - confined feeding operation
DTNB – 5, 5'-dithiobis-2-nitro-benzoic acid
EDC - endocrine disrupting compounds
EE2 - ethinylestradiol
ELISA - enzyme-linked immunosorbent assay
EPEA - Environmental Protection and Enhancement Act
ER - estrogen receptor
FHMN - fathead minnow, *Pimephales promelas*
FSH - follicle stimulating hormone
GtH – gonadotropin hormone
GnRH - gonadotropin releasing hormone
GSI - gonadosomatic index
HPG – hypothalamic-pituitary-gonadal
HDPE - high density polyethylene
LDPE - low density polyethylene
LH - luteinizing hormone
LNID - Lethbridge Northern Irrigation District
LSI - liver somatic index
NRCB - Natural Resources Conservation Board
PCP - personal care product
PD - Piyami Drain
PVC - polyvinylchloride
QPCR - quantitative polymerase chain reaction
UK - United Kingdom
Vtg - vitellogenin
WWTP - wastewater treatment plant

CHAPTER 1. ENDOCRINE DISRUPTING COMPOUNDS: SOURCES, BIOTRANSFORMATION AND REPRODUCTIVE EFFECTS IN FISH – BACKGROUND AND LITERATURE REVIEW

1.1 Introduction

In many areas of Canada, wastewater effluents from domestic, industrial and agricultural sources are discharged into the aquatic environment with little or no regulation. In Alberta, few regulations existed until 1993 and 2002, when the Environmental Protection and Enhancement Act (EPEA) and Part 2 of the Agricultural Operations Practices Act (AOPA) respectively, came into effect. However, in Alberta the destination of effluents from many regulated and non-regulated sources is still the aquatic environment. Water availability in Southern Alberta, and in many other areas of the world, has become a concern as the demands on our aquatic resources are rapidly increasing while the quality is decreasing (Byrne et al. 2006).

Endocrine disrupting compounds (EDCs), chemicals that interfere with an organism's endogenous endocrine pathways (hormonal systems), have been detected in surface waters (Sosiak & Hebben, 2005) and groundwater (Arnon et al, 2008), as well as in sediments (Ternes et al. 2002a). They exert their effects by mimicking or blocking the action of natural hormones, or by disrupting their synthesis (Walker et al. 2005). EDCs that infiltrate water bodies may have the ability to specifically affect the reproductive systems of aquatic organisms by disrupting natural steroid hormone pathways. There is substantial evidence that EDCs have specific effects on the reproductive systems of aquatic species such as mollusks (Matozzo & Marin, 2005), amphibians (Kloas & Lutz,

2006) and fish (Kidd et al. 2007; Ankley et al. 2002; Servos 1999; Jobling et al. 1996; Purdom et al. 1994; Jobling & Sumpter, 1993).

EDCs include plasticizers (Barse et al. 2007), surfactants (Routledge & Sumpter, 1996), PCBs (Vaccaro et al. 2005), pesticides (Walsh et al. 2000), personal care products (PCP) (Schreurs et al. 2005), as well as medicinal products (pharmaceuticals) for human and animal use, and their metabolites (Ankley et al. 2002). Many of these chemicals are introduced into the aquatic environment via migration from utilities infrastructure, landfill sites, as well as industrial wastewater, and rural and urban domestic wastewater systems.

Substantial attention has been directed at the fate of estrogen-mimicking compounds in wastewater treatment plant (WWTP) effluent after scientists discovered intersex gonads (gonads containing both sperm and eggs) in fish exposed to WWTP effluents in the United Kingdom (UK) (Purdom et al. 1994). A variety of compounds within WWTP effluents, including pharmaceuticals, phenolic compounds (Jobling & Sumpter, 1993) and conjugated estrogen metabolites (excreted by pregnant women or those taking birth control pills) have since been characterized as being estrogenic in non-mammalian vertebrates (Purdom et al. 1994; Tyler et al. 1999).

Although effects at the species level have been investigated, mode of action of EDCs, multiple compound effects and interspecies differences in sensitivity have yet to be fully understood. The effects on species population dynamics have were investigated by Kidd et al. (2007), reporting the collapse of a fathead minnow, *Pimephales promelas*, population after 7 years of treating a whole lake with a common estrogenic EDC, 17 α-

ethinylestradiol (5-6 ng/L). However, understanding of whole ecosystem effects of EDCs and of cumulative effects remains limited (Weber et al. 2008; Dube et al. 2002).

In addition to WWTP effluents, agricultural runoff (confined feeding operation effluent, pesticides, and other chemicals) also introduce EDCs, both synthetic and natural, to the aquatic environment. Even though these sources have received less attention than WWTP effluents, there is a growing body of evidence demonstrating that runoff, especially from pesticide applications and confined feeding operations (CFOs), has an effect on the reproductive systems of oviparous species (Lorenzen et al. 2004; Ankley et al. 2002; Irwin et al. 2001). Steroid hormones excreted into the environment in the urine and feces of farm animals (Hanselman et al. 2003) have the potential, along with a suite of veterinary pharmaceuticals recently detected in Alberta waters (Forrest et al. 2006), to contaminate the aquatic systems through ground infiltration or surface runoff. With 34% of Alberta's cattle feedlot capacity (not including other livestock) in the immediate Lethbridge area (CanFax, 2008), there is an urgent need to determine whether individual or collective runoff in "feedlot alley", is affecting the reproductive systems of fish in Southern Alberta.

Research on these substances and their effects on aquatic organisms is an important area of study, since aquatic species are chronically exposed to waterborne chemicals, and their responses can be used as biomarkers for water quality. Even though there are chemical data on the occurrence of EDCs in Southern Alberta rivers (Forrest et al. 2006; Sosiak & Hebben, 2005; Metcalfe et al. 2003), and reproductive effects have been reported in longnose dace (Jeffries et al. 2008), the effects of these compounds on the reproductive systems of fathead minnow (FHMN) in Southern Alberta have not been

determined. Research in Southern Alberta is also urgently needed to determine the point source and non-point source impacts on the status of water quality and health of aquatic species, as public concern is escalating regarding water quantity and quality in the semiarid climate.

1.2 Sources of Endocrine Disrupting Compounds

1.2.1 Rural and Urban Domestic Wastewater

Wastewater effluent is one of the main contributors of EDCs to the environment. Some EDCs commonly found in domestic wastewater effluent include PCPs, steroid hormones (natural and synthetic) and pharmaceuticals. Pharmaceuticals encompass numerous chemicals created for human or veterinary use. Human use pharmaceuticals may be classified by therapeutic use, physiological or chemical action, the body system they affect, or as prescription or non-prescription (Clayton, 2001). Human use pharmaceuticals or their biotransformed metabolites are incorporated into rural and urban domestic wastewater and are of particular concern, since they are designed to affect specific physiological systems, but they may also affect non-target organisms with similar systems. An aging and growing human population results in greater use of pharmaceuticals and greater potential impacts. A review by Daughton & Ternes (1999) reports that pharmaceuticals such as steroids (estrogens and androgens), sympathomimetics, and antidepressants have a myriad of reproductive effects (induction of spawning and parturition, accelerated testicular maturation) on aquatic organisms (fish, bivalves, crustaceans). However, it is important to note, since many municipalities in Alberta process wastewater from industrial facilities, industrial chemicals (discussed

later) are also commonly detected in WWTP effluent (Sosiak & Hebben, 2005).The degree and type of WWTP processing is also a factor in the degree to which EDCs are degraded and their ultimate environmental fate (Servos et al. 2005).

Rural domestic wastewater is collected in a perforated underground tank (septic tank), allowing solids to settle out, while liquid can seep into the ground. Degrading products (e.g. bacteria, enzymes) are sometimes added to the tank to aid in decomposition of solid material, however, solid material can build up and tanks may have to be cleaned out by a vacuum truck and disposed.

Urban domestic wastewater enters a municipal or county collection system and is sometimes processed through a WWTP. The degree of treatment is determined by the plant technology and complexity. In Alberta (but not all Canadian provinces), treatment of urban wastewater is mandatory. The degree of treatment is regulated by Alberta Environment and is based on the population of contributors (AENV, 2009). After treatment, the wastewater is either released to land, stored in a lagoon or released to surface water (Arnon et al. 2008). However, wastewater stored in lagoons or released to land may leach into the water table if lagoon structures are not sound or if soil structure is conducive to leaching (Heberer, 2005; Arnon et al. 2008). Despite these efforts to treat domestic sewage, WWTPs are not efficient at removing all chemicals from the wastewater (Metcalfe, 2004), allowing EDCs to be transported into the aquatic environment.

1.2.2 Agricultural Sources

Veterinary pharmaceuticals are used in agriculture to prevent or manage disease, promote growth or alter physiological functions (eg reproductive cycles) (Health Canada, 2005). Endogenous reproductive hormones are naturally excreted by livestock. However, livestock operations that administer supplementary synthetic hormones could further contribute to the contamination of surface waters since agricultural use pharmaceuticals (Health Canada, 2001) augment endogenous hormones or their biotransformed metabolites that are excreted in waste (urine or feces). CFOs may also concentrate livestock effluent (thus EDCs) by collecting it into designated collection sites (ponds or piles). As of 2002, all new CFO's in Alberta are required by the Natural Resource Conservation Board under the *Agricultural Operation Practices Act* to contain animal excrements. However, CFO's built prior to this date are exempt from this requirement until such time that they want to modify or expand their operation. Modifications to existing operations require compliance to present regulations (NRCB, 2007). Excrement from a CFO remains in the collection site or is applied to agricultural land as fertilizer. Endogenous hormones and pharmaceuticals, or their biotransformed metabolites, may leach to the groundwater or water table due to ground infiltration or from improper or lack of pond containment. They may also be carried to surface water after a rain event in overland flow. Overland flow may also contain pesticide formulation residues.

Pesticide use is most prevalent in the prairie provinces of Canada (Tuduri et al. 2006) and herbicides are the class of pesticide most commonly used (Byrtus, 2000). Pesticides are classified by target organism (e.g. insecticides target insects) and each classification is then further broken down. For herbicides, classification is by mode of

action. Pesticides are known endocrine disruptors and have been shown to specifically disrupt the reproductive system of many organisms (McKinlay et al. 2008). Pesticides are often detected in surface waters (Anderson, 2005) and groundwater. These chemicals are introduced to the water through a variety of routes, overspray and ground seepage, but contributions via overland flow and runoff during precipitation events are considerable. Pesticides are comprised of active ingredients and non-active ingredients to create a pesticide formulation; either part of the formulation may cause endocrine disruption. Active ingredients are known to cause reproductive problems in fish such as delayed sexual maturation, abnormal gonad development (Kime, 1995), Vitellogenin (Vtg) production (Xie et al. 2005), or altered sex differentiation, reduced spawning and gamete production (Ankley et al. 2001). However, it is not uncommon for the non-active ingredient to be an endocrine disruptor, even if the active ingredient is not (Servos, 1999). Surfactants are one example of a chemical commonly used in the pesticide formulation, to act as an adjuvant or a chemical that helps the formulation stick to the foliage. Some common pesticide surfactants are alkylphenols and their polyethoxylates, which have been shown to have endocrine disrupting effects in organisms (Servos, 1999). Surfactants are also commonly used in industrial applications.

1.2.3 Industrial Sources

Surfactants and plasticizers are chemicals commonly found in industrial effluents. A surfactant is a “surface acting agent” that is used to reduce the tension (interfacial) between two media, one hydrophobic and one hydrophilic, that inherently repel each other. Surfactants have both a hydrophobic and hydrophilic group making them soluble

in both media, enabling mixing of the two. Surfactants have many applications in industries such as construction, oil and gas, agriculture, pesticide application, plastics and textiles; they are also used as building block chemicals for fragrances, antioxidants and fire retardant materials. Common surfactants are alkylphenols and their polyethoxylates such as nonylphenol, nonylphenol ethoxylate, octylphenol, and octylphenol ethoxylate. Alkylphenol ethoxylates increase in toxicity as their chain length decreases (Jobling & Sumpter, 1993) and though degradation usually results in a decrease in chain length, full biodegradation also results in reduced concentration (Servos 1999; Jobling & Sumpter, 1993).

Plasticisers are chemicals used to soften plastic to make it more pliable. The most commonly used plasticizers are phthalates. They are used in a wide range of products from toys and babycare items, insect repellants, perfumes, life-saving medical devices and cling wrap, to flooring, wall coverings, make up, and the manufacture of clothing and footwear. Phthalates are used to soften mainly polyvinylchloride (PVC) plastics and small quantities of plasticisers are also used in paints, adhesives and as solvents in pesticides. Many surfactants and plasticizers not only enter the aquatic environment through effluents from manufacturing but also from product leaching and landfill seepage. They are present in WWTP effluents and have been identified as reproductive endocrine disruptors. Gray & Metcalfe (1997) provide evidence for intersex in Japanese medaka with exposure to nonylphenol, and Jobling & Sumpter (1993) characterized estrogenicity of alkylphenol ethoxylates in rainbow trout.

Bisphenol-A (BPA) is used in the production of polycarbonate plastics and epoxy resins. It is an ingredient used to make PVC plastic, food can lining, baby bottles, water

bottles, medical and dental devices, and numerous electronics. BPA and metabolites may enter the aquatic environment similarly to surfactants and plasticizers. BPA binds to the estrogen receptor (USEPA, 2005) in fish and reproductive effects were demonstrated in FHMN exposed to BPA (Sohoni et al. 2001).

1.3 Biotransformation

Endogenous or introduced compounds are not always fully metabolized in the body and some portion of the parent compound may be excreted. Compounds can also be degraded and/or transformed in the body by enzymes (Phase I, Phase II). Pharmaceuticals are created either as pro-drugs that require metabolic transformation in the body before becoming active, or conversely as the active form where the chemical can act immediately once in the body and metabolic conversion terminates the desired effects of the drug (Cunningham, 2004; Parkinson, 2003). The unchanged parent chemicals (Heberer & Adam, 2005) or their metabolites are discharged into the environment, however the understanding of their fate and their impact on non-target species is still very limited. Recent studies carried out in Canada have detected pharmaceuticals in our WWTP effluent (Ternes, 1999), surface waters and sediments (Forrest et al. 2006; Sosiak & Hebben, 2005). Presence of pharmaceuticals in effluent indicates that WWTPs remove some but not all pharmaceuticals and their metabolites. It is apparent that studies on only parent compounds may not be suitable for determining the impact of chemicals on non-target organisms. Unfortunately fate of the thousands of pharmaceuticals being used is not often known and further testing is needed (Cunningham 2004; Parkinson, 2003; Randall, 2002).

Pharmaceuticals (parent molecule or metabolites) in domestic or animal sewage entering the wastewater treatment plant or collection pond, may again undergo chemical changes. Microbes and fecal enzymes can further metabolize the metabolites (e.g. glucuronides can be deconjugated to parent compound form during the sewage treatment process) or metabolites may adsorb to biomass, sediments (Cunningham, 2004) or soils (Heberer & Adam, 2005). The fate of compounds depends on their properties and the environment, including pH and temperature.

1.4 Contamination of Drinking Water

Wastewater from wastewater treatment plants and agricultural operations in watershed headwaters eventually become the raw water source for downstream communities. Water treatment plants are projected to purify this water to make it suitable for consumption in downstream communities. However, thousands of new chemicals are developed annually and technology is failing to develop detection and removal methods for these compounds. The public and the scientific community are unsure of the contaminant loads and the implications of their possible presence in the drinking water supply. EDCs have the potential to impact aquatic species (Daughton & Ternes, 1999), as well as humans exposed through drinking water (Webb et al. 2005). Water treatment plant types vary in design but none were originally intended to remove these compounds from the water and at least some compounds escape from even the most technologically advanced plants (Webb et al. 2005; Ternes et al. 2002b). Recent studies carried out in Canada show pharmaceuticals are in our drinking water (Forrest et al. 2006; Sosiak & Hebben, 2005; Webb et al. 2005). Some of the greatest current challenges of aquatic

ecotoxicology are to: 1) determine whether the concentrations of human and veterinary use pharmaceuticals detected in our surface waters have adverse effects on non target species; 2) set safe concentrations guidelines; and 3) elucidate the mechanisms of action through which these chemicals exert their adverse effects on non-target species.

1.5 Reproductive Physiology

Fish reproductive systems are dependent on endogenous sex hormones for normal function and behavior (Redding & Patino, 1993) via two main physiological systems, activational and organizational. Activational effects result in changes in morphology, function and behavior (e.g. influence of sex steroids on seasonal morphology and behavior), but changes disappear upon removal of stimulus. Organizational system disruptions result in permanent morphological changes (e.g. gender-specific brain and gonad characteristics) that persist after removal of the stimulus. Disruption of either system by exogenous estrogenic compounds could result in changes to essential reproductive function and behavior, having a considerable effect on the individual's reproductive fitness and species population dynamics (Hiramatsu et al. 2005; Kidd et al. 2007).

Fish reproductive hormones are synthesized and regulated through the hypothalamic-pituitary-gonadal axis. In response to environmental stimuli (photoperiod, temperature, food availability) and endogenous endocrine signals, the hypothalamus synthesizes and releases gonadotropin releasing hormone (GnRH) to the pituitary which stimulates the release of gonadotropins GtH I and GtH II (Kime, 1995), hormones homologous to the mammalian gonadotropin hormones (follicle stimulating hormone,

FSH, and luteinizing hormone, LH). GtH I initiates gametogenesis and steroidogenesis, whereas GtH II is responsible for final maturation of gametes (Hiramatsu et al. 2005; Kime, 1999; Kime, 1998). Gonadotropins drive the reproductive process by stimulating gonadal tissues to produce steroid hormones (estrogens, androgens and progestins), which stimulate other tissues to affect reproductive processes and which also exert positive or negative feedback. In females, GtH I stimulates follicular thecal cells to produce testosterone which is later aromatized to estrogen in the granulosa layer. Estrogen binds to estrogen receptors (ER) in the liver to stimulate the production of the Vitellogenin protein via Vtg mRNA. Vitellogenin is an egg yolk precursor (phospholipoprotein) that is transported through the blood back to the oocytes where it binds to receptors on the oocyte and is integrated to provide nutrients for growth of the embryo (Hiramatsu et al. 2005). When GtH I declines, GtH II increases (Arcand-Hoy & Benson, 1998) stimulating final maturation of oocytes and ovulation by binding to granulosa cells, which synthesize and release progestins (Arcand-Hoy & Benson, 1998). In males GtH I is responsible for gametogenesis by stimulating production of spermatogonia in the seminiferous tubules and of androgens by the interstitial cells of Leydig. GtH II is high throughout spawning when levels of androgens (and GtH I) are lower but progestins are high (Hiramatsu et al. 2005; Kime, 1999; Arcand-Hoy & Benson, 1998).

Males do not normally produce high enough concentrations of estrogen to trigger the redundant gene in the liver to produce Vtg, however, when exposed to exogenous estrogens, males can be triggered to produce Vtg (Matozzo et al. 2008; Kime, 1998; Tyler et al. 1996) and, even develop intersex testis, where oocytes are mixed with sperm

(Kidd et al. 2007). Genotypic males exposed to exogenous estrogens during an early developmental window can change into phenotypic females (Parrott & Blunt 2005), skew the sex ratio (Panter et al. 2006) and influence population dynamics (Ankley et al. 2008; Kidd et al. 2007). Other effects include a decrease in spawning activity, spawning success and male secondary sex characteristics (Parrott & Blunt 2005).

1.6 Test Species – *Pimephales promelas*

The fathead minnow (FHMN), *Pimephales promelas*, a teleost fish, has been selected as a suitable sentinel species to evaluate the reproductive status of fish exposed to potential pollutants in the field. The FHMN was chosen because of their prevalent distribution in southern Alberta (Nelson & Paetz 1992) and the extensive background information available for this fish (Van Aerle et al. 2004), including a procedure to measure Vtg by RT-PCR (Lattier et al. 2002). The FHMN is a small fish from the Cyprinidae family, it has a short life cycle and is easy to culture for laboratory studies (Jensen et al. 2001). Both the USEPA (2006) and Environment Canada (1997) use the FHMN for reproductive toxicity testing.

The FHMN is a sexually dimorphic oviparous fish that lives approximately two years and spawns in the spring through summer (Nelson & Paetz, 1992). It spawns multiple times per breeding season, every 3 to 4 days (Watanabe et al. 2007; Jensen et al. 2001) for females while males can breed continuously. Males develop a fatpad (for cleaning the nest), cranial nuptial tubercles, and parr marks during the breeding season and court females to spawn in shallow water on the underside of rocks, leaves or a solid surface (Nelson & Paetz, 1992). The ratio of males to females is 1:1 (Parrot & Blunt

2005; Parrot & Wood 2002; Zerulla et al. 2002; Lange et al. 2001) and males weigh 4 to 5 g and females 2 to 3 g (Watanabe et al. 2007)

1.7 Reproductive Status

Reproductive status in fish can be assessed by measuring specific biomarkers in the field or in the laboratory, to provide insight regarding an individual's ability to reproduce (Hiramatsu et al. 2005). Biological data can provide information regarding species fitness and future population trends. The biomarkers used to assess the reproductive status of fish include morphological measurements (gonad size, gonad morphology, gonad maturity, sex ratio) and biochemical measurements on plasma or tissues. Analysis of these biomarkers can indicate whether water quality is having an adverse effect on fish reproductive systems.

1.7.1 Biochemical Markers

The principal biochemical marker of exogenous estrogenic effects is an increased level of Vtg in male and juvenile fish (Hiramatsu et al. 2005; Tyler et al. 1996). Vtg is a yolk precursor protein produced by the liver of reproductively maturing females in oviparous vertebrates in response to estrogen. Vtg proteins are transported to growing oocytes via the blood. Growing oocytes take up Vtg and process it into yolk proteins which are then stored in the ooplasm. Vtg appears naturally in female fish in response to endogenous estrogens, but not in males or juveniles since they have low estrogen concentrations. However, exogenous estrogen will induce Vtg production in males and juveniles, and is therefore an indicator of estrogen exposure (Hiramatsu et al. 2005).

A number of assays have been developed to measure Vtg. Two popular methods include enzyme-linked immunosorbent assays (ELISAs) (Allen et al. 1999; Tyler et al. 1999; Tyler et al. 1996) which measure Vtg proteins, and quantitative RT-PCR (Islinger et al. 2002; Lattier et al. 2002; Lattier et al. 2001) which measures Vtg mRNA. Liver Vtg mRNA will be analyzed in this study.

1.7.2 Morphometric markers

Physical markers of adversely impacted reproductive fitness include lower GSI (Jobling et al. 1996), the ratio between the gonadal weight and the total body weight, and intersex gonads which negatively impact gamete production (Jobling et al. 2002). Gonadal histology is used to examine the morphology of the gonads, determine gonad maturity, diagnose intersex, and evaluate sex ratios in fish. Another physical marker used to assess fitness in fish is the condition factor (CF), calculated as $K=W\ (100)/L^3$ where L is the fork length and W is the total body weight, measured to assess the growth capacity of the fish (Moyle & Chech, 1996). Several environmental factors (including exposure to EDCs) can affect the ability of the fish to grow.

Exposure to pollutants other than EDCs can also influence growth. Pesticides are extensively used in Southern Alberta and there is evidence that these chemicals impact growth capacity and have endocrine disrupting (reproductive) effects (Ngoula et al. 2007). Organophosphate and carbamate pesticides inhibit acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine. Inhibition of AChE is a well validated marker of pesticide exposure (Sturm et al. 2007; Dorval et al. 2005;

Stenerson 2004). Although pesticide concentrations are monitored in S. Alberta rivers, the effects of pesticides on the reproductive systems of FHMNs and the interactive effects of pesticides and other chemicals in WWTP effluents or agricultural runoff are not presently understood.

Together with the biochemical indicators (hormone concentrations, Vtg, AChE activity) of effects and exposure, the morphological indicators (GSI, sex ratio, gonadal histology) can be used to assess the reproductive status of the fish.

1.8 Objectives of the study

The objectives of this study were to determine: 1) through the use of physiological, morphological and biochemical markers (Liver Vtg mRNA expression, GSI, LSI, CF, number of tubercles and AChE), if agricultural runoff and WWTP effluents in Southern Alberta have an effect on the reproductive systems of FHMN exposed in the field; 2) in a laboratory setting using physiological, morphological and biochemical markers, the effects of WWTP effluent on the reproductive system of laboratory-reared FHMN; 3) with standard histological procedures if FHMN exposed to WWTP effluent or agricultural runoff in Southern Alberta's aquatic systems, have abnormal gonads, skewed sex ratio or intersex. 4) whether there is a seasonal variation in physiological, morphometric and biochemical responses to agricultural runoff.

Hypothesis

Fish exposed to agricultural runoff and/or WWTP effluent have decreased reproductive fitness, characterized by abnormal GSI, LSI and CF, higher liver Vtg mRNA expression, abnormal sex ratios and abnormal gonadal morphology.

CHAPTER 2. REPRODUCTIVE EFFECTS OF WASTEWATER TREATMENT PLANT EFFLUENT IN FATHEAD MINNOW, *PIMEPHALES PROMELAS.*

2.1 Introduction

Increasing attention has been given to the effects of wastewater treatment plant (WWTP) effluents on aquatic organisms (National Research Council, 1999), including fish (Mills & Chichester 2005). Reproductive effects linked to the presence of estrogenic chemicals in WWTP effluents were reported in roach (Liney et al. 2005; Jobling et al. 2002), mollusks (Jobling et al. 2004), and fathead minnow (FHMN) (Ankley & Villeneuve, 2006; Panter et al. 1998). Many chemicals can persist or undergo biotransformation through the treatment process and, though WWTPs are working toward improving their efficiency, most facilities lack the technology required to remove all the chemicals from the effluent (Servos et al. 2005; Birkett & Lester, 2003). Chemicals of concern include nonyphenols (Servos et al. 2003; Servos 1999), bisphenol-A (Sohoni et al. 2001), and residual or metabolites of hormonal birth control products (Kidd et al. 2007).

Chemicals may target reproductive hormone pathways by disrupting endogenous estrogen-dependent processes. In physiologically normal fish, gonadotropin releasing hormone (GnRH) is released from the hypothalamus and stimulates the pituitary to produce the gonadotropins GtH I and GtH II, hormones homologous to follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins are transported in blood to the gonads; in females they stimulate the theca and granulosa cells of the ovary to ultimately produce estrogen. Estrogen then stimulates the liver to produce a

phospholipoprotein, Vitellogenin (Vtg), which is carried in the blood back to the ovary, and is incorporated into the oocytes, to become the egg yolk providing nutrients to the growing embryo. In males, gonadotropins target the testis and stimulate the production of testosterone and consequently sperm. Males do not normally produce high enough levels of estrogen to trigger the liver to produce Vtg (Schmid et al. 2002). However, when exposed to exogenous estrogens, females can produce very high levels of Vtg and males can also be triggered to start producing Vtg (Matozzo et al. 2008; Kime, 1998; Tyler et al. 1996) and undergo feminization (Parrott & Blunt 2005). Oocytes can develop in the gonads to cause intersex testis (Gray & Metcalfe 1997), where oocytes are mixed with sperm, and Vtg carried through the blood can be taken up by oocytes in the testis. Genotypic males exposed to exogenous estrogens during an early developmental window can change into phenotypic females, skew the sex ratio, and influence population dynamics (Ankley et al. 2008; Kidd et al. 2007; Panter et al. 2006).

Effects of WWTP effluent have been documented in European rivers since a classic study (Purdom 1994) sparked research interest in what local fishermen had been seeing in their catch. Substantial research has since ensued to characterize estrogenic effects in fish in European rivers (Allen et al. 1999; Harries et al. 1997; Harries et al. 1996). Research in Canada followed, with studies by Rickwood et al. (2008), Kidd et al. (2007), Servos et al. (2005), Kavanagh et al. (2004), and Gray & Metcalfe (1997). Recently in Southern Alberta, contaminants from WWTPs, including phenolics and hormones, have been detected in surface water, groundwater streams and sediments (Jeffries et al. 2008; Chen et al. 2006; Sosiak & Hebben 2005). However, more research is needed to evaluate the effects of effluents on Southern Alberta aquatic species.

Pesticides have also been detected in Southern Alberta's surface water (Koning et al. 2006; Tuduri et al. 2006) and although inhibition of acetylcholinesterase (AChE) is commonly used as biomarker of exposure to organophosphate and carbamate pesticides (Sturm et al. 2007; Stenerson 2004), interactions between estrogenic chemicals and pesticides have not been investigated.

The objectives of this study were to determine if: 1) WWTP effluent from the Southern Alberta cities of Lethbridge and Medicine Hat affect the reproductive systems of wild FHMNs, *Pimephales promelas;* 2) fish exposed to effluent in the laboratory exhibit effects mirroring those in the field; and 3) FHMN were exposed to AChE inhibiting pesticides.

Fathead minnows were exposed to WWTP effluent in the laboratory and in the field, and a suite of biochemical and morphological markers were analyzed to test the hypotheses that: 1) fish exposed to WWTP effluent have abnormal GSI, higher liver Vtg mRNA, abnormal sex ratios and gonadal morphology, and 2) fish exposed to WWTP effluent are exposed to AChE inhibiting pesticides characterized by a decrease in head AChE concentrations.

2.2 Materials and Methods

2.2.1. Fish and Laboratory Exposures

Adult male and female FHMNs (average body weight 2.09 $\pm$ 0.04 g, ~5 months old) were obtained from Aquatic BioSystems, Inc. (Fort Collins, CO, USA). Upon arrival, fish were placed in four tanks (aged, aerated tap water, room temperature, 2 tanks for males, 2 tanks for females) to recover from transport. The following day, males (M)

and females (F) were randomly distributed (1:1 M:F) among 12 tanks (7M and 7F/tank, 50 L tanks filled with 20 L aged dechlorinated City of Lethbridge tap water, static system, 25% renewal weekly, 16L:8D photoperiod, 18.8 $\pm$ 0.1^{o}C, 7.2 $\pm$ 0.1 pH) for a 3-4 week acclimation before exposure to effluent. Each tank was aerated and supplied with a 5" clay pot and floating vegetation made of a size 7 laboratory cork stopper (Fisher 7-781K Pittsburg PA,USA) wrapped with cut green (80% acrylic, 20% lambs wool, Lion Brand) yarn for shelter. Fish were fed flake food (TetraMin) *ad libitum* during acclimation.

WWTP Effluent Exposure (21 days): Fish were exposed to City of Lethbridge (Class IV wastewater treatment plant) ultraviolet-treated final wastewater treatment plant effluent at 0, 10, 25, 50 and 100% concentrations (2 tanks/treatment, 7M:7F per tank) or 10 ng/L 17 alpha-ethinylestradiol (EE2) (positive control) or 0.19 µL/L ethanol (solvent control). Effluent was transported daily from the treatment plant in a 53 L high density polyethylene (HDPE) tank (Duramax Flo N'Go); all containers used for storage or mixing were HDPE, low density polyethylene (LDPE) plastic or glass. Aged, aerated tap water was used for diluting the effluent (all tanks were static, with 50% renewal every second day, 18.7 $\pm$ 0.2^{o}C, 7.2 $\pm$ 0.1 pH, 16L:8D). Fish were fed 0.10 g flake food (TetraMin) twice a day. After 21 days fish were sacrificed and sampled.

An initial solution of EE2 was made by adding 0.0506 g of 17 alpha-ethinylestradiol, minimum 98% HPLC (Sigma E4876-1G) to 1 ml 99% anhydrous ethanol (Commercial Alcohols Inc.) to make 50.6 mg/ml EE2 (Solution A), as described

by (Parrott & Blunt, 2005). This solution was diluted again with ethanol to make 50.6 µg/L EE2 solution (Solution B; 0.1 ml of Solution A added to 99.9 ml ethanol), then diluted again with deionized reverse osmosis water to prepare a solution of 0.0506 ng/µL EE2 solution (Solution C; 1ml of Solution B added to 999 ml of deionized water). This final solution C (4 ml) was added by pipette to 20 L of dechlorinated (City of Lethbridge) tap water in the EE2 exposure tank, for a nominal concentration of 10 ng/L EE2. All steps except the addition of EE2 were repeated to make the ethanol solvent treatment tank solution (0.19 µL ethanol/L)

Water analysis: Water samples were collected in 1L HDPE Nalgene plastic bottles (VWR 16126-134), triple rinsed, filled with exposure tank water and frozen (-20 ^{o}C). Samples were taken from EE2, ethanol, 0% and 100% effluent tanks for analysis of EE2 and estrogenic chemicals (in progress).

2.2.1.1 Sampling of Fish in Laboratory Exposures

Fish were sampled starting at 13:00 hrs in a staggered schedule (3 tanks/day) to ensure all the fish were sampled at similar times of day (both in the laboratory and also in the field). All fish were removed simultaneously from the tank and immediately anesthetized in MS 222 (0.1g/L tricaine methane sulphonate; MPBiomedicals). Fork length and total body weight were recorded and fish were sacrificed by decapitation. Sex, gonad, liver weight and head tubercle number were recorded. Condition factor (CF), liver somatic index (LSI) and gonadosomatic index (GSI) were calculated. Head (AChE activity), liver (Vtg mRNA) and one gonad were dissected, placed in Eppendorf tubes

and stored at -80 ^{o}C until analysis. The other gonad was preserved in Bouin's fixative for histology. Carcasses were stored at -20 ^{o}C.

2.2.2 River Study Sites

Two rivers were sampled in Southern Alberta, Canada. The South Saskatchewan and Oldman rivers were sampled upstream and downstream from WWTPs in Medicine Hat (Class III plant) and Lethbridge (Class IV plant), respectively (Figure 2.2). The South Saskatchewan River originates from the confluence of the Oldman and the Bow rivers (Figure 2.1). Sites were selected based on proximity to WWTPs, accessibility and presence of FHMNs. The river sites were sampled in 2006 (July-September) and in 2007 (May-August). Water pH and temperature were measured at each site (Table 2.1).

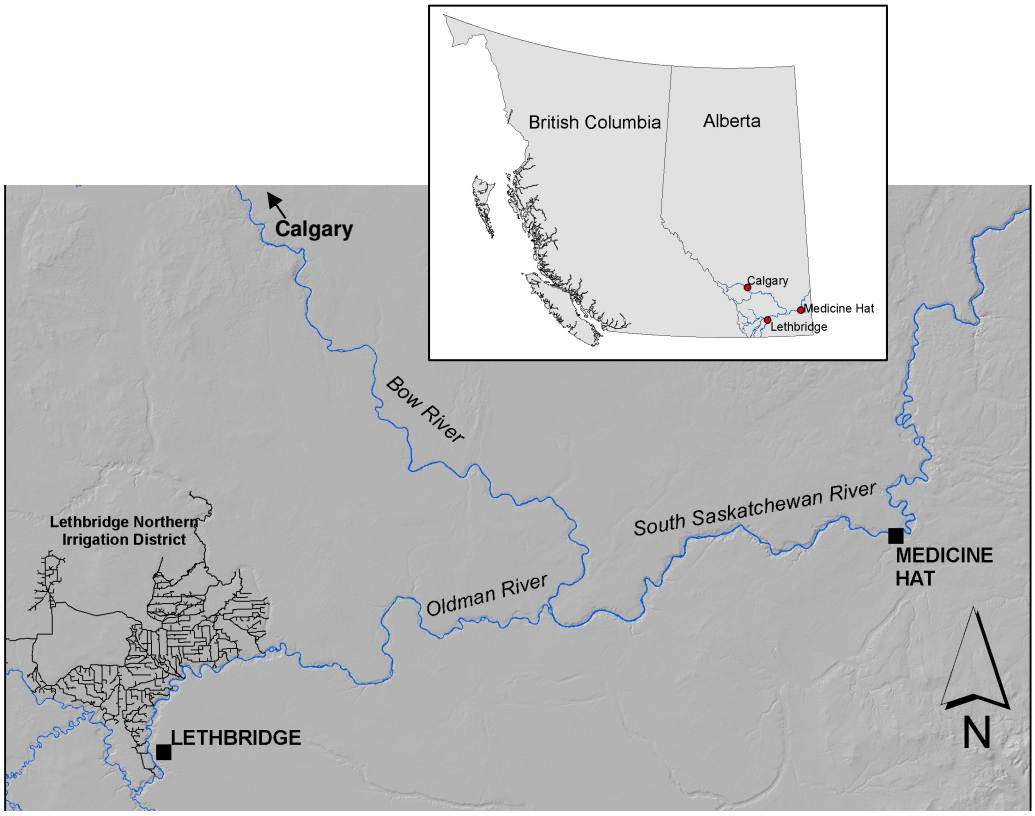


Figure 2.1-Map of Oldman, Bow and South Saskatchewan rivers in Southern Alberta

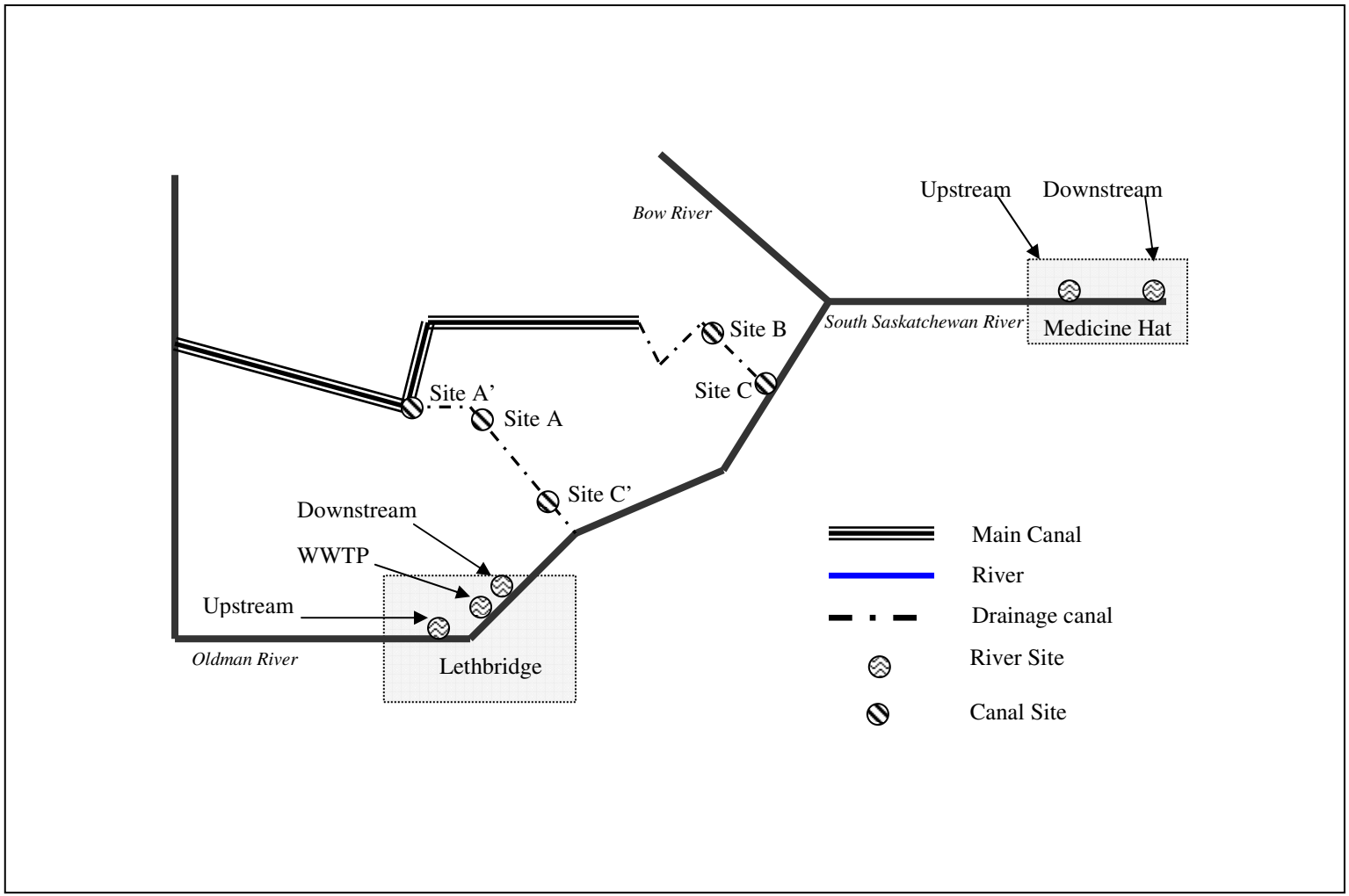


Figure 2.2-Schematic of sampling sites upstream and downstream of WWTPs in Lethbridge and Medicine Hat. Lethbridge Northern Irrigation District (LNID) canal sites sampled are also shown in light grey.

Table 2.1 Characteristics of river sites sampled in 2006 and 2007.

	Site	Legal Land Location	N[a] 2006	Sampling Dates	Temp (°C)	pH	N[a] 2007	Sampling Dates	Temp	pH
Lethbridge	Upstream	NE 25-8-22W4	29	Aug 2, 3	20.0±0.2	NA	21	July 18	25.2	7.4
	WWTP	NW 12-9-22W4	26	July 12,13,19	21.2±0.7	NA	23	July 23,24,25	25.0±0.7	7.7±0.2
	Downstream	NW 31-9-21W4	21	Aug 10,16, 17	20.0±1.0	NA	25	May 15,16	15.8±1.0	7.5±0.3
Medicine Hat	Upstream	NE 31-12-5W4	22	Aug 21,22,27,29 Aug 31, Sept 2	20.8±1.2	8.4+0.28	22	Aug 8,9	24.4±0.5	7.2±0.1
	Downstream	NE 9-13-5W4	24	Aug 23,30	20.3±0.5	8.4+0.06	2	Aug 14,15	20.2±0.9	7.8±0.2

[a] Number of fish sampled/site
NA = Not available

2.2.2.1 Sampling at River Sites

Fish (N = 20-40 per site) were captured in the morning with a Smith Root LR-24 electrofisher, nets or seine and held in aerated river water until sampled. Fish were sampled either in the field or laboratory (if site was close to university) starting at 13:00 hrs. They were anesthetized (MS-222 and site water), and tubercle number (2007 only), fork length, total body weight, gonad and liver weight were recorded. Tissue samples of liver, gonad (1) and head were dissected, frozen in liquid nitrogen and stored at -80 ^{o}C; carcasses were stored at -20 ^{o}C. One gonad was placed in Bouin's fixative for standard histology.

2.2.3 Biochemical and Morphological Analyses

2.2.3.1 Liver Vitellogenin

Liver Vtg mRNA was measured in collaboration with Dr. E. R. Nelson and Dr. H. R. Habibi at the University of Calgary. Extraction of RNA and cDNA synthesis was carried out as previously described in Nelson & Habibi (2006). Briefly, total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen). An aliquot (4 µg) of total RNA was then used for cDNA synthesis, using an oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's protocol.

Real time quantitative polymerase chain reaction (QPCR) was used to quantify relative Vtg mRNA levels, as previously described (Nelson et al. 2007). A BIO-RAD iCycler iQ Multicolour Real-Time PCR Detection System was used with the following conditions per well: 0.5 µl of cDNA, 0.26 µM of each primer, 0.2 mM dNTPs, Sybr green and Taq polymerase in buffer (10 mM Tris-HCL [pH 9.0], 50 mM KCl, 1.4 mM

MgCl2, 20 nM fluorescein) to a total volume of 25 µl. As an internal control, β-actin was also amplified as described previously. Primers were as follows:

Vtg: [5'- gaagtgcgcatggtggcttgtatt-3'] and [5'- agctgccatatcaggagcagtgat-3'], β-actin: [5'-CCTCCATTGTTGGCACC-3'] and [5'-CCTCTCTTGCTTTGAGCCTC-3']. Cycling was as follows: 3 minutes at 94^{o}C followed by 30-50 cycles of 10 seconds at 94^{o}C and 40 s at 54.3^{o}C. Each experimental group was run in triplicate to ensure consistency. Validation experiments found the primers to amplify only one product as determined by melt curve analysis and gel-electrophoresis, and primer efficiencies were determined to be 96.7% for Vtg and 95% for β–actin.

2.2.3.2 Acetylcholinesterase

Acetylcholinesterase (AChE) activity was measured with a modified protocol of Ellman et al. (1961) and Chuiko (2000). Heads were homogenized in a phosphate buffer at a 1:3 ratio with a Power Max AHS 200 (VWR). Homogenate was centrifuged 3 times at 13 000 RPM for 3 minutes at 5^{o}C and supernatant was collected. Final supernatant was diluted with phosphate buffer at a 1:10 ratio for AChE assay (and further diluted at a 1:15 ratio with protein dilution solution for Bradford protein assay). Supernatant (4 µL) was pipetted in triplicates into a 96 well plate with 120 µL of Tris-ISO-OMPA (Sigma T5030), followed by incubation of samples for 10 minutes at room temperature on a shaker. Then 10 µL of DTNB (Sigma D8130) followed by 10 µL of AChI (Sigma A5751) were added to the wells and the samples were incubated for another 10 minutes on a shaker at room temperature. The microplate was read in a microplate reader (Molecular Devices, MAXline Emax Precision) using Softmax Pro software every 2

minutes for 10 minutes at 405 nm. Concentration of AChE was determined from the slope of the rate of change in absorbance. Internal standards were assayed on each plate to ensure the accuracy of the assay (Normal Serum Control, TC-TROL [N], Teco Diagnostics, 1 U/mL and 2 U/mL eel acetylcholinesterase, Sigma C3389), with assay characteristics as shown in Miller et al. (2009). Activity of AChE was expressed as units/mg protein (measured with a spectrophotometer at 595 nm, using the Bradford method).

2.2.3.3 Histology

Gonads were placed in a cassette (VWR CA87002-424) and fixed in Bouin's fixative for at least three days. Tissues were then washed in water and then in a series of increasing water and ethanol (Commercial Alcohols) concentrations up to 70%. Tissues were stored in 70% ethanol, then they were dehydrated in an ethanol series, clearing agent Safeclear (Fisher 044-192) or Citrosolve (Fisher 22-143975), and embedded in Paraplast Plus (Fisher 23-021-400). Tissues were sectioned (microtome) longitudinally at 5-10 μm, mounted on slides with gelatin (Fisher G8), stained with Eosin (Fisher 245-658) and Hematoxylin (Fisher 245-656), and mounted with Permount (Fisher SP15-100).

2.2.3.4 Gonad Scoring

Gonadal maturity of females was determined using a grid system modified from Wolf et al. (2004). Pictures of gonads were taken using a microscope-mounted camera (Canon Powershot A640); three tissue pictures were taken for each sample in a standardized way at 200 X, the camera zoom remaining constant. A 30 point grid was centered over the picture using Gimp 2.4.6 software. Oocyte maturity stage was scored

(perinucleolar, early vitellogenic, mid-late vitellogenic, mature/spawning, and atretic) at each gridpoint.

Male and female gonads from field samples and laboratory exposures were examined histologically and anomalies in structure (presence of parasites, intersex, proteinaceous masses and other anomalies) were recorded.

2.2.4 Statistical Analysis

Data were analysed using JMP IN 5.1 (SAS Institute Inc.) with an ANOVA and a post hoc Tukey Kramer HSD. All data is shown without transformation. However, all statistical tests were performed on natural log transformed data (to maintain consistency), unless otherwise noted (gonad maturity scores, anomaly and parasite counts and tubercle counts were not log transformed). All statistical tests were based on an alpha of 0.05. Sex ratio statistics were performed using a Chi^2 analysis and a 50:50 expected ratio.

2.3 Results

2.3.1 Chemical and Physical Characteristics of the WWTP Effluent

It is important to note that the average volume of the Oldman river comprised of WWTP effluent was 2.69% for the duration of the laboratory exposure (Figure 2.3 inset), and averaged 1.00% in all of 2006 and 1.44% in 2007 (Figure 2.3). The average volume of the South Saskatchewan river comprised of city of Medicine Hat WWTP effluent

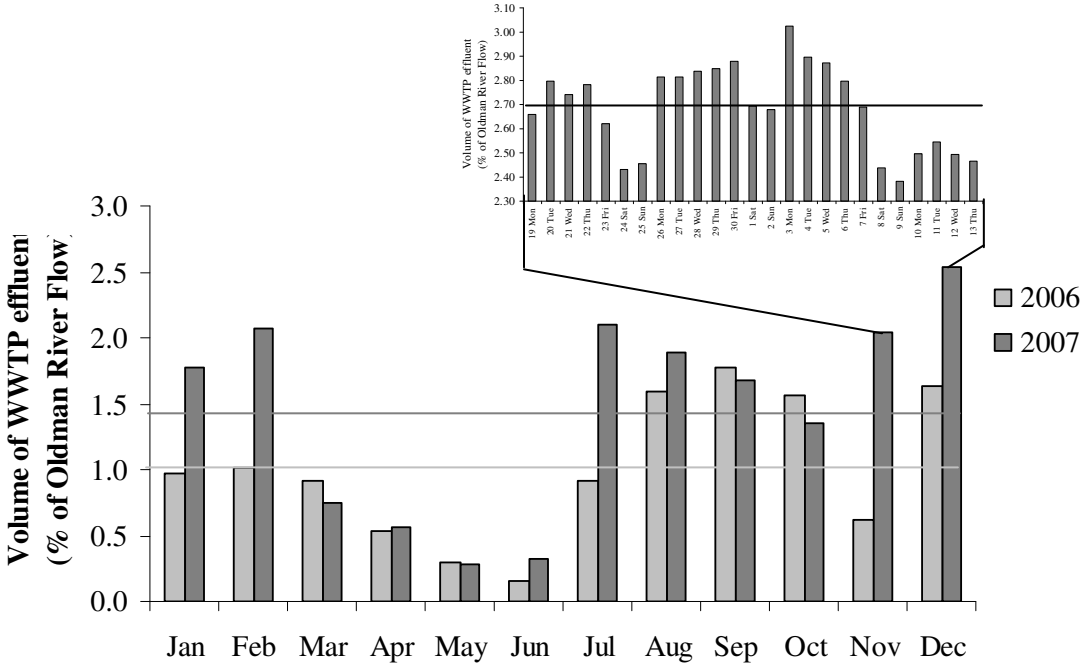
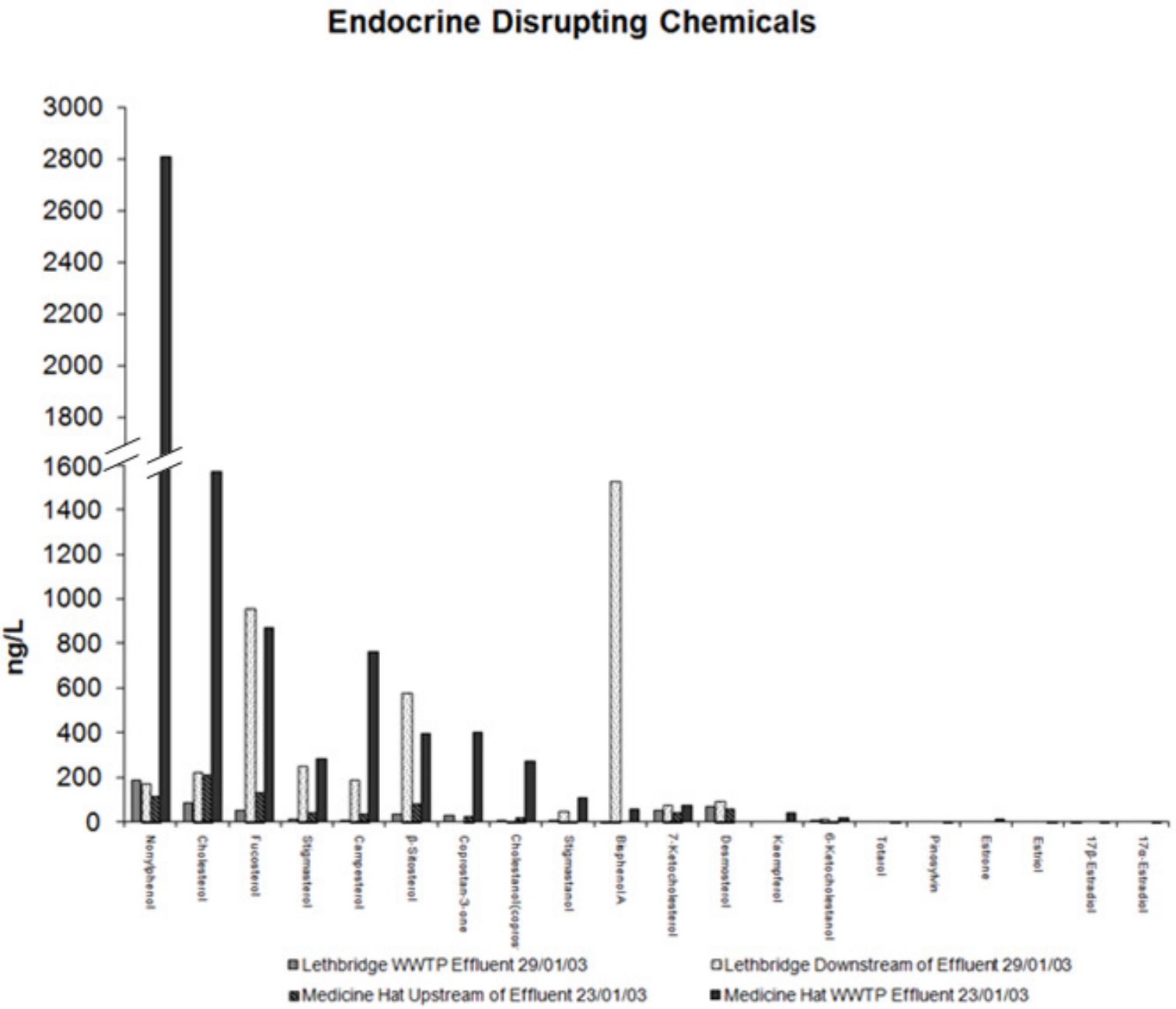


Figure 2.3. Volume of Lethbridge WWTP effluent (expressed as a percentage of Oldman river flow) released into the Oldman river in 2006, 2007 and during the laboratory exposure period (November 19 - December 13, 2007, inset). The average volume was 1.00%, 1.44% and 2.69% respectively.

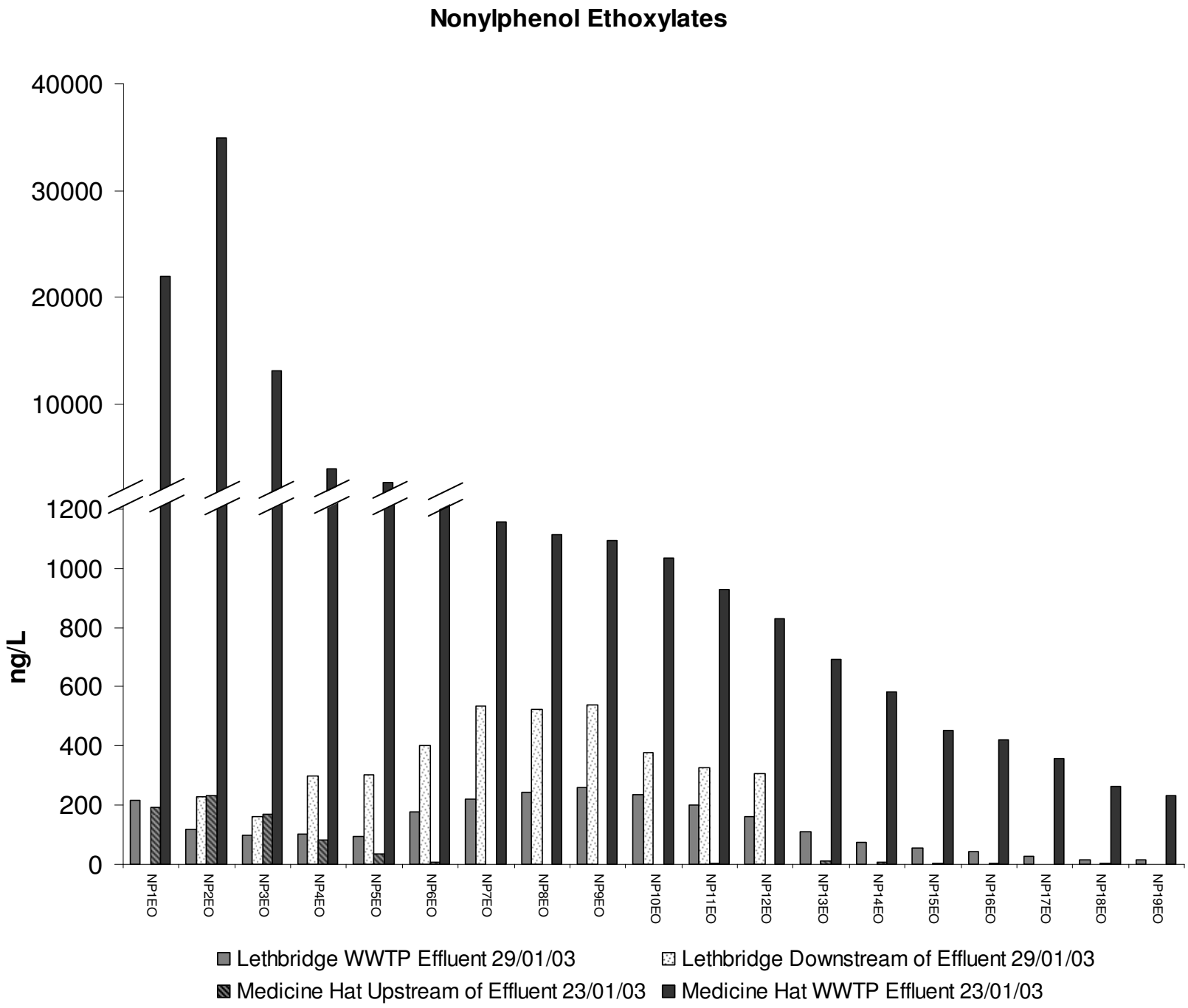
flow was 0.22% in 2006 and 0.23% in 2007 (data not shown). These data are crucial for establishing links between the exposures to WWTP effluent in the laboratory and the field studies carried out at the river sites receiving the effluent. Moreover, data are available for chemicals detected in the Oldman and South Saskatchewan rivers by Alberta Environment in 2002-2003 (Sosiak & Hebben, 2005) (Fig. 2.4). Endocrine disrupting compounds (EDCs) detected in the Oldman river were generally at higher concentrations downstream of WWTP effluent sites compared to within source effluent (Fig. 2.4).

In Medicine Hat, EDCs were at generally higher concentrations within source effluent compared to upstream of the WWTP effluent site (Figure 2.4 A). Nonylphenol ethoxylates, phthalate esters and mono-phthalate esters were at higher concentrations downstream of the Lethbridge WWTP effluent site compared to source effluent and they were also detected in Medicine Hat WWTP effluent at higher concentrations in the whole treated WWTP effluent compared to the upstream site (Figure 2.4 B). Nonylphenol concentrations in Medicine Hat WWTP effluent were also higher than in Lethbridge WWTP effluent (Figure 2.4 C). However it is interesting to note that chemicals were in fact present in the upstream waters of Medicine Hat. The City of Lethbridge WWTP effluent used in the laboratory exposures has been partially characterized (Table 2.2) and the concentrations fell within Alberta Environments guidelines for release of effluent to surface water (AENV, 2006).

A)


Endocrine Disrupting Chemicals
ng/L
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Nonylphenol
Cholesterol
Fucosterol
Stigmasterol
Campesterol
β-Sitosterol
Coprostan-3-one
Cholestanol(copros
Stigmastanol
BisphenolA
7-Ketocholesterol
Desmosterol
Kaempferol
6-Ketocholestanol
Totarol
Pinosylvin
Estrone
Estriol
17β-Estradiol
17α-Estradiol
Lethbridge WWTP Effluent 29/01/03
Lethbridge Downstream of Effluent 29/01/03
Medicine Hat Upstream of Effluent 23/01/03
Medicine Hat WWTP Effluent 23/01/03

B)


Nonylphenol Ethoxylates
40000
30000
20000
10000
1200
1000
800
600
400
200
0
ng/L
NP1EO
NP2EO
NP3EO
NP4EO
NP5EO
NP6EO
NP7EO
NP8EO
NP9EO
NP10EO
NP11EO
NP12EO
NP13EO
NP14EO
NP15EO
NP16EO
NP17EO
NP18EO
NP19EO
Lethbridge WWTP Effluent 29/01/03
Lethbridge Downstream of Effluent 29/01/03
Medicine Hat Upstream of Effluent 23/01/03
Medicine Hat WWTP Effluent 23/01/03

C)

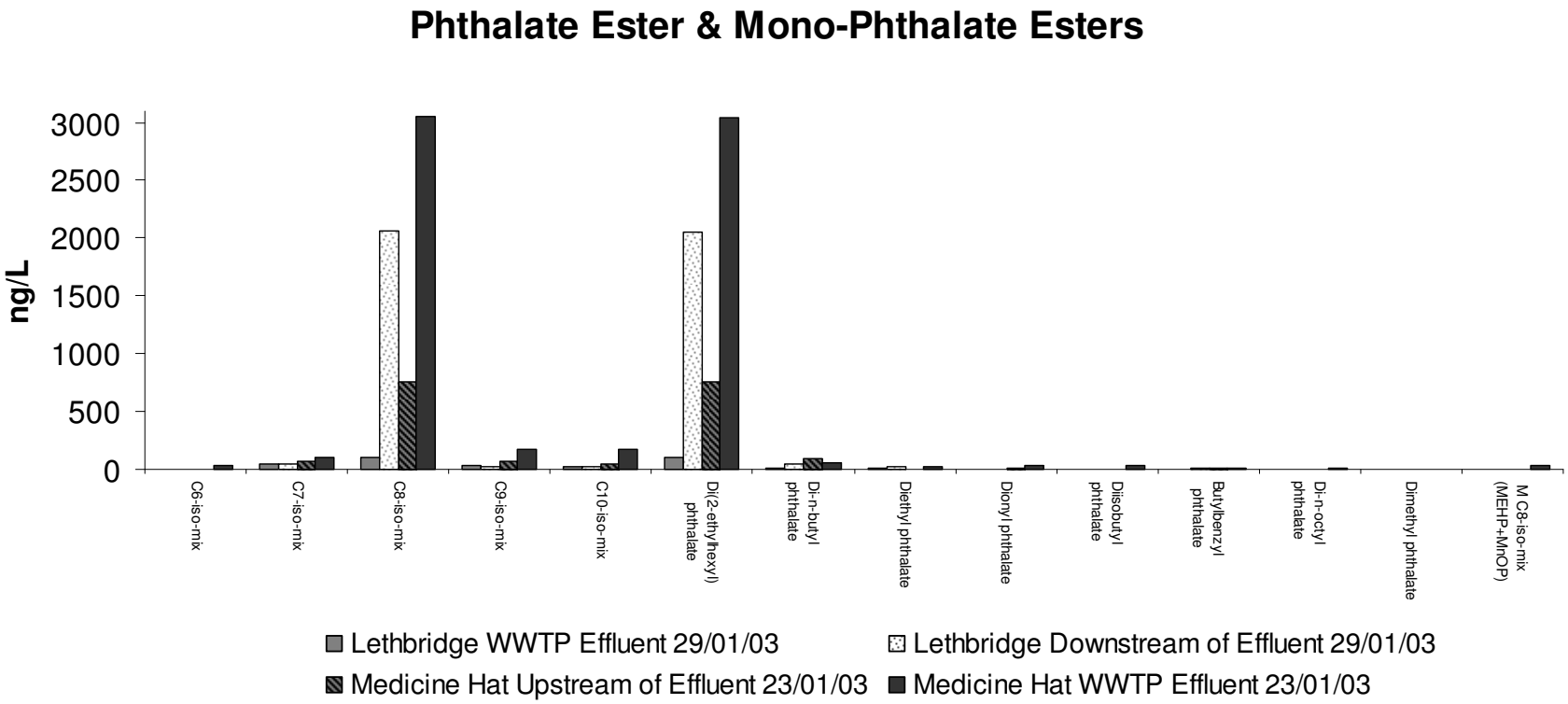


Figure 2.4. Concentrations of A) endocrine disrupting compounds, (B) nonylphenol ethoxylates and (C) phthalate ester & mono-phthalate esters in Southern Alberta WWTP effluents and contributing/receiving waters (2003). Adapted from Sosiak and Hebben (2005).

Table 2.2. Lethbridge WWTP effluent volume and chemical characteristics[a] for days effluent was used in laboratory exposures (Nov 19-Dec 13, 2007).

Lethbridge WWTP effluent	Averages	SE
Effluent Volume (m^3 x 1000/day)	33.99	0.29
pH	7.61	0.07
Total Suspended Solids (mg/L)	5.68	0.30
Final Ammonia Nitrogen, NH3 N(mg/L)	4.52	0.83
Nitrite/Nitrates, NO2/NO3 (N mg/L)	3.36	0.30
Total Kjeldahl Nitrogen, TKN (mg/L)	3.14	0.34
Final Total Phosphorous (mg/L)	0.47	0.05
Carbonaceous Biochemical Oxygen Demand 5 (mg/L)	3.16	0.27
Biological Oxygen Demand 5 (mg/L)	5.72	0.47
Calcium Carbonate, $CaCO_3$ (mg/L)	222.00	5.66
Fecal #/100mL	21.38	7.16
Total Coliform/100mL	99.38	33.70

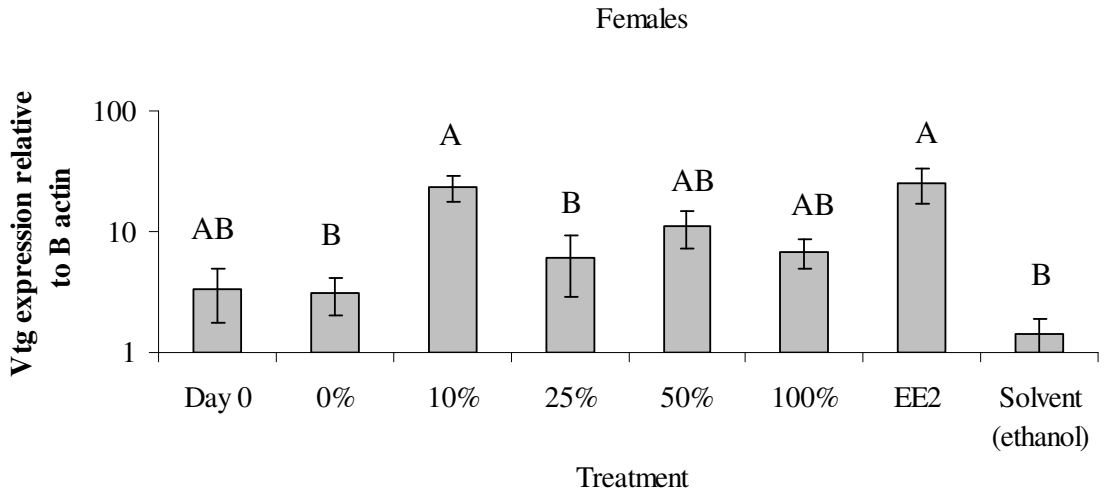
[a], data obtained from City of Lethbridge WWTP

2.3.2 Laboratory Exposure to WWTP Effluent

Liver Vitellogenin

Liver Vtg expression in females exposed to WWTP effluent were generally higher than expression in controls (Day 0, 0% effluent and ethanol) and significantly higher ($F_{7, 80} = 4.67$, p=0.0002) in fish exposed to EE2 and 10% WWTP effluent (Figure 2.5 A). Liver Vtg expression in males exposed to WWTP effluent were generally higher than expression in controls (Day 0, 0% and ethanol) and significantly higher ($F_{7, 79} = 2.90$, p=0.0095) in fish exposed to EE2 (Figure 2.5 B).

(A)



(B)

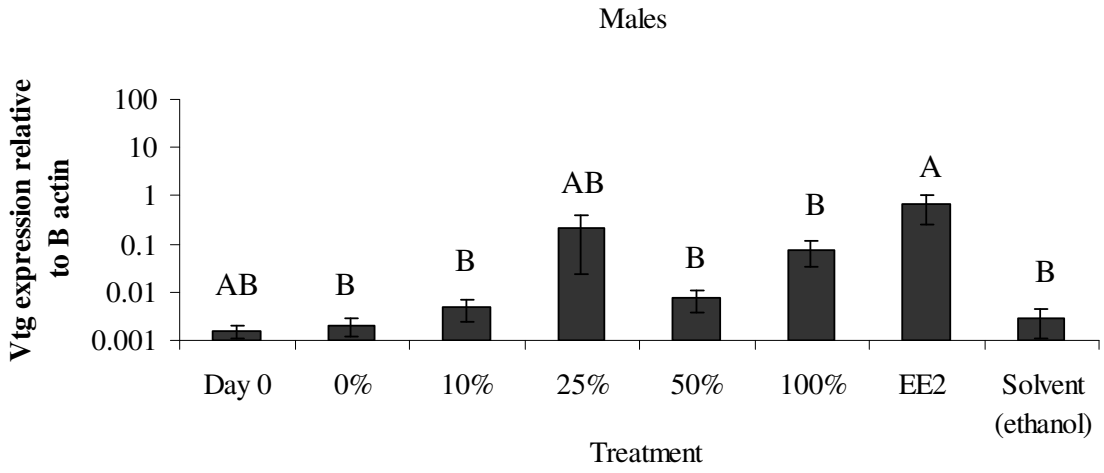


Figure 2.5. Vitellogenin, Vtg (mean±SE) in (A) female and (B) male FHMN exposed for 21 days to control water (0% effluent), WWTP effluent (10, 25, 50 or 100% effluent), 10 ng/L ethinylestradiol (EE2) or the solvent (0.1976 µl/L ethanol). Day 0 represents fish sampled at the start of the exposure. Significant difference indicated by different letters (N=5-14). Note different scales in y axes.

Reproductive status and growth indices

Compared to day 0 fish, female gonads tended to have a lower percentage of mature (M) oocytes in fish exposed to 10%, 25%, 100% effluent and EE2, whereas there was a significantly lower ($F_{7, 280}$ = 2.13, p=0.0409) percentage of mature (M) oocytes in fish exposed to 10% WWTP effluent (Figure 2.6). However, oocyte maturity of fish exposed to WWTP effluent or EE2 was not different from non-exposed fish (0%).

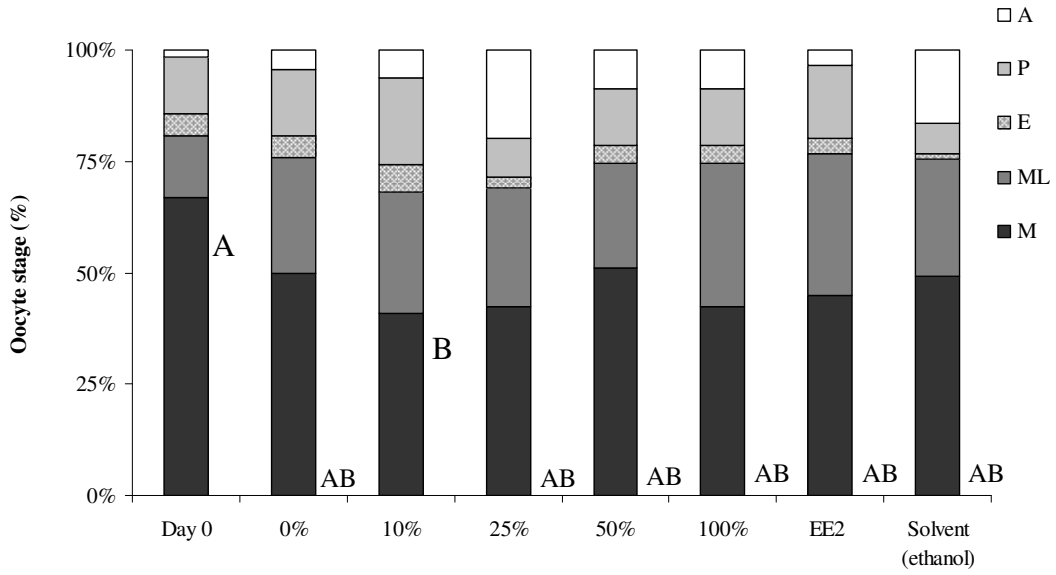


Figure 2.6. Gonad maturity score (average percent) in female FHMN exposed for 21 days to control water (0% effluent), WWTP effluent (10, 25, 50, or 100% effluent), 10 ng/L ethinylestradiol (EE2) or the solvent (0.1976 µl/L ethanol). Day 0 represents fish sampled at the start of the exposure. Significant difference indicated by different letters. (N=5-14). Scoring method adapted from Wolf et al. (2004). A= atretic, P = perinucleolar, E= early development, ML= mid-late development, M= mature.

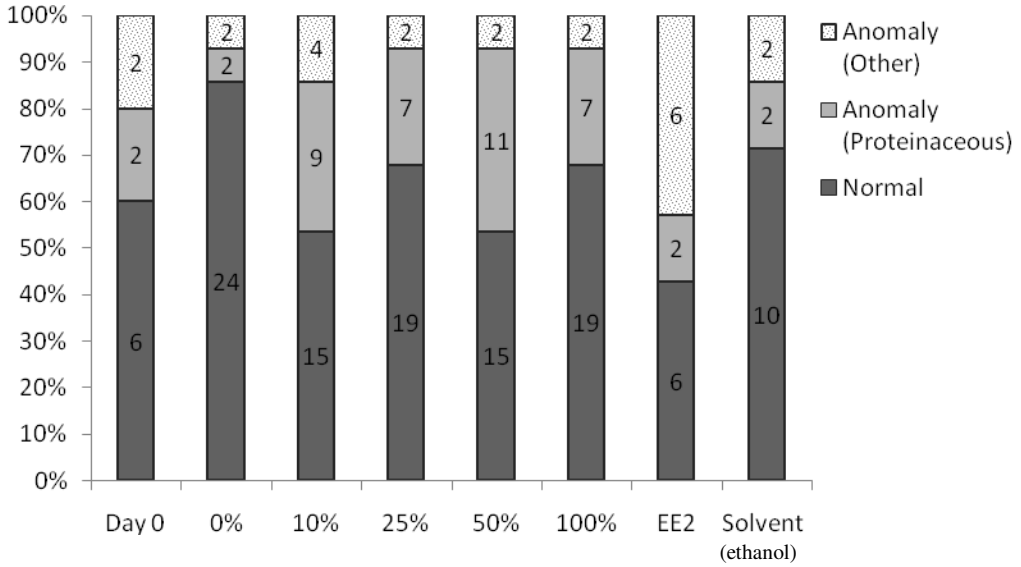


Figure 2.7 Histopathology survey of anomalies (males and females grouped) in FHMN exposed for 21 days to control water (0% effluent), WWTP effluent (10, 25, 50 or 100% effluent), 10 ng/L ethinylestradiol (EE2) or the solvent (0.1976 µl/L ethanol). Day 0 represents fish sampled at the start of the exposure (N=10-28).

Histopathology survey of gonadal anomalies (Figure 2.7) shows that the gonads of fish exposed to 10, 25, 50 or 100% WWTP effluent or EE2 had more total gonadal anomalies (Figure 2.8) in comparison to control fish (Day 0, 0 % effluent or Solvent), though not significantly ($F_{7, 170} = 1.69$, p=0.1143).

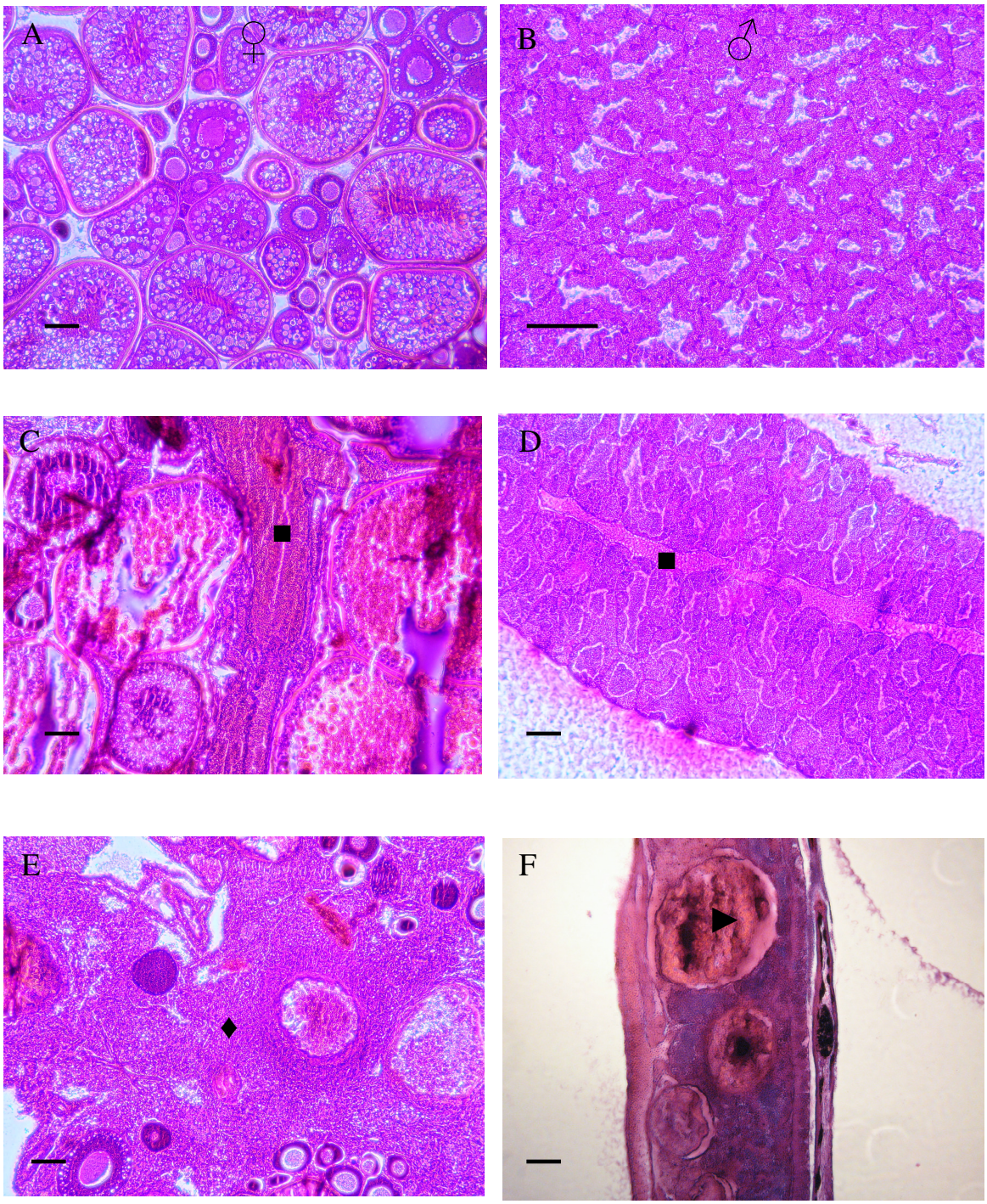


Figure 2.8 Gonadal histopathology of FHMN exposed in the WWTP laboratory exposure experiment. A) Normal Female B) Normal Male C) Female with ■ Anomaly (proteinacious mass) D) Male with ■ Anomaly (proteinacious mass) E) Female with ♦ Anomaly (other) F) Male with ▶ Anomaly (other). Scale bar = 100 µm.

There were no significant differences ($F_{7, 78} = 0.8092$, p=0.5823) in tubercle numbers between treatment groups but the numbers were lowest on fish exposed to EE2, 100% effluent and 25% effluent (Figure 2.9).

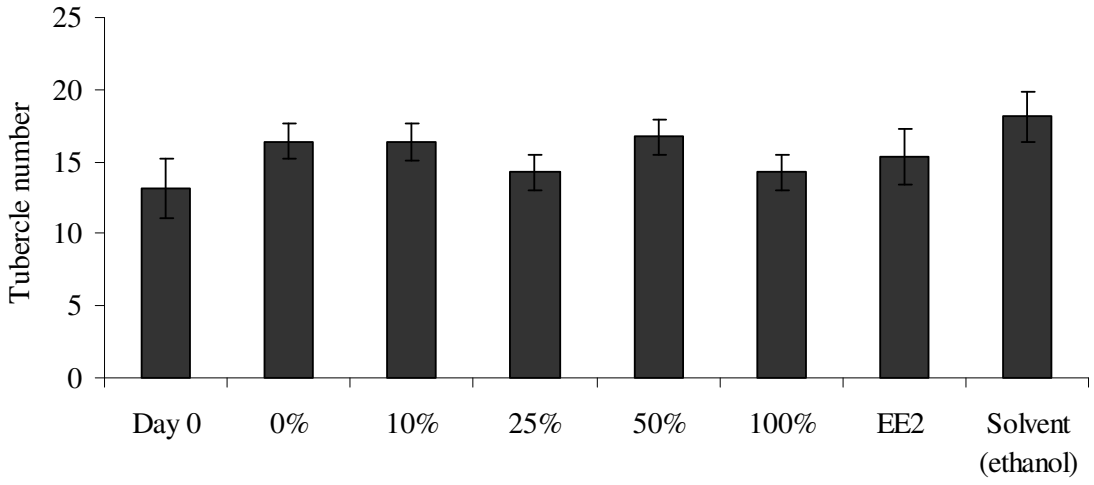
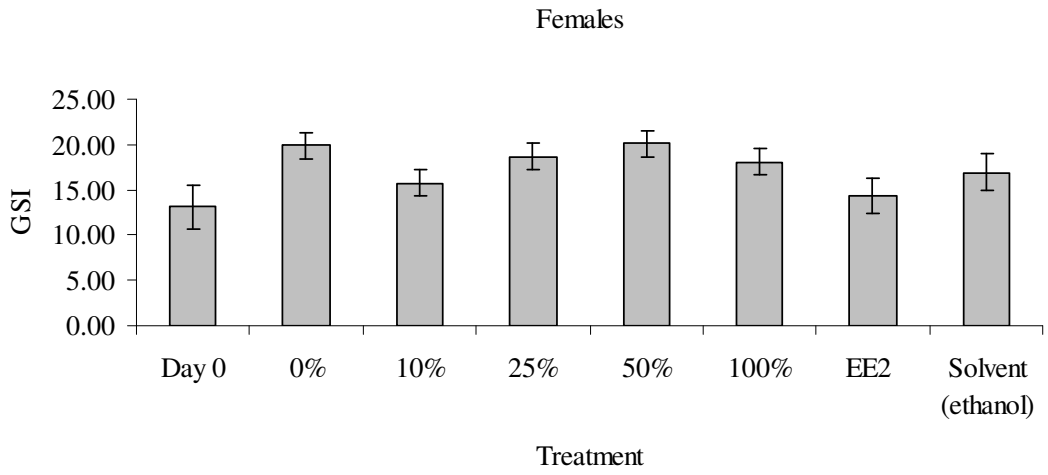


Figure 2.9. Tubercles (number, mean ± SE) on male FHMN exposed for 21 days to control water (0% effluent), WWTP effluent (10, 25, 50 or 100% effluent), 10 ng/L ethinylestradiol (EE2) or the solvent (0.1976 µl/L ethanol). Day 0 represents fish sampled at the start of the exposure (N=5-14).

Female GSI did not differ significantly ($F_{7, 82} = 2.01$, p=0.0634) between groups but it was lowest in Day 0, 10% effluent and EE2 exposed fish (Figure 2.10A). Male GSI did not differ significantly ($F_{7, 82} = 1.08$, p=0.3841) between groups either, but it was lowest in 25% effluent and EE2 exposed fish (Figure 2.10B).

(A)



(B)

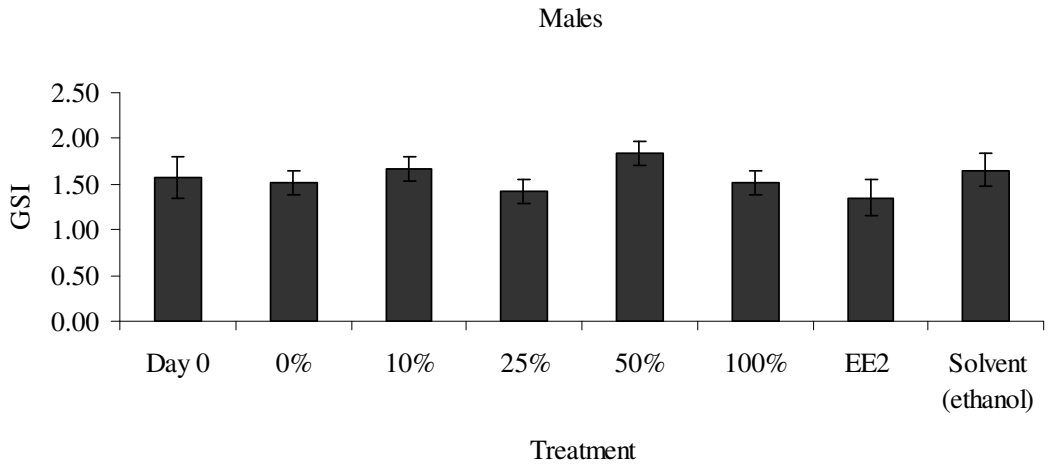


Figure 2.10. Gonadosomatic index, GSI (mean±SE) in (A) female and (B) male FHMN exposed for 21 days to control water (0% effluent), WWTP effluent (10, 25, 50 or 100% effluent), 10 ng/L ethinylestradiol (EE2) or the solvent (0.1976 µl/L ethanol). No statistically significant difference found (N=6-8).

There were no significant differences in LSI ($F_{7, 170}$ = 2.11, p=0.0446) and CF ($F_{7, 170}$ = 1.36, p=0.2268) between groups but they were lowest in Day 0 fish and EE2 exposed fish (Table 2.3).

Table 2.3. Morphological and biochemical endpoints (mean ± SE) in FHMN exposed to WWTP effluent or EE2 for 21 days.

Treatment	Sample Size (N)	LSI[a]	CF[b]	AChE[c]
Day 0	10	2.27 ± 0.23	1.33 ± 0.06	-
0% Effluent	28	2.98 ± 0.14	1.47 ± 0.04	22.14 ± 0.97
10% WWTP Effluent	28	2.92 ± 0.14	1.46 ± 0.04	-
25% WWTP Effluent	28	2.60 ± 0.14	1.45 ± 0.04	-
50% WWTP Effluent	28	2.71 ± 0.14	1.51 ± 0.04	-
100% WWTP Effluent	28	2.96 ± 0.14	1.45 ± 0.04	24.84 ± 0.97
EE2 10 ng/L	14	2.47 ± 0.20	1.37 ± 0.05	21.70 ± 1.37
Solvent (ethanol)	14	2.78 ± 0.20	1.47 ± 0.05	-

No statistical differences (based on Ln transformed data) found between treatments.
[a] Liver Somatic Index = liver weight / total weight x 100.
[b] Condition Factor = total weight x 100/fork length^3.
[c] Head Acetylcholinesterase activity (µM/mg protein)

AChE activity

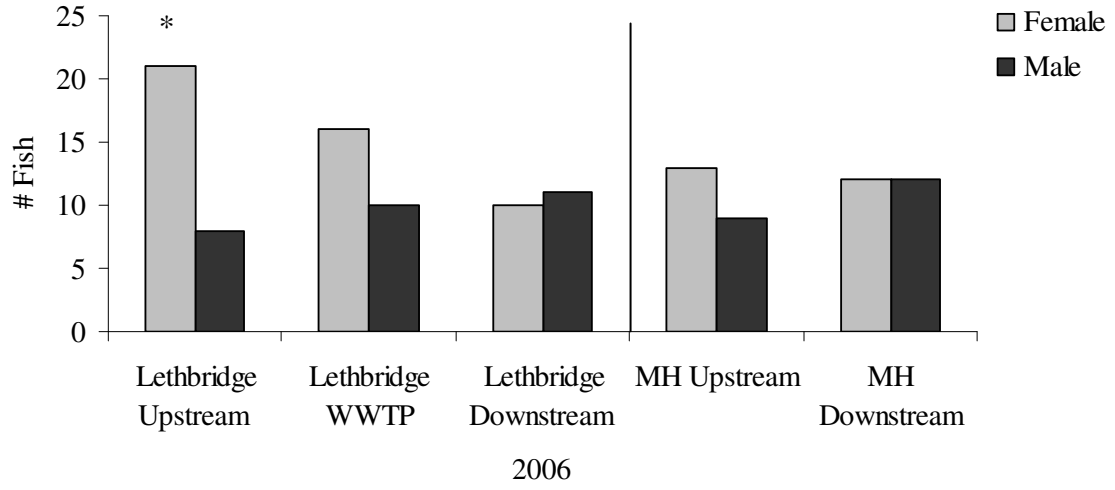
Acetylcholinesterase activity was only analyzed in fish exposed to 0% effluent, 100% effluent and EE2, and results showed no significant differences ($F_{2, 67}$ = 2.56, p=0.0851) between groups (Table 2.3).

2.3.3 Field Results 2006 and 2007

Sex ratio

In 2006, more females ($p < 0.05$, based on 50:50 sex ratio (Parrot & Blunt 2005; Parrot & Wood 2002; Zerulla et al. 2002; Lange et al. 2001) than males were sampled at the Lethbridge Upstream site (Figure 2.11A). In 2007, there were no significant differences in numbers of males and females collected (Figure 2.11B).

(A)



(B)

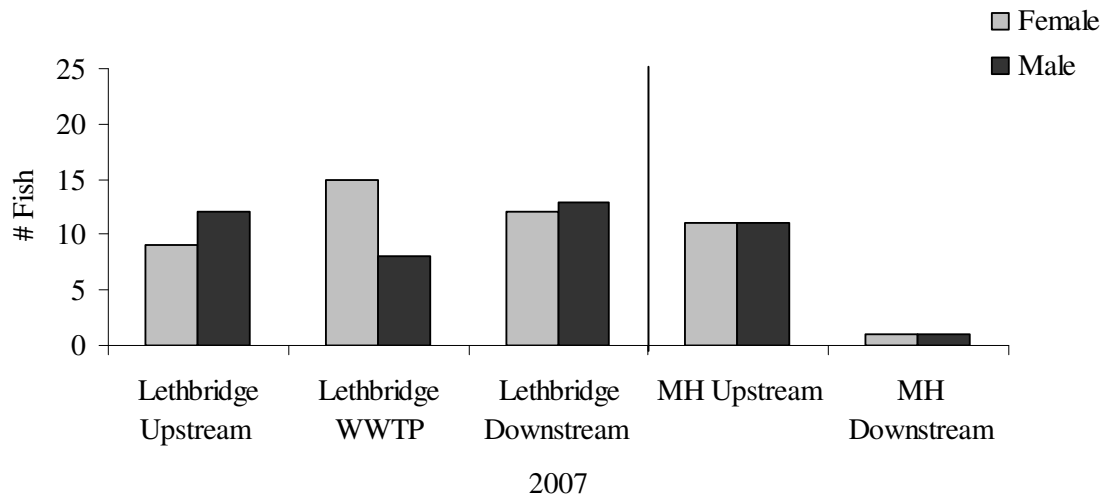


Figure 2.11. Numbers of female and male FHMN captured from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat (MH) in (A) summer 2006 and (B) summer 2007 (n = 21-29/site). Significant difference based on Chi^2 analysis indicated with *.

Liver Vitellogenin

In 2006, female liver Vtg expression was higher ($F_{2, 45} = 5.50$, p=0.0073) in fish sampled at the Lethbridge WWTP site compared to fish sampled at the other Lethbridge sites. In Medicine Hat the female fish sampled at the downstream site had higher ($F_{1, 23} =$

5.29, p=0.0309) Vtg expression compared to upstream. Male Vtg levels were not significantly different among sites in Lethbridge or Medicine Hat (Figure 2.12A). In 2007, Vtg levels were not significantly different among sites for either sex (Figure 2.12B).

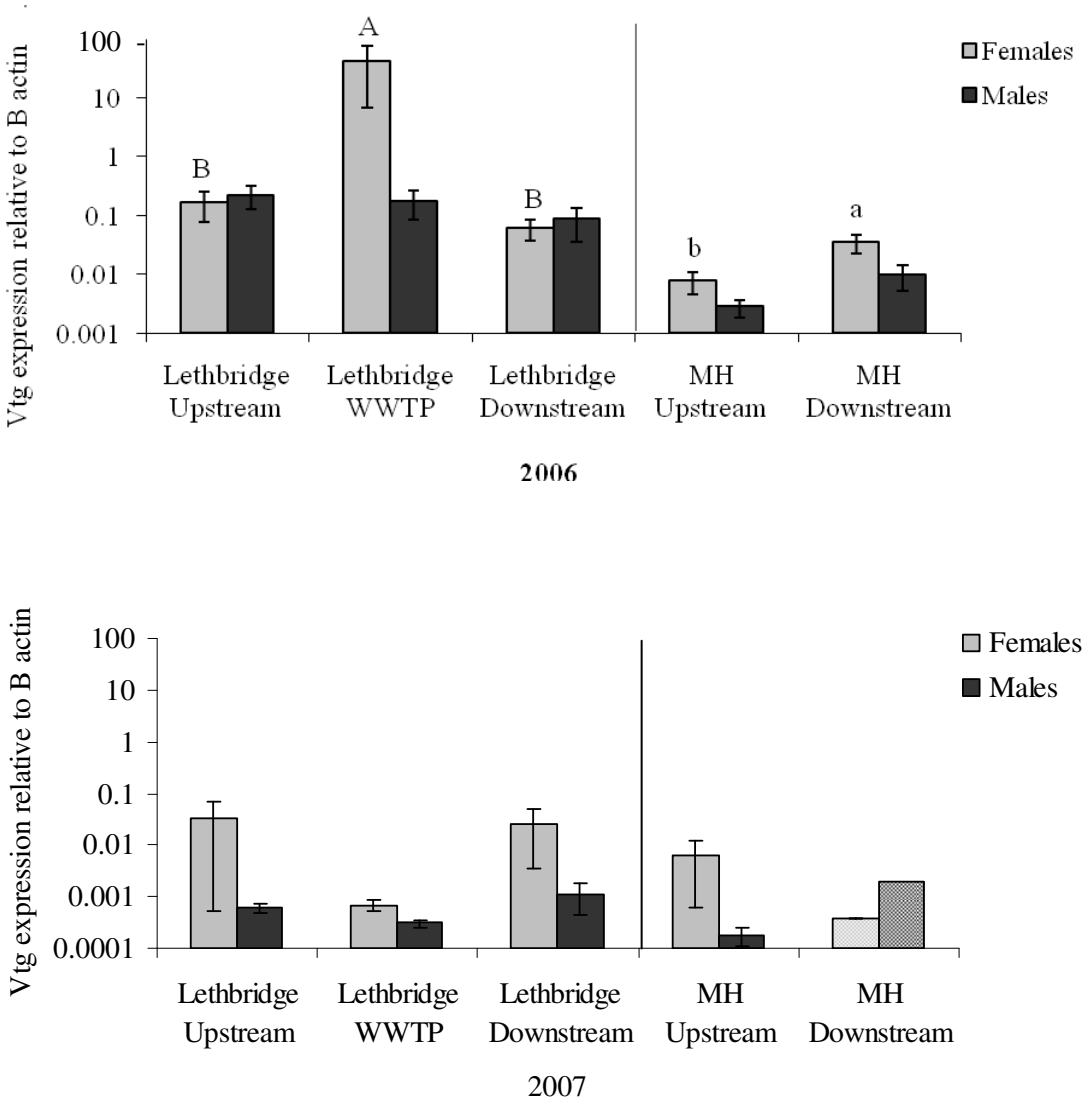


Figure 2.12. Vitellogenin, Vtg (mean±SE) in (A) female and (B) male FHMN sampled from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat (MH) in summer (A) 2006 and (B) 2007 (n = 21-29/site). Capital letters and small letters indicate significant differences within cities sampled. Cross hatch indicates sample size was too small to use for statistical analysis (n=1).

Reproductive status and growth indices

In 2006, female GSI was higher ($F_{1, 20} = 27.03$, p=<0.0001) at the Lethbridge WWTP site compared to fish sampled at the upstream site (no data for the downstream site). The number of male GSI measures was too small for a statistical comparison. In Medicine Hat, GSI among sites was not significantly different for males or females (Table 2.4). In 2007, female GSI was higher ($F_{2, 33} = 6.43$, p=0.0044) at the Lethbridge downstream site compared to fish sampled at the other Lethbridge sites. Male GSI was not different among sites (Table 2.4). In Medicine Hat statistical analysis or comparison could not be done (fish were not captured at the downstream site).

In 2006, LSI was higher ($F_{2, 52} = 3.04$, p=0.0561) in fish sampled at the Lethbridge upstream site compared to the downstream site and in Medicine Hat LSI was higher ($F_{1, 40} = 51.90$, p=<0.0001) in fish sampled at the downstream site compared to the upstream site (Table 2.4). In 2007 LSI in fish sampled at the Lethbridge downstream site was not different from fish sampled at the other Lethbridge sites (Table 2.4). In Medicine Hat statistical analysis or comparison could not be done.

Condition factor in 2006 was higher ($F_{2, 72} = 10.59$, p=<0.0001) at the Lethbridge upstream and WWTP sites compared to fish sampled at the downstream site. In Medicine Hat fish sampled at the downstream site had higher ($F_{1, 44} = 26.13$, p=<0.0001) CF compared to fish sampled upstream (Table 2.4). In 2007, CF of fish sampled at the Lethbridge Upstream site was higher ($F_{2, 66} = 3.71$, p=0.0297) compared to downstream (Table 2.4). In Medicine Hat statistical analysis or comparison could not be done.

Table 2.4. Morphological and biochemical endpoints (mean ± SE) in FHMN sampled from rivers (up and downstream from WWTP) in Lethbridge and Medicine Hat, in summer 2006 and 2007 (n = 21-29).

Site	n	LSI[a]		GSI Females[b]		GSI Males[b]	Condition Factor[c]	
				2006				
Lethbridge Upstream	29	3.14 ± 0.30	A	2.38 ± 0.76	B	-	1.20 ± 0.02	A
Lethbridge WWTP	26	2.89 ± 0.26	AB	8.26 ± 1.20	A	1.25 ± 0.27	1.19 ± 0.03	A
Lethbridge Downstream	21	1.86 ± 0.06	B	-		-	1.07 ± 0.02	B
Medicine Hat Upstream	22	2.76 ± 0.19	b	2.43 ± 0.34		0.76 ± 0.08	1.07 ± 0.02	b
Medicine Hat Downstream	24	4.98 ± 0.23	a	2.03 ± 0.11		0.82 ± 0.07	1.19 ± 0.02	a
				2007				
Lethbridge Upstream	21	2.40 ± 0.13		2.88 ± 0.59	B	0.50 ± 0.07	1.14 ± 0.02	A
Lethbridge WWTP	23	2.29 ± 0.10		2.12 ± 0.15	B	0.51 ± 0.06	1.12 ± 0.02	AB
Lethbridge Downstream	25	2.68 ± 0.14		4.76 ± 1.04	A	0.77 ± 0.19	1.08 ± 0.02	B
Medicine Hat Upstream	22	3.45 ± 0.19		1.94 ± 0.16		0.64 ± 0.19	1.12 ± 0.01	
Medicine Hat Downstream	2	-		-		-	-	

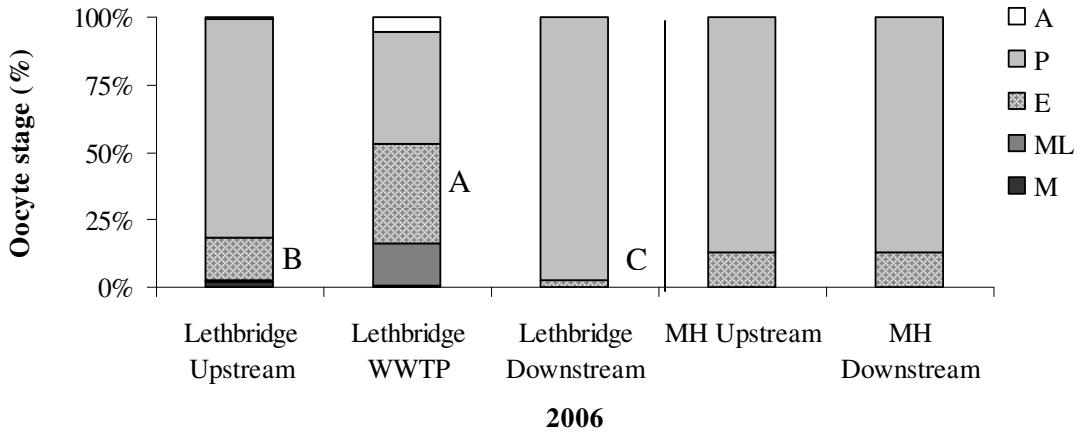
Statistical differences between treatments indicated by different letters. Capital letters and small letters indicate significant differences within cities sampled.

[a] Liver Somatic Index = liver weight / total weight x 100.

[b] Gonadosomatic Index = gonad weight / total weight x 100.

[c] Condition Factor = total weight x 100/fork length^3.

A)



B)

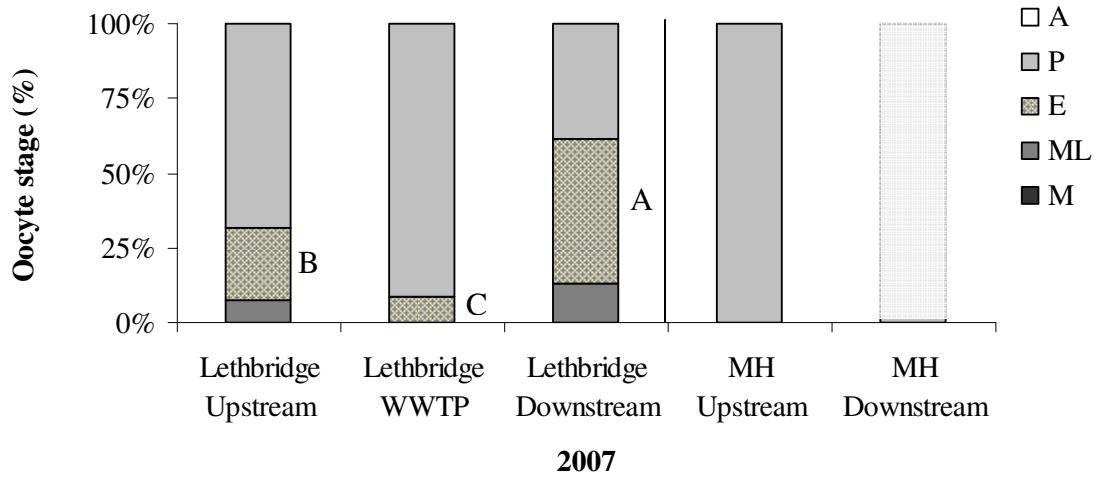


Figure 2.13. Gonad maturity score (average percent) in female FHMN sampled from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat (MH), in summer (A) 2006 and (B) 2007 (n = 21-29). Capital letters indicate significant differences among groups sampled. Scoring method adapted from Wolf et al. (2004). A= atretic, P = perinucleolar, E= early development, ML= mid-Late development, M= mature. White bar indicates sample size was too small to use for statistical analysis (n=1).

In 2006, female gonads were more mature ($F_{2, 132} = 32.39$, p=<0.0001) in fish sampled at the Lethbridge WWTP site and least mature in fish sampled at the Lethbridge downstream site. Maturity did not differ between females sampled at Medicine Hat sites

(Figure 2.13A). In 2007, female gonads were more mature ($F_{2, 105}$ = 52.80, p=<0.0001) in fish sampled at the Lethbridge downstream site and least mature in fish sampled at the Lethbridge WWTP site (Figure 2.13B). In Medicine Hat statistical analysis or comparison could not be done.

Both years there were no differences between sites for gonadal anomalies or parasitization (Figure A1 and A2 in appendix). In both years, there were however more anomalies in female fish than males. Parasitism did not seem to affect Vtg levels.

There were no significant differences in tubercle numbers between fish sampled at Lethbridge or Medicine Hat sites (Figure 2.14).

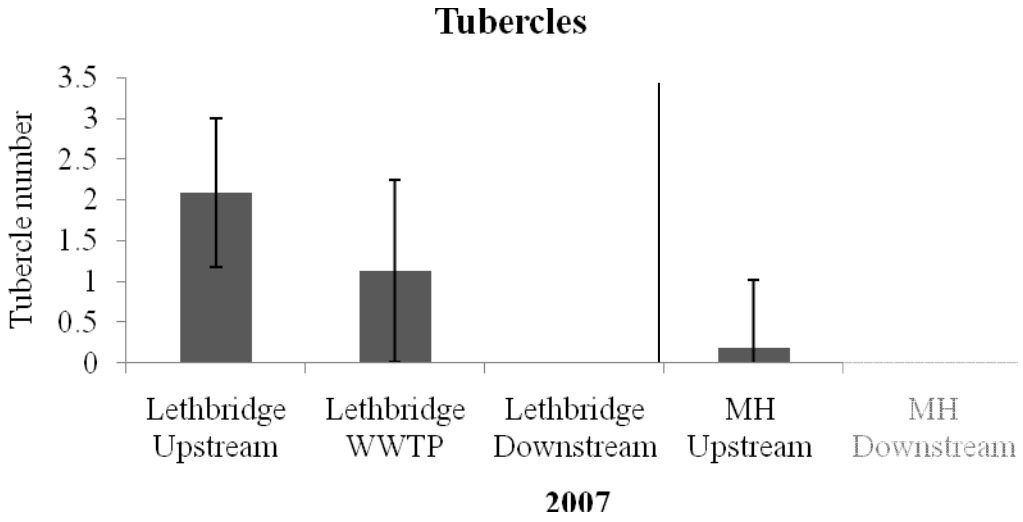


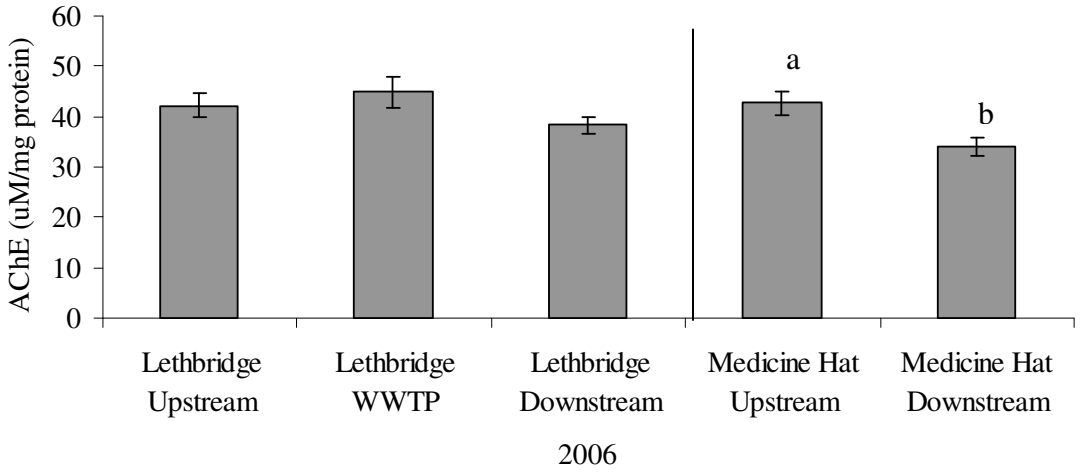
Figure 2.14. Tubercle number (mean ± SE) on male FHMN sampled from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat (MH), in summer 2007 (n= 21-25). No differences among groups sampled. White area indicates sample size was too small to use for statistical analysis (n=1).

AChE Activity

In 2006, AChE activity levels were not significantly different among fish sampled at Lethbridge sites. AChE levels were higher ($F_{1, 44}$ = 8.74, p=0.0050) in fish sampled at the Medicine Hat upstream site compared to fish sampled at the downstream site (Figure

2.15A). In 2007 AChE levels were significantly higher ($F_{2, 66} = 4.90$, p=0.0104) in fish sampled at the Lethbridge downstream site compared to the Lethbridge upstream site (Figure 2.15B). In Medicine Hat statistical analysis or comparison could not be done.

A)



B)

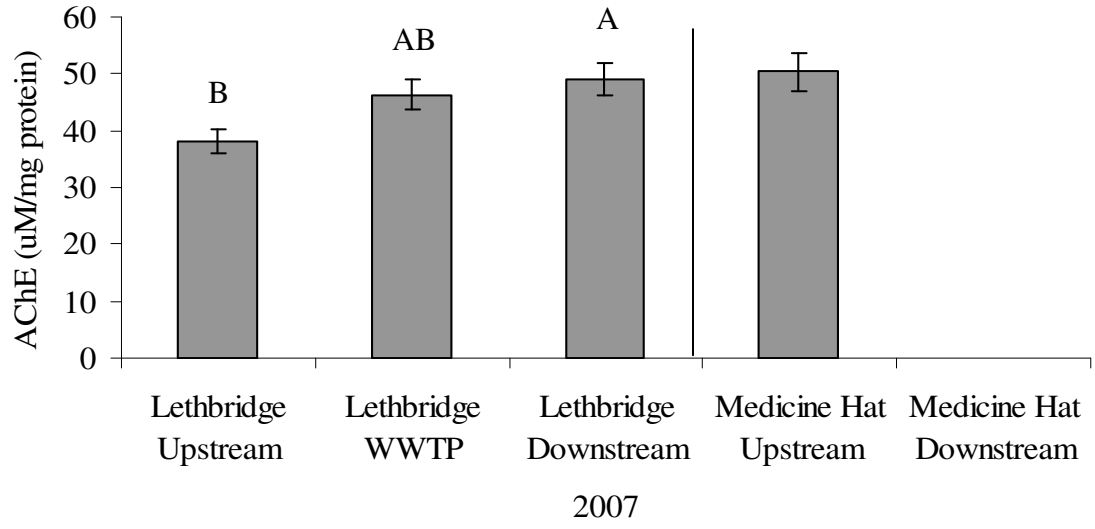


Figure 2.15. Head AChE activity (uM/mg protein) (mean±SE) in FHMN sampled from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat, in summer (A) 2006 and (B) 2007 (n = 21-29).Capital letters and small letters indicate significant differences within cities sampled.

2.4 Discussion

The objectives of this study were to assess the effects of WWTP effluent on the reproductive systems of FHMNs sampled upstream and downstream from the Lethbridge and Medicine Hat WWTPs, and hatchery-reared FHMNs exposed for 21 days to Lethbridge WWTP effluent in the laboratory. The study was designed to determine if fish exposed to the WWTP effluent in the laboratory mirror effects found in the field. A key study by Alberta Environment (Sosiak & Hebben, 2005) reported EDCs (nonylphenol ethoxylates, phthalate esters and mono-phthalate esters) in Southern Alberta rivers, including the Oldman and South Saskatchewan, and in WWTP effluents from the cities of Medicine Hat and Lethbridge (Figure. 2.4). A number of the chemicals detected are known endocrine disruptors that specifically affect the reproductive system (Mills & Chichester, 2005; Servos 1999; Daughton & Ternes, 1999).

Reproductive EDCs in WWTP effluent originate from a variety of sources including industrial use of surfactants and plasticizers, urban use of pesticides, as well as human production of steroid hormones, and human use of pharmaceuticals such as birth control. These chemicals can undergo substantial changes within the body and in the wastewater treatment process, resulting in a chemical released into the environment that may be degraded, transformed or combined into a compound that has an adverse effect on non-target aquatic organisms. In WWTPs, numerous compounds combine to create an effluent that is a constantly changing chemical mixture, as characterized by Sosiak & Hebben (2005), and each treatment plant receives input from a unique combination of sources, producing a distinct effluent.

Laboratory Study

To determine if effluent from the Lethbridge WWT plant affected the reproductive systems of FHMN, effluent was collected and laboratory-reared fish were exposed for 21 days in vivo. The ten percent effluent concentration was chosen for the laboratory exposure to reflect the 10:1 river flow:effluent discharge ratio limit, outlined in Alberta Environments (AENV) Standards and Guidelines for Municipal Waterworks, Wastewater, and Storm Drainage Systems (2006). At the time the laboratory exposure was performed, 2.69% of the Oldman river was comprised of the City of Lethbridge WWTP effluent. However, the long term average quantity of water flowing through the Oldman river is declining (Byrne et al. 2006) and if a drought year similar to the one experienced in 1988 (Water Survey of Canada, 2009) were to occur, with the City of Lethbridge current WWTP effluent discharge rates, effluent concentrations could increase to 7.6%. Although effluent released to the Oldman river (Table 2.2) currently meets release guidelines (AENV, 2009) set by Alberta Environment, regulations do not require monitoring of EDCs nor are environmental limits established for most of these chemicals.

In the laboratory exposure, male and female FHMNs exposed to EE2 and lower concentrations (10- 25%) of WWTP effluent had increased in liver Vtg mRNA expression and a decrease in tubercle number, GSI and percentage of mature oocytes. An increase in gonadal abnormalities was observed in fish exposed to all concentrations of WWTP effluent as well as EE2. All these responses are indicative of endocrine disruption (Arcand-Hoy & Benson, 1998; Guillette et al. 1995; Sumpter & Jobling, 1995). However, in both males and females, all endpoints measured did not progress in a

dose-dependent manner. Exposure of fish to higher concentrations of WWTP effluent did not result in a proportional increase in observed reproductive effects, but resulted in Vtg expression not different from controls. Similar patterns of reproductive effects of environmental pollutants have been reported in other studies (Nichols et al 2001, Giesy et al. 2000). One explanation for this pattern of responses is that one of the numerous chemicals in the effluent was toxic to the liver (Weber et al. 2004) and whereas lower concentrations of effluent were compatible with normal liver function and Vtg expression increased, higher concentrations became toxic and hindered the liver's ability to produce Vtg (Jobling & Sumpter, 1993). Negative feedback loops in the HPG (hypothalamic-pituitary-gonadal) axis, variation in estrogen receptor binding affinities to various chemicals, and steroid binding protein limiting factors are other mechanisms for non-linearity of reproductive effects, as proposed by Wei et al. (2007); Nichols et al. (2001); Folmar et al. (2000); and Giesy et al. (2000).

Moreover, it should be noted that liver Vtg mRNA was measured as opposed to actual circulating Vtg protein. Vtg mRNA is a snapshot in time of gene expression (Hiramatsu et al. 2005) which may have been affected by liver damaging chemicals whereas measurement of Vtg protein may offer better insight into cumulative effects of the exposure, as it remains in the blood longer (Hiramatsu et al. 2005). After exposure to EDCs, studies report Vtg mRNA reaching a plateau or decreasing, whereas Vtg protein persisted (Schmid et al. 2002; Bowman et al. 2000; Korte et al. 2000) or followed a linear dose response (Folmar et al. 2000). Increased Vtg mRNA half life (Bowman et al. 2000) and Vtg protein half life (Schmid et al. 2002, Korte et al. 2000) are explanations for the

continued response of Vtg protein. This could mean that for this study, even though Vtg mRNA did not increase in a linear dose response, Vtg protein may have.

With significant increase of female and male Vtg expression at 10 and 25% WWTP, respectively, after 21 days, one might expect that even lower concentrations would still induce Vtg. To understand how fish reproductive systems are affected by exposure to environmentally relevant WWTP effluent concentrations, further research is needed to determine Vtg induction rates at 1-10% WWTP effluent dilution. It is important to note however that dilutions 25% and higher can be detected in the acute and chronic mixing zones from the effluent discharge point of open pipe systems (New York State, 1996).

Future studies with WWTP effluent in the laboratory could be improved by use of a continuous flow exposure system rather than a static system (with renewal of only 50% effluent every 48 hrs), increased replicates of tanks and sample size (n), and thus increased statistical power. Additional modification could include incorporation of shorter (96 hrs) and longer (28 days) sampling intervals, since a shorter duration of sampling may provide information regarding the initial Vtg mRNA response of the liver to the effluents whereas a longer exposure may lead to more pronounced morphological effects. Despite these limitations, our study provided evidence that Lethbridge WWTP effluent has an adverse effect on the reproductive systems of laboratory-reared FHMNs. The activity of AChE, a marker for exposure to organophosphate and carbamate pesticides, did not differ between fish exposed to different concentrations of the WWTP effluent. Therefore, we inferred that, at the time of exposure, the Lethbridge WWTP

effluent did not contain concentrations of pesticides high enough to inhibit AChE activities.

Field Study

To determine if the effects observed in the laboratory exposure to WWTP mirror effects observed in the field, a large field study was undertaken in 2006 and 2007. Wild FHMNs were caught upstream and downstream of the city of Medicine Hat and the city of Lethbridge WWTPs, in the South Saskatchewan and Oldman rivers respectively. Since flow was higher in 2006 compared to 2007 (Water Survey of Canada, 2009), the percentage of the river(s) comprised of WWTP effluent was less in 2006 than 2007. For the Oldman, the average volume of flow in 2006 and 2007 was 1.00 and 1.44% respectively, whereas in the South Saskatchewan the average volume of flow was 0.22 and 0.23% respectively. Although these concentrations were much lower than the lowest laboratory exposure concentration (10%), the release processes may result in relatively high concentrations of effluent near the discharge, particularly for the City of Lethbridge WWTP effluent that is discharged through an end of pipe system, which does not promote uniform mixing.

The City of Lethbridge WWTP site fish were sampled within a 250 m^2 area around the discharge pipe, within the acute mixing zone where concentrations of effluent are quite high (AENV, 2006). In the acute mixing zone (New York State, 1996) concentrations similar to the higher concentrations used in the exposure (25, 50, 100%) may be detected. Vtg expression at the WWTP site in Lethbridge was increased in 2006 but not in 2007, when Oldman river flows were lower and WWTP effluent concentrations

may have reached high levels. This pattern could be reflective of what was found in the laboratory study at higher WWTP effluent concentrations. Even though fish are mobile and may not spend all their time directly in the effluent stream, Hemmer et al (2002) observed Vtg increases that lasted 4 - 8 days in fish exposed intermittently to 17β estradiol or para-nonylphenol. Panter et al (2000) also reported that intermittent exposure of FHMN to 120 ng/L estradiol resulted in plasma Vtg levels not different from continuously exposed FHMN, and that Vtg levels remained high after 21 days depuration; therefore, short term exposure could still have an effect on fish reproductive systems.

The City of Medicine Hat discharges its effluent into the South Saskatchewan River through a diffuser pipe, a design that promotes even mixing of WWTP effluent within the river and prevents areas of high effluent concentrations. A WWTP effluent sampling site, similar to the WWTP site in Lethbridge, was not found presumably because the fish did not have a similar nutrient-rich point around which to congregate. In 2006, Vtg expression was significantly increased in females sampled at the Medicine Hat downstream site compared to upstream, while downstream males had slightly higher Vtg than upstream, though not significantly. In 2007, only two FHMNs were caught at the Medicine Hat downstream site. No explanation for this absence of FHMN downstream of the WWTP effluent site can be provided, as there was an abundance of FHMN in 2006, as well as other species of fish including minnows caught in 2007. However, at all river sites FHMN were the least abundant species. High concentrations of some chemicals present in the effluent (Sosiak & Hebben 2005) may have caused mortality to FHMN.

Nonylphenol and bisphenol-A, have been reported to cause mortality (Hemmer et al 2001) to FHMN at high concentrations.

Increased Vtg expression in FHMN was expected, based on chemical analyses of the Oldman river and S. Saskatchewan river (Sosiak & Hebben 2005), and confirmed results of regional studies with other fish species (Jeffries et al. 2008). Future studies will determine whether high concentrations of WWTP effluent have a negative effect on the liver and impair its ability to produce Vtg.

Our study was designed to test the possibility that pesticides or their surfactants may influence the reproductive effects of WWTP effluents on FHMN. In 2006, AChE levels were significantly lower in the Medicine Hat downstream fish compared to upstream, indicating that fish were exposed to pesticides downstream from the WWTP. No differences were observed in the Lethbridge sampled fish in 2006, however in 2007 AChE was significantly lower at the Lethbridge Upstream site compared to the downstream. Input from two golf courses upstream from this site could be causing the inhibition of the AChE activities – however the effects on the reproductive system were not. Given the importance of pesticide use, particularly in agricultural regions such as S. Alberta, pesticides are a factor that should be thoroughly investigated in future studies.

2.5 Conclusions

Adverse reproductive effects were detected in laboratory reared fish exposed for 21 days to 10 and 25% of Lethbridge WWTP effluent, but higher concentrations did not have a significant effect. Similar dose-response curves have been reported in other studies and could be attributed to hepatic toxicity at high concentrations, negative

feedback in the HPG axis, variation in estrogen receptor binding affinities or steroid binding protein limiting factors. Differences in half life of Vtg mRNA and Vtg proteins could also have contributed to the non-linearity of the dose response curves of Vtg induction in the present study. It is interesting however to note that microscopic reproductive anomalies were detected in all groups treated with a toxicant. More research is needed to determine reproductive effects at current environmentally relevant concentrations of WWTP effluent at 1-10%.

In the field study, effluents from both Lethbridge and Medicine Hat had an effect of the reproductive systems of fish. Effects were more pronounced in 2006 when precipitation was higher and effluent was more diluted compared to 2007 when precipitation was lower and effluent was more concentrated in the rivers. It was interesting to note an absence of FHMN (but an abundance of other minnows) at the downstream Medicine Hat WWTP site in 2007.

Analyses of AChE activity in FHMN exposed to WWTP effluent in the laboratory provided evidence that effluent from Lethbridge did not contain organophosphate or carbamate pesticides at concentrations high enough to inhibit AChE. Results from the field study were inconclusive, since inhibition of AChE was not linked to WWTP effluent release.

Overall, our study provided evidence that WWTP effluent in Southern Alberta has the potential to introduce EDCs into receiving water and affect the reproductive systems of exposed fish.

CHAPTER 3. REPRODUCTIVE ENDPOINTS OF FATHEAD MINNOWS, *PIMEPHALES PROMELAS*, EXPOSED TO AGRICULTURAL RUNOFF IN IRRIGATION CANALS IN SOUTHERN ALBERTA.

3.1 Introduction

Southern Alberta's growing human population, intense agricultural irrigation practices and high feedlot density contribute natural and synthetic chemical pollutants to aquatic systems (Hanselman et al. 2003). Although past interest has focused on the endocrine disrupting effects of human domestic wastewater treatment plant (WWTP) effluents (Ankley and Villeneuve 2006; Sosiak and Hebben 2005; Daughton and Ternes 1999), increasing attention is now being given to the effects confined feeding operation (CFO) effluents are having on aquatic organisms (Orlando et al. 2004). Estrogenic chemicals in the effluents, including natural and synthetic hormones (Matthiessen et al. 2006) and growth promoting agents excreted from animals as waste (Schiffer et al. 2001), are associated with reproductive effects in fish (Jensen et al. 2006). Supplemental hormones are used in feedlots to increase weight gain, and control the reproductive cycle for propagation and to suppress the reproductive cycle to prevent mating and parturition injuries in a crowded feedlot (Health Canada 2005). In Canada, only three natural (progesterone, testosterone and estradiol-17ß) and three synthetic (trenbolone acetate, zeranol and melengestrol acetate) hormonal growth promoters are approved for use (Health Canada 2005). Although supplemental hormonal growth promoters are only approved for use in beef cattle (Health Canada 2005), natural (endogenous) hormonal steroids are still present in excrement of other livestock animals like poultry, swine, and

especially dairy cattle (usually pregnant), with the latter two excreting more estrogen per year than humans (Johnson et al 2006).

New or modified CFOs in Alberta require containment for animal wastes since 2002, when the Natural Resources Conservation Board (NRCB) became responsible for Part 2 of the Agricultural Operations Practice Act (AOPA) (NRCB 2006). However, many operations are grandfathered until they are brought to the attention of NRCB or they make apply to upgrade. They may not currently have containment for waste, and thus contribute to aquatic contamination through overland flow (NRCB 2008). Even though Lorenzen (2006) suggested that steroids may not reach the aquatic ecosystem due to the potential of chemicals to bind to soils, Arnon et al. (2008) reported that dairy farm waste, leaching through wastewater lagoons, introduced hormonal contamination to groundwater. Also, CFOs following best management practices are permitted to spread manure onto agricultural lands as a fertilizer and estrogenic compounds have been detected in streams flowing through livestock farms (Matthiessen et al. 2006). Moreover, there are no regulations against cattle entering or finding relief in a waterbody, providing a direct source of contamination to the aquatic environment.

Interference with and disruption of endogenous reproductive hormone pathways in fish by introduced androgenic and estrogenic-like compounds is a major concern (Burkholder et al. 2007). Female fish normally produce a phospholipoprotein, Vitellogenin (Vtg), by the liver in response to ovarian estrogen. Males do not produce high enough levels of endogenous estrogen to turn on their liver Vtg production gene, although it is present and functional (Schmid et al. 2002; Kime, 1998). When exogenous estrogens are introduced, they induce males and females to produce substantially higher

than normal levels of Vtg (Matozzo et al. 2008; Tyler et al. 1996), potentially leading to intersex gonads (Kidd et al. 2007). Other conditions influenced by exogenous hormones include skewed sex ratios, altered secondary sex characteristics and decreased breeding (Watanabe et al. 2007; Parrott and Wood 2002). The fathead minnow (*Pimephales promelas*), a sentinel species used extensively in reproductive toxicity testing (Ankley et al. 2001; Ankley and Villeneuve 2006), is found in S. Alberta, yet the effects of regional endocrine disrupting compounds (EDCs) on fathead minnow (FHMN) reproductive status has not yet been assessed. Southern Alberta has the largest potential to introduce endocrine disrupting chemicals from feedlots into the environment (Canfax 2008). Alberta dairy farms (645 in total) average 114 animals per farm (Alberta Milk 2008). In addition, 60% of Alberta cattle feedlots have capacity for 1000-5000 head, whereas 7% of cattle feedlots lots have > 20 000 head capacity (but represent 38% of Alberta's total feedlot cattle) (Canfax 2008). Alberta pork and poultry industry numbers register 2 020 000 animals (Alberta Agriculture Food and Rural Development, 2006) and 560 farms respectively (Alberta Agriculture Food and Rural Development, 2002). In the Oldman river watershed basin alone, there are almost 600 CFOs (Oldman Watershed Council 2009). The Alberta livestock animal population is greater than the human population, and therefore cumulative livestock waste per year amounts to more than human waste production and could potentially be a greater concern for aquatic contamination than WWTP effluent. Moreover, S. Alberta agriculture relies heavily on pesticides, and these chemicals, including cholinesterase-inhibiting pesticides (organophosphates and carbamates), also have the potential to cause reproductive effects in aquatic organisms and to contribute to contamination of surface and ground water (Kamrin 1997).

For the last century, Albertans in the agricultural industry have relied on the irrigation districts to provide the essential element, water, to the producers in the arid South. The Lethbridge Northern Irrigation District (LNID) is a main canal conveyance system of water from the Oldman River to the agricultural sector north of Lethbridge. The Battersea Drain is just one of many irrigation return flow drainage canals that specifically flow into the Oldman River downstream of Lethbridge. Studies undertaken by the Battersea Drain Watershed Group (Oldman Watershed Council, 2009) determined that the quality of water declined during wetter years due to the increased amount of runoff carrying high nutrient and fecal bacterial loads from non point sources (eg livestock excrement). In 2006, a year with higher precipitation and more frequent pesticides detections compared to 2007, a drier year, were reported in the Battersea drain (Unpublished data, Alberta Agriculture Food and Rural Development). Forrest et al. (2006) reported the Battersea drain as having the highest number of agricultural pharmaceutical compounds (not including steroids) detected and the most types of compounds detected as compared to watershed basins throughout Alberta. These compounds, and endocrine disruptors including reproductive steroid hormones, can presumably be associated with livestock waste, be carried in agricultural runoff and accumulate in the return flow canals. Even though endocrine disrupting effects have been detected in the Oldman river (Jeffries et al. 2008), there are no quantitative data to characterize and assess the contributing influence of the irrigation canals and return flows. The potential for agricultural and CFO intensive Southern Alberta to contribute endocrine disruptive compounds to the abundant irrigation canals and affect the reproductive systems of aquatic organisms is considerable and has yet to be investigated.

The objectives of this study were to: 1) assess the reproductive endpoints of wild FHMN in return flow drain canals in southern Alberta, and 2) investigate the effects of agricultural runoff (CFO effluent, pesticides, and other chemicals) and season on the reproductive systems of the fish. Liver Vtg mRNA, GSI, LSI, CF, number of tubercles, sex ratio, and gonadal morphology, including intersex, were used to assess the reproductive status, whereas head AChE activity was used as a biomarker of exposure to organophosphate and carbamate pesticides (Stenerson 2004). The study was designed to test the hypothesis that fish exposed to agricultural runoff have decreased reproductive fitness characterized by abnormal GSI and LSI, higher liver Vtg mRNA, abnormal sex ratios and gonadal morphology, and lower CF.

3.2 Materials and Methods

3.2.1 Canal Study Sites

The Lethbridge Northern Irrigation District is fed by the Oldman river at a diversion point forty miles west of Lethbridge. LNID water follows a number of main flow paths, mostly providing water to the agricultural industry, with some water eventually returning to the Oldman river (closer to Lethbridge), in return flow drainage canals (Figure 3.1). Main flow source waters and return flow drainage canals were sampled. Sites were selected based on accessibility and presence of FHMNs. Three canal sites were sampled in June-July 2006; the same sites and additional two canal sites were sampled in May-June 2007 (Figure 3.2). Two of the canal sites were sampled a second time in the fall (September-October) 2006 and 2007 to investigate seasonal variation in

the reproductive endpoints of the FHMNs. Water pH and temperature were measured at each site (Table 3.1).

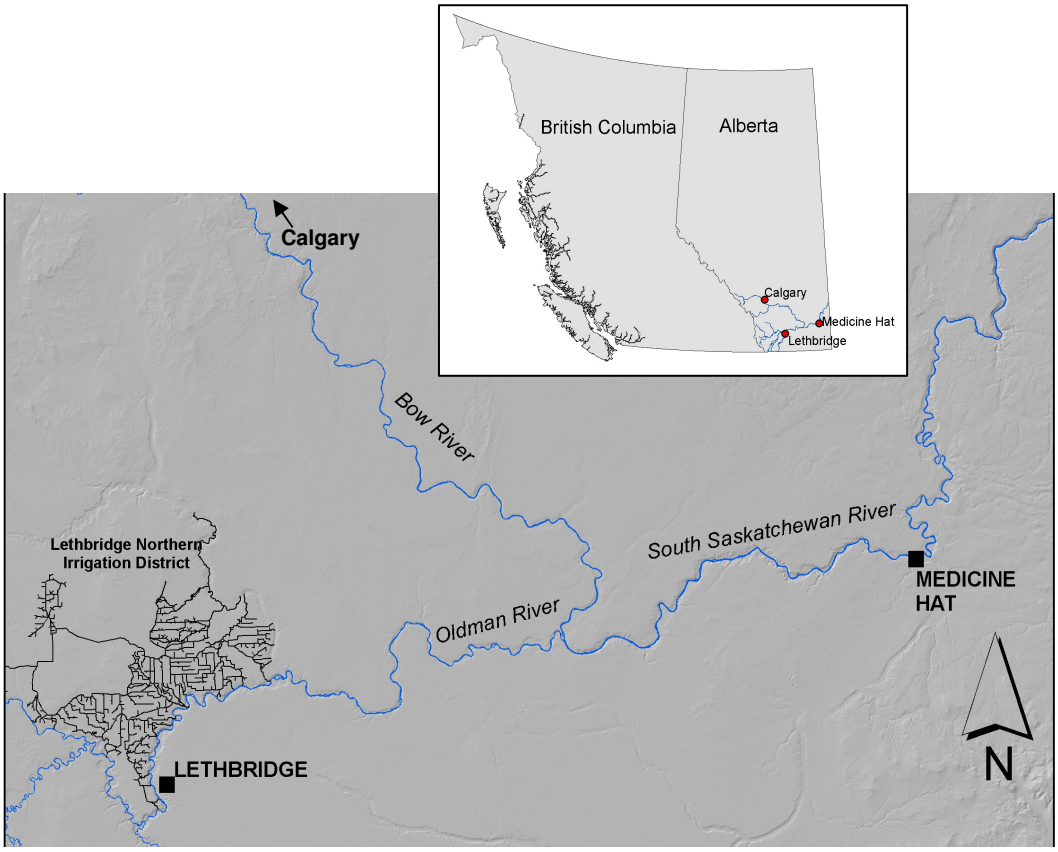


Figure 3.1-Map of Lethbridge Northern Irrigation District, Oldman, Bow and South Saskatchewan rivers in Southern Alberta

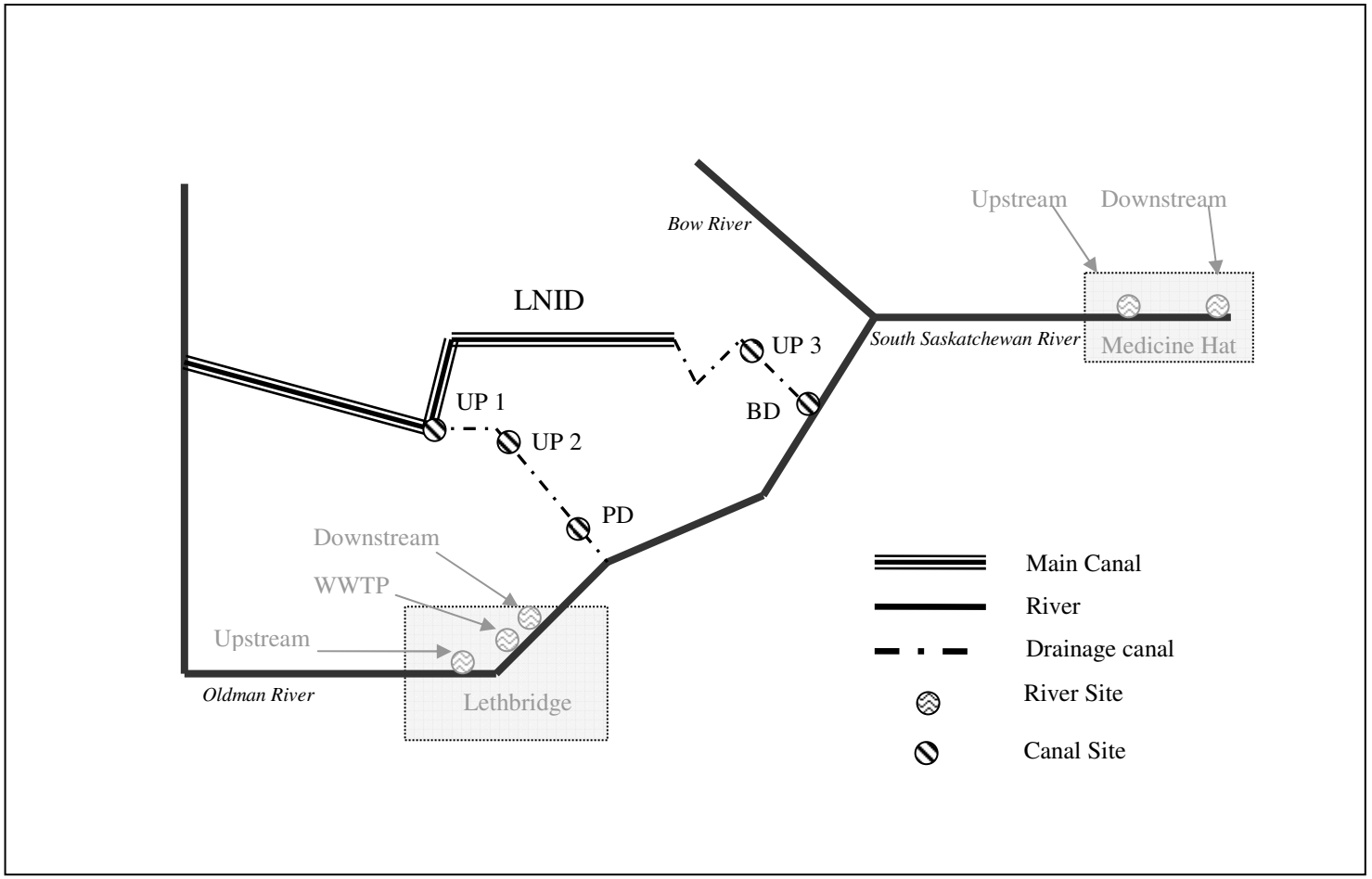


Figure 3.2-Schematic of sampling sites at the source waters, Upstream 1, 2, 3 (UP 1, UP 2, UP 3) and return flow drainage canals, Pyami Drain (PD) and Battersea Drain (BD) in the Lethbridge Northern Irrigation District (LNID), an area with numerous confined feeding operations. River sites sampled up and downstream from WWTPs are also shown in light grey.

Table 3.1 Characteristics of canal sites sampled in summer and fall 2006 and 2007.

Site	Legal Land Location	N[a] **2006**	Sampling Dates	Temperature	pH	N[a] **2007**	Sampling Dates	Temperature	pH
UP1	NE 14-11-22W4	-	-	-	-	20	June 1, 11	21.4[b]	7.4[b]
UP2	SW 13-11-22W4	32	July 18	21.2	8.6	21	May 31	18.1	7.6
UP2 (Fall)	SW 13-11-22W4	31	Sept 26, 29	11.8	8.6	21	Oct 9	10.3	6.9
UP 3	NE 8-11-20W4	-	-	-	-	25	June 21, 22	19.0[b]	7.1[b]
PD	SE 22-10-21W4	21	June 29, 30	20.8[b]	8.6[b]	24	June 13	17.1	8.1
BD	NW 36-10-20W4	35	June 26, 27	26.8[b]	7.5[b]	24	May 7	15.0	8.5
BD (Fall)	NW 36-10-20W4	30	Oct 5, 12	8.2[b]	7.9[b]	25	Sept 26, Oct 5	8.3[b]	6.4[b]

[a] Number of FHMNs sampled/site

[b] Average

3.2.2 Sampling at Canal Sites

Fish (N = 20-40 per site) were captured with a Smith Root LR-24 electrofisher in the morning and held in aerated canal water until sampling. Fish were sampled either in the field or laboratory (if site was close to university) starting at 1PM. They were anesthetized (MS-222 and site water), and tubercle number (in 2007 only), fork length, total body weight, gonad and liver weight were recorded. Condition factor (CF), liver somatic index (LSI) and gonadosomatic index (GSI) were calculated. Tissue samples of liver, one gonad and head were dissected and frozen in liquid nitrogen and stored at -80 ^{o}C, carcasses were stored at -20 ^{o}C. The other gonad was placed in Bouin's fixative for standard histology.

3.2.3 Biochemical and Morphological Analyses

3.2.3.1 Liver Vitellogenin

Liver Vtg mRNA was measured in collaboration with Dr. E. R. Nelson and Dr. H. R. Habibi at the University of Calgary. RNA extraction and cDNA synthesis was carried out as previously described in Nelson and Habibi (2006). Briefly, total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen). Four micrograms of total RNA was then used for cDNA synthesis using an oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's protocol.

Real time quantitative polymerase chain reaction (QPCR) was used to quantify relative Vtg mRNA levels, as previously described (Nelson et al. 2007). A BIO-RAD iCycler iQ Multicolour Real-Time PCR Detection System was used with the following conditions per well: 0.5 µl of cDNA, 0.26 µM of each primer, 0.2 mM dNTPs, Sybr green and Taq polymerase in buffer (10 mM Tris-HCL [pH 9.0], 50 mM KCl, 1.4 mM MgCl2, 20 nM

fluorescein) to a total volume of 25 µl. As an internal control, β-actin was also amplified as described previously. Primers were as follows:

Vtg: [5'- gaagtgcgcatggtggcttgtatt-3'] and [5'- agctgccatatcaggagcagtgat-3'], β-actin: [5'-CCTCCATTGTTGGCACC-3'] and [5'-CCTCTCTTGCTTTGAGCCTC-3']. Cycling was as follows: 3 min at 94^{o}C followed by 30-50 cycles of 10 s at 94^{o}C and 40 s at 54.3^{o}C. Each experimental group was run in triplicate to ensure consistency. Validation experiments found the primers to amplify only one product as determined by melt curve analysis and gel-electrophoresis, and primer efficiencies were determined to be 96.7% for Vtg and 95% for β –actin.

3.2.3.2 Acetylcholinesterase

Acetylcholinesterase (AChE) activity was measured with a modified protocol of Ellman et al. (1961) and Chuiko (2000). Heads were homogenized in a phosphate buffer at a 1:3 ratio with a Power Max AHS 200 (VWR). Homogenate was centrifuged 3 times at 13 000 RPM for 3 minutes at 5^{o}C and supernatant was collected. Final supernatant was diluted with phosphate buffer at a 1:10 ratio for AChE assay (and further diluted at a 1:15 ratio with protein dilution solution for Bradford protein assay). Supernatant (4 µL) at triplicate was pipetted into a 96 well plate with 120 µL of Tris-ISO-OMPA (Sigma T5030), followed by incubation of samples for 10 minutes at room temperature on a shaker. Then 10 µL of DTNB (Sigma D8130) followed by 10 µL of AChI (Sigma A5751) were added to the wells and the samples were incubated for another 10 minutes on a shaker at room temperature. The microplate was read in a microplate reader (Molecular Devices, MAXline Emax Precision) using Softmax Pro software every 2

minutes for 10 minutes at 405 nm. AChE concentration was determined from the slope of the change in absorbance. Internal standards were run on each plate to ensure the accuracy of the assay (Normal Serum Control, TC-TROL [N], Teco Diagnostics, 1 U/mL and 2 U/mL eel acetylcholinesterase, Sigma C3389), with assay characteristics as shown in Miller et al. (2009). AChE activity values were expressed as units/mg protein (measured with a spectrophotometer at 595 nm using the Bradford method).

3.2.3.3 Histology

Gonads were placed in a cassette (VWR CA87002-424) and fixed in Bouin's fixative for at least three days. Tissues were washed in water and then in a series of increasing water and ethanol (Commercial Alcohols) concentrations up to 70%. Tissues were stored in 70% ethanol, then they were dehydrated in an ethanol series, clearing agent Safeclear (Fisher 044-192) or Citrosolve (Fisher 22-143975), and embedded in Paraplast Plus (Fisher 23-021-400). Tissues were cut (longitudinally) at 5-10 μm, mounted on slides with gelatin (Fisher G8), stained with Eosin (Fisher 245-658) and Hematoxylin (Fisher 245-656), and mounted with Permount (Fisher SP15-100). Sex was determined and a visual survey was completed to check for gonadal abnormalities (presence of parasite, intersex, proteinaceous mass and other anomalies).

3.2.4 Statistical Analysis

Data were analyzed with JMP IN 5.1, using an ANOVA with a post hoc Tukey Kramer HSD. All data are shown without transformation. However, all statistical tests were performed on natural log transformed data (to maintain consistency), unless otherwise noted (gonadal anomalies and tubercle counts were not log transformed). Log

transformation promotes normality and most data sets were not normal. All statistical tests were based on an alpha of 0.05. Sex ratio statistics were performed using a Chi^2 analysis and a 50:50 expected.

3.3 Results

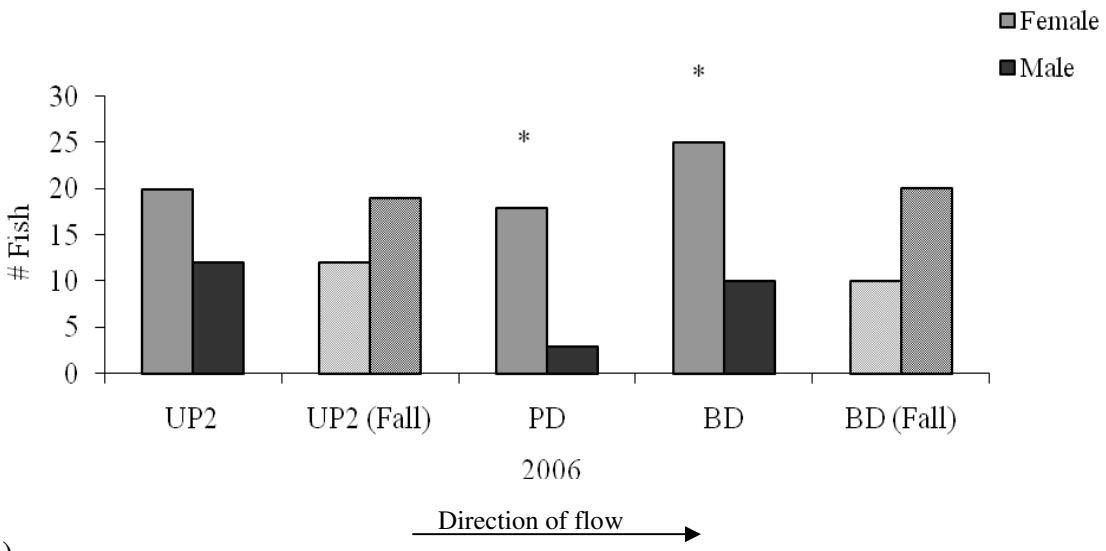
Data provided by the Oldman Watershed Council (2006) and Alberta Agriculture (Unpublished data, Alberta Agriculture Food and Rural Development) indicate that there was more rainfall and higher fecal coliform counts in the irrigation canals in 2006 than in 2007. This evidence is interpreted, in absence of chemical data, as evidence for higher exposure to potential endocrine disruptors in 2006 than in 2007.

Field Results 2006/07

Sex ratio

In summer 2006, based on an expected 50:50 sex ratio (Parrot and Blunt 2005; Parrot and Wood 2002; Zerulla et al. 2002; Lange et al. 2001), more females than males ($p < 0.05$), were caught at the two return flow drainage canals (PD and BD). In the fall, more males were caught at both sampled sites (UP2 and BD); however the differences were not statistically significant (Figure 3.3 A). In summer 2007, a female-skewed ratio although only significant at UP3, was also noted at UP2 and PD. In the fall, more males were sampled at UP2 and BD, similar to 2006 results, though the differences in sex ratio were not statistically significant (Figure 3.3 B).

A)



B)

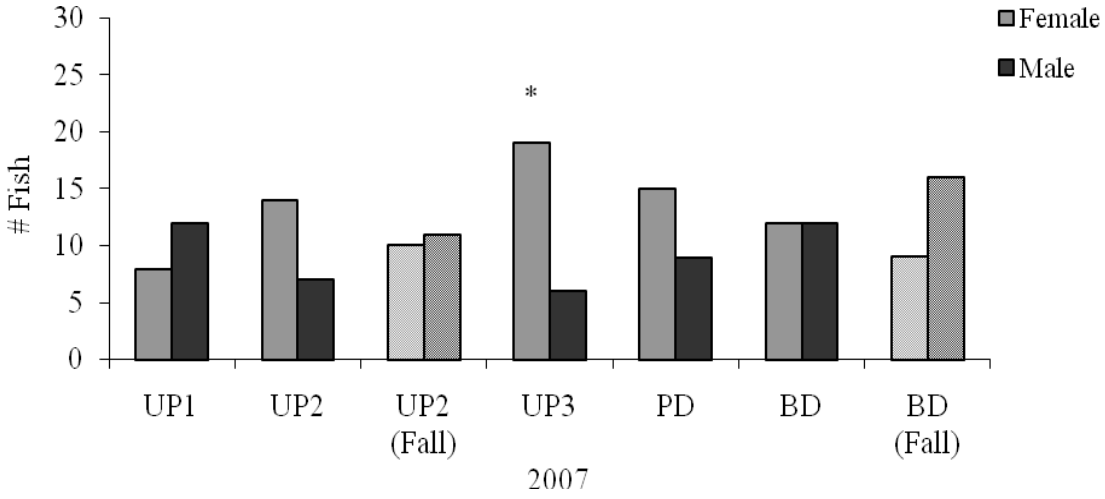


Figure 3.3 Numbers of female and male FHMN caught in LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall (A) 2006 and (B) 2007.Significant difference, based on Chi^2 analysis and a 50:50 sex ratio, indicated with *. Cross hatch signifies fall sampling.

Liver Vitellogenin

In summer 2006, liver Vtg expression for both females ($F_{2, 56}$ = 19.80, p=<0.0001) and males ($F_{2, 18}$ = 4.99, p=0.0189) was higher in BD sampled fish compared to UP2 and PD (Figure 3.4 A&B). In fall 2006, though not significant, female fish had higher Vtg expression at BD compared to UP2 (Figure 3.4 A), whereas Vtg expression in males was significantly lower ($F_{1, 33}$ = 5.57, p=0.0243) at BD compared to UP2 (Figure 3.4 B). Seasonally, Vtg expression in both females ($F_{1, 33}$ = 7.80, p=0.0086) and males ($F_{1, 27}$ = 9.17, p=0.0054) was higher in the summer than fall at BD, except in males at UP2 where Vtg was lower ($F_{1, 24}$ = 10.34, p=0.0037) in the summer compared to the fall (Figure 3.4 A & B).

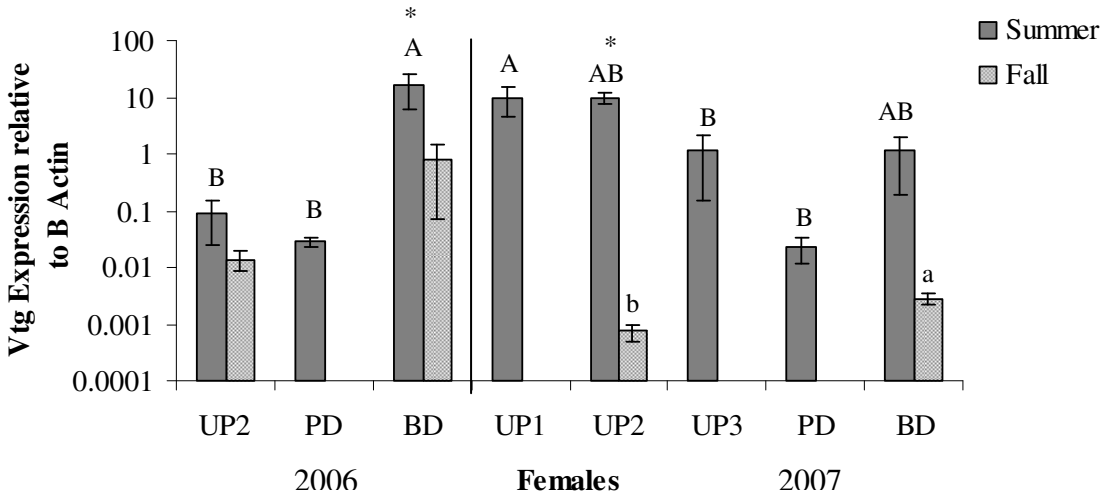
In summer 2007, female liver Vtg expression was lower ($F_{4, 61}$ = 4.01, p=0.0060) at PD, and also at UP3 compared to UP1 (Figure 3.4 A) while there were no significant differences in male Vtg expression among different sites (Figure 3.4 B). In fall 2007, female Vtg expression was higher ($F_{1, 16}$ = 7.74, p=0.0133) at BD than UP2 (Figure 3.4 A) and males showed a similar trend (Figure 3.4 B). Seasonally, Vtg expression at UP2 tended to be higher in the summer than fall in both females ($F_{1, 22}$= 20.79, p=0.0002) and males, with a similar trend found in females at BD (Figure 3.4 A & B).

Reproductive status and growth indices

Female GSI in summer 2006 was higher ($F_{2, 27}$ = 16.41, p=0.0001) in fish at PD and BD compared to UP2, whereas in the fall, there was no significant difference between sites (Figure 3.5 A). Male GSI was also higher at BD compared to UP2 in the summer ($F_{1, 4}$ = 14.51, p=0.0189), as well in the fall ($F_{1, 18}$ = 9.93, p=0.0055) (Figure 3.5

B). Seasonally, in both female and male fish GSI was higher in the summer compared to the fall, significantly ($F_{1, 22}$ = 86.24, p=<0.0001), ($F_{1, 12}$ = 27.35, p=0.0002) at BD (Figure 3.5 A&B).

A)



B)

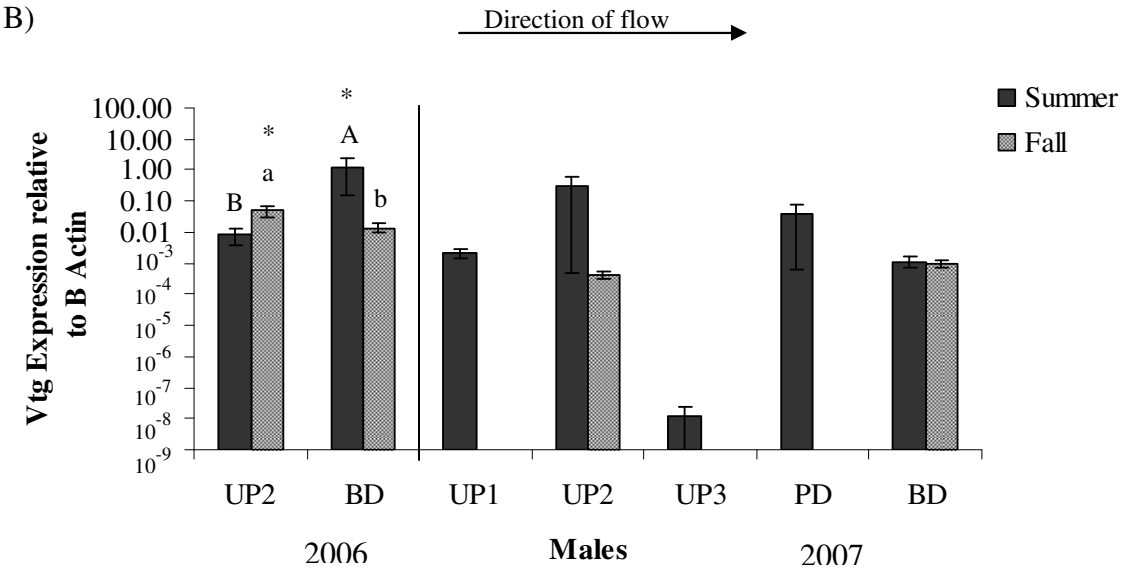
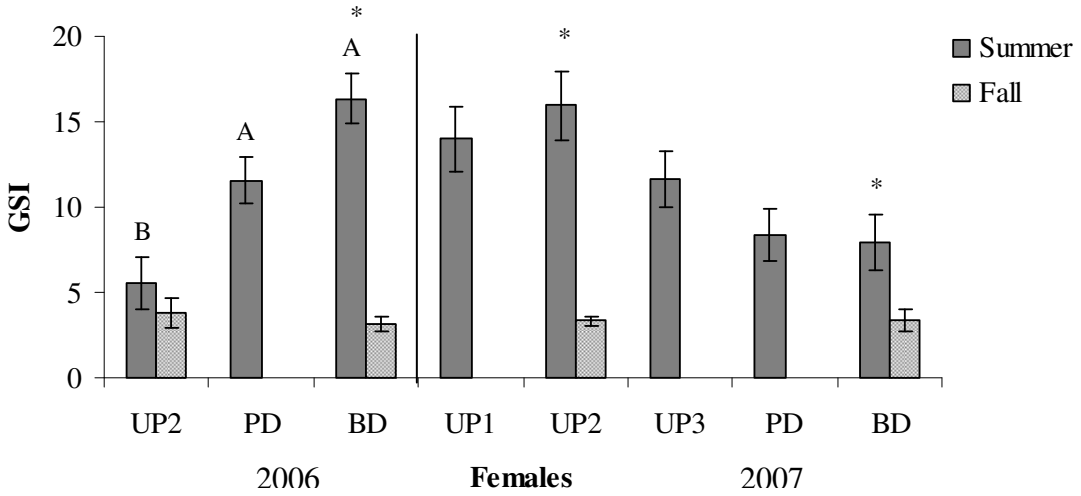


Figure 3.4 Vitellogenin, Vtg (mean±SE) in (A) female and (B) male FHMN sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall 2006 and 2007 (N=20-35/site). Capital letters and small letters indicate significant differences between sites in summer and fall respectively. Significant difference between seasons indicated with *(number of fish sampled indicated in Fig. 3.4).

In summer 2007, there were no significant differences in GSI in females sampled at the different sites, although a trend for higher GSI at UP1, UP2 and UP3 than at PD and BD was observed. In the fall, there was no significant difference between sites (Figure 3.5 A). In the summer, male GSI was significantly higher ($F_{4, 40} = 2.98$, p=0.0304) at UP1 compared to UP2, with no significant difference in the fall (Figure 3.5 B). Seasonally, as in 2006, GSI of female (UP2 $F_{1, 22} = 22.42$, p=0.0001; BD $F_{1, 19} = 8.65$, p=0.0084) and male fish were higher ($F_{1, 26} = 5.68$, p=0.0247) in the summer compared to the fall, although for males at UP2 the difference was not statistically significant (Figure 3.5 A&B).

Based on histopathology of gonadal anomalies (Figure 3.7) in the fall of 2006 site UP2 had significantly ($F_{1, 58} = 8.51$, p=0.0050) more FHMNs with anomalies compared to BD (Figure 3.6A). It is also interesting to note that in 2006, UP2 was also significantly ($F_{1, 58} = 19.33$, p=<0.0001) parasitized (*Posthodiplostomum minimum*) in the fall (Figure A3 in appendix). Seasonally, there were more parasites ($F_{1, 58} = 14.32$, p=0.0004) and anomalies ($F_{1, 58} = 5.80$, p=0.0192) in the fall compared to the summer at UP2 (Figure 3.6A and A3). In 2007, there were no differences between sites for gonadal anomalies or parasitization (Figure 3.6B and Figure A3). In both years, there were however more anomalies in female fish than males (twice as many in 2006, three times as many in 2007), and in both years females were parasitized more than males. Parasitism did not seem to affect Vtg levels.

A)



Direction of flow

B)

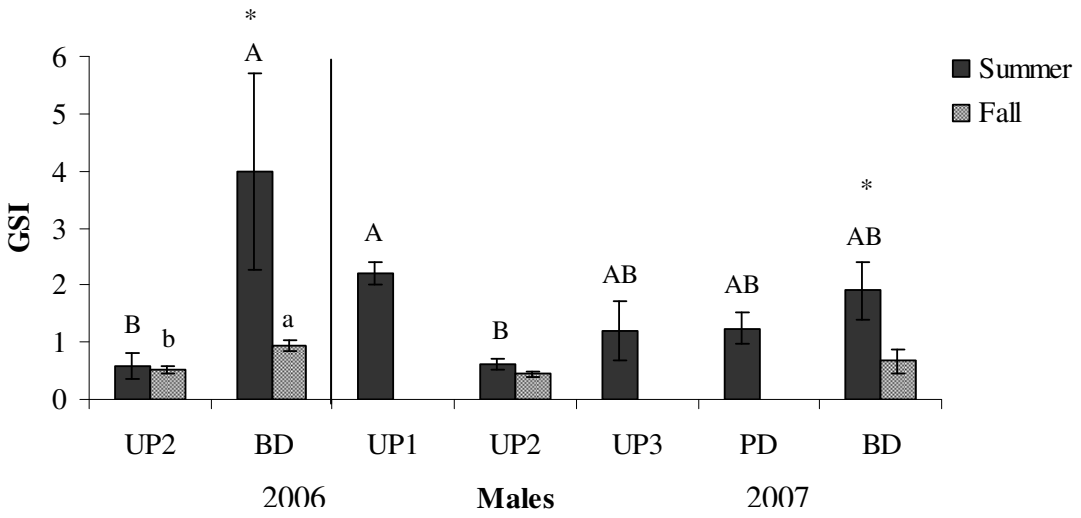


Figure 3.5 Gonadosomatic index, GSI (mean±SE) in (A) female and (B) male FHMN sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall 2006 and 2007 (N=20-35). Capital letters and small letters indicate significant differences between sites in summer and fall respectively. Significant difference between seasons indicated with *.

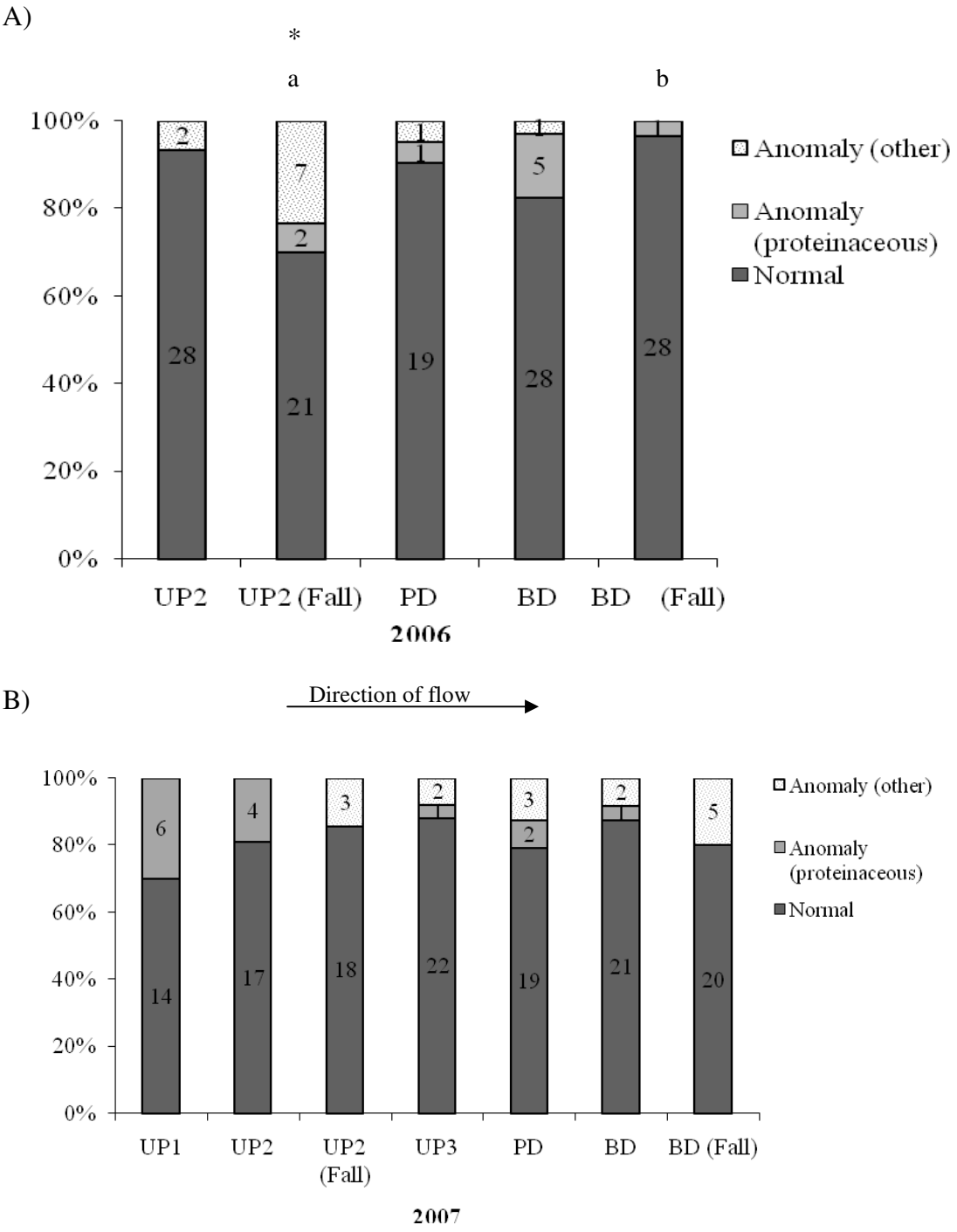


Figure 3.6 Histopathology survey of anomalies (percent, males and females grouped) in FHMN sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall (A) 2006 and (B) 2007 (N=20-34). Small letters indicate significant differences of total anomalies (proteinaceous and other) among groups sampled in the fall. Significant difference between seasons indicated with *.

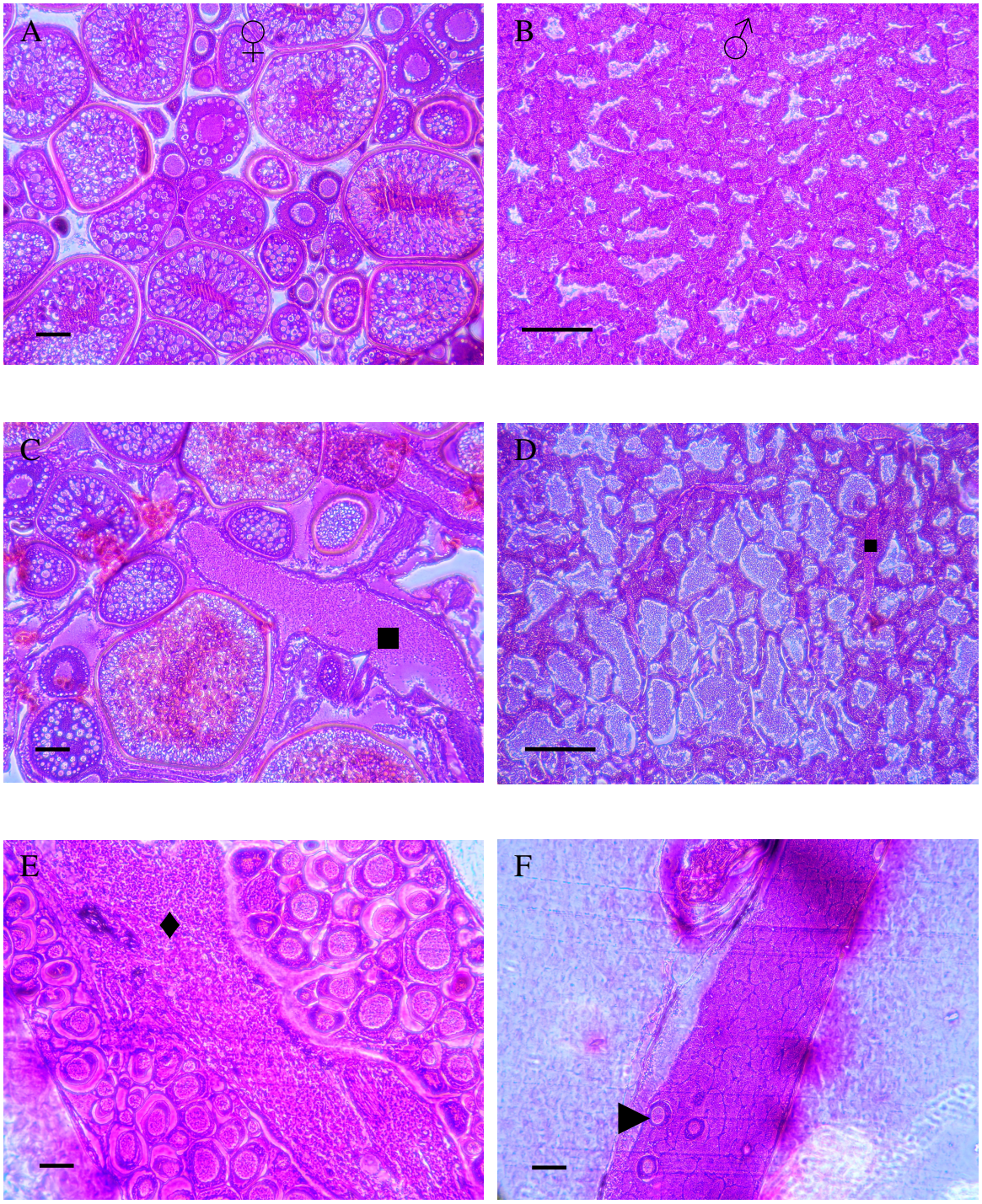


Figure 3.7 Gonadal histopathology of FHMN sampled from LNID in 2006 and 2007 (N=20-34). A) Normal Female B) Normal Male C) Female with ■ Anomaly (proteinacious) D Male with ■ Anomaly (proteinacious) E) Female with ♦ Anomaly (other) F) Male with ▶Anomaly (other). Scale bar = 100µm.

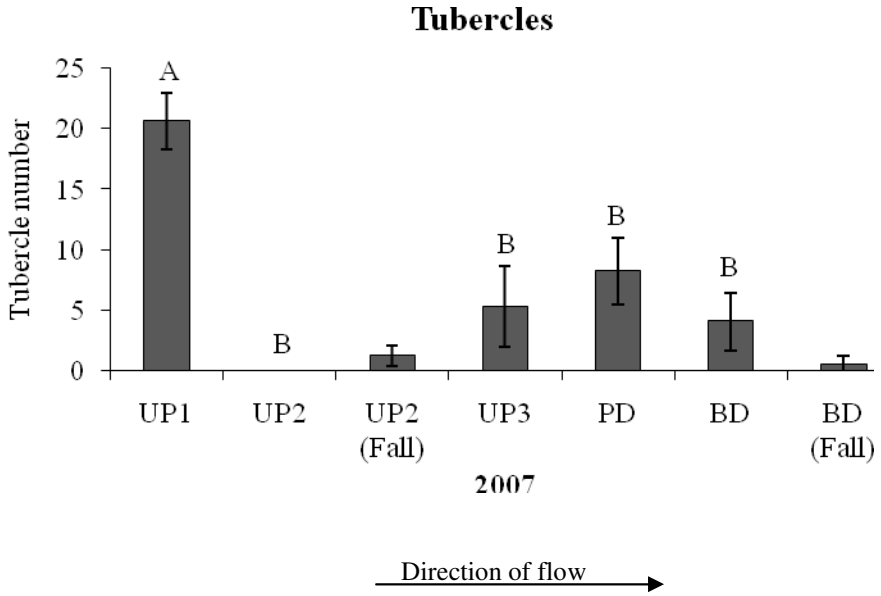


Figure 3.8 Tubercles (number, mean ± SE) on male FHMN sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall 2007 (N=7-16). Capital letters indicate significant differences of tubercles among sites sampled within the summer.

Tubercle numbers in summer 2007 were greater ($F_{4, 41} = 9.60$, p=<0.0001) at UP1 compared to all other sites. No seasonal tubercle differences were detected in 2007 (Figure 3.8). Data was not available for 2006.

In summer 2006, there were no significant differences in LSI (females and males from each site grouped together), although LSI tended to be higher at BD compared to UP2 (Table 3.2). No differences were detected in the fall. Seasonally, LSI was higher in the fall compared to the summer at UP2 ($F_{1, 50} = 17.75$, p=0.0001). In summer 2007, LSI at UP2 was significantly higher ($F_{4, 109} = 10.87$, p=<0.0001) compared to UP3 and PD, with no differences in the fall. No seasonal differences in LSI were detected in 2007 (Table 3.2).

Table 3.2. Liver Somatic Index, LSI (mean ± SE) in FHMN sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall 2006 and 2007 (N=20-35).

Site	n	LSI[a]	Site Difference	Seasonal Difference
		2006		
UP2	32	2.83 ± 0.5	A	*
UP2 (Fall)	31	4.76 ± 0.33	a	
PD	21	ND	-	
BD	35	4.20 ± 0.67	A	
BD (Fall)	30	4.79 ± 0.29	a	
		2007		
UP1	20	4.81 ± 0.92	AB	
UP2	21	4.89 ± 0.29	A	
UP2 (Fall)	21	4.36 ± 0.25	a	
UP3	25	3.59 ± 0.19	BC	
PD	24	2.88 ± 0.11	C	
BD	24	4.38 ± 0.14	AB	
BD (Fall)	25	4.28 ± 0.24	a	

Capital letters and small letters indicate significant differences between sites in summer and fall respectively. Significant difference between seasons indicated with * ($p<0.05$).

[a] Liver Somatic Index = liver weight / total weight x 100.

[#]males and females from a site grouped

Condition factor (females and males grouped) in summer 2006 was higher ($F_{2, 98} = 25.38$, p=<0.0001) at BD compared to UP2 and PD, with no significant differences in the fall. Seasonally, CF was higher ($F_{1, 68} = 18.58$, p=<0.0001) in the summer compared to the fall at BD (Figure 3.9). In summer 2007, CF was higher ($F_{4, 109} = 8.30$, p=<0.0001) at UP1 compared to UP3, PD and BD, with no significant differences in the fall. Seasonally, CF was higher in the summer compared to the fall at both UP2 ($F_{1, 40} = 25.90$, p=<0.0001) and BD ($F_{1, 47} = 8.06$, p=0.0067) (Figure 3.9).

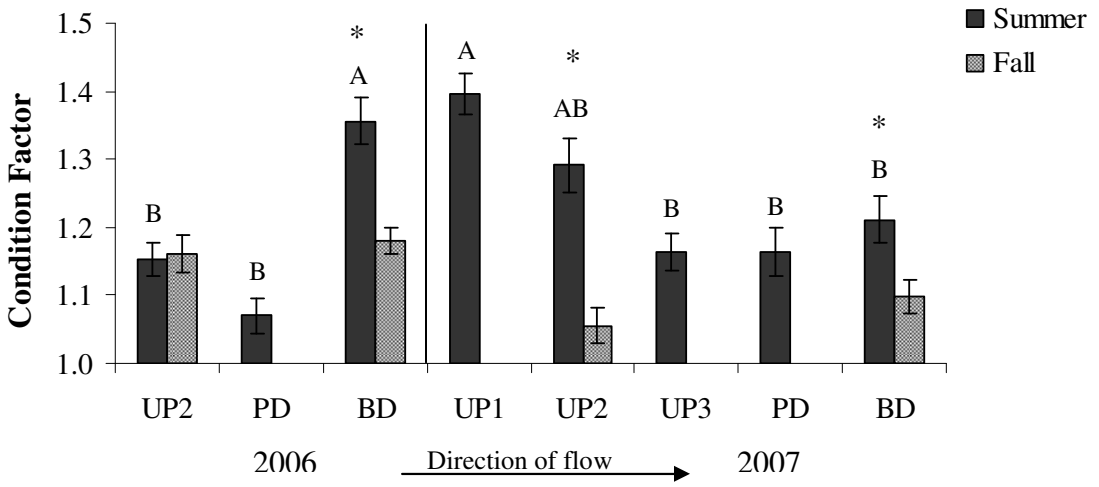


Figure 3.9 Condition Factor (mean±SE) in FHMN (males and females grouped) sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall 2006 and 2007 (N=20-35). Capital letters and small letters indicate significant differences between sites in summer and fall respectively. Significant difference between seasons indicated with *.

AChE activity

Head AChE activities in fish sampled in summer 2006 were higher ($F_{2, 89} = 38.61$, p=<0.0001) at PD compared to UP2, while both were also significantly higher than BD. In the fall, AChE activity at UP2 was higher ($F_{1, 61} = 9.81$, p=0.0027) than at BD. Seasonally, higher AChE activities were measured in the fall compared to the summer, significantly ($F_{1, 58} = 4.25$, p=0.0436) at UP2. In summer 2007 AChE activities were higher ($F_{4, 109} = 2.56$, p=0.0425) at BD compared to PD, with no significant differences in the fall. There were no seasonal differences in AChE activities between summer and fall in 2007 (Figure 3.10).

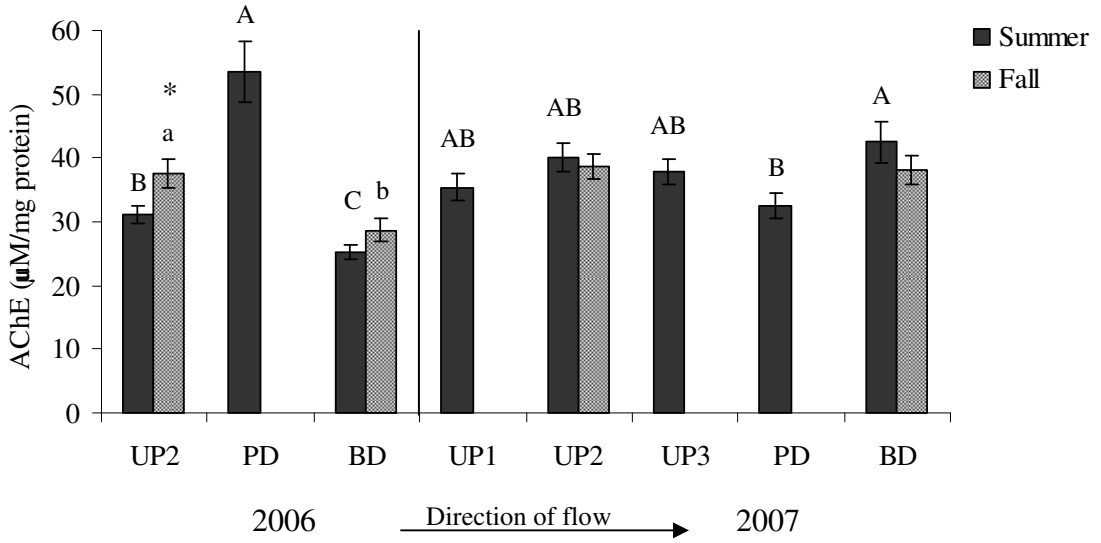


Figure 3.10 Head AChE activity (mean±SE) in FHMN (females and males) sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall 2006 and 2007 (N=20-35). Capital letters and small letters indicate significant differences between sites in summer and fall respectively. Significant difference between seasons indicated with *.

3.4 Discussion

The objectives of this study were to assess the effects of agricultural runoff and season on the reproductive systems of wild FHMNs in return flow drain canals in southern Alberta. The LNID irrigates an area of approximately 710 Km^2, including the 73 Km^2 Battersea Drain (BD) and the 267 Km^2 Pyami Drain (PD) which collect runoff from the Battersea Watershed and the Pyami Watershed, respectively. Water from both drain canals exits to the Oldman river. Although limited chemical data were available for the canals and drains, indirect evidence, specifically fecal coliform counts and precipitation, were consulted as surrogates of potential exposure to agrichemicals (Byrne et al. 2006). Head AChE activity was used as a marker of exposure to organophosphate and carbamate pesticides (Stenerson 2004). Gonadal size and morphology, liver Vtg and sex ratio were

used to assess the reproductive status of the fish sampled in the canals, similar to other studies (Kidd et al. 2007; Watanabe et al. 2007; Parrott and Wood 2002; Tyler et al. 1996).

Sampling sites in this study were selected in relation to agricultural point and non-point sources. The upstream sites (UP1 and UP2) are located on the periphery of the irrigation district, UP3 is located in the middle, whereas the two drain sites (BD and PD) are located downstream from these sources. It was expected that an accumulation of contaminants from the various agricultural point and non-point sources would affect the quality of water and in turn the reproductive systems of wild FHMNs (Orlando et al. 2004). Most significant effects were expected in fish sampled in the Battersea drain return flow canal, particularly in 2006, the year when water quality was lower compared to 2007 (Oldman Watershed Council, 2009). Intense precipitation events facilitate a more direct route of introducing contaminants into surface waters, as materials normally steadfast on the land wash away (Kjaer et al. 2007). In 2006, increased detections of nutrients, fecal coliforms and pesticides indicated reduced water quality. Induction of liver Vtg, a sensitive marker of exposure to estrogens and an indicator of endocrine disruption and gonadal anomalies, particularly in male fish, were expected at sites potentially impacted by endocrine disruptors (Ankley et al. 2001; Tyler et al. 1996). In 2006, both male and female FHMN had increased Vtg expression and higher GSI at the Battersea Drain site compared to the UP2 site. The Vtg response, combined with a statistically significant female skewed sex ratio, were strong indicators of exposure to estrogenic endocrine disruptors in 2006 (Mills and Chichester 2005). Additionally, FHMN AChE levels in 2006 were lower at the BD site, suggesting pesticide exposure

(Sturm et al. 2007; Stenerson 2004). Increased CF and LSI at BD site in 2006 was unexpected but may signify an increased availability of nutrients (associated with overland flow).

In 2007, Vtg, GSI, LSI and CF were generally higher at the UP1 and UP2 sites with a slight decline in values in return flow sites (BD and PD). This pattern of responses may be characteristic of a watershed that is not highly impacted by EDCs. Water quality data were consistent with a decrease of nutrient, fecal coliform and pesticides detections that indicate a water quality improvement in 2007 (Oldman Watershed Council, 2009).

In addition to demonstrating that reproductive endpoints, specifically Vtg induction, gonadal size and morphology in FHMN are sensitive markers of endocrine disruption linked to agricultural activities, this study also investigated the importance of seasonal variation (Ma et al. 2005). A general decline in Vtg, GSI, and CF in fall compared to summer most likely reflects a seasonal difference linked to cessation of the breeding season (Redding and Patino, 1993). Interestingly in 2006, AChE levels increased in the fall, suggesting lower exposure to pesticides (Stenerson 2004) in the fall compared to summer. In contrast, AChE values were essentially unchanged between seasons in 2007, the year when water quality was improved. Though not significant, a male skewed sex ratio was noted in the fall of both years at both sites (UP2, BD), suggesting a post breeding female die off. The presence of parasites in the gonads was an unexpected occurrence and though it did not seem to significantly affect Vtg levels, it should be considered in future projects.

3.5 Conclusions

Adverse reproductive effects were detected in wild FHMNs in years when water quality in irrigation drain canals decreased. Water quality measures, including nutrient load and fecal counts as well as pesticide detections, were indicative of increased overland flow which may also introduce harmful concentrations of EDCs into the aquatic environment. Seasonal sampling provided valuable data regarding the effects of seasons in the physiological, biochemical and morphological responses of fish. A decline in reproductive endpoints in fall compared to summer highlighted the importance for future monitoring programs to incorporate the seasonal fluctuations into experimental design models. This study demonstrated that FHMN, while a robust species, was sensitive to EDCs at environmentally relevant concentrations, as reported in other studies (Kidd et al. 2007). It also provided further evidence that Vtg induction is a practical biomarker for use in the detection of estrogenic contamination. AChE activity is a useful tool for systems where there are a number of possible contaminant sources, including mixtures of pesticides and endocrine disruptors. AChE results also highlighted the importance of factoring season into design. Overall, our study provided evidence that agricultural runoff has the potential to introduce EDCs into irrigation canals during intense precipitation years which can affect the reproductive systems of fish in canal systems.

CHAPTER 4. GENERAL CONCLUSIONS

4.1 Overview

Chapter 1 (background and literature review) outlined how wastewater treatment plant (WWTP) effluent and agricultural runoff contribute endocrine disrupting compounds (EDCs) to the aquatic environment. EDCs interfere with hormonal pathways and can specifically affect the reproductive systems of fish. There is an established focus of research on estrogen-mimicking compounds from WWTPs, with additional attention directed to contribution from agricultural runoff containing pesticides, effluents from confined feeding operations (CFO), and other chemicals used in agriculture.

EDCs are introduced into the rural and urban wastewater systems as endogenous hormones (e.g. from pregnant women) and synthetic hormones (from pharmaceuticals, including estrogen therapy and birth control) excreted from the human body. Industrial effluents (commonly directed to municipal WWTPs) contain industrial chemicals such as surfactants, plasticizers and Bisphenol-A, that have been characterized as EDCs. These chemicals can also leach from the products they are used to make, for example plastic bottles or polyvinylchloride pipe. Product leachate can also be concentrated in landfill seepage.

Agricultural practices may also introduce EDCs to the aquatic environment, as natural hormones (e.g. from pregnant livestock) and synthetic hormones (veterinary pharmaceuticals) excreted from livestock. In addition to excreted hormones, some pesticides may also contribute EDCs to the aquatic environment through overspray, ground seepage, and runoff during precipitation events. Some of the active and non-active (surfactants) ingredients of pesticide formulations are known to have reproductive

effects, however the interactive effects of pesticides and chemicals in WWTP effluents or agricultural runoff are not presently understood.

Chemicals can undergo biotransformation by enzymes within the body, by processes used in a WWTP, or processes in the environment. Biotransformation can terminate the endocrine disrupting effect of a chemical or enhance it. Despite substantial research efforts, the fate of EDCs compounds in the environment is not well monitored or understood.

The effects of EDCs compounds on the reproductive systems of fathead minnow (FHMN) in Southern Alberta have not been determined and there is an urgent need to investigate the point source and non-point source impacts on the status of water quality and reproductive health of aquatic species.

Research Objectives

The objectives of this project were to determine, through the use of physiological, morphological and biochemical markers, if agricultural runoff or WWTP effluents in Southern Alberta have an effect on the reproductive systems of FHMN exposed in the field or in the laboratory, and to characterize seasonal variation of the responses.

Hypothesis

The sampling protocols and the experiments were designed to test the hypothesis that fish exposed to agricultural runoff or WWTP effluent have decreased reproductive fitness characterized by abnormal GSI, LSI and CF, higher liver Vitellogenin (Vtg) mRNA expression, abnormal sex ratios and abnormal gonadal morphology.

4.2 SUMMARY OF FINDINGS

The objectives of the study described in Chapter 2 ("Reproductive effects of wastewater treatment plant effluent in fathead minnow, *Pimephales promelas"*) were to determine if: 1) WWTP effluent from the Southern Alberta cities of Lethbridge and Medicine Hat affect the reproductive systems of wild FHMNs; 2) fish exposed to effluent in the laboratory exhibit effects mirroring those in the field; and 3) pesticides influence the reproductive effects of WWTP effluent.

In 2006, liver Vtg expression in females sampled downstream from the WWTP in Medicine Hat was significantly higher compared to upstream, and a similar trend was detected in male fish. Therefore, results indicate that the WWTP effluent had estrogenic effects in FHMN. In 2007, only two FHMNs were caught at the Medicine Hat downstream site and, despite extensive capture efforts, no explanation for this absence of FHMN at the downstream site can be provided. Our study was designed to test the possibility that pesticides or their surfactants may influence the reproductive effects of WWTP effluents in FHMN. In 2006, the activity of acetylcholinesterase (AChE), a marker for exposure to organophosphate and carbamate pesticides, was significantly lower in the Medicine Hat downstream fish compared to upstream, suggesting that fish were exposed to pesticides downstream from the WWTP.

Around Lethbridge, Vtg expression was higher at the WWTP site compared to upstream in 2006, but not in 2007 when Oldman river flows were lower and WWTP effluent concentrations may have reached high levels. A similar pattern, with lower concentrations of WWTP effluent stimulating Vtg mRNA expression more than high concentrations, was observed in the laboratory study. The inhibition of the activity of

AChE was measured and no differences in AChE activities were observed in fish sampled at the Lethbridge sites in 2006; however, in 2007 AChE was significantly lower at the Lethbridge Upstream site compared to downstream. Input from two golf courses upstream from this site could be causing the inhibition of the AChE activities – however the effects on the reproductive system were not clear. Given the intensity of pesticide use, particularly in agricultural regions such as S. Alberta, pesticides are a factor that should be thoroughly investigated in future studies.

In the laboratory exposure, male and female FHMNs exposed to ethinylestradiol, EE2 (used as a positive control for Vtg induction) and lower concentrations (10- 25%) of WWTP effluent displayed an increase in liver Vtg mRNA expression and a decrease in tubercle number, GSI and percentage of mature oocytes. An increase in gonadal abnormalities was observed in fish exposed to all concentrations of WWTP effluent, as well as EE2. All these responses were indicative of endocrine disruption (Arcand-Hoy et al. 1998; Guillette et al. 1995; Sumpter & Jobling, 1995). However, in both males and females, all endpoints measured did not progress in a dose dependent manner and exposure of fish to higher concentrations of WWTP effluent did not result in a proportional increase in observed reproductive effects. The non linearity of the dose response curve in studies of EDCs has been reported by others (Nichols et al. 2001; Folmar et al. 2000; and Giesy et al. 2000) and is one of the most interesting and significant aspects of this experiment. Based on AChE analyses from the laboratory exposure we inferred that, at the time of exposure, the Lethbridge WWTP effluent did not contain concentrations of pesticides high enough to inhibit AChE activities.

This study provided evidence that effluents from both Lethbridge and Medicine Hat affected the reproductive systems of fish. In the field, effects were more pronounced in 2006 when precipitation was higher and effluent was more diluted compared to 2007, when precipitation was lower and effluent was more concentrated in the rivers. In the laboratory, exposure to WWTP effluent at lower concentrations induced higher Vtg expression. Based on AChE, fish may be exposed to pesticides in the field, but WWTP effluent is likely not a point source. Overall, this study provided evidence that WWTP effluent in Southern Alberta has the potential to introduce EDCs into receiving water and affect the reproductive systems of exposed fish.

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The objectives of the study described in Chapter 3 ("Reproductive endpoints of fathead minnows, *Pimephales promelas*, exposed to agricultural runoff in irrigation canals in Southern Alberta") were to assess the effects of agricultural runoff, and season on the reproductive systems of wild FHMNs in return flow drain canals in Southern Alberta.

In summer 2006, both male and female FHMN showed increased Vtg expression and higher GSI at the drain (BD) site, compared to the upstream (UP2) site. The Vtg responses, combined with a female skewed sex ratio, were strong indicators of exposure to estrogenic endocrine disruptors (Mills and Chichester 2005) at the BD site in 2006. Additionally, FHMN AChE levels in 2006 were lower at the BD site, suggesting pesticide exposure (Sturm et al. 2007; Stenerson 2004). Increased CF and LSI at BD site in 2006 was unexpected, but may signify increased availability of nutrients (associated with overland flow).

In 2007, Vtg, GSI, LSI and CF were generally higher at the UP1 and UP2 sites, with a slight decline in values in return flow sites (BD and PD). This pattern of responses may be characteristic of a watershed that is not highly impacted by EDCs. Water quality data concurred with a decrease of nutrient, fecal coliform and pesticides detections that indicate a water quality improvement in 2007 (Oldman Watershed Council, 2009). There was higher AChE at the BD site, indicating less exposure to AChE inhibiting pesticides in 2007.

Histopathology survey of gonadal anomalies in 2006 detected more anomalies in the fall compared to the summer and in the fall, with more anomalies at the upstream site. It is also interesting to note that in the fall of 2006 the upstream site was also highly parasitized (*Posthodiplostomum minimum*). In 2007 there were no differences between sites for gonadal anomalies or parasitization. In both years, number of anomalies and parasitism was higher in female fish than males. However, parasitism did not affect Vtg levels.

This study also investigated the importance of seasonal variation. A general decline in Vtg, GSI, and CF in fall compared to summer most likely reflected a seasonal difference linked to cessation of the breeding season (Redding and Patino, 1993). In 2006, AChE levels increased in the fall, suggesting lower exposure to pesticides in the fall compared to summer. In contrast, AChE activities were unchanged between seasons in 2007, the year when water quality was improved.

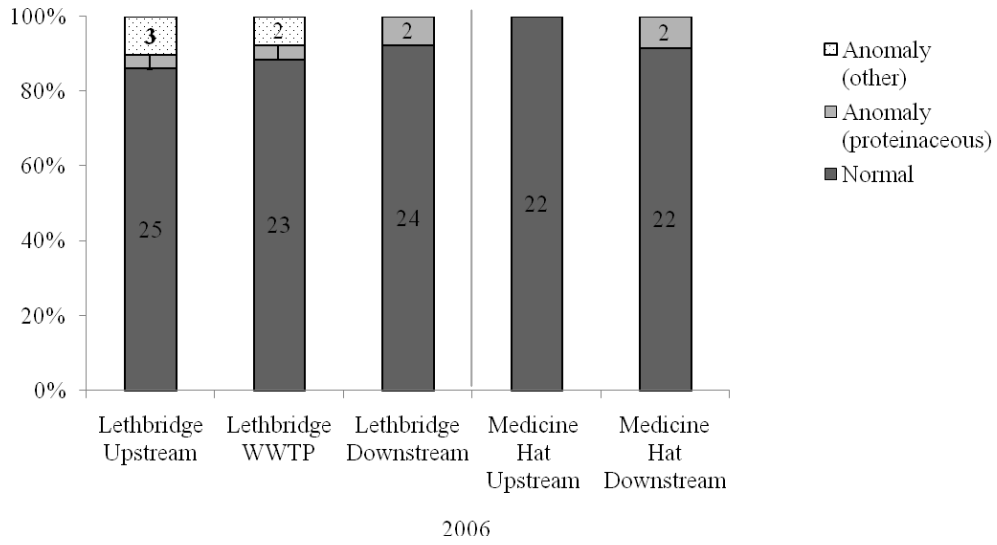
Overall, this study provided evidence that agricultural runoff has the potential to introduce endocrine disrupting compounds and pesticides into irrigation canals during

intense precipitation years and affect the reproductive systems of fish in the canal systems.

This research project demonstrated that FHMN, while a robust species, was sensitive to EDCs at environmentally relevant concentrations, as reported in other studies (Kidd et al. 2007). It also provided further evidence that Vtg induction was a practical biomarker of estrogenic contamination. AChE activity was a useful tool for systems where there are a number of possible contaminant sources, including mixtures of pesticides and endocrine disruptors. The results of AChE also highlighted the importance of factoring season into the experimental design. The presence of parasites in the gonads was an unexpected occurrence and though it did not seem to significantly affect Vtg levels, it may have affected the occurrence of gonadal anomalies and should be considered in future projects.

Appendix

A)



B)

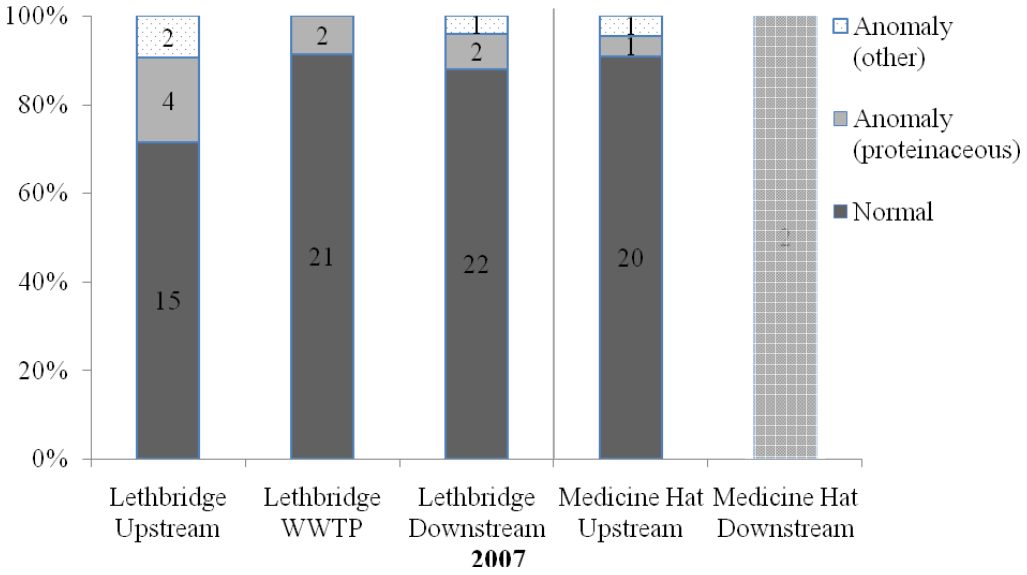


Figure A.1 Histopathology survey of anomalies (percent, males and females grouped) in FHMN sampled from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat, in summer (A) 2006 and (B) 2007 (n = 21-29). White bar indicates sample size was too small to use for statistical analysis.

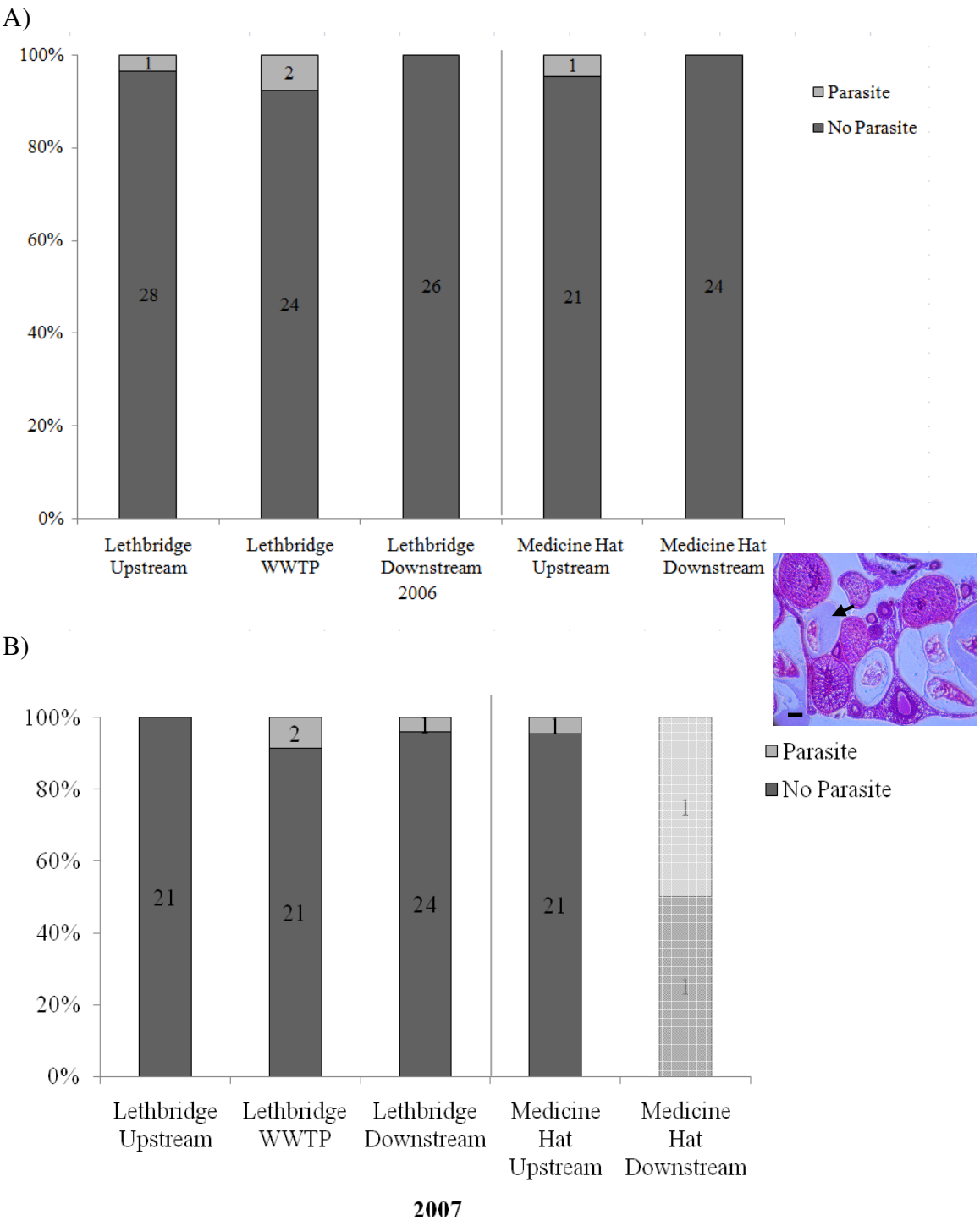


Figure A.2 Histopathology survey of parasitized gonads (percent, males and females grouped) in FHMN sampled from rivers (up and downstream from WWTP) near Lethbridge and Medicine Hat, in summer (A) 2006 and (B) 2007 (n = 21-29). White bar indicates sample size was too small to use for statistical analysis. Inset histology picture of parasites (arrow) within female gonad. Scale bar = 100 µm.

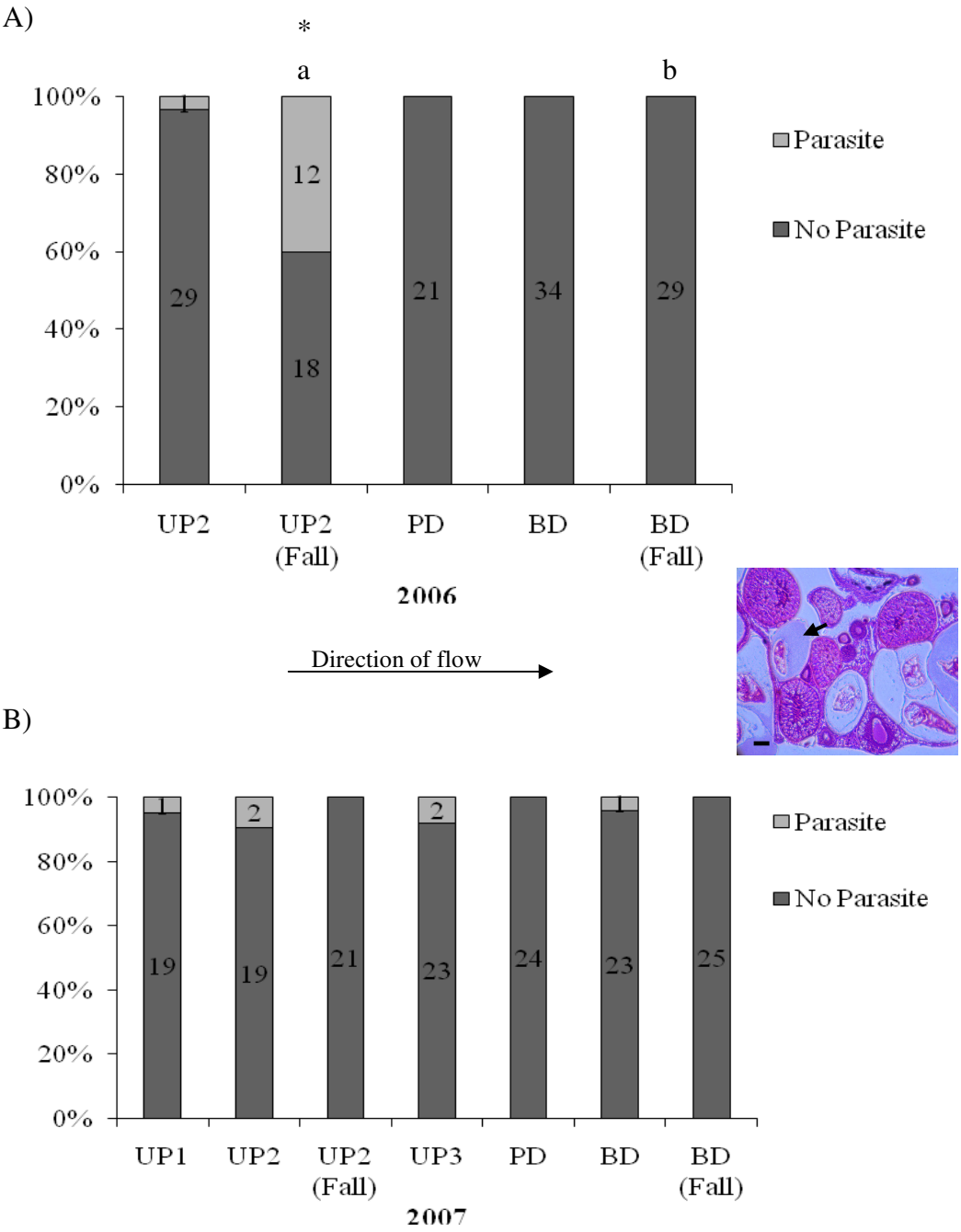


Figure A.3 Histopathology survey of parasitized gonads (percent, males and females grouped) in FHMN sampled from LNID source waters (UP 1, UP 2, UP 3) and return flow drainage canals (PD and BD), in summer and fall (A) 2006 and (B) 2007 (N=20-34). Small letters indicate significant differences of parasitism among groups sampled in the fall. Significant difference between seasons indicated with *. Inset histology picture of parasites (arrow) within female gonad. Scale bar = 100 µm.

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