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Estrogenic environmental contaminants alter the mRNA abundance profiles of genes involved in gonadal differentiation of the American bullfrog

Stephanie E. Wolff [a], Nik Veldhoen [b], Caren C. Helbing [b], Claire A. Ramirez [a], Janae M. Malpas [a], Catherine R. Propper [a,*]

[a] Department of Biological Sciences, S. Beaver St., Northern Arizona University, Flagstaff, AZ 86011, USA
[b] Department of Biochemistry and Microbiology, University of Victoria, P.O. Box 1700, STN CSC, Victoria, British Columbia V8W 2Y2, Canada

HIGHLIGHTS

- Wastewater effluent is a mix of anthropogenic chemicals that may feminize gonads.
- Estrogenic compounds alter activity of genes responsible for gonadal differentiation.
- Estradiol and octylphenol affect *cyp19a1* and *nr5a1* mRNA levels differently.
- Wastewater effluent exposure increases *cyp19a1* mRNA abundance in developing gonads.

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ABSTRACT

Wildlife and human populations are exposed to anthropogenic mixtures of chemicals in the environment that may adversely influence normal reproductive function and development. We determined the effects of exposure to estrogenic chemicals and wastewater effluent (WWE) on developing gonads of the American bullfrog, *Rana* (*Lithobates*) *catesbeiana*, a species whose widespread distribution make it an ideal model for environmental monitoring of endocrine effects of chemical contaminants. Premetamorphic bullfrog tadpoles were exposed to treatment vehicle, 17β-estradiol (E_2; 10^{-9} M) or 4-*tert*-octylphenol (OP; 10^{-9} M, 10^{-8} M, and 10^{-7} M). Additionally, gonadal differentiation was evaluated in bullfrog tadpoles from a WWE-containing site versus those from a reference location receiving no WWE. In both studies, phenotypic sex, steroidogenic factor-1 (*nr5a1*), and aromatase (*cyp19a1*) mRNA levels using quantitative real-time PCR were determined. Exposure to E_2 or OP did not alter sex ratios. In controls, both *nr5a1* and *cyp19a1* transcript levels exhibited sexual dimorphism, with males demonstrating higher levels of *nr5a1* and females greater abundance of *cyp19a1*. However, E_2 exposure increased *cyp19a1* mRNA abundance in testes and decreased levels in ovaries, eliminating the sexual dimorphism observed in controls. E_2-exposed males exhibited increased *nr5a1* transcript levels in the testes compared to controls, while females demonstrated no E_2 effect. OP treatment had no effect on female *cyp19a1* mRNA abundance, but exposure to 10^{-7} M OP increased testicular transcript levels. Treatment with 10^{-9} and 10^{-8} M OP, but not 10^{-7} M, resulted in decreased abundance of *nr5a1* transcript in both ovaries and testes. Animals from the field had sexually dimorphic gonadal levels of *cyp19a1*, but both sexes from the WWE site exhibited elevated *cyp19a1* transcript abundance compared to the reference location. Individual chemical compounds and anthropogenic wastewater effluent dispersed within the environment influence the levels of gonadal mRNA encoding key proteins involved in gonadal differentiation.

1. Introduction

Freshwater habitats are often the receiving environment for chemical byproducts related to municipal, industrial, and agricultural practices (reviewed in Luo et al., 2014). Many of these pollutants have been confirmed to disrupt various aspects of vertebrate endocrine function (Guillette, 2006; Kloas et al., 2009; Propper, 2005) and are identified as endocrine-disrupting chemicals (EDCs)[1] (Kavlock et al., 1996). However,

* Corresponding author at: Department of Biological Sciences, S. Beaver St., Northern Arizona University, Flagstaff, AZ 86011, USA.
E-mail address: Catherine.Propper@nau.edu (C.R. Propper).

[1] List of abbreviations: 4-*tert*-octylphenol (OP); 17β estradiol (E_2), endocrine disrupting chemical (EDC); JD Dam (JD); Rio de Flag (RDF); steroidogenic factor 1 (SF-1); wastewater effluent (WWE); parts per trillion (ppt).

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in an environmental context, these compounds exist as complex mixtures that vary depending upon factors such as biological transformation, local geochemistry, and specific land and water use (Sharma et al., 2009).

One source of anthropogenic pollution is from residual bioactive agents present in wastewater effluent (WWE) released into the environment following the primary and secondary treatment of municipal sewage (Sharma et al., 2009). Rivers and streams are the principal receiving environments for WWE, which can contain potent EDCs, including pharmaceuticals, alkylphenols, personal care products, pesticides and other contaminants that may interact with wildlife at sensitive life stages and negatively influence normal developmental processes (Kolpin et al., 2002; Propper, 2006). Abnormalities, such as sex reversal or the occurrence of testicular oocytes, have been noted in aquatic vertebrate species exposed to WWE, highlighting the deleterious impact on sex hormone action during development (Jobling et al., 1998; Liney et al., 2006; Sowers et al., 2009; Vajda et al., 2008; Woodling et al., 2006; reviewed in Quanrud and Propper, 2010).

Many different anthropogenic chemicals found within WWE are demonstrated, or predicted, to contribute to the estrogenic activity detected in receiving environments. An example is the alkylphenolic pollutant, 4-*tert*-octylphenol (OP) (Sharma et al., 2009; Ying et al., 2002) that is detected in the plasma and adipose tissue within human populations (Calafat et al., 2008; Inoue et al., 2000; Lopez-Espinosa et al., 2009; Ying et al., 2002). Across a wider range of vertebrate species the observed biological effects of OP exposure are multifold and may include altered gene and/or protein expression in gonadal and pituitary tissues (Lee et al., 2006; Majdic et al., 1997; Mayer et al., 2003; Rhee et al., 2009), abnormal steroid production (Mikkila et al., 2006; S.A. Myllymaki et al., 2005), sperm abnormalities, changes in testicular function (Herath et al., 2004; Kinnberg and Toft, 2003; Rey et al., 2009; Blake et al., 2004), and impaired sexual differentiation and fertility (Rasmussen et al., 2002; Seki et al., 2003; Karels et al., 2003). Thus, investigation of the biological outcome following treatment of a sentinel species with OP represents a useful model for exposure to estrogenic substances prevalent within the environment.

Anurans are established sentinels for investigating how EDCs impact reproductive development and function (Berg et al., 2009; Huang et al., 2005; Kloas and Lutz, 2006; Lutz and Kloas, 1999). Exposure of anurans at crucial developmental time points to the sex hormone 17β-estradiol (E_2), or environmental pollutants exhibiting estrogen-like activity, results in feminization of the gonads (Hayes, 1998; Hayes et al., 2010; Hogan et al., 2008; Hu et al., 2008; Kloas et al., 1999; Levy et al., 2004; Pettersson et al., 2006), and impairment of testicular function and oviduct development (Gyllenhammar et al., 2009; Pettersson et al., 2006). These effects following chemical exposure suggest a common root in dysregulation of estrogen-dependent signaling and modulation of associated gene networks involved in amphibian gonadal differentiation.

Molecular targets of estrogenic EDC action can include altered expression and function of steroidogenic factor 1 (SF-1), an orphan nuclear receptor encoded by *nr5a1* that functions as a transcription factor. The role of SF-1 has been elucidated primarily in mammalian and teleost species and is critical to steroidogenesis and subsequent differentiation of the gonads (Orlando and Guillette, 2001; reviewed in Schimmer and White, 2010). SF-1 is one of several transcription factors that regulate expression of a number of genes encoding proteins involved in the steroidogenic pathway, including steroidogenic acute regulatory protein (StAR), hydroxysteroid dehydrogenases (HSDs), and cytochrome P450 family 19 (CYP19; also known as aromatase) (Hoivik et al., 2010; reviewed in Schimmer and White, 2010). The latter enzyme is encoded by *cyp19a1* and is crucial in the production of endogenous estrogen. Thus, individual chemicals or complex mixtures comprised of environmental contaminants that are found to interfere with normal expression of *nr5a1* or SF-1 protein activity could have profound effects on gonadal differentiation and reproductive function across a diverse range of wildlife species. In the American bullfrog, *Rana* (*Lithobates*) *catesbeiana*, exposure to OP alters SF-1 protein levels and accelerates sexual differentiation in developing gonads (Mayer et al., 2003), demonstrating the potential of EDC exposure to influence expression of this gene critical to gonadal development.

In the present study, we evaluated the impact of short-term exposure of *R. catesbeiana* tadpoles to the estrogenic substances OP and E_2 by examining gonad morphology and the levels of gonadal *nr5a1* and *cyp19a1* mRNA. Because exposure to complex mixes found in WWE has also been demonstrated to impact gonadal morphology in both fish and amphibians, we additionally determined whether tadpoles exposed in situ within a pond receiving WWE display altered mRNA abundance profiles relative to a reference location that receives no point or non-point sources of pollution. Although OP itself was not detected in the WWE under investigation, several other estrogen-like compounds, including the closely related alkylphenol and nonylphenol were present (Propper, 2006). Our observations reinforce the ability of certain environmental contaminants to alter aspects of sex differentiation in wildlife species and suggest the need for further functional evaluation of the complex chemical mixture already found within human tissues (CDC, 2013).

2. Materials and methods

2.1. Animals and exposures

Animal collection was performed under an Arizona State Game and Fish collecting permit to C. Propper and all animal treatments were approved by, and followed the guidelines of, Northern Arizona University (NAU) IACUC Protocols #06-011 and #09-006 to C. Propper. OP (CAS # 140-66-9) and E_2 (CAS # 50-28-2) were purchased from Sigma-Aldrich (St. Louis, MO).

For Experiment 1, *R. catesbeiana* tadpoles at Gosner stage 34 were collected in one day in late June 2008 by seine net from JD Dam Lake (South Kaibab National Forest, Coconino County, AZ, USA) and brought to the NAU Animal Care Facility. JD Dam Lake is located in an area with no significant population center or agriculture, and is restricted to minimal non-motorized recreational use. Animals were kept at laboratory conditions (12 light:12 dark cycle; 21 °C) for 24h prior to treatment. All animals were maintained in the same conditions and treated in 40 L glass aquaria containing 35 L of artificial pond water prepared from reverse osmosis water with the addition of salts; 350 mg/L NaCl, 70 mg/L $CaCl_2$, and 10 mg/L $NaHCO_3$ (Sigma Aldrich, St. Louis, MO).

Chemical solutions were made on the first day of treatment and stored at −20 °C thereafter. Static exposures to each chemical were performed in triplicate (8–9 animals/replicate) for a total of N = 26/treatment and included nominal final concentrations of 10^{-9} M E_2 or 10^{-9} M, 10^{-8} M, and 10^{-7} M OP prepared in 95% ethanol and applied from stock chemical preparations for a final dilution of 3.5×10^{6} (10uL/tank). Control animals received an equivalent amount of 95% EtOH vehicle. All animals were treated for 96h, since previous studies demonstrated as little as 24h of treatment with OP was sufficient to induce shifts in SF-1 protein concentration (Mayer et al., 2003). Because concentrations may change during the 96 hour exposure time, after the first 48h of treatment, 90% of the water in each tank was changed with re-application of test chemicals, also at 90% of the original nominal concentrations. This dosing regimen insured that the animals were exposed to no more than the nominal concentration throughout the time course of the experiment, and that they received between 90 and 100% of the nominal concentration during most of the 96h exposure. Forty-eight hours following the second chemical administration, animals were euthanized by immersion in 0.2% tricaine methane sulfonate (MS-222; Finquel, Argent Laboratories) adjusted to pH 7.0 with KOH. Following euthanasia, body mass and snout-vent length (SVL) measurements were immediately obtained for each treated animal prior to tissue collection.

Following opening of the abdominal cavity, the gonads of each tadpole were photographed using a Nikon Coolpix 7600 (Nikon Inc.,