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1 Effects of 17 α -ethynylestradiol on early-life development, sex differentiation and
2 vitellogenin induction in mummichog (Fundulus heteroclitus)

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Abstract

Fertilized mummichog eggs retrieved from 17 α -ethynylestradiol (EE₂) exposed adult fish were raised in concentrations of EE₂ ranging from 0 to 100ng/L (100ng/L EE₂ estimated to have actual average exposure concentrations of 30% of nominal; 0.1 to 10ng/L were below detect throughout 24-h exposure period) for 61 weeks post-hatch. Eggs exposed at 100ng/L hatched sooner, the larvae were longer, and survival of juvenile fish from hatch to study termination was greater than all other treatments, though fewer hatched at this treatment. Sex ratios were skewed (>80% female phenotype) at 100ng/L EE₂, and some gonadal male fish displayed female secondary sex characteristics. Condition factor, gonadosomatic index (GSI), and liver somatic index (LSI) were found to decrease in both sexes between 52 and 61 weeks post-hatch. Female fish had increased hepatic vitellogenin (VTG) at 52 weeks post-hatch. When exposed to 1, 10 and 100ng/L EE₂, female fish had a higher proportion of vitellogenic follicles in the ovarian tissue. Males exposed at 100ng/L may have had disruption at some endpoints (GSI, VTG) that is masked due to reduced sample size compared to other treatments. Fish exposed to concentrations of EE₂ at or below 10ng/L showed inconsistent effects on development and reproductive potential. This study indicates the potential for population-level effects at the high range of environmental EE₂ at concentrations equivalent to those at which consistent effects in fecundity in the adult mummichog reproductive test have been measured. This work demonstrates that chronic EE₂ exposure causes developmental effects at concentrations similar to those which cause effects in the shorter-term adult mummichog reproductive test. Effects are at higher concentrations than have been noted for freshwater model species. Whether this is

- 59 because of species sensitivity or due to differences between freshwater and saltwater
- 60 availability of EE₂ or its uptake requires further study.

ACCEPTED MANUSCRIPT

61 Key Words

62 Ethynylestradiol, *Fundulus heteroclitus*, vitellogenin, sex differentiation, development,

63 lifecycle, bioassay, endocrine disruption

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Introduction

Endocrine disrupting substances (EDSs) are exogenous substances or mixtures that alter function of the endocrine system and consequently cause adverse effects in individuals or their progeny (OECD, 1999). Effects of pulp mill effluents in Canada (Hewitt et al. 2008) and sewage in Britain (Jobling and Tyler 2003) provide some of the best examples of endocrine disruption in wild fish. Recently, in a whole-lake experiment, chronic exposure of fathead minnow to low concentrations (5-6ng/L) of 17 α -ethynylestradiol (EE₂) caused feminization and intersex in males, induced vitellogenesis, altered oogenesis in females, and caused a near collapse of the population (Kidd et al. 2007). EDSs may interfere with any part of endocrine control, including production, release, transport, metabolism, receptor binding, action or elimination. The sum total of these effects can be measured in fish laboratory bioassays designed to assess reproduction, development and growth (Patyna et al., 1999; Zillioux et al., 2001; Parrott and Wood, 2002; Seki et al., 2004). Chronic exposure to xenobiotics can induce changes to organisms not noted by shorter-term exposures (Parrott and Blunt, 2005), including factors that may be manifested at the population level, such as lower recruitment (Ankley et al., 2001).

Lifecycle bioassays or multigenerational bioassays have been developed in freshwater fish species, including the fathead minnow, *Pimephales promelas* (Ankley et al., 2001; Lange et al., 2001), Japanese medaka, *Oryzias latipes* (Seki et al., 2003) and zebrafish, *Danio rerio* (Olsson et al., 1999). Partial lifecycle and short-term bioassays have been developed for estuarine and marine species; examples include sheepshead minnow, *Cyprinodon variegates* (Folmar et al., 2000; Zillioux et al., 2001; Hemmer et

al., 2008) and mummichog, *Fundulus heteroclitus* (MacLatchy et al., 2003; Boudreau et al., 2004; Peters et al. 2007; Bosker et al., 2009). Effects of EDSs on species living in salt water may be quite different than those in fresh water because of differences in biological availability of the contaminants based on physical water chemistry and fish physiology (e.g., in contaminant uptake). Therefore, studies focused on the effects of EDSs on estuarine species are warranted, especially given the extent of coastal activity by humans and the importance of estuaries as spawning and nursery grounds (Oberdörster and Cheek, 2001).

Mummichog are the numerically dominant fish species in salt marshes along the east coast of Canada and the United States (Armstrong and Child, 1965), and have demonstrated sensitivity to EDSs in laboratory (MacLatchy et al., 2003; Peters et al., 2007), artificial stream (Dubé et al., 2002), and field (Leblanc et al., 1997) assessments. The mummichog is a good candidate for lifecycle bioassay development due to its size, ease of husbandry and ability to manipulate its reproductive cycles. Mummichog are relatively sedentary, exhibiting small home ranges in the wild (Skinner et al. 2005) and they are potentially exposed to environmental EDSs throughout their life cycle.

EE₂ has been chosen as a model EDS for developing bioassays due to its environmental relevance as well as its confirmed effects on the reproductive endocrine system via estrogen receptor-mediated pathways (OECD, 1999; Ankley et al., 2001; Metcalfe et al., 2001; MacLatchy et al., 2003). EE₂ is one component of sewage effluent associated with increased incidence of intersex in male fish exposed downstream of sewage treatment plants (STPs) (Jobling and Tyler, 2003). It is a synthetic pharmaceutical (birth control pill and hormone replacement therapy) that is not broken

down in sewage treatment; concentrations of EE₂ present in Canadian STPs are usually between 1-10ng/L EE₂, although levels have been documented as high as 42ng/L (Desbrow et al., 1998; Ternes et al., 1999). In earlier short-term (7- or 15-day) exposure studies using EE₂, adult mummichog displayed endocrine impacts at low, environmentally-relevant concentrations, as well as similar responses at higher pharmaceutical concentrations (MacLatchy et al., 2003). In longer-term (21- or 28-day) EE₂ exposures, reproductive cycling was shifted in females, sex steroid production and circulating levels were altered and at environmentally-relevant (approximately 20% of nominal 100ng/L exposures), fecundity and fertility were reduced (Peters et al., 2007).

The objective of this study was to determine the impact of chronic EE₂ exposure on offspring development. Embryos derived from mummichog parents exposed during pre-spawning and spawning phases to EE₂ were continuously exposed to EE₂ for 15 months, through their development to pre-spawning juveniles/adults. Embryonic/larval endpoints (time to hatch, hatch success, length at hatch), larval/juvenile endpoints (growth, survival, vertebral abnormalities) and yearling endpoints (liver vitellogenin, gonad and liversomatic indices, condition factor, sex ratios) were evaluated for anomalies. This study, in conjunction with our previous studies (MacLatchy et al., 2003, Boudreau et al., 2004; MacLatchy et al., 2005; Boudreau et al., 2005; Sharpe et al., 2004; Peters et al., 2007) furthers our ability to understand the effects of EDSs on various life stages of mummichog. This study demonstrates that developmental stages of mummichog are sensitive to EE₂ at exposure levels similar to those that interfere with reproduction in adult mummichog (Peters et al. 2007), and that the concentrations at

which effects occur are higher than those noted in freshwater model species (Länge et al. 2001; Andersen et al., 2003).

Materials and Methods

Chemicals

The 17 α -ethynylestradiol (EE₂; 98% purity) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). EE₂ was stored at -20°C in 100% ethanol (Les Alcools de Commerce, Boucherville, QC, Canada) at stock concentrations of 3ng/mL, 30ng/mL, 300ng/mL and 3000ng/mL EE₂ for adult exposures and 10ng/mL, 100ng/mL, 1000ng/mL and 10000ng/mL EE₂ for larval and juvenile exposures. Unless otherwise indicated, chemicals and reagents were purchased from Sigma-Aldrich and laboratory supplies from Fisher Scientific (Nepean, ON, Canada).

Experimental Conditions

Collection and breeding protocols for adult mummichog used in this study have previously been described (Peters et al., 2007). Offspring were maintained at the same exposure conditions as their parental groups at 0, 0.1, 1, 10, or 100ng/L EE₂, in static conditions with daily water changes and treatment renewal for the 61-week study period. As developing embryos, larvae, fry and juveniles grew, photoperiod was adjusted to simulate seasonal day length: 16:8 h light:dark at initiation (July); 14:10 h light:dark at week 14 (October); 12:12; light:dark at week 20 (November); 14:10 light:dark at week 32 (February); 15:9 light:dark at week 40 (April); and 16:8 light:dark from week 46 (May) to termination of the experiment. Temperature was held at room temperature, which

decreased slightly from 18-21°C in the summer (weeks 1-20) to 16-18°C for the winter (weeks 21-40), returning to 18-21°C for the remainder of the study.

Larvae were fed live, newly hatched *Artemia* sp. nauplii (Bohai Bay Salt Ponds *Artemia* Cysts, Aquatic Ecosystems, Apopka, FL, USA) enriched with Roti-rich™ (Aquatic Ecosystems) twice daily (1 mL of concentrated *Artemia* per L of water) and Fry Food (Rolf C. Hagen, Montreal, QC, Canada) to satiation once daily for 14 weeks. Beginning at 8 weeks, freeze-dried Red Grubs (Rolf C. Hagen) were used to supplement the Fry Food diet as the juveniles were weaned from the *Artemia*. Flaked Staple Food (Rolf C. Hagen) was introduced as the primary feed at 22 weeks, fed 2-3 times daily, supplemented by Red Grubs or Cichlid Food (Rolf C. Hagen) once daily.

Exposures

Naturally-spawned, fertilized mummichog eggs were collected from adults exposed to nominal exposure concentrations of 0, 0.1, 1, 10, or 100ng/L EE₂ for 21 and 28-days as previously reported (Peters et al., 2007). The fertilized eggs were transferred to glass Petri dishes at an initial density of 30 eggs per dish and held in 50 mL of 20‰ salinity EE₂-treated water. Each dish was examined daily at 4X magnification, and dead embryos and hatched larvae were removed. The water was then removed from each dish and replaced with 50 mL of water treated with the appropriate amount of EE₂. Time to hatch, survival to hatch and length at hatch were monitored over the hatching period.

Upon hatch, larvae were maintained in 50mL beakers containing the appropriate EE₂ concentrations and held at a maximum density of 10 larvae per beaker. Once the yolk sac was absorbed (1-3 days) and swimming began, the larvae were randomly

allocated to one of four aerated, static 37-L aquaria per treatment. Daily water renewals were done by completely replacing the water and adding new treatment solution in each tank after removing fish with a dip net and placing them in a temporary holding aquarium; fish were returned to their original treatment tanks following the water change. Upon initiation of the growout phase, each aquarium contained 5 L of EE₂-treated 20‰ saline water (dissolved oxygen > 80% saturation). At five weeks, the volume of water in each aquarium was increased to 10 L to accommodate growing larvae. Beginning at 10 weeks, volumes of water were adjusted separately for each tank to minimize the effect of density differences among the tanks due to differential survival. Water volume was set to 1 L per 1 g total wet weight of the fish in the aquarium and was adjusted every 3-4 weeks for the remainder of the experiment. Each tank was replaced 100% daily throughout the exposure period. Due to a dosing error in week 14, replicate tanks for 0 ng/L and 100 ng/L EE₂ were reduced to three tanks for subsequent endpoints.

Growth was determined weekly for the initial seven-week period, and then approximately every three weeks for the remainder of the experiment by collecting total length measurements (to the nearest mm) from 25 fish removed, measured and returned to each aquarium. Survival was determined from the number of fish in each tank on each measurement day, and calculated as the proportion of the original number of fish in the tank. Vertebral abnormalities were assessed at weeks 15, 48 and 61 and ranged from mild (one or two slight bends in the spine) to severe (one or more bends in the spine that dramatically altered body shape and/or affected swimming ability) and included scoliosis (lateral curvature) and lordosis (dorsoventral curvature) (Boudreau et al., 2004).

At week 48 (May 2004), all fish were assessed for weight (to 0.001g), standard and total length (mm), vertebral abnormalities and external sex. Sex was determined based upon colouration and secondary sex characteristics including yellow bellies, dark dorsal fin spot, silver vertical stripes, white or yellow spots in males, and brown or green backs and pale bellies in females. At week 52 (22 June 2004), less than a week following the full moon, 20 fish were randomly sampled from each aquarium except one 1ng/L aquarium and one 100ng/L aquarium where 0 and 15 fish were selected respectively, due to low numbers of fish in these tanks. These fish were anaesthetized (buffered 0.05% tricaine methane sulfonate; Syndel Laboratories, Vancouver, BC, Canada), assessed for weight (to 0.001g), standard and total length (mm), vertebral abnormalities and sex based upon secondary characteristics and gonadal assessment at 4X magnification.

Vitellogenin (VTG) in the liver was assessed at week 52 using an ELISA modified from MacLatchy et al. (2003). Liver tissue was dissected out, weighed, frozen on dry ice, and then stored at -80°C. For each mg of liver tissue, 1 µL of aprotinin (1KIU/µL) was added to the microfuge tube. Tissue was thawed and homogenized with a Kontes Pellet Pestle hand-held homogenizer. A small amount of the slurry (5 µL) was diluted 100X with Tris buffered saline containing Tween and bovine serum albumin and assayed as per MacLatchy et al. (2003). Prior to analysis, the method was tested by spiking liver slurry samples with a known concentration of VTG. Recovery of the spiked amount of VTG [recovered VTG = $0.549 + 1.071 * (\text{spiked VTG})$; $R^2=0.774$; $p<0.001$] was 88%. Interassay variability was 7% and intra-assay variability was 6%.

Gonadal tissue collected at week 52 was fixed in 10% buffered formalin and stored in 70% ethanol. Tissues were embedded in paraplast and sectioned at 7 µm using

a rotary microtome. Tissue sections were stained with Mallory stain and examined with a light microscope at 10X magnification for female tissue and 40X magnification for male tissue. For each fish that had sufficient tissue, six fields of view (at different depths into the gonad) were assessed for intersex and to determine developmental stage at week 52. Using the terminology for developmental stage of Blazer (2002), the number of previtellogenic and vitellogenic follicles were counted in females and the developmental stage(s) of the testes were determined for each field of view.

At week 61 (August 2004), four days prior to the full moon, all remaining fish were sampled and assessed for weight (0.001g), standard and total length (mm), vertebral abnormalities and sex based upon secondary characteristics and gonadal assessment at 4X magnification. At 52 weeks, the fish were not reproductive and by 61 weeks were beginning to regress; therefore, a breeding trial could not be carried out and the experiment was terminated.

EE₂ Analysis

Analysis of the concentration of EE₂ in the water was unavailable for this study due to insufficient water volumes collected for analysis. Therefore, a subsequent two-week exposure of adult mummichog (with equivalent volume of water and mass of fish as per juvenile exposures) was conducted in duplicate aquaria at nominal concentrations of 0 and 100ng/L EE₂. Volumes of water extracted and analyzed were 1L. EE₂ levels were extracted and analyzed as previously described (Peters et al., 2007) with additional method standardization to ensure EE₂ recovery in salt water. The water concentrations for the 100ng/L EE₂ treatment aquaria were highest at the point of delivery (74.7ng/L)

and decreased with time to 66.8ng/L at 1 h and 30.7ng/L at 6 h; at 12 and 24 h EE₂ levels were undetectable (detection limit at 10ng/L). Because of the inability to determine exposure concentrations at exposures <10ng/L nominal, all exposure concentrations are given as nominal values.

Statistics

Statistical analyses were conducted using SigmaStat 3.0 (SPSS, Chicago, IL, USA) or Systat 9.0 (Systat Software Inc., Richmond, CA, USA). Significant differences ($p < 0.05$) among the data were assessed separately for each sex when it was possible to determine gonadal sex. Prior to parametric analysis, assumptions of normality and variance homogeneity were tested on morphological data using normal probability plots and Levene's test. Where the data failed to meet the assumptions, they were $\log_{10}$ transformed and the assumptions retested. If the data still failed to meet the assumptions, an equivalent non-parametric test was used. Measures of time to hatch, hatch success rate, and length at hatch were assessed using single factor ANOVA, using Petri dishes as the replication units. Mortality, vertebral abnormalities and liver VTG were assessed using single factor ANOVA, with aquarium as the unit of replication. Vitellogenin data were examined for outliers using Dixon's test prior to analysis. Analyses of weight and length were performed using a nested analysis of variance (ANOVA) followed by Tukey's or Dunn's tests, with aquarium as the factor nested within treatment group. Kruskal-Wallis was used when the data failed to meet parametric assumptions. Differences in gonadal and liver weights relative to body weights (gonadosomatic index; GSI; [gonad wt/body wt]·100; and liversomatic index; LSI; [liver wt/body wt]·100), body

weight to standard length (condition factor; CF; $[\text{body wt}/\text{standard length}^3] \cdot 100$), and total length over time (growth) were determined using analysis of covariance (ANCOVA). The interaction term significance was set at $p=0.1$ (Environment Canada, 2005). Differences in morphological variables between sampling dates were assessed using A. Student's t-test. Sex ratios and gonadal development ratios were assessed using a chi-square analysis for proportions, with expected proportions set at 50:50, followed by an angular transformation and Tukey-type multiple comparison (Zar, 1999). Values are reported as means $\pm$ standard error (SE).

Results

Eggs exposed to 10ng/L and 100ng/L EE₂ hatched sooner than eggs exposed to 0ng/L EE₂ ($p=0.018$; Fig. 1). Eggs exposed to 100ng/L EE₂ had significantly lower hatch success than those in 0, 0.1, and 1ng/L ($p<0.001$; Fig. 1). Larvae hatched at 1 and 100ng/L EE₂ were significantly longer than those at 0, 0.1 and 10ng/L ($p<0.001$; Fig. 1).

Weight at length (condition factor) for both sexes decreased between week 48 (data not shown) and week 61 in all treatment groups. There were no differences in weight at length in male or female fish between 0ng/L and any other treatment at weeks 48, 52 (data not shown) or 61 (Table 1).

Post-hatch survival was found to be significantly higher for 100ng/L exposed fish than any other treatment ($p<0.001$; Fig. 2). At week 15 the proportion of fish exhibiting vertebral abnormalities at 0.1, 10, and 100ng/L were significantly lower than at 0 and 1ng/L ($p=0.021$; Fig. 3). These differences among treatments were not retained at 48 nor 61 weeks (data not shown).

Male fish exposed to 100ng/L EE₂ had significantly lower GSI at 52 weeks (p=0.016; 1.42±0.15 at 0ng/L vs 0.60±0.23ng/L at 100ng/L EE₂) but not at 61 weeks post-hatch (Table 1) and between week 52 and week 61, male GSI decreased in all treatments except 100ng/L EE₂ (data not shown). No differences were found in male LSI among treatments at weeks 48, 52 (data not shown) or 61 (Table 1). There was no difference in female GSI among treatments at 52 weeks (p=0.641; range of 2.55±0.14 at 0.1ng/L to 4.81±0.99 at 100ng/L) or 61 weeks (Table 1) though female GSI decreased between week 52 and week 61 in fish exposed to 0, 0.1, and 1ng/L EE₂. No differences in female LSI were found among fish exposed at 0ng/L and any other treatment (weeks 48 and 52, data not shown; week 61, Table 1).

At 52 weeks post-hatch, there was no difference among treatments in liver VTG in males (p=0.732; data not shown). There was an increase in liver VTG in females exposed to 100ng/L EE₂ compared to all other treatments (p<0.001; approximately 10-fold increase from 1500-2500µg/mg VTG at 0-10ng/L EE₂ to 14,000 to 17,000µg/mg VTG at 100ng/L EE₂).

At 52 weeks post-hatch, sex ratios based on secondary sexual characteristics were significantly different at 100ng/L, with females accounting for greater than 90% of the fish at this concentration (p<0.001; Table 2). Secondary sex characteristics differed in a similar fashion at the highest exposure at 48 and 61-weeks post-hatch. Visual examination of the gonads at 52 weeks post-hatch found sex ratios to be significantly different at 100ng/L EE₂; the proportion of females was 86.8% (p<0.001; Table 2).

Histological analysis of the gonads found sex ratios to be significantly different at 100ng/L EE₂; the proportion of females was 86.1% (p<0.001; Table 2). There were

significantly more vitellogenic oocytes in female fish exposed to 1, 10 and 100ng/L EE₂ when compared to the group exposed to 0ng/L EE₂ ($p < 0.001$; Table 3). There was only a single case of intersex, where a male fish (tissue was primarily prespermatogenic) exposed to 0.1ng/L EE₂ had three previtellogenic oocytes within the tissue. The proportion of male gonadal tissue at each maturation stage did not differ among treatments ($p > 0.05$; Table 3).

Discussion

Previous studies in our laboratories (MacLatchy et al., 2003; Boudreau et al., 2004; Peters et al., 2007) examined the effects of EE₂ exposure on adult and larval mummichog. This present work addresses questions that arose from these studies, including whether a standardized, multigenerational protocol could be developed for EDS studies to correlate physiological effects with population and/or reproductive impacts in mummichog as has been suggested for other fish species (Patyna et al., 1999; Ankley et al., 2001). The eggs used in this study were spawned and fertilized in a short-term reproductive bioassay in which adult mummichog were exposed to concentrations of EE₂ ranging from 0 to 100ng/L (nominal) for 21 and 28 days (Peters et al., 2007). The rearing of these eggs to sexual maturity provides a more complete understanding of the total impact of chronic and lifecycle EE₂ exposure in an estuarine species. Long-term exposure to the highest concentration (100ng/L nominal; approximately 30% of exposure concentration) resulted in a higher proportion of fish with female secondary sex characteristics and gonads. As well, female livers contained higher VTG levels at this concentration. These EE₂-induced changes occurred only at the highest concentrations

and parallel the physiological and reproductive effects observed in the parents of these offspring (decreased circulating steroids, increased VTG, decreased egg production and decreased fecundity/fertility; Peters et al., 2007) during shorter exposure periods.

In the progeny generation, hatching success was reduced when parents and eggs were exposed to 100ng/L EE₂. Decreases in both spawning output and fertilization success during adult exposure to 100ng/L EE₂ have been demonstrated in mummichog in our laboratory (Peters et al., 2007). The effects on progeny may be a threshold effect, as there is no indication of similar difficulties in spawning, fertilization or hatching at EE₂ levels <10ng/L (this study; Peters et al., 2007). However, a concentration-dependent response may exist between exposure concentrations of 10 and 100ng/L EE₂.

Sheepshead minnow exposed to EE₂ showed reduced egg production at 20 and 200ng/L and reduced hatching at 200ng/L (Zillioux et al., 2001). Hatching success decreased in a dose-dependent manner in larval mummichog exposed to estradiol (E₂) (Urushitani et al., 2002) and estrogenic alkylphenols caused complete larval mortality at concentrations $\geq 10\mu\text{M}$ and hatching success was <10% below this concentration (Kelly and Di Giulio, 2000).

As EE₂ concentrations increased, there was a decrease in the egg incubation period prior to hatch. Shortened time to hatch has been documented in zebrafish exposed to the weakly estrogenic pesticide methoxychlor (Versonnen et al., 2004) and brown trout (*Salmo trutta*) exposed to wood-sterols (Lehtinen, et al., 1999). Larvae that emerged in the present study were significantly longer when exposed to 1 and 100ng/L EE₂.

Exposure to EE₂ prior to spawning and fertilization may affect the developing oocyte before the development of the protective chorion and associated membranes of the egg

that prevent exogenous compounds from reaching the developing embryo (Anadu et al., 1999).

Survival from hatch to the conclusion of the study was found to be significantly higher for 100ng/L EE₂-exposed fish than any other treatment. Fathead minnow exposed to EE₂ from fertilization showed no differences in survival among treatments (Länge et al., 2001). Mummichog exposed for 60 days and reared in clean water showed increased survival at 10ng/L, no change in survival at 1000ng/L and decreased survival at 10,000ng/L EE₂ (Boudreau et al., 2004). After 10 weeks of the growout period, volumes in all the aquaria were adjusted to compensate for fish numbers in an attempt to eliminate potential density-dependent effects. Boudreau et al. (2004) suggested that decreasing density by increasing volume of water and/or decreasing number of fish reduced mortalities in mummichog. It is also possible that the decreased mortality post-hatch in the 100ng/L treatment was the result of increased embryonic mortality, which weeded-out weaker fish.

There were no differences in male or female condition (length at weight) throughout the study and differences in vertebral abnormalities observed among treatments at week 15 were not present at week 48 or 61. The proportion of 0ng/L fish exhibiting vertebral abnormalities decreased over time, and may be attributable to the mortality of these fish. The growth of soft-tissue that hides underlying abnormalities has previously been suggested as a mechanism that decreases observable deformities (Boudreau et al., 2004). Whether any of the endpoints measured in relation to time to hatch, size at hatch, juvenile growth, abnormalities, etc., are significant from a population/biological standpoint requires more study, especially in a field situation. It

has long been known that size at hatch, growing season length, and food availability all significantly affect long-term survival in wild fish populations (Andersen, 1988; Cargnelli and Gross, 1996) making any size deficiency an issue in the wild.

VTG production in the liver naturally occurs in female fish once a threshold level of E_2 is reached in the hepatic estrogen receptors (Denslow et al., 1999). VTG is a sensitive biomarker of estrogen exposure; exogenous estrogen induces VTG synthesis in female and male and/or juvenile fish (Denslow et al., 1999) in both freshwater (Panter et al., 2002) and saltwater (MacLatchy et al., 2003) species. VTG induction as an indicator of decreased reproductive potential has recently been assessed in fathead minnow (Miller et al., 2007) and high levels can cause kidney problems in fish (Folmar et al., 2001).

There was no difference in liver VTG in male fish at week 52. However, the mean VTG for males exposed to 100ng/L EE_2 was more than twice the mean of male fish exposed to 0ng/L EE_2 . There were only five fish identified as male in the 100ng/L EE_2 treatments, compared to 23-39 fish in each of the other treatments. The small sample size for male fish at this treatment level may be masking any effect on VTG. Alternatively, during a long exposure, feedback and regulatory physiological mechanisms may allow the fish to achieve homeostasis and prevent a VTG response (Länge et al., 2001). Females exposed to 100ng/L EE_2 had increased liver vitellogenin compared to all other treatments.

Medaka exposed to two estrogenic alkylphenols for 60 days post-hatch had concentration-dependent increases in hepatic VTG in both sexes (Seki et al., 2003).

Whole body VTG increased in fathead minnow exposed to EE_2 for 142 days post-hatch (Länge et al., 2001). Adult female mummichog had elevated plasma VTG at 100ng/L EE_2 in a 15-day exposure (MacLatchy et al., 2003), indicating consistent effects through

different life stages in this species. While liver sizes in fish generally increase during vitellogenesis (Kleinkauf et al., 2004), there was no correlation between LSI and liver VTG in this study (data not shown).

At 52 weeks post-hatch, males exposed to 100ng/L EE₂ had significantly lower GSI than all other treatments and at week 61, males exposed to 100ng/L EE₂ had GSIs less than 60% of each of the other treatments. Male GSI decreased between week 52 and week 61 in 0, 0.1, 1 and 10ng/L EE₂ treatments. Few males showed the brilliant yellow of sexual maturity and it is possible that male GSI was decreasing due to the annual reproductive cycle (week 61 was 26 August 2004). GSI in mummichog decreases as the breeding season ends (Cochran, 1987). Male fish exposed to 100ng/L EE₂ did not demonstrate the reduction in GSI between weeks 52 and 61 observed in the other treatments. Male zebrafish exposed for 24 days at 10 and 25ng/L EE₂ showed reductions in GSI, due to a predominance of immature sperm (Van den Belt et al., 2002). No differences among treatments were found within each male gonadal maturation stage. However, the high variability, especially in the 100ng/L treatments, where only five male fish had enough gonadal tissue to embed and section for analysis, may be masking an effect.

There was no difference in GSI among treatments in female fish at 52 (June) or 61 (August) weeks post-hatch. However, female GSI decreased between week 52 and week 61 in fish exposed to 0, 0.1, and 1ng/L EE₂, and did not change in female fish exposed to 10 and 100ng/L EE₂. These changes in GSI, and the increased proportion of vitellogenic oocytes in female fish exposed to 1, 10 and 100ng/L EE₂, indicate that higher levels of EE₂ may cause differences in gonadal development during the spawning season.

In the parental generation, there was also indication that EE₂ exposure could cause disruption to reproductive cycles in female mummichog. Adult females exposed to higher concentrations of EE₂ (1-100ng/L) had altered gonadal steroid production and completed the spawning cycle earlier than fish exposed to 0 and 0.1ng/L EE₂ (Peters et al., 2007).

Sex ratios based upon secondary sexual characteristics, visual examination of the gonads and histological analysis demonstrated skewed sex ratios at 100ng/L. Early life exposure of mummichog to EE₂ weakly skewed the sex ratio towards females, even when raised following 60 days of post-hatch EE₂ exposure in clean water (Boudreau et al., 2004). Medaka eggs injected with EE₂ have shown sex reversal of genetic males upon growout (Papoulias et al., 1999) while medaka exposed to EE₂ from two days post-hatch had gonadal intersex at 2ng/L (Balch et al., 2004). Fathead minnow have been completely feminized in lifecycle exposures to 4ng/L EE₂ (Länge et al., 2001) and 3.2ng/L (Parrott and Wood, 2002). Mummichog sex ratios appear to be affected at higher exposure concentrations (>10ng/L EE₂) than other species (<5ng/L EE₂). Gonadal sex change in fish can be induced by exposure to estrogens during windows of sensitivity related to gonadal differentiation and development (Nakamura et al. 1998). The fathead minnow has a period of enhanced sensitivity to EE₂ between 10 and 15 days post-hatch, when male fish are more likely to develop female characteristics (Van Aerle et al., 2002) and 6-day exposures with medaka have demonstrated that exposure while an embryo, newly hatched fry or 1 week post-hatch are sufficient to alter sex ratios and/or develop intersex gonads at sexual maturity (Koger et al., 2000). In mummichog, the window of

sensitivity to exogenous steroids during differentiation has not been precisely identified but most likely occurs from 2-4 weeks after hatching (Urushitani et al., 2002).

EE₂ is typically found in low concentrations (0.1-10ng/L with amounts as high as 42ng/L in some cases) in Canadian sewage effluents (Desbrow et al., 1998; Ternes et al., 1999). In the low range of environmentally-relevant concentrations, exposure to EE₂ during different life stages results in minimal, if any, adverse effects on reproductive endpoints (this study; MacLatchy et al., 2003; Boudreau et al., 2004). However, exposure at the high end of the range (30% of nominal 100ng/L EE₂ exposure) negatively affected larval and juvenile endpoints, including hatch success, liver VTG, male GSI, sex ratios and female gonadal development in mummichog. Because these effects occurred at the high range (>10ng/L actual exposure), long-term full lifecycle exposures in mummichog do not appear to offer advantages from a testing and sensitivity perspective over the shorter-term adult reproductive test (e.g., Peters et al., 2007).

More studies are required to determine the relative sensitivities of different fish species, including those typically classified as “model” species. In the present study, the 100ng/L exposure averaged approximately 30% (30ng/L) over the course of a 24-h day; all other exposures were ≤10ng/L nominal and therefore significantly less in actuality. This makes comparison to other studies that had exposure concentrations <10ng/L or >10ng/L possible. Fathead minnow have failed development of secondary sex characteristics, altered mating behaviour, intersex and induced VTG in males at 1-5 ng/L EE₂ (Länge et al., 2001; Lattier et al., 2002; Parrott and Blunt, 2005); Japanese medaka display intersex and reduced copulations at 2-10ng/L EE₂ (Balch et al., 2004); and zebrafish have reduced spawning, fertilization, intersex and GSI at 5-10ng/L EE₂ (Van

den Belt et al., 2001; Van den Belt et al., 2002; Nash et al., 2004). In contrast to freshwater species, in which effects are observed at concentrations $\leq 10\text{ng/L}$ EE₂, sheepshead minnow have reduced reproductive success at EE₂ between 20 and 200ng/L (Zillioux et al., 2001) while mummichog have altered steroid synthesis, VTG induction, reproductive success and sex differentiation at $>10\text{ng/L}$ EE₂ (MacLatchy et al., 2003; Peters et al., 2007; this study). Clearly, all of these species show significant effects from estrogen exposure during lifecycle exposure. Estuarine species, however, may have a different level of sensitivity, and/or their uptake may be altered (e.g., differences in gill vs. gut uptake), and/or the fate of contaminants may be different in saltwater than freshwater systems. Clearly, further investigation is required to determine the roles of physiology and physical environment on biological effects of EDSs in estuarine species before determining whether zebrafish or other freshwater species with shorter life cycles have significant test advantages and can be used as surrogate test species for regulating EDS exposures in saltwater environments.

In mummichog, population-level adaptation to bio-accumulative, persistent contaminants such as halogenated aryl hydrocarbons has been observed in field and lab studies (Nacci et al., 1999); the mechanism for such adaptation is associated with a consistent lack of inducibility of genes that are normally inducible by aryl hydrocarbon receptor agonists (Meyer et al., 2003). In the highly-polluted New Bedford Harbor (Massachusetts, USA), reproductively-active males and females demonstrate different physiological (e.g., lower estrogen and androgen levels) and molecular (e.g., higher brain P450 aromatase B expression) states compared to reference-site fish. Despite these effects, gonadal sex differentiation, reproduction and embryonic/larval development are

within normal ranges even following multi-generational exposure to estrogenic contaminants (Greytak et al., 2003). Further studies are required to complete the picture linking physiological/molecular effects and population-level effects in fish exposed to EDSs; better understanding of these links may help explain differences in species sensitivities.

In conclusion, this is the first lifecycle exposure of mummichog to EE₂ and demonstrates that continued development of bioassay protocols in this cyclical, lunar spawner of the east coast of North America can provide useful models for the study of EDSs. The concentration levels at which effects occurred in the offspring were greater than have been shown for freshwater species and at the same approximate level shown to have effects in the mummichog adult reproductive test (>10ng/L). Of most concern in this study are endpoints with the potential to affect the population structure. Chronic exposure of successive generations of mummichog to EE₂ resulted in reduced spawning (Peters et al., 2007), fertilization and hatching, as well as altered sex ratios and gonadal development. This implies fewer fish being added to the population with each successive year; coupled with altered GSIs in adult and juvenile males, problems could compound with time. Severe effects of EE₂ on a population of fathead minnow have recently been demonstrated in a whole-lake experiment (Kidd et al. 2007), demonstrating cause for concern of environmentally-relevant EE₂ concentrations on fish populations.

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Figures

Figure 1. (A) Mean time to hatch of mummichog eggs exposed to 0, 0.1, 1, 10, or 100ng/L 17 α -ethynylestradiol (EE₂). (B) Mean hatch success of mummichog eggs exposed to 0, 0.1, 1, 10, or 100ng/L EE₂. (C) Mean length at hatch of mummichog larvae exposed to 0, 0.1, 1, 10, or 100ng/L EE₂. Bars represent means $\pm$ SE. Means showing different letters are significantly different ($p < 0.05$). $n = 39-51$ Petri dishes per treatment.

Figure 2. Mean survival of mummichog exposed to 0, 0.1, 1, 10, or 100ng/L 17 α -ethynylestradiol from hatch to 61 weeks post-hatch. $n = 3-4$ tanks per treatment.

Figure 3. Mean prevalence of vertebral abnormalities in juvenile mummichog exposed to 0, 0.1, 1, 10, or 100ng/L 17 α -ethynylestradiol at week 15 post-hatch. Bars represent means $\pm$ SE. Means showing different letters are significantly different ($p < 0.05$). $n = 3-4$ tanks per treatment.

Table 1: Mean (± 1 SE) weight, length, liver somatic index (LSI), gonadosomatic index (GSI), and condition factor for juvenile male and female *Fundulus heteroclitus* exposed to 17 α -ethynylestradiol (EE₂) from fertilization at 61 weeks after hatch. Different letters identify significantly different treatments within variables ($p < 0.05$).

Sex	Treatment (ng/L)	n	Weight (g)	Length (cm)	LSI (%)	GSI (%)	Condition (%)
Male	0	26	0.85 $\pm$ 0.06 ^a	3.76 $\pm$ 0.08 ^a	2.35 $\pm$ 0.16 ^a	0.86 $\pm$ 0.12 ^a	1.57 $\pm$ 0.07 ^a
	0.1	37	0.71 $\pm$ 0.05 ^a	3.55 $\pm$ 0.08 ^a	1.97 $\pm$ 0.12 ^a	0.77 $\pm$ 0.08 ^a	1.50 $\pm$ 0.05 ^a
	1	51	0.85 $\pm$ 0.05 ^a	3.70 $\pm$ 0.07 ^a	1.98 $\pm$ 0.13 ^a	0.78 $\pm$ 0.07 ^a	1.60 $\pm$ 0.04 ^a
	10	34	0.83 $\pm$ 0.06 ^a	3.72 $\pm$ 0.07 ^a	2.33 $\pm$ 0.17 ^a	0.82 $\pm$ 0.10 ^a	1.54 $\pm$ 0.06 ^a
	100	12	0.65 $\pm$ 0.07 ^a	3.53 $\pm$ 0.15 ^a	2.27 $\pm$ 0.35 ^a	0.45 $\pm$ 0.13 ^a	1.46 $\pm$ 0.10 ^a
	p-value		0.155	0.497	0.242	0.395	0.418
Female	0	27	0.94 $\pm$ 0.05 ^{ab}	3.91 $\pm$ 0.08 ^a	2.45 $\pm$ 0.19 ^{ab}	2.78 $\pm$ 0.77 ^a	1.56 $\pm$ 0.05 ^{ab}
	0.1	42	0.85 $\pm$ 0.05 ^a	3.82 $\pm$ 0.08 ^a	2.59 $\pm$ 0.15 ^{ac}	1.78 $\pm$ 0.09 ^a	1.49 $\pm$ 0.07 ^a
	1	45	0.98 $\pm$ 0.05 ^{ab}	3.93 $\pm$ 0.08 ^a	2.05 $\pm$ 0.14 ^b	2.07 $\pm$ 0.19 ^a	1.58 $\pm$ 0.04 ^{ab}
	10	38	0.91 $\pm$ 0.06 ^{ab}	3.83 $\pm$ 0.07 ^a	2.85 $\pm$ 0.10 ^a	3.62 $\pm$ 0.79 ^a	1.57 $\pm$ 0.04 ^{ab}
	100	47	1.11 $\pm$ 0.06 ^b	4.04 $\pm$ 0.07 ^a	2.24 $\pm$ 0.11 ^{bc}	3.56 $\pm$ 0.81 ^a	1.65 $\pm$ 0.06 ^b
	p-value		0.025	0.371	<0.001	0.364	0.024

Table 2. Proportion (mean $\pm$ 1SE) of juvenile mummichog exhibiting female secondary sex characteristics and/or female gonads at 52 weeks post-hatch after exposure to 0, 0.1, 1, 10 or 100ng/L EE₂. Differing superscripts identify significantly different treatments ($p < 0.05$). n=3-4 tanks per treatment.

Treatment (ng/L)	N	Mean % $\pm$ SE		
		Secondary Sex Characteristics	Gonadal Sex	Histological Sex
0	3	0.514 $\pm$ 0.104 ^a	0.495 $\pm$ 0.095 ^a	0.583 $\pm$ 0.084 ^a
0.1	4	0.462 $\pm$ 0.078 ^a	0.480 $\pm$ 0.045 ^a	0.430 $\pm$ 0.043 ^a
1	3	0.594 $\pm$ 0.075 ^a	0.567 $\pm$ 0.058 ^a	0.602 $\pm$ 0.033 ^{ab}
10	4	0.580 $\pm$ 0.084 ^a	0.525 $\pm$ 0.103 ^a	0.570 $\pm$ 0.076 ^a
100	3	0.928 $\pm$ 0.043 ^b	0.868 $\pm$ 0.076 ^b	0.861 $\pm$ 0.071 ^b

Table 3. Developmental stages of gonadal tissue for female and male juvenile mummichog exposed to 0, 0.1, 1, 10 or 100ng/L EE₂ at 52 weeks post-hatch. Proportion (mean $\pm$ 1SE) of fish with vitellogenic gonads (females) and spermatogenic gonads (males) is shown. Differing superscripts identify significantly different treatments ($p < 0.05$) within each gonadal stage. n=2-4 tanks per treatment.

Treatment (ng/L)	n	Females	n	Males
		Vitellogenic		Spermatogenic
0	3	0.467 $\pm$ 0.014 ^a	3	0.590 $\pm$ 0.117 ^a
0.1	4	0.519 $\pm$ 0.024 ^{ab}	3	0.703 $\pm$ 0.104 ^a
1	3	0.526 $\pm$ 0.040 ^{bc}	3	0.515 $\pm$ 0.039 ^a
10	4	0.565 $\pm$ 0.008 ^d	4	0.431 $\pm$ 0.067 ^a
100	3	0.504 $\pm$ 0.039 ^{bc}	2	0.195 $\pm$ 0.195 ^a

Figure 1

Figure 2

Figure 3