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ISBN: 978-0-494-75377-4
Our file Notre référence
ISBN: 978-0-494-75377-4

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Canada

Improving understanding of endocrine-active compounds in pulp and paper mill condensates using a mummichog (*Fundulus heteroclitus*) bioassay

by

Robert John Gary Rutherford

B. Sc, University of Guelph, 2007

THESIS

Submitted to the Department of Biology

Faculty of Science

in partial fulfilment of the requirements for the

Master of Science in Integrative Biology

Wilfrid Laurier University

2011

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Abstract

Since the late 1990s an investigation of cause (IOC) study at Irving Pulp and Paper Ltd (IPP), a bleached kraft mill in Saint John, NB, Canada, has successfully identified an in-mill waste stream (5th effect evaporator condensates) as containing endocrine disrupting substances (EDSs). These EDSs affect levels of reproductive steroids in a native estuarine fish, the mummichog (*Fundulus heteroclitus*). To confirm the minimal concentration at which whole condensates impact reproductive endpoints, adult mummichog were exposed to 2% and 4% (v/v) whole condensates for 14 days. Plasma testosterone was significantly depressed in males in the 4% (v/v) treatment, confirming previous work done with IPP condensates. Also, significant induction of hepatic cytochrome P450 genes 1 and 3 were found in female fish exposed to both condensate concentrations. To chemically isolate suspect EDSs, a solid phase extraction protocol was developed by collaborating researchers. The protocol generates four chemically-distinct fractions, along with residual condensates. Fractionation was completed using 4% (v/v) whole condensates and the resultant fractions, and 4% (v/v) whole condensates, were exposed to adult mummichog in the reproductive endocrine bioassay. There was no significant response in fish exposed to 4% (v/v) whole condensates, making interpretation of specific fraction treatments difficult. While fish were in different reproductive periods in the two bioassays, which may account for some of the differences between the experiments, a more compelling explanation is the variability in the chemical make-up of the condensates. Chemical fingerprinting of both condensate batches by collaborating researchers found a higher androgenic potency in the fractionation condensates. Mummichog were then exposed to model androgens methyltestosterone (100 and 1000 ng/L; MT) and dihydrotestosterone (10 and 100 μg/L; DHT) to determine if androgenic responses paralleled condensate responses. Plasma testosterone was

significantly depressed by 10 μg/L DHT in males. Females had significant depression of plasma estradiol when exposed to both DHT concentrations and 1000 ng/L MT. Female mummichog had lower thresholds of effects than males and were more responsive to androgen exposure. Comparison of effects seen in fish exposed to pulp mill condensates and those exposed to model androgens indicate androgens in the condensates may not be the source of reproductive endocrine effects in the condensate-exposed mummichog.

Acknowledgements

First I must extend my thanks and love to my family and friends for their encouragement and guidance throughout my entire school career. Thanks especially to my mother, Gail, and my father, Gary, for teaching me from a young age that learning can be fun, and science is always cool.

My university support group, Esteban Gillio Meina, Allison Van Slack, Ibrahim Chehade, everyone else in the MacLatchy lab and especially Andrea Lister. Sharing problems with you guys always led to solutions, and I am appreciative of that. For all the guidance and tactical meetings that Andrea had with me, she deserves extra special mention. Without you I have no doubts I would still be standing in the fish lab trying to set up my first exposure! Thanks also to Dr. Glen Van Der Kraak and his technician Jacquie Matsumoto for providing me with lab space and expertise as I analyzed gene expression endpoints. My times at Guelph were always informative and fun.

Thanks are also due to my chemistry counterpart Phillip Scott and his colleagues Craig Milestone and Dr. Mark Hewitt. Without your tireless work everything we biologists did would not have been possible. Also thanks to all personnel at Irving Pulp and Paper Ltd, without whom this thesis would not have been possible. Thanks especially to Heather Munn and Jasna Krstic for answering a multitude of emails on a variety of subjects, and ensuring prompt sampling and shipping of the condensates. Also to my committee members Jim McGeer and Scott Smith, thank you for your support and imparted knowledge over the course of my thesis. Your feedback was integral to the success of my thesis.

To my supervisor Deborah MacLatchy, thank you is inadequate. Your constant support, ideas and assistance were invaluable to me and I have gained immeasurable knowledge and life experiences from working with you. No matter how hectic your schedule was, I always knew that you were just an email away with help and guidance. Of course I must mention your master bleeding skills which always made my sampling days run that much smoother.

Finally I must especially thank my partner, Mark Najder, for enduring over two years of endless conversation about mummichog and lab practices. Your support and sacrifices have not gone unnoticed. Even when I was feeling I was "the worst scientist in the world", I knew that you were in my corner.

Funding was provided by the National Sciences and Engineering Research Council (NSERC) of Canada and Irving Pulp and Paper Ltd.

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Chapter 1: General introduction

1.1 Effects of pulp mill effluents on fish reproductive status

Canada is the world's premier exporter of wood pulp and manufacturer of newsprint (Natural Resources Canada, 2002). Over 250 pulp and paper mills are located in Canada, producing 17 million tonnes of paper and 23 million tonnes of pulp annually (Langlois *et al.*, 1997; Munkittrick, 2004). Production of pulp and paper has been a major Canadian economic activity for over 100 years, with discharge of industrial effluents into aquatic receiving environments as a major by-product of this industry for the same period. Fish exposed to final effluents experience multiple adverse reproductive effects including increased age to sexual maturity (Kovacs *et al.*, 1995; McMaster *et al.*, 2006), reduced gonad size (Leblanc *et al.*, 1997; Janz *et al.*, 2001), skewed sex ratio (Örn *et al.*, 2006), altered secondary sexual characteristics (Larson and Forlin, 2002; Parrott *et al.*, 2004), reduced egg production (Borton *et al.*, 2000; Rickwood *et al.*, 2006), and depressed reproductive steroid levels (Dubé and MacLatchy, 2000; van den Heuvel and Ellis 2002; Sepulveda *et al.*, 2003). Effluent toxicity was not regulated in Canada prior to 1971, resulting in discharge of untreated waste effluent directly into the environment. Environments downstream of effluent discharge suffered extensive habitat degradation and fish populations were subjected to massive die-offs (McLeay, 1987). The Canadian Pulp and Paper Effluent Regulation of the Fisheries Act was introduced in 1971 (Sinclair, 1990), requiring all new mills to monitor and limit a number of final effluent factors including biochemical oxygen demand (BOD), total suspended solids (TSS) and acute lethality (McLeay, 1987). These regulations helped improve final effluent quality and lessen impacts in receiving environments, however, this Act covered less than 10% of all Canadian mills, resulting in a majority of mills still discharging untreated effluent into Canadian receiving waters.

Studies done in Sweden in the late 1980's found significant impacts on body size, rate of growth, metabolic disorders, suppressed immune function and physiological deformities in wild fish exposed to bleached kraft pulp mill (BKPM) effluent (Andersson *et al.,* 1988). Additionally, ethoxyresorufin-O-deethylase enzyme (EROD) was induced (Andersson *et al.,* 1988). EROD, measured as hepatic induced mixed function oxygenase (MFO) enzyme activity, is an indication of exposure to a wide array of organic contaminants and is an extremely sensitive indicator of contaminant uptake in fish (Munkittrick *et al.,* 1994). Effects were seen in fish 10 km downstream of mill discharge and were hypothesized to occur due to impairment of hormonal control, especially during sexual maturation (Andersson *et al.,* 1988).

Similar Canadian studies investigated effects on fish of mill discharge downstream of a BKPM in Jackfish Bay, ON in the early 1990's (McMaster *et al.,* 1991; Munkittrick *et al.,* 1991; 1998). Observed effects paralleled those found in the Swedish studies and included increased age to sexual maturity, alteration of secondary sexual characteristics, significant MFO induction and depressions of reproductive hormones (McMaster *et al.,* 1991, Servos *et al.,* 1996). Based on these studies, typical effects of many pulp mill effluents were confirmed, including smaller egg sizes, decreased gonad size, increased age to maturation and depressed levels of reproductive hormones (Munkittrick *et al.,* 1998). These contaminants, collectively termed endocrine disrupting substances (EDSs), elicit effects via endocrine-mediated pathways. Specific EDSs in PME have not been identified due to the complex nature of PME (Hewitt *et al.,* 2002) and the varied responses of fish species exposed to PME (Munkittrick, 2004). Identification of mechanisms of interaction of the causative compounds is difficult due to this variation (Van Der Kraak *et al.,* 1998). Mechanistic bioassays have been established to identify endocrine-active compounds in PME and methods of interaction(s) of EDSs with the hypothalamo-pituitary-

gonad (HPG) axis in fish (MacLatchy *et al.,* 2005). Chemical fractionation and characterization techniques have also been developed to identify causative compounds (Hewitt *et al.,* 2002; Belknap *et al.,* 2006).

Estrogenic effects of PME have been observed in multiple studies, however, the consistency of these findings has been irregular, even within a single effluent (van den Heuvel and Ellis, 2002). Evidence of estrogenic effects in PME include the induction of vitellogenin in male fish (Hornug *et al.,* 2003), a yolk-precursor protein normally expressed only in female fish. Estrogenic responses, including vitellogenin induction, have been observed in rainbow trout (*Oncorhynchus mykiss*) caged downstream of a bleached kraft mill in Chile (Orrego *et al.* 2005; 2006). Specific flavonoids identified within PME are estrogenic (Belknap *et al.,* 2006) and may result in vitellogenin induction. Further work with Chilean bleached kraft mill effluent found that rainbow trout injected with effluent extract had elevated plasma testosterone and 17β-estradiol, as well as significant aromatase induction (Orrego *et al.,* 2010). These estrogenic effects could be related to specific compounds within the effluent acting as estrogen receptor agonists (Orrego *et al.,* 2010). Studies at North American pulp and paper mills have found fewer estrogenic responses in exposed fish. A study done in North Carolina exposed bluegill (*Lepomis macrochirus*) to effluent from a bleached kraft mill for eight months and found no estrogenic responses (Cheek *et al.,* 2004). Similarly, largemouth bass (*Micropterus salmoides*) exposed to bleached kraft mill effluent in Florida had no estrogenic responses (Sepulveda *et al.,* 2003). However, estrogenic responses have been observed at some North American mills. Canadian mills of several process types induced vitellogenin in rainbow trout hepatocytes after exposure to effluent extracts (Marlatt *et al.,* 2006). Currently, more consistent North American findings have been linked to androgenic compounds within PME.

Androgenic effects in fish exposed to PME have been reported since the mid-1980's (Denton *et al.,* 1985). Evidence for androgenic disruption include male-skewed sex ratios (Forlin *et al.,* 2004), masculinisation of female fish (Orlando *et al.,* 2007), down-regulation of aromatase activity (Li *et al.,* 2006) and increased plasma testosterone levels (Palowski *et al.,* 2004). A recent study on Swedish PME found that 37 identified compounds had the ability to bind with ovarian androgen receptors and cause androgenic effects in Atlantic croaker (*Micropogonias undulatus*; Larsson *et al.,* 2006). Progesterone, androstenedione and androstadienedione have been identified in water and sediment downstream of a BKPM (Carson *et al.,* 2008). These potent androgens are precursors to testosterone in the steroidogenic pathway and may be present in PME due to bacterial degradation of plant sterols (Carson *et al.,* 2008).

An amendment to the 1971 Canadian Pulp and Paper Effluent Regulations was made in 1992, making the regulations applicable to all Canadian mills. The industry made significant changes in mill operation to eliminate use of molecular chlorine, a suspected inducer of reproductive problems in fish; this resulted in the elimination of furans and polychlorinated dioxins from mill effluent (Hewitt *et al*., 2008). Concurrently, secondary effluent treatment systems were installed at most mills, in which microorganisms detoxify and degrade organic material through cellular respiration (Smook, 1994). This resulted in a reduction in BOD, chemical oxygen demand (COD), TSS, concentration of chlorinated organic compounds, acute lethality of effluent on fish and an overall improvement of effluent quality (Stromberg *et al.,* 1996; Rickwood *et al.,* 2006). The bleaching process was originally suspected as the source of EDSs in PME, however, despite implementation of new treatment technologies, there are continued reports of effects on fish reproduction downstream of Canadian mills (Hewitt *et al.,* 2008). This implies that EDSs are not linked to the bleaching process and are not eliminated

through secondary effluent treatment systems. The effects observed after the installation of effluent treatment systems indicated that current regulations do not completely protect aquatic receiving environments (Courtenay *et al.,* 2002) and a national monitoring program was needed to aid in the long-term goal of improving effluent quality via science-based policy.

1.2 Environmental effects monitoring program

Within the 1992 amendments of the Canadian Pulp and Paper Effluent Regulation, the environmental effects monitoring (EEM) program was enacted. This program requires all mills to monitor receiving water quality, total effluent toxicity and fish and benthos populations on a cyclical basis (Munkittrick, 2004). EEM was the first regulatory program in Canada requiring field biomonitoring on a national scale (Walker *et al.,* 2002). The EEM program aims to determine if regulations implemented in 1992 were sufficient to protect Canadian aquatic environments. Integration of analyses from different regions of Canada requires standardization of methodologies and sampling techniques and the EEM program provides these with respect to long-term monitoring of aquatic community structures (Munkittrick, 2004).

In early 1996, the first EEM cycle was completed, with over 115 individual studies incorporated (Munkittrick, 2004). A review of these results led to specific recommendations which allowed for greater site flexibility and specificity during the second cycle (Environment Canada, 1997). Pulp mills located on estuarine receiving environments were found to be lacking in adequate surveys, due to insufficient numbers of fish caught and problems determining the degree to which fish were exposed to effluent in these locations (Courtenay *et al.,* 2002).

Recommendations for Cycle 2 suggested one possibility was the use of a sedentary, small-bodied fish in on-site mesocosm studies to alleviate these concerns (Courtenay *et al.,* 2002).

Analysis of results collected during following cycles indicate that effects on fish habitats range from eutrophication to food limitation; with decreases in gonad size and increases in condition factor, liver size, and size-at-age (all signs of metabolic disruption) being the consistent overall national trend in exposed fish (Munkittrick *et al.,* 2002). Currently in its sixth cycle, which began in 2010, the EEM has shown a national pattern of eutrophication responses in fish concomitant with metabolic or endocrine disruption. This natural response pattern of Canadian pulp mills on aquatic receiving environments has reinforced the need for investigation of cause (IOC) research to identify the sources of these effects and to support process changes that will eliminate causative compounds from release.

1.3 Reproductive controls in fish

The hypothalamo-pituitary-gonadal (HPG) axis has evolved in fish to maintain reproductive capacities (McMaster *et al.,* 1995). Integration of internal and external cues trigger mechanisms within the HPG axis to maximize reproductive capacity during times of variable environmental conditions (McMaster *et al.,* 1995). EDSs are exogenous substances or mixtures that alter the function(s) of the endocrine system through HPG interaction and can cause adverse effects in an organism, its progeny or subpopulations (Vos *et al.,* 2000). Exposure of sexually-mature adults to EDSs can result in reduced reproductive capacity and lower population recruitment (McMaster *et al.,* 1995). EDSs may affect an organism by mimicking or blocking endogenous hormones, disrupting the natural pathway of hormone synthesis, altering the

expression of hormone receptors, or antagonizing naturally-synthesized hormones (Sonnenschein and Soto, 1998).

Integration of external and internal cues to trigger responses in the HPG axis is a multiple- step process which begins in the brain of fish. Receptors in the brain interpret environmental cues and stimulate the release of gonadotropin releasing hormone (GnRH) from the hypothalamus (Lethimonier *et al.,* 2004). The pituitary gland is the target of GnRH, which stimulates the production and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH; Ohkubo *et al.,* 2010). These two gonadotropins interact with the gonadal tissue to stimulate growth or steroid production (Mateos *et al.,* 2002). Initiation of gametogenesis and steroidogenesis is controlled by FSH, while LH aids in the maturation of gametes (Mateos *et al.,* 2002). The steroidogenic pathway within fish gonadal cells is a multi-step enzymatic path with the synthesis of testosterone, the dominant sex steroid, as a final product (Nagahama, 1994). Cholesterol is the precursor to testosterone and is mobilized into the mitochondrion via steroidogenic acute regulatory protein (StAR) and hydroxycholesterol analogues (Kim *et al.,* 1997). Conversion of cholesterol to testosterone involves intermediates such as pregnenolone, progesterone and androstenedione, facilitated by the enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase (P450c17) and C17,20-lyase (P450c17) respectively (Figure 1.1). Testosterone is a precursor for 17β-estradiol (via aromatase) in both male and female fish, and for 11-ketotestosterone (via 11β-hydroxysteroid dehydrogenase) in males (Figure 1.1). 11-Ketotestosterone controls expression of secondary sexual characteristics, spawning behaviour and sperm cell maturation. Production of vitellogenin in the liver of female fish is controlled by 17β-estradiol binding to cellular receptors (Ankley *et al.,* 2001). Feedback loops, both positive and negative, within the HPG axis maintain homeostatic conditions for hormone production

levels by: 1) limiting of synthesis and release of sex steroids; 2) timing of gamete production; 3) control of gonad growth and maintenance; and 4) environmental cues triggering spawning behaviour (Kumar and Trant, 2001; Mateos *et al.,* 2002; Yaron *et al.,* 2003).

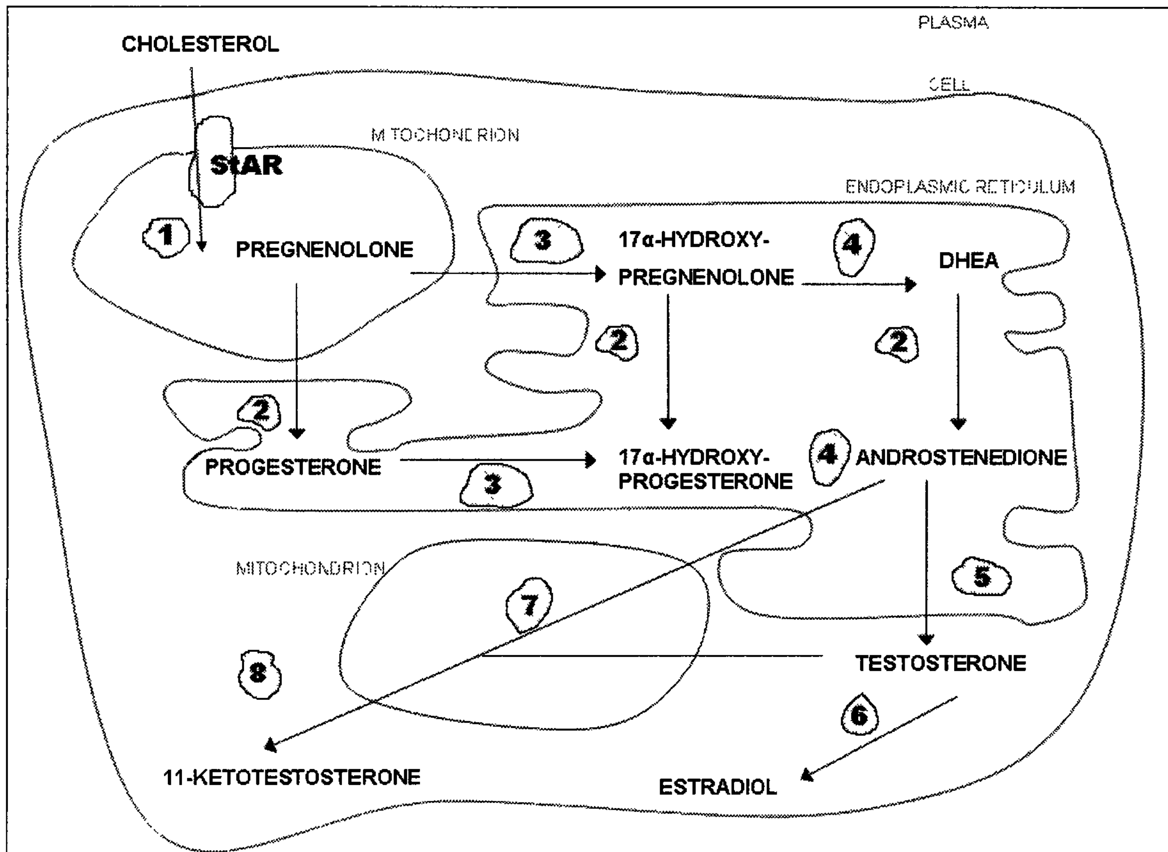

Figure 1.1 The steroidogenic pathway within gonadal cells (modified from Nagahama, 1994; Leusch and MacLatchy, 2003). StAR = steroidogenic acute regulatory protein; 1 = cytochrome P450 side-chain cleavage; 2 = 3β-hydroxysteroid dehydrogenase; 3 = 17α-hydroxylase; 4 = C17,20-lyase (P450c17); 5 = 17β-hydroxysteroid dehydrogenase; 6 = cytochrome P450 aromatase; 7/8 = 11β-hydroxysteroid dehydrogenase.

1.4 PME effects on fish reproduction

Interference with the HPG axis from effluent originating at BKPMs has induced reproductive effects in fish (Van Der Kraak *et al.,* 1992). Lower levels of LH have been found in white sucker (*Catostomus commersonii*) living downstream of a BKPM in Jackfish Bay, ON (Van Der Kraak *et al.,* 1992). This indicates altered function of the pituitary, and exposed fish also had reduced levels of testosterone production compared to control fish (McMaster *et al.,* 1995). The steroidogenic pathway is influenced downstream of cholesterol, and the reduced production of steroid hormones was attributed to the inhibition of specific enzymes responsible for the conversion of intermediates to testosterone, or lower availability of endogenous cholesterol substrate (McMaster *et al.,* 1995). Recent work on the impact of effluents on steroidogenic activity has shown (via alteration of aromatase expression) that aromatase is affected in fish exposed to PME (Orrego *et al.,* 2010).

In order to test effects of pulp mill effluents on a broad scale, standardized fish bioassays were developed using the fathead minnow (*Pimephalus promelas*; Parrot and Wood, 2002). This model species is used to test pulp mill effluent discharge into freshwater environments, however, the EEM program also called for use of a species of fish inhabiting estuarine environments to address the difficulties encountered during the first cycle in determining effects of pulp mills discharging into coastal receiving environments (Courtenay *et al.,* 2002). The mummichog (*Fundulus heteroclitus*) is an Atlantic coast species inhabiting estuarine environments, and a validated endocrine fish bioassay was developed for this species in 2003 (MacLatchy *et al.,* 2003; 2005). This bioassay provides the foundation for work in this thesis.

1.5 Mummichog reproduction

The mummichog (*Fundulus heteroclitus*) is a species of killifish inhabiting the coastal estuaries of eastern North America. It is abundant in salt marshes, and readily available for laboratory study. Mummichog physiology, reproductive cycles and embryological development are well studied (Hsiao *et al.,* 1994; Burnett *et al.,* 2007). Their natural range, size and adaptability make laboratory holding and manipulation possible (MacLatchy *et al.,* 2003). Mummichog have been shown to be sensitive to hormonally-active substances (MacLatchy *et al.,* 2003; Sharpe *et al.,* 2004; Peters *et al.,* 2007) making this species a good candidate to study EDSs (MacLatchy *et al.,* 2003). This species has been used in many artificial stream (Dubé and MacLatchy, 2000), field (Courtenay *et al.,* 2002) and laboratory (Shaughnessy *et al.,* 2007; Melvin *et al.,* 2009) studies aimed at determining effects of pulp and paper mill effluent on fish reproductive endocrine status. This species has been suggested as a model estuarine species for researching EDSs (MacLatchy *et al.,* 2005).

The mummichog is a hardy fish, tolerant of a broad range of temperatures (-1.5°C to 36°C), dissolved oxygen concentrations and salinities (0.0 ppt – 120 ppt; Scott and Scott, 1988). Environmental and endogenous cues regulate spawning events, which can number from one to eight times per year, depending on environmental conditions (Taylor, 1986; McMullin *et al.,* 2009). Two distinct subspecies of mummichog, the southern *Fundulus heteroclitus heteroclitus* and the northern *Fundulus heteroclitus macrolepidotus*, have variable spawning events (McMullin *et al.,* 2009). The northern subspecies, used in this thesis, typically experience a single spawning per year, triggered by water temperature and photoperiod (McMullin *et al.,* 2009). Southern mummichog are repeat spawners and usually begin to breed in March (early spring), and end in September (early autumn; Kneib, 1986).

In certain populations lunar cycling patterns are observed (Hsiao *et al.,* 1994), but in other populations some females are continually gravid for the duration of the reproductive season, lacking lunar controls (Taylor, 1986). Lunar control of spawning in the southern subspecies is stronger than in northern counterparts (McMullin *et al.,* 2009). Laboratory-held mummichog have been shown to be daily spawners with a weak lunar pattern (Bosker *et al.,* 2009). A lunar pattern of cycling has not been observed in the northern subspecies, and researchers believe that photoperiod and water temperature are responsible for synchronizing reproductive cycles in these populations (Shimizu, 1997; McMullin *et al.,* 2009). Laboratory control of reproductive cycling is possible through the manipulation of photoperiod and water temperature.

1.6 Irving Pulp and Paper Ltd. processing and condensate generation

The three main components of wood are cellulose, hemicellulose and lignin. These chemical structures are present in both softwood (derived from gymnosperms) and hardwood (derived from angiosperms) trees. Other components of wood include resin, fatty acids, phenols, plant sterols and terpenes, all of which are present in small quantities and are extracted during the pulping process (Smook, 1994; Biermann, 1996). The overall goal of the pulping process is to convert wood chips into a fibrous solution termed pulp. The successful separation of cellulose and hemicellulose fibers from all other wood components, with a minimum of fiber loss, is the primary objective of the pulping process (LaFleur, 1996). To achieve this in the kraft process, lignin is dissolved by a chemical mixture of sodium sulphide (Na_2S) and hot caustic soda ($NaOH$) called white liquor (Biermann, 1996). Wood chips are placed in a digester and heated at temperatures between 160°C and 180°C and pressure of 90-100 psi. White liquor is then added

to dissolve lignin and some hemicellulose, leaving the less soluble cellulose behind. Digested pulp (termed brownstock) remaining in the digester is then washed and screened to remove residual chemical compounds used in lignin dissolution. These washed chemicals become weak black liquor (WBL) which consists mainly of cooking chemicals, residual lignin and wood extractives (Biermann, 1996). Oxygen delignification and/or chlorine dioxide (ClO_2) bleaching then remove residual lignin from the brownstock. This kraft process is the most commonly used method for pulping in Canada.

WBL is then subjected to a recovery phase to recoup up to 99.5% of the spent cooking chemicals, as these are of economic value. A series of six multiple effect evaporators (MEE) are used at the Irving Pulp and Paper Ltd. (IPP) mill in New Brunswick, Canada, to heat WBL and evaporate water, producing strong black liquor. Original WBL consists of ~15-20% solids while strong black liquor is ~50-70% solids. Strong black liquor is then sent to a recovery boiler for further chemical recovery (McLeay, 1987). The six MEEs use a counter-current steam flow to carry vaporized components in sequence. Each MEE operates at a different temperature, increasing as the evaporation progresses. Vapours produced in each evaporator consist mainly of volatile and semi-volatile organic black liquor compounds, and are used to heat the liquor in the next MEE. The vapour condenses as it heats up the liquor in the next step as it loses energy to exert this effect. The condensates differ among the evaporators due to the varying temperatures of evaporation; however, vapours generally consist of low molecular weight volatile compounds such as terpenes, ketones, aldehydes, dissolved gases, phenolics and alcohols (mainly methanol; Blackwell *et al.,* 1979; LaFleur, 1996).

To reduce boiling points of chemicals during recovery, vacuums are used at the 3rd, 4th, 5th and 6th evaporators. For example, the 5th multiple effect evaporator has an inlet temperature of

90°C and an outlet temperature of 78°C. Chemicals with a boiling point of 90°C are therefore present in the vapour of the 5th MEE, however, chemicals with a higher boiling point are also present due to the use of a vacuum. Therefore the temperature of each MEE is only an estimate of the boiling points of the compounds included in their respective condensate. Vapour from the 6th MEE is condensed and sent to a foul condensate container, where it is steam stripped and then sent to the mixed chemical sewer.

Prior to 1997, condensates from the 5th and 6th MEEs were recycled within the mill for use as wash water. This permitted chemical mixing between the condensates and the bleaching chemicals, influencing final effluent quality. High BOD and COD were identified within 5th effect condensates and the bleaching plant, leading to implementation of a within-mill condensate waste treatment system (Dubé and MacLatchy, 2000). To meet EEM regulations established in 1992, IPP enacted a pollution prevention policy with the installation of in-plant technology, without the use of secondary effluent treatment, to meet environmental regulations on effluent quality. A reverse-osmosis (RO) system was installed in 1998 for treating 5th effect evaporator condensates prior to reuse within the mill as wash water (Dubé and MacLatchy, 2000).

1.7 Reverse osmosis system

Typically used to treat and purify water, the reverse osmosis system removes small particles (≤ 0.001 µm) and dissolved salts using an osmotic gradient. Pressure is placed on the solute side of a semi-permeable membrane, with the solvent flowing on the opposite side. IPP is the only mill in the world to employ this treatment system on its condensates. Currently, the 5th

effect evaporator (RO feed) condensates are subjected to pressure and forced through the semi-permeable membrane to remove contaminants at a rate of 4164 L/min. Two distinct waste streams are generated from this technique: the RO permeate (~99% of flow) is clean condensates which passed through the membrane; and the RO concentrate (~1% of the flow) which is composed of chemicals that did not cross the membrane. The RO permeate is used as wash water within the mill before ultimately being discharged into the mixed chemical sewer, where it combines with the other in-mill waste streams. The RO concentrate is incinerated in the bark boiler (Dubé and MacLatchy, 2000). The installation of the RO treatment system has eliminated the need for a secondary effluent treatment system on the full final effluent waste stream because it reduces COD, total carbon and BOD to meet effluent quality regulations (Dubé *et al.*, 2000). Installation of the RO system gave researchers the ability to study effluent impacts on fish populations before and after installation, which had never been investigated before (Dubé and MacLatchy, 2000). Effects on fish reproduction were no longer apparent in effluent treated by RO, indicating that EDSs may be eliminated through this wastestream treatment system (Dubé and MacLatchy, 2000).

1.8 Investigation of cause (IOC) at IPP

Since the late 1990's investigation of cause (IOC) studies at IPP have been implemented as part of the EEM program. The goals of IOC are focused on identifying where within the mill process potential EDSs originate (Hewitt *et al.*, 2002), identifying the suspected EDSs and developing appropriate process changes to eliminate the sources of EDSs (MacLatchy *et al.*, 2010).

Assessment of in-mill waste streams for potential EDSs was first accomplished at IPP using a mesocosm (artificial stream) study in which mummichog were exposed to environmentally-relevant concentrations (0.5%-5% v/v) of multiple in-mill waste streams prior to RO installation (Dubé and MacLatchy, 2001). Females exposed to the condensate waste stream had depressed *in vitro* 17β-estradiol production and increased liver size, indicating that this waste stream was responsible for nonlethal mummichog responses (Dubé and MacLatchy, 2001). At 1% (v/v) final effluent concentration both male and female mummichog had significantly depressed plasma testosterone levels.

After RO installation in 1998, 1% (v/v) combined effluent no longer caused depression of plasma testosterone in mummichog (Dubé and MacLatchy, 2000), indicating that RO treatment had improved final effluent quality. EDSs were still present in the effluent, as mummichog exposed to 5% (v/v) concentration of combined effluent still exhibited decreased plasma testosterone levels. This indicates that the RO treatment does not completely remove EDSs from the final effluent (Dubé and MacLatchy, 2000). The results of this study confirm that the 5th effect chemical recovery condensates (RO feed) is a source of EDSs at IPP. These were the first studies to successfully identify an EDS source within a mill capable of causing endocrine disruption in fish.

Isolation and identification of specific EDSs within PME is hindered due to high concentrations of lignin, which make studying small biologically-active molecules difficult (Hewitt *et al.*, 2008). Condensates are less chemically complex than PME and do not contain lignin, making them easier to chemically isolate. Efforts to analyze biological and chemical effects of condensates has been the primary focus of the IOC since 2000. MacLatchy *et al.*

(2001) confirmed bioactivity of whole condensates and RO concentrate stream, but not the RO permeate stream.

A two-stage solid phase extraction (SPE) method was developed to isolate compounds from 5th effect chemical recovery condensates into chemically-distinct fractions (Hewitt *et al.,* 2002). A seven-day adult mummichog endocrine bioassay tested the ability of these distinct fractions to impact steroid hormones. Treatments were whole condensates, extracts from suspended solids (> 1 μm), SPE-1 methanol, SPE-1 ethyl acetate (both from the first SPE, styrene divinylbenzene), SPE-2 extract (from second SPE, reversible graphitized carbon), and residual condensates. Fish exposed to suspended solids, SPE-1 methanol, SPE-2 extract had depressed plasma testosterone levels. Fish exposed to residual condensates had no significant changes compared to control, indicating that all bioactive compounds were recovered during SPE (Hewitt *et al.,* 2002).

To isolate and identify the EDSs responsible for depression of plasma testosterone, a reverse-phase high pressure liquid chromatography (HPLC) process was developed to fractionate the most potent fraction (SPE-2). A seven-day adult mummichog exposure was conducted with six HPLC fractions of SPE-2, at 1% (v/v) condensate equivalents. The unfractionated SPE-2 (positive control; whole condensates) response in mummichog was not as pronounced as the response seen in earlier bioassays. To test consistency of responses, the bioassay was repeated with 1.5% (v/v) condensate equivalents. Results proved inconsistent with previous studies as well (Shaughnessy *et al.,* 2007).

To confirm that the SPE-2 fraction did contain EDSs, mummichog were exposed to a range of SPE-2 concentrations (0.5 %, 1%, 2% and 4%). Significant plasma testosterone

depression in males exposed to 4% (v/v) condensates was observed, but no response in females was found. The male findings confirmed that EDSs were present in the SPE-2 fraction. A new bioassay was conducted at 4% (v/v) condensate equivalents of HPLC SPE-2 fractions. There was no depression of plasma testosterone in any fraction compared to control. In two fractions there was an increase in plasma testosterone. Mummichog response to positive control (4% (v/v) whole condensates) returned a significant decrease in plasma testosterone in males, indicating loss of activity during the fractionation protocol (Shaughnessy *et al.,* 2007).

Using a set of predetermined criteria based on previous experiments where RO feed and RO concentrate depressed plasma steroids, candidate compounds associated with plasma testosterone depressions were identified (Dube and MacLatchy, 2001; MacLatchy *et al.,* 2001; Belknap *et al.,* 2006; MacLatchy *et al.,* 2010). A total of 39 compounds associated with hormone activity were identified in the SPE extracts (Belknap *et al.,* 2006), six of which were identified as potential EDSs. Of the six potential EDSs, three chemical compounds (hydroxylated diterpenoids, sesquiterpenois, and a lignin-derived stilbene) were hypothesized to be associated with plasma testosterone depression.

To ascertain why SPE-2 exhibited endocrine-disrupting abilities but the HPLC fractions did not, examination of the fractionation protocol was undertaken. EDSs were lost during evaporation of the mobile phase (a mixture of acetontitrile and water) in preparation of solvent exchange. Most of the known compounds in condensates are partially volatized in conditions similar to those required to evaporate water. Also, samples dried using nitrogen showed decreased levels of phenolics and diterpenes (such as manool; MacLatchy *et al.,* 2010). This work was carried out by Dr. Craig Milestone.

To eliminate the evaporation of the mobile phase and subsequent loss of compounds, normal phase HPLC replaced reverse phase HPLC. This allowed a non-aqueous method for the fractionation of SPE-2, but the results were unsuccessful. Elution of the graphitized carbon SPE-2 cartridge with solvents of decreasing polarity resulted in a washing effect, still not resolving the loss of bioactivity. That the graphitized carbon had the ability to irreversibly bind organic compounds (Hennion, 2000) also affected results. Focus was then shifted to the SPE-1 cartridge, with experimentation revealing many compounds being left on this cartridge and thus not included in the mummichog *in vivo* testing. With this information, a new fractionation protocol was developed to eliminate residual compounds on the cartridges (C. Milestone in Scott, 2010).

Reduced recoveries of condensate extracts were addressed with the replacement of the Biotage ENV+ cartridge, used in the original SPE protocol, with the Waters Oasis HLB cartridge. This new cartridge contains a mixed mode resin to encompass both hydrophilic and lipophilic extraction capabilities, and is tolerant of a broader range of pHs and potential solvents compared to the original Biotage ENV+ cartridge. These properties generate higher recovery of compounds present in IPP condensates. Due to this increased extraction capability, the breakthrough volume for the current protocol is now less than half of the volume required for the previous protocol using the Biotage ENV+ cartridge. Accordingly, the loading and eluting rates for this new cartridge have been slowed to maximize the Waters Oasis HLB extraction properties. Another modification from the previous protocol was the eluting solvents. Originally, the presence of specific chemical compounds (such as geranyl linalool) in all fractions prevented the generation of chemically-distinct fractions. Four solvents of differing polarity (dichloromethane (DCM), hexane, methanol (MeOH) and ethyl acetate) were tested for their ability to elute known compounds and it was found only DCM and MeOH were required to elute

all compounds from the new cartridge. From these modifications by C. Milestone, a new protocol generating four chemically-distinct fractions was created for *in vivo* testing with mummichog.

1.9 Thesis research as "integrative biology"

The present work attempts to integrate changes in function at the molecular, tissue, and whole-organism levels of biological organization as a method to understand the point(s) in the reproductive endocrine system that EDSs alter. Previous work within the MacLatchy lab has exposed mummichog to model anti-/androgens, anti-/estrogens and effluents from various pulp mills in efforts to identify the mechanistic locations of impact these compounds have on mummichog reproduction and development. The current thesis adds knowledge to this research area by focusing on responses of mummichog to one particular waste stream (condensates) and to model androgens, and by applying molecular tools to determine effects on gene expression.

The work within this thesis also integrates biology and chemistry in an effort to identify compounds with the ability to alter the reproductive status of mummichog. Bioassay-driven fractionation of whole condensates involves a chemistry lab at Environment Canada in Burlington, ON and a biology lab at Wilfrid Laurier University working together to identify EDSs within IPP condensates. Gene expression analysis was completed in collaboration with Dr. Glen Van Der Kraak's lab at the University of Guelph. This lab works to determine whole organism and mechanistic responses in zebrafish (*Danio rerio*) to a variety of EDSs, pharmaceuticals and model compounds. In the past, collaborations between MacLatchy and Van Der Kraak labs have compared species responses to effluent from a bleached kraft pulp mill.

1.10 Objectives

Working in collaboration with another Laurier graduate student (Phillip Scott) and research scientist Dr. Mark Hewitt at Environment Canada in Burlington, ON, the primary objective of this thesis is to test the potential of refined condensate fractions from Irving Pulp and Paper Ltd. to depress sex steroid levels in mummichog. Mechanistic effects of condensate exposure will be investigated using gene expression analysis to determine where EDSs are impacting the steroidogenic pathway. Successful identification of EDSs within the condensates will continue the IOC process begun at IPP in 1997. Cause and effect relationships have been difficult to establish due to species-specific response patterns (Van Der Kraak *et al.,* 1998) and effluent variability (Hewitt *et al.,* 2008). In chapter two, *in vivo* testing of whole condensates and fractions generated from the new protocol are tested in mummichog for their ability to alter reproductive endpoints. In chapter three, the focus is on the reproductive responses of mummichog exposed to the model androgens methyltestosterone and dihydrotestosterone. In chapter four (general discussion) the results from chapters two and three are compared to determine whether the major bioactive compounds of condensates are androgenic in nature; the results are also put into perspective with our current understanding of the chemical make-up of condensates and their bioactivity in fish, and the field of integrative biology.

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Chapter Two: Bioactivity in the mummichog of endocrine-active compounds in kraft mill condensates

2.1 Abstract

In the mid 1990s, investigation of cause studies began at a bleached kraft pulp mill located in Saint John, NB, Canada with the goal to identify waste stream(s) in the mill containing endocrine-active contaminants. Linking of the 5th effect chemical recovery condensate stream to endocrine effects led to a development of a solid phase extraction (SPE) technique to isolate EDSs in the condensate stream. To determine if the condensates continue to be endocrine-active in exposed mummichog (*Fundulus heteroclitus*), a 14-day concentration-response experiment demonstrated that a 4% (v/v) concentration of whole condensates significantly depressed circulating plasma testosterone in male mummichog, with no effects on other plasma steroid hormones measured. Hepatic CYP1A and CYP3A were also significantly induced in exposed fish. A follow-up exposure returned inconsistent results. The 4% (v/v) concentrations did not alter plasma steroid levels and neither did SPE fractions derived from the condensates. While fish were in different reproductive periods, which may account for some of the differences between the experiments, a more compelling explanation is the variability in the chemical make-up of the condensates.

2.2 Introduction

In Canada, federal government regulations for pulp and paper mill effluent toxicity were implemented in 1992 through the environmental effects monitoring (EEM) program. In many cases, effluent treatment systems were installed to satisfy regulations which required mills to monitor total effluent toxicity and fish and benthos populations (Munkittrick, 2004). Effluent treatment protocols were designed to reduce biological oxygen demand (BOD), total suspended solids and chemical oxygen demand (COD), and to create an overall improvement of effluent quality (Stromberg *et al.,* 1996; Rickwood *et al.,* 2006). Despite the improvements observed in effluent lethal and sub-lethal toxicity during the past 10-15 years, reports of effects on fish reproduction downstream of Canadian mills have continued throughout this period (Hewitt *et al.,* 2008). These effects are attributed to endocrine disrupting substances (EDSs) within the effluent, though the responsible compounds are currently unknown (Hewitt *et al.,* 2008). Endocrine disruption in fish exposed to pulp mill effluent (PME) has been identified in countries other than Canada (Larson *et al*., 2000; Parks *et al*., 2001; Van Der Kraak *et al*., 2001; Goksoyr, 2006).

An investigation of cause (IOC) study began in 1997 at Irving Pulp and Paper (IPP), a bleached kraft mill in Saint John, New Brunswick. The goal was to investigate the reproductive impairments in fish downstream of effluent discharge (MacLatchy *et al.,* 2010). A mesocosm study exposed mummichog (*Fundulus heteroclitus*), an estuarine killifish residing in the discharge environment, to 1% concentrations of multiple in-mill waste streams and found increased liver size, decreased plasma testosterone (T) levels and decreased *in vitro* production of 17β-estradiol (E_2) in females exposed to the condensate waste stream (Dubé and MacLatchy, 2000), indicating this waste stream as the possible source of EDSs within IPP (Dubé, 2000).

Installation of a reverse osmosis (RO) treatment system of the 5th effect evaporator waste stream condensates in 1998 permitted studies to determine if this treatment system removed endocrine impacts of the final effluent on the fish. Mummichog exposed to an environmentally-relevant concentration of final effluent (1% v/v) following RO installation had no plasma testosterone (T) depression (Dubé and MacLatchy, 2000), however mummichog exposed to a 5% concentration of RO- treated final effluent had significantly depressed plasma T, indicating that RO treatment reduced, but did not eliminate, potential EDSs from the effluent. The results from these mesocosm studies indicated that the chemical recovery condensates were a possible source of EDSs at IPP, and that RO treatment improves effluent quality through the removal of some EDSs from final effluent (Dubé and MacLatchy, 2000; MacLatchy *et al.,* 2010).

Laboratory follow-up studies confirmed chemical recovery condensates as the primary EDS source at IPP. Mummichog exposed for seven days to RO feed (condensates prior to RO filtration) had significant depression of plasma testosterone (Dubé and MacLatchy, 2001). Removal of EDSs by RO treatment was tested with a 21-d bioassay, exposing mummichog to either RO feed or RO permeate (condensates post RO) streams. Fish exposed to RO permeate had no significant effects on reproductive status, while fish exposed to RO feed had significant reduction of plasma T (Dubé and MacLatchy, 2001). These studies were the first to determine a linkage between reproductive endocrine effects and an in-mill waste stream source, and to demonstrate reduction of potential EDSs by an effluent treatment system. Further laboratory studies confirmed that the EDSs were removed by the RO into what is known as the concentrate waste stream (burned in bark boiler) and were not present in the permeate (MacLatchy *et al.,* 2001).

Development of a solid-phase extraction technique was completed to fraction RO feed condensates and to test each fraction for the ability to impact hormonal activity in fish (Hewitt *et al.,* 2002). Mummichog exposed for seven days to these fractions showed significant plasma T depression. This study was one of the first to isolate chemicals from pulp mill condensates that are associated with reproductive impairment in fish (Hewitt *et al.,* 2002).

Based on the findings of this study, a reverse-phase high pressure liquid chromatography (HPLC) technique was developed to further fractionate RO feed condensates (Belknap *et al.,* 2006). Testing of six fractions generated through the HPLC protocol in the mummichog 14-day endocrine bioassay resulted in inconsistent findings at a 1% (v/v) concentration with previous studies (Shaughnessy *et al.,* 2007). A wider range of fraction concentrations were tested and reproductive endocrine effects observed in male mummichog at a 4% (v/v) concentration. A mummichog endocrine bioassay was run using the six HPLC fractions at 4% (v/v), however, there were no significant responses in mummichog (Shaughnessy *et al.,* 2007). The 4% (v/v) whole condensate treatment caused significant plasma T depression compared to control, indicating that there was a loss of bioactivity during the fractionation protocol (Shaughnessy *et al.,* 2007). Bioactivity loss was later shown to happen during evaporation of the mobile phase in preparation for solvent exchange (Scott, 2010).

A new SPE protocol was recently created by Dr. Craig Milestone (Scott, 2010), eliminating problems from the first protocol (Belknap *et al.,* 2006). A total of four distinct chemical fractions are generated (Figure 2.1; Scott, 2010) in the new protocol. Each fraction contains chemically-distinct compounds and is eluted based on compound size and polarity. Residual condensates are collected as the final fraction. The goal of the present research was to test these fractions for bioactivity using the mummichog endocrine bioassay. Prior to testing the

fractions, a whole condensate exposure was undertaken to determine the present-day concentration at which IPP condensates are bioactive. Depression of plasma T is an indicator of endocrine disruption in fish, and the concentration response bioassay would confirm the lowest concentration at which this response is observed. To extend our understanding of potential mechanisms of bioactivity, the following endpoints were assessed: liver somatic and gonad somatic indices, plasma T, 11-ketotestosterone (11-KT) and E_2 (whole organism endpoints); gonadal steroid production of T, 11-KT and E_2 (tissue-level endpoints); and gonadal steroidogenic acute regulatory protein (StAR) and aromatase gene expression, and hepatic cytochrome P450 genes 1 (CYP1A) and 3 (CYP3A) gene expression (molecular-level endpoints). StAR and aromatase are key steps in the steroidogenic pathway (Stocco and Clark, 1996; Rasheeda *et al.,* 2010) while CYP1A and CYP3A are indications of contaminant exposure in fish (Stegeman and Hahn, 1994). CYP3A is responsible for the facilitated excretion of contaminants through the addition of a hydroxyl group to the chemical structure, increasing the hydrophilic state of the molecule, thus ensuring removal from the organism (Danielson, 2002). This is the first time this full suite of endpoints has been assessed in mummichog exposed to pulp mill condensates and furthers previous work focused on identifying the source of EDSs in IPP condensates (MacLatchy *et al.,* 2010).

2.3 Materials and Methods

2.3.1 Condensate collection

IPP alternates between hardwood (maple and birch) and softwood (pine, fir, spruce) furnish using a five-stage $D_{100}E_{op}DED$ bleaching sequence (D_{100} = chlorine dioxide, oxygen bleaching; E_{op} = caustic, peroxide, and oxygen extraction; D = chlorine dioxide bleaching; E = caustic extraction) to produce approximately 990 air dried tonnes of pulp daily. Condensates were collected during softwood production runs, allowing at least five days of softwood manufacture to occur before condensate collection to ensure no contamination with residual hardwood furnish (J. Krstic, IPP, personal communication). On April 22, 2009, 130L of condensates were collected in a solvent-rinsed barrel from the RO feed. The condensates were shipped overnight to Wilfrid Laurier University, Waterloo ON, Canada for the concentration-response bioassay. Condensates were stored at 4°C to prevent potential breakdown of chemical compounds. Condensates for the fractionation bioassay were collected July 27th, 2009, from the RO feed and shipped overnight to Environment Canada (Burlington, ON) for fractionation. Extraction of the condensates occurred upon arrival, to reduce risk of chemical modification (Scott, 2010).

2.3.2 Fractionation regime for condensates

Condensate fractionation was conducted as per Scott (2010; Figure 2.1). Briefly, whole condensates had pH adjusted, were fortified with 2% (v/v) methanol and passed through 1 µm glass fibre filters (Whatman, Clifton, NJ, USA). Filters were then air-dried and non-polar compounds extracted using dichloromethane via a Soxhlet apparatus for 24h (Figure 2.1, fraction 1; filter paper non-polar (FP-NP)). To recover polar compounds, methanol was then used on the

filters (Figure 2.1, fraction 2; FP polar (FP-P)). Oasis HLB cartridges (Waters Ltd., Mississauga, ON) were then conditioned (two bed volumes each of water, methane and dichloromethane) and then loaded at a rate of 100 mL condensate/500 mg packing material. Flow rate was 5 mL/min for this solid-phase extraction (SPE) phase. Condensates that passed through the SPE cartridges were collected for *in vivo* testing (Figure 2.1, fraction 5; residual condensate (RC)). Cartridges were dried with an air vacuum (20 mm Hg for 1 h/g) and then washed with dichloromethane (40 mL/g) to elute the non-polar fraction (Figure 2.1, fraction 3; SPE non-polar (SPE-NP)). Finally the cartridges were washed with methanol (40 mL/g) to isolate the polar fraction (Figure 2.1, fraction 4; SPE polar (SPE-P)).

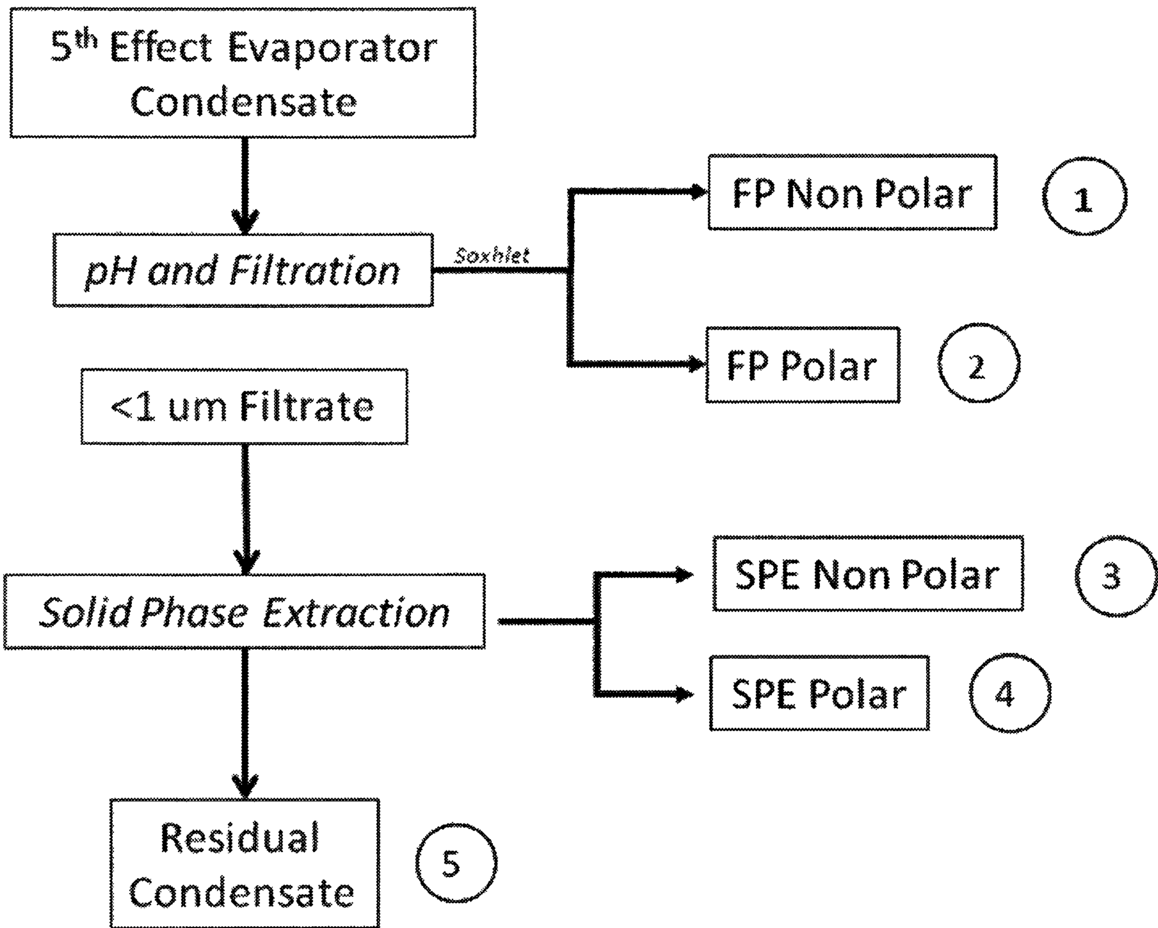

Figure 2.1 Protocol for condensate fractionation as developed by C. Milestone (Scott, 2010). Five distinct chemical fractions are generated for biological testing. FP = filter paper, SPE = solid phase extraction.

2.3.3 Mummichog collection

Mummichog were collected in April, 2009 from an uncontaminated reference estuary in Miramichi, NB (N 47° 02, W 65° 09) by seine netting and transferred to Wilfrid Laurier University in aerated plastic totes. Fish were housed in recirculating, 425L holding tanks from Aquabiotech (Coaticook, QC, Canada) at natural photoperiod, 16 parts per thousand (ppt) salinity (City of Waterloo well water mixed with Crystal Sea Salts; Marine Enterprise International, Inc., Baltimore, MD, USA) and dissolved oxygen (DO) > 80%. Fish were fed crushed commercial trout pellets (Corey Feed Mills, Fredericton, NB) daily to satiation. Standardized conditions for ammonia (< 0.5 ppm), nitrite (< 0.1 mg/L), nitrate (<0.1 mg/L), salinity (15.5-16.5 ppt), temperature (16-18°C) and DO were maintained by routine water quality tests. Partial water changes were done as needed to keep recirculating tanks within parameters. Minimal mortalities occurred in stock tanks (< 5%). Fish were acclimitized to lab conditions for a minimum of two weeks before each experiment.

2.3.4 Concentration-response bioassay

A short-term bioassay designed for mummichog (MacLatchy *et al.,* 2003) was used for exposures. Prior to commencing the concentration-response bioassay, four adult mummichog of each sex were weighed (to 0.01g) and randomly allocated to 24 glass aquaria, each containing 16L of 16ppt water. Each glass aquarium was randomly assigned an experimental treatment group, consisting of n=8 aquaria for each of control, 2% (v/v) condensates or 4% (v/v) condensates. Sample sizes (replicate aquaria) of n=8 have been shown to decrease variability and increase power in the standardized adult fish reproductive test (Bosker *et al.*, 2010). Fish were allowed to acclimate to the experimental aquaria for one week before beginning treatments.

Water quality measurements (salinity, DO, temperature, conductivity) were recorded daily using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Fish were fed crushed commercial trout pellets (approximately 1% of total body weight), and each aquarium was aerated to maintain DO levels above 80%. Tank temperatures were 17°C ± 1.5 °C for both bioassays. Photoperiod for all bioassays was maintained at 14h L:10h D (late spring conditions).

On May 1st 2009 treatments began under static conditions with 24h water and effluent renewal. Treatments were run for 14 consecutive days.

2.3.5 Fractionation bioassay

A 14-day adult mummichog bioassay was conducted beginning August 5th 2009. Prior to commencement, three adult mummichog of each sex were weighed (nearest 0.01 g) and randomly allocated to 56 20L glass aquaria. Fish were acclimated to the experimental aquaria for one week prior to the exposure. The protocol for the fractionation bioassay was identical to the protocol for the concentration-response bioassay. Treatments were all 4% (v/v) concentration and consisted of a negative control (methanol), five distinct chemical fractions (FP-NP, FP-P, SPE-NP, SPE-P, RC) and a positive control (4% whole condensates). Half of the aquaria began treatment on August 5th 2009 and were sampled August 19th, while the remaining aquaria began treatment on August 6th and were sampled August 20th. Sampling protocol and reproductive endpoints measured were similar to those described for the concentration-response bioassay.

2.3.6 Sampling protocol

Upon completion of the bioassay, fish were anaesthetized with 0.05% tricaine methane sulfonate (Sigma-Aldrich, Oakville, ON), weighed (g) and measured for length (mm). Blood was collected from caudal vasculature using heparinised 26 3/8 gauge needles (Beckton-Dickenson,

Franklin Lakes, NJ, USA). Blood was centrifuged (4000 *x g,* 12 min, 4°C) to isolate plasma. Plasma was frozen at -20°C until later ether extraction and radioimmunoassay (RIA) for plasma steroid determination (Dubé and MacLatchy, 2001). Following blood collection, fish were killed by spinal severance and the liver and gonads excised and weighed (0.01g) for determination of somatic indices. Gonadosomatic and liversomatic indices were calculated as GSI or LSI = (tissue weight (g)/ [total weight (g)- tissue weight (g)])*100 (McMaster *et al.,* 1995). Condition factor (CF) was calculated as CF= (total weight (g)/ standard length^3)*100. Ovarian tissue and female livers were snap frozen with liquid nitrogen for gene expression analysis. Testes were placed in Medium 199 (Sigma-Aldrich) for *in vitro* gonadal steroid production (McMaster *et al,.* 1995). Full details of the sampling protocol are available in MacLatchy *et al.* (2005).

2.3.7 Radioimmunoassay

Circulating plasma T, E_2 and 11-KT hormone levels were determined by RIA. Plasma was thawed and steroid hormones were isolated from blood proteins using ether extraction (McMaster *et al.,* 1992). A triple extraction was done to ensure maximum hormone recovery. Steroid hormones were re-suspended in 1 mL of phosgel buffer and frozen at -20°C until analyzed by RIA. Protocols from MacLatchy *et al.* (2005) were followed for RIA determination of steroid hormones. ^{3}H-Labelled testosterone and ^{3}H-labelled estradiol were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC). ^{3}H-Labelled 11-KT was purchased from M. McMaster's lab (Environment Canada, Burlington, ON). Unlabelled T, E_2 and 11-KT were purchased from Sigma-Aldrich. Testosterone and estradiol antibodies were purchased from MP Biomedicals (Solon, OH, USA). 11-KT antibody was purchased from AssayPro (St. Charles, MO, USA). All antibodies had less than 0.1% cross-reactivity with closely-related steroids.

Intra-assay and inter-assay variability were minimal (all intra-assay variability < 6%, all inter-assay variability <10%).

2.3.8 In vitro analysis

Following the method developed by McMaster *et al.* (1995) and optimized by MacLatchy *et al.* (2003), testes tissue was minced with a scalpel. A minimum of two gonad pieces from the same fish were placed in each incubation well, already containing Medium 199 (Sigma-Aldrich). A total of 18-22mg of gonad tissue was added to each well. Depending on the size of the gonad upon dissection, 1-3 replicate wells per fish were run. Immediately prior to beginning of incubation, old Medium 199 was removed from the tissue samples and 1 mL of fresh medium added. Samples were incubated at 18°C for 24h. Following incubation, tissue and medium were separated using a pipette, and the medium was frozen at -20°C until RIAs were run for T, E_2 , and 11-KT.

2.3.9 Gene expression

Total RNA from ovarian and liver tissue was extracted using TRIzol solution (Invitrogen, Carlsbad, CA, USA). Manufacturer's instructions were followed with minor modifications. Sections of tissue (50-100mg pieces) were added to 800 µL TRIzol and homogenized. Homogenized samples were incubated at room temperature for 10 min and then had 160 µL chloroform added. Samples were then shaken vigorously for 15 s, and incubated at room temperature for 3 min. Following incubation, samples were centrifuged (12,000 *x g*, 15 min, 4°C) followed by transfer of the upper phase into a new tube containing 400 µL isopropyl alcohol. Samples were shaken, incubated at room temperature for 10 min, then centrifuged (12,000 *x g*, 10 min, 4°C). Supernatant was removed and the RNA pellet washed with 800 µL 75% ethanol,

followed by a final centrifugation (12,000 *x g*, 5 min, 4°C). Ethanol was then removed, the RNA pellet allowed to air dry and then reconstituted in 30-50 μL GIBCO water depending on pellet size. Samples were stored at -80°C.

Samples were thawed and 3 μL of each total RNA sample were added to 147 μL GIBCO water for RNA quantification (Ings and Van Der Kraak, 2006). Quality was determined from absorbance at 260nm and 280nm (ideal ratio = 2.0, Ings and Van Der Kraak, 2006). Following quantification, 5 μL of each total RNA sample was combined with GIBCO water to standardize all samples to a concentration of 1 μg/μL. Standardized samples were then quantified at absorbance of 260nm to confirm concentration.

A total volume of 2 μL of standardized total RNA was then combined with 1 μL 10x reaction buffer (Invitrogen, Carlsbad, CA, USA), 1 μL AMP-D1 (Sigma, St. Louis, MO) and 6 μL GIBCO water for DNase treatment. Samples were incubated for 15 min at room temperature, and then 1 μL of stop solution was added to halt the reactions. Samples were then incubated at 70°C for 5 min, and then buried in ice until cool. Random primers were then added (0.2 ng; Promega, Madison, WI, USA) and samples were incubated at 70°C for 5 min then buried in ice until cool. A total volume of 25 μL for each sample was achieved through the addition of (concentrations represent final values): 5x RT buffer (50mM Tris-HCL, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen), RNasin (25U; Promega), dNTPs (0.5mM; Roche Molecular Biochemicals, Laval, QC), DDT (10mM; Invitrogen), M-MLV reverse transcriptase (200U; Invitrogen), and GIBCO water (Ings and Van Der Kraak, 2006). Reverse transcription (RT) reaction was completed by sample incubation at 37°C for 60 min, followed by 5 min at 90°C to inactivate the enzymes (Ings and Van Der Kraak, 2006). Resulting cDNA product was stored at -20°C until Real Time PCR amplification. To ensure no discrepancies within tissues, all ovarian

samples were processed in the RT simultaneously, and all liver samples were processed simultaneously in the next batch. This avoids inappropriate comparisons between treatments in the event there were discrepancies during the two RT reaction batches (Lister and Van Der Kraak, 2009).

Genes of interest were ovarian steroidogenic acute regulatory protein (StAR) and aromatase (Aroma), and liver cytochrome P450 genes 1 (CYP1A) and 3 (CYP3A). Primer Express software v. 2.0 (Applied Biosystems, Forster City, CA, USA) was used to design primers which span exon-exon boundaries in the mRNA to prevent genomic DNA amplification (Ings and Van Der Kraak, 2006). Accession numbers and primer sequences are shown in Table 2.1.

Table 2.1 List of primers and their sequences used in this study (Fwd = forward primer, Rev = reverse primer)

Gene		Sequence 5' - 3'	Accession Number
StAR	Fwd	CAGAGCTGAGAACGGGCCTAC	CN983145.1
	Rev	CTTTGGGATCCAGCCCTTC	
EF1α	Fwd	ACCAGAAAGTACTACGTGACCATC	AY430091
	Rev	TCAGCCTGGGAGGTACCG	
Aromatase A (CYP19A1- Ovarian)	Fwd	TGCCCCTCGACGAGAAAG	AY713118.1
	Rev	GTAGATGTCGGGTTTGATCAGCA	
CYP3A30	Fwd	GAACAACCCATCAGACCCGTT	AF105068
	Rev	AAGCGACGGCGAGGAAG	
CYP1A1	Fwd	GAGGACCGGAAGCTCGATG	AF026800.1
	Rev	ACCAGCTCCGAAGAGGTCGT	

The relative standard curve/SYBR green method was used with primer pair efficiency being determined through the creation of a standard curve by a serial dilution of RT product in 50ng/mL yeast RNA (Ings and Van Der Kraak, 2006). Each PCR reaction well was composed of: 5 μL RT product (5X diluted in GIBCO water), 2.5 μL forward and reverse primers (0.4 μM), and 10 μL SYBR green PCR Master Mix (SYBR green dye, dNTPs, Passive Reference I, AmpliTaq®Gold DNA polymerase; Applied Biosystems; Ings and Van Der Kraak, 2006). cDNA product was amplified using ABI Prism 7000 sequence detection system (Applied Biosystems) with the following cycling conditions for all genes: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C (Ings and Van Der Kraak, 2006). Duplicate reactions were performed for each sample and then averaged.

To proceed with gene expression comparison, genes of interest were normalized to elongation factor 1-α (ef1α) an endogenous control gene with steady expression regardless of treatments (Lister and Van Der Kraak, 2009).

2.3.10 Data analysis

Sexes were separated for statistical analysis. Prior to statistical analysis, Dixon tests for the presence or absence of outliers were conducted (MacLatchy *et al.*, 2005) and outliers were removed from further analysis. Statistical analysis was performed using Statistica© 6.0 (Statsoft INC, Tulsa, OK, USA) and Sigmaplot 11.0 (Systat Software INC, Chicago, IL, USA). Assumptions of normality and homogeneity of variance were tested using Levene's test prior to parametric analysis. A one-way ANOVA ($p \leq 0.05$) was used to test for differences among treatments. A Dunnett's post hoc test was conducted if applicable to determine treatment differences. If data did not fit the assumption of normality, log transformations were conducted

and data were re-tested. Comparisons of liver weight and gonad weight were done using ANCOVA, with total fish weight as the co-variate. ANCOVAs for total body weight were conducted with standard length as the co-variate to evaluate condition.

2.4 Results

2.4.1 Concentration response

Fish had naturally recrudescing gonads during the bioassay. No significant treatment differences were found for liver weight, gonad weight, body weight, length or condition factor for this bioassay (Table 2.2).

Mean plasma testosterone was significantly depressed in male mummichog exposed to 4% (v/v) whole condensates compared to control (p=0.045; Figure 2.2). There was no significant change in plasma testosterone in females (p=0.20; Figure 2.3). No change in plasma estradiol occurred in either males (Figure 2.2) or females (Figure 2.3) at any treatment concentration compared to control (p=0.71 and p=0.28 for males and females, respectively). Mean plasma 11-KT was not changed (p=0.65; Figure 2.2) nor was *in vitro* production of T (p=0.14), E_2 (p=0.23) or 11-KT (p=0.53) in male mummichog (Figure 2.4). Expression of StAR and aromatase in ovarian tissue was not significantly impacted compared to controls (p= 0.82 and p=0.45, respectively; Figure 2.5). Expression of CYP1A in female liver was significantly increased compared to control for both 2% and 4% treatments (p=0.0025). CYP3A expression was also significantly increased in 2% and 4% treatments compared to control (p=0.006; Figure 2.5).

Table 2.2 Mean (± 1SE) length, weight, gonadosomatic indices (GSI) and liversomatic indices (LSI) of male and female mummichog (*Fundulus heteroclitus*) exposed to various concentrations (v/v) of whole condensates collected from 5th effect evaporators at IPP. No significant differences were detected within variables ($p \leq 0.05$).

		Treatment		
Sex	**Variable**	**Control**	**2%**	**4%**
M	Length (mm)	73 ± 0.8	74.8 ± 1.3	74.2 ± 1.2
	Weight (g)	4.4 ± 0.1	4.5 ± 0.3	4.3 ± 0.2
	GSI (%)	1.4 ± 0.1	1.1 ± 0.1	1 ± 0.1
	LSI (%)	4.4 ± 0.2	4.2 ± 0.2	3.9 ± 0.2
F	Length (mm)	83 ± 0.9	80 ± 1.0	80 ± 1.2
	Weight (g)	6.8 ± 0.5	5.7 ± 0.2	5.6 ± 0.3
	GSI (%)	4.7 ± 0.8	3.9 ± 0.4	4.5 ± 0.7
	LSI (%)	5.9 ± 0.7	5.2 ± 0.2	5.2 ± 0.3

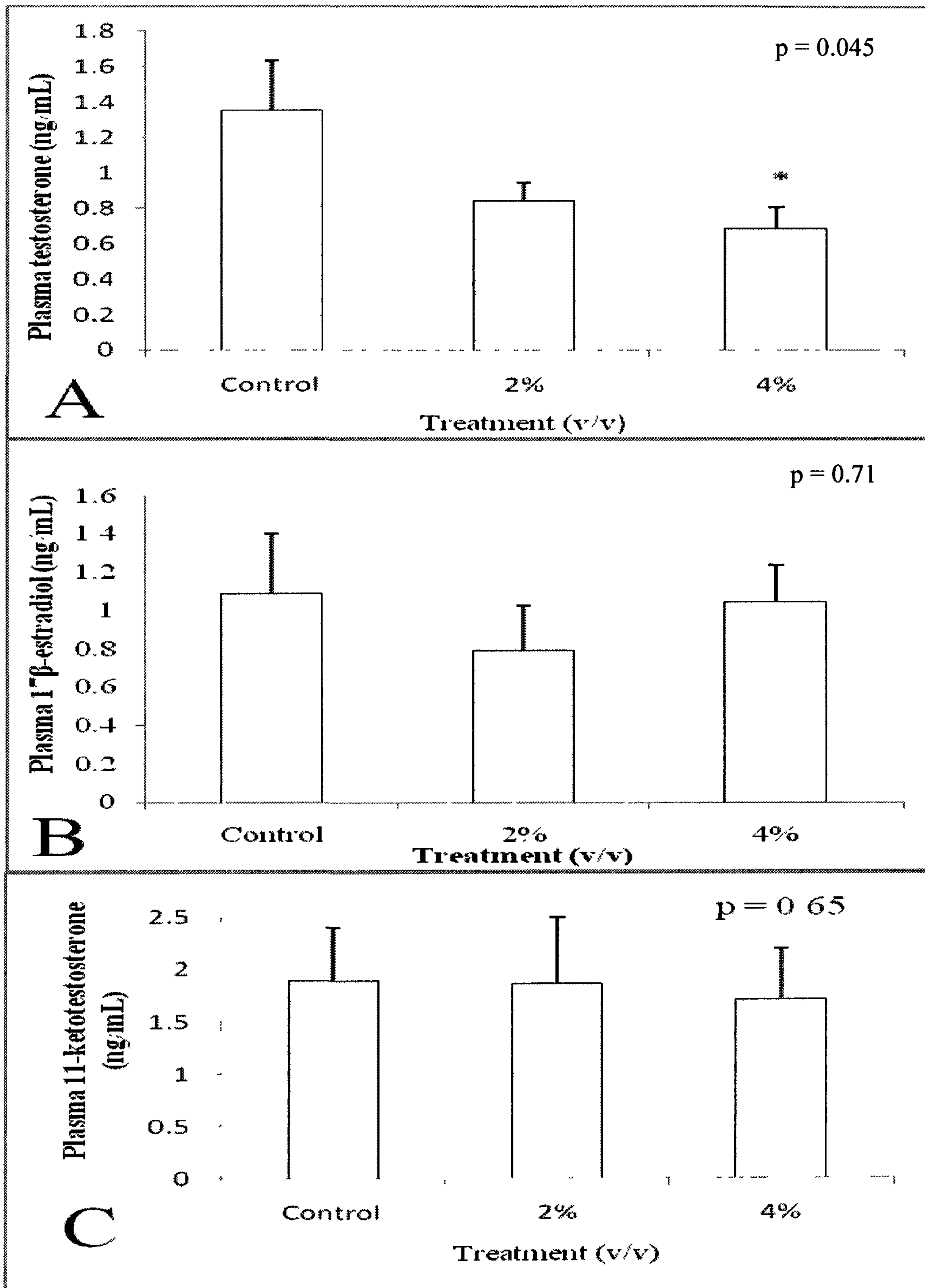

Figure 2.2 Mean (± 1SE) plasma testosterone (A), plasma 17β-estradiol (B) and 11-ketotestosterone (C) levels (ng/ml) in male mummichog exposed to 0% (control), 2% or 4% (v/v) whole condensates for 14 days in May 2009. Bar with an asterisk (*) is significantly different from control group ($p \leq 0.05$).

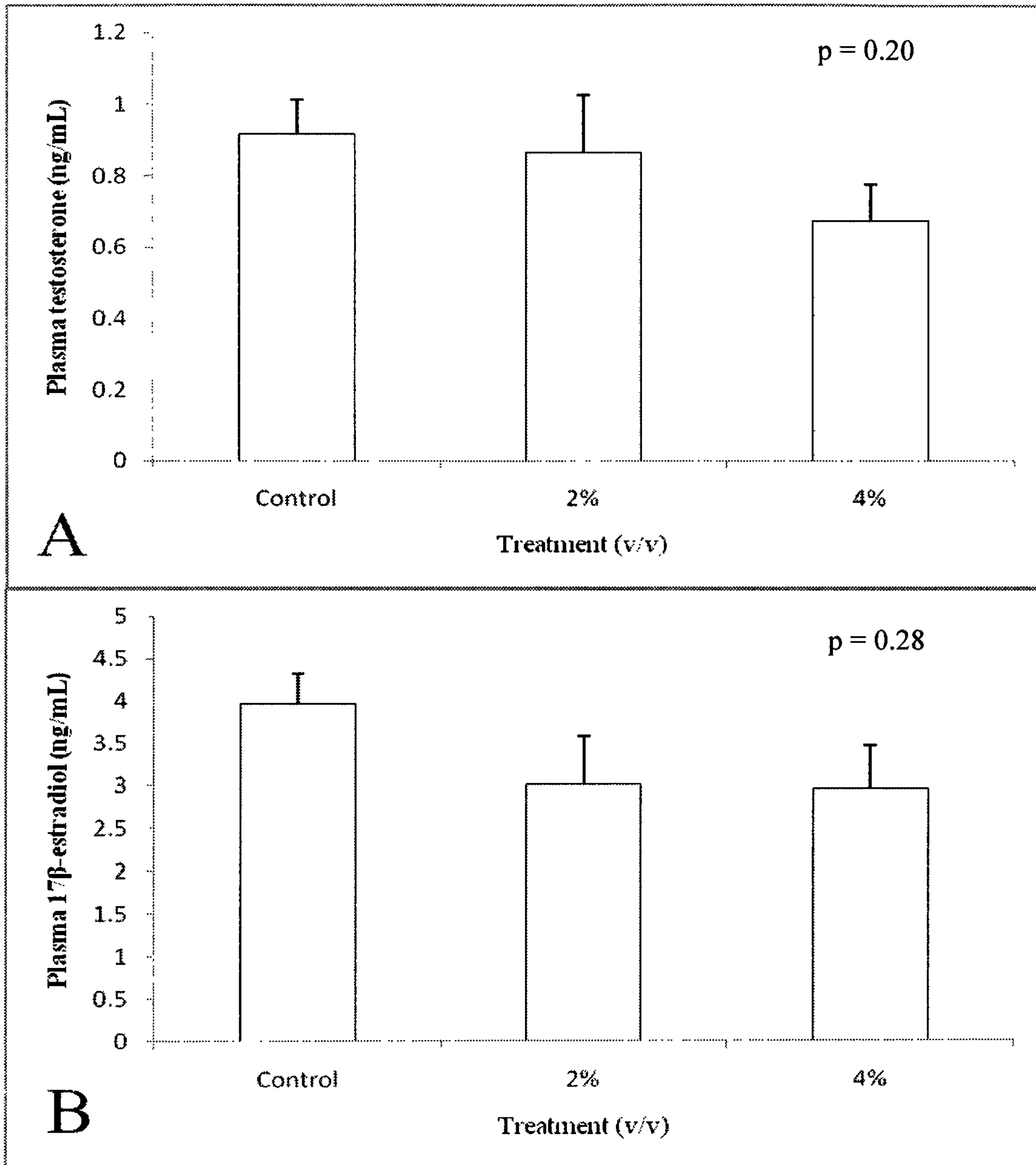

Figure 2.3 Mean (± 1SE) plasma testosterone (A) and plasma 17β-estradiol (B) levels (ng/ml) in female mummichog exposed to 0% (control), 2% or 4% (v/v) whole condensates for 14 days in May 2009.

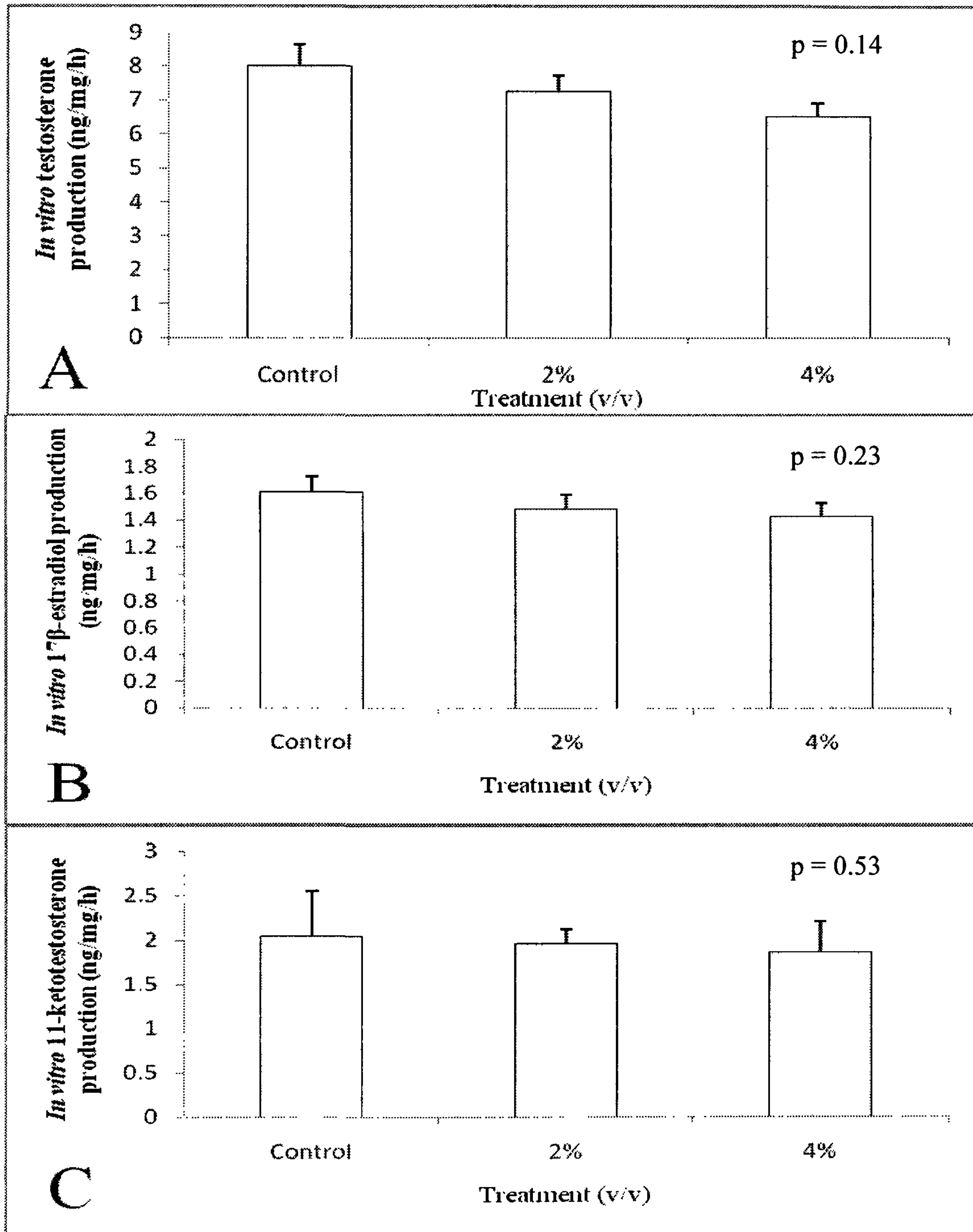

Figure 2.4 Mean (± 1SE) gonadal *in vitro* production of testosterone (A), 17β-estradiol (B) and 11-ketotestosterone (C) levels (ng/mg/h) in male mummichog exposed to 0% (control), 2% and 4% (v/v) whole condensates for 14 days in May 2009. Gonads incubated for 24h at 18°C.

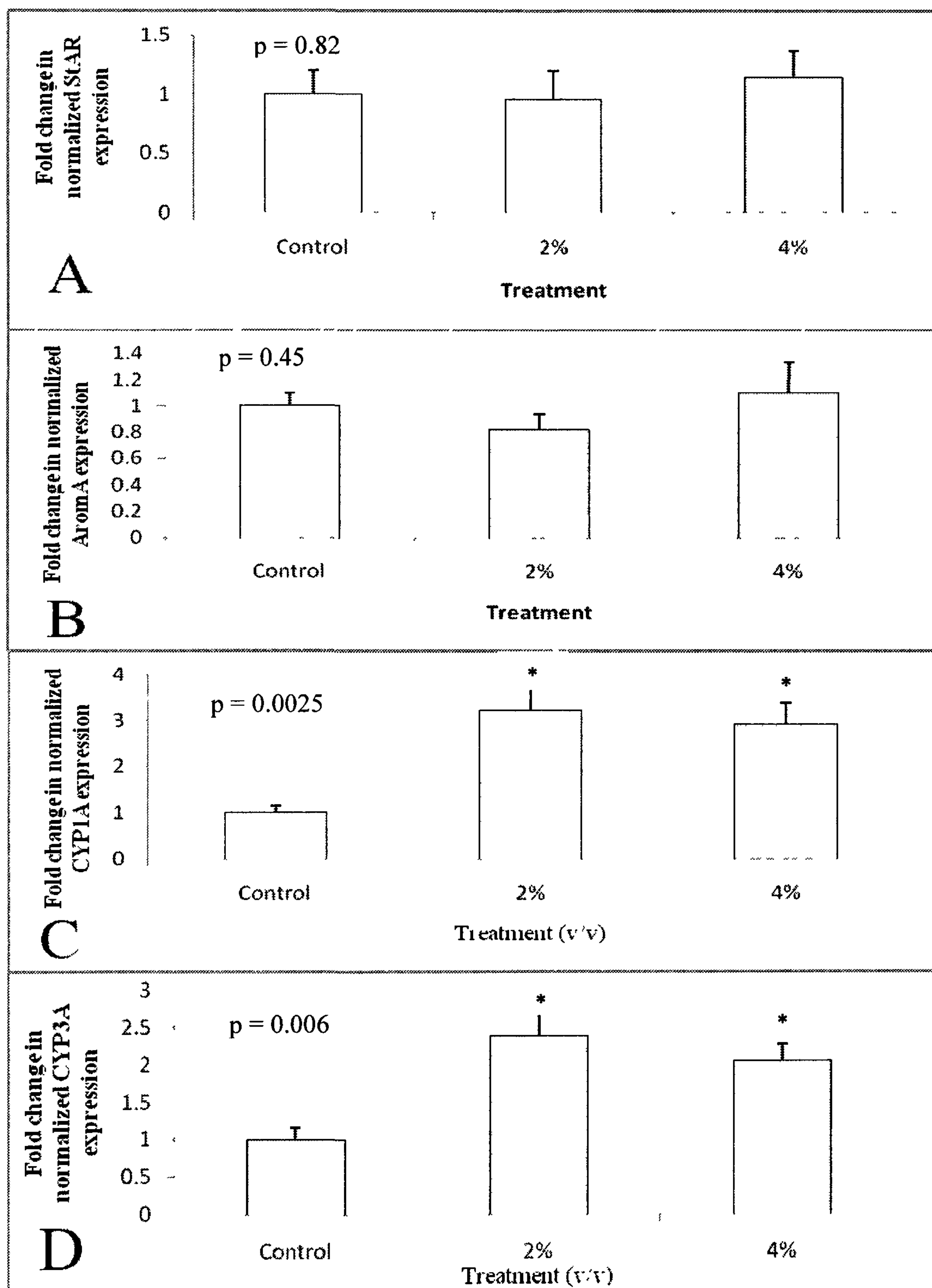

Figure 2.5 Ovarian expression of steroidogenic acute regulatory protein (StAR) (A) and aromatase (AromA) (B) and hepatic expression of cytochrome P450 genes 1 (CYP1A) (C) and 3 (CYP3A) (D) in female mummichog exposed to 0% (control), 2% or 4% (v/v) whole condensates for 14 days in May 2009. Expression levels were normalized to elongation factor 1-α (ef1α). Bars with an asterisk (*) are significantly different from the control group.

2.4.2 Fractionation bioassay

Fish gonads were naturally regressing during the bioassay. No significant treatment differences were found in liver weight, gonad weight, body weight, length or condition factor among treatments in this bioassay (Table 2.3).

No significant responses in plasma T levels were observed in males ($p=0.12$; Figure 2.6) or females ($p=0.064$; Figure 2.7). Responses of plasma E_2 were not significantly different in either male (Figure 2.6) or female (Figure 2.7) cohorts ($p= 0.11$ and $p = 0.63$, respectively). Plasma 11-KT in males was not significantly different compared to control ($p = 0.69$; Figure 2.6).

In vitro analysis was confined to comparing the 4% (v/v) whole condensates and control treatments based on the results in the prior concentration-response exposure. No significant responses were observed in either mean *in vitro* T or E_2 in male mummichog ($p = 0.16$ and $p = 0.78$ respectively; Figure 2.8).

Table 2.3 Mean (± 1SE) length (mm), weight (g), gonadosomatic indices (GSI) and liversomatic indices (LSI) of male and female mummichog (*Fundulus heteroclitus*) exposed to various treatments for 14 days in August 2009. No significant differences were detected within variables. Control is the reference treatment (4% methanol), 4% treatment represents positive control of 4% (v/v) whole condensates. All other treatments are chemically-distinct fractions isolated from whole condensates (FP-NP = filter paper non-polar, FP-P = filter paper polar, SPE-NP = solid phase extraction non-polar, SPE-P = solid phase extraction polar, RC = residual condensates)

		Treatment						
Sex	**Variable**	**Control**	**4%**	**FP-NP**	**FP-P**	**SPE-NP**	**SPE-P**	**RC**
M	Length(mm)	80.3 ± 1.3	78.8 ± 1.1	79.4 ±1.5	83.3 ± 1.5	80.2 ± 0.9	82.3 ± 1.3	82 ± 1.3
	Weight (g)	6.2 ± 0.4	5.2 ± 0.2	5.6 ± 0.4	6.3 ± 0.5	5.6 ± 0.2	5.7 ± 0.3	6.2 ± 0.3
	GSI (%)	0.68 ± 0.06	0.62 ± 0.04	0.84 ±0.06	0.69 ± 0.07	0.71 ± 0.04	0.60 ± 0.04	0.84 ± 0.05
	LSI (%)	6.5 ± 0.3	6.2 ± 0.3	6.7 ± 0.3	5.9 ±0.3	6.2 ± 0.3	6 ± 0.4	6.7 ± 0.3
F	Length(mm)	80.5 ± 2.4	77.7 ± 1.5	79.5 ± 1.8	75 ± 1.3	76.1 ± 1.8	76.9 ± 1.5	76.9 ± 1.8
	Weight(g)	6.6 ± 0.7	5.0 ±0.4	5.9 ± 0.5	4.7 ± 0.3	5.2 ± 0.4	4.9 ± 0.3	5.2 ± 0.4
	GSI (%)	1.8 ± 0.1	1.8 ±0.1	1.8 ± 0.05	1.9 ± 0.1	1.9 ± 0.05	1.8 ± 0.1	1.9 ± 0.1
	LSI (%)	7.1 ± 0.3	6.4 ± 0.3	6.9 ± 0.3	6.7 ± 0.3	6.3 ± 0.3	6.2 ± 0.3	6.4 ± 0.3

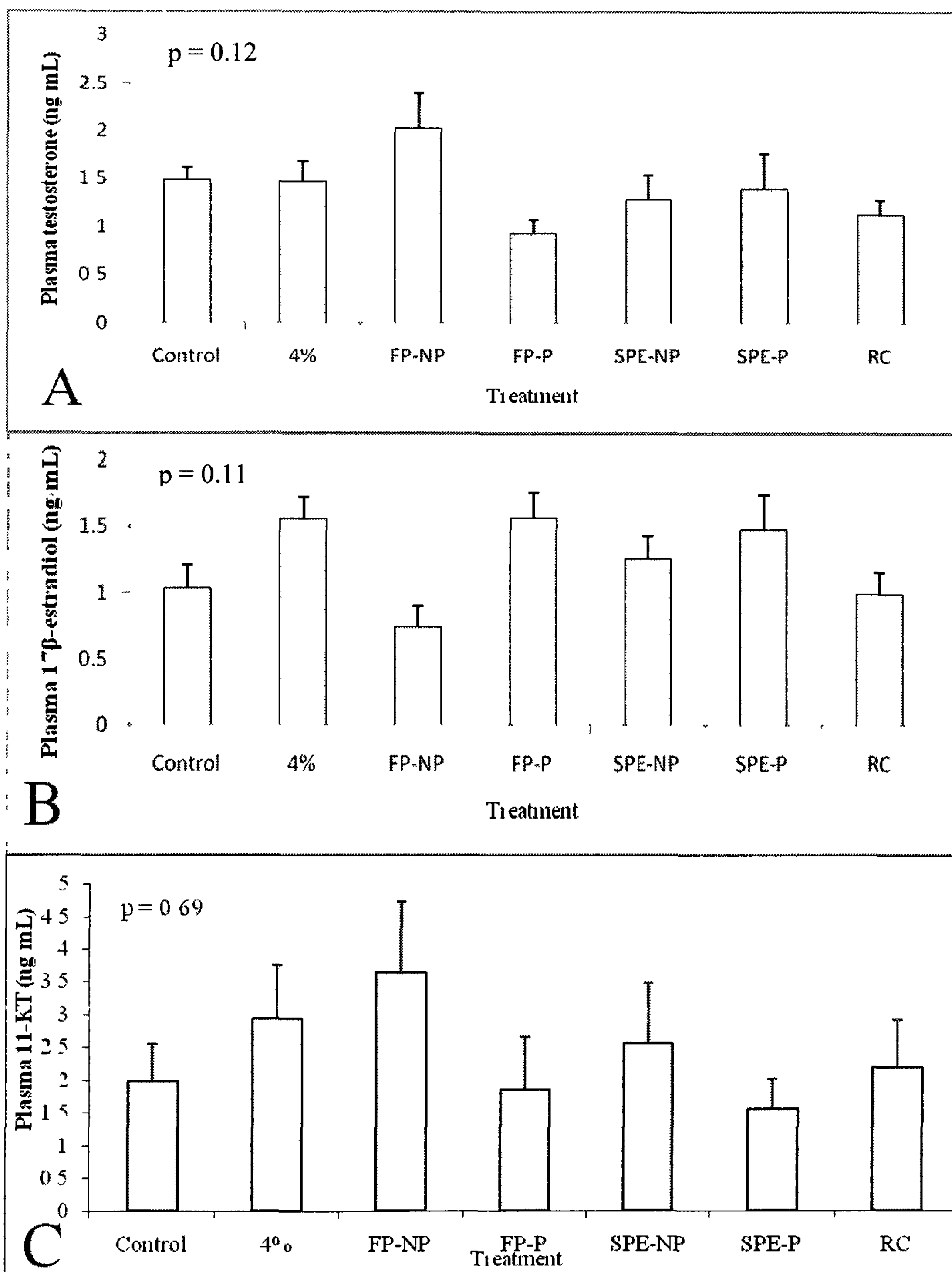

Figure 2.6 Mean (± 1SE) plasma testosterone (A), 17β-estradiol (B) and 11-ketotestosterone (11-KT; C) levels (ng/ml) in male mummichog exposed to 5th effect evaporator condensates or condensate fractions for 14 days in August 2009. Control is the reference treatment (4% methanol), 4% treatment represents positive control of 4% (v/v) whole condensates. All other treatments are chemically-distinct fractions isolated from whole condensates (FP-NP = filter paper non-polar, FP-P = filter paper polar, SPE-NP = solid phase extraction non-polar, SPE-P = solid phase extraction polar).

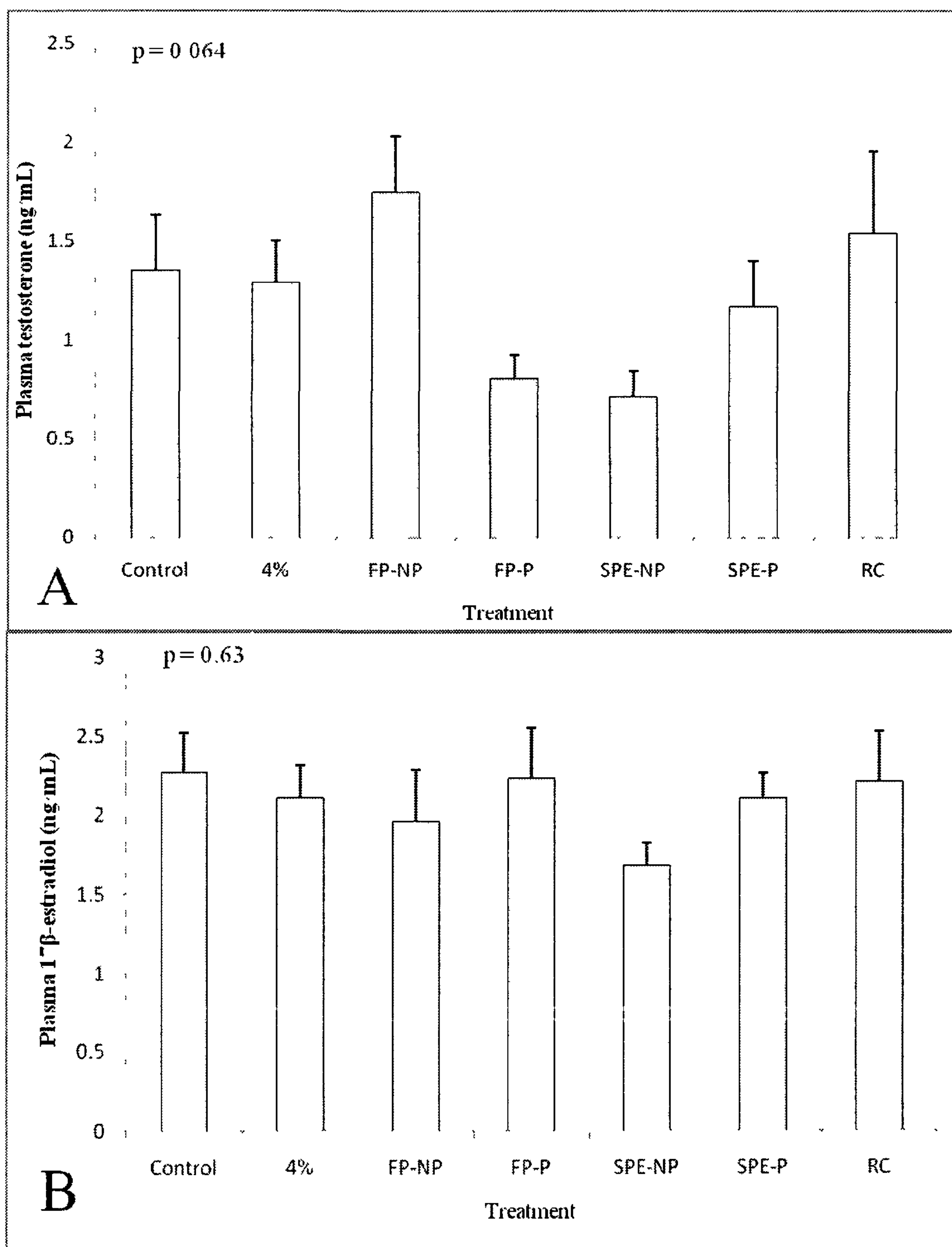

Figure 2.7 Mean (± 1SE) plasma testosterone (A) and 17β-estradiol (B) levels (ng/ml) in female mummichog exposed to 5th effect evaporator condensates or condensate fractions for 14 days in August 2009. Control is the reference treatment (4% methanol), 4% treatment represents positive control of 4% (v/v) whole condensates. All other treatments are chemically-distinct fractions isolated from whole condensates (FP-NP = filter paper non-polar, FP-P = filter paper polar, SPE-NP = solid phase extraction non-polar, SPE-P = solid phase extraction polar).

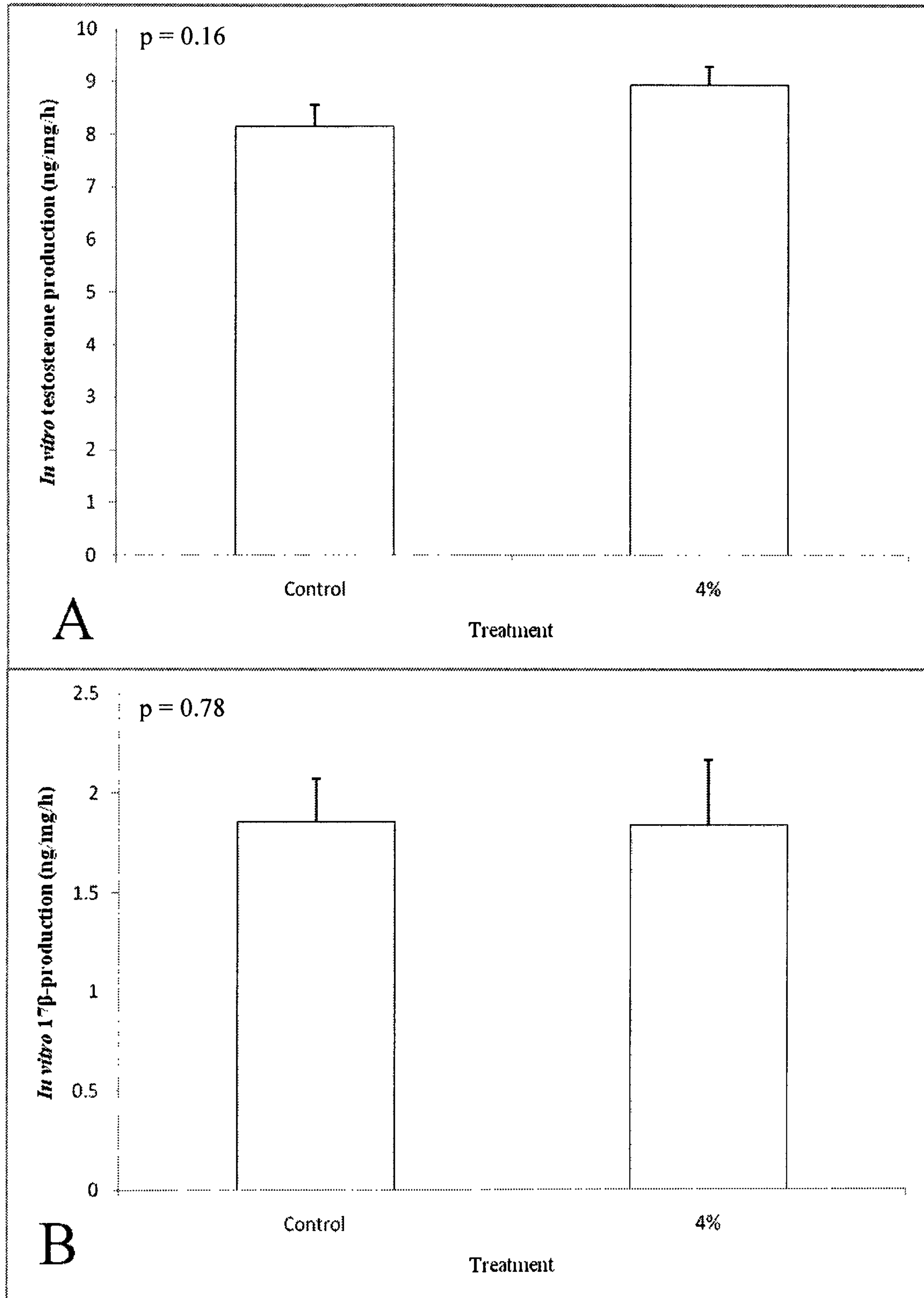

Figure 2.8 Mean (± 1SE) gonadal *in vitro* production of testosterone (A) and 17β-estradiol (B) levels (ng/mg/h) in male mummichog exposed to 4% methanol (control) and 4% (v/v) whole condensates for 14 days in August 2009. Gonads incubated for 24h at 18°C.

2.5 Discussion

These studies were focused on furthering previous work that showed condensates from a bleached kraft pulp mill in Saint John, New Brunswick, caused depression of plasma and *in vitro* steroid hormones. The concentration-response experiment exposed adult mummichog of both sexes to multiple concentrations of RO feed condensates to determine plasma and *in vitro* hormonal responses. This study confirmed significant plasma testosterone depression in mummichog exposed to 4% (v/v) whole condensates as shown previously (Shaughnessy *et al.,* 2007). The application of real-time polymerase chain reaction (RT-PCR) to investigate gene expression of enzymes of interest is a novel tool used in this study to examine potential mechanisms of action of condensates. Induction of hepatic CYP1A and CYP3A genes indicate that compounds in whole condensates may require detoxifying. The fractionation study used a more robust fractionation protocol compared to past studies. However, the positive control (4% (v/v) RO feed) failed to elicit a significant response in plasma reproductive hormones and no effects of the fractions were observed.

Depression of plasma testosterone in male mummichog exposed to 4% (v/v) RO feed was found in the concentration-response bioassay. In concert with previous work on the condensates, endocrine-active compounds are present in the RO feed. Depression of plasma testosterone is a common response in fish exposed to pulp mill effluent discharge (Munkittrick *et al.,* 1994, McMaster *et al.,* 1996, Rickwood *et al.,* 2006) as well as in exposures of fish to the IPP condensates (Dubé and MacLatchy, 2000; Hewitt *et al.,* 2002; Shaughnessy *et al.,* 2007). Previous studies on the condensates showed a 1% (v/v) concentration was capable of depressing circulating plasma testosterone levels by up to 58% (Dubé and MacLatchy, 2000; Hewitt *et al.,* 2002). However, a more recent study by Shaughnessy *et al.* (2007) returned results more

comparable to the current study, as a concentration of 4% (v/v) RO feed caused testosterone depression in males. There are no known mill changes in the years between the studies to account for the differences in endocrine activity of the condensates. It has been proposed that the potency of condensates at IPP have decreased since studies conducted prior to 2001 (Hewitt *et al.*, 2002; Shaughnessy *et al.*, 2007).

Female mummichog in the concentration-response experiment showed no significant plasma testosterone depression at any concentration compared to control, which confirms findings in Shaughnessy *et al.* (2007) of sex-based differences in responses. Based on results from Dubé and MacLatchy (2001) and Hewitt *et al.* (2002) in which both male and female mummichog exposed to the IPP 5th effect condensates had plasma testosterone depression, it appears that while IPP condensate potency has decreased since studies done in the late 1990's/ early 2000's, it is currently of the same potency and make-up as the condensates used in the 2007 Shaughnessy study. Based on this confirmation, future fractionation experiments were conducted with a 4% (v/v) concentration of chemically-distinct fractions to test for ability to depress plasma testosterone and other steroid hormones.

Gene expression analysis was used to identify potential locations of interactions between the gonad and endocrine-active compounds in the RO feed. There was no significant response in either StAR or aromatase expression, indicating that if alteration of the steroidogenic pathway is occurring, it is not through changes in gene expression at either of these two loci. StAR is one of the proteins responsible for the mobilization of cholesterol through the mitochondrial membrane (Stocco and Clark, 1996), is highly conserved across vertebrate classes (Bauer *et al.*, 2000) and is hypothesized to be the rate-limiting step in the steroidogenic pathway in mammals (Sugawara *et al.*, 1997). Studies on the effects of EDSs, such as the pesticide methoxychlor and

ethinylestradiol, on StAR expression in fish gonads, report decreased expression (Vang *et al.*, 2007; Blum *et al.*, 2008), however, impacts on StAR expression in fish exposed to pulp mill effluent has not been reported. Aromatase is responsible for the conversion of androgens (mainly testosterone) to estrogens (mainly 17β-estradiol; Rasheeda *et al.*, 2010). Fish exposed to EDSs in pulp mill effluent exhibit a range of responses in aromatase expression, from induction (Orrego *et al.*, 2010) to reduction (Kortner *et al.*, 2009a, Kortner *et al.*, 2009b).

Hepatic cytochrome P4501A (CYP1A) and cytochrome P4503A (CYP3A) were significantly induced in both the 2% and 4% RO feed treatments. CYP1A has been comprehensively characterized due to its sensitive induction in fish exposed to environmentally-relevant concentrations of a host of contaminants including dioxins, furans and polycyclic aromatic hydrocarbon (PAHs; Stegeman and Hahn, 1994). In the concentration-response bioassay CYP1A was significantly induced, a three-fold increase for both 2% and 4% (v/v) RO feed. Previous studies done with mummichog have also noted a higher expression of CYP1A in fish exposed to creosote-contaminated sediments (Meyer *et al.*, 2002) and waters polluted with polychlorinated biphenyls and halogenated aromatic hydrocarbons (Oleksiak, 2008). Similarly, CYP3A was significantly induced in both RO feed treatments compared to control. This specific family of genes is responsible for hepatic drug metabolism and detoxification (Danielson, 2002). The increases in CYP1A and CYP3A expression indicate that whole condensates contain certain chemical compounds that the liver identifies as harmful and attempts to detoxify.

The fractionation regime made improvements on previous work done to develop a fractionation protocol for IPP condensates, so as to optimize chemical compound recovery from the SPE cartridges (Scott, 2010). This new protocol included improvements in the solid phase cartridges, elution solvent and solvent order, eluting methods and loading capacity (Scott, 2010).

The fractions derived from this more robust protocol, as well as a positive control of 4% (v/v) RO feed condensates, demonstrated no effects on plasma reproductive steroid levels.

In the concentration-response experiment, a 4% (v/v) RO feed concentration caused 49.7% and 26.4% decreases in mean plasma testosterone in males and females, respectively. Similar testosterone depression has been reported in mummichog at the same RO feed concentration (Shaughnessy *et al.*, 2007). In the fractionation exposure, however, the same 4% (v/v) RO feed concentration returned mean plasma testosterone responses of 24.6% depression in males and a 13.0% increase in females, although neither of these were significant. This lack of response makes interpretation of specific fraction bioactivity difficult.

The discrepancy in 4% RO feed condensate effects between the two studies may be attributed to a variety of factors, including: i) the different gonadal state of the fish during the two bioassays, and ii) temporal fluctuations in the active substances themselves. Comparison of GSI and reproductive state between the bioassays reveals that in the concentration-response experiment gonads were more mature. Mean plasma testosterone levels were comparable between the two bioassays. In male mummichog, the average plasma testosterone levels were 1.4 ng/mL and 1.5 ng/mL for the concentration-response and fractionation bioassays, respectively. In female fish, mean plasma testosterone was 0.91 ng/mL and 1.4 ng/mL for the two assays, with the fractionation exposure having the greater mean value. Previous work with mummichog has revealed that regressed fish (such as those in the fractionation exposure) still typically respond with plasma testosterone depression when exposed to IPP condensates although the effects may be on a reduced scale (Hewitt *et al.,* 2002; MacLatchy *et al.*, 2005). Seasonal fluctuations in mummichog hormones have been reported (Shimizu, 1997), with most mummichog reproductively active from mid-March to mid-August and regressed by September. Laboratory

manipulation of reproductive cycles in mummichog can help to lessen the fluctuations seen in wild populations (MacLatchy *et al.*, 2003) and facilitate experiments outside of the spring and summer periods.

While alteration of the chemical constituents and the potential loss of bioactive compounds from the condensates over the duration of the bioassay may be one reason results differed between the two exposures, this is not likely. Work done by Shaughnessy *et al.* (2007) involving daily sampling of the RO feed at IPP with comparison to a single RO feed batch sampling found that potential compound degradation did not have an impact on reproductive endpoints examined in the study. Plasma testosterone was significantly depressed at the 4% (v/v) level in both daily RO feed sampling and single RO feed sampling (Shaughnessy *et al.*, 2007). As well, the fractions have been chemically tested over time to ensure that they remain stable (C. Milestone, personal communication).

Historically, effluent and condensate composition has been shown to change depending on tree species harvested, time of year of harvesting, soil composition and a host of other factors (Christianson-Heiska *et al.*, 2008). Studies done across mills have found variability between mills and within mills in effluent composition depending on timing of effluent sampling, with some finding that effluent composition varies on a weekly basis (Rickwood *et al.*, 2006a; Rickwood *et al.*, 2006b). Analysis of IPP condensate variability has found that conductivity of condensates (an indicator of likeness between effluent samplings) is variable, and that condensate composition is not temporally consistent (Belknap *et al.*, 2006). Chemical analysis of condensates used in the concentration-response and fractionation studies was completed by P. Scott at Environment Canada (Scott, 2010). Differences in compounds previously confirmed to be in condensates was ascertained by gas chromatography mass spectrometry. Minor variations

were found in manool, 4-ethylguaiacol, squalene and isoeugenol concentrations. Geranyl linalool levels were 5.9 mg/L and 9 mg/L for the concentration-response and fractionation condensates, respectively. Androgen equivalents were also determined within the two condensates, as past studies with condensates have indicated possible links between androgen levels and steroid hormone impacts in mummichog (MacLatchy *et al.*, 2004). Testosterone equivalents (TEQ) is a measure of androgenicity, a theoretical testosterone equivalency concentration within a sample. Larger TEQ values indicate a larger concentration of androgen equivalents, thus samples with a high TEQ are more androgenic than those with a low TEQ. Condensates from both bioassays were separated into five distinct fractions (the same as the treatments in the fractionation bioassay) and androgenicity measured in each fraction using an androgen receptor (AR) binding assay (Scott, 2010). Residual condensates were not tested for androgenicity as they were not compatible with the AR binding assay due to their reconstitution in water. In both condensate batches, the FP-NP and SPE-NP were the most androgenic of the fractions. FP-NP and SPE-NP from the concentration-response condensates were approximately six and 2.5 times lower in TEQ, respectively, compared to the equivalent fraction in the fractionation condensates (Scott, 2010).

Overall the concentration-response bioassay confirmed that 4% (v/v) RO feed depressed plasma testosterone in adult mummichog. Hepatic CYP1A and CYP3A induction indicates that the RO feed does contain compounds requiring biotransformation and perhaps detoxification, although whether these contaminants are the endocrine-active constituents remain unknown. Variability between RO feed condensates in the bioassays was detected through the lack of response in the fractionation bioassay of the 4% (v/v) treatment compared to control. AR binding ligands revealed a difference in androgen strength between the two condensate batches (Scott,

2010), leading to questions regarding the potential role of androgens as the source of bioactivity in condensates.

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Chapter Three: Model androgen effects in the mummichog (*Fundulus heteroclitus*)

3.1 Abstract

Mummichog (*Fundulus heteroclitus*) were exposed to 100 ng/L or 1000 ng/L of the aromatizable androgen methyltestosterone (MT) or to 10 μg/L or 100 μg/L of the non-aromatizable androgen dihydrotestosterone (DHT) in a static, 24-h exposure to assess the impact of androgens on plasma hormones and gene expression of key proteins in the steroidogenic pathway. Female mummichog had lower thresholds of effects than males and were more responsive to androgen exposure. Plasma testosterone was significantly depressed by 10 μg/L DHT in males. Females had significant depression of plasma 17β-estradiol (E_2) when exposed to both DHT concentrations and 1000 ng/L MT. *In vitro* production of E_2 was depressed in females exposed to 100 μg/L DHT and both MT concentrations. A 2.5-fold increase in ovarian aromatase expression was detected in the 1000 ng/L MT treatment. DHT altered plasma steroid levels more than MT, however, MT decreased *in vitro* production of hormones in more treatments than DHT. The expression of aromatase was not reduced compared to control in any treatment of either androgen, leading to speculation that other genes in the steroidogenic pathway are changed by androgen exposure and responsible for the variation in steroid hormone responses.

3.2 Introduction

Androgenic compounds are discharged into the aquatic environment from various sources, including agricultural runoff and pulp mill effluent (Ellis *et al.,* 2003). Binding of exogenous compounds to androgen receptors (ARs) may induce functional or organizational changes in fish (Gray *et al.,* 1997). Fish exposed to androgenic substances exhibit intersex gonads (Arlsan *et al.,* 2009), male-skewed sex ratios in eggs and fry exposed during development (Forlin *et al.,* 2004), expression of male secondary sexual characteristics in females (Orlando *et al.,* 2007), and complete masculinisation of females (Li *et al.,* 2006). Evidence for direct receptor-mediated androgenic effects includes the mechanisms by which methyltestosterone (MT) exposure in the Atlantic croaker (*Micropogonias undulatus*) increases testosterone production in gonadal tissue (Khan *et al.,* 1999). Species-specific isoforms of androgen receptors have been found in goldfish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*) and some other fish species (Wells and Van Der Kraak, 2000). These isoforms can result in varied responses to androgenic compounds between species, making speculation of direct receptor-mediated effects difficult in fish where AR binding has not been specifically studied (Sharpe *et al.,* 2004).

Aromatase depression in fish exposed to model androgens is widely reported (Kitano *et al.,* 2000; Fenske and Segner, 2004; Hornug *et al.,* 2004; Li *et al.,* 2006). It is hypothesized as the main point of interaction between androgenic compounds and the steroidogenic pathway (Li *et al.,* 2006). Aromatase converts testosterone to 17β-estradiol (E_2) in steroidogenic tissue in both male and female fish. Decreased ability to aromatize testosterone may result in increased plasma testosterone levels, decreased plasma E_2, and expression of male secondary sexual characteristics in female fish (Palowski *et al.,* 2004; Li *et al.,* 2006). Reduction of aromatase inhibits the ability

of endogenous estrogens to be synthesized and thus can result in masculinisation of fish over time through the accumulation of testosterone (Kitano *et al.*, 2000; Hoffman *et al.*, 2008). Other enzymes of the steroidogenic pathway are upregulated due to androgenic exposure including 11β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase (Hoffman *et al.*, 2008). In zebrafish (*Danio rerio*), these enzymes were each increased at least 2.5 times above control expression during waterborne exposure to dihydrotestosterone (DHT; Hoffman *et al.*, 2008). The increased expression of these enzymes results in faster metabolism of cholesterol to synthesize testosterone and leads to increased levels of plasma testosterone.

Some androgenic compounds, such as MT, have the ability to exert both estrogenic and androgenic responses (Ankley *et al.*, 2000). Aromatization of these androgens can lead to estrogenic responses such as elevated plasma E_2 levels and induction of the vitellogenin 1 gene expression in male liver. Non-aromatizable androgens, such as DHT, can be converted to 11-ketotestosterone (11-KT) in male fish, and propagate male secondary sexual characteristics (Howell *et al.*, 1980). Appearance of these traits (such as elongated anal fins) in females is evidence of androgen interaction (Orlando *et al.*, 2007).

Androgenic effects of pulp mill effluent (PME) have been reported in Canada, Sweden, New Zealand and the USA (Sodergren 1992; Parks *et al.*, 2001; van den Heuvel and Ellis, 2002; Hewitt *et al.*, 2005). Identification of androgenic effects due to PME exposure began in the 1980's when it was shown that several species of fish living downstream of discharge of a bleached kraft mill (BKM) were masculinised (Denton *et al.*, 1985). Male-skewed sex ratios have been reported in fish embryos downstream of BKM discharge in Sweden (Forlin *et al.*, 2004). After a temporary mill shutdown in 1999, sex bias disappeared and was restablished when

operation of the mill commenced in 2000 (Larsson and Forlin, 2002), indicating that effects were not bioaccumulative. Female mosquitofish (*Gambusia holbrooki*) collected from a PME-contaminated section of the Fenholloway River in Florida displayed masculinisation with an elongated male-like anal fin and rudimentary gonopodium development (Orlando *et al.,* 2007). PME from the Fenholloway River contains chemical mixtures which bind androgen receptors and induce *in vitro* androgen-dependant gene expression, the presumed mechanism of action of masculinisation of female mosquitofish found in this river (Parks *et al.,* 2001)

Larsson *et al.* (2006) tested a Swedish PME for the presence of fish gonadal AR ligands. Competitive binding assays for AR were done with ovaries of Atlantic croaker (*Micropogonias undulatus*) and 37 compounds with potential AR binding capabilities were found. A majority of these were polar compounds. Progesterone was present in the primary effluent, but was removed with biological treatment. Similarly progesterone and androstenedione were detected downstream of a different mill (Carson *et al.,* 2008). The presence of androgens and other steroids may be due to bacterial metabolization of β-sitosterol (a plant sterol) commonly found in PME (Carson *et al.,* 2008). Progesterone and androstenedione are precursors to testosterone in the steroidogenic pathway, and the presence of these precursors could cause endocrine disruption in fish living downstream of pulp mills. Similarly, fish exposed to PME from three separate Canadian pulp mills showed androgenic responses (Hewitt *et al.,* 2005). Liver extracts of exposed fish were tested in the goldfish AR assay and found to contain ligands for these receptors. Primary treated effluent showed greater androgenic ligand concentration than final effluent, however, both still elicited androgenic responses in fish (Hewitt *et al.,* 2005). Recently, androgenic activities of 11 pulp and paper mills in Canada, Brazil and New Zealand were compared, with two Canadian mills having the highest androgen activities (Scott, 2010).

Androgen activity was not linked to effluent treatment type, mill location or mill process (Scott, 2010).

Because androgenic compounds in PME have been identified as a potential cause of endocrine-mediated effects on fish living downstream, research is needed to better understand the effects and modes of action of model androgen compounds in mummichog. Previous androgen studies with mummichog have shown this species to be responsive to androgen exposure at a range of concentrations (Sharpe *et al.,* 2004). A short-term adult mummichog reproductive endocrine bioassay was used to test the effects and mechanisms of action of exposure to an aromatizable (methyltestosterone) and a non-aromatizable (dihydrotestosterone) androgen. Gene expression analysis was undertaken to provide information on the role of aromatase in mummichog during exposure to model androgens. This study extends previous work in mummichog to elucidate the potential effects and mechanisms of action of endocrine-active compounds in estuarine environments.

3.3 Materials and Methods

3.3.1 Model androgen concentrations

Dihydrotestoterone and methyltestosterone were purchased from Steraloids (Newport, Rhode Island, USA). Nominal concentrations were selected which had elicited reproductive responses in fish in previous studies (Panter *et al.*, 2004, Sharpe *et al.*, 2004). Treatments included control (100 µL ethanol), 10 µg/L DHT, 100 µg/L DHT, 100 ng/L MT and 1000 ng/L MT. Stock solutions of each androgen were created by dissolving in ethanol. Concentrations of stock solutions were created so that addition of 100 µL ethanol solution to a 15L aquarium gave desired nominal concentrations.

3.3.2 Mummichog collection

Mummichog were collected in April 2010 from an uncontaminated reference estuary in Miramichi, NB (N 47°02, W 65°09) by seine netting and transferred to Wilfrid Laurier University in aerated plastic totes. Fish were housed in recirculating, 425L holding tanks from Aquabiotech (Coaticook, QC, Canada) at natural photoperiod, 16 ppt salinity (City of Waterloo well water mixed with Crystal Sea Salts; Marine Enterprise International, Inc., Baltimore, MD) and dissolved oxygen (DO) > 80%. Fish were fed crushed commercial trout pellets (Corey Feed Mills, Fredericton, NB) daily to satiation. Standardized conditions for ammonia (< 0.5 ppm), nitrite (< 0.1 mg/L), nitrate (<0.1 mg/L), salinity (15.5-16.5 ppt), temperature (16-18°C) and DO were maintained by routine water quality tests. Partial water changes were done as needed to keep recirculating tanks within standardized parameters. Minimal mortalities occurred in stock tanks (< 5%). Fish were housed in holding tanks for two weeks before experimentation.

3.3.3 Model androgen bioassay

One week prior to treatment commencement, three adult mummichog of each sex were removed from stock tanks, weighed (nearest 0.01g) and randomly allocated into one of 30, 20L glass aquaria to acclimate. Each glass aquarium held 15 L of 16 ppt water. Experimental treatment groups of control (ethanol), 10 μg/L DHT, 100 μg/L DHT, 100 ng/L MT and 1000 ng/L MT were randomly assigned to each aquarium. Each treatment was composed of six replicate aquaria. During the acclimation period, water renewal was done every 24h to fully acclimate fish to experimental conditions.

Water quality measurements (salinity, DO, temperature, conductivity) were recorded daily using a YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Fish were fed crushed commercial trout pellets (approximately 1% of total body weight), and each aquarium was aerated to maintain dissolved oxygen levels above 80%. Tank temperatures were 18°C ± 1°C. Photoperiod was maintained at 14h L:10h D (late spring conditions).

Treatments began on May 12th 2010 and continued for 15 consecutive days.

3.3.4 Sampling protocol

At the conclusion of the bioassay fish were anaesthetized with 0.05% tricaine methane sulfonate (Sigma-Aldrich, Oakville, ON, Canada), weighed (g) and measured for length (mm). Blood was collected from the caudal blood vessels using a heparinised syringe fitted with a 26 3/8 gauge needle (Beckton-Dickenson). Blood was centrifuged (4000 *x g,* 12 min, 4°C) to isolate plasma. Plasma was frozen at -20°C until later ether extraction and radioimmunoassay (RIA) for plasma steroid determination (Dubé and MacLatchy, 2001). After blood collection fish, were killed by spinal severance and the liver and gonads excised and weighed (0.01g) for

determination of somatic indices. Gonadosomatic and liversomatic indices were calculated as GSI or LSI = (tissue weight (g)/ [total weight (g)- tissue weight (g)])*100. Condition factor (CF) was calculated as CF= (total weight (g)/ standard length^3)*100. Gonadal tissue from one fish of each sex from each aquarium was placed into 1 mL of Medium 199 (Sigma-Aldrich) for *in vitro* analysis of steroid production (McMaster *et al.* 1995). The gonads from the four remaining fish were snap frozen with liquid nitrogen for gene expression analysis. Full details of the sampling protocol are available in MacLatchy *et al.* (2005).

3.3.5 Radioimmunoassay (RIA)

Circulating plasma testosterone (T), 17β-Estradiol (E_2) and 11-ketotestosterone (11-KT) levels were determined by RIA. Plasma was thawed and steroid hormones were isolated from blood proteins using a triplicate ether extraction method (McMaster *et al.* 1992). Steroid hormones were resuspended in 1 mL of phosgel buffer and frozen at -20°C until measured by RIA (MacLatchy *et al.*, 2005). ^{3}H-Labelled testosterone and ^{3}H-labelled estradiol were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC). ^{3}H-Labelled 11-KT was purchased from M. McMaster's lab (Environment Canada, Burlington, ON). Unlabelled T, E_2 and 11-KT were purchased from Sigma-Aldrich. Testosterone and estradiol antibodies were purchased from MP Biomedicals (Solon, OH, USA). 11-KT antibody was purchased from AssayPro (St. Charles, MO, USA). All antibodies had less than 0.1% cross-reactivity with closely-related steroids. Intra-assay and inter-assay variabilities were minimal in all hormones in both bioassays (all intra-assay variability < 6%, all inter-assay variability <10%), falling within acceptable levels (MacLatchy *et al.*, 2003).

3.3.6 In vitro analysis

Following the method developed by McMaster *et al.* (1995) and optimized in MacLatchy *et al.* (2003), gonadal tissue was minced with a scalpel. A minimum of two gonad pieces from the same fish were placed in each incubation well, already containing Medium 199 (Sigma-Aldrich). A total of 18-22mg of gonad tissue was added to each well. Depending on the size of the gonad upon dissection, 1-3 replicate wells per fish were run. Immediately prior to beginning the incubation, old Medium 199 was removed from the tissue samples and 1 mL of fresh medium added. Samples were incubated at 18°C for 24h. Following incubation, tissue and medium were separated using a pipette, and the medium was frozen at -20°C until RIAs were run for T, E_2 , and 11-KT.

3.3.7 Gene expression

Total RNA from gonadal and liver tissue was extracted using TRIzol solution (Invitrogen, Carlsbad, CA, USA). Manufacturer's instructions were followed with minor modifications. Tissue (50-100mg) was added to 800 µL TRIzol and homogenized. Homogenized samples were incubated at room temperature for 10 min and then 160 µL of chloroform was added. Samples were shaken vigorously for 15 sec, then incubated at room temperature for 3 min. Samples were then centrifuged (12,000 *x g*, 15 min, 4°C) followed by transfer of the upper phase into a new tube containing 400 µL isopropyl alcohol. Samples were shaken, incubated at room temperature for 10 min, then centrifuged (12,000 *x g*, 10 min, 4°C). Supernatant was removed and the RNA pellet washed with 800 µL of 75% ethanol, followed by a final centrifugation (12,000 *x g*, 5 min, 4°C). Ethanol was then removed, the RNA pellet allowed to air

dry and then reconstituted in 30-50 μL GIBCO water depending on pellet size. Samples were stored at -80°C.

Samples were thawed and 3 μL of each total RNA sample was added to 147 μL GIBCO water for RNA quantification (Ings and Van Der Kraak, 2006). Quality was determined from absorbance at 260 nm and 280 nm (ideal ratio = 2.0, Ings and Van Der Kraak, 2006). Following quantification, 5 μL of each total RNA sample were combined with GIBCO water to standardize all samples to a concentration of 1μg/μL. Standardized samples were then quantified at absorbance of 260 nm to confirm concentration.

Standardized total RNA (2 μL) was then combined with 1 μL 10x reaction buffer (Invitrogen, Carlsbad, CA, USA), 1 μL AMP-D1 (Sigma Aldrich) and 6 μL GIBCO water for DNase treatment. Samples were incubated for 15 minutes at room temperature, and then 1 μL of stop solution was added to halt the reaction. Samples were then incubated at 70°C for 5 min, and then buried in ice until cool. Random primers were then added (0.2 ng; Promega, Madison, WI, USA) and samples were incubated at 70°C for 5 min then buried in ice until cool. A total volume of 25 μL for each sample was achieved through the addition of (concentrations represent final values): 5x RT buffer (50mM Tris-HCL, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen), RNasin (25U; Promega), dNTPs (0.5mM; Roche Molecular Biochemicals, Laval, QC), DDT (10mM; Invitrogen), M-MLV reverse transcriptase (200U; Invitrogen), and GIBCO water (Ings and Van Der Kraak, 2006). The reverse transcription (RT) reaction was completed by sample incubation at 37°C for 60 min, followed by 5 min at 90°C to inactivate the enzymes (Ings and Van Der Kraak, 2006). Resulting cDNA product was stored at -20°C until Real Time PCR amplification. To ensure no discrepancies within tissues, all ovarian samples were processed in the RT simultaneously, all testes processed simultaneously in the next batch and all liver samples were

processed simultaneously in the next batch. This avoids inappropriate comparisons between treatments in the event there were discrepancies during the two RT reaction batches (Lister and Van Der Kraak, 2009).

Levels of expression for gonadal steroidogenic acute regulatory protein (StAR) and aromatase, hepatic cytochrome P450 genes 1 (CYP1A) and 3 (CYP3A) and hepatic vitellogenin 1 (VTG1) were measured. Primer Express software v. 2.0 (Applied Biosystems, Forster City, CA) was used to design primers which span exon-exon boundaries in the mRNA to prevent genomic DNA amplification (Ings and Van Der Kraak, 2006). Accession numbers and primer sequences are shown in Table 3.1.

Table 3.1 List of primers and their sequences used in this study (Fwd = forward primer, Rev = reverse primer)

Gene		Sequence 5' - 3'	Accession Number
Vitellogenin 1	Fwd	GACAACGTCACTGTGAAGGTCAAC	UO7055.2
	Rev	CCTCTCCGCTTTGTCTTGATCT	
StAR	Fwd	CAGAGCTGAGAACGGGCCTAC	CN983145.1
	Rev	CTTTGGGATCCAGCCCTTC	
EF1α	Fwd	ACCAGAAAGTACTACGTGACCATC	AY430091
	Rev	TCAGCCTGGGAGGTACCG	
Aromatase A (CYP19A1- Ovarian)	Fwd	TGCCCCTCGACGAGAAAG	AY713118.1
	Rev	GTAGATGTCGGGTTTGATCAGCA	
CYP3A30	Fwd	GAACAACCCATCAGACCCGTT	AF105068
	Rev	AAGCGACGGCGAGGAAG	
CYP1A1	Fwd	GAGGACCGGAAGCTCGATG	AF026800.1
	Rev	ACCAGCTCCGAAGAGGTCGT	

The relative standard curve/SYBR green method was used with primer pair efficiency being determined through the creation of a standard curve by a serial dilution of RT product in 50ng/mL yeast RNA (Ings and Van Der Kraak, 2006). Each PCR reaction well was composed of: 5 μL of RT product (5X diluted in GIBCO water), 2.5 μL of forward and reverse primers (0.4 μM), and 10 μL of SYBR green PCR Master Mix (SYBR green dye, dNTPs, Passive Reference I, AmpliTaq®Gold DNA polymerase; Applied Biosystems; Ings and Van Der Kraak, 2006). cDNA product was amplified using ABI Prism 7000 sequence detection system (Applied Biosystems) with the following cycling conditions for all genes: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C (Ings and Van Der Kraak, 2006). Duplicate reactions were performed for each sample and then averaged.

To proceed with gene expression comparison, genes of interest were normalized to an endogenous control gene with steady expression regardless of treatments (Lister and Van Der Kraak, 2009). Elongation factor 1-α (EF1α; Table 3.1) expression levels were examined and all other genes normalized to it.

3.3.8 Data analysis

Sexes were separated for statistical analysis. Prior to statistical analysis, Dixon tests for the presence or absence of outliers were conducted (MacLatchy *et al.*, 2005) and outliers were removed from further analysis. Statistical analysis was performed using Statistica© 6.0 (Statsoft INC, Tulsa, OK, USA) and Sigmaplot 11.0 (Systat Software INC, Chicago, IL, USA). Assumptions of normality and homogeneity of variance were tested using Levene's test prior to parametric analysis. A one-way ANOVA ($p \leq 0.05$) was used to test for differences among treatments. A Dunnett's post hoc test was conducted if applicable to determine treatment

differences. If data did not fit the assumption of normality, log transformation was conducted and data were re-tested. Comparisons of liver weight and gonad weight were done using ANCOVA, with total fish weight as the co-variate. An ANCOVA for total body weight was conducted with standard length as the co-variate to evaluate condition.

3.4 Results

There were no significant differences among treatments in fish length, body weight, gonad weight, condition factor or liver weight for this bioassay (Table 3.2). Fish were naturally recrudescing during the bioassay.

Significant depression of mean plasma testosterone occurred in males in the DHT 10 treatment ($p = 0.038$; Figure 3.1). There was a significant increase in mean plasma testosterone in females in the DHT 100 treatment ($p = 0.031$; Figure 3.2). There was no effect on plasma E_2 in males ($p = 0.51$; Figure 3.1). Female fish had significant depressions in plasma E_2 in DHT 10, DHT 100 and MT 1000, three of four treatment groups ($p = 0.0006$; Figure 3.2). Male mean plasma 11-KT was not changed in any treatment ($p = 0.28$; Figure 3.1).

In vitro testosterone production was not significantly depressed in either males or females ($p = 0.17$, 0.29, Figures 3.3 and 3.4, respectively). Gonadal E_2 production was not significantly impacted in males ($p = 0.51$; Figure 3.3). Female E_2 production was depressed in all treatments except for DHT 10 ($p = 0.0017$; Figure 3.4). Production of 11-KT in male gonads was significantly depressed in DHT 10, MT 100 and MT 1000 ($p = 0.0089$; Figure 3.3).

StAR expression levels did not change in either male or female gonad tissue across any treatment ($p = 0.12$, 0.5, Figures 3.5 and 3.6; respectively). Aromatase expression was not altered in any treatment for males ($p = 0.46$; Figure 3.5), however, in ovarian tissue aromatase expression experienced a 2.5 fold increase in MT 1000 compared to control ($p = 0.044$; Figure 3.6).

In both males and females, expression levels of hepatic CYP1A did not differ significantly from control ($p = 0.22$, 0.17, Figures 3.5 and 3.6; respectively). Similar results were

seen for hepatic CYP3A expression ($p = 0.14$, 0.19, Figures 3.5 and 3.6; respectively). VTG1 expression levels were significantly induced in male DHT 100 treatment compared to control ($p < 0.001$; Figure 3.5). Conversely in female mummichog DHT 100 caused a significant reduction in expression ($p = 0.022$; Figure 3.6). No other treatments had significant impacts on hepatic VTG1 expression.

Table 3.2 Mean (± 1SE) length, weight, gonadosomatic indices (GSI) and liversomatic indices (LSI) of male and female mummichog (*Fundulus heteroclitus*) exposed to either the non-aromatizable androgen dihydrotestosterone (DHT) or the aromatizable androgen methyltestosterone (MT). Treatments are DHT 10 µg/L (DHT 10) and 100 µg/L (DHT 100) and MT 100 ng/L (MT 100) and 1000 ng/L (MT 1000). No significant differences were detected within variables ($p \leq 0.05$).

		Treatment				
Sex	**Variable**	**Control**	**DHT 10**	**DHT 100**	**MT 100**	**MT 1000**
M	Length (mm)	83.8 ± 4.9	84.3 ± 1.5	86.4 ± 2.2	88.4 ± 2	87.6 ± 2.3
	Weight (g)	6.5 ± 0.41	5.8 ± 0.24	6.3 ± 0.45	6.8 ± 0.5	6.2 ± 0.38
	GSI (%)	2.3 ± 0.16	2.4 ± 0.19	2.4 ± 0.21	2.1 ± 0.2	2.4 ± ±0.13
	LSI (%)	3.3 ± 0.26	3.4 ± 0.22	3.7 ± 0.31	3.3 ± 0.4	3.8 ± 2.7
F	Length (mm)	91.7 ± 2.4	90.6 ± 2.2	91.3 ± 2.5	94.9 ± 2.8	90 ± 1.4
	Weight (g)	8.2 ± 0.64	7.9 ± 0.63	7.6 ± 0.51	8.2 ± 0.88	7.5 ± 1.3
	GSI (%)	8.1 ± 0.58	8.1 ± 0.57	7.4 ± 0.49	8.2 ± 0.49	7.7 ± 0.9
	LSI (%)	5.1 ± 0.38	5.5 ± 0.49	5 ± 0.35	5.2 ± 0.41	5.3 ± 0.36

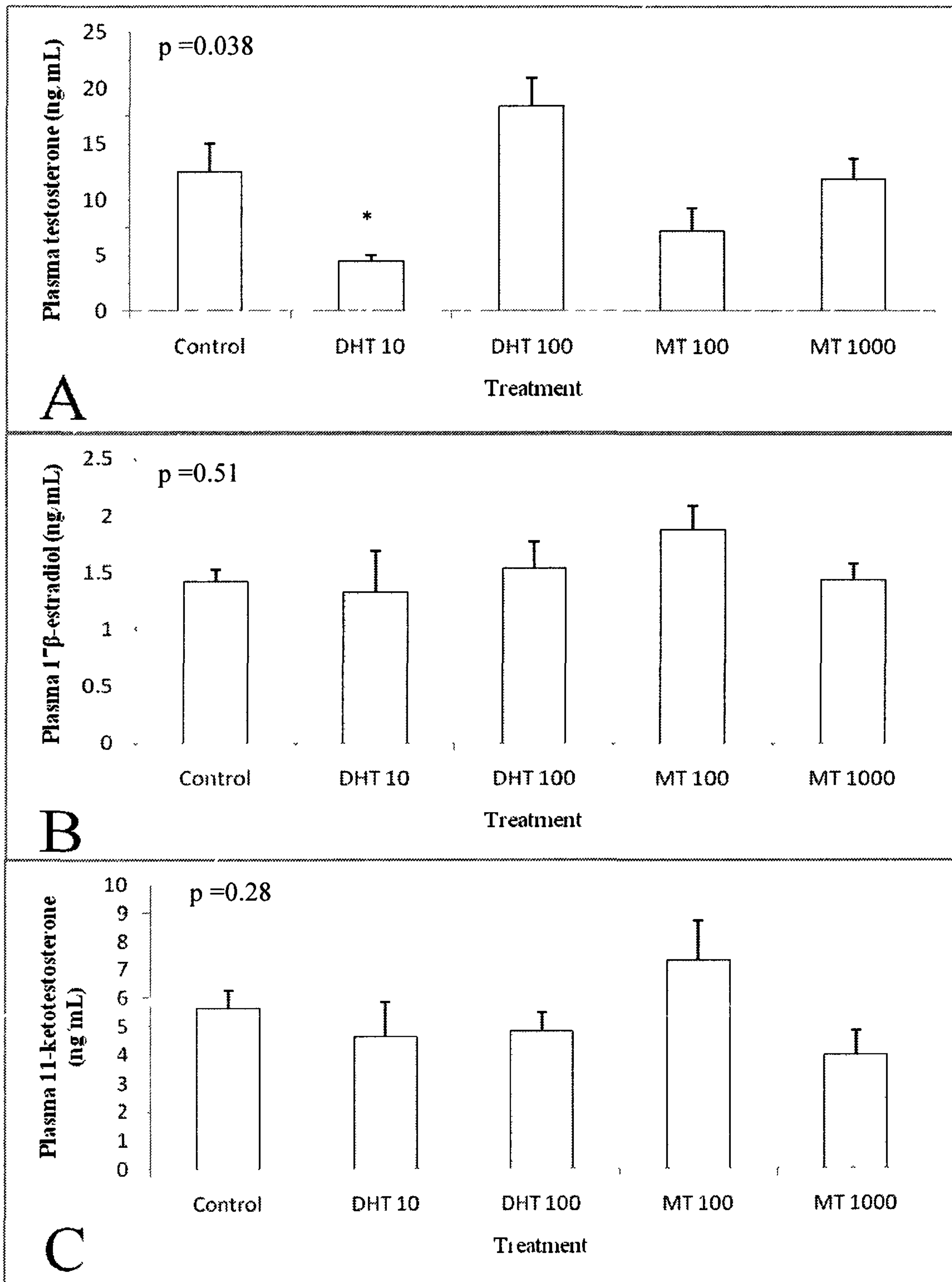

Figure 3.1 Mean (± 1SE) plasma testosterone (A), 17β-estradiol (B) and 11-ketotestosterone (C) levels (ng/ml) in male mummichog exposed to control (ethanol), non-aromatizable androgen dihydrotestosterone (concentrations of 10 µg/L (DHT 10) and 100 µg/L (DHT 100)) and aromatizable androgen methyltestosterone (concentrations of 100 ng/L (MT 100) and 1000 ng/L (MT 1000)) . Bars with an asterisk (*) are significantly different from control group.

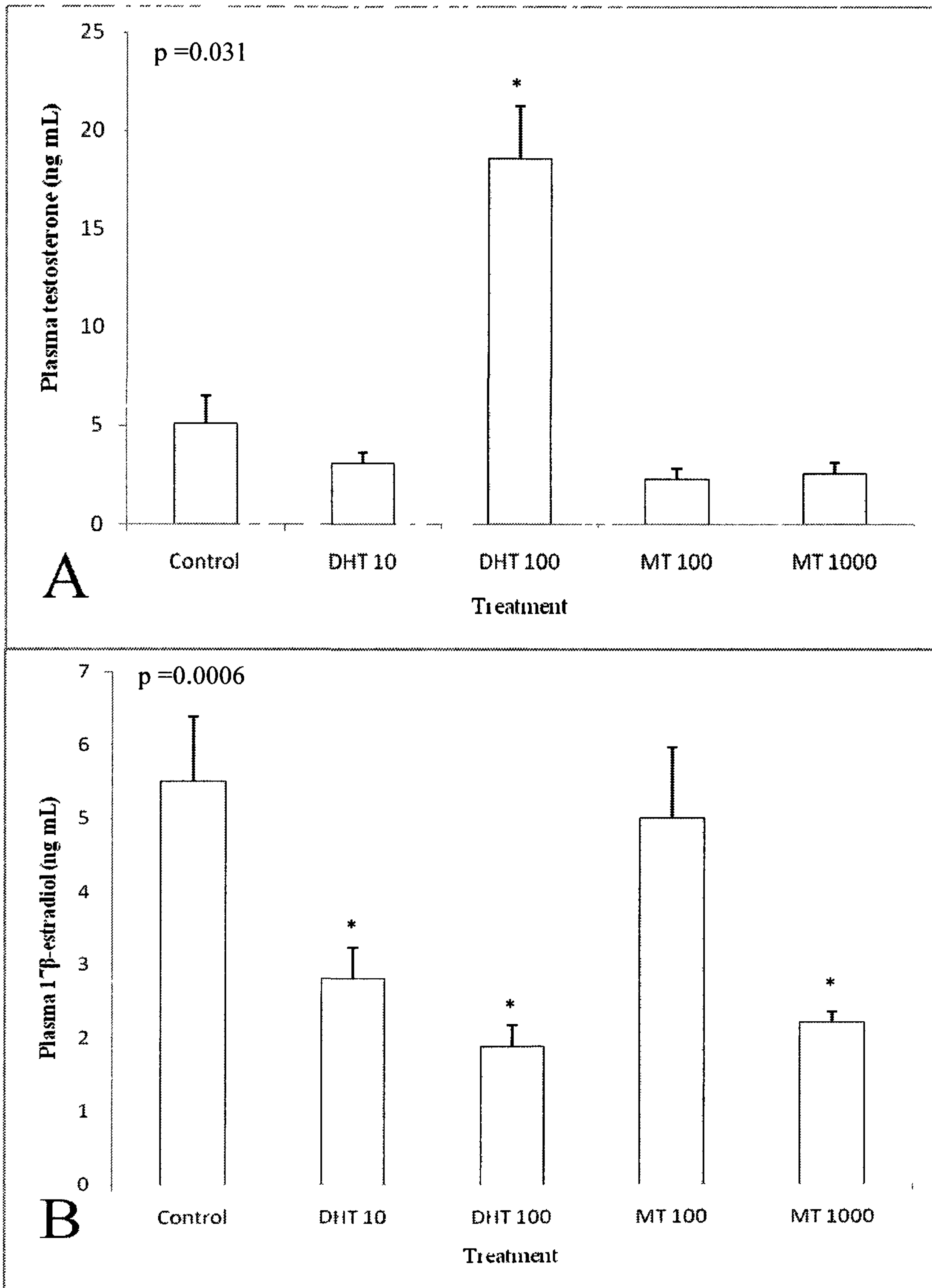

Figure 3.2 Mean (± 1SE) plasma testosterone (A) and 17β-estradiol (B) levels (ng/ml) in female mummichog exposed to control (ethanol), non-aromatizable androgen dihydrotestosterone (concentrations of 10 µg/L (DHT 10) and 100 µg/L (DHT 100)) and aromatizable androgen methyltestosterone (concentrations of 100 ng/L (MT 100) and 1000 ng/L (MT 1000)). Bars with an asterisk (*) are significantly different from control group.

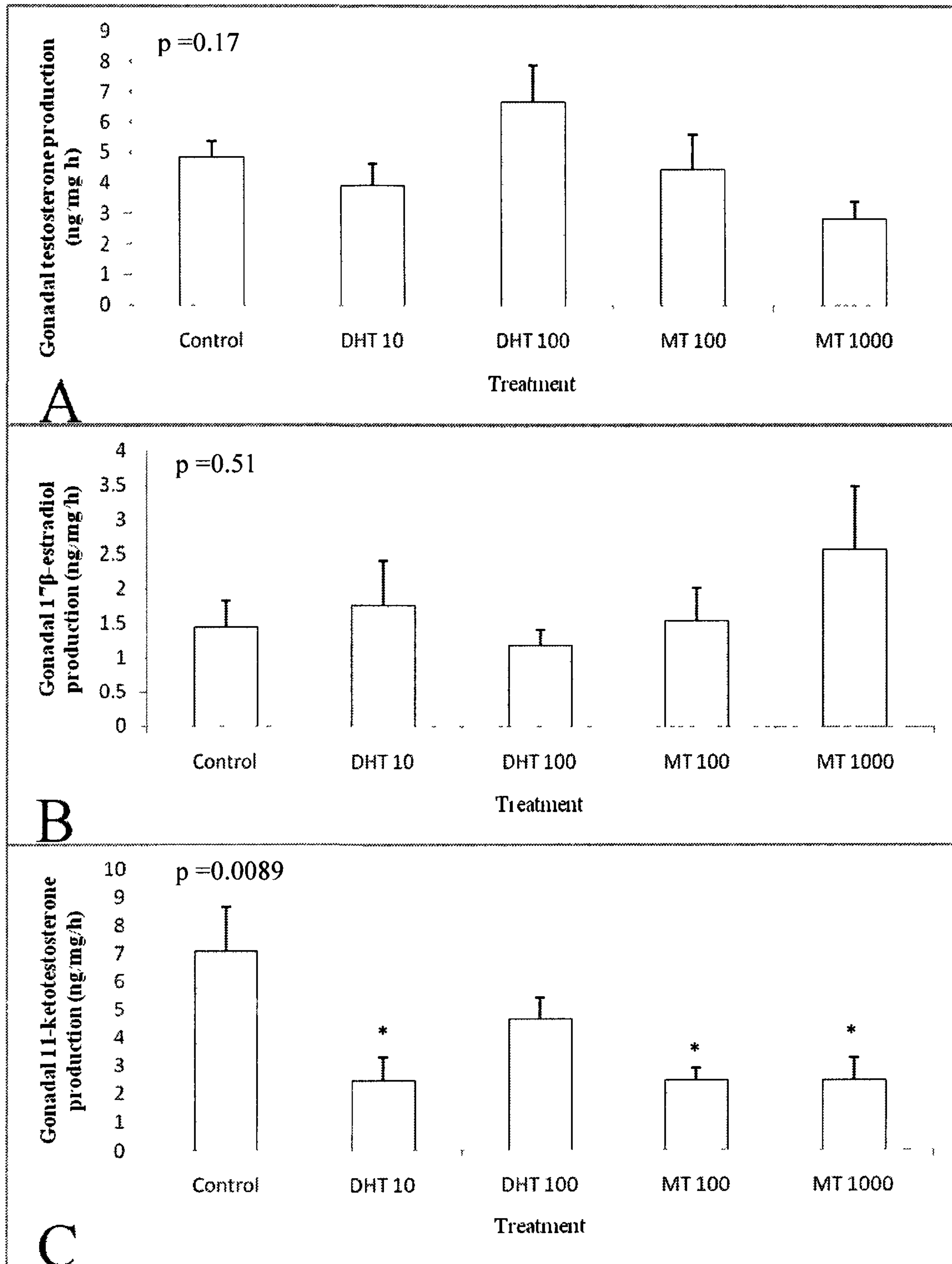

Figure 3.3 Mean (± 1SE) gonadal *in vitro* production of testosterone (A), 17β-estradiol (B) and 11-ketotestosterone (C) (ng/ml) in male mummichog exposed to control (ethanol), non-aromatizable androgen dihydrotestosterone (concentrations of 10 μg/L (DHT 10) and 100 μg/L (DHT 100)) and aromatizable androgen methyltestosterone (concentrations of 100 ng/L (MT 100) and 1000 ng/L (MT 1000)). Bars with an asterisk (*) are significantly different from control group.

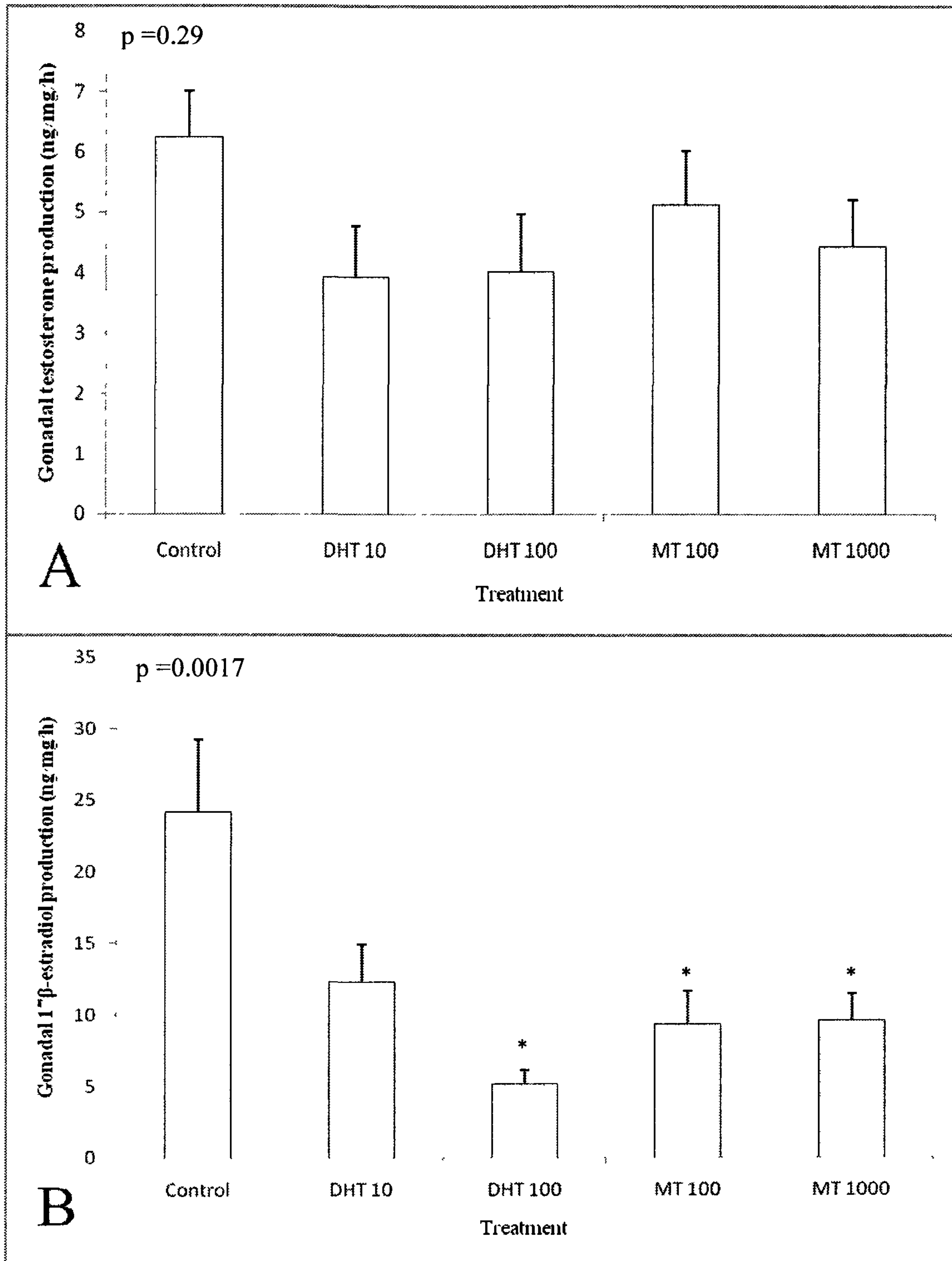

Figure 3.4 Mean (± 1SE) gonadal *in vitro* production of testosterone (A) and 17β-estradiol (B) (ng/ml) in female mummichog exposed to control (ethanol), non-aromatizable androgen dihydrotestosterone (concentrations of 10 μg/L (DHT 10) and 100 μg/L (DHT 100)) and aromatizable androgen methyltestosterone (concentrations of 100 ng/L (MT 100) and 1000 ng/L (MT 1000)). Bars with an asterisk (*) are significantly different from control group.

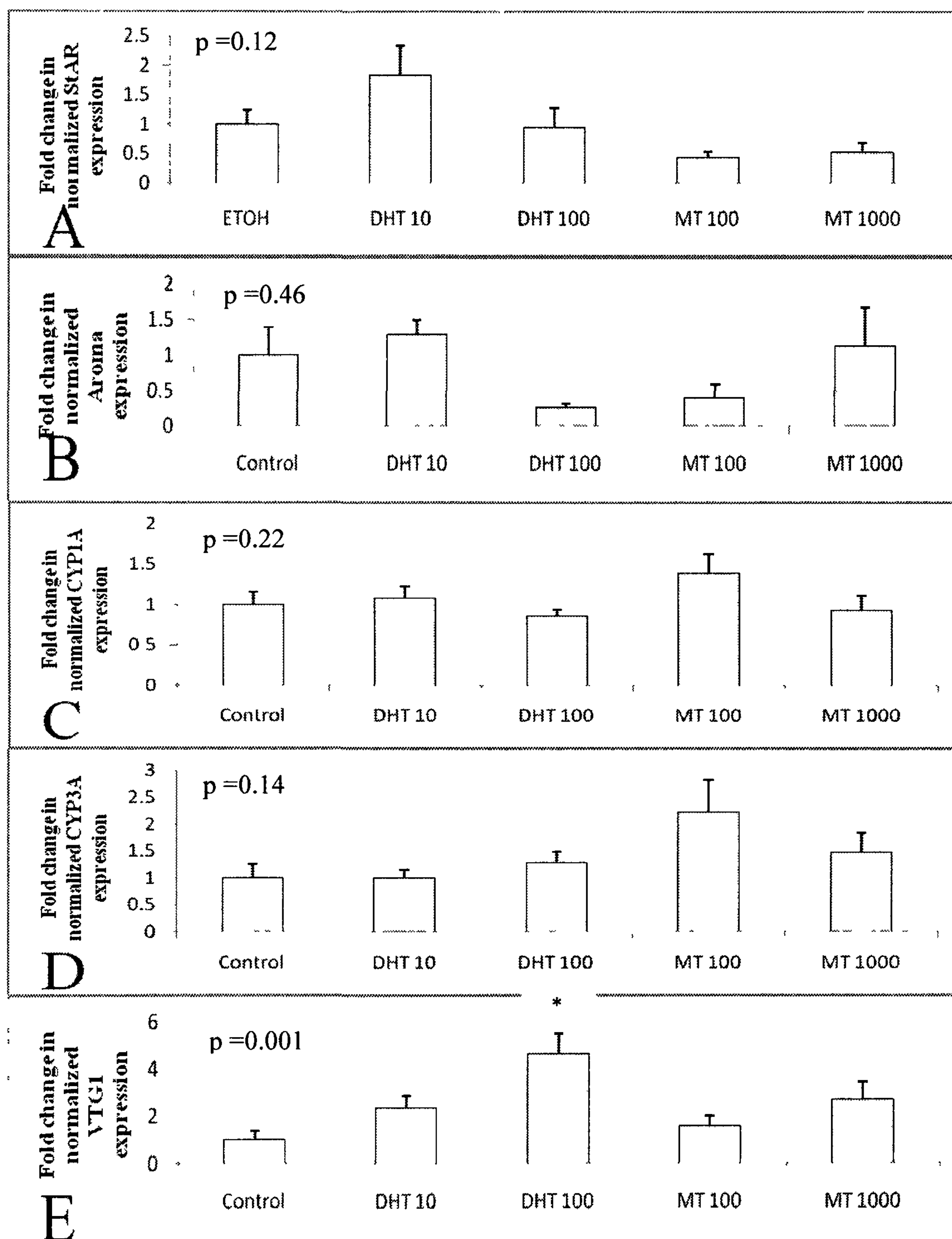

Figure 3.5 Gonadal expression (± 1SE) of steroidogenic acute regulatory protein (StAR) (A) and aromatase (B) and hepatic expression of cytochrome P4501A (CYP1A) (C), cytochrome P4503A (CYP3A) (D) and vitellogenin 1 (VTG1) (E) in male mummichog exposed to control (ethanol), non-aromatizable androgen dihydrotestosterone (concentrations of 10 μg/L (DHT 10) and 100 μg/L (DHT 100)) and aromatizable androgen methyltestosterone (concentrations of 100 ng/L (MT 100) and 1000 ng/L (MT 1000)). Expression levels were normalized to elongation factor 1-α (EF1α). Bars with an asterisk (*) are significantly different from control group.

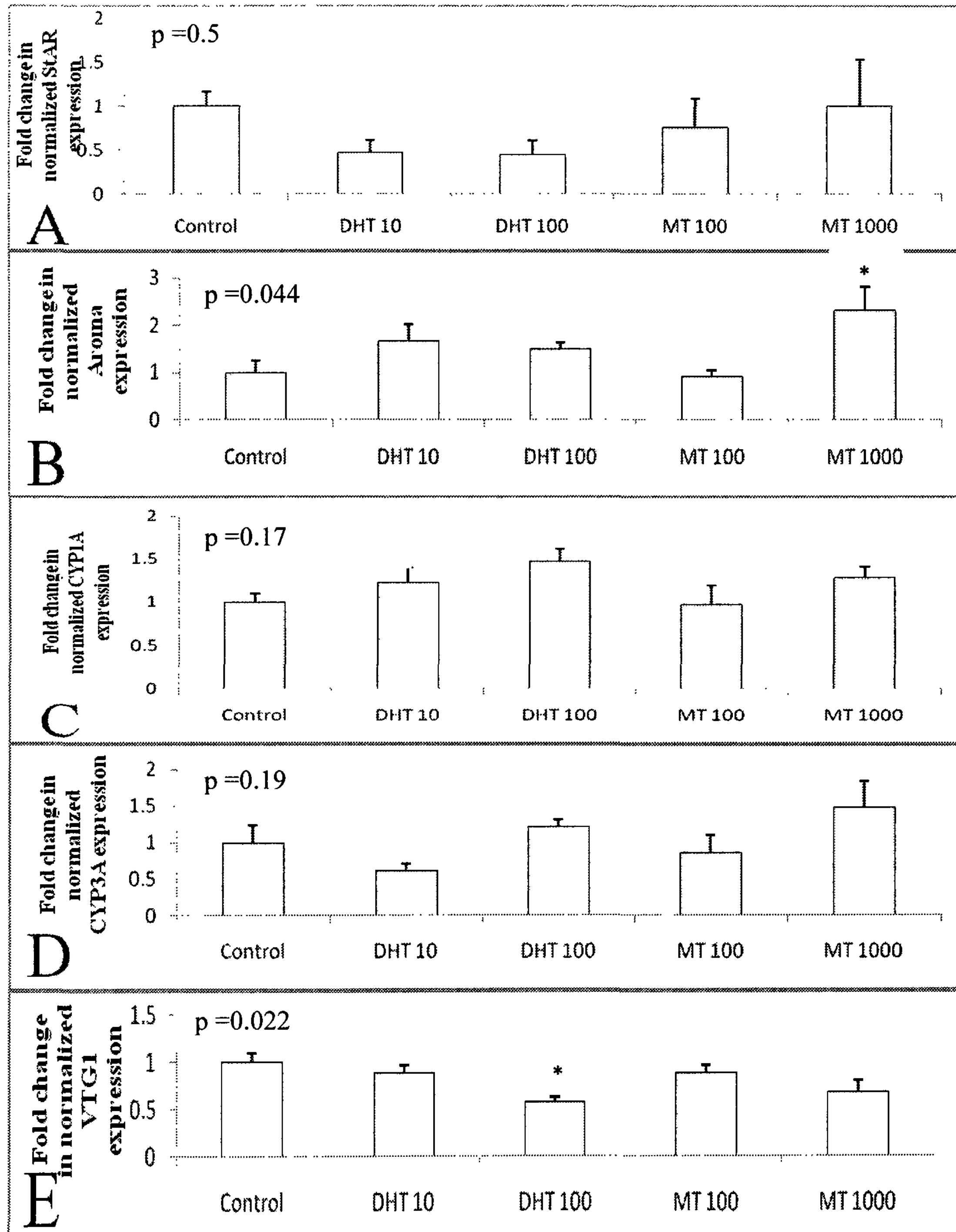

Figure 3.6 Gonadal expression (± 1SE) of steroidogenic acute regulatory protein (StAR) (A) and aromatase (B) and hepatic expression of cytochrome P4501A (CYP1A) (C), cytochrome P4503A (CYP3A) (D) and vitellogenin 1 (VTG1) (E) in female mummichog exposed to control (ethanol), non-aromatizable androgen dihydrotestosterone (concentrations of 10 μg/L (DHT 10) and 100 μg/L (DHT 100)) and aromatizable androgen methyltestosterone (concentrations of 100 ng/L (MT 100) and 1000 ng/L (MT 1000)). Expression levels were normalized to elongation factor 1-α (EF1α). Bars with an asterisk (*) are significantly different from control group.

3.5 Discussion

Mummichog responses to model androgens varied depending on androgen concentration and type (aromatizable vs. non-aromatizable). Fish exposed to DHT had greater responses than those exposed to MT. The potential aromatization of MT means that both potential estrogenic and androgenic effects could result from exposure. Female responses were more pronounced than male responses across all treatments, indicating that female mummichog are more susceptible to androgen perturbation compared to males. Gene expression was used to identify the potential mechanism of effect in the steroidogenic pathway. Significant induction of aromatase was found in ovarian tissue from fish exposed to MT 1000 treatment, contrary to reports in other fish species where androgen exposure causes a decrease in aromatase expression. Hepatic gene expression of vitellogenin 1 (VTG1) was increased in males and decreased in females exposed to DHT.

DHT was more potent than MT in causing changes in plasma steroid levels (Table 3.3). Plasma T in males was significantly depressed upon exposure to 10 μg/L DHT although there was no effect on plasma T levels in males exposed to 100 μg/L DHT. In females exposed to DHT there was a significant increase in plasma testosterone at a concentration of 100 μg/L. Findings from a study which exposed female zebrafish (*Danio rerio*) to various low concentrations of waterborne DHT (ranging from 0.1 to 4.9 μg/L) showed an increase in plasma testosterone production (Hoffman *et al.,* 2008), similar to what was seen in the current exposure. It was hypothesized that this increase was due to the reduction of expression of P450aromatase levels caused by DHT exposure (Li *et al.,* 2006). Both DHT treatments also depressed plasma 17β-estradiol levels in females in the current study. This depression of E_2 in female mummichog

exposed to DHT in the current study has been documented previously in zebrafish (Hoffman *et al.*, 2008).

Exposure to methyltestosterone did not significantly affect plasma testosterone in either sex (Table 3.3). Exposure of mummichog to high levels of waterborne MT (250 and 1000 ng/L) for seven days showed depressed plasma T and E_2 in females (Sharpe *et al.*, 2004). A follow-up study exposing fish to lower concentrations (1-100 ng/L) of MT for a longer duration (14 days) found significant depression of plasma T in females at concentrations of 10 ng/L and higher, however, only the bioassay of longer duration found significant impacts on male plasma steroids (Sharpe *et al.*, 2004). The reason for the lack of response from the current study compared to the work done by Sharpe *et al.* (2004) has not been identified, as in both experiments fish were naturally recrudescing, and method of exposure and exposure length were identical. Exposure concentrations for the current study were higher (100 and 1000 ng/L) than those used in the 14-day exposure by Sharpe *et al.* (2004) so it is unlikely it is related to exposure length, concentration or the bioassay protocol. Differences in the photoperiod between the current bioassay and Sharpe *et al.* (2004) may account for the differential responses. The current bioassay used 14 h L: 10 h D, whereas Sharpe (2004) had a photoperiod of 16 h L: 8 h D. The longer light period may have impacted gonadal state of mummichog, keeping them more developed compared to the current bioassay, which may impact results. Additionally stock solutions differed between the bioassays, with the current exposure dosing tanks with 100 µl of solution to achieve desired concentrations and Sharpe *et al.* adding 35 µl of stock to achieve treatment concentration. Fish were fed to satiation in the current exposure, but fed 3% of daily body weight in the previous study. These minor differences may account for some differential responses seen between the bioassays. The effect of potential degradation or loss of MT within

the aquaria, causing lack of responses, is unknown. The lack of response of plasma steroids in fish exposed to either concentration of MT may be due to the length of time the bioassay was conducted. The modes of action of this androgen on the hypothalamo-pituitary-gonadal (HPG) axis have been postulated to include receptor binding mechanisms, alteration of steroidogenesis or interference with endogenous hormone transport (Lister and Van Der Kraak, 2001; Sharpe *et al.,* 2004; Denny *et al.,* 2005), each of which are time-sensitive interactions. Identifying the length of exposure most appropriate for measuring changes in the reproductive endocrine system is challenging, especially as it is possible that compensatory mechanisms may be capable of resetting homeostatic levels of plasma steroids (Sharpe *et al.,* 2004).

Gonadal *in vitro* production of T and E_2 was not impacted by either MT or DHT exposure in male mummichog (Table 3.3). A concentration of 100 ng/L MT was sufficient to cause *in vitro* depression of T in male mummichog previously (Sharpe *et al.,* 2004). In the present study, 11-KT production was decreased at both concentrations of MT, and DHT 10. This confirms findings by Sharpe *et al.* (2004) that 100 ng/L MT depresses gonadal production of 11-KT. In the current study, *in vitro* incubations were done without the use of 3-isobutyl 1-methylxanthine (IBMX), a phosphodiesterase inhibitor. IBMX prevents cAMP breakdown, resulting in increased basal steroid production (McMaster *et al.,* 1995). IBMX was used in incubations for Sharpe *et al.* (2004), possibly enhancing basal steroid production. This may account for some differences in responses between the studies.

Contrary to other studies, *in vitro* testosterone production was not depressed in any treatment in female mummichog (Table 3.3). Female gonadal production of E_2 was depressed in both MT treatments, and DHT 100. Depression of E_2 production has been found in female

mummichog exposed to 100 ng/L MT (Sharpe *et al.,* 2004) and this study confirms this concentration as capable of depressing E_2.

Overall, whole organism endpoints were more influenced from exposure to DHT, while mechanistic endpoints were more altered by MT. Interaction between DHT and the HPG may be outside the gonads, while MT has demonstrated effects within gonadal cells. When sea bass (*Dicentrarchus labrax*) were implanted with DHT, the levels of follicle stimulating hormone (FSH) decreased 4- to 15-fold compared to control fish (Mateos *et al.,* 2001). Increased levels of luteinizing hormone (LH) within the pituitary, indicated that LH synthesis was under partial androgen control (Mateos *et al.,* 2001). With the depression of FSH, a key hormone controlling steroidogenesis, possible plasma steroid fluctuations may not necessarily be reflected in gonadal incubations due to feedback controls external to the gonad.

In the current study, there were no effects on StAR expression in any treatment (Table 3.3). This lack of response implies that cholesterol is mobilized at the same rate in fish exposed to androgens as in control fish although other proteins also mobilize cholesterol across the mitochondria in gonads (e.g., peripheral-type benzodiazepine receptor; Hauet *et al.,* 2002). Injection of the physiological androgen 11-KT into Atlantic cod (*Gadus morhua*) caused a dose-dependant response in StAR expression. Lower doses (0.05-0.5 mg/kg) caused a decrease in StAR expression, whereas higher doses ($\geq$ 5 mg/kg) had no effect on StAR expression compared to control (Kortner *et al.,* 2009). This suggests induction of negative feedback loops with higher androgen doses (Kortner *et al.,* 2009). Disruption of StAR may represent the first event in a series of event cascades associated with EDS-induced depressions of plasma hormones (Arukwe, 2008), however, the lack of response in StAR in the current study indicates other interactions with androgens are responsible for altering hormone levels.

Induction of VTG1 protein in male fish has been reported in other species exposed to androgens (Blum *et al.*, 2004) and is an indication that male fish are experiencing estrogenic endocrine disruption (Riley *et al.*, 2002). Estrogenic responses from androgenic substances are possible through aromatization of the androgen. In the current study, MT appears to be confirmed as an aromatizable androgen. Increases in plasma E_2, decreased 11-KT production and increased VTG1 expression in males exposed to MT is evidence for aromatization of MT. Depression of plasma E_2 in females is another indication of MT aromatization. These simultaneous estrogenic and androgenic effects are reported in other studies (Ankley *et al.*, 2000; Parrot and Wood, 2002) and are attributed to the successful aromatization of MT to 17α-methylestradiol (ME2; Simpson *et al.*, 1994). Increased plasma ME2 levels have been reported in fathead minnow (*Pimephales promelas*) exposed to MT for 7 days (Hornug *et al.*, 2004), with estrogenic effects being due to ME2 synthesis. As MT aromatization to ME2 has not been confirmed in mummichog, future exposures to MT should measure plasma ME2 levels.

In the current study, VTG1 was induced in males exposed to DHT 100 (Table 3.3). The cause of VTG1 induction in this treatment is unknown, as DHT is presumed to be non-aromatizable. There were no impacts on gonadal T or E_2 production for males in this treatment, and plasma steroid levels were unaltered compared to the control. Female mummichog exposed to DHT 100 concentrations had a significant reduction of VTG1 expression (Table 3.3), which can be an early indication of masculinisation (Li *et al.*, 2006). Females exposed to DHT 100 also had lower plasma and gonadal production levels of E2, indicating that DHT is non-aromatizable.

Induction of aromatase occurred in ovarian tissue from the MT 1000 treatment (Table 3.3). A 2.5-fold increase over control was observed in this treatment. DHT exposure caused 1.75-fold and 1.5-fold increases in ovarian aromatase expression for the lower and higher

concentrations, respectively, although these were not significant. Aromatase induction after exposure to model androgens has not been previously reported (Kitano *et al.,* 2000; Li *et al.,* 2006). There was no significant depression of aromatase in ovarian tissue in any treatment, contrary to many previous studies. Aromatase expression in testes was down-regulated by 60% in MT 100 and 70% in DHT 100 treatments, however, high variability in the control group meant these were not significant. The differential responses in aromatase expression between sexes may be due to gender-specific mode(s) of interaction of the androgens.

There is no correlation in the current study between aromatase expression and plasma or *in vitro* T or E_2 levels (Table 3.3). The upregulation of aromatase found in ovarian tissue from the MT 1000 treatment did not coincide with increased E_2 plasma levels or *in vitro* production. Levels of E_2 were actually significantly decreased in both of these endpoints. Additionally, levels of plasma and *in vitro* T were not altered in the MT 1000 group. These findings suggest that aromatase may not be the sole location of interaction in the steroidogenic pathway of exogenous androgens in mummichog. Exposure of fathead minnow (*Pimephales promelas*) to the model androgen flutamide induced 11β-hydroxysteroid dehydrogenase (11β-HSD), responsible for synthesis of 11-KT, indicating that androgens may have an inhibitory action on negative feedback pathways (Filby *et al.,* 2007). Additionally, MT exposure has induced expression of 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in zebrafish (*Danio rerio*) (Hoffman *et al.,* 2008). Increased expression of these genes may account for the unaltered level of *in vitro* testosterone production, as cholesterol is being metabolized to testosterone, and ultimately 11-KT, at a faster rate than it is metabolized to E_2. Future studies may wish to measure 11-KT in female gonadal cells as an indicator of early masculinisation.

There was no effect on CYP1A or CYP3A expression in fish exposed to either androgen (Table 3.3). The lack of response in these genes, responsible for hepatic drug metabolism and detoxification (Danielson, 2002), indicate that neither exogenous androgen was identified as requiring detoxification. The induction of both CYP genes has been found in mummichog exposed to waters polluted with polychlorinated biphenyls and halogenated aromatic hydrocarbons (Oleksiak, 2008), and is a sensitive indicator when fish are exposed to a range of environmental contaminants (Stegeman and Hahn, 1994).

Table 3.3 Summary of mummichog response to model androgens dihydrotestosterone (DHT) and methyltestosterone (MT). Symbols include: no observed effects (-), significant depression (↓) or significant induction (↑) compared to control. Endpoints measured include: testosterone (T), 17β-estradiol (E_2), 11-ketotestosterone (11-KT), gonadal steroidogenic acute regulatory protein (StAR), aromatase and hepatic cytochrome P450 genes 1 (CYP1A) and 3 (CYP3A) and vitellogenin 1 (VTG 1). 11-KT endpoints were not measured in females.

Sex	Treatment	Plasma T	Plasma E_2	Plasma 11-KT	*In vitro* T	*In vitro* E_2	*In vitro* 11-KT	StAR	Aromatase	CYP1A	CYP3A	VTG 1
Male	DHT 10 μg/L	↓	–	–	–	–	↓	–	–	–	–	–
	DHT 100 μg/L	–	–	–	–	–	–	–	–	–	–	↑
	MT 100 ng/L	–	–	–	–	–	↓	–	–	–	–	–
	MT 1000 ng/L	–	–	–	–	–	↓	–	–	–	–	–
Female	DHT 10 μg/L	–	↓		–	–		–	–	–	–	–
	DHT 100 μg/L	↑	↓		–	↓		–	–	–	–	↓
	MT 100 ng/L	–	–		–	↓		–	–	–	–	–
	MT 1000 ng/L	–	↓		–	↓		–	↑	–	–	–

Overall, female mummichog had greater responses in more endpoints than male mummichog when exposed to both model androgens. Plasma T and E_2, *in vitro* E_2 and VTG1 expression were all significantly decreased through exposure to specific androgens. Previous work in mummichog has shown that females are more responsive than males when exposed to MT, and have generally lower threshold levels for effects (Sharpe *et al.*, 2004). Higher threshold levels for males may make them more resistant to changes in endpoints when exposed to the same concentrations as female mummichog.

In summary, exposure to DHT and MT in a short-term bioassay altered steroid production in mummichog. Data indicate that aromatization of MT caused estrogenic as well as androgenic effects. Threshold levels of effects are much lower in females, possibly due to sex-specific modes of interaction between androgens and the reproductive endocrine system in mummichog. Gene expression analysis shows that aromatase was not significantly altered in most treatments and that other areas of the steroidogenic pathway or HPG axis must be impacted by androgen exposure to account for observed effects in plasma steroids. Future work should incorporate potential effects on gonadal enzyme activity and/or gene expression of genes such as 11β-HSD, 17β-HSD and 3β-HSD. Bioassay duration should be both shortened and extended to further elucidate time-sensitive endpoints such as altered plasma steroid levels and gene expression. The increased understanding about the effects of model androgens in the mummichog endocrine bioassay developed here provides valuable information to better understand the potential effects of more complex effluents that may contain androgenic compounds, such as agricultural runoff and PME.

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Chapter Four: General discussion and conclusion

4.1 Summary of results

Identification of specific endocrine-active chemicals or chemical compounds in pulp mill effluent (PME) has been hindered for numerous reasons. Variation of chemical constituents can occur on a weekly basis, and is influenced by tree species harvested, time of year of harvesting, soil composition during tree growth and many other factors (Christianson-Heiska *et al.*, 2008; Hewitt *et al.*, 2008). Effluent mixtures are highly complex and contain high levels of lignin, making isolation of small molecules difficult. Additionally, bioavailability of endocrine-active compounds, as well as specific uptake and metabolism of these compounds in fish, is not well understood (Munkittrick, 2004). A major challenge hindering this area of research is that fish responses are inconsistent after exposure to endocrine-disrupting substances (EDSs) within PME (Munkittrick, 2004). Despite these difficulties, many of which affected the studies in this thesis, progress was made in the goal of identifying bioactive compounds in Irving Pulp and Paper Ltd.'s 5th effect evaporator condensate waste stream.

The investigation of cause (IOC) studies at Irving Pulp and Paper Ltd. (IPP), a bleached kraft pulp mill in Saint John, New Brunswick, Canada, has been one of the first and most intensive studies to link an in-mill waste stream (5th effect evaporator condensates) with steroid depression in fish (Dubé and MacLatchy, 2001). Subsequent development of a two-stage solid phase recovery (SPE) method to isolate different fractions has been helpful in identifying chemical constituents of the condensates (Belknap *et al.*, 2006; Scott, 2010). *In vivo* bioassays using the early fractions derived from this SPE protocol found loss of bioactivity of the fractions during the fractionation process (Shaughnessy *et al.*, 2007). Development of a novel fractionation regime was then completed by C. Milestone, eliminating the steps in which bioactivity was lost in the previous protocol (Scott, 2010). As part of this thesis, two exposures

were conducted in 2009, a concentration-response bioassay (Exposure 1) and a fractionation bioassay (Exposure 2). Exposure 1 exposed mummichog to varying concentrations of whole condensates to confirm the minimal concentration at which reproductive responses were observed. This bioassay confirmed previous work that 4% (v/v) concentration of whole condensates elicited responses in mummichog (Shaughnessy *et al.,* 2007). Males exposed to this volume had depressed plasma testosterone, while females showed no response (Tables 4.1 and 4.2, respectively). Gene expression analysis was used to determine the potential locations of interactions between the gonad and the endocrine-active compounds in the RO feed. Lack of response in StAR and aromatase indicate that active compounds within the condensates interact at other loci within the steroidogenic pathway. Additionally, induction of both hepatic CYP1A and CYP3A in females (Table 4.2) at 2% and 4% (v/v) whole condensates, indicates that condensates contain certain chemical compounds that are identified as harmful and which the liver attempts to detoxify. From these findings, Exposure 2 was conducted with five chemically-distinct fractions derived from the new fractionation protocol (Scott, 2010) and a positive control of 4% (v/v) concentration of whole condensates. None of the fractions depressed plasma testosterone in either sex, however, the whole condensate treatment also had no effect on the reproductive steroids, making interpretation of biological activity in each fraction impossible.

The discrepancy between Exposure 1 and Exposure 2 responses to 4% (v/v) whole condensates could be attributed to either the different gonadal states of the fish during exposure or temporal fluctuations in the active substances themselves. Comparison of gonadosomatic indices between the exposures revealed that fish from Exposure 1 had more mature gonads. However, average plasma testosterone levels from control fish in Exposure 1 were comparable to control fish from Exposure 2. Additionally, past studies done with regressed mummichog have

found them to be responsive (Hewitt *et al.,* 2002; MacLatchy *et al.,* 2005). Previous work done with condensates has shown them to resist chemical degradation over a short period of time, so loss of activity is likely not due to chemical degradation during exposure (Shaughnessy *et al.,* 2007). Chemical fingerprinting was completed for condensates from both exposures, and minor variations in a number of chemical compounds were detected (Scott, 2010). Androgenic constituents of the two condensate batches also differed (Table 4.3; Scott, 2010). Condensate batches from both bioassays were fractionated according to the newly-developed protocol (Scott, 2010), and each fraction subjected to goldfish (*Carassius auratus*) androgen receptor (AR) binding assays to determine androgenic potential. Testosterone equivalents (TEQ), a measure of androgenic potency, was found to be highest in the fractionation bioassays condensate filter paper non-polar (FP-NP) fraction (Table 4.3). This was approximately seven times more androgenic than the same fraction in the concentration-response bioassay. Manool, one of the chemical compounds with detectable differences between the two exposures, was found to account for approximately 22-30% of total androgenic activity (Scott, 2010). Because variability in the chemical make-up of the condensates was linked to differences in bioactivity (binding to androgen receptors *in vitro*), it was determined that studies on model androgen effects in mummichog were warranted at this stage in the condensate IOC studies.

In May 2010, a third reproductive endocrine bioassay was undertaken to assess the effects of the model androgens methyltestosterone (MT) and dihydrotestosterone (DHT). The objectives of this bioassay were to determine mummichog responsiveness to androgen exposure, determine if aromatizable androgens elicit different effects than nonaromatizable androgens, and compare responses seen in fish exposed to model androgens with those exposed to pulp mill condensates. Mummichog responses were varied, with DHT eliciting more reproductive impacts

than MT. The most impacted steroid was 17β-estradiol in both plasma and *in vitro* analysis (Table 4.2). Female mummichog were more sensitive to androgen exposure than males, confirming the hypothesis that females have a lower effects-threshold to androgens (Sharpe *et al.*, 2004). Mechanistic investigations found that aromatase was not altered after exposure to either androgen, indicating that interaction between the steroidogenic pathway and androgens must be at another location (Table 4.1 for males, 4.2 for females). Neither CYP1A nor CYP3A were induced in any treatment compared to control, indicating that exogenous androgens are not identified as requiring detoxification (Table 4.1 for males, 4.2 for females).

Comparison of whole condensates and model androgens is hampered due to the potential of interaction between chemicals within condensates, which may enhance or mask effects which would be observed during exposure to model compounds. However, responses to key reproductive endpoints allow preliminary comparison between mummichog exposed to whole condensates and those exposed to model androgens. Plasma testosterone depression is the major indication of exposure to whole condensates in mummichog, however exposure to model androgens primarily impacts plasma and *in vitro* 17β-estradiol. The induction of CYP1A and CYP3A occurred in fish exposed to whole condensates, but no induction was found in fish exposed to model androgens. This indicates that the chemical compounds within condensates require transformation and detoxification, while model androgens are not similarly identified. Additionally, male mummichog are affected by condensate exposure, while females have greater sensitivity to androgens than males.

Overall, as shown through androgen receptor binding assays, condensates do possess androgenic compounds (Scott, 2010), but these compounds may not be the bioactive compounds responsible for endocrine effects in mummichog exposed to condensates. Two pieces of

evidence support this: (1) the condensates and model androgen responses in mummichog do not parallel each other (Tables 4.1 and 4.2); and (2) the condensates with greater androgenicity content (Scott, 2010; Exposure 2) did not cause reproductive endocrine effects in fish, while the exposure with lesser androgen content (Exposure 1) did (Table 4.3).

Table 4.1 Summary of reproductive responses of male mummichog exposed to whole condensates (WC), chemically distinct fractions isolated from whole condensates (FP-NP = filter paper non-polar, FP-P = filter paper polar, SPE-NP = solid phase extraction non-polar, SPE-P = solid phase extraction polar) or model androgens dihydrotestosterone (DHT) and methyltestosterone (MT). Symbols include: no observed effects (-), significant depression (↓) or significant induction (↑) compared to control. Endpoints measured include: testosterone (T), 17β-estradiol (E_2), 11-ketotestosterone (11-KT), gonadal steroidogenic acute regulatory protein (StAR), aromatase and hepatic cytochrome P450 genes 1 (CYP1A) and 3 (CYP3A) and vitellogenin 1 (VTG 1). Original data from Shaughnessy *et al.* (2007) and Chapters two and three of this thesis. Shaded grey areas indicate endpoints for which there is no data.

Bioassay	Plasma T	Plasma E	Plasma 11-KT	*In vitro* T	*In vitro* E	*In vitro* 11-KT	StAR	Aromatase	CYP1A	CYP3A	VTG 1
Shaughnessy et al. (2007)											
0.5 % WC	-										
1% WC	↓										
2% WC	-										
4% WC	↓										
Concentration-response (Ch. 2)											
2% WC	-	-	-	-	-	-					
4% WC	↓	-	-	-	-	-					
Fractionation (Ch. 2)											
4% WC	-	-	-	-	-						
FP-NP	-	-	-								
FP-P	-	-	-								
SPE-NP	-	-	-								
SPE-P	-	-	-								
RC	-	-	-								
Model Androgen (Ch. 3)											
DHT 10 μg/L	↓	-	-	-	-	↓	-	-	-	-	-
DHT 100 μg/L	-	-	-	-	-	-	-	-	-	-	↑
MT 100 ng/L	-	-	-	-	-	↓	-	-	-	-	-
MT 1000 ng/L	-	-	-	-	-	↓	-	-	-	-	-

Table 4.2 Summary of reproductive responses of female mummichog exposed to whole condensates (WC), chemically distinct fractions isolated from whole condensates (FP-NP = filter paper non-polar, FP-P = filter paper polar, SPE-NP = solid phase extraction non-polar, SPE-P = solid phase extraction polar) or model androgens dihydrotestosterone (DHT) and methyltestosterone (MT). Symbols include: no observed effects (-), significant depression (↓) or significant induction (↑) compared to control. Endpoints measured include: testosterone (T), 17β-estradiol (E_2), gonadal steroidogenic acute regulatory protein (StAR), aromatase and hepatic cytochrome P450 genes 1 (CYP1A) and 3 (CYP3A) and vitellogenin 1 (VTG 1). Original data from Shaughnessy *et al.* (2007) and Chapters two and three of this thesis. Shaded grey areas indicate endpoints for which there are no data.

Bioassay	Plasma T	Plasma E	*In vitro* T	*In vitro* E	StAR	Aromatase	CYP1A	CYP3A	VTG 1
Shaughnessy et al. (2007)									
0.5 % WC	-								
1% WC	-								
2% WC	-								
4% WC	-								
Concentration-response (Ch. 2)									
2% WC	-	-			-	-	↑	↑	
4% WC	-	-			-	-	↑	↑	
Fractionation (Ch. 2)									
4% WC	-	-							
FP-NP	-	-							
FP-P	-	-							
SPE-NP	-	-							
SPE-P	-	-							
RC	-	-							
Model Androgen (Ch. 3)									
DHT 10 μg/L	-	↓	-	-	-	-	-	-	-
DHT 100 μg/L	↑	↓	-	↓	-	-	-	-	↓
MT 100 ng/L	-	-	-	↓	-	-	-	-	-
MT 1000 ng/L	-	↓	-	↓	-	↑	-	-	-

Table 4.3 Summary of endocrine-related responses in mummichog from concentration-response and fractionation bioassays conducted in this thesis. Comparison of androgenic equivalents (testosterone equivalents (TEQ)) of condensates achieved through AR binding assay (Scott, 2010). Symbols include no observed effect (-) and significant depression (↓) compared to control.

	Current Thesis Effects on plasma testosterone endpoints		Scott (2010)
Bioassay	Male	Female	TEQ (ng/L)
Concentration-response	↓	–	
FP-NP			~ 100,000
FP-P			–
SPE-NP			~ 4,200
SPE-P			–
Fractionation	–	–	
FP-NP			~ 700,000
FP-P			–
SPE-NP			~ 10,500
SPE-P			–

4.2 Future Work

Future work can focus on two distinct objectives: 1) furthering bioassay-directed fractionation to isolate and characterize endocrine-active compounds from IPP condensates and 2) increasing knowledge of impacts of model androgens on mummichog.

To pursue objective one, reconfirmation of the minimum concentration of whole condensates required to elicit reproductive changes in mummichog may be required. Past work has shown that IPP condensates decreased in potency between the late 1990's and the mid 2000's (Shaughnessy *et al.,* 2007). In the months between the current concentration-response bioassay, which confirmed 4% (v/v) whole condensates as capable of depressing plasma testosterone, and the fractionation bioassay, a decrease in potency occurred confirming that temporal variability in the condensate constituents continues to be a challenge with identifying the endocrine-active compounds. However, variability could be a valuable investigative tool going forward as it allows testing of condensates with differences. Future work should aim to undertake biological studies with condensates of known chemical constituents.

Objective two can be furthered by varying exposure lengths (< 14 days and > 14 days) to fully encompass all possible changes within the hypothalamo-pituitary-gonadal axis. Determination of manool effects on the mummichog reproductive endocrine system should be undertaken to determine if this androgen is a possible endocrine-active component of pulp mill condensates. Manool was shown in Scott (2010) to be responsible for up to 33% of the androgenicity of IPP condensates. A bioassay exposing mummichog to varying concentrations of manool will help elucidate if the androgenic properties of manool are linked to reproductive effects in fish. Additionally, the linkage between steroid hormone depression in mummichog and

the *in vitro* goldfish AR binding assay results have not yet been substantiated. Development of an AR binding assay for mummichog would aid in establishing links between androgenic compounds and *in vivo* hormone depression in mummichog.

4.3 Reflections on thesis as "integrative biology"

Measured endpoints incorporated multiple levels of biological organization, from the molecular (gene expression) to the whole organism (e.g., plasma steroids, gonad size), involving a broad spectrum of protocols and tools designed to elucidate mechanistic responses in mummichog exposed to condensates or model compounds. The thesis included collaboration with a chemistry lab at Environment Canada, and with another reproductive physiology lab at University of Guelph. The findings within this thesis can be integrated into the broader fields of toxicology, reproductive biology and conservation biology for future collaborations aimed at improving the health of Canadian water systems.

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