Ecotoxicology (2015) 24:321–329
DOI 10.1007/s10646-014-1380-x

# Transcriptional changes in African clawed frogs (*Xenopus laevis*) exposed to 17α-ethynylestradiol during early development

**Amber R. Tompsett · Eric Higley · Sara Pryce · John P. Giesy · Markus Hecker · Steve Wiseman**

Accepted: 2 November 2014 / Published online: 27 November 2014


**Abstract** Although the past two decades have witnessed a significant increase in the number of studies investigating effects of estrogenic chemicals on amphibians, to date little is known about specific molecular interactions of estrogens with the hypothalamus–pituitary–gonadal–hepatic axis in developing amphibians. Here, tissue-specific functional sets of genes, derived previously from studies of fishes exposed to endocrine active chemicals, were evaluated in *Xenopus laevis* exposed to 17α-ethynylestradiol (EE2) throughout their early development. Specifically, transcriptional responses of *X. laevis* exposed to 0.09, 0.84, or 8.81 μg EE2/L were characterized during sexual differentiation [31 day post hatch (dph)] and after completion of metamorphosis during the juvenile stage (89 dph). While at 31 dph there were no consistent effects of EE2 on abundances of transcripts,at 89 dph *X. laevis* exhibited significant alterations in expression of genes involved in steroid signaling and metabolism, synthesis of cholesterol, and vitellogenesis. Specifically, expression of androgen receptor, farnesyl diphosphate synthase, estrogen receptor α, and vitellogenin A2 was significantly greater (>2-fold) than in controls while expression of farnesoid x-activated receptors α and β was significantly less (>2-fold reduction) than in controls. These results support the hypothesis that sets of genes derived from studies in teleost fish can be extrapolated for use in amphibians during the juvenile stage but not in sexually undifferentiated individuals. Furthermore, changes in abundances of transcripts of the here utilized sets of genes in animals sampled post sexual differentiation were in accordance with developmental effects and alterations of gonadal histology reported in a parallel study. This set of genes might be useful for predicting potential adverse outcomes at later life-stages.

**Keywords** Amphibian · Endocrine · Vitellogenin · Estrogen · Sexual differentiation · Adverse outcome pathways

## Introduction

Because potent estrogens can interfere with sexual differentiation, sexual development, normal development of early

A. R. Tompsett · E. Higley · S. Pryce · S. Wiseman
Toxicology Centre, University of Saskatchewan, 44 Campus Drive, Saskatoon, SK S7N 5B3, Canada

J. P. Giesy
Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, SK, Canada

J. P. Giesy
Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA

J. P. Giesy
Department of Biology & Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, Kowloon, Hong Kong, SAR, China

J. P. Giesy
School of Biological Sciences, University of Hong Kong, Hong Kong, SAR, China

J. P. Giesy
State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, People's Republic of China

J. P. Giesy · M. Hecker (✉)
School of the Environment and Sustainability, Toxicology Centre, University of Saskatchewan, 44 Campus Drive, Saskatoon, SK S7N 5B3, Canada
e-mail: markus.hecker@usask.ca

life stages prior to metamorphosis, and homeostasis of the endocrine system, they are among the most-studied endocrine-active substances (Chang and Witschi 1955; Witschi et al. 1958; Hogan et al. 2008; Tompsett et al. 2012). Estrogenic substances, such as 17β-estradiol (E2) and 17α-ethynylestradiol (EE2), enter the aquatic environment through sources such as the discharge of liquid effluents from wastewater treatment facilities (Ankley et al. 2007), urban runoff (Jarošová et al. 2014) and runoff containing animal manure (Hanselman et al. 2003). Estrogens are commonly detected in the environment in the low nanogram per liter range (reviewed by Corcoran et al. 2010), and biological responses to these concentrations of estrogens in various aquatic vertebrate species, including fish and frogs, have been demonstrated (Jobling et al. 1998; Park and Kidd 2005; Kidd et al. 2007; Pettersson and Berg 2007).

Amphibians such as the African clawed frog (*Xenopus laevis*) are relatively sensitive to exposure to estrogens throughout their ontogeny. During determination of sex, and differentiation and development of gonads and secondary sexual characteristics [between Nieukwoop-Faber (NF) stages 50 and 54], exposure of *X. laevis* to potent estrogens causes disordered sexual development and even reversal of the male phenotype of genetic males to a female phenotype (Chang and Witschi 1955; Villalpando and Merchant-Larios 1990; Miyata et al. 1999; Lutz et al. 2008; Tompsett et al. 2012). However, exposure of *X. laevis* to estrogens after the period of sexual differentiation does not cause reversal of phenotypic sex or alter development of gonads or secondary sexual characteristics, but it can affect other biological parameters. After sexual differentiation, the estrogen receptor (ER) gene of *X. laevis* becomes auto-inducible (Tata et al. 1993) and can be up-regulated by the presence of endogenous and exogenous estrogens. Therefore, exposure to estrogens after NF stage 62 has the potential to activate genes and pathways that are responsive to estrogens.

Among vertebrates, effects of exposure to endocrine active compounds, including estrogens, tend to be most pronounced in organs that are part of the hypothalamic–pituitary–gonadal–liver axis (HPGL) (Watanabe et al. 2009; Villeneuve et al. 2012). Recently, an extensive graphical systems model of components of this axis in teleost fish was constructed, which included derivation of tissue-specific sets of genes affected by endocrine active compounds (Villeneuve et al. 2012). These functional sets of genes were derived from data from multiple experiments with three different endocrine-active compounds, and it has been shown that quantification of expression of these genes is useful to predict tissue-specific responses to selected EDCs. Because of some similarities of reproductive endocrine systems between fish and amphibians, the set of genes proposed by Villeneuve et al. (2012) are potentially suitable to explore molecular toxicity pathways along the HPGL axis of amphibians exposed to endocrine-active compounds, including estrogens.

The current study utilized chronic exposure of *X. laevis* to the synthetic estrogen, 17α-ethynylestradiol (EE2), to determine effects on abundances of transcripts of estrogen-responsive genes during sexual differentiation (31 dph) and after completion of metamorphosis (89 dph). *X. laevis* were exposed to nominal doses of 0.1, 1, or 10 μg EE2/L from before hatch through the larval and early post-metamorphic period. Sexual differentiation and development of genetic male individuals was affected, as has been described previously (Tompsett et al. 2012). Abundances of transcripts of 15 genes from the functional sets of genes that was proposed by Villeneuve et al. (2012) as indicative of exposure of fishes to estrogens, were quantified in *X. laevis* to determine whether this set of genes would be indicative of endocrine disrupting phenotypes in amphibians. The study also investigated whether developmental stage (time of sexual differentiation [NF53] vs metamorphic climax [NF62]) had an influence on patterns of response obtained for the selected set of genes. Abundances of transcripts of ten genes involved in steroid signaling and metabolism, two genes involved in cholesterol biosynthesis, and three genes involved in vitellogenesis were quantified (Table 1). Concentrations of EE2 were based upon their ability to elicit effects on phenotype, but the least dose was only 3-fold greater than the range of estrogen equivalents (about 5–30 ng/L) that would be expected in a natural watershed (reviewed by Kidd et al. 2007), but was 3-fold less than the maximal concentrations of EE2 (about 0.3 μg/L) detected in streams in the US (Kolpin et al. 2002a, 2002b).

## Materials and methods

### Experimental animals

Prior to commencement of research, approval for the use of animals and all experimental procedures was obtained from the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan (Animal Use Protocol #20090066). Sexually-mature, adult *X. laevis* were purchased from Boreal Laboratories (St. Catharine's, ON, Canada) and acclimated to laboratory conditions [18 ± 2 °C water temperature; 12:12 light–dark cycle; fed Nasco frog brittle (medium nuggets) (Salida, CA, USA) ad libitum daily] for 1 month. After acclimation, pairs of *X. laevis* were spawned and eggs were collected as described previously (Tompsett et al. 2012).

### Exposure study design

The exposure was conducted as has been detailed previously (Tompsett et al. 2012). Briefly, each treatment group

**Table 1** Primer sets for quantification of expression of genes expected to be affected by exposure to estrogens

| Gene identity | Abbreviation | Accession number | Function | Forward primer | Reverse primer |
|---|---|---|---|---|---|
| Aryl hydrocarbon receptor 1α | *ahr1a* | AY635782.1 | SSM | CATGGTGACTCCCCAGTCTT | GAGCTGCCATGACTGCATTA |
| Aryl hydrocarbon nuclear translocator 1 | *arnt1* | NM_001090153.1 | SSM | CTCCTCCCAGAAGCACAAAG | AGCCTCTCGCTGTTCGATAA |
| Cytochrome P450 1a1 | *cyp1a1* | NM_001097072.1 | SSM | GATCCGAACCTGTGGAAAGA | CAATGGCTTCACCAACACAC |
| Estrogen sulfotransferase | *est* | NM_001095815.1 | SSM | AATGCCAACGTGACACCATA | ATCAGCTGGATGGGAAGATG |
| Retinoid X receptor β | *rxrb* | BC108460.1 | SSM | TAAGGGCTGGATGGAATGAG | GCCTGCACTGTGTGCACTAT |
| Pregnane X receptor | *pxr* | NM_001090137.1 | SSM | GGTGTCTGCTGGTTGGTTTT | AGTTGTGGGGCTTGATTTTG |
| Glucocorticoid receptor | *gr* | NM_001088062.1 | SSM | TGACATATCTGCCCAACCAA | CCAGGTCCCGTAGTAGGTCA |
| Farnesoid x-activated receptor α | *fxra* | NM_001088774.1 | SSM | TGGAAGCACATCCACAAGAG | TAAGGGATCCCATGCTTCTG |
| Farnesoid x-activated receptor β | *fxrb* | NM_001090085.1 | SSM | TGAAGGCTGCAAAGGTTTCT | CTGACACTTTCTGCGCATGT |
| Androgen receptor | *ar* | U67129.1 | SSM | ATGCTTGTGTGCCAATACCA | TGCAACATGGATGAAGGAAA |
| 7-dehydrocholesterol reductase | *dhcr7* | NM_001086117.1 | CB | ATATTCCTGTTGGCGTTTGC | GCGCTTTGCCAGAGTATAGG |
| Farnesyl diphosphate synthase | *fdps* | NM_001096644.1 | CB | CGACGATACGAGGAGGAGAG | GTATCCCAGCATGCCTCACT |
| Estrogen receptor α | *era* | NM_001089617.1 | VTGS | CGACTGGCTCAGCTTCTTCT | AGGGGACAACATTCTTGCAC |
| High density lipoprotein binding protein | *hdlbp* | NM_001086696.1 | VGTS | CACAGGCTATGAGCGAAACA | TTCCGTACAGCTTTGCCTCT |
| Vitellogenin A2 | *vtga2* | NM_001159281.1 | VTGS | CTGACTCCTCCCCAAATTCA | CTGTACCAGGTGCTGCAAGA |
| Glyceraldehyde 3-phosphate dehydrogenase | *gapdh* | NM_001087098.1 | RF | CAGAGGTGCAGGTCAGAACA | GGAAAGCCATTCCGGTTATT |

*SSM* steroid signaling and metabolism, *VTGS* vitellogenesis, *CB* cholesterol biosynthesis, *RF* reference

consisted of triplicate tanks. The appropriate nominal concentration of EE2 was added to each tank in an ethanol carrier, and the final concentration of ethanol in treatment tanks was 0.0025 %. To validate that expected nominal values were approximated, concentrations of EE2 in exposure water were monitored periodically during the exposure via high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) by use of methods that have been described elsewhere (Chang et al. 2010; Tompsett et al. 2012). A 50 % static water renewal was performed daily and basic water quality variables were monitored daily (temperature, DO, pH, conductivity) or weekly (nitrate nitrogen, nitrite nitrogen, ammonia nitrogen). Tadpoles and frogs were fed ad libitum daily with Nasco frog brittle products.

*Xenopus laevis* were exposed to EE2 from before hatch until 89 dph. At 31 dph, a subset of tadpoles that were undergoing sexual differentiation was euthanized by an overdose of MS-222 (Sigma, Oakville, ON, Canada). The tail of each tadpole was frozen at −20 °C for use in an assay to determine genetic sex (Yoshimoto et al. 2008), and the body of the tadpole was flash frozen in liquid nitrogen and stored at −80 °C for use in qPCR assays. At 89 dph, the remaining *X. laevis* were euthanized. A tissue sample from a leg of each froglet was frozen at −20 °C to use for determination of genetic sex. The liver was removed from each froglet, flash frozen in liquid nitrogen, and stored at −80 °C for use in the qPCR assays. The gonadal phenotype of each individual was determined as reported previously (Tompsett et al. 2012).

## Molecular analyses

At total of 20 tadpoles and 48 juveniles of *X. laevis* were used for quantification of abundances of transcripts by use of qPCR. Tadpoles were limited to genetic male individuals because effects of EE2 were expected to be most pronounced in males. Juvenile frogs were assigned to one of four groups based on genetic and phenotypic sex (Tompsett et al. 2012): genetic and phenotypic female, genetic and phenotypic male, genetic male and abnormal phenotypic male, or genetic male and mixed sex phenotype.

For quantitative polymerase chain reaction (qPCR), total RNA was isolated from tadpoles of *X. laevis* by use of an RNeasy® Plus Mini Kit (Qiagen, Mississauga, ON, Canada) and from samples of livers by use of an RNeasy® Lipid Tissue Mini Kit (Qiagen) according to the protocol provided by the manufacturer. Total RNA was quantified and integrity determined, and cDNA was synthesized, as previously described (Tompsett et al. 2013a). Primers for use in qPCR were designed for genes of interest (Table 1) based on published sequences for *X. laevis* mRNAs (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) by use of Primer3 software (Rozen and Skaletsky 2000). Primers were designed to amplify

products of PCR between 100 and 150 base pairs in length by use of standard parameters, and forward and reverse DNA oligonucleotides (oligos) were purchased (Invitrogen, Burlington, ON, Canada). For all primer sets, efficiency curves were constructed and qPCR was performed as has been described previously (Tompsett et al. 2013a). For each target gene, abundance of transcripts was quantified according to the Mean Normalized Expression (MNE) method of Simon (2003) with glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) as a reference gene.

### Statistical analysis

Statistical tests were performed by use of IBM SPSS 19 software (IBM, Armonk, NY). Treatment means are expressed as mean ± S.E. throughout. Statistical significance was defined as $p \leq 0.05$. Data from qPCR assays were first assessed for normality and homogeneity of variances by use of Shapiro–Wilk and Levene's tests, respectively. Then, they were evaluated for statistical equivalency between control and solvent control treatments and pooled where appropriate (all cases in the current study). Data were analyzed with ANOVA tests with post hoc Tukey's tests when the assumptions of normality and equal variances were met. Data for transcripts that deviated from normality and/or homogeneity of variances were analyzed with nonparametric Kruskal–Wallis tests to determine differences among treatments in abundances of transcripts. Where the p value of the Kruskal–Wallis test was significant, post hoc testing was performed by use ofthe nonparametric, Mann–Whitney U tests to compare each treatment group to the control group. Pearson correlation analysis was conducted to identify possible relationships between parameters in *X. laevis* exposed to 89 dph (no correlation analyses were conducted with tadpoles exposed until 31 dph as no significant differences among treatment groups for any of the measured parameters occurred).

## Results

Water quality parameters were all within an acceptable range for culture of *X. laevis* over the course of the experiment and have been reported previously (Tompsett et al. 2012, 2013b). Validated concentrations of EE2 in treatment tanks have also been reported previously (Tompsett et al. 2012) and were 0.09 ± 0.005, 0.84 ± 0.06, and 8.81 ± 1.25 µg EE2/L immediately following water changes.

Exposure to EE2 for 31 dph did not affect abundances of transcripts of several target genes. There were no significant differences in abundances of transcripts of aryl hydrocarbon receptor 1α (*ahr1α*), cytochrome P450 1a1 (*cyp1a1*), estrogen sulfotransferase (*est*), estrogen receptor α (*erα*), or farnesyl diphosphate synthase (*fdps*) in tadpoles that were genetic males. The abundance of transcripts of vitellogenin A2 (*vtga2*) was less than the method detection limit. Since abundances of those six transcripts did not change in *X. laevis* at this stage in development, quantification of abundances of remaining target transcripts was not performed.

In contrast, exposure to EE2 for 89 d had a significant effect on abundances of transcripts involved in steroid signaling and metabolism. Abundances of transcripts of farnesoid X-activated receptor α (*fxra*), farnesoid X-activated receptor β (*fxrb*), and androgen receptor (*ar*) were significantly altered in livers from *X. laevis* exposed to EE2 (Kruskal–Wallis, p = 0.002, 0.006 and 0.014 for *fxra, fxrb,* and *ar*, respectively). Abundances of transcripts of *fxra* were −2.8-fold to −8.4-fold less in abnormal males, mixed sex, and female individuals exposed to 0.84 µg EE2/L and in abnormal males and females exposed to 8.81 µg EE2/L, compared to the abundance in control individuals (Table 2). For *fxrb*, changes in abundances of transcripts ranged from −8.5-fold to −3.4-fold in abnormal male and female individuals exposed to 0.84 µg EE2/L and female individuals exposed to 8.81 µg EE2/L, relative to the abundance in control individuals (Table 2). For *ar*, fold-changes in abundances of transcripts ranged from +5.8-fold to +15-fold, in all individuals exposed to EE2, except mixed sex individuals exposed to 8.8 µg EE2/L, relative to control individuals (Table 2; Fig. 1). There were no changes in abundances of transcripts of *ahr1a, cyp1a1,* aryl hydrocarbon nuclear translocator 1 (*arnt1*), *est,* retinoid X receptor β (*rxrb*), glucocorticoid receptor (*gr*), or pregnane X receptor (*pxr*).

Abundances of transcripts of two genes involved in synthesis of cholesterol, 7-dehydrocholesterol reductase (*dhcr7*) and *fdps*, were evaluated in livers from individuals exposed to EE2 until 89 dph. There was no change in abundances of transcripts of *dhcr7*. There were significant differences in abundances of transcripts of *fdps* among treatments (Kruskal–Wallis, p = 0.013). Abundances of transcripts of *fdps* were significantly greater by +2.6-fold to +4.0-fold, in males, abnormal males, and females exposed to 0.09 µg EE2/L and in abnormal males and females exposed to 0.84 µg EE2/L, relative to control individuals (Table 2).

Abundances of transcripts of genes involved in synthesis of vitellogenin were significantly greater in livers from *X. laevis* exposed to EE2 until 89 dph than abundances in control individuals. Abundances of transcripts of *era* or *vtga2* were significantly different among treatments (Kruskal–Wallis, $p = 0.003$ and 0.002 for *era* and *vtga2*, respectively), but abundances of transcripts of high-density

**Table 2** Summary of fold-changes in relative abundances of transcripts in livers from *Xenopus laevis* exposed to 17α-ethynylestradiol during development

| 17α-ethynylestradiol concentration | | 0.09 μg/L | | | | 0.84 μg/L | | | 8.81 μg/L | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenotype | | M | AM | MS | F | AM | MS | F | AM | MS | F |
| Transcript | Biological function | Fold-change[a] (±S.E.) | | | | | | | | | |
| *Fxra* | SSM | – | – | – | – | −3.4 (±0.1) | −8.4 (±0.1) | −3.8 (±0.1) | −2.8 (±0.2) | – | −8.1 (±0.1) |
| *Fxrb* | SSM | – | – | – | – | −3.9 (±0.1) | – | −3.4 (±0.1) | – | – | −8.5 (±0.1) |
| *Ar* | SSM | +11 (±2.3) | +6.7 (±3.2) | +7.5 (±0.7) | +6.9 (±1.5) | +6.9 (±2.3) | +8.5 (±3.2) | +7.4 (±1.1) | +15 (±6.5) | – | +5.8 (±2.1) |
| *Era* | VTGS | +12 (±2.1) | +7.2 (±3.5) | +8.8 (±3.9) | +7.3 (±1.5) | +6.8 (±1.7) | – | +4.5 (±0.9) | +6.6 (±3.9) | – | – |
| *vtga2* | VTGS | +102 (±22) | +86 (±51) | +71 (±36) | +84 (±16) | +52 (±15) | +87 (±30) | +38 (±11) | +61 (±31) | – | +32 (±14) |
| *Fdps* | CB | +2.9 (±0.8) | +2.8 (±0.4) | – | +4.0 (±0.5) | +2.7 (±0.8) | – | +2.6 (±0.3) | | – | – |

*M* male, *AM* abnormal male, *MS* mixed sex, *F* female, *SSM* steroid signaling and metabolism, *VTGS* vitellogenesis, *CB* cholesterol biosynthesis

–, the fold-change was not significant

[a] Data are presented for those transcripts that were significantly altered (Kruskal–Wallis, $p \leq 0.05$; post hoc Mann–Whitney U, $p \leq 0.05$; $n = 3$–6 frogs in each category) in individuals exposed to EE2 compared to control individuals

lipoprotein binding protein (*hdlbp*) were not different in livers from *X. laevis* exposed to EE2. For *era*, abundances of transcripts were significantly greater by +4.5-fold to +12-fold, in all EE2 treatment groups, except mixed sex individuals exposed to either 0.84 or 8.81 μg EE2/L, relative to control individuals (Table 2; Fig. 2). Abundances of transcripts of *vtga2* were +32-fold to +102-fold greater in all EE2 treatment groups, except mixed sex individuals exposed to 8.81 μg EE2/L, relative to control individuals (Table 2; Fig. 3).

There were significant correlations between abundances of transcripts of several pairs of genes as well as significant correlations between abundances of several transcripts with previously reported size of male (including normal and abnormal males as well as mixed sex individuals) and female froglets at metamorphosis, and with time to metamorphosis (Tompsett et al. 2012) (Table 3). In females, there was a significant and positive correlation between days to metamorphosis (DTM) and expression of *ar* ($r = 0.570$; $p = 0.014$) and *era* ($r = 0.512$; $p = 0.030$), and in males DTM was positively correlated with *ar* ($r = 0.445$; $p = 0.014$) and *vtga2* ($r = 0.380$; $p = 0.039$). Furthermore, in females expression of the *ar* was positively correlated with *era* ($r = 0.789$; $p = 0.000$), *fdps* ($r = 0.500$; $p = 0.035$) and *vtga2* ($r = 0.703$; $p = 0.001$), and the *era* with *fxra* ($r = 0.509$; $p = 0.031$), *fxrb* ($r = 0.484$; $p = 0.042$), *fdps* ($r = 0.697$; $p = 0.001$) and *vtga2* ($r = 0.824$; $p = 0.000$). Also, *fxra* and *fxrb* were highly correlated in females ($r = 0.980$; $p = 0.000$). In males, significant and positive correlations occurred between the expression of *ar* and *era* ($r = 0.692$; $p = 0.000$) and *vtga2* ($r = 0.654$; $p = 0.000$). Expression of *era* was positively correlated with *vtga2* ($r = 0.797$; $p = 0.000$), and *fxra* with *fxrb* ($r = 0.792$; $p = 0.000$). When mixed sex frogs were excluded from the correlational analysis among males, the above effects were comparable with the exception that there was also a positive correlation between *fdps* and *ar* ($r = 0.451$; $p = 0.040$) and *era* ($r = 0.472$; $p = 0.031$).

## Discussion

There were similarities between expressions of genes observed in *X. laevis* exposed to EE2 and those that have been reported to be modulated by estrogens in fishes. Abundances of transcripts of genes involved with metabolism of steroids, synthesis of cholesterol, and vitellogenesis, which have been linked to fecundity and feminization of fishes, were detectable in livers of juvenile frogs after 89 days of constant exposure to EE2. These results suggest that quantification of alterations in abundances of transcripts previously identified in teleost fishes exposed to endocrine

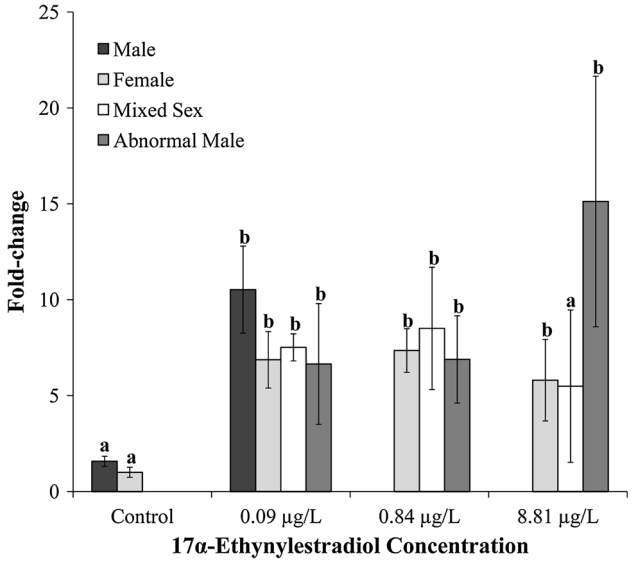


**Fig. 1** Fold-changes in abundances of transcripts of androgen receptor (*ar*) in livers from *Xenopus laevis* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of transcripts of *ar* among treatments after exposure. Significant differences from control treatments are denoted by *different letters* (Kruskal–Wallis, $p = 0.014$; $n = 3$–6 frogs in each category)

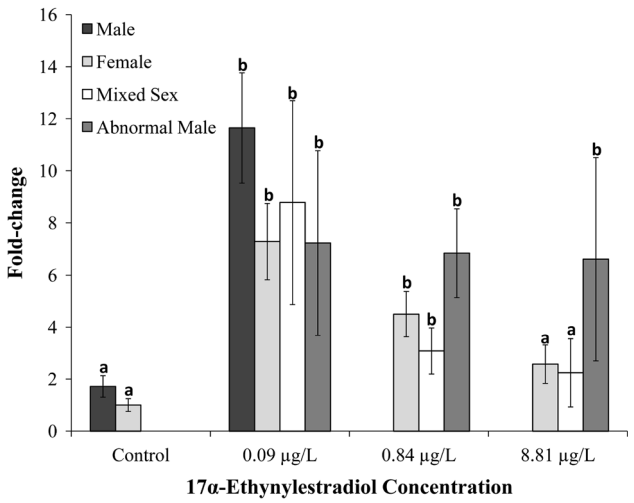


**Fig. 2** Fold-changes in abundances of transcripts of estrogen receptor α (*era*) in livers from *Xenopus laevis* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of transcripts of *era* among treatments after exposure. Significant differences from control treatments are denoted by *different letters* (Kruskal–Wallis, $p = 0.003$; $n = 3$–6 frogs in each category)

active chemicals might also be suitable endpoints in sub-chronic and chronic studies of effects of environmental estrogens on amphibians. However, there were differences between the results of this study with *X. laevis* and those reported for fishes. Abundances of transcripts of a number of genes that were observed to be modulated in fishes, were not

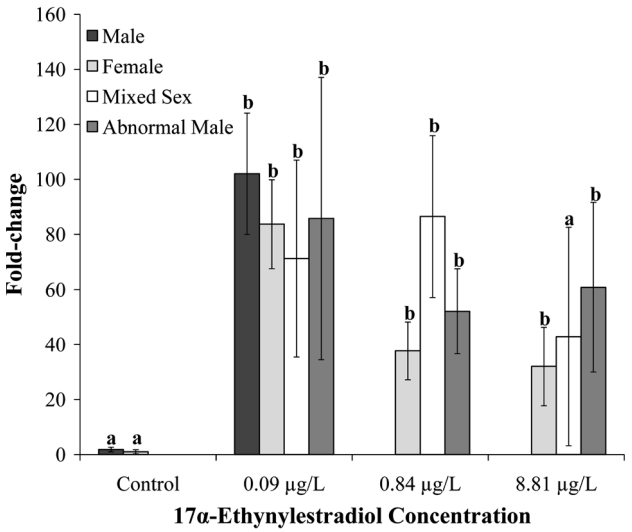


**Fig. 3** Fold-changes in abundances of transcripts of vitellogenin A2 (*vtga2*) in livers from *Xenopus laevis* chronically exposed to 17α-ethynylestradiol. Fold-changes are expressed as mean ± S.E. There were significant differences in abundances of transcripts of *vtga2* among treatments after exposure. Significant differences from control treatments are denoted by *different letters* (Kruskal–Wallis, $p = 0.002$; $n = 3$–6 frogs in each category)

affected in tadpoles of *X. laevis* during the period of sexual differentiation (31 dph), which indicates that the suitability of the sets of genes identified in teleost fish might be limited by developmental stage. It is likely that the lack of response of some genes in tadpoles exposed to EE2 was linked to their lack of auto-inducible ERs at this life stage (Tata et al. 1993), which would limit their ability to respond to exogenous estrogen via ER-mediated pathways.

Farnesoid X receptors (FXRs) are involved in transport and metabolism of bile acid in vertebrates, and their natural ligands are bile acids, which are end products of the metabolism of cholesterol (Reschly et al. 2008). Non-mammalian vertebrates, such as fish, secrete metabolites of steroids and xenobiotics into bile acids produced in the liver during detoxification (Gibson et al. 2005). Similar mechanisms are common to mammals, which also transcriptionally regulate some nuclear receptors, including RXRs, PXRs, and FXRs, as a mechanism of hepato protection (Zollner and Trauner 2009; Henriquez-Hernandez et al. 2007). In addition, lesser abundance of transcripts of *fxr* in the human liver has been linked to the presence of primary liver tumors (Martinez-Beccerra et al. Martinez-Becerra et al. 2012). In the current study, abundances of transcripts of both *fxra* and *fxrb* were significantly less in some phenotype groups exposed to 0.84 and 8.81 µg EE2/L compared to abundances in control groups. Lesser abundances of transcripts of these receptors might indicate that the hepatoprotective functions served by these receptors were affected.

**Table 3** Significant correlations between biological endpoints and abundances of transcripts in male and female *Xenopus laevis* exposed to EE2

| Transcript | Sex | Days to metamorphosis | *era* | *fdps* | *vtga2* | *fxra* | *fxrb* |
|---|---|---|---|---|---|---|---|
| *ar* | Female | r = 0.570* | r = 0.789** | r = 0.500* | r = 0.703** | – | – |
| | Male | r = 0.445* | r = 0.692** | r = 0.451*[a] | r = 0.654** | – | – |
| *era* | Female | r = 0.512* | – | r = 0.697** | r = 0.824** | r = 0.509* | r = 0.484* |
| | Male | – | – | r = 0.472*[a] | r = 0.797** | – | – |
| *fxra* | Female | – | – | – | – | – | r = 0.980** |
| | Male | – | – | – | – | – | r = 0.792** |
| *vtga2* | Male | r = 0.380* | – | – | – | – | – |

–, the correlation was not significant

* Statistically significant correlation ($p \leq 0.05$)

** Statistically significant correlation ($p \leq 0.01$)

[a] Mixed sex animals were excluded from analyses

The balance of estrogens and androgens is an important part of endocrine homeostasis (Rivas et al. 2002). Although the two classes of hormones have differing functions in reproduction, formation of endogenous E2 is dependent upon aromatization of testosterone (T) by the aromatase enzyme (CYP19). Although E2 and T are ligands for the ER and AR, respectively, there is evidence that the ER and AR are not exclusively agonized by E2 and T, and there is the potential for crosstalk between the two pathways (Eick and Thornton 2011). For example, in the human prostate, 3β-adiol, a metabolite of dihydrotestosterone, is known to be an agonist of ERβ (Muthusamy et al. 2011). In the current study, exposure to EE2, an agonist of the ER, resulted in greater abundance of transcripts of *ar* in the liver. While this is not a response that is typically associated with exposure to estrogens, the chronic nature of the exposure and the potential for interactions between the AR- and ER-mediated pathways could explain the effect. The endocrine system is regulated by feedback loops, and it is possible that the greater abundance of transcripts of *ar* is an indication that frogs exposed to EE2 were attempting to maintain homeostatic balance between AR and ER signaling pathways via greater transcription of *AR*. This is also supported by the significant correlation between the abundances of transcripts of *ar* and *era* in froglets of both sexes.

Transcripts of *fdps* code for the enzyme that catalyzes the formation of farnesyl diphosphate, a precursor of sterols, carotenoids, and ubiquinones, which is an intermediate product in formation of cholesterol. In the current study, the abundance of transcripts of *fdps* was significantly greater in some groups of individuals exposed to 0.09 or 0.84 μg EE2/L. However, the abundance of transcripts of *dhcr7*, which codes for the enzyme that catalyzes the final step in the synthesis of cholesterol, was not affected by exposure to EE2. In other non-mammalian vertebrates, exposure to estrogens affects synthesis of cholesterol. In female fathead minnows (*Pimephales promelas*) exposed for 4–8 days to EE2 total cholesterol in the liver was significantly increased (Ekman et al. 2009). Concentrations of cholesterol were also greater in livers of goldfish (*Carassius auratus*) exposed to E2 for 5 months (Sharpe and MacLatchy 2007). In addition, *X. laevis* produced greater amounts of fatty acids and cholesterol after exposure to estrogen (Smith et al. 1978). In the current study, concentrations of cholesterol were not monitored, so it is unclear whether synthesis of cholesterol was ultimately affected by exposure to EE2, especially since those transcripts that encode enzymes involved in cholesterol metabolism that were monitored displayed differential responses to EE2.

Expression of both *era* and *vtga2* are rapidly up-regulated in response to estrogens, even in genetic males. This occurs via binding of an activated ER complex to an estrogen response element (ERE) in the promoter region of these genes (reviewed by Rotchell and Ostrander 2003). In the current study, abundances of transcripts of *era* and *vtga2* were significantly greater in sexually immature or male individuals exposed to EE2, even though these individuals do not normally express these genes. However, the abundance of transcripts of *hdlbp* was not changed by exposure to EE2. The lack of change in the abundance of transcripts of *hdlbp*, which codes for a protein that binds to and stabilizes vitellogenin, could be linked to the overexpression of *vtga2* itself, which would render the stabilizing protein unnecessary.

Greater abundance of transcripts of *vtga2 i*n the current study complements the greater amount of vitellogenin protein that was described previously for these same *X. laevis* (Tompsett et al. 2012). Histological analysis of the kidney-gonad complexes of these *X. laevis* revealed proteinaceous material in and around gonads and kidneys that was identified, by use of immunohistochemistry, as vitellogenin. Thus, even though these frogs showed evidence of vitellogenin protein deposits in the kidney-gonad complex,

abundances of *vtga2* transcripts were still elevated, suggesting that disordered vitellogenesis was still occurring. Furthermore, it was (Tompsett et al. 2013a) hypothesized that the increased energy demand associated with the significant induction of vitellogenesis in both male and female frogs exposed to EE2 resulted in a delay of metamorphosis. This study reported a significant positive correlation between the expression of *vtga2* and DTM in male *X. laevis*, which would support this hypothesis further.

The current study illustrated that quantification by use of qPCR of the expression of sets of genes previously identified as being differentially expressed in teleost fishes exposed to endocrine active chemicals was a useful point of departure for detecting changes in estrogen-responsive genes in *X. laevis* after long-term (89 dph) exposure to EE2. For studies utilizing chemicals like EE2, this is especially useful since environmentally realistic exposures are likely to be chronic due to the constant input of estrogens into the aquatic environment via sources such as effluents from wastewater treatment plants and runoff from agricultural operations. While the gene set quantified here, which was chosen based on sets of genes derived from exposures of teleost fishes to endocrine active chemicals (Villeneuve et al. 2012), was found to be useful at the 89 days time-point, it was also determined that it was not an effective tool for *X. laevis* that were 31 dph. Quantification of abundances of transcripts by next generation sequencing technology, such as RNA seq, could be a viable alternative for determining effects of exposure to estrogens at this early life stage, or for the development of sets of genes appropriate for determining effects of estrogens at early life stages (Tompsett et al. 2013b).

**Acknowledgments** The authors wish to acknowledge the support by the Natural Science and Engineering Research Council of Canada (Discovery Grants), Grants from Western Economic Diversification Canada (Project # 6578 and 6807), and an instrumentation Grant from the Canada Foundation for Innovation to J. Giesy and M. Hecker. J. Giesy and M. Hecker were supported by the Canada Research Chair program. Furthermore, John Giesy was supported by Distinguished visiting Professorship in the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, the 2012 "High Level Foreign Experts" (#GDW20123200120) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. The authors would like to thank J. Doering, S. Beitel, B. Tendler, and T. Tse for their assistance.

**Conflict of interest** The authors declare no conflict of interest in context with the presented work.

## References

Ankley G, Brooks B, Huggett D, Sumpter J (2007) Repeating history: pharmaceuticals in the environment. Environ Sci Technol 41:8211–8217

Chang C, Witschi E (1955) Breeding of sex-reversed males of *Xenopus laevis* Daudin. Proc Soc Exper Biol Med 89:150–152

Chang H, Wan Y, Naile J, Zhang X, Wiseman S, Hecker M, Lam M, Giesy J, Jones P (2010) Simultaneous quantification of multiple classes of phenolic compounds in blood plasma by liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr A 1217:506–513

Corcoran J, Winter M, Tyler C (2010) Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit Rev Toxicol 40:287–304

Eick G, Thornton J (2011) Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Mol Cell Endocrinol 334:31–38

Ekman D, Teng Q, Villeneuve D, Kahl M, Jensen K, Durhan E, Ankley G, Collette T (2009) Profiling lipid metabolites yields unique information on sex- and time-dependent responses of fathead minnows (Pimephales promelas) exposed to 17 alpha-ethynylestradiol. Metabolomics 5:22–32

Gibson R, Smith M, Spary C, Tyler C, Hill E (2005) Mixtures of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents. Environ Sci Technol 39:2461–2471

Hanselman T, Graetz D, Wilkie A (2003) Manure-borne estrogens as potential environmental contaminants: a review. Environ Sci Technol 37:5471–5478

Henriquez-Hernandez L, Flores-Morales A, Santana-Farre R, Axelson M, Nilsson P, Norstedt G, Fernandez-Perez L (2007) Role of pituitary hormones on 17 alpha-ethinylestradiol-induced cholestasis in rat. J Pharm Exp Therap 320:695–705

Hogan N, Duarte P, Wade M, Lean D, Trudeau V (2008) Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (*Rana pipiens*): identifying critically vulnerable periods of development. Gen Comp Endocrinol 156:515–523

Jarošová B, Erseková A, Hilscherová K, Loos R, Gawlik B, Giesy JP, Bláha L (2014) Europe-wide monitoring of estrogenicity in wastewater treatment plant effluents: the need for the effect-based monitoring. Environ Sci Pollut Res. doi:10.1007/s11356-014-3056-8

Jobling S, Nolan M, Tyler C, Brighty G, Sumpter J (1998) Wide spread sexual disruption in wild fish. Environ Sci Technol 32:2498–2506

Kidd K, Blanchfield P, Mills K, Palace V, Evans R, Lazorchak J, Flick R (2007) Collapse of a fish population following exposure to a synthetic estrogen. Proc Nat Acad Sci 104:8897–8901

Kolpin D, Furlong E, Meyer M, Thurman E, Zaugg S, Barber L, Buxton H (2002a) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000. Environ Sci Technol 36:1202–1211

Kolpin D, Furlong E, Meyer M, Thurman E, Zaugg S, Barber L, Buxton H (2002b) Response to comment on "Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: a national reconnaissance". Environ Sci Technol 36:4007–4008

Lutz I, Kloas W, Springer T, Holden L, Wolf J, Krueger H, Hosmer A (2008) Development, standardization, and refinement of procedures for evaluating effects of endocrine active compounds on development and sexual differentiation of *Xenopus laevis*. Anal Bioanal Chem 390:2031–2048

Martinez-Becerra P, Monte I, Romero M, Serrano M, Vaquero J, Macias R, Del Rio A, Grane-Boladeras N, Jimenez F, San-Martin F, Pastor-Anglada M, Marin J (2012) Up-regulation of FXR isoforms is not required for stimulation of the expression of genes involved in the lack of response of colon cancer to chemotherapy. Pharm Res 66:419–427

Miyata S, Koike S, Kubo T (1999) Hormonal sex reversal and the genetic control of sex differentiation in Xenopus. Zool Sci 16:335–340

Muthusamy S, Andersson S, Kim HJ, Butler R, Waage L, Bergerheim U, Gustafsson JA (2011) Estrogen receptor beta and 17-beta-hydroxysteroid dehydrogenase type 6, a growth regulatory pathway that is lost in prostate cancer. Poc Nat Acad Sci 108:20090–20094

Park B, Kidd K (2005) Effects of the synthetic estrogen ethinylestradiol on early life stages of mink frogs and green frogs in the wild and in situ. Environ Toxicol Chem 24:2027–2036

Pettersson I, Berg C (2007) Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in *Xenopus tropicalis* and *Rana temporaria*. Environ Toxicol Chem 26:1005–1009

Reschly E, Ai N, Welsh W, Ekins S, Hagey L, Krasowski M (2008) Ligand specificity and evolution of liver X receptors. J Steroid Biochem Mol Biol 110:83–94

Rivas A, Fisher J, McKinnell C, Atanassova N, Sharpe R (2002) Induction of reproductive tract developmental abnormalities in the male rate by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance. Endocrinol 143:4797–4808

Rotchell J, Ostrander G (2003) Molecular markers of endocrine disruption in aquatic organisms. J Toxicol Environ Health Part B 6:453–495

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Sharpe R, MacLatchy D (2007) Lipid dynamics in goldfish (*Carassius auratus*) during a period of gonadal recrudescence: effects of beta-sitosterol and 17 beta-estradiol exposure. Comp Biochem Physiol C 145:507–517

Simon P (2003) Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19:1439–1440

Smith D, Penning T, Ansari A, Munday K, Akhtar M (1978) Oestrogen-induced cholesterol and fatty acid biosynthesis in *Xenopus laevis* liver during vitellogenic response. Biochem J 174:353–361

Tata J, Baker B, Machuca I, Rabelo E, Yamauchi K (1993) Autoinduction of nuclear receptor genes and its significance. J Steroid Biochem Mol Biol 46:105–119

Tompsett A, Wiseman S, Higley E, Pryce S, Chang H, Giesy J, Hecker M (2012) Effects of 17-alpha-ethynylestradiol on sexual differentiation and development of the African clawed frog (*Xenopus laevis*). Com Biochem Physiol C 156:202–210

Tompsett A, Wiseman S, Higley E, Giesy J, Hecker M (2013a) Effects of exposure to 17alpha-ethynylestradiol during larval development on growth, sexual differentiation, and abundances of transcripts in the liver of the wood frog (*Lithobates sylvaticus*). Aquat Toxicol 126:42–51

Tompsett A, Wiseman S, Higley E, Giesy J, Hecker M (2013b) Effects of exposure to 17alpha-ethynylestradiol during sexual differentiation on the transcriptome of the African clawed frog (*Xenopus laevis*). Environ Sci Technol 47:4822–4828

Villalpando I, Merchant-Larios H (1990) Determination of the sensitive stages for gonadal sex-reversal in *Xenopus laevis* tadpoles. Int J Develop Biol 34:281–285

Villeneuve D, Garcia-Reyero N, Martinovic-Weigelt D, Li Z, Watanabe K, Orlando E, LaLone C, Edwards S, Burgoon L, Denslow N, Perkins E, Ankley G (2012) A graphical systems model and tissue-specific functional sets of genes to aid transcriptomic analysis of chemical impacts on the female teleost reproductive axis. Mut Res 746:151–162

Watanabe K, Li Z, Kroll K, Villeneuve D, Garcia-Reyero N, Orlando E, Sepulveda M, Collette T, Ekman D, Ankley G, Denslow N (2009) A computational model of the hypothalamic-pituitary-gonadal axis in male fathead minnows exposed to 17alpha-ethinylestradiol and 17beta-estradiol. Toxicol Sci 109:180–192

Witschi E, Foote C, Chang C (1958) Modification of sex differentiation by steroid hormones in a tree frog (*Pseudacris nigrita triseriata* Wied). Proc Soc Exp Biol Med 97:196–197

Yoshimoto S, Okada E, Umemoto H, Tamura K, Uno Y, Nishida-Umehara C, Matsuda Y, Takamatsu N, Shiba T, Ito M (2008) A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. PNAS 105:2469–2474

Zollner G, Trauner M (2009) Nuclear receptors as therapeutic targets in cholestatic liver diseases. Br J Pharmacol 156:7–27