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

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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 for endocrine effects of chemical contaminants. Premetamorphic bullfrog tadpoles were exposed to treatment vehicle, 17 β -estradiol (E₂; 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 E₂ 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, E₂ exposure increased *cyp19a1* mRNA abundance in testes and decreased levels in ovaries, eliminating the sexual dimorphism observed in controls. E₂-exposed males exhibited increased *nr5a1* transcript levels in the testes compared to controls, while females demonstrated no E₂ 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

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dispersed within the environment influence the levels of gonadal mRNA encoding key proteins involved in gonadal differentiation.

Keywords

aromatase; steroidogenic factor 1; endocrine disruption; gonadal differentiation; octylphenol; wastewater effluent; amphibian

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, Jr., 2006; Kloas et al., 2009; Propper, 2005) and are identified as endocrine-disrupting chemicals (EDCs)¹ (Kavlock et al., 1996). However, 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; Snyder et al., 2001). 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; Myllymaki et al., 2005b), 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

¹**List of Abbreviations:** 4-*tert*octylphenol (OP); 17 β estradiol (E₂); endocrine disrupting chemical (EDC); JD Dam (JD); Rio de Flag (RDF); steroidogenic factor 1 (SF-1); wastewater effluent (WWE); parts per trillion (ppt)

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 17 β -estradiol (E₂), 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., 2007; 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, Jr., 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 comprising 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 for 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 E₂ 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, 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 CRP. OP (CAS # 140-66-9) and E₂ (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 24 hours 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 CaCl₂, and 10 mg/L NaHCO₃ (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 E₂ 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 3.5×10⁶ (.). Control animals received an equivalent amount of 95% EtOH vehicle. All animals were treated for 96 hours since previous studies demonstrated as little as 24 hours 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 48 hours 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 96 hour exposure. Forty-eight hours following the second chemical administration, animals were euthanized by immersion in 0.2% tricaine methane sulfonate (MS-222; Fiquel, 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, Melville, NY). Gross phenotypic sex was determined through a combination of direct visual inspection and evaluation of photographic images of treated animals. The right gonad was then removed and placed in RNAlater tissue preservative solution (Life Technologies Inc., Grand Island, NY, USA) and stored at 4°C for 24 hours with further storage at -80°C prior to total RNA isolation. The left gonad was placed in 10% neutral buffered formalin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and stored at room temperature for histological evaluation.

For Experiment 2, *R. catesbeiana* tadpoles were collected using a seine net from two sites in Coconino County, AZ; JD Dam Lake (GPS coordinates: 35.066471 N -112.027568 W; Elevation 1968 m; approximately 8.21 hectares of surface area) acted as the reference site and the Rio de Flag WWE pond (23.9 miles NE of JD Dam Lake (GPS coordinates: 35.183559 N -111.631271 W; Elevation 2072 m; 0.53 hectares) represented the test location. The Rio de Flag pond is fed by Class A+ (Arizona Department of Environmental Quality Grading) reclaimed wastewater originating from the City of Flagstaff's Rio de Flag municipal treatment plant as well as from rainwater run-off. A United States Geological Survey (USGS) evaluation of this water resource determined that it contains anthropogenic compounds at similar frequencies and levels to those found by broader USGS studies (Kolpin et al., 2002; Propper, 2006). JD Dam Lake is remotely located in the South Kaibab National Forest (47.5 km from the nearest town of Williams, Arizona) and receives only rain water runoff from the local region with little input from either point or non-point pollution sources. It has no motorized boating with occasional on-lake fishing and limited foreshore camping and fishing. All animals were collected in the same year in late June over a 23-day period and their median stage was Gosner stage 35 (ranging from Gosner stage 34 to 40). On the same day as the field collection, tadpoles were transported to the NAU Animal Care Facility and euthanized as described above, with body mass and SVL measurements immediately collected from each animal. Following opening of the abdominal cavity, the right gonad was preserved in RNAlater solution (Life Technologies) as described above, while the remaining kidney-gonad structure was placed in Bouin's fixative (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours followed by transfer to 70% ethanol and storage at room temperature.

2.2 Nucleic acid preparation and quantitative real-time polymerase reaction (qPCR) analysis

In Experiment 1, total RNA was isolated from RNAlater-preserved gonad tissue using TRIzol reagent (Life Technologies) according to the manufacturer's instructions and quantified using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE); the absence of protein contaminants was also confirmed. Total cDNA was prepared from 1 µg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) as per the manufacturer's recommended protocol and diluted 20-fold prior to qPCR analysis. Gene-specific amplification was achieved using *cyp19a1*, *nr5a1*, and ribosomal protein L8 (*rpl8*) qPCR primers designed and validated for *R. catesbeiana* (Table 1) (Gunderson et al., 2011). Reactions were assembled and run on a MX3005P Real-Time PCR System (Agilent Technologies Canada Inc, Mississauga, ON, Canada) as described previously (Gunderson et al., 2011).

In Experiment 2, total RNA was extracted from gonad tissue using an RNA Mini Kit as described by the manufacturer (Life Technologies). Total cDNA was prepared from 1 µg total RNA using the Superscript III First Strand Synthesis System following the manufacturer's protocol (Life Technologies). All cDNA samples were diluted 20-fold prior to qPCR analysis on an iQ5 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each diluted cDNA sample (2 µL) was included in a 25 µL reaction prepared using iQ SYBR Green SuperMix (Bio-Rad) containing 10 pmol of each primer. The thermocycle

conditions included an enzyme activation step at 95°C for 2 minutes, followed by 40 cycles of DNA template denaturation at 95°C for 15 sec (*cyp19a1*, *nr5a1*) or 30 sec (*rpl8*), primer annealing at temperatures shown in Table 1 for 30 seconds, and elongation at 65°C for 45 sec (*cyp19a1*, *nr5a1*) or 68°C for 1 min (*rpl8*).

For both experiments, each sample was run in quadruplicate and the average of the cycle threshold (C_t) values was used to calculate relative fold difference values reflective of *nr5a1* and *cyp19a1* transcript levels using the comparative C_t method (2^{-C_t}) (Schmittgen and Livak, 2008) with *rpl8* representing the endogenous reference mRNA used in data normalization (Gunderson et al. 2011). Data in Experiment 1 are expressed relative to female control animals and in Experiment 2 relative to reference site females.

2.3 Gonad histology and sex phenotype

Gonad tissue samples were prepared for histology using a Shandon Citadel 2000 automated tissue processor (Thermo Fisher). Paraffin embedded tissue was sectioned to 10 μ m with a Leica RM2255 microtome (Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained using a Hematoxylin and Phloxine-Eosin regressive staining method (Presnell, 1997). For each animal, gonad morphology and associated phenotypic sex were determined through a combination of inspection of dissection photographs and evaluation of gonad histology using a Nikon binocular light microscope. Male gonads appear as a whole structure with easily definable seminiferous tubules while females have an obvious ovarian cavity surrounded by oocytes (Swingle, 1926). Dissection photographs and histology of the gonads were further examined for malformations that include oocytes in testicular tissue, testicular tissue within ovaries, one ovary and one testis, and malformed testes or ovaries (Hayes et al., 2006; Mackenzie et al., 2006). Animals with gonad tissue containing any of these abnormalities were scored as intersex (Mackenzie et al., 2006).

2.4 Statistical Analysis

For experiment 1, all statistics are based on evaluations following nominal exposures to E2 and OP. For both experiments, sex ratios among treatments were evaluated with a binomial test determining whether the proportion of females differed from an expected 50:50 sex ratio. For gene transcript profiling, relative fold difference data were $\log_{10}$ transformed and evaluated for normality and equality of variance in JMP 9.0.2 (SAS Institute Inc, Cary, NC, USA) using the Levene's test for unequal variances. If data were normally distributed and exhibited equality of variance, analysis was conducted using a two-way ANOVA followed by Tukey's *post hoc* tests. When either normality or equality of variance criteria was not met, treatments were compared using Kruskal-Wallis non-parametric test followed by Mann-Whitney U test to compare treatments to controls. Pair-wise correlation analysis between *cyp19a1* and *nr5a1* mRNA levels was conducted within each sex and treatment condition.

3. RESULTS

3.1 Experiment 1: Short-term exposure of tadpoles to estrogenic chemicals

There were no significant differences detected across the 96 hour treatments with either OP or E₂ related to gross morphological endpoints (Table 2) or sex ratio (Fig. 1). In untreated tadpoles, sexual dimorphism was detected in gonad mRNA abundance profiles. Females demonstrated increased *cyp19a1* abundance compared to males (Fig 2; P<0.0001), and males exhibited increased levels of *nr5a1* mRNA compared to females (Fig. 2; P=0.028). Gonad transcript abundance profiles were altered following the short-term chemical exposures. Treatment with E₂ lowered *cyp19a1* mRNA abundance in females (Mann-Whitney; P=0.01) relative to control females (Fig. 3) and elevated *cyp19a1* mRNA levels in males (Mann-Whitney; P=0.01) relative to control males. The sexual dimorphism in *cyp19a1* mRNA levels noted in control animals was no longer apparent following E₂ treatment (Fig. 3; P=0.13). E₂ exposure led to a significant sex-by-treatment interaction effect for *nr5a1* transcript levels (P=0.004). Post-hoc analysis demonstrated that E₂-exposed males had greater *nr5a1* mRNA levels than control males (P<0.05; Fig. 3). Female *nr5a1* transcript abundance did not differ between control and E₂ exposed animals, and treatment with E₂ did not impact sexually dimorphic abundance of *nr5a1*.

Exposure to OP also resulted in altered gonadal transcript abundance profiles, with *cyp19a1* mRNA levels displaying a sex-by-treatment interaction (P=0.014). Post-hoc tests revealed that OP had no effect on female *cyp19a1* transcript abundance (Fig. 4). In contrast, exposure to the highest OP concentration resulted in an increase in *cyp19a1* transcript abundance in males (Fig. 4). *Nr5a1* transcript levels were dependent on sex (P<0.0001) and treatment (P<0.0001). For both sexes, the two lowest OP exposure concentrations were associated with a decrease in *nr5a1* mRNA abundance (Fig. 4).

We determined whether *cyp19a1* and *nr5a1* transcript levels displayed a correlation within each treatment group for both males and females. In control females there was a positive correlation between profiles of these two gene transcripts (P=0.0003; Table 3). Exposure to E₂ and OP at the lowest concentration eliminated this correlation. However, a positive association between the two mRNA abundance profiles was also significant in females following exposure to the two highest OP **nominal** doses (P = 0.03; P<0.01). In males, there was no correlation between gene transcript levels within any treatment group, except for a positive correlation at the highest OP concentration suggesting a feminized expression pattern (Table 3).

3.2 Experiment 2: Biological effects of field-based WWE exposure

There was no significant difference in developmental stage between animals collected from the two field sites (Mann-Whitney, P=0.7), and Gosner stage (P=0.68) and SVL (P=0.12) among animals collected at each site was not altered during the collection period. Weight did increase over the collection period, likely the result of increased time available for feeding during the summer season (P=0.001) (data not shown). However, body mass and SVL were greater in animals from the WWE site (RDF) compared to the reference location (JDD) (P<0.0001 and P=0.0020, respectively; Table 4). In evaluating the SVL to weight

ratio, it appears that the WWE-exposed tadpoles grew in length faster than they gained weight. The proportion of sexes from the reference site did not differ from the predicted 50:50 ratio ($P=0.5$; Fig. 5A), while there was a significant skew toward females at the site receiving WWE ($P=0.01$; Fig. 5A).

Cyp19a1 mRNA abundance in the gonad was significantly different between sexes ($P<0.001$) and collection sites ($P=0.034$; Fig. 5B). There was no significant interaction between sex and collection site indicating that both sexes had elevated *cyp19a1* abundance levels in the RDF site receiving WWE. Gonadal *nr5a1* transcript abundance levels in RDF animals were generally below the qPCR assay detection limits making statistical comparisons not possible.

4. DISCUSSION

Estrogenic chemical compounds either individually or present in complex mixtures, such as WWE, disseminated into the natural environment can affect vertebrate gonadal development through a range of mechanisms of action culminating in an alteration of steroidogenic pathways essential in sexual development and differentiation. *R. catesbeiana* gonadal *nr5a1* and *cyp19a1* mRNA abundance profiles suggest that exposure to such EDCs interferes with anuran estrogen-dependent sexual differentiation. To our knowledge, this is the first demonstration that wild populations of an aquatic vertebrate living in surface waters receiving WWE demonstrate altered expression of genes known to be responsible for gonadal differentiation.

The abundance profiles of *cyp19a1* and *nr5a1* transcripts in the larval tadpole are sexually dimorphic, with ovaries exhibiting increased levels of *cyp19a1* and testes exhibiting elevated *nr5a1* mRNA. Sexual dimorphism during development with respect to gonadal *cyp19a1* mRNA levels is also reported in other amphibian species (Dumond et al., 2008; Okada et al., 2009; Urbatzka et al., 2007). In *Rana rugosa* tadpoles, developing ovaries exhibit both increased immunopositive SF-1 protein expression and *cyp19a1* mRNA levels with the capacity to synthesize E_2 (Isomura et al., 2011; Maruo et al., 2008; Nakamura, 2009). Similar findings for SF-1 protein expression are found in *R. catesbeiana* (Mayer et al., 2002).

The ability of E_2 to disrupt aromatase expression and impact vertebrate gonadal development has been demonstrated in laboratory experiments across diverse vertebrate clades. Male *R. rugosa* exposed to E_2 during development show increased *cyp19a1* mRNA expression and elevated aromatase activity in the developing gonads (Ohtani et al., 2003). Exposure of zebrafish (*Danio rerio*) larvae to E_2 results in increased gonadal *cyp19a1* transcripts, (Hinfrey et al., 2006). Our present observations, along with the consistent finding that E_2 feminizes (Hayes, 1998) and anti-estrogens and aromatase inhibitors masculinize amphibian gonads (Duarte-Guterman et al., 2009; Mackenzie et al., 2003; Olmstead et al., 2009), demonstrate the importance of appropriately timed synthesis and action of estrogen with respect to amphibian gonadal differentiation. Our observations also suggest that exposure to E_2 , OP or WWE containing estrogen-disruptive compounds may

impact endogenous estradiol synthesis through affecting aromatase expression, especially in males.

The E₂-associated regulation of vertebrate gonadal development through modulation of genes involved in gonadal differentiation differs among species and between sexes within species, suggesting interplay between genetic and environmental factors. For example, in red-eared slider turtles (*Trachemys scripta elegans*) raised at a male-promoting temperature, E₂ treatment induces a decrease in *nr5a1* mRNA in developing gonads, while exposure of animals to female-promoting temperatures shows no effect on the levels of *nr5a1* transcripts (Fleming and Crews, 2001). Also in turtles (*Malaclemys terrapin* and *Chelydra serpentina*) exposed to a combination of male-promoting temperatures and estrogenic chemicals (E₂ or OP) display increased gonadal aromatase expression and activity (Place et al., 2001). In contrast, a similar chemical exposure at female-promoting temperature results in reduced aromatase expression and activity. Our results demonstrate differential impacts of exogenous E₂ on the sexes of larval *R. catesbeiana* for gonadal *cyp19a1* and *nr5a1* mRNA abundance. *Cyp19a1* displays reduced transcript abundance in females and elevated levels in males, while *nr5a1* exhibits higher mRNA abundance, but only in developing testes. Combined, these studies suggest that the disruptive nature of water borne estrogenic chemicals involves sex-dependent modulation in SF-1 and aromatase availability and/or activity which can lead to altered endogenous E₂ synthesis, resulting in dysregulation of gonadal differentiation under long-term exposure regimes.

OP may interfere with gonadal differentiation in a manner not requiring direct modulation of estrogen receptor (ER) activity to which it displays low binding affinity (Bonefeld-Jorgensen et al., 2007). Additionally, the effects of OP within the developing gonad are detected at exposure concentrations below what is expected to impact receptor-mediated mechanism of action. Such chemical-associated increases in *R. catesbeiana* mRNA that encode the rate-limiting enzyme in estradiol production is predicted to promote feminization through aberrant production of endogenous estradiol rather than *via* a direct effect of OP on receptor-mediated signaling. In bank voles, *Clethrionomys glareolus*, long-term (60 day) exposure to OP up-regulates testicular aromatase and ER α levels (Hejmej et al., 2011). Myllymaki *et al.* (2005a) found that *in vitro* exposure of rat ovarian follicles to OP did not impact aromatase activity, but did affect E₂ and testosterone secretion. In male rats treated with OP decreased levels of SF-1 expression occur during gonad development (Majdic et al. 1997; Saunders et al. 1997). We observed that the two lowest OP exposure concentrations reduced *nr5a1* mRNA levels in *R. catesbeiana* gonadal tissue of both sexes, with only the highest exposure concentration affecting levels of *cyp19a1* transcripts in testes. Together, these results suggest that there are sex-specific regulatory mechanisms downstream of SF-1's action on aromatase and that compounds affecting aromatase expression and/or activity may be acting directly on the aromatase promoter or through epigenetic actions that are also sex specific. Variation in the extent of biological disruption of OP on aromatase status is likely to be influenced by factors such as the species under consideration, developmental stage, and/or exposure dose and duration. Further, the actions of both E₂ and OP on gonadal gene expression may be mediated through effects on the hypothalamus and pituitary. Regardless of the precise mode of action, it is clear that, in general terms, OP

affects the estrogen-dependent signaling required for normal reproductive development in vertebrates.

One potential phenotypic outcome of exposure to estrogenic chemical contaminants is hermaphroditism or sex reversal, with gonads presenting intersex characteristics or developing into a gonad that does not match the predicted genetic determination mechanism. Short-term (96 hour) exposure to either E₂ or OP during early sexual differentiation of the gonad does not induce immediate sex reversal or lead to rapid development of hermaphroditism/intersex in *R. catesbeiana* tadpoles. However, in the Northern leopard frog, *Rana(Lithobates) pipiens*, exposure to E₂ or nonylphenol throughout larval development leads to an increase in the number of intersex gonads (Mackenzie et al., 2003). Similar impacts occur in a number of teleost species exposed to water borne EDCs, with medaka (*Oryzias latipes*) displaying an increased rate of intersex gonads, guppies (*Poecilia reticulata*) exhibiting sex ratio shifts towards female suggestive of sex reversal, and eelpout (*Zoarces viviparus*) showing an increase in the frequency of ovarian components within male testes (Rasmussen et al., 2002; Seki et al., 2003; Toft and Baatrup, 2003). Correlative evidence in humans suggests a strong link between exposure to OP and nonylphenolic compounds and idiopathic infertility (Chen et al., 2013), suggesting that, although these compounds do not induce overt sex reversal, exposure leads to adverse outcomes for gonadal function. Combined, these results demonstrate that long-term exposure of vertebrates during early development to estrogenic EDCs, or complex chemical contaminant sources with estrogen-disruptive activity, affects adult gonadal function. Information obtained from wildlife will help augment our understanding of potential human health impacts associated with exposure to alkylphenolic compounds.

A demonstration of perturbation in the biological status of the gonad was demonstrated with the assessment of wild *R. catesbeiana* tadpoles residing in a municipal WWE receiving environment. Animals of both sexes from the WWE site had elevated levels of gonadal *cyp19a1* transcripts compared to those from a reference pond. The female-skewed sex ratio present in the WWE-exposed animal population suggests the occurrence of disruption in normal steroidogenic endocrine function during critical periods of sexual differentiation and development. Modulation of *cyp19a1* mRNA levels has also been noted in a cell culture-based steroidogenesis assay examining marine coastal waters contaminated with WWE (Gracia et al., 2008), while abnormal sex ratios of aquatic vertebrates have been found associated with a number of WWE sources around the world (Jobling et al., 2002; Jobling et al., 1998; Jobling et al., 2006; Vajda et al., 2008; van Aerle et al., 2001; Woodling et al., 2006).

Although the RDF WWE examined does not contain detectable levels of E₂ or OP (below low ppt; City of Flagstaff, personal communication), our present observations suggest an estrogenic or anti-androgenic capability of this complex anthropogenic mixture. We recently demonstrated that high removal efficiencies at wastewater treatment facilities that lead to no chemical detection of EDCs, do not necessarily equate to lack of WWE biological impact (Osachoff et al., 2014). This caveat is again supported by the present results demonstrating that chemical detection profiles established following wastewater treatment do not allow for straightforward inferences related to biological effects. Typical detection limits for many

chemicals are above the level where biological effects can be observed (Osachoff et al, 2014). With these studies, the link between disruption of expression of genes involved in E₂ production and steroidogenesis, with a resultant influence on the sex status of wild anuran populations, is reinforced.

Chemical contaminant exposure events at sensitive life stages in a variety of wild aquatic animal species can lead to feminization of the developing gonad through disruption of gene expression networks crucial for steroidogenesis. The concentrations of many chemical compounds found in wastewater are within the range of those detected in human tissues (CDC, 2013; Kolpin et al., 2002). It is, therefore, crucial to establish their bioactive properties in the context of WWE with the help of vertebrate sentinel species and recognize the potential risk for altered reproductive outcomes resulting from changes in gene expression during critical periods of human development. Continued development of effective biological assays that screen for such activity is vital in the effort to mitigate EDC action across all vertebrate taxa.

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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

Figure 1.

Phenotypic sex distribution of *R. catesbeiana* tadpoles exposed to estradiol (E₂) or 4-tert-octylphenol (OP) for 96 hours. Gonad morphology was scored as female (white), male (black), or intersex (grey) following chemical treatment. The bevel indicates decreasing nominal OP concentrations of 10⁻⁷, 10⁻⁸, and 10⁻⁹ M. No statistical differences in sex distribution were found across treatment groups.

Figure 2. Sexual dimorphism in (A) *cyp19a1* and (B) *nr5a1* mRNA levels in the gonad of developing *R. catesbeiana* tadpoles. Mean relative fold difference values are shown by the bar height, while standard error of the mean are depicted by whiskers. For this and all subsequent figures, the number of animals evaluated within each group is shown above the bars. An asterisk indicates significant difference ($P < 0.05$; student t-test) between the sexes in relative gene transcript abundance levels.

Figure 3.

Comparison of relative abundance in *cyp19a1* and *nr5a1* mRNA in the *R. catesbeiana* gonad following exposure to estradiol (E₂). Mean relative fold difference values are shown by the bar height, while standard error of the mean are depicted by whiskers. The number of animals evaluated within each group is shown above the bars. An asterisk indicates significant difference ($P < 0.05$; Tukey's post-hoc test) between control (white bars) and E₂-treated (grey bars) animals for either sex.

Figure 4.

Comparison of relative abundance in *cyp19a1* and *nr5a1* mRNA in the *R. catesbeiana* gonad following 4-*tert*-octylphenol (OP) exposure. Mean relative fold difference values are shown by the bar height, while standard error of the mean are depicted by whiskers. The bevel indicates increasing nominal concentrations of 10⁻⁹ M, 10⁻⁸ M, and 10⁻⁷ M OP. Animals treated with vehicle control (C) were included. The number of animals evaluated within each group is shown above the bars. An asterisk indicates a significant difference (P<0.05; Tukey's post-hoc test) compared with control animals.

Figure 5.

Evaluation of *R. catesbeiana* tadpoles collected from the JD Dam reference location (JDD) and the Rio de Flag wastewater receiving site (RDF). (A) Phenotypic sex distribution of female (white bars), male (black bars) and intersex (grey bars) animals at both locations as determined by gonad histology. An asterisk indicates significant deviation ($P < 0.01$) from the expected 50:50 male:female sex ratio. (B) Comparison of gonad *cyp19a1* mRNA levels between female (white bars) and male (grey bars) tadpoles collected from the two field locations. Bar height represents mean relative fold difference values compared to JDD and

whiskers denote the standard error of the mean. An asterisk indicates the main effect in mRNA abundance ($P < 0.001$) between the sexes at each location, while main effect between collection sites is identified by '+' ($P < 0.034$).

Table 1

Characteristics of the qPCR assay performed on *R. catesbeiana*.

Gene transcript	NCBI GenBank Acc. #	Primer Name	Primer Sequence	Annealing Temp (°C)	Amplicon Size (bp)
<i>rpl8</i>	AY452063	L8-2 up UL8 dn	5'-CAGGGGACAGAGAAAAAGGTG-3' 5'-TGAGCTTTCTTGCCACAG-3'	55	270
<i>cyp19a1</i>	GU062745	Q1331 up Q1331 dn	5'-CCGCTGAGAAATGTGAACC-3' 5'-GCATGACCAAGCCTACCA-3'	62	249
<i>mr5a1</i>	GU062744	USF21 up 424 dn	5'-GATCCCGTGCGAATGGTAT-3' 5'-CGAGCCCCACTCTACTATG-3'	58	527

Table 2

Gross metrics of *R. catesbeiana* tadpoles following chemical exposures.

		Treatment ^b					
		Control	10 ⁻⁹ M E ₂	10 ⁻⁹ M OP	10 ⁻⁸ M OP	10 ⁻⁷ M OP	
Female ^a	N	15	17	18	17	16	
	Mass (g)	15.86 (0.460)	14.88 (0.457)	15.35 (0.345)	15.41 (0.537)	16.41 (0.385)	
	SVL (mm)	46.84 (0.706)	46.11 (0.837)	46.39 (0.644)	45.02 (1.01)	46.84 (0.899)	
Male ^a	N	8	7	7	9	5	
	Mass (g)	16.51 (0.651)	15.01 (0.596)	15.98 (0.624)	15.37 (0.496)	14.47 (1.49)	
	SVL (mm)	46.85 (1.08)	47.31 (0.677)	47.31 (1.23)	44.92 (1.20)	46.12 (2.06)	

^a Mean body mass and snout-vent length (SVL) are shown with standard error of the mean in parentheses.

^b Chemical treatments include estrogen (E₂) and 4-*tert*-octylphenol (OP).

Table 3

Correlation analysis of mRNA abundance profiles in *R. catesbeiana* gonads^a.

	Chemical Treatment				
	Control	10 ⁻⁹ M E ₂	10 ⁻⁹ M OP	10 ⁻⁸ M OP	10 ⁻⁷ M OP
Female R ²	0.84	0.27	0.46	0.68	0.67
P-value	0.0003*	0.35	0.20	0.03*	0.01*
Male R ²	0.3	0.8	-0.25	0.13	0.94
P-value	0.43	0.11	0.63	0.76	0.05*

^a An asterisk denotes significant correlation (P<0.05) between gonad *nr5a1* and *cyp19a1* mRNA.

Table 4

Gross metrics of *R. catesbeiana* tadpoles collected at the reference (JD Dam Lake; JDD) and wastewater treatment (Rio de Flag; RDF) locations.

		JDD	RDF
Female ^a	N	11	16
	Body mass (g)	24.2 (0.826)	33.05* (1.36)
	SVL (mm)	54.9 (0.821)	59.5* (0.808)
Male ^a	N	8	6
	Body mass (g)	24.7 (1.20)	33.4* (2.02)
	SVL (mm)	54.1 (1.11)	59.1* (1.44)

^a Mean body mass and snout-vent length (SVL) with standard error of the mean shown in parentheses. An asterisk denotes significant difference (P<0.05; Tukey's Post hoc test) compared with the reference site.