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The effects of 17α-ethynylestradiol (EE_2) on gonadal development and differentiation in the estuarine killifish, *Fundulus heteroclitus*

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Abstract

Endocrine disrupting substances (EDSs) comprise a wide variety of chemicals that perturb normal endocrine function including developmental and reproductive processes in vertebrates. The synthetic estrogen 17α-ethynylestradiol (EE_2) is a commonly-used model EDS because of its environmental relevance and its effects on the reproductive endocrine system. Early life-stage exposure of fish to estrogenic EDSs causes effects such as intersex (ovotestes in males) and feminization. This study aims to confirm the period of gonadal differentiation in the estuarine killifish or mummichog (*Fundulus heteroclitus*) and to determine the sensitivity of gonadal development to EE_2. Artificially-regressed mummichog were spawned, and fertilized eggs were collected and exposed to EE_2 (0, 10, 50 and 250ng/L) within 8 h of spawning. Embryos and larvae were continually exposed in petri dishes and beakers (26˚C) and sampled weekly from hatch date to 10 weeks post-hatch (wph) to determine histologically the sensitivity of gonadal development to EE_2. Exposure to EE_2 (10-250ng/L) resulted in a concentration-dependent increase in skeletal abnormalities and mortalities; larval lengths proved insensitive to EE_2 exposure. Complete gonadal differentiation in mummichog occurred by 3 wph in control groups, whereas exposure to EE_2 accelerated gonadal differentiation as early ast 1 wph in all EE_2 exposed groups. Sex ratios were skewed (>80% female phenotype) within all groups treated with EE_2. This study demonstrates that early onset of exposure elicits effects on developing mummichog as exposure of EE_2 prior to establishment of endogenous differentiation pathways influenced and altered sex differentiation, resulting in feminized groups of fish at environmentally-relevant and higher concentrations.

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Chapter 1

General Introduction

1.1 Endocrine-Disrupting Substances

Endocrine-disrupting substances (EDSs), comprise exogenous substances or mixtures that are able to disrupt the function(s) of the endocrine system in organisms, their progeny and/or populations. EDSs can elicit responses even at low concentrations and are of increasing concern in the environment (Damstra et al., 2002; Dietrich and Krieger, 2009). These substances alter endocrine function by mimicking, altering, or inhibiting the action of endogenous hormones responsible for endocrine homeostasis (Zillioux et al., 2001). EDSs have been found to occur in industrial wastes, agricultural runoff, and municipal wastewater effluents (Sumpter and Jobling, 1995; Jobling et al., 1998; Routledge et al., 1998; Desbrow et al., 1998). For example, a component of the birth control pill (17α-ethynylestradiol; EE_2; a synthetic estrogen and demonstrated EDS) enters aquatic ecosystems via sewage discharge and is found at low parts per trillion concentrations (<5 ng/L; Larsson et al., 1999; Palace et al., 2006). Environmental EDSs have been shown to disrupt developmental, endocrine and reproductive processes in the laboratory and in the field. Endocrine disruptions in aquatic species can result in altered reproductive endocrine status (Leblanc et al., 1997; MacLatchy et al., 2003) and secondary sex characteristics (Parrott et al., 2004), reduced gonad size and egg production (Parrott et al., 2004), as well as developmental abnormalities during early life stages (Maack and Segner, 2004; Boudreau et al., 2004; 2005). Results from field studies by Kidd et al. (2007) on fathead minnow (*Pimephales promelas*) showed that whole-lake exposure to EE_2 at 5-6 ng/L decreased the reproductive success and sustainability of fish populations.

For the most part, bioassays on the effects of EDSs have been developed for freshwater fish, with fewer studies on marine and estuarine species (Boudreau et al., 2005). Estuaries are unique areas that receive both industrial (Durhan et al., 2002) and municipal (Desbrow et al., 1998) effluents. Effects of EDSs on marine and estuarine species may differ from those on freshwater fish because of differences that may occur in biological availability of the contaminants based on physical water chemistry and fish physiology (Peters et al., 2010). For this reason, EDS studies focusing on estuarine species are necessary as these areas serve as breeding and nursery grounds for many teleost fish (Boudreau et al., 2005).

EDSs have the potential to cause significant effects on fish development. The reproductive steroids are critical in the development of gonads into either testes or ovaries (Yamamoto, 1969; Dietrich and Krieger, 2009). The precise period of development at which differentiating gonads are exposed to steroids has major implications. This time period is particularly relevant as even the shortest exposure of the developing teleost to EDSs may have irreversible effects on the sex and reproductive state of the individual (Maack and Segner, 2004), which may over time have adverse effects on the entire fish population.

Various researchers have shown that teleost fish are sensitive to contaminants during their early life stages of development (Van Aerle et al., 2002; Örn et al., 2003; Boudreau et al., 2004; Maack and Segner, 2004; Peters et al., 2010). Mechanisms by which these chemicals cause their effects are not well understood. Therefore, baseline studies are required to begin establishing the mechanistic targets of these chemicals and their effects on the developing teleost.

1.2 Mummichog (*Fundulus heteroclitus*)

Mummichog (*Fundulus heteroclitus*) has been developed as a model fish species for EDS studies (MacLatchy et al., 2003; Peters et al., 2007; Burnett et al., 2007; Bosker et al., 2009). This species has a long history of use in ecological and physiological research (Antz, 1986). It is a small bodied, (semi-) lunar and/or temperature-dependent, asynchronous-spawning teleost fish that inhabits a large geographical range along the east coast of North America, extending from Florida, USA, to Newfoundland, Canada (Armstrong and Child, 1965; Burnett et al., 2007; McMullin et al., 2009). Mummichog are commonly found in coastal salt marshes and creeks that experience wide fluctuating levels of salinity, oxygen, pH and temperature (Burnett et al., 2007). They are at risk of exposure to EDSs throughout their lifecycle due to the release of effluents into their environment. As well, their sensitivity to hormonally-active substances has influenced their selection as a fish model to study EDSs (MacLatchy et al., 2003; Sharpe et al., 2004; Boudreau et al., 2004; 2005; Peters et al., 2010).

1.3 Fish Reproductive Endocrinology

In vertebrates, reproduction is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis is an interaction between the hypothalamus, which produces and secretes gonadotropin releasing hormone (GnRH), the pituitary gland, which is responsible for the production and secretion of the gonadotropic hormones (GtHs), and the gonads, responsible for the production and secretion of the sex steroid hormones (Bieniarz and Epler, 1992; Nagahama, 1994; Dietrich and Krieger, 2009). GnRH does not travel from the hypothalamus to the anterior pituitary via the hypophyseal portal system

(as found in mammals) but rather, through direct innervations between the pituitary and hypothalamic neurons; thus, stimulating the gonadotropes to release GtHs (Dietrich and Krieger, 2009). The primary stimuli that regulate these feedback mechanisms are environmental cues, such as photoperiod and temperature (Jin et al., 2009). These cues stimulate various sensory receptors including those in the pineal gland and are integrated within the brain to initiate and regulate reproductive endocrine signalling (Watanabe, 2009).

The GtHs are found in two distinct forms, follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH is responsible for gonadal growth and gametogenesis. LH is responsible for gonadal maturation and spawning (Kawauchi et al., 1989). LH targets the gonads (granulocytes in females, Leydig cells in males), where it acts to stimulate the production of the sex steroids: estrogens, androgens and progesterone. 17β-estradiol (E_2) is the main estrogen in females, whereas testosterone (T) and 11-ketotestosterone (11-KT) are the main androgens in males (Borg, 1994; MacLatchy and Van Der Kraak, 1995). Production of these steroid hormones is mediated by the conversion of cholesterol and intermediate steroids via a process known as steroidogenesis (Leusch and MacLatchy, 2003).

Steroidogenesis is a multi-step biosynthetic pathway involving a number of intermediates and enzymatic conversions to produce the final product(s), the sex steroid hormones (Leusch and MacLatchy, 2003). The pathway is dependent on the delivery of the substrate, cholesterol, from the cytoplasm, across the mitochondrial membrane, to the inner mitochondrial membrane (IMM), referred to as cholesterol mobilization. This process requires the use of a membrane transporter such as steroid acute regulatory

protein (StAR) to move cholesterol across the membrane (Melamed and Sherwood, 2005). Once in the IMM, the cytochrome P450 side-chain cleavage enzyme (P450scc) hydroxylates carbons 20 and 22 and removes a six-carbon residue side chain (C22-C27), giving rise to pregnenolone. Pregnenolone is then the precursor for the synthesis of the steroid hormones T, E_2 and 11-KT (Melamed and Sherwood, 2005) via a multi-step enzymatic process. The movement of cholesterol and P450scc conversion are the presumed rate-limiting step of the steroidogenic pathway in mammals (Sugawara et al., 1997; Stocco, 2000); however, this may not be true in fish (Nakamura et al., 2005).

1.4 Effects of Estrogenic EDSs on Reproductive Endocrine Status

Estrogenic EDSs have the ability to disrupt the steroidogenic pathway, resulting in altered hormone production (Hogan et al., 2010), by closely mimicking the structure of E_2 and binding to its receptors. Researchers studying 17β-sitosterol, a phytoestrogen in pulp mill effluents, have illustrated steroid depression in exposed fish (MacLatchy and Van Der Kraak, 1995; Gilman et al., 2003). This depression was explained by the inhibition of cholesterol mobilization within the steroidogenic pathway (Leusch and MacLatchy, 2003) and further supported by the decrease in transcript levels of StAR protein (Sharpe et al., 2007). There has been increasing evidence suggesting that estrogens have inhibitory effects on the steroidogenic pathway (MacLatchy and Van Der Kraak, 1995; MacLatchy et al., 1997; Leusch and MacLatchy, 2003; Hogan et al., 2010).

Estrogens exhibit their effects via receptor-mediated processes. For instance, synthetic estrogen will mimic and compete with endogenous E_2 for binding to the estrogen receptor (ER; Hogan et al., 2010). Binding of estrogen, whether synthetic or

naturally-produced, to the ER will allow transcription of the estrogen-responsive genes and their translation into proteins (e.g., vitellogenin; MacLatchy et al., 2003; Hogan et al., 2010). While most vertebrates have two subtypes of ER, ERα and ERβ, teleost fish have three, one ERα and two ERβ`s (Hogan et al., 2010). These three subtypes are encoded by different genes and exhibit different tissue patterns (Hogan et al., 2010). Results by Urushitani et al. (2002) indicate that treatment of mummichog with exogenous estrogens induces one of the three ER receptors, specifically, ERα.

1.5 Sexual Differentiation and Gonadal Development

Understanding sexual differentiation and gonadal development is crucial for research involving EDSs as these endocrine-active substances have been found to disrupt developmental processes in various species such as fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), zebrafish (*Danio* rerio) and mummichog (Nimrod and Benson, 1998; Van Aerle et al., 2002; Örn et al., 2003; Boudreau et al., 2004; 2005; Peters et al., 2010). Two key events are responsible for gonadal development of each individual, which are i) sex determination and ii) sex differentiation. Sex determination according to Piferrer (2001) is defined as the set of genes responsible for the formation of the gonads. Dietrich and Krieger (2009) defined sexual differentiation as the process by which the genetic information is phenotypically expressed. In other words, it involves a series of events where the primordial undifferentiated gonads differentiate into their respective genetically-encoded testes or ovaries.

Early differentiation in teleost fish begins within the primordial gonad (primary gonad, composed of both somatic and germ cells). It is a process which involves a series

of events that include the migration of the primordial germ cells along with the formation of the gonadal ridges, followed by the differentiation of the gonad into its respective testis or ovary, dependent on the encoded genotype (Nakamura, 1998; Piferrer, 2001; Strüssmann and Nakamura, 2002; Dietrich and Krieger, 2009). Research on gonadal differentiation and development of teleost fish to date has determined that gonads of female individuals differentiate sooner than those of males (Piferrer, 2001; Strüssmann and Nakamura, 2002); however, the explanation for this remains unknown. Fathead minnow sexual differentiation in females occurs as early as 10-25 days post hatch (dph), whereas it begins at 90 dph for males (Van Aerle et al., 2004).

Sexual differentiation may occur through two pathways in fish (Piferrer, 2001). The first pathway involves differentiation directly from the primordial gonad into either a testis or ovary. The second pathway involves differentiation of all gonads into an ovary-like gonad; later, half of the fish undergo sexual reversal with degenerating oocytes evident along with an increase in number of stromal cells which leads to formation and maturation of testes as seen in species such as zebrafish (Piferrer, 2001; Örn et al., 2003). Mummichog gonadal differentiation has been suggested to follow the first pathway as reported by Shimizu et al. (2008). Gaps remain in the literature as to the critical period of differentiation and the mechanistic processes involved, even in well-studied fish species.

1.6 Role of Sex Steroids in Gonadal Differentiation

It has long been established that the brain-pituitary-gonad axis is intact during sexual differentiation in fish and that GnRH, FSH, and sex steroid hormones to the greatest degree, fluctuate during this period in development (Feist and Schreck, 1996).

Genes responsible for steroid biosynthesis are expressed differently in somatic cells of testes than ovaries resulting in the production of sex-specific steroid hormones (Nakamura et al., 1998). Testosterone in fish is a hormone that is not directly involved in the mechanism of sex differentiation; however, it plays a critical role as a precursor of both 11-KT and E_2 (Nakamura et al., 1998; Baroiller et al., 1999). 11-KT and E_2 have direct effects on germ cell development as the former is the major androgen which regulates development of testes in fish, whereas, the latter is responsible for inducing and maintaining ovarian development (Devlin and Nagahama, 2002; Sandra and Norma, 2010). 11-KT triggers Sertoli cells to synthesize activin β_B (a protein complex) which binds to type I and II receptors on spermatogonia A leading to the initiation of mitosis, generating spermatogonia B, thereby inducing premeiotic spermatogonial proliferation (Ge et al., 1997; Nagahama et al., 1997; Dietrich and Krieger, 2009). E_2, on the other hand, has been shown to induce feminization by either enhancing gonadal differentiation by increasing aromatase (grey mullet; *Mugil cephalus*; Chang et al., 1999) or repressing activity of genes associated with development of testes (e.g., 11β-hydroxylase; rainbow trout, *Oncorhynchus mykiss*; Govoroun et al., 2001).

These sex steroids bring about their actions by binding to steroid-specific receptors (Sandra and Norma, 2010). ERs and androgen receptors (ARs) have been detected in early fish gonads and receptor-mediated mechanisms are the presumed modes of action of steroids on gonadal differentiation (Fitzpatrick et al., 1994; Chang et al., 1999; Devlin and Nagahama, 2002; Hossain et al., 2008; Sandra and Norma, 2010). ARs are controlled by the presence of androgens (e.g., 11-KT), whereas, ERs are positively controlled in developing fish by the presence of estrogens (e.g., E_2; Sandra and Norma,

2010). This positive control ensures sufficient levels of these receptors are present during changes in sex steroid levels suggesting that ERs are important in the regulation of early gonadal differentiation (Menuet et al., 2002; Sandra and Norma, 2010; Leet et al., 2011).

E_2 in embryonic and larval fish comes from two sources. In early-stage embryos, estrogens are originally present within the yolk as deposited in the spawned eggs (Devlin and Nagahama, 2002; Guiguen et al., 2009); this estrogen depletes as the embryo develops. The second sources of reproductive steroids are the gonads and extra-gonadal steroid-synthesizing tissue (e.g., interrenal, brain; Baroiller et al., 1999; Devlin and Nagahama, 2002). Synthesized sex steroids cannot be stored; thus, the expression and activity of enzymes responsible for synthesizing these sex specific steroids are important in their regulation (Piferrer and Guiguen, 2008). For example, in genetically female cells, the aromatase enzyme converts testosterone to E_2; in genetically male cells, the aromatase gene remains inactive, while other steroidogenic enzymes actively convert testosterone to 11-KT (Baroiller et al., 1999; Guiguen, 1999; Devlin and Nagahama, 2002). Sex steroids are critical in sex differentiation and the production of sex steroid-producing cells exists both prior to and during morphological development of the gonad (Nakamura et al., 1998).

1.7 17α-Ethynylestradiol (EE_2)

The estrogenic compound 17α-ethynylestradiol (EE_2) has been chosen as a model EDS for lifecycle bioassays due to its environmental relevance and its effects on the reproductive endocrine system via ER–mediated pathways (MacLatchy et al., 2003; Peters et al., 2007; Ankley et al., 2009). EE_2 is a synthetic pharmaceutical that is found in

contraceptive pills and is used for hormone replacement therapies (Desbrow et al., 1998). It is one component of sewage effluents that is not broken down during sewage treatment (Ternes et al., 2009). Its concentrations in Canadian sewage treatment plants are usually between 1-10ng/L EE_2 (Desbrow et al., 1998). This is a concern because the exposure of estrogenic EDSs at these concentrations may have potential adverse effects on fish reproductive systems and fish populations as demonstrated by various lab and field studies (Colborn et al., 1994; MacLatchy et al., 2003; Kidd et al., 2007; Peters et al., 2007).

Feminization has been evident in many laboratory studies following a minimal exposure to EE_2. Studies on zebrafish by Örn et al. (2003) showed that following an exposure of 1ng/L of EE_2, the sex ratios were skewed towards the female sex, with increases in female phenotypes and female-specific proteins, mainly vitellogenin. Van Aerle et al. (2002) found similar effects following an exposure of 10ng/L EE_2 to fathead minnow.

In a number of studies, mummichog have been exposed to estrogens during their early-life stages and its effects on morphological development, including gonadal development, were assessed (Urushitani et al., 2002; Boudreau et al., 2004; Peters et al., 2007; 2010). Peters et al. (2010) studied the effects of EE_2 on development of mummichog; in this study, exposure of the developing mummichog from pre-fertilization (adult generation) through to sexual development (offspring generation) at 100 ng/L (nominal; actual concentration was approximately 30% of exposure concentration) for 52 weeks resulted in skewed sex ratios (86.1% females). EDSs will most likely cause gonadal sex change in fish if exposed during the sensitive periods related to gonadal

differentiation and development. For mummichog, this sensitive period is yet to be characterized; however, Urushitani et al. (2002) have suggested that it occurs between 2-4 weeks after hatch.

The exact mechanisms by which these outcomes occur have yet to be clearly understood. Thus, it is warranted to characterize the process, pattern and timing of gonadal differentiation during the early stages of mummichog development. Establishing an understanding for this process will greatly enhance our knowledge on the developmental progression of the embryos and the sensitivity of gonadal differentiation to EDSs.

1.8 Integrative Biology

Concern about EDSs on the environment generates a requirement for a coordination of efforts from sub-disciplines of biology including ecology, toxicology, endocrinology and molecular biology. The choice of mummichog as a model organism for this study is considered to be ideal for an integrative approach as it has previously been used in numerous studies in the aforementioned sub-disciplines (MacLatchy et al; 2003, 2006; Burnett et al., 2007; Fangue et al., 2008; Greytak et al., 2007, 2010; Hogan et al., 2010; Peters et al., 2010). Techniques used in this study involve examination at the tissue (i.e., histology) and organismal (i.e., steroid concentration) levels. From these data, the potential of consequences at the population level can be hypothesized.

This study is expected to provide a greater understanding of the effects of EE_2 on mummichog morphology and development. Mummichog is considered a model species for evolutionary, ecological and physiological studies due to its adaptation to its widely

fluctuating environment. Including endpoints at various levels of biological organization allows the integration of the information to propose consequences of EE_2 exposure in wild populations.

1.9 Objectives

Research linking the pattern of reproductive ontogeny (development of organism from embryo to adult) and the effects of EDSs at the developmental level has identified differentiation as a very sensitive period in many fish species (van Aerle et al., 2002; Örn et al., 2003; Aoki et al., 2011). The first objective of this study is to confirm the critical period during gonadal differentiation at which the gonads develop into testes or ovaries in mummichog.

The second objective is to determine the sensitivity of mummichog gonadal development to EE_2. Endpoints will include hatch rates, hatch success, larval abnormalities and mortalities during larval growth (to 10 wph) as well as gonadal differentiation (as determined by histology).The null hypotheses are:

H_0: EE_2 has no effects on hatch success, growth, mortality or skeletal development.

H_0: EE_2 has no effect on the timing and outcome of gonadal differentiation.

1.10 References

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Chapter 2

Sensitivity of Early-life Stages of Mummichog

(*Fundulus heteroclitus*) to 17α-Ethynylestradiol

2.1 Abstract

The objectives of this study were to confirm the period of gonadal differentiation in the estuarine killifish or mummichog (*Fundulus heteroclitus*) and to determine the sensitivity of gonadal development to EE_2. Artificially-regressed mummichog were spawned, and fertilized eggs were collected and exposed to EE_2 (0, 10, 50 and 250ng/L) within 8 h of spawning. Embryos and larvae were continually exposed in petri dishes and beakers (26˚C) and sampled weekly from hatch date to 10 weeks post-hatch (wph) to histologically determine the sensitivity of gonadal development to EE_2. Exposure to EE_2 (10-250ng/L) resulted in a concentration-dependent increase in skeletal abnormalities and mortalities; larval lengths proved insensitive to EE_2. Histological analyses of mummichog gonads showed complete gonadal differentiation occurred by 3 wph in control groups, whereas exposure to EE_2 (10, 50 and 250ng/L) accelerated gonadal differentiation as ovarian tissue was evident by 1 wph. Timing of gonadal differentiation was concentration dependent (0 < 10 < 50 < 250ng/L EE_2). Sex ratios were skewed (>80% female phenotype) within all groups treated with EE_2. Whole-body E_2 concentrations were found to be higher at 4 wph compared to 8 wph; however, no treatment-related responses were detected in EE_2-exposed groups. Environmentally-relevant levels of EE_2 used in this exposure indicate that mummichog developing in contaminated estuaries could be at risk for impaired development.

Keywords: 17α-Ethynylestradiol, Endocrine Disruption, Gonadal Differentiation, Skeletal Abnormalities, *Fundulus heteroclitus*, Embryos, Larvae

2.2 Introduction

Endocrine-disrupting substances (EDSs) include a variety of anthropogenic chemicals with estrogen- or androgen-like properties which have similar modes of action to those of endogenous hormones (Örn et al., 2003). EDSs can enter the environment by means of industrial wastes, agricultural runoff and municipal wastewater effluents (Sumpter and Jobling, 1995; Desbrow et al., 1998; Jobling et al., 1998; Routledge et al., 1998; Ternes et al., 1999). For example, 17α-ethynylestradiol, EE_2, a component of the birth control pill and hormone replacement therapy, enters aquatic ecosystems via sewage discharge and is found at low parts per trillion concentrations (<5ng/L; Larsson et al., 1999; Palace et al., 2006). In fish, EDSs have the ability to alter endocrine function within individuals and/or their progeny (Zillioux et al. 2001; Damstra et al., 2002; Dietrich and Krieger, 2009). Effects of these substances have been evident in field (Kidd et al., 2007) and lab studies, resulting in disruption of developmental (Maack and Segner, 2004, Boudreau et al. 2004; 2005) and reproductive endocrine (MacLatchy et al., 2003) processes. In aquatic species, effects of EDSs may result in altered secondary sexual characteristics (Parrott et al., 2004); reduced gonad size (Janz et al., 2001) and egg production (Parrott et al., 2004); developmental abnormalities (Boudreau 2004; 2005; Peters et al., 2010); and altered gonadal differentiation (Örn et al., 2003).

Effects of endocrine disruption on early-life developmental processes in fish have been studied less than effects on reproduction. Mummichog (*Fundulus heteroclitus*), a species of teleost fish that is dominant in coastal salt marshes and estuaries along the east coast of North America (Armstrong and Child, 1965), has demonstrated sensitivity to hormonally-active substances in the field (Leblanc et al. 1997) and in the lab (MacLatchy

et al., 2003; Peters et al., 2007; 2010). It is considered an ideal candidate for early-life development and gonadal differentiation studies due to its size; its ease of breeding, grow out and lab husbandry; and the ability to manipulate its reproductive cycles (Burnett et al., 2007; Lister et al., 2011). In addition, due to the extent of human activity in coastal regions (Oberdörster and Cheek, 2000), it is expected that mummichog are exposed to environmental EDSs throughout their lives in many areas.

EE_2 is an environmentally-relevant model EDS with strong affinity for the estrogen receptor (Peters et al., 2007; Dietrich and Krieger, 2009; Hogan et al., 2010). It is persistent in the environment, has the ability to bioconcentrate in fish tissue and is more stable than its natural counterpart, 17β-estradiol (Thorpe et al., 2003; Soares et al., 2009; Aoki et al., 2011). Levels in the environment have been found to range between 1-10ng/L EE_2 downstream of Canadian sewage treatment plants (STPs; Desbrow et al., 1998) and are documented to be as high as 42 ng/L EE_2 (Ternes et al., 1999). Exposures of fathead minnow (*Pimephales promelas*) and pearl dace (*Magariscus margarita*) to low levels of EE_2 during a whole-lake experiment demonstrated the potential for adverse effects on fish reproductive systems and populations (Palace et al., 2006; Kidd et al., 2007).

Research linking the pattern of reproductive ontogeny to the effects of EDSs has identified differentiation as a very sensitive period in many fish species (van Aerle et al., 2002; Örn et al., 2003; Aoki et al., 2011). Exposure of EE_2 to teleost fish during early-life stages results in morphological abnormalities (Boudreau et al., 2004), increased mortalities (Kidd et al., 2007; Peters et al., 2010), accelerated ovarian differentiation

(Aoki et al., 2011) and skewed sex ratios (van Aerle et al., 2002; Örn et al., 2003; Peters et al., 2010).

In mummichog, early-life exposure to estrogenic EDSs causes abnormal development and feminization (Urushitani et al., 2002; Boudreau et al., 2004; Peters et al., 2007; 2010); however, the sensitive periods for these effects are not well understood. Peters et al. (2010) showed that the proportion of mummichog exposed to 100 ng/L EE_2 (nominal; actual concentration was approximately 30% of exposure concentration) had skewed sex ratios (86.1%) in the direction of the female sex; however, those fish were exposed for 61 wph prior to assessment of their gonadal status. In addition, mummichog embryonic development has been well documented (Armstrong and Child, 1965) and gonadal differentiation is suspected to occur between 2-4 weeks post hatch (wph; Urushitani et al., 2002) although, the precise period has yet to be identified. Further investigation is therefore warranted on the early-life development of mummichog in order to achieve a greater understanding of the pattern and timing of gonadal differentiation and the effects of a model estrogen, EE_2, on this differentiation.

Adult mummichog were regressed and recrudesced and embryos derived from these parents were collected within 8 h of spawn and randomly assigned to one of four treatments – 0 (control), 10, 50 and 250ng/L EE_2 – and continually exposed up to 10 wph. Endpoints examined included embryonic hatch success, as well as larval/juvenile growth, mortality, skeletal abnormalities and gonadal differentiation. This study will enhance our knowledge of the reproductive ontogeny of mummichog and the effects of EDSs on gonadal developmental.

2.3 Materials and Methods

2.3.1. Mummichog collection and husbandry

Mummichog were collected from an uncontaminated site near Shediac, NB, Canada (N47°16', W64°30') using a beach seine, and transferred to Wilfrid Laurier University in an aerated plastic tote. The fish were housed in recirculating, 425L holding tanks (Aquabiotech; Coaticook, QC, Canada). Conditions were maintained to meet optimal holding requirements (16 ppt salinity, >85% dissolved oxygen, 18˚C and summer seasonal photoperiod). Mummichog were fed crushed commercial trout pellets (Corey Feed Mills, Fredericton, NB), blood worms (*Glycera dibranchiata*; Hikari Inc., Hayward, CA, USA) and mysis shrimp (*Mysis relicta*; Hikari Inc.). Well water was mixed with Crystal Sea Marine Mixed Buffered Salt (Enterprises International, Baltimore, MD, USA) to achieve the desired salinity. Daily water quality tests were conducted to ensure that all conditions were met; nitrite and ammonia levels were tested weekly (means 0.17 and 0.11 mg/L, respectively). Water changes were regularly performed to maintain conditions within desired levels.

2.3.2. Chemicals

17α-Ethynylestradiol (EE_2) was purchased from Sigma-Aldrich, Canada (Oakville, ON), dissolved in 100% ethanol (EtOH) and stored at -20°C at a stock concentration of 1 mg/mL EE_2. Working stock solution was prepared for the exposure at 10 µg/mL EE_2, stored at -20°C and 100 µl was alliquoted daily into 4 L H_2O (16 ppt) to prepare the working concentration solution of 250ng/L EE_2 (0.0025% EtOH) for the highest concentration used in the exposure. The 250ng/L EE_2 stock was diluted 5x to

achieve 50ng/L EE_2 (0.0005% EtOH) and 25x to achieve the 10ng/L EE_2 (0.0001% EtOH) concentrations for the remaining concentrations used. A 100µl aliquot of 100% EtOH was dissolved in 4 L H_2O (16 ppt) for the exposure control. Homogenizing buffer (PBS) and EDTA were also all purchased from Sigma-Aldrich.

2.3.3. Environmental manipulation

To provide a supply of fertilized eggs for the exposure, environmental conditions were manipulated to artificially regress and then recrudesce male and female mummichog (MacLatchy et al., 2003; Bosker et al. 2010). With fish (sexes separated) in the recirculating holding tanks, temperature and photoperiod were lowered to 8°C and 8h Light:16h Dark by lowering 1°C daily and reducing light 2 h every second day. Fish were maintained under these conditions for 30 days and then temperatures and light were increased (1°C daily; 2 h light every second day) to stimulate recrudescence to a final temperature of 26°C and photoperiod of 16h L:8h D.

2.3.4. Experimental design and exposure

Adult fish (sexes separate) were distributed to seven static, filtered tanks (20 L; four female tanks and three male tanks; AquaClear 50 Power Filter, Baie d'Urfé, QC). Following two weeks of acclimation, sexes were combined (four males and four females) and fertilized eggs were collected from the tanks by collecting them from the bottom of the tank below a mesh screen (Peters et al., 2007; Bosker et al., 2009). Fertilized eggs were collected within 8 h of spawn, randomized and transferred to 250 mL glass dishes at an initial density of 120 eggs in 16 ppt saline water per dish (n=6). The bottom of the

dishes were placed in water baths and maintained at temperatures of 26 ± 1 °C with a photoperiod of 16h L:8h D. Dishes were randomly assigned to one of four treatments: 0 (control), 10, 50 and 250ng/L EE_2 (See Appendix A). Survival and time of hatch were monitored over the duration of the hatching period.

Upon hatch, larvae were transferred to 500 mL glass dishes containing the treatment concentration from its respective dish at a density of 35 ± 5 larvae per dish (n=5). Excess larvae were transferred into similar conditions (n=3 for control and 250ng/L; n=4 for 10ng/L and 50 ng/L) for preliminary studies on whole-body 17β-estradiol (E_2) concentrations. Dishes containing larvae were submerged in water baths at a temperature and a photoperiod similar to those of the embryos. Daily water and treatment renewals were performed. Once the yolk sac was absorbed (4 d post hatch), larvae were fed newly hatched *Artemia* sp. *nauplii* (Ocean Star International, UT, USA) twice daily. At 5 wph, the marine larval diet, Otohime B2 (360-620 micron diet; Reed Mariculture Inc., CA) supplemented the juvenile diet as they were weaned from *Artemia*.

Larval and juvenile growth (length measured to the nearest mm) and vertebral abnormalities, calculated as the proportion affected, were assessed on a weekly basis from 1 wph to 10 wph. Analyses were performed from images produced using a Nikon SMZ1500 stereomicroscope equipped with PaxCam Arc digital camera and Pax-it imaging software (Villa Park, IL, USA). Vertebral abnormalities were analyzed based on previously established criteria for scoliosis (lateral curvature) and lordosis (dorsoventral curvature; Boudreau et al., 2004; Peters et al., 2010). Larval and juvenile mortalities were assessed on a daily basis.

2.3.5. Tissue sampling and histological analysis

For histological sex evaluation, larval and juvenile mummichog were sampled on a weekly basis (n=10 per treatment) from 1 wph to 10 wph. Prior to fixation, specimens were sectioned by cutting in front of the operculum and just behind the anal fin, leaving the abdominal region, which was fixed in 10% buffered formalin purchased from Sigma-Aldrich. Tissues were sent to the Ontario Veterinary College (Susan Lapos, University of Guelph, ON) for tissue processing, sectioning and staining. Tissues were paraffin-embedded, transverse (cross) serial sectioned at 5 µm, hemotoxylin and eosin stained and mounted on slides using Tissue Tek permanent mounting medium (Sakura Finetek, CA, USA). Slides were evaluated using a light microscope equipped with a PaxCam Arc digital camera and Pax-it imaging software. For each slide, nine to 12 fields of view were analyzed (dependent on tissue availability) to confirm the phenotypic sex of the individual. Sections were analyzed for germ cells, undifferentiated gonads, ovaries, testes and potential intersex.

Histological analyses of the gonads were categorized into five distinct groups: i) undefined, ii) germ cells, iii) undifferentiated, and well defined, iv) testes, or v) ovaries. Undefined included those in which no gonads were evident as either the gonads had yet to develop (e.g., during the first wph) or histological slide sections were missing the area in which the gonads were located. Germ cells were categorized by the existence of the primordial germ cells and gonadal ridges (Fig. 2.5; Nakamura et al., 1998; Piferrer, 2001; Dietrich and Krieger, 2009). The difference between germ cells and undifferentiated is minute and subjective. Prior to sexual differentiation, there exists a process of germ cell proliferation resulting in the gradual enlargement of the gonad and it is during this period

that gonads are classified as undifferentiated (Dietrich and Krieger, 2009). Testes were initially identified by the presence of spermatogonia. During later stages of development, testes were examined for the presence of spermatocytes, sperm ducts and a vascular system (lobular vs. tubular; Fig 2.7; Dietrich and Krieger, 2009). Ovaries were identified during early stages by the presence of the ovarian cavity and perinucleolar oocytes (Fig 2.8).

2.3.6. Whole-body homogenates

Larval and juvenile mummichog were collected at 4 and 8 wph for analysis of whole-body E_2. Fish in groups of three were transferred to 7mL cryovials and stored at -80°C. For analysis, tubes were transferred from the freezer onto ice, fish were weighed frozen, placed in aluminum foil and transferred onto dry ice. Samples were crushed and transferred into 1.5mL micro-centrifuge tubes containing homogenizing buffer (HB; PBS, pH 7.4, 1mM EDTA) in the ratio of 2:1 (HB volume:weight). Samples were sonicated on ice (twice for 5 s), vortexed and methanol (MeOH) was added in the ratio of 4:1 (MeOH volume:HB volume). Samples were incubated at 4°C for 1 h, vortexing periodically, and centrifuged (3,000 *x g*, 5 min, 4°C). Supernatant was collected in 7mL glass tubes and the process of methanol addition and centrifugation was repeated twice more with a reduced incubation period (30 min/cycle) each time. The homogenate was dried using nitrogen gas (N_2) and reconstituted with 1mL acetate buffer (50 mM, pH 4.0), left at room temperature for 30 min and stored at -20°C freezer for further analysis.

2.3.7. Steroid extractions & enzyme immunoassay analysis

Following tissue sonication, tubes containing acetate buffer were thawed on ice and prepared for solid phase extractions (SPE). SPE columns (DSC-18, 100mg; Sigma-Aldrich) were conditioned by the addition of 1 mL MeOH, followed by 1 mL acetate buffer (pH 4.0). Each entire sample was added to the column and was allowed to flow through until complete dryness. Once dry, 1 mL hexane was added to the column and the total volume collected was discarded and new 7mL glass scintillation vials were obtained to collect potential steroids within the column. Ethyl acetate (1% MeOH) was added to the column twice (1mL/cycle), collecting potential steroids from the column, and the eluate was evaporated under N_2 gas. Upon evaporation, precipitate was reconstituted with 1 mL enzyme immunoassay (EIA) buffer and 50 µl of total sample was analyzed using an EIA assay kit (Cayman Chemicals Co., Kit #582251, Cedarlane Labs, Burlington, ON) to measure E_2 concentrations following manufacturer's protocol. EIA plates were read at 405nm for 60 – 90 minutes.

2.3.8. Water sampling and EE_2 analysis

In order to confirm actual EE_2 concentrations, water samples were collected three times throughout the exposure in duplicates (at 0 and 8 h) and pre-treated with deuterated EE_2. Analysis was performed in collaboration with Dr. Mark Servos's laboratory at University of Waterloo (Waterloo, ON). Briefly, EE_2 was solid phase extracted, eluted in methanol, evaporated under N_2, reconstituted in 500 µL methanol and stored at -20°C until analysis (<1 week). Prior to analysis, samples were derivatized in order to be volatile as required for gas chromatography mass spectroscopy (GC-MS). Following

derivatization, samples were analyzed on an Agilent 6890N gas chromatograph (GC) with an Agilent 5975B mass spectrometer (MS) detector equipped with an Agilent HP-5ms (30 m x 0.25 mm x 0.25 µm) column (Agilent Technologies, Mississauga, ON). The ions monitored for EE_2 identification and quantification were 425.3 and 429.3 m/z. Concentrations of EE_2 were found to be below the detection limits (50ng/L EE_2) for the control and 10ng/L samples; however, levels were found to be in the expected range of concentration for 50 and 250ng/L EE_2 (actual mean values of 57 ± 3.9 and 229 ± 12.3ng/L EE_2, respectively). Therefore, since the values were close to the desired concentrations, results are described in terms of the nominal concentrations.

2.3.9. Statistical analysis

Statistical analysis was performed using Sigmaplot 11.0 (Systat Software Inc., Chicago, IL) and SPSS Statistics 17.0 (IBM Corporation, Armonk, NY, USA). Prior to statistical analysis, values calculated as percentages were arc sined and assessed as a proportion. These included hatching rates, abnormalities, mortalities and histological assessments of the gonads. Assumptions of normality and homogeneity of variance were tested using Shapiro-Wilk's and Levene's tests, respectively. Data were $\log_{10}$ transformed and re-tested when they failed to meet the assumptions for normality. Larval and juvenile lengths were assessed using two-way analysis of variance ($p < 0.05$) to test differences among treatments and weeks. Hatching rates and mortalities were assessed using one-way analysis of variance ($p < 0.05$) to test the differences among the treatments. Abnormalities were analyzed conducting a Kruskal-Wallis test as normality did not meet the assumptions. Histological data were assessed using two-way analysis of variance.

Tukey's post hoc test was conducted when applicable to determine between-treatment differences. Whole-body E_2 data were examined for outliers using Dixon's test prior to analysis. Analyses of whole-body E_2 data were then conducted using a two-way analysis of variance and Tukey's post hoc test was used when applicable.

2.4 Results

2.4.1. Embryo hatch success

Embryos in 50ng/L EE_2 hatched significantly later (1 day) than control group compared to those exposed to any other treatment (p=0.021; Fig. 2.1A). All embryos hatched between 10-12 days. Percent successful hatch was not different amongst the treatments (p=0.171; Fig 2.1B). Average percent successful hatch for all treatments combined was 42.4%.

2.4.2. Larval and juvenile development

Weekly analysis of larval and juvenile lengths showed no significant differences among treatments (p=0.661; Fig. 2.2). Abnormalities observed in larval and juvenile mummichog (Fig. 2.3) included vertebral scoliosis (lateral curvature) and lordosis (dorsoventral curvature). Percent abnormalities increased in a concentration-related manner; with the increase in EE_2 concentration, there was a higher incidence of abnormalities evident (Control 16%; 10ng/L 20%; 50ng/L 30%; 250ng/L 46% abnormalities). There were significant differences observed between the control and 10ng/L EE_2 in comparison to the highest concentration, 250ng/L EE_2 (p=0.006).

Mortalities in larval and juvenile mummichog increased in a concentration-related manner with a significant difference between the 250ng/L EE_2 and the control (p=0.042). Percentage of mortalities ranged from 18 ± 0.229% in controls to 33 ± 0.967% in 250ng/L EE_2.

2.4.3. Gonadal differentiation and development

Initial signs of sexual development in the control group were evident at 1 wph with 77.8% showing primordial germ cells (22.2% undefined; Fig. 2.4). Sexual differentiation was observed at 2 wph with 50% showing ovarian tissue and 20% exhibiting testis tissue (Fig. 2.5A). Complete sexual differentiation was evident at 3 wph with 30% males and 70% females (Fig. 2.5A; see Appendix B). At the end of complete differentiation (3 wph), testis tissue with spermatogonia distributed in an intratubular fashion and perinucleolar occytes (Fig. 2.6 and 2.7, respectively) were clearly distinguishable. An increase in proportion of males was evident as weeks progressed as 40% were classified as males and 60% as females in controls at 5 wph (Appendix E). At 7 wph, the ratio of males to females was 1:1 (Fig. 2.5A). Proportion of females were found to be greater (70%) at 10 wph compared to males (30%; Fig. 2.5A).

EE_2-treated larvae showed evidence of sensitivity to the chemical as sexual differentiation with defined gonads was evident at 1 wph in all EE_2-treated groups (see Appendix C & D). Larvae exposed to 10 and 50ng/L EE_2 had on average 30% undifferentiated gonads and 70 and 60% differentiated ovaries by 1 wph, respectively (Fig. 2.5B & C). The 250ng/L EE_2 exposed group showed almost complete differentiation with 88.9% exhibiting female phenotype (11% undefined) by 1 wph (Fig. 2.5D). At 2 wph, complete differentiation was evident in all of the exposed groups (Fig 2.5B-D). By 8 wph, the 10ng/L EE_2 group had 100% ovaries. Groups exposed to 50 and 250ng/L EE_2 showed complete ovarian differentiation ($p<0.001$; Fig. 2.5C & D) although some of the gonads examined at 50ng/L were germ cells (20%). Larvae sampled at 7 wph had skewed sex ratios among the treatments as 10ng/L treatment displayed 10%:60%

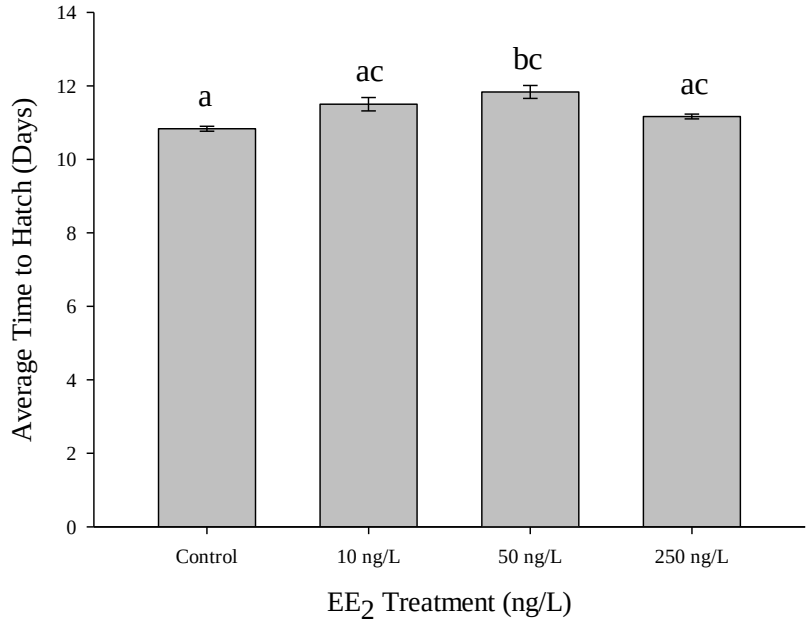
(male:female; 30% undefined), while 90% of the 50 and 250ng/L EE_2 treated fish displayed a female phenotype (10% undefined; Fig. 2.5 B-D). Complete feminization was evident at 10 wph in all EE_2 treated groups (10 and 50ng/L revealed 20 and 30% undefined, respectively; Fig 2.5 B-D). No intersex was observed in any of the treatments. Proportions of females were significantly higher in all of the groups exposed to EE_2 ($p<0.001$; Fig. 2.5C-D). All of the groups had a number of fish in which sex could not be determined and these were categorized as undefined.

2.4.4. Quantifying E_2 concentrations

Whole-body E_2 concentrations measured at both 4 and 8 wph showed no significant difference among the treatments ($p=0.783$; Fig. 2.8). There was a significant difference in E_2 within all groups between weeks 4 and 8 post hatch ($p<0.001$). However, control groups at both weeks had a greater concentration of E_2 (pg/mg tissue) present compared to treatments. In general, E_2 concentrations were found to decrease from 4 to 8 wph. Cross reactivity between E_2 and EE_2 was not a factor in this study (0.05%).

2.4.5. Figures and illustrations

(A)



(B)

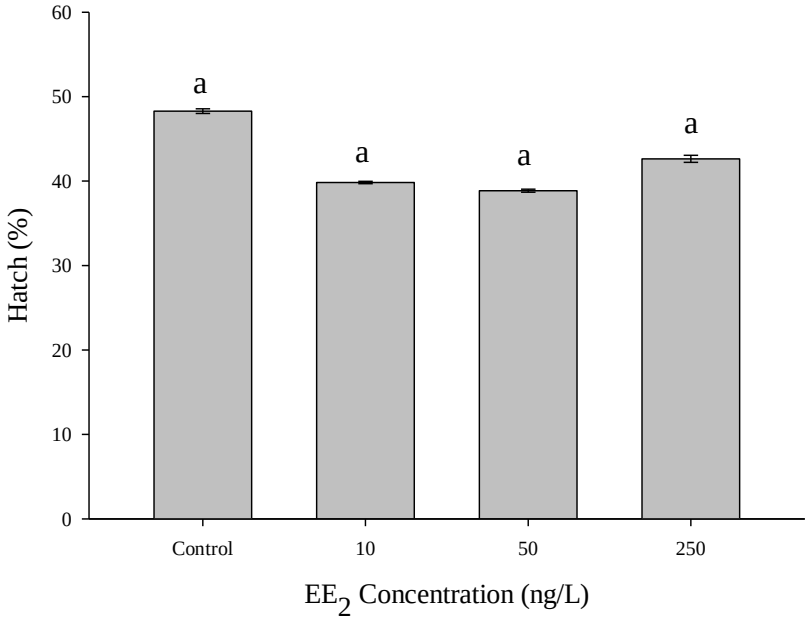


Figure 2.1 (A) Mean days to hatch of mummichog eggs exposed to 0, 10, 50 and 250ng/L 17α-ethynylestradiol (EE_2; p=0.021). **(B)** Percentage of successful hatch of mummichog eggs exposed to 0, 10, 50 and 250ng/L EE_2 (p=0.171). Bars showing different letters are significantly different. Values represent means ± SE. *n*=6 dishes per treatment.

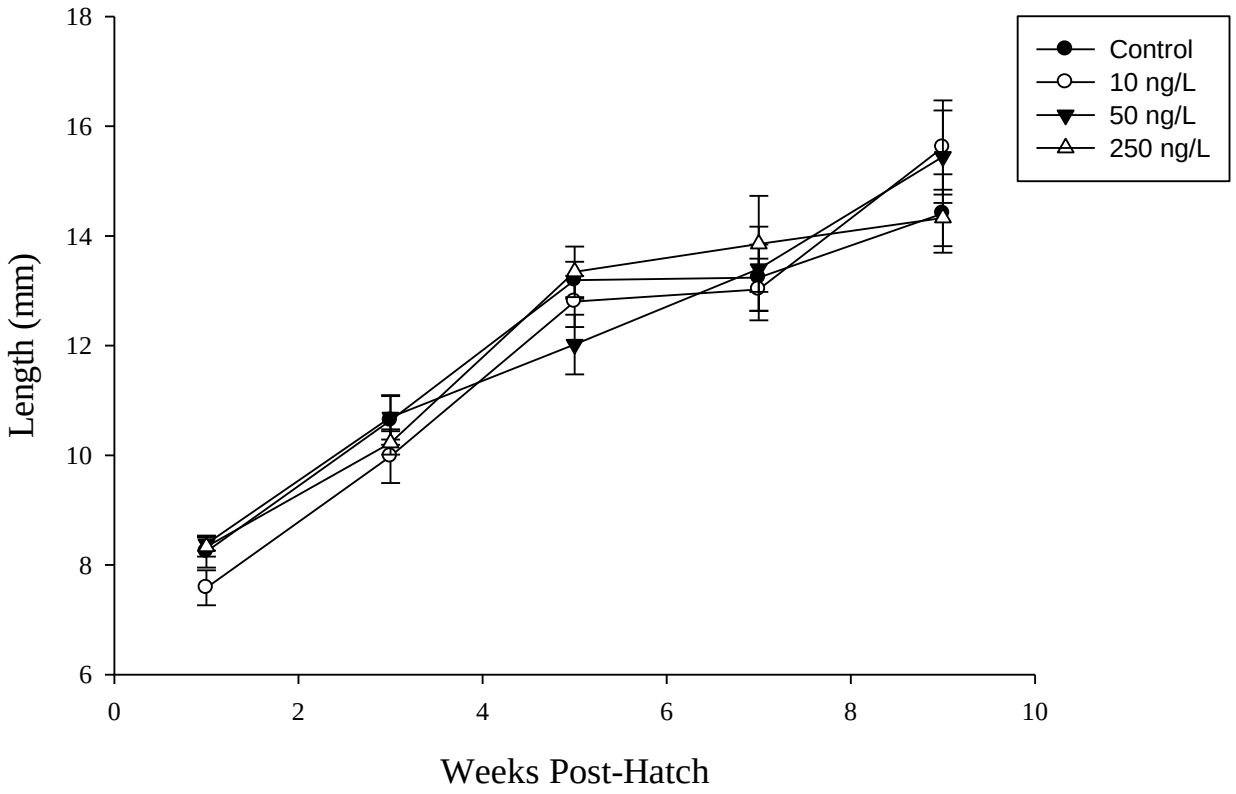


Figure 2.2 Average lengths of larval and juvenile mummichog exposed to 0, 10, 50 and 250ng/L EE_2 (p=0.661). Symbols represent means ± SE. *n*=10 mummichog per treatment (120 eggs/dish).

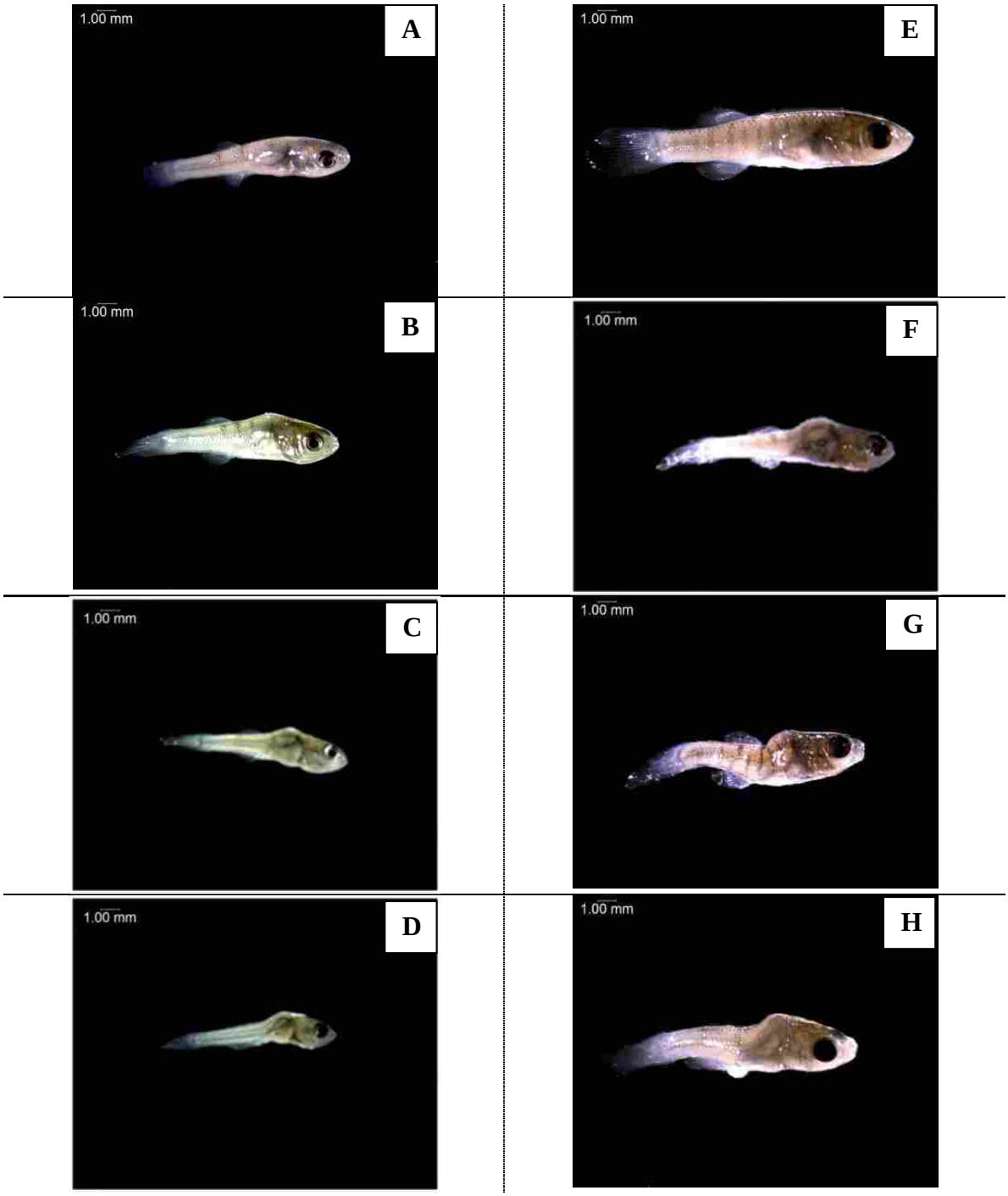


Figure 2.3 Groups A-D are mummichog sampled at 4 wph exposed to 0, 10, 50 and 250ng/L EE_2, respectively. Groups E-H are 8 wph old mummichog exposed to 0, 10, 50 and 250ng/L EE_2, respectively. (A & E) Control mummichog showing undisrupted skeletal development. (B & F) Mummichog exposed to 10ng/L EE_2 showing lordosis. (C & G) Mummichog exposed to 50ng/L EE_2 with lordosis (left) and severe lordosis (right). (D & H) Juvenile mummichog exposed to 250ng/L EE_2 showing lordosis (left) and severe lordosis with scoliosis (right). Abnormalities occurred in all groups; these are provided as representative illustrations.

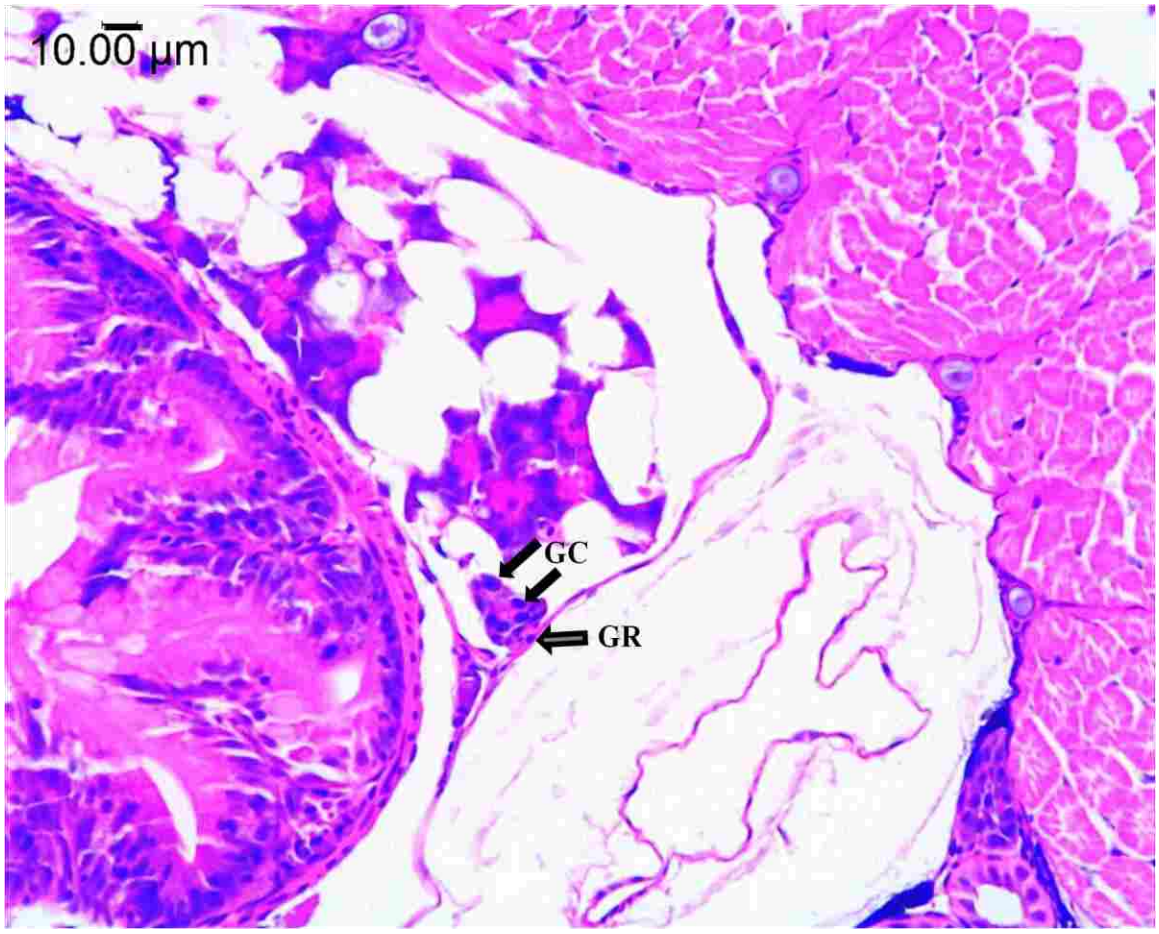


Figure 2.4 Light micrograph of a mummichog sexually undifferentiated primordial gonad (Control; 1 wph) showing germ cells (GC) and the gonadal ridge (GR).

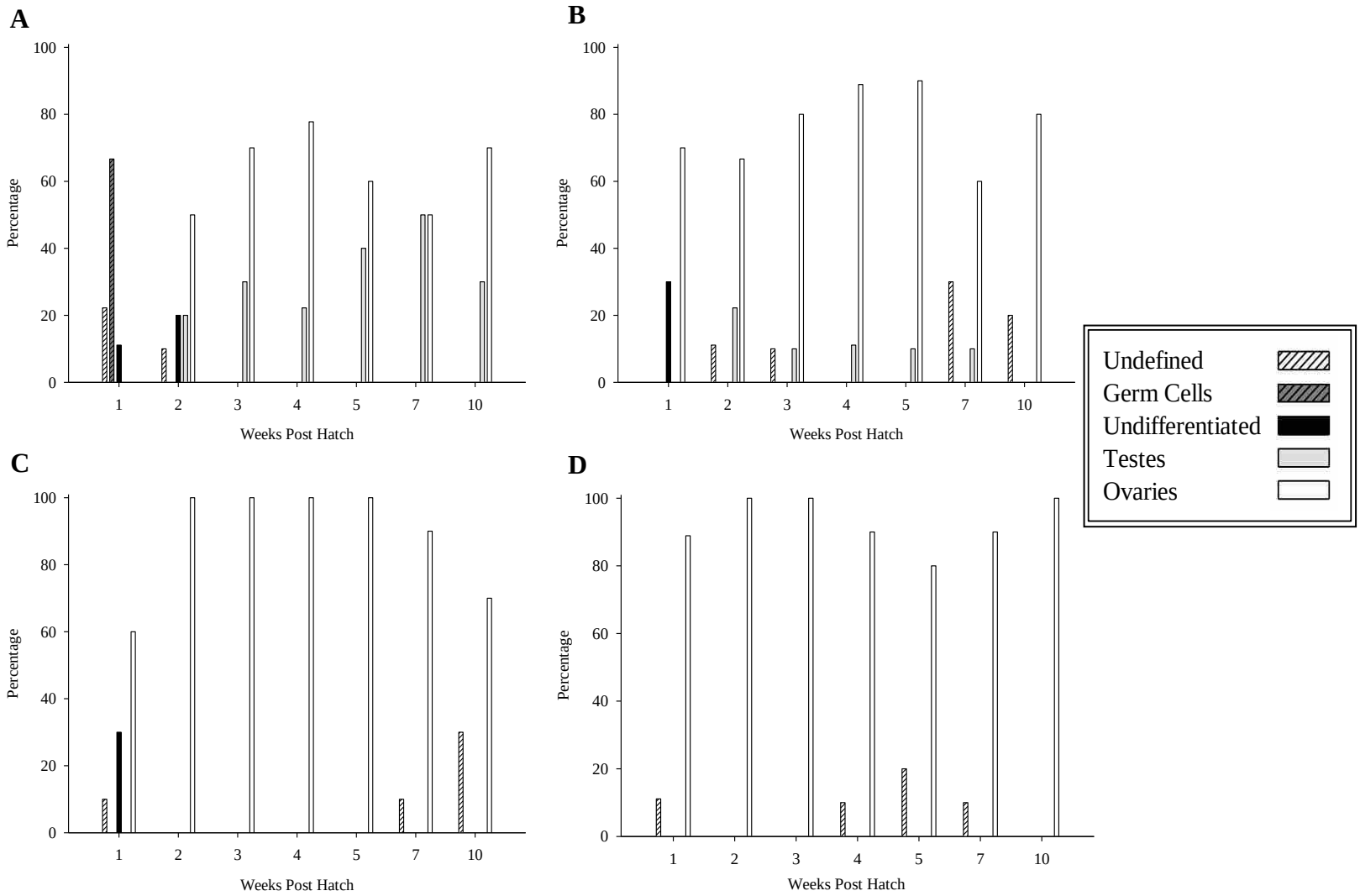


Figure 2.5 Larvae and juvenile mummichog sexual differentiation throughout the exposure period. (A) Control group; (B) 10ng/L EE_2; (C) 50ng/L EE_2; (D) 250ng/L EE_2.

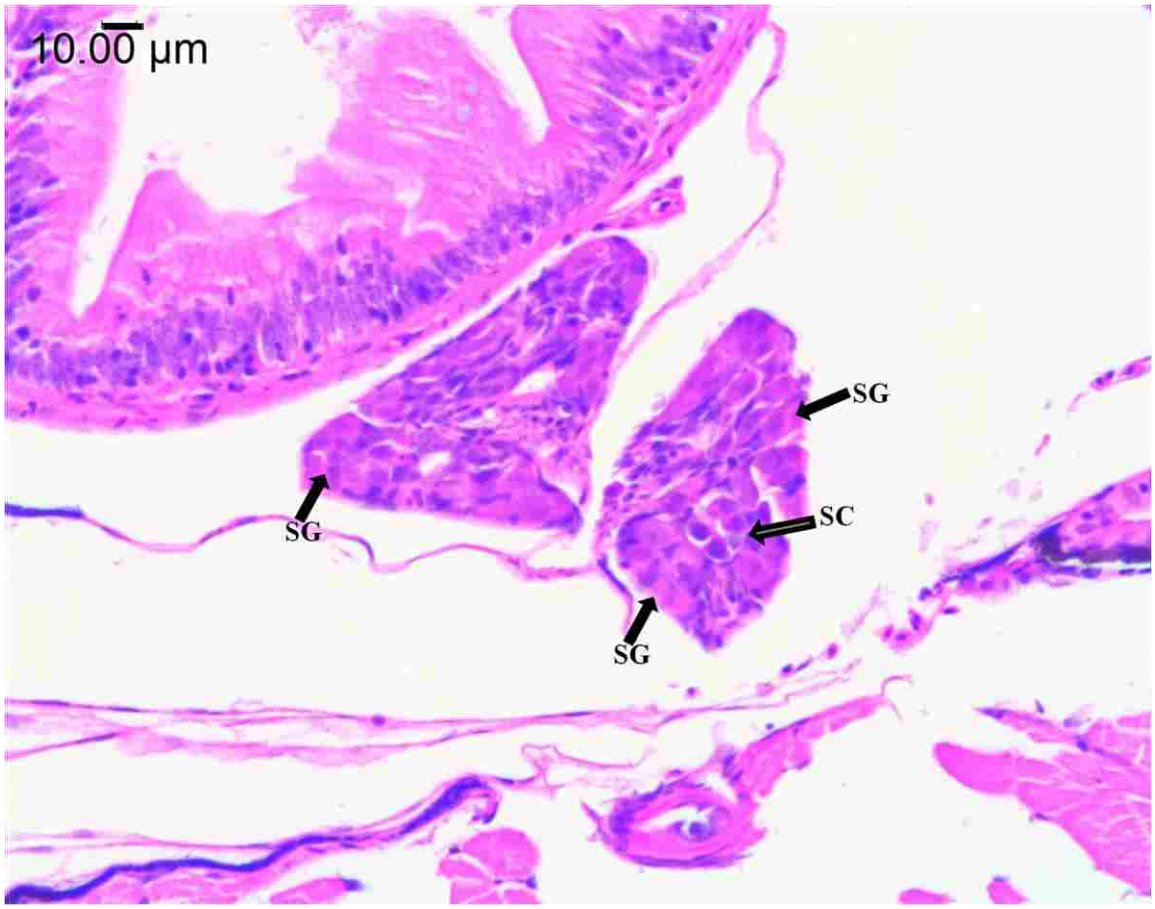


Figure 2.6 Mummichog testis tissue with an intratubular distribution of the spermatogonia (Control; 5 wph). SG: spermatogonia; SC: spermatocytes.

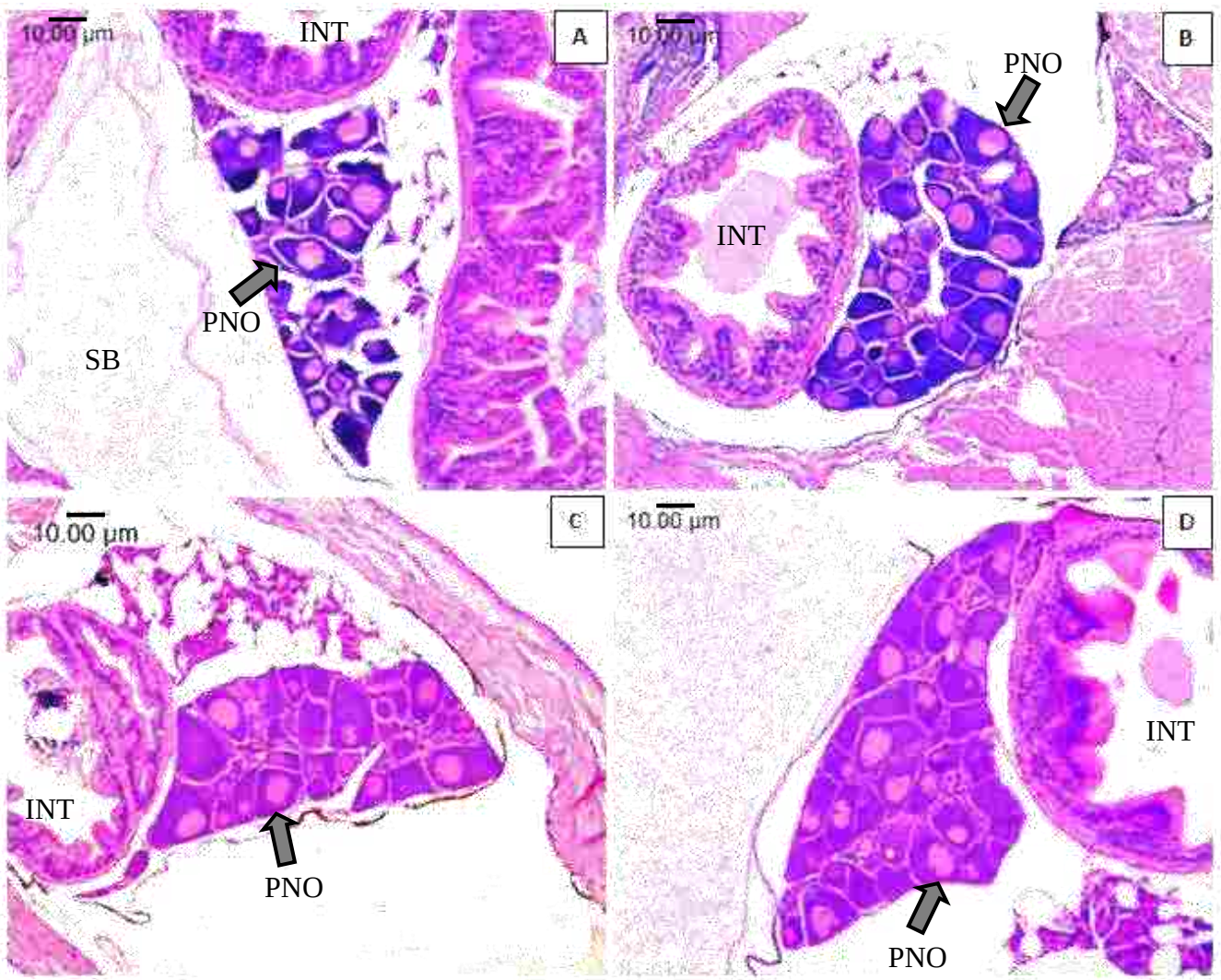


Figure 2.7 Light micrographs of mummichog ovaries at 5 wph showing perinucleolar oocytes. (A) Control group; (B) 10ng/L EE_2; (C) 50ng/L EE_2; (D) 250ng/L EE_2. Bar: 10.00µm . INT: intestines; SB: swim bladder; PNO: perinucleolar oocyte.

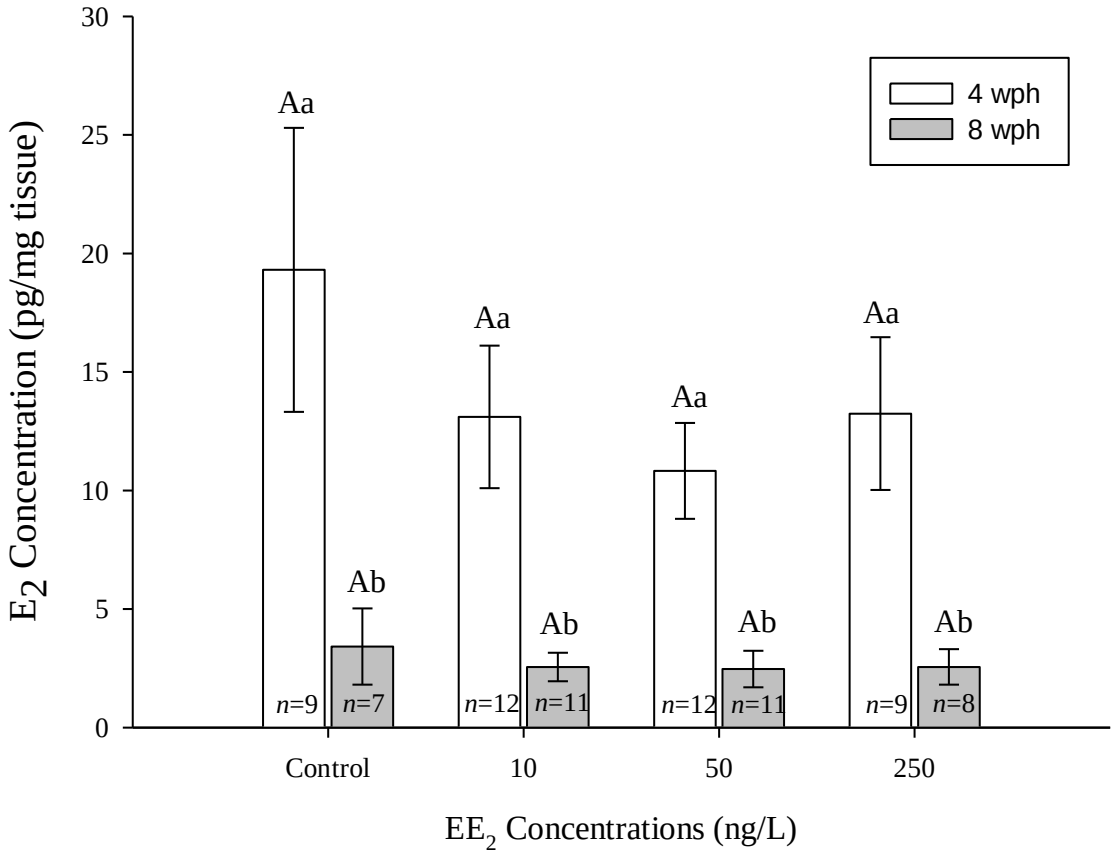


Figure 2.8 Mean whole-body 17β-estradiol (E_2) concentration in 4- and 8-week old mummichog larvae exposed to 0, 10, 50 and 250ng/L 17α-ethynylestradiol (EE_2; p=0.783). Upper case letters represent significant differences within a given week. Lower case letters represent significant differences between the 4 and 8 wph within treatments. Bars represent means ± SE. *n* value analyzed for each group is given at the base of the respective bar.

2.5 Discussion

The primary focus of this study was to enhance our knowledge and understanding of the effects of an estrogenic EDS on the development and differentiation process of mummichog. Eggs from adult mummichog were collected within 8 h of spawn and exposed immediately to EE_2 concentrations of 0, 10, 50 and 250ng/L for up to 10 wph. Results from this study demonstrate a significant concentration-dependent increase in skeletal abnormalities, skewed sex ratios (favouring females), and accelerated sexual differentiation in mummichog exposed to EE_2. Complete gonadal differentiation in control mummichog was evident by 3 wph; whereas, gonadal differentiation was evident as early as 1 wph for those exposed to EE_2. These results add to previous studies on mummichog gonadal differentiation (Urushitani et al., 2002) and the effects of EE_2 on development (Boudreau et al., 2004), including gonadal development (Peters et al., 2010) and indicate a strong potential for environmental estrogens to alter reproductive status in this species.

The number of days to hatch were not altered in a concentration-dependent fashion as time to hatch for embryos exposed to 50ng/L EE_2 was significantly greater than the control group; however, found to be non-significant at 250ng/L. In a previous study, increased EE_2 concentration (10 and 100ng/L) reduced the time to hatch (Peters et al., 2010). Results from previous studies have revealed a significant concentration-related decrease in hatching success in sheepshead minnow (*Cyprinodon variegatus* Lacépède; Zillioux et al., 2001) and mummichog (Peters et al., 2010) exposed to 200 and 100ng/L EE_2, respectively. In Boudreau et al. (2004), hatch success was unaffected by EE_2. In the present study, hatch success was unaffected. Protective membranes, including the

chorion, present prior to fertilization, and the vitelline and previtelline, formed following fertilization (Armstrong, 1965), reduce the ease of diffusion of EDSs into the embryo (Anadu et al., 1999). The differences in results observed in Zillioux et al. (2001) and Peters et al. (2010) compared to Boudreau et al. (2004) and the present study could be due to the methodologies used in the studies. Fish embryos in the previous two studies by Zillioux and Peters were exposed to EE_2 during spawning, prior to formation of the protective membranes. Embryos in the Boudreau and present studies were transferred to an EE_2-treated environment following fertilization. This later exposure allowed the formation of the protective membranes which could have supplied some protection to the embryos. However, the developmental effects of EE_2 demonstrated in the present study indicate EE_2 was taken up by the embryos.

Morphological analysis of larval and juvenile lengths throughout the duration of the exposure showed an increase in length; however, there were no significant differences among the treatments. Previous studies on mummichog by Peters et al. (2007) also demonstrated no significant differences among larval growth in treatments of 0, 0.1, 1, 10 and 100 ng/L EE_2 (nominal; actual concentrations 30% of nominal) exposed for 3 and 4 wph. Exposure of fathead minnow to 10ng/L EE_2 for 4 wph showed no significant effect on growth (van Aerle et al., 2002). A 28- and 56-day exposure of larval fathead minnow to EE_2 showed a significant decrease in lengths at concentrations of 16 and 64ng/L EE_2 for 28 days and 4, 16 and 64ng/L EE_2 for the 56-day exposure (Länge et al., 2001). Thus, it appears that a longer exposure period and/or greater exposure concentrations are required to impede the growth of fathead minnow. The lack of effects on growth in the present study is consistent with the studies on mummichog by Peters et al. (2007; 2010)

as compared to fathead minnow. Although previous studies on fish have shown estrogens affect growth by depressing insulin-like growth factor (IGF-1) expression, perhaps through a genomic mechanism, mediated by estrogen receptor (ER) regulation, there were no indications during the period studied here in mummichog that such mechanisms were operating. In addition to any potential effects of EE_2, other variables, such as food availability and temperature, affect growth (Radtke and Dean, 1979). All larvae were raised at uniform temperatures in the present study and food was not limiting (potentially offsetting effects of EE_2 on growth) thus minimizing these variables as factors through the study design.

The effect of increasing EE_2 concentrations on skeletal abnormalities confirms previous studies in fathead minnow (Länge et al., 2001) and mummichog (Boudreau et al., 2004). In the present study, as the concentration increased, the proportion of abnormalities, including scoliosis and lordosis, increased and was significant at the higher concentrations (50 and 250ng/L EE_2). Previous studies on mummichog (Boudreau et al., 2004; 25 and 60 days post hatch (dph) exposure to 1,000 and 10,000ng/L EE_2) and fathead minnow (Länge et al., 2001; 120 dph to >16ng/L EE_2; Warner and Jenkins, 2007; 25-26 dph to 0.1-100µg/L EE_2) showed that abnormalities were significant at the highest concentrations used. Results in the present study emphasize that EE_2 has a significant impact on skeletal development as abnormalities were found to be significant at 50 and 250ng/L, concentrations much lower than demonstrated in Boudreau et al. (2004). In addition, Urushitani et al. (2002) exposed mummichog to 10^{-8} M (28.5×10^4 ng/L) E_2 for 12 weeks and observed incomplete ossification in vertebrae, cranial bones and other bones in fry. Urushitani et al. (2002) suggested that the early-life exposure of a strong

estrogen mimic such as EE_2 leads to a direct ER-mediated effect on bone development by causing incomplete bone ossification. Further investigation is necessary to determine the impacts of exogenous estrogens (e.g. EE_2) on skeletal growth and development through direct ER-mediated mechanisms as well as interactions with other endocrine systems (e.g., growth hormone-IGF-1 mediated mechanisms).

Mortality of mummichog larvae and juveniles throughout the duration of the study was found to be significantly higher in the 250ng/L EE_2-exposed group. Mortalities were also significantly greater at the highest concentration (100ng/L EE_2) compared to the other groups in Peters et al. (2010). Studies using fathead minnow showed no significant difference in survival among the treatments (0.2, 1, 4, 16 and 64ng/L EE_2; Länge et al., 2001) in the range similar to the two lower concentrations (10 and 50ng/L) used in the present study. Therefore, there is some indication that higher but not lower concentrations of EE_2 causes mortality through direct (e.g., toxic) or indirect (e.g., developmental abnormalities) mechanisms. It has been suggested that fish survival, primarily during early-life development, could be susceptible to density-dependent effects (Boudreau et al., 2004). Although treatment-related densities were kept as similar as possible in the present study, increasing the volume of water or minimizing density within a tank/dish could potentially limit mortalities and encourage fish survival among all treatments.

In the present study, histological examination of the control gonads revealed that initial signs of development were evident at 1 wph as both germ cells and undifferentiated gonads were present (66.7% and 11.1%, respectively). Gonadal differentiation was observed by 2 wph with the presence of testes and ovaries (20% and

50%, respectively); however, undifferentiated gonads were also present at that time point (20%). Complete gonadal differentiation into ovaries or testes occurred by 3 wph with 70% exhibiting ovarian tissue and 30% showing testes. This is supported by Urushitani et al. (2002) as they found that sexual differentiation occured between 2-4 wph in mummichog raised in 25°C under natural photoperiod (9-14 h light). As well, Shimizu et al. (2002) found that both testes and ovaries differentiate by 3 wph in mummichog (20°C and 16h L:8h D). Research on gonadal differentiation of some teleost species has determined that gonads of female individuals differentiate sooner than those of males (Piferrer, 2001; Strüssmann and Nakamura, 2002). This is evident as complete sexual differentiation in zebrafish (*Danio rerio*) was found to occur by 4-5 wph for females and 6 wph for males (Örn et al., 2003) and fathead minnow females are sexually differentiated by 2 wph and males by 3 wph (Uguz, 2008). Although other fish species indicate that testes differentiate much later than ovaries, this time difference is not evident in mummichog in either the present or previous studies. It is well established that fish differentiate by two distinct pathways (Piferrer, 2001; Strüssmann and Nakamura, 2002). The first pathway involves differentiation directly from the primordial gonad into either a testis or ovary as exhibited by mummichog (Shimizu et al., 2002; Urushitani et al., 2002); whereas the second pathway involves differentiation of all gonads into an ovary-like gonad and later half of the fish undergo sexual reversal leading to testes formation, as exhibited by zebrafish (Örn et al., 2003).

In comparison to the control larvae, EE_2-exposed developing gonads revealed both an earlier differentiation period and a significantly skewed ratio in favour of the female sex. At 1 wph, exposure of mummichog to 10 and 50ng/L EE_2 revealed the

presence of undifferentiated gonads and differentiated ovarian tissue. Germ cells and testes were not evident within these groups at this sampling time. Complete sexual differentiation was evident in these groups by 2 wph as both testes and ovaries were present in individuals exposed to 10ng/L EE_2 and only ovarian differentiation was present in the 50ng/L EE_2 group. Exposure of mummichog to 250ng/L EE_2 showed complete ovarian differentiation present by 1 wph as the gonads all appeared to have ovarian cavities and/or perinucleolar oocytes. Therefore, exposure of mummichog to EE_2, including environmentally-relevant concentrations (10ng/L), promotes early ovarian differentiation. These results are in agreement with other studies as exposure of 11-month-old juvenile grey mullet (*Mugil cephalus*) to EE_2-treated diets for 4 weeks found initial signs of sexual differentiation to be accelerated as early as 26 days following transfer to an EE_2-free environment for those previously exposed to 0.04 µg/g EE_2 and 0 days for 4 µg/g EE_2 exposed individuals. On the contrary, sexual differentiation of control groups were found to occur 280 and 350 days following transfer (Aoki et al., 2011). In addition, Aoki et al. (2011) found complete female sexual differentiation to have occurred as early as 26 days following transfer of grey mullet exposed to 4 µg/g (Aoki et al., 2011).

Following the period of complete sexual differentiation, the proportion of females was found to be approximately equivalent to males (60% to 40%, respectively) in the control group at 5 wph. Mummichog exposed to 10ng/L EE_2 showed a skewed sex ratio of 90% females to 10% males at the same time. In addition, mummichog exposed to the higher concentrations of EE_2, 50 and 250ng/L, displayed skewed sex ratios of 100% and 80% (20% undefined), respectively, in favour of the female sex at 5 wph with no

evidence of male gonadal tissue observed. Mummichog larvae sampled at 7 wph had a 1:1 (male:female) sex ratio within the controls and skewed sex ratios among the treatments as 10ng/L treatment displayed 10%:60% (male:female; 30% undefined), while 90% of the 50 and 250ng/L EE_2 treated fish displayed a female phenotype (10% undefined). It is important to note that no intersex was observed in any of the treatments. These results were further supported at 10 wph as complete feminization was evident in all EE_2 treated groups (10 and 50ng/L revealed 20 and 30% undefined, respectively). Although gonadal differentiation within the control group at 10 wph favoured female sex (70%:30%, male:female), the reason for this result is unclear as it could have occurred by sampling chance as at week 7 the ratio was 50:50 female:male. Peters et al. (2010) observed sex ratios to be skewed by 86.1% in favour of the female phenotype following exposure to 100ng/L EE_2 nominal concentration (actually ~30ng/L) for 52 wph. Results from the present study parallel those from previous studies on other species as following a full life cycle exposure of 4ng/L EE_2 on fathead minnow, Länge et al. (2001) observed 84% females at 56 dph and complete feminization at 172 dph. In addition, skewed female sex ratios and complete feminization was evident in zebrafish exposed from 20-60 dph to 2, 5 and 10ng/L EE_2; however, the reason was unclear for the appearance of males at 25ng/L EE_2 (Örn et al., 2003). Results from the present study, in relation to other studies, suggest that early-life exposure of fish to EE_2 promotes skewed sex ratios, favouring females.

In developing fish, production of sex-specific steroid hormones are critical in sex differentiation. The genes responsible for the biosynthesis of these hormones are expressed differently in somatic cells of testes than ovaries (Nakamura et al., 1998). 11-

Ketotestosterone (11-KT), a major androgen responsible for differentiation of testes binds to androgen receptors (ARs) directly within the gonads inducing premeiotic spermatogonial proliferation (Ge et al., 1997; Nagahama et al., 1997; Devlin and Nagahama, 2002; Dietrich and Krieger, 2009). On the contrary, E_2 stimulates oocyte development through direct effects on germ cells (Devlin and Nagahama, 2002; Sandra and Norma, 2010). E_2 up-regulates ER numbers in developing occytes via positive feedback, ensuring sufficient levels are present for the circulating levels of the hormone (Mommsen and Lazier, 1986; Devlin and Nagahama, 2002; Menuet et al., 2002; Sandra and Norma, 2010; Leet et al., 2011). The presence of exogenous sex steroids (e.g., EE_2) can lead to alterations in sex-specific receptors and affect the sex of the individual (Devlin and Nagahama, 2002; Leet et al., 2011). The presence of EE_2 prior to gonadal differentiation competes with and mimics E_2, binding to the ERs, promoting oocyte development and leading to feminization (Länge et al. 2001; Devlin and Nagahama, 2002; Örn et al., 2003; Leet et al., 2011).

Exogenous hormones have the potential to disrupt reproductive processes in fish prior to differentiation and even after sex differentiation has occurred (Devlin and Nagahama, 2002; Leet et al., 2011). The major factors responsible for inducing sex reversal in fish species include i) timing of exposure, ii) duration of exposure, and iii) concentration of the hormone used (Yamamoto, 1969; Nakamura et al., 1998; Piferrer, 2001). Timing of the exposure is critical as sexually undifferentiated fish are considered to be susceptible to exogenous hormones compared to differentiated fish as there exists a labile period (period of physiological sex differentiation) of uncharacterized events in the gonads that are undetectable prior to the first histological signs of sex differentiation

(Piferrer, 2001). Thus, the presence of the exogenous hormones prior to differentiation is critical as these hormones target the sex steroid-specific receptors (e.g. ERs or ARs) mimicking endogenous hormones and disrupting normal expression levels of genes involved in sex differentiation (Leet et al., 2011). The mechanism of action of these exogenous hormones involves direct interaction with hormone receptors, changes in gene expression, amount of receptors present, and alterations of hypothalamus-pituitary-gonadal axis feedback mechanisms (Piferrer, 2001; Leet et al., 2011). For example, ERs are present in both developing testes and ovaries (Devlin and Nagahama, 2002; Piferrer, 2001; Sandra and Norma, 2010). EE_2 binds to ERs and initiates ER-mediated mechanisms that disrupt normal expression of genes involved in sex differentiation, promoting the transcription of estrogen-responsive genes, leading to female gonadal development (Devlin and Nagahama, 2002; Leet et al., 2011). Previous studies exposing EE_2 during early-life stages of grey mullet (Chang et al., 1999; 20 mg/kg EE_2 in feed for four months) and Japanese medaka (*Oryzias latipes,* Scholz and Gutzeit, 2000; 1, 10 and 100 ng/L EE_2 for two months) stimulated aromatase in both males and females; whereas, the exposure of 17α-methyltestosterone (synthetic male androgen; 20 mg/kg feed for four months) suppressed aromatase activity (Chang et al., 1999). These results provide evidence that the timing and presence of specific steroid hormones during gonadal development have critical effects. More research is required in mummichog to elucidate the mode(s) of action of endogenous and exogenous estrogen on gonadal development.

Previous studies have shown that gonadal steroid production concentrations in mummichog adults are decreased by exposure to EE_2 (MacLatchy et al., 2003; Peters et al., 2007). Although whole-body E_2 decreased from 4 to 8 wph in control mummichog,

no treatment-related responses were detected. It is assumed that EE_2's high affinity for the estrogen receptor stimulates a strong negative feedback response, inhibiting further production of the naturally-circulating estrogenic hormone, E_2 (Peters et al., 2007; Dietrich and Krieger, 2009; Hogan et al., 2010). In addition, E_2 in embryos and larvae is produced by developing steroidogenic tissue (Dietrich and Krieger, 2009) or could be of external origin (Desbrow et al., 1998). The lack of effect could be due to unresponsiveness of the system at this stage; however, high variability among the individuals sampled (perhaps due to the range in differentiated stages) cannot be eliminated as a reason. A study by Feist and Schreck (1996) observed that steroid hormone concentrations in whole-body rainbow trout (*Oncorhynchus mykiss*) were high upon hatch and during the period of gonadal differentiation and were reduced following differentiation. It is believed that the relative increase in steroid concentration upon differentiation may be involved in promoting sexual differentiation (Feist and Schreck, 1996). In the present study, whole-body E_2 concentrations were greater at 4 wph compared to 8 wph among all treatments, generally following the pattern described by Feist and Schreck (1996). Further studies will aid in gaining a clearer understanding of the effects of EE_2 on developing mummichog.

2.6 Conclusion

EE_2 in Canadian STPs has been found to be at concentrations between 1-10ng/L and as high as 42ng/L (Desbrow et al., 1998; Ternes et al., 1999). The present study clearly demonstrates that EE_2 significantly disrupts sexual differentiation and development in mummichog as skeletal abnormalities, mortalities, accelerated sexual

differentiation and skewed sex ratios favouring females were evident. Histological analyses of mummichog gonads showed complete gonadal differentiation to occur by 3 wph in control groups, whereas exposure to EE_2 accelerated gonadal differentiation as early as 1 wph. Environmentally-relevant concentrations accelerated gonadal differentiation and skewed sex ratios suggesting that these concentrations have the potential to cause adverse effects in the environment. Future studies are recommended to determine the mechanisms by which environmentally-relevant concentrations of estrogenic EDSs impact skeletal and gonadal development.

2.7 References

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Chapter 3

General Discussion and Integration

3.1 Discussion Overview

Sexual differentiation in fish is a delicate developmental process that occurs during the early-life stages of the individual (Strüssmann and Nakamura, 2002). Endocrine disrupting substances (EDSs) in aquatic environments have the potential to cause significant effects on fish development and the timing, duration and concentration of these exogenous chemicals are major factors influencing the scale of the effects on individuals and/or populations (Yamamoto, 1969; Nakamura et al., 1998; Pifferrer, 2001).

More bioassays on the effects of EDSs have been developed for freshwater fish, compared to the fewer studies on marine and/or estuarine species (Boudreau et al., 2005). Estuarine species may have a different threshold of sensitivity to these EDSs and may have differing uptake processes of these chemicals. In addition, the fate of the contaminants in saltwater systems may differ from those in fresh water (Peters et al., 2010). Therefore, EDS studies focusing on estuarine species are necessary as these areas serve as breeding and nursery grounds for many teleost fish (Boudreau et al., 2005).

Previous studies in the field and the lab have examined the effects of EDSs, specifically estrogenic contaminants, on teleost fish during both their adult and developmental stages. Estrogenic compounds exist in aquatic environments at concentrations which are sufficient to induce an effect on the individual and/or its progeny (Damstra et al., 2002; Dietrich and Krieger, 2009). For example, in the lab, endocrine disruptors have been found to alter secondary sexual characteristics (Parrott et al., 2004), reduce gonad size and egg production (Parrott et al., 2004; Peters et al., 2007), cause developmental abnormalities during early-life stages (Boudreau et al., 2004; 2005),

and alter sex ratios (Peters et al., 2010) in various teleost fish species. In the field, studies by Kidd et al. (2007) demonstrated a decrease in reproductive success and sustainability of fathead minnow (*Pimephales promelas*) populations following a whole-lake exposure to 5ng/L EE_2. Results obtained from the present study add to the knowledge of EDSs, specifically EE_2, on early-life exposure to mummichog (*Fundulus heteroclitus*) as skeletal abnormalities, mortalities, accelerated sexual differentiation and skewed sex ratios favouring females were evident.

For the present study, mummichog were exposed to EE_2 within 8 h of spawn at concentrations that ranged from environmentally relevant, 10ng/L EE_2, up to 50 and 250ng/L EE_2. The timing and concentrations used in this early-life exposure were important factors in the outcomes of the study. Accelerated gonadal differentiation and skewed sex ratios (>80% favouring female sex) were evident in all EE_2 treated groups, suggesting that the presence of steroid hormones during gonadal development of mummichog are critical in determining the fate of the sex of the individual. These results are comparable with those described in previous literature on both freshwater and estuarine species. For example, studies using freshwater species found significant differences with sex ratios at low concentrations as complete sexual feminization was exhibited at exposures of 4.2ng/L (Länge et al., 2001) and 3.2ng/L EE_2 (Parrott and Wood, 2002) using fathead minnow; whereas, feminization was evident at concentrations of 1ng/L and 2ng/L EE_2 using zebrafish (*Danio rerio*; Örn et al., 2003). In contrast to freshwater species, mummichog were found to have altered sex ratios at concentrations of 100ng/L EE_2 (actually ~30ng/L) as sex ratios were skewed ~86.1% favouring the female phenotype (Peters et al., 2010). The binding of EE_2 to estrogen receptors (ERs;

present in both testes and ovaries), inducing gene expression leading to feminization is believed to play a major role in the mechanism of action of EE_2 (Piferrer, 2001; Devlin and Nagahama, 2002); however, the mode(s) of action require further research for complete understanding (see section 3.2).

Studies on the effects of EDSs on mummichog enhance our knowledge on the sensitivity of this species to environmental contaminants. As well, it adds to our knowledge on the mechanisms that allow the species to remain reproductively viable within contaminated environments (Lister et al., 2011). Research using mummichog as a model species for ecotoxicological studies strengthens our understanding about the effects of EDSs in estuaries.

3.2 Future Directions

Future work contributing to the results from this study should focus on two distinct objectives.

The first objective should focus on reducing sampling periods, specifically during the first four weeks post hatch (wph). The significance of doing so will allow the identification of the period of gonadal differentiation. As well, since this study exhibited earlier sexual differentiation to those exposed to EE_2 (e.g., complete differentiation by 1 wph for those exposed to 250ng/L), increasing sampling points within the first few weeks will allow determination of the precise period when differentiation occurs in those exposed compared to those in the controls. Because this study suggested that EE_2 has significant effects on mummichog during its early-life development, and the mechanism is not clearly understood, determining the precise period of gonadal differentiation will

support further mechanistic studies by reducing time and effort spent during periods that would yield limited increases in understanding.

There is sufficient evidence from previous literature to conclude that a mechanistic relationship exists between steroid hormones and fish development (Yamamoto, 1969; Piferrer, 2001; Devlin and Nagahama, 2002). Thus, the second objective should focus on determining ontogeny of ERs along with the pattern of gonadal steroidogenic gene expression (e.g., aromatase) in early-life stages of both control and EE_2-exposed mummichog. These studies should help elucidate modes of action of EE_2 on gonadal differentiation of mummichog. This will also enhance our knowledge and understanding of the modes of action of EDSs on sexual differentiation in fish. In addition, studies on androgens should also be initiated to understand their potential to also affect sex differentiation in mummichog.

3.3 Thesis Relation to Integrative Biology

Recently, increases in interdisciplinary studies merging various fields of biology have proven vital in understanding the action and impact of EDSs from molecular to population levels (Hayes, 2005). Our knowledge gained from previous studies in comparative endocrinology has made teleost fish attractive model organisms to predict effects of EDS in the lab and the field. This has been demonstrated in this study as the presence of EE_2 during early-life gonadal development of mummichog promoted earlier sex differentiation and lead to feminization, an outcome that has been exhibited in other teleost fish (Länge et al., 2001; Örn et al., 2003; Aoki et al., 2011) as well as amphibians (Hogan et al., 2008). In addition, EE_2 has been found to have effects on morphological

development leading to skeletal deformities in various teleost fish species, including mummichog (Boudreau et al., 2004; Länge et al., 2001; Warner and Jenkins, 2007; this study), and the effects have been suggested to occur through direct ER-mediated mechanisms (Urushitani et al., 2002). The present study adds to previous work integrating various biological sub-disciplines including ecology, toxicology, cellular/molecular biology, endocrinology and others to increase our ability to understand and predict the impacts of EDSs on fish. Specifically, the present study integrated endocrinology, toxicology and histology.

Mummichog is considered a model species in evolutionary, ecological and physiological studies due to its adaptation to its variable environment. Analyzing the effects of EE_2 on early-life gonadal differentiation and development of mummichog enhanced our understanding at various levels of biological organization. Histological techniques used in this study provided insight into the effects of EE_2 at a tissue and organ level; whereas, the morphological and whole-body E_2 levels provided information at the system and organismal levels. Although population effects were not examined in the present study, the endpoints examined at the lower biological levels of organization provide the opportunity to make inferences about potential effects at the population level. Specific to the results of this study, EE_2 exposures at environmentally-relevant concentrations caused high levels of skeletal deformities and feminization, both of which have the potential to have negative effects on population sustainability.

3.4 References

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Appendix A: Experimental design schematic

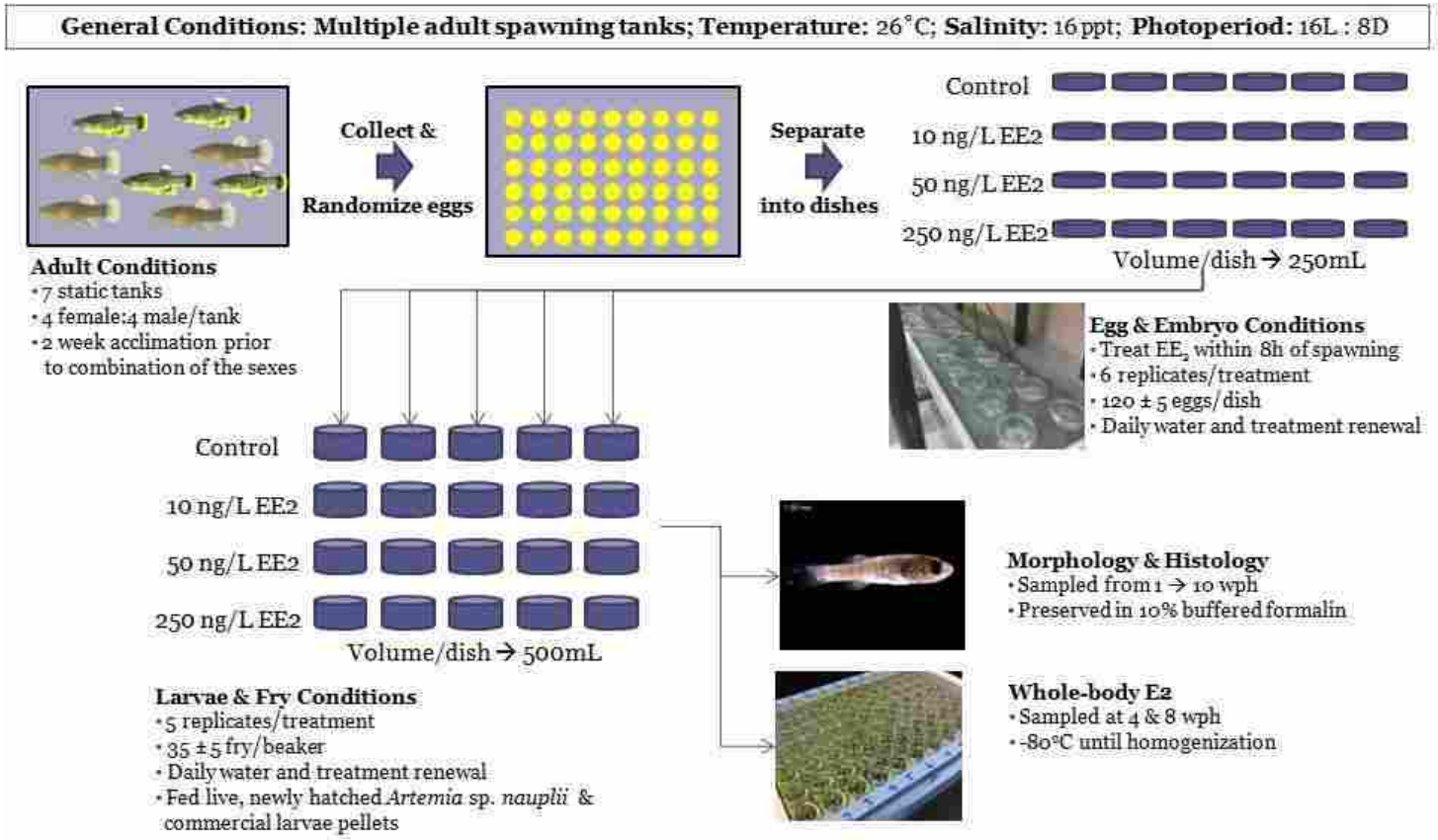

Figure A. Schematic of the experimental design and exposure used in this study. Schematic depicts adult, egg, embryo and larvae conditions throughout the exposure.

Appendix B: Control mummichog gonadal differentiation

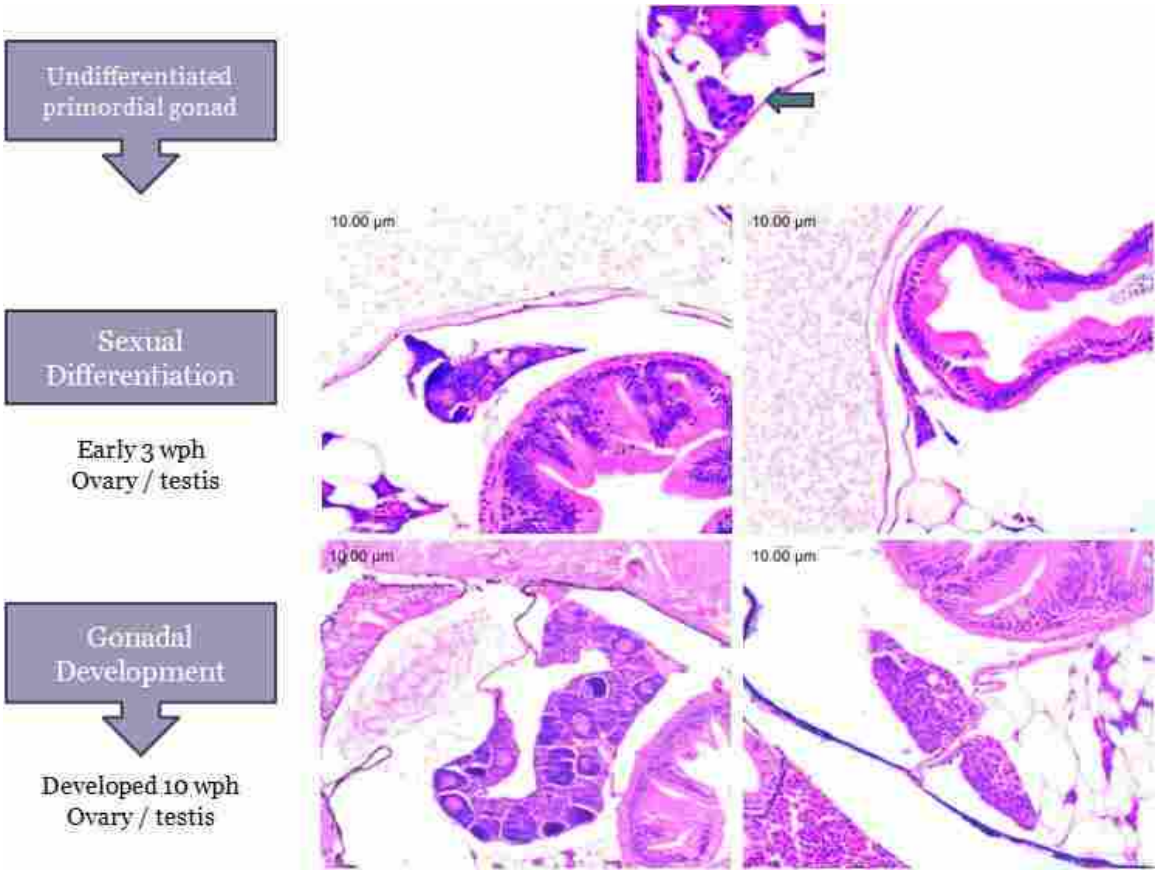

Figure B. Light micrograph of mummichog gonadal differentiation (Control) illustrating progress from undifferentiated primordial gonad to differentiated, developed gonads.

Appendix C: EE_2-exposed mummichog gonadal differentiation

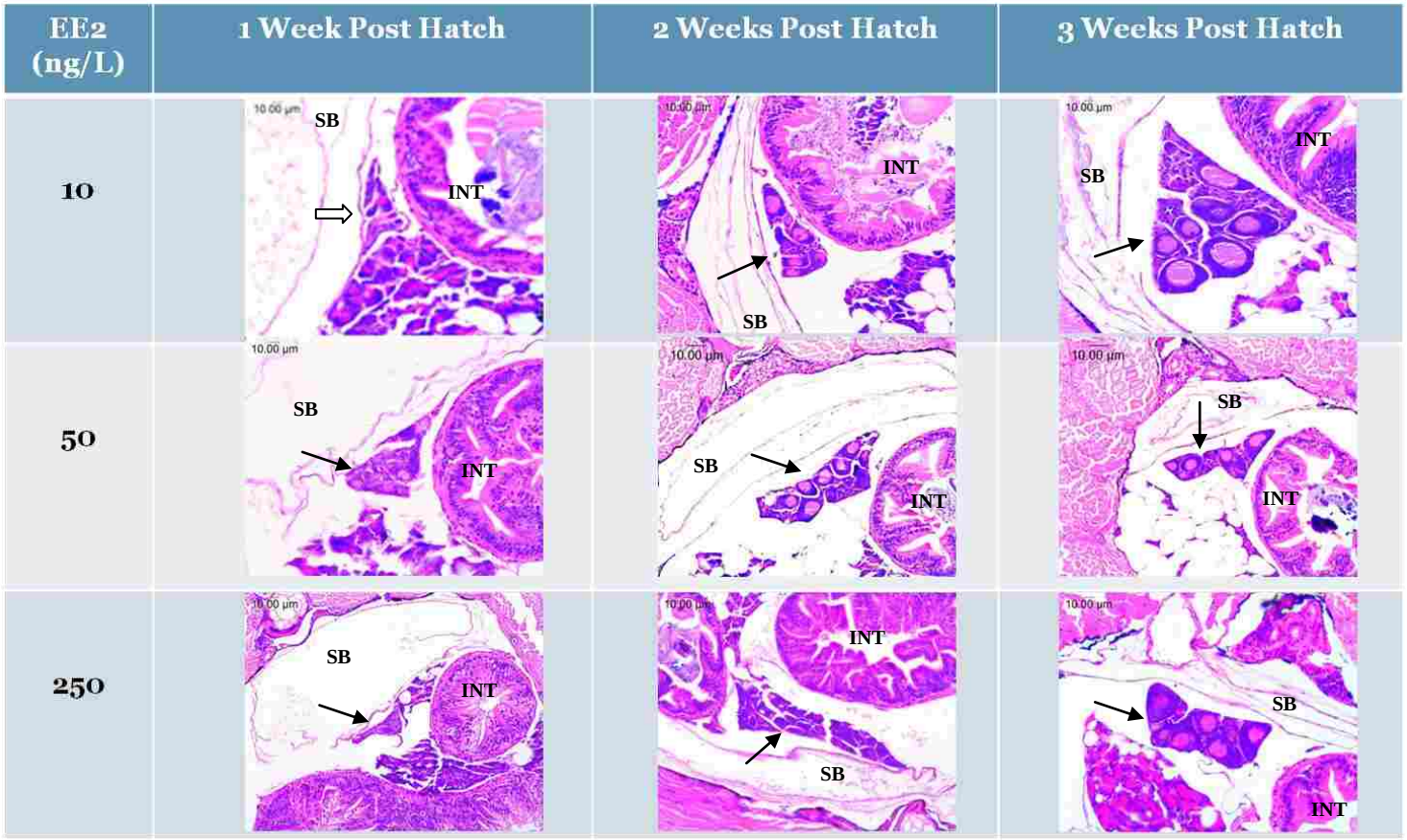

Figure C. Light micrograph of mummichog gonadal differentiation at from 1-3 wph exposed to 10, 50 and 250ng/L EE_2. SB: swim bladder; INT: Intestine; Black arrows: ovarian gonads; White arrow: undifferentiated gonad.

Appendix D: Mummichog ovarian development

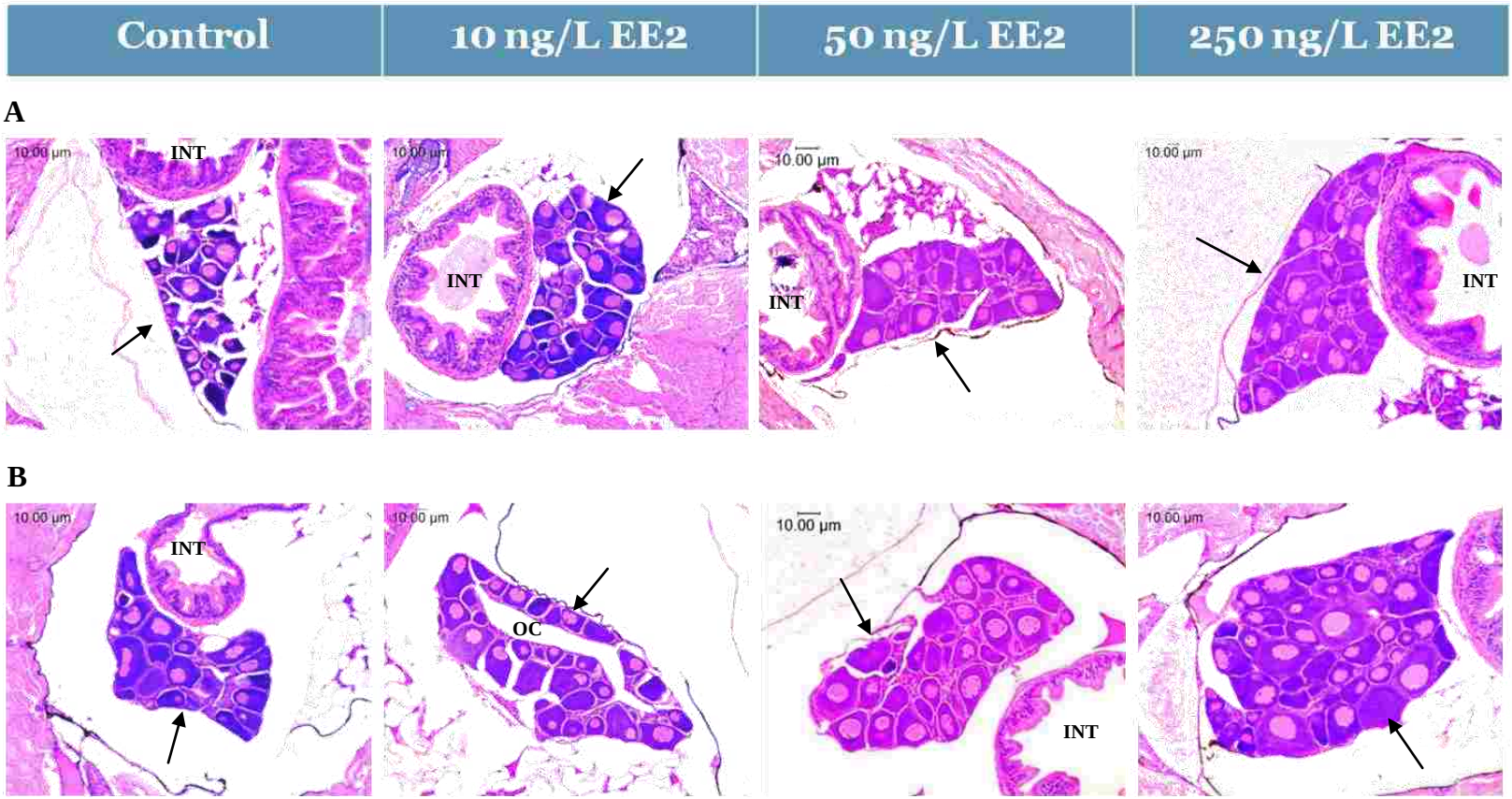

Figure D. Light micrograph of mummichog ovarian development exposed to control, 10, 50 and 250ng/L EE_2. Row A) 5 wph; row B) 9 wph. Bar: 10.00µm. INT: intestine; OC: ovarian cavity; Arrows: ovary.

Appendix E: Mummichog testicular development

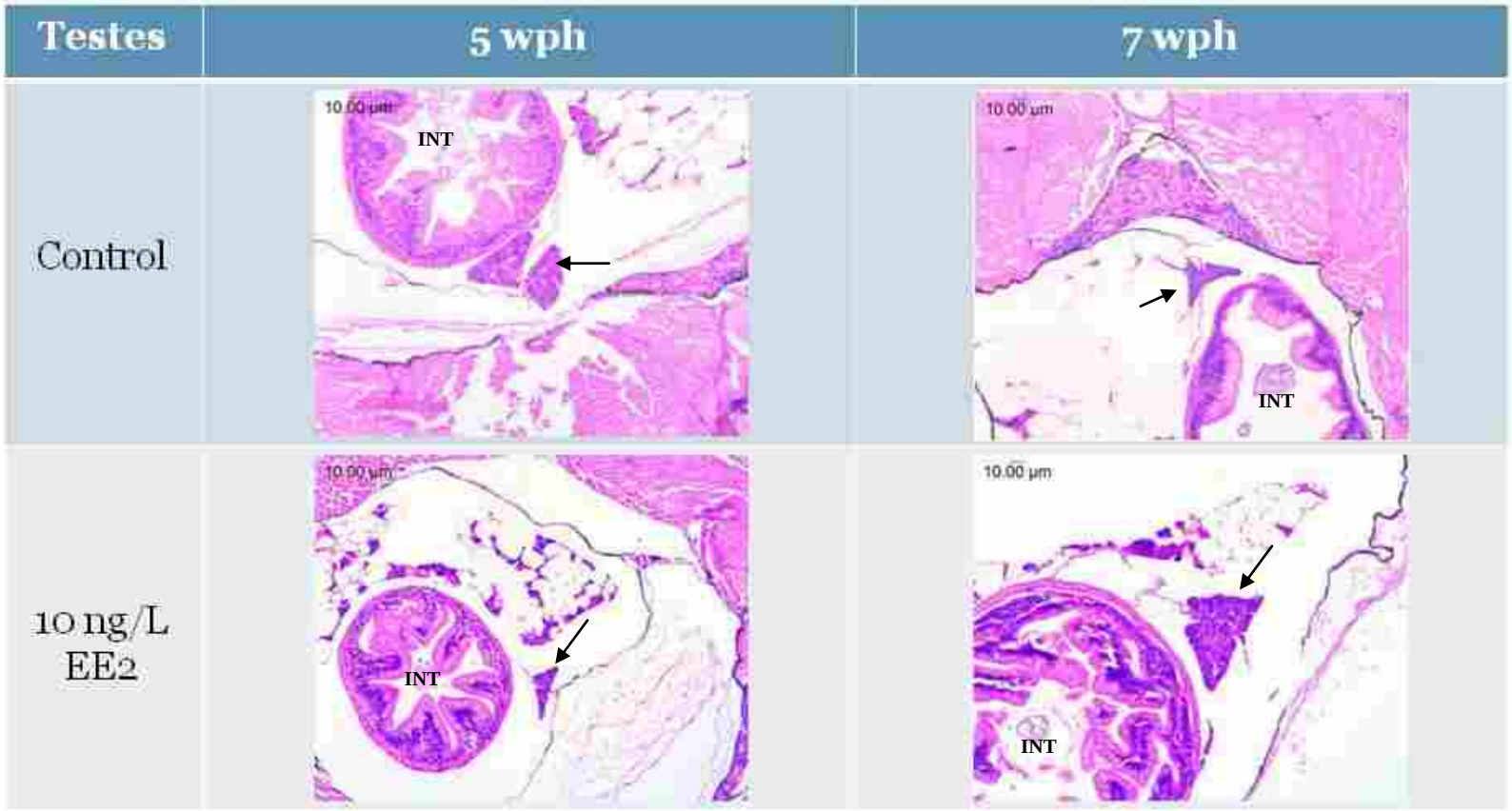

Figure E. Light micrograph of mummichog testicular tissue development exposed to control and 10ng/L EE_2 at 5 and 7 wph. Bar: 10.00µm. INT: intestine; Arrows: testes.