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Effects of exposure to 17α-ethynylestradiol during larval development on growth, sexual differentiation, and abundances of transcripts in the liver of the wood frog (*Lithobates sylvaticus*)

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ABSTRACT

Populations of amphibians are in decline in certain locations around the world, and the possible contribution of environmental contaminants, including estrogenic compounds, to these declines is of potential concern. In the current study, responses of the wood frog (*Lithobates sylvaticus*) to exposure to 17α-ethynylestradiol (EE2), the synthetic estrogen used in oral contraceptives, during the larval period were characterized. Exposure of *L. sylvaticus* to 1.08, 9.55, or 80.9 μg EE2/L had no effects on survival, growth, or metamorphic endpoints monitored in the current study. However, there were significant effects of exposure to EE2 on phenotypic sex ratios. In general, lesser proportions of *L. sylvaticus* developed as phenotypic males and greater proportions developed as phenotypic females or with mixed sex phenotypes at all concentrations of EE2 tested. Utilizing the data collected in the current study, the EC_{50} for complete feminization of *L. sylvaticus* was determined to be 7.7 μg EE2/L, and the EC_{50} for partial feminization was determined to be 2.3 μg EE2/L. In addition, after chronic exposure, abundances of transcripts of vitellogenin A2, high density lipoprotein binding protein, and 7-dehydrocholesterol reductase were 1.8–280-fold greater in livers from *L. sylvaticus* exposed to EE2 compared to controls. Overall, there were significant effects of exposure to all concentrations of EE2 tested, the least of which was within about 2-fold of estrogen equivalent concentrations previously measured in the environment.

1. Introduction

Declining populations of amphibians are of concern worldwide (McCallum, 2007). Several factors have been considered as possible contributing causes to these declines. These factors include exposure to chemical contaminants in the aquatic environment (Linder et al., 2003). One class of contaminants that is of concern for amphibians is chemicals that can modulate endocrine function. Some of these chemicals can be released by human activities and might affect endocrine systems of aquatic vertebrates. Among the endocrine active chemicals, environmental estrogens have been hypothesized to be one group of compounds that might affect populations of amphibians, due to their ability to feminize and/or demasculinize development of the gonads, thereby affecting the homeostasis of the endocrine system and potentially affecting reproductive fitness (Chang and Witschi, 1955; Hogan et al., 2008; Witschi, 1951). Estrogenic substances, such as 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), can enter aquatic environments through sources like the discharge of liquid effluents from wastewater treatment facilities (Ankley et al., 2007) and runoff that contains animal manure (Hanselman et al., 2003), which has the potential to result in exposure of aquatic wildlife, including fish and amphibians.

The wood frog (*Lithobates sylvaticus*) is native to areas of North America. It ranges further north than any other species of frog in North America and is the only species of amphibian found north of the Arctic Circle in this part of the world. *L. sylvaticus* is considered common throughout much of its range, but it is not generally used in toxicological studies that focus on the effects of endocrine active chemicals. Other native species of North American frogs in the family Ranidae, like green frogs (*Lithobates clamitans*) (McDaniel et al., 2008; Park and Kidd, 2005), bullfrogs (*Lithobates catesbeianus*) (Gunderson et al., 2011; Veldhoen and Helbing, 2001), and northern or southern leopard frogs (*Lithobates pipiens* or *Lithobates sphenocephalus*) (Hogan et al., 2008; Langlois et al., 2010; Storrs and Semlitsch, 2008; Tsai et al., 2005), are more commonly

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used. However, considering its extensive range in comparison with other North American species, additional toxicological data pertaining to *L. sylvaticus* would be relevant to assessments of risks posed by estrogenic chemicals in a variety of ecosystems.

L. sylvaticus is sensitive to exposure to estrogens around the time of sexual differentiation. While the exact timing of sexual differentiation in *L. sylvaticus* is unknown, in other ranid species, the process begins during pre-metamorphosis (before Gosner stage 30) and is completed before the end of mid-metamorphosis (Gosner stage 36) (Gosner, 1960; Hogan et al., 2008). Exposure to concentrations of potent estrogens in the 10–100 μg/L range at this time results in reversal of the phenotypic sex of genetic males, meaning they develop as phenotypic females (Witschi, 1951). While mechanisms responsible for reversing the phenotypic sexual characteristics of males to those of females in *L. sylvaticus* are not clear, previous research with the African clawed frog (*Xenopus laevis*) indicates that reversal of sex is due to the failure of primordial germ cells to migrate after exposure to estrogen (Hu et al., 2008). Thus, the reversal of phenotypic sex is due to demasculinizing effects of estrogen, which would not normally be present in great quantities in genetic males. Although *L. sylvaticus* has an XY system of sex determination, while *X. laevis* has a ZW system of sex determination, it is possible that a similar mechanism is responsible for reversal of phenotypic sex in both species.

Exposure to estrogens after sexual differentiation has been completed cannot cause sex reversal, but it can affect other biological parameters in amphibians. During larval development, the estrogen receptor (ER) gene becomes auto-inducible, which means it is able to be up-regulated by the presence of endogenous or exogenous estrogens (Tata et al., 1993). Once the ER becomes auto-inducible, exposure to estrogens activates typical estrogen-responsive pathways that are commonly used in studies of estrogenic compounds in a variety of species of vertebrates (Garcia-Reyero et al., 2009; Watanabe et al., 2009; Villeneuve et al., 2012). Estrogenic compounds, including endogenous hormones, pharmaceuticals, and synthetic chemicals, share a similar mode of action, which is the ability to bind to and agonize the estrogen receptor, thereby inducing expression of estrogen-responsive genes (Boelsterli, 2003). Induction of these genes presumably leads to *in vivo* effects observed in aquatic vertebrates exposed to estrogenic substances, including reproductive failure, formation of mixed sex gonads, and greater production of the yolk-precursor protein vitellogenin (reviewed by Sumpter and Johnson, 2008). In vertebrates, effects on estrogen-responsive genes are most pronounced in organs that are part of the hypothalamic–pituitary–gonad-liver axis (Villeneuve et al., 2012), and previous studies have demonstrated that alterations of the endocrine system can be reliably detected by monitoring expression of genes in livers of amphibians (Duarte-Guterman et al., 2010). As described by Villeneuve et al. (2012), in teleost fish, the specific genes in the liver affected by exposure to estrogens can be classified into three functional groups: genes involved in steroid metabolism and signaling, genes involved in vitellogenesis, and genes involved in cholesterol biosynthesis. Due to the scarcity of available data on similar processes in amphibians, and the great degree of conservation of the endocrine system among vertebrates, these functional groups were considered to be suitable for use in *L. sylvaticus*.

EE2 is the synthetic estrogen used in human oral contraceptives, and it is structurally and functionally similar to E2 *in vivo*. The initial work with *L. sylvaticus* exposed to EE2 during sexual differentiation indicated that concentrations of 1 μg EE2/L had no effect on sex ratios, 10 μg EE2/L partially feminized/demasculinized male larvae, and concentrations of 100 μg EE2/L completely feminized/demasculinized male larvae (Witschi, 1951). However, a later study indicated that exposure of *L. sylvaticus* tadpoles to 1 or 10 μg EE2/L had no effects on sexual differentiation, although that study suffered from some design flaws, including no replication of EE2 treatments (Mackenzie et al., 2003). Thus, although the threshold for feminization or demasculinization of *L. sylvaticus* by EE2 is unclear, exposure to 100 μg EE2/L significantly affected sexual differentiation of genetic males. This concentration elicits effects on phenotype, but it is ~6-fold less than the LC_{50} (~568 μg EE2/L) and ~2-fold less than the threshold for biological effects on growth (~225 μg EE2/L) for *L. sylvaticus* (Hogan et al., 2006).

The current study was designed to utilize the model estrogen EE2 to cause disordered sexual development in *L. sylvaticus*, and to further characterize concentrations of EE2 that cause feminization/demasculinization in this species. In addition, abundances of transcripts of genes involved in steroid metabolism and signaling, synthesis of cholesterol, and vitellogenesis were evaluated in livers from individual *L. sylvaticus* after long-term (up to 100 d) exposure to EE2. Based on previously published results, nominal concentrations of EE2 chosen for the current study, 1, 10, and 100 μg EE2/L, were expected to cause few, moderate, and severe effects (complete reversal of male phenotypes to female phenotypes), respectively. Concentrations chosen for the current study were based upon their ability to elicit effects on phenotype, but the least dose was only 2-fold greater than maximal estrogen equivalent concentrations measured in surface waters in the United States (~0.5 μg estrogen equivalents/L) (Kolpin et al., 2002a,b).

2. Materials and methods

2.1. L. sylvaticus

Before research commenced, all experimental procedures were approved by the University Committee on Animal Care and Supply (UCACS) at the University of Saskatchewan (Protocol #20100036). Collection of *L. sylvaticus* egg masses for scientific research was approved by the Saskatchewan Ministry of Environment (Permit #10FW059). On April 8, 2010, 6 egg masses of *L. sylvaticus* were collected from a communal deposition site from a pond in a relatively pristine non-agricultural area near Saskatoon, SK, Canada. Egg masses were immediately transferred to the Aquatic Toxicology Research Facility at the University of Saskatchewan and acclimated to laboratory conditions (~19 °C; light:dark cycle 16:8) and water (filtered City of Saskatoon municipal water). Eggs began hatching on April 12, 2010, and most larvae were free-swimming on April 15, 2010, at which point exposure to EE2 was initiated.

Healthy larvae (15 per tank) were placed into 6 L of laboratory water with the appropriate nominal concentration of EE2 (1, 10, or 100 μg/L; Sigma, Oakville, ON, Canada) dissolved in an ethanol carrier (Commercial Alcohols 95% ethyl alcohol, Toronto, ON, Canada). The final concentration of ethanol in treatment tanks, including solvent controls, was 0.0025%. All treatments were replicated in triplicate tanks. Tadpoles were fed *ad libitum* daily with a slurry of Nutrafin Flake Food and Nutrafin Max Spirulina Flakes (Rolf C. Hagen, Montreal, QC, Canada).

Each day, a 50% static water renewal was performed on each tank. Basic water quality measurements (temperature, DO, pH, conductivity) were collected daily with an YSI Quatro Multi-Parameter probe (Yellow Springs, OH, USA). Mortality of tadpoles was recorded daily. Concentrations of ammonia nitrogen, nitrate nitrogen, and nitrite nitrogen were monitored weekly with Lamotte colorimetric kits (Chestertown, MD, USA).

2.2. Analysis of 17α-ethynylestradiol concentrations

Concentrations of EE2 in exposure water were monitored periodically during the experiment via high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) to