Marine Environmental Research 69 (2010) 178–186

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

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

Rebecca E.M. Peters [a], Simon C. Courtenay [b], L. Mark Hewitt [c], Deborah L. MacLatchy [a,*]

[a] Department of Biology and Canadian Rivers Institute, University of New Brunswick, Saint John, NB, Canada E2L 4L5
[b] Fisheries and Oceans Canada at the Canadian Rivers Institute, Department of Biology, University of New Brunswick, Fredericton, NB, Canada E3B 6E1
[c] National Water Research Institute, Environment Canada Burlington, ON, Canada L7R 4A6

ARTICLE INFO

Article history:
Received 27 December 2007
Received in revised form 6 September 2009
Accepted 7 October 2009

Keywords:
Ethynylestradiol
Fundulus heteroclitus
Vitellogenin
Sex differentiation
Development
Lifecycle
Bioassay
Endocrine disruption

ABSTRACT

Fertilized mummichog eggs retrieved from 17α-ethynylestradiol (EE_2) exposed adult fish were raised in concentrations of EE_2 ranging from 0 to 100 ng/L (100 ng/L EE_2 estimated to have actual average exposure concentrations of 30% of nominal; 0.1–10 ng/L were below detect throughout 24-h exposure period) for 61 weeks post-hatch. Eggs exposed at 100 ng/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 100 ng/L EE_2, 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 100 ng/L EE_2, female fish had a higher proportion of vitellogenic follicles in the ovarian tissue. Males exposed at 100 ng/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 EE_2 at or below 10 ng/L showed inconsistent effects on development and reproductive potential. This study indicates the potential for population-level effects at the high range of environmental EE_2 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 EE_2 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 because of species sensitivity or due to differences between freshwater and saltwater availability of EE_2 or its uptake requires further study.

1. 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 (5–6 ng/L) of 17α-ethynylestradiol (EE_2) 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; Länge et al., 2001), Japanese medaka, *Oryzias laripes* (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

* Corresponding author. Present address: Department of Biology and Canadian Rivers Institute, Wilfrid Laurier University Waterloo, ON, Canada N2L 3C5. Tel.: +1 519 884 0710x2221; fax: +1 519 746 2472.
E-mail address: dmaclatchy@wlu.ca (D.L. MacLatchy).

0141-1136/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2009.10.002

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.

EE_2 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). EE_2 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 EE_2 present in Canadian STPs are usually between 1 and 10 ng/L EE_2, although levels have been documented as high as 42 ng/L (Desbrow et al., 1998; Ternes et al., 1999). In earlier short-term (7- or 15-day) exposure studies using EE_2, 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) EE_2 exposures, reproductive cycling was shifted in females, sex steroid production and circulating levels were altered and at environmentally-relevant (~20% of nominal 100 ng/L exposures), fecundity and fertility were reduced (Peters et al., 2007).

The objective of this study was to determine the impact of chronic EE_2 exposure on offspring development. Embryos derived from mummichog parents exposed during pre-spawning and spawning phases to EE_2 were continuously exposed to EE_2 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, 2005; Boudreau et al., 2004, 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 EE_2 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).

2. Materials and methods

2.1. Chemicals

The 17α-ethynylestradiol (EE_2; 98% purity) was purchased from Sigma–Aldrich, Canada (Oakville, ON, Canada). EE_2 was stored at −20°C in 100% ethanol (Les Alcools de Commerce, Boucherville, QC, Canada) at stock concentrations of 3, 30, 300 and 3000 ng/mL EE_2 for adult exposures and 10, 100, 1000 and 10000 ng/mL EE_2 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).

2.2. 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 100 ng/L EE_2, 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× daily, supplemented by Red Grubs or Cichlid Food (Rolf C. Hagen) once daily.

2.3. Exposures

Naturally-spawned, fertilized mummichog eggs were collected from adults exposed to nominal exposure concentrations of 0, 0.1, 1, 10, or 100 ng/L EE_2 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 EE_2-treated water. Each dish was examined daily at 4× 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 EE_2. Time to hatch, survival to hatch and length at hatch were monitored over the hatching period.

Upon hatch, larvae were maintained in 50 mL beakers containing the appropriate EE_2 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 EE_2-treated 20‰ saline water (dissolved oxygen >80% saturation). At 5 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