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Author(s): Sahoo, Tarini Prasad; Oikari, Aimo

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Use of Early Juvenile Zebrafish *Danio rerio* for *In-Vivo* Assessment of Endocrine Modulation by Xenoestrogens

Tarini P Sahoo* and A Oikari

Division of Environmental Science and Technology, University of Jyväskylä, Jyväskylä, Finland

Abstract

Reliable and cost-effective early-life stage (ELS) bioassays incorporating practical experimentation without compromising scientific relevance are crucial in chemical risk assessment. This study investigated the use of 20 days-post-fertilization life stage (20dpfZF) of zebrafish *Danio rerio* to screen environmental chemicals known to be estrogenic in adult fish. Firstly, studies with key genes in steroidogenesis were conducted; the brain isoform of aromatase gene (*cyp19a1b*) being the most prominently expressed biomarker. Regulation of mRNA levels of molecular biomarkers, vitellogenin 1 gene (*vtg1*) and *cyp19a1b* were selected to assess the endocrine modulation by xenoestrogens, 17 α -ethinylestradiol (EE2), 4-*n*-nonylphenol (NP), 4-*t*-octylphenol (OP) and bisphenol A (BPA). Groups of 20dpfZF (n=15) as three replicates were exposed to chemicals over a five-day period in aerated static setups. Exposure of 20dpfZF to sediment spiked with EE2 (nominal 3 μ g g⁻¹dw) was also conducted to assess the sensitivity of this life-stage to sediment with estrogenic potency. Whole body homogenates of exposed juveniles showed the estrogenic potential of chemicals in the order: EE2 > OP > BPA > NP. Higher relative expression of *cyp19a1b* was noticed at lower ambient concentrations of EE2, although *vtg1* showed more pronounced expression to it. The 20dpfZF responded in a dose-related way to sediment spiked with EE2, expanding its use as a general aquatic animal model. The suitability of 20dpfZF as an *in vivo* model, along with stable expression of reference genes was established. In addition to consistent expression pattern of key target genes on xenoestrogenicity, it serves as a practical screening model for the risk assessment of environmental chemicals and samples with estrogenic potential.

Keywords: Brain aromatase; Early-life stage; Vitellogenin1; Xenoestrogenicity; Zebrafish

Introduction

Diverse groups of natural and synthetic compounds known to have an endocrine bioactive potential are constantly introduced into the environment. These endocrine disrupting compounds (EDCs) have been found to exhibit significant effects on reproduction by acting on endocrine functions [1,2]. Given the extent of organic pollutants released into aquatic environment, teleosts among vertebrate taxa qualify as candidates to investigate endocrine modulation [2-6]. The choice of zebrafish *Danio rerio* (Hamilton, 1822) as a toxicological model presents practical advantages, including cost-effective maintenance, asynchronous breeding, and rapid development of the embryo-larval stages. Moreover, the sexual differentiation in the brain determining the fate of the gonads in teleosts, contrasts with amphibians and mammals [7]. In general, signaling cues in the form of endogenous hormones and external factors stimulate the pituitary to release gonadotropins, which in turn stimulate the gonads to synthesize and release endogenous hormones, sex steroids. Under endocrine control, the hypothalamus-pituitary-gonadal (HPG) axis is further extended to the liver (HPGL), the site for synthesis of the precursor of yolk protein, vitellogenin (*Vtg*), common to oviparous vertebrates. The hepatic synthesis of these proteins is stimulated by the binding of physiological estrogens or xenoestrogens to estrogen receptors (ERs) in the liver (Figure 1). In zebrafish, seven isoforms of *vtg* have been identified, with dominant expression of *vtg1* compared to *vtg2-7* [8]. Males possessing the normally unexpressed *vtg* genes when exposed to exogenous estrogen or their mimics, up-regulate vitellogenesis [1]. Consequently, alteration of endocrine signaling along the HPGL axis and subsequent modulation of gametogenesis and other reproductive processes from teleost responses indicates mechanisms of EDC action [9].

Bio-chemical pathways have been used to link health and reproductive status in wildlife to environmental chemical exposures. Steroid biosynthetic enzymes catalyze the conversion of cholesterol

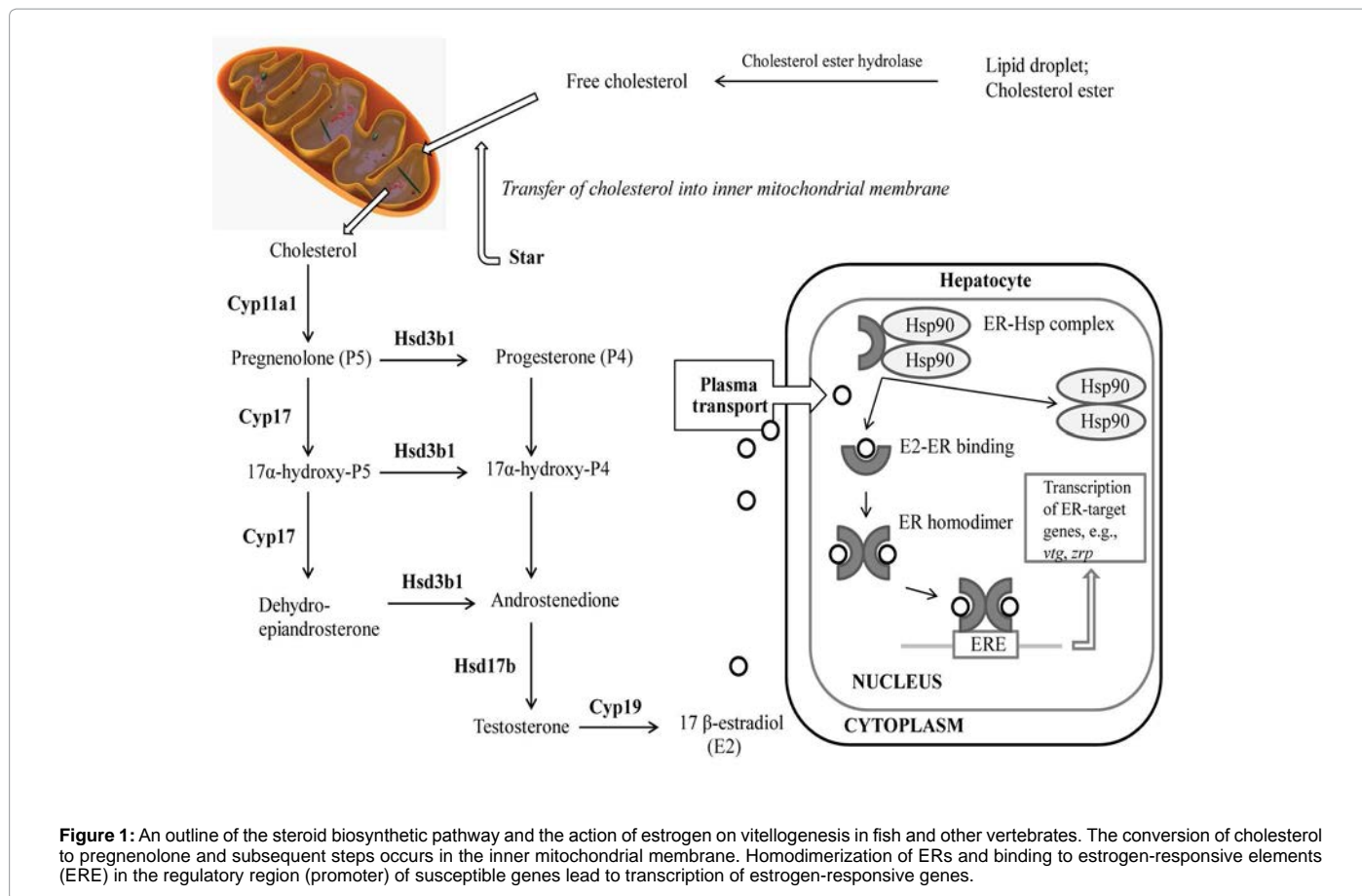
into active sex hormones (estradiol and testosterone) mainly in brain, kidney and gonads [10]. Key enzymes in steroidogenesis include steroidogenic acute regulatory (Star) protein, cytochrome P450 side-chain cleavage (P450_{sc}) or Cyp11a1, 3- β -hydroxysteroid dehydrogenase (Hsd3b1), and cytochrome P450 aromatase (P450_{arom}) or Cyp19a1 (Figure 1). Other studies have also discussed regulation in the steroidogenic pathway with gene expression analyses with adult fathead minnow [11] and zebrafish (*in vitro*, [12]; *in vivo*, [13,14]). Immunohistochemical studies showed the significance of (endogenous) sex steroids on gonadal sex differentiation in teleosts [9,15]. Other studies did not support the involvement of endogenous steroids in the gonadal sex differentiation in fish species such as medaka [16]. Therefore, it was interesting to compare in this study the expression patterns of key steroidogenic enzymes in early juvenile zebrafish, if any. Importantly, the synthesis and release of sex steroids is controlled by the aromatization of C19 androgens (testosterone) to C18 estrogens (17 β -estradiol) by aromatase. In teleosts, the expression of tissue-specific isoforms of P450_{arom} genes (gonad, *cyp19a1a*; brain, *cyp19a1b*) [17], with significantly higher relative expression of the brain isoform, has been used to indicate estrogenic stimulation of sex steroid synthesis [18-23]. Besides induction of *vtg1*, alterations of steroid biosynthesis and plasma steroids are biomarkers indicative of estrogenic action [24].

*Corresponding author: Tarini Prasad Sahoo, Division of Environmental Science and Technology, University of Jyväskylä, Jyväskylä, 40014, Finland, Tel: 358408053900; Fax : 358142602321 ; E-mail: tarini.sahoo@jyu.fi

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Along with *vtg1*, gene expression of *cyp19a1b*, a central steroidogenic enzyme, present suitable molecular markers to detect and analyze the impact of estrogenic exposure, considering the short ovarian cycles and estrogen-responsive embryonic stages in zebrafish. The post-embryonic life-stage (20 days-post-fertilization zebrafish; *20dpfZF*) was used for a five-day exposure to 17 α -ethinylestradiol (EE2), a common benchmark of xenoestrogens. Although ranging in nano- and micro-molar concentrations in ambient water (0.2-40.0 ng L⁻¹; [25]) and sediment (< 0.5-22.8 μ g kg⁻¹ dry mass; [26]) sources respectively, EE2 is a potent xenoestrogen known to have adverse effects on the endocrine function of aquatic organisms.

The objective of this study was to evaluate a life-stage incorporating practical and experimental advantages for screening environmental estrogens, assessed by gene expression analyses. Pilot exposures to EE2 were carried out to select the key steroidogenic enzyme, *cyp19a1b* to complement *vtg1* as a panel of estrogen-responsive biomarkers for subsequent studies. Besides EE2, the sensitivity of this life-stage was evaluated with water-borne exposures of other xenoestrogens with varying potencies-bisphenol A (BPA), 4-*n*-nonylphenol (NP) and 4-*tert*-octylphenol (OP). The screening potential of *20dpfZF* was also verified with exposure to sediment spiked with EE2, and is discussed in context to regulatory requirements with the use of adult animals in toxicological research.

Materials and Methods

Chemicals and exposure stock solution

2,2-bis(4-hydroxyphenyl)propane (BPA, purity 99+%),

17 α -ethynyl,3,5(10)-estratriene-3, 17 β -diol (EE2, minimum 98% by HPLC), and 4-(1,1,3,3-tetramethylbutyl)phenol (OP, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-*n*-Nonylphenol (NP, 98+ %) was obtained from Alfa Aesar (Karlsruhe, Germany). Silylation reagent N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Fluka Chemie (Buchs, Switzerland). Internal standards 17 α -ethynylestradiol-d4 (EE2-d4, 97-98%) and 2,2-bis(4-hydroxyphenyl) propane-d16 (BPA-d16, 98%) were purchased from LGC Standards AB (Borås, Sweden). The solvents, methanol (MeOH) and acetonitrile, supplied by Merck Chemicals (Darmstadt, Germany) were of HPLC grade. Separate chemicals were dissolved in methanol, and stocks were stored at -20°C until use, within two weeks.

Experimental animals

Adult male and female zebrafish (*Danio rerio*) (wild type; WT) were obtained from Institute of Medical Technology, University of Tampere (Tampere, Finland). Fish were acclimatized to temperatures (mean \pm standard deviation, 26 \pm 1°C) and photoperiod (14 h light/10 h dark) for at least two weeks prior to setup for breeding. Animal husbandry was carried out along established guidelines (Westerfield, 2007). Zebrafish adults were fed twice daily with frozen chironomid larvae (Ruto frozen fish food, Zevenhuizen, Holland). Following breeding between sexually mature adults (~8 month old), fertilized eggs (0.5-1.5 hours-post-fertilization; hpf) were collected and reared through early post-embryonic stages for 20 days (*20dpfZF*) in rearing water or embryo medium (Westerfield, 2007). Post-hatch zebrafish from 6 dpf through 25 dpf were fed with fry food (SDS diets, UK).

Chemical exposure and sampling

Chemical exposures were conducted in glass jars containing 250 mL of exposure medium, consisted of gently aerated embryo-rearing water (pH 7.2) for the duration of the experiment, to avoid change in water quality. Water-borne exposures (I and II) were carried out in a semi-static manner, replacing 100% of the medium daily. Water quality parameters were measured on days 1 and 4. The mean temperature in the exposure jars was in the range 24–26°C. The content of dissolved oxygen (DO, mg L⁻¹) was 7.6 ± 0.2 (mean DO ± sd) and pH 7.2 ± 0.2 (mean ± sd). Chemicals dissolved in the carrier solvent, methanol (MeOH), were administered to the exposure water, the solvent concentration being 0.02% or less. Preliminary exposures also included water controls (data not shown), i.e., without solvent to confirm similarity with that of the maximum solvent concentration (0.02%). The first set of exposures (Exposure I) were performed with groups of 30 fish (*20dpfZF*), replicated three times in parallel, exposed to 0.02% MeOH with 0, 5, 25 and 50 ng EE2 L⁻¹, to determine expression and selection of suitable of steroidogenic gene(s). The second set of exposures (Exposure II) were performed with groups of 25 fish (*20dpfZF*), replicated three times, exposed to 0.02% MeOH with 0, 5, 25, and 50 ng EE2 L⁻¹, 0, 100, 500, and 1000 µg BPA L⁻¹, 0, 10, 50, and 100 µg NP L⁻¹, and 0, 10, 50, and 100 µg OP L⁻¹. Although the results used in discussion represent measurement from three parallel replicates, the pattern of relative expression of target genes were also verified with two independent exposures conducted earlier. Fish were sampled at the end of five days of exposure. Each group of sampled animals (n = 15) was collectively weighed in rna-free micro centrifuge tubes (Starlab, Germany), and snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Experiments were conducted according to and licensed by the Finnish authority for animal experiments (ESAVI-2010-07885/Ym-23).

Sediment exposure

The reference sediment (0–10 cm depth; dry weight, dw 5.2%; total organic content, TOC 4.7%) was sampled from Lake Palosjärvi (located in Central Finland) using an Eckman grab sampler. Chemical spiking of it was performed according to standard guidelines for whole-sediment toxicity testing [27,28]. Briefly, nominal concentration 3 µg g⁻¹ dw of EE2-spiked sediment was prepared by gradually administering EE2 (dissolved in 100% methanol) to sediment slurry with minimum amount of water mixed for ~7 h. Following the addition of EE2, the headspace of sample container was filled with an inert gas (N₂) and sealed for equilibration at 4°C for a minimum of 30 d before the assays. The exposure series (Exposure III) were prepared in 600 mL glass beakers in a ratio 1:4 (dw/v) of sediment and overlying water (3°dH or 54 mg CaCO₃ L⁻¹), added slowly to avoid any suspension of particulates to the water column. The exposure beakers were allowed to stand for ~16 h at 24–26°C before the experimental fish (n=25) were added. The exposure series consisted of control sediment (Ref-Sed; i.e., the reference Palosjärvi sample) compared with EE2-spiked samples; Sed 1 (3 d), and Sed 2 (5 d). The exposures of animals were conducted in aerated static systems (no water renewal), sampled at the end of 3 d for Ref-Sed, Water-EE2 (150 ng L⁻¹), Sed 1+ Salt and Sed 1, and at the end of 5 d for Sed 2 (Figure 4). Two controls treatments, each accompanying the respective 3- and 5 d exposures were included to provide for relative quantification per sampling time point. To maintain the water quality, the fish were not fed during the experiment. To assess the possible role of the influence of electrolyte content of overlying water, another sediment exposure Sed1 + Salt included E3 salts (embryo medium; 6°dH or 107 mg CaCO₃ L⁻¹) at levels used for rearing of embryo-larval stages of zebrafish up to *20dpfZF*. For comparison of estrogenic effects, water-

borne exposure of *20dpfZF* to 150 ng EE2 L⁻¹ for 3d was also included as positive control. Experimental conditions and fish sampling (n=3 pools of at least 15 fish each) was the same as in water-borne exposures.

Solid-phase extraction (SPE) of water samples

Ethinylestradiol was extracted from water samples by a SPE method [29] modified for the analyses in the present study (see supplementary information). Similar extraction was employed with NP and OP samples (3–10 mL) using BPA-d16 as the internal standard.

GC-MS analysis of EE2

The gas chromatography–mass spectrometry (GC-MS) was performed with HP 6890 gas chromatograph (Hewlett-Packard, Walbronn, Germany) equipped with HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). The method included a derivatization [30] by silylation with BSTFA, and pyridine to increase the stability of derivatized products and improve extraction recoveries of the target compounds (see supplementary information). Concentration of EE2 was calculated using relative spectral peak-areas of analytes, internal standard, and the determined response factor. Specifically, the results were based on determination of di-TMS derivatives of EE2 and EE2-d4.

LC-MS /MS analysis of BPA, NP, and OP

The analysis was performed with Waters Alliance 2795 (MA, USA) LC and the determination of target compounds was performed in negative ion electro spray mode (ESI-) and a Quattro Micro triple-quadrupole mass spectrometer (MS/MS) (Waters, MA, USA) with electro spray interface was used as detector. The method described by Revilla-Ruiz et al. [31], with minor modifications, was conducted with extracted (NP and OP) and un-extracted (BPA) water samples. Data acquisition was performed with multiple reactions monitoring (MRM) mode and the corresponding parameters for the target compounds are presented in supplementary (Table SI).

Isolation of RNA and reverse transcription

Total RNA was extracted from homogenized pooled fish samples (15 animals with total weight ca. 16 mg) using the Tri Reagent (Molecular Research Center, Inc.) according to manufacturer's instructions, and the quality and quantity were determined on a NanoDrop ND-1000 UV-visible spectrophotometer. Absorbance measurements including 260:280 ratios (1.8 to 2.0) and the additional 260:230 > 260:280 ratios indicated a secondary measure of nucleic acid purity [32]. The integrity of RNA was also verified using the Agilent RNA 6000 Nano Assay Protocol with an Agilent 2100 Bioanalyzer (Waldbronn, Germany). RNA integrity number (RIN, 1–10) generated for analyzed samples was checked within range 7–10 for all samples. The total RNA extracted was reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, USA), and the cDNA templates were diluted ten times for use in real-time PCR assays.

Real-time PCR

Real-time PCR was performed using iQ SYBR Green supermix (Bio-Rad, USA) and run on a Bio-Rad CFX96™ real-time PCR detection system (Bio-Rad, USA). Gene-specific primers (Table SII) for house-keeping genes (beta-actin (*β-actin*) and elongation factor 1 alpha (*ef1a*)) as well as target genes (*star*, *cyp11a1*, *hsd3b1*, *cyp19a1b*) were designed using Amplifx (v 1.5.4), and the given oligonucleotides (desalt purified) were purchased from Sigma-Aldrich (UK). The primer pair sequences were checked with the program Mfold (<http://mfold.bioinfo.rpi.edu/>)

cgi-bin/dna-form1.cgi) to predict the potential for amplicon secondary structures that may prevent efficient amplification. To evaluate qPCR efficiency, amplification efficiency with standard curve analysis (PCR efficiency, 90-110%; slope, -3.1 to -3.6; $R^2 > 0.98$) was performed, and optimization of qPCR reactions and protocol was carried out. The qPCR reaction mix was optimized for a 25 μ L reaction mix consisting of 12.5 μ L of SYBR green supermix, 2.5 μ L of each of the primer pairs (200 nM), 2.5 μ L sterile water, and 5 μ L of cDNA template (100 ng μ L⁻¹). The PCR protocol comprised 10 min polymerase activation at 95°C, followed by 40 cycles of 30 sec at 95°C, and 30 sec at 60°C. Each sample was assayed in duplicate, and assays requiring multiple runs included inter-run calibrators (IRCs) on each of the plates to facilitate sample maximization and restricting reference gene reactions to a single plate. The inter-run calibration was performed to correct for variation, allowing comparison between plates within the same analysis.

Data analysis

The expressions of target genes were normalized to the expression of two reference genes, (β -actin, *ef1a*). The choice of reference genes was based on their stable expression across samples of different treatment. The determined stability parameter (M value) for the reference genes were used to select suitable reference genes with M values < 0.5; the lower the M-value, that higher the stability [33]. The relative gene expression levels were calculated from the experimental amplification data (Cq values; quantification cycles) using the $2^{-\Delta\Delta CT}$ method [34]. Statistical significance ($P < 0.05$) of log-transformed data from Exposures I and III was determined by a one-way analysis of variance (ANOVA) followed by Tukey HSD *post hoc* test. Data from Exposure II was analyzed using Welch's test followed by Games-Howell *post hoc* test. All analyses were performed using IBM SPSS (v 19).

Results

Water quality and concentrations of xenoestrogens in experiments

No significant difference in water quality was found between exposure jars or treatments during the exposure period. Regarding to nominal concentration added in experimental waters, actual chemical concentrations (mean \pm sd) are presented in Table SIII. However, all measured concentrations of NP were not detected. Since both NP and OP samples were extracted simultaneously with the same method, the undetected NP samples may have resulted from issues with the extraction method. For clarity, while the measured concentrations commonly ranged 20-50% below expected, revealing effects, the nominal values are used in further discussion.

Exposure I: Responses of steroidogenic enzyme mRNA levels

The mean M-values of reference genes, β -actin and *ef1a* across the corresponding concentration series of exposures (BPA, NP, OP, and EE2) were 0.004, 0.1, 0.3, 0.06; and 0.01, 0.04, 0.06, 0.09 respectively, showing stable expression of the respective housekeeping genes. For normalization of gene expression, the mean M-values of both reference genes combined and considered together for the chemical exposures were 0.01, 0.07, 0.06, and 0.07 respectively, suggesting the stable expression of this reference gene pair across chemical treatment at the studied life-stage.

Figure 2 shows the normalized gene expression levels of *cyp11a1*, *cyp19a1b*, *hsd3b1*, and *star* in *20dpfZF* exposed to EE2 for five days (20-25 dpf). Among the studied genes, only *cyp19a1b* showed pronounced expression levels compared with *cyp11a1*, *hsd3b1*, and *star*, (Tukey

HSD, $P < 0.001$). Noticeable induction of *cyp19a1b* resulted formal EE2 exposures (5, 25 and 50 ng L⁻¹), averaging 13.5, 18.7 and 22.8-fold changes respectively, with gradual increase with increasing concentrations (5-50 ng L⁻¹). In preliminary studies with similar experimental conditions, *cyp19a1b* showed ~2-fold increase at even lower exposures (1 ng L⁻¹) (data not shown). There was no induction of *star* in exposure groups, with expression even in the highest EE2 exposure, 50 ng EE2 L⁻¹, being similar with that of control and 5 ng EE2 L⁻¹. Although 25 ng EE2 L⁻¹ revealed ~1.3-fold change, the altered *star* expression was not significantly different from either the control or respective expression levels of *cyp11a1* and *hsd3b1* (Tukey HSD, $P > 0.05$). Similarly, no statistically significant effects were observed with the mRNA levels of *cyp11a1* and *hsd3b1* that actually showed decreased average expression compared to solvent control. Overall, there was a statistically significant difference between expression of *cyp19a1b* and the other steroidogenic enzymes (*star*, *cyp11a1* and *hsd3b1*) as determined by one-way ANOVA for EE2 exposures 5 ng L⁻¹ ($F(3, 8) = 91.740$, $P < 0.001$), 25 ng L⁻¹ ($F(3, 8) = 61.130$, $P < 0.001$) and 50 ng L⁻¹ ($F(3, 8) = 133.082$, $P < 0.001$). A Tukey HSD *post hoc* test revealed the significant up-regulation of only *cyp19a1b* expression among the studied steroidogenic enzymes ($P < 0.001$) compared to *star-cyp11a1* ($P = 0.848$) and *star-hsd3b1* ($P = 0.982$).

Exposure II: Changes in *cyp19a1b* and *vtg1* mRNA levels to xenoestrogens in water

Figure 3a shows the relative expression of *cyp19a1b* and *vtg1* in *20dpfZF* exposed to EE2 from 20 through 25 dpf. The mRNA levels of *cyp19a1b* exposed to 5, 25, and 50 ng EE2 L⁻¹ were induced to 13, 19, and 24-fold respectively, relative to control treatment. For the same concentrations, relative expression levels of *vtg1* were 5, 143, and 1272-fold. Despite the larger extent of *vtg1* expression across the exposure treatment, *cyp19a1b* showed more sensitive induction (13-fold; Games-Howell, $P < 0.05$) at the lowest range (5ng EE2 L⁻¹) compared to *vtg1* (5-fold; Games-Howell, $P < 0.05$). As presumed, the pattern of expression of *cyp19a1b* at all concentrations of EE2 was similar to samples studied for steroidogenic enzyme mRNA levels (Figure 2d); with gradual increase from 5 to 50 ng EE2 L⁻¹. However, at higher treatments levels (25 and 50 ng EE2 L⁻¹), the relative induction of *vtg1* (Games-Howell, $P < 0.01$, 0.001) was noticeably higher than *cyp19a1b* (Games-Howell, $P < 0.05$), albeit both the genes showing significant relative expression. Almost 15% mortality was observed in groups exposed to the highest nominal concentration of EE2 (Figure S1).

Whole-body *cyp19a1b* showed increased expression levels at 100, 500 and 1000 μ g BPA L⁻¹ compared to *vtg1* (Figure 3b). Expression of *vtg1* was down-regulated (in average -1.2-fold change) by lower concentrations of BPA (100 and 500 μ g L⁻¹), but was significantly up-regulated at the highest concentration (2.7-fold change; Games-Howell, $P < 0.05$). The two higher concentrations of BPA L⁻¹ showed significant induction of *cyp19a1b* transcripts, 2.9 and 4.4-fold (Games-Howell, $P < 0.05$) respectively. Clearly, *cyp19a1b* was more sensitive compared to *vtg1* across a range of BPA exposures, further revealing that BPA induces a weak estrogenic effect when compared to EE2.

In *20dpfZF* exposed to 10, 50 and 100 μ g NP L⁻¹, the relative expression levels of both *cyp19a1b* and *vtg1* showed no statistically significant change (Games-Howell, $P > 0.05$), showing a clear down-regulation for both genes (Figure 3b). Compared to NP, the expression pattern was different with OP exposure; *cyp19a1b* showing an up-regulatory pattern for all three concentrations compared to control (Figure 3b). The relative induction of *vtg1* was significant only at 100

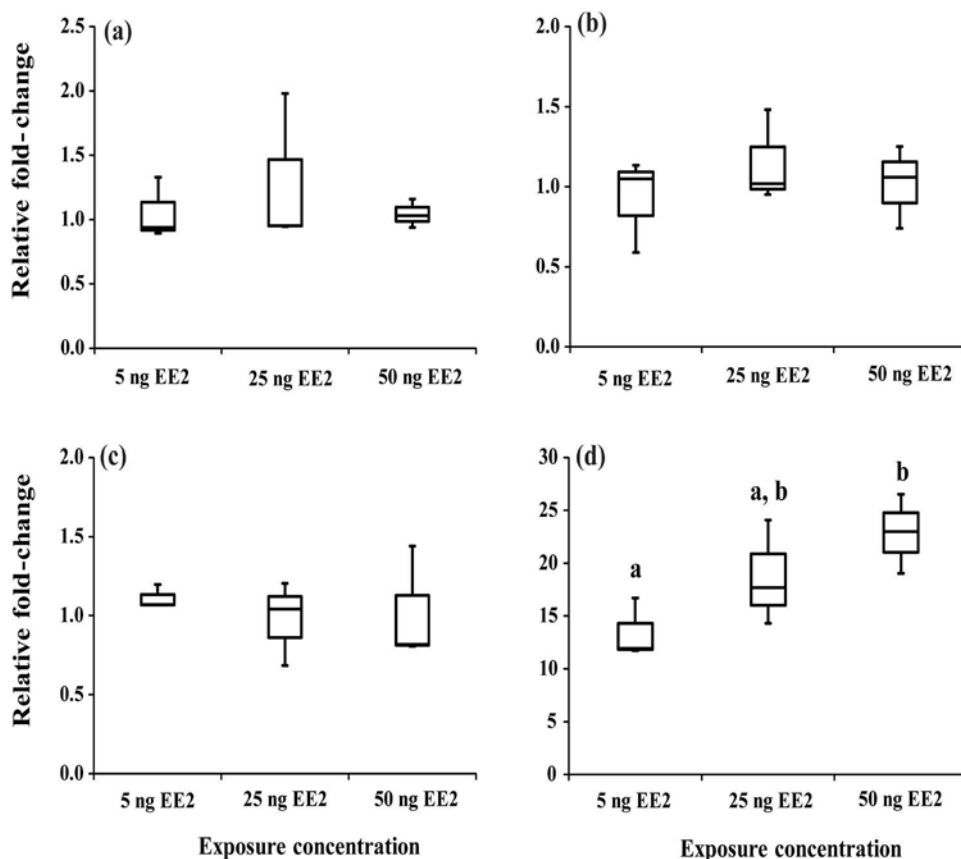


Figure 2: Exposure I: Changes in mRNA levels of steroidogenic enzymes. Gene expression of *star* (a), *cyp11a1* (b), *hsd3b1* (c) and *cyp19a1b* (d) in *20dpfZF* exposed to concentrations of EE2 for five days (from 20 to 25 dpf). Box plots represent median (—), 25th and 75th percentiles (lower and upper end of boxes), minimum and maximum values (end of the whiskers) for pooled whole-body homogenates (n=3 pools of at least 20 fish each). Target genes were normalized to geometric mean of two reference genes (*β-actin*, *ef1a*). Nominal exposure concentrations include 0, 5, 25 and 50 ng EE2 L⁻¹. Means with different letters are significantly different (P < 0.05) as determined by One-way ANOVA followed by Tukey's HSD *post hoc* test.

µg OP L⁻¹ (2.5-fold; Games-Howell, P < 0.05). For all the concentrations, *cyp19a1b* again showed higher sensitivity for the induction, i.e., 1.9, 1.7, and 2.5-fold. With OP, as in the case of EE2 and BPA, *cyp19a1b* was a more sensitive biomarker of estrogenicity than *vtg1*. Overall, it is noteworthy that NP compared to EE2, BPA, and OP had a much less estrogenic effect in the *20dpfZF* model. There was statistically significant difference between exposure groups determined by Welch's test for both *cyp19a1b* (F (11, 9.197) = 422.008, P < 0.001) and *vtg1* (F (11, 9.355) = 666.860, P < 0.001). Both 50 and 100 µg OP L⁻¹ affected the survival of exposed fish to ~20 and 30% mortality (Figure S1).

Exposure III: Changes in *cyp19a1b* and *vtg1* mRNA levels to sediment spiked with EE2

In relevant contexts of *in vivo* exposures, *20dpfZF* showed preferential use of water-column depending on exposure type (with and without sediment). While each group of 25 animals in the water-only units appeared to distribute randomly across the water column, the fish in units with sediment headed for the sediment-water interface, perhaps seeking food. Actually, by actively ingesting contaminated sediment, the possibility to study diverse exposure scenarios with *20dpfZF* (i.e. contaminants taken up by ingestion) was obvious (Figure 5b). We suggest that the same is not possible with sessile and non-feeding life stages.

One-way ANOVA analysis of normalized gene expression showed significant induction of both *cyp19a1b* (F (4, 10) = 1096.501, P < 0.001) and *vtg1* (F (4, 10) = 2471.846, P < 0.001) of *20dpfZF* when exposed to sediment spiked with nominal 3 µg EE2 g⁻¹dw over three to five days (Figure 4a, 4b). Between the exposures (Sed1 and Sed2) conducted over three and five days, Tukey's HSD *post hoc* test showed that fish exposed to sediments with EE2 exhibited higher induction of *cyp19a1b* than the water-borne exposure (nominal 150 ng EE2 L⁻¹) at 63- and 50-fold change, respectively (Figure 4a). Significant up-regulation of Sed 2 fish with 74-fold change, compared to EE2-water exposure, indicated the influence of the extended exposure duration. For the 3d exposures (Sed1+Salt, Sed1), which differed only in respect to the electrolyte content, showed higher induction (72-fold) compared to that of Sed1, although not significantly different (Tukey's HSD, P = 0.473).

The expression of *vtg1* showed very pronounced up-regulation patterns, increasing from EE2 in water (181-fold) to Sed 1 (500-fold) and Sed 2 (1152-fold) (Figure 4b). Unlike *cyp19a1b*, *vtg1* showed significantly lower induction in Sed 1 + Salt (198-fold) when compared to Sed 1 (500-fold), possibly as an indication of the difference in the fraction of EE2 taken up by the fish (Tukey's HSD, P < 0.001). These may be considered consistent with differences in the partitioning and bioavailability of EE2 between static water and sediment exposures systems, based on ambient characteristics.

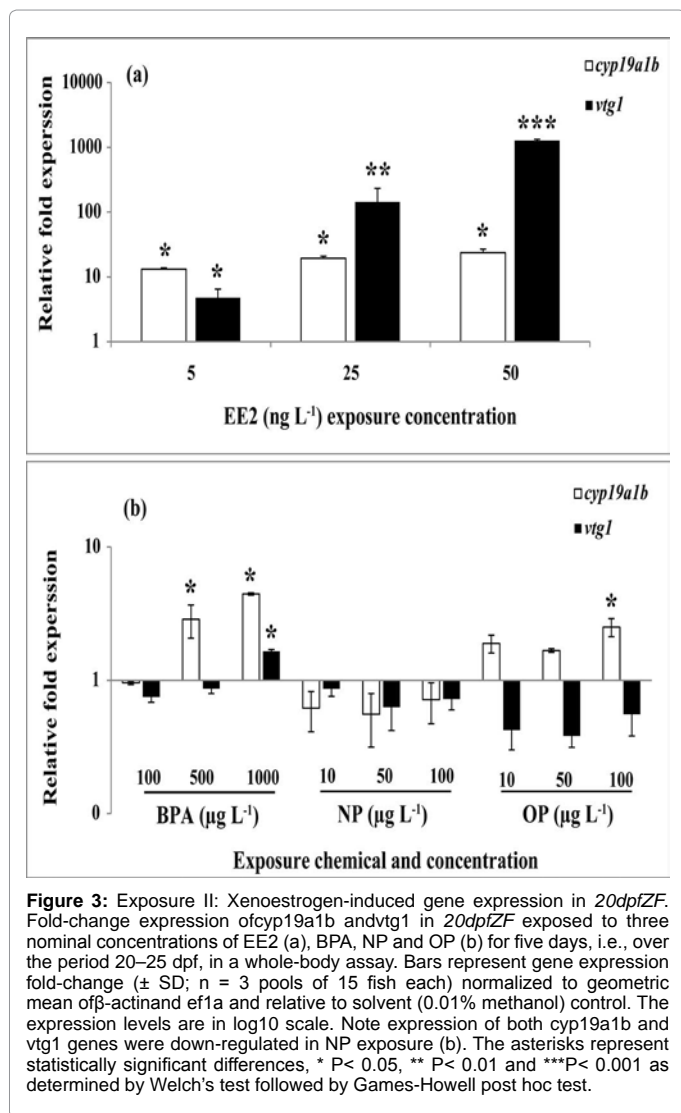


Figure 3: Exposure II: Xenoestrogen-induced gene expression in 20dpfZF. Fold-change expression of *cyp19a1b* and *vtg1* in 20dpfZF exposed to three nominal concentrations of EE2 (a), BPA, NP and OP (b) for five days, i.e., over the period 20–25 dpf, in a whole-body assay. Bars represent gene expression fold-change (\pm SD; n = 3 pools of 15 fish each) normalized to geometric mean of β -actin and ef1a and relative to solvent (0.01% methanol) control. The expression levels are in log₁₀ scale. Note expression of both *cyp19a1b* and *vtg1* genes were down-regulated in NP exposure (b). The asterisks represent statistically significant differences, * P < 0.05, ** P < 0.01 and ***P < 0.001 as determined by Welch's test followed by Games-Howell post hoc test.

Discussion

Among aquatic vertebrates, the zebrafish has been widely applied in investigating biological responses in development [35], endocrine disruption [3,4,36], and reproduction [37] among other effects. As adult organisms, the fish has been established as a reliable and sensitive organism in studies of comparative toxicology. On the other hand, in line with alternative animal testing requirements, ELS bioassays are aimed to substitute more advanced stages for toxicity testing [37–39]. The feasibility to expose ELS includes a large sample size, not possible with adult fish. Moreover, an early *in vivo* model presents an integrated organism system compliant with regulatory mandates of vertebrate animal testing. The reliability of gene level responses [40,41], including estrogenic and the other steroidogenic ones, is suggested to give a mechanistic understanding on impacts of environmental chemicals, their dynamics of effects, and most importantly, extrapolations used in environmental risk assessment. Introduction of the 20dpfZF clearly satisfied several scientific, practical, and cost-effective requirements, although the main focus of this study was to select and evaluate the life-stage as a suitable screening model for xenoestrogenicity. The information regarding the gene-level response determined here in

water-based exposures could be compared to a sediment exposure carrying a potent xenoestrogen with substantial trait of hydrophobicity, EE2 (log K_{ow} 4.15) [42].

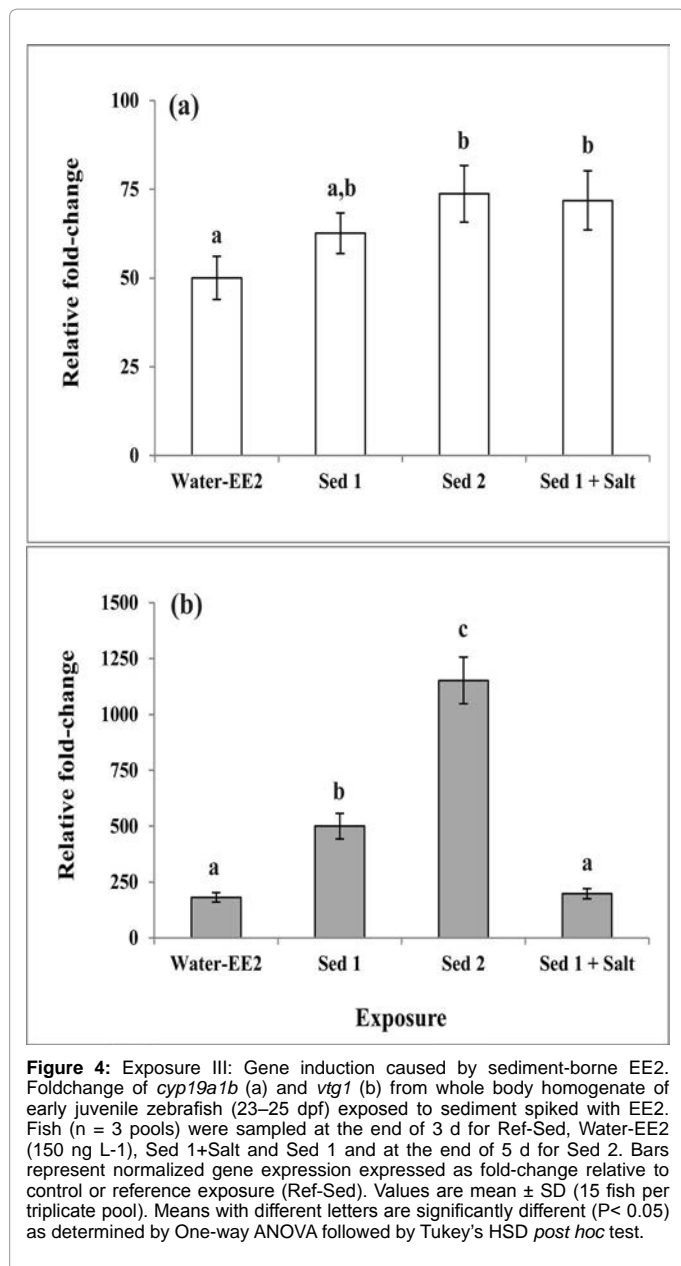
Exposure of zebrafish (20–25 dpf) to EE2, BPA, NP, and OP was used to evaluate regulation of *cyp19a1b* and *vtg1* transcript levels. The choice of *cyp19a1b* as a core endpoint for this life-stage was determined from concentration-specific transcript abundance compared to other steroidogenic enzymes (*star*, *cyp11a1* and *hsd3b1*; Figure 1). The presence of ERE in the promoter region of *cyp19a1b*, but absent on *cyp19a1a*, has been shown to explain the estrogen-responsiveness of *cyp19a1b* [7,18,43].

Exposure I: Selection of steroidogenic endpoint for xenoestrogenicity

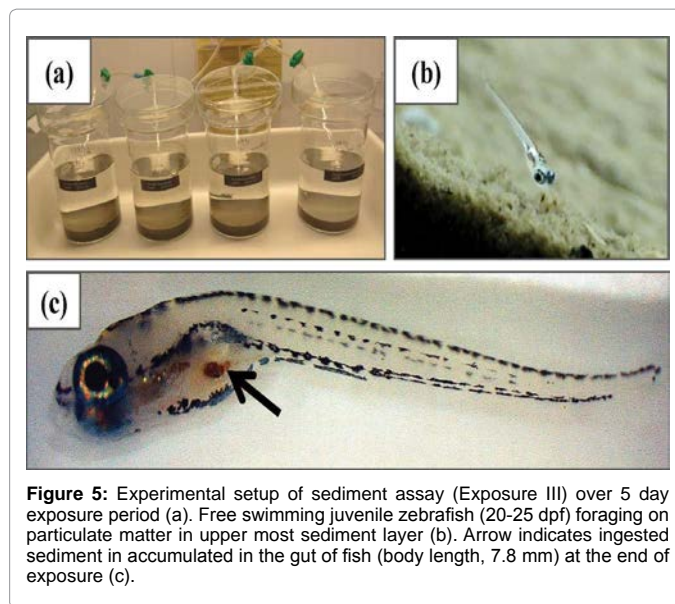
The steroid biosynthetic pathway can be a susceptible target because of its key enzymes, each capable of setting off a cascade of events. For instance, in adult zebrafish exposed to water-soluble fraction of crude oil, Arukwe et al. [13] demonstrated a negative relationship between altered steroidogenic responses and xenobiotic biotransformation processes. The same study showed down-regulation of important steroidogenic enzymes including *star* and *cyp11a1*, and up-regulation of *hsd3b1* along with increasing contaminant level. Levi et al. [14] showed hepatic expression of *star* in non-vitellogenic female zebrafish. In the present study we found no change of *star* mRNA levels except some tendency for down-regulation relative to the control group at exposure levels 5 and 25 ng EE2 L⁻¹ (Figure 2a). Filby et al. [11] also showed down-regulation of *star* mRNA in adult male fathead minnow exposed to 10 ng EE2 L⁻¹ but no significant changes in exposed females. *In vitro* cultures of zebrafish ovarian follicles at different maturation stages exposed to human chorionic gonadotropin revealed an increase in expression of *star*, *cyp11a1* and *hsd3b1* with mature stages, in contrast with a decreasing pattern without exposure [12]. In this study, the mRNA levels of *cyp11a1* and *hsd3b1* were relatively lower for all exposure concentrations with lowest relative expression for 25 ng EE2 L⁻¹ at 59% and 45% respectively (Figure 2b). Lower expression levels of *star*, *cyp11a1*, and *hsd3b1* in fish exposed to EE2, a potent agonist for estrogenic effects, suggest effective regulatory processes in effect. In comparison to three other transcripts, *cyp19a1b* showed a clear dose-specific up-regulation pattern. Filby et al. [11] that showed up-regulation of only *cyp19a1a* but not *cyp19a1b* in male fathead minnow exposed to EE2, possibly owing to the pronounced expression of the brain isoform (*cyp19a1b*) in earlier life-stages. The use of *cyp19a1b* signal has also been employed in transgenic zebrafish specifically as estrogenic-sensitive screens [44,45]. Because *cyp19a1b* is a key player in steroid biosynthesis at stage of 20dpfZF, involved in conversion of androgen to estrogen, it qualified as a compelling signature of xenoestrogen-induced effects.

Exposure II: *cyp19a1b* and *vtg1* mRNA regulation by four potential xenoestrogens

In the present study, modulation of *cyp19a1b* and *vtg1* mRNA expression was used as biomarker for estrogenic effect in the identified responsive ELS of zebrafish (Figure 3). Earlier, in mature adults, estrogen-mediated expression of *cyp19a1b* has been described among other teleosts, e.g. in Atlantic salmon [46], mangrove killifish [47], medaka [48], and zebrafish [18–20]. Expression of *cyp19a1b* in developing zebrafish (3–4dpf) was shown to be a sensitive indicator of estrogen-induced effects even earlier than 20dpfZF [49]. Giving further support to our research, Muncke and Eggen [50] described the induction of *vtg1* mRNA as a reliable endpoint for 1–5 dpf zebrafish, presenting alternatives for adult animal testing.



Our juvenile zebrafish (20 dpf) exposed to EE2 showed inductions of *cyp19a1b* and *vtg1* in a concentration-dependant manner, *vtg1* being more distinct than *cyp19a1b*. Importantly, however, the expression levels of *cyp19a1b* were increased in all exposure groups relative to control, serving as an excellent benchmark. For comparison, in adult male zebrafish exposed to EE2 for three weeks, induction of *cyp19a1b* mRNA [51] and protein levels [4] were also evident. Our data with *20dpfZF* showed around 13-fold *cyp19a1b* induction at nominal concentration of 5 ng EE2 L⁻¹ and more at higher concentrations, keeping with range of expression in adults. Even somewhat younger animals than in our study, 17 dpf zebrafish exposed from 0.3 to 30 ng L⁻¹ EE2 showed statistically significant induction in a dose-dependent manner in three days [19,52]. Although significant induction of *vtg1* mRNA was observed in very early zebrafish embryos (2 dpf) [50] exposed to 1000 ng EE2 L⁻¹, the same authors also showed significant up-regulation of *vtg1* mRNA in zebrafish embryos exposed to 25 ng L⁻¹ for 5 dpf [41].



In *20dpfZF*, *cyp19a1b* was up-regulated in a concentration-dependent manner above 500 µg BPA L⁻¹ (P < 0.05) and *vtg1* at 1000 µg BPA L⁻¹ (P < 0.05). While in adult fish the induction of Vtg has been documented well [4], post-hatched and early juvenile zebrafish exposed to BPA for 5 d induced *vtg1* at higher concentrations, 2280 µg BPA L⁻¹ [41]. Our results showed sensitivity of *20dpfZF* exposed to 1000 µg BPAL⁻¹, comparable with adult male zebrafish exposed for three weeks that showed lowest observed effect concentration (LOEC) for vitellogenin protein induction for similar exposure concentration [4]. Thereby, the estrogenic responses of *20dpfZF* present a cost-effective life-stage compared to the adult fish as well as further developed metabolic capacity when compared to embryonic stages.

Unlike BPA, NP showed clear down-regulation of estrogenic response in *20dpfZF*. This is in contrast to significant effects observed in adult [4,53] as well as larval [54] stages of zebrafish and other small bodied fish models, Japanese medaka [55] and fat head minnow [56]. However, other studies this far with zebrafish embryos exposed to NP showed no significant induction of *vtg1* mRNA [36,41], indicating low sensitivity to the chemical. Our results confirmed the low sensitivity of juvenile life-stages to NP as shown by Jin et al. [36]. It also contrasts with the significant induction of aromatase by juvenile zebrafish (17 dpf) exposed to NP for 3d [52]. In our study, the mRNA of both *cyp19a1b* and *vtg1* were down-regulated. In essence, the observed discrepancies can be related to the difference in composition of the selected chemical. Previous studies [4,54] with NP included a technical grade of NP (CAS 84852-15-3); a mixture of isomers. In our study, the linear form of 4-*n*-NP (CAS 104-40-5) that is devoid of branched alkyl side-chains present in most of the isomers, showed slightly reduced estrogenic potency (relative to E2), compared to the higher efficacy of technical grade NP [57]. From metabolite studies of 4-*n*-NP in fish, biotransformation pathway involving β-oxidation of the alkyl side-chain results in metabolites, lacking the alkyl side-chain, which largely accounts for the lesser estrogenic potential of 4-*n*-NP *in vivo* [58].

Among the three alkylphenols in the present study, only OP significantly induced *vtg1* in *20dpfZF*. Earlier studies with adult zebrafish showed the weak estrogenic potential of OP at similar concentrations as used by us [4]. Thus, considering the differences in sensitivity between

life stages, the dose-dependent induction of both *vtg1* and *cyp19a1b* observed in *20dpfZF* is indicative of a suitable window to predict xenoestrogenicity as the key biomarker. It presents a viable and cost-effective life-stage to assess all related chemicals with weak estrogenic potential. In all, from the exposure series with *20dpfZF*, a clear ranking of the estrogenic potency was obtained, with EE2 being the most potent agonist, followed by OP, BPA, and 4-*n*-NP in that order.

Exposure III: *cyp19a1b* and *vtg1* mRNA regulation by sediment spiked with EE2

Gene induction data from the sediment assay with spiked EE2 indicated applicability and distinct sensitivity of the *20dpfZF* in *in vivo* bioassays (Figure 4a and 4b). Due to its physicochemical properties ($\log K_{ow} = 4.15$; $\log K_{oc} = 3.8$; sorption constant, $1/n = 0.83$; sorption coefficient, $\log K_f = 1.72$) [42], EE2 was understood to have sorbed to the sediment that consequently acts as a sink for the sediment-water interactions. While the *vtg1* gene expression as shown in Exposure I (Figure 3) indicating the pronounced potency of EE2 even at 50 ng L^{-1} to produce nearly 1400-fold change, a reduced effect was seen in the water-borne exposure to $150 \text{ ng EE2 L}^{-1}$, the positive control (Figure 4b). This can be explained by the static nature of the water-only exposure without renewal of media. Thereby the exposure via water in the static system would result in decline of available EE2 in water phase. Thus the exposure scenario was probably different. In the sediment acting as a sink, thereby partitioning EE2 to water phase to maintain its concentrations towards the sediment-water equilibrium of EE2, was taken up by the fish. Of the two biomarkers, *vtg1* was more sensitive than *cyp19a1b* to sedimental EE2 and indicative even of some influence of electrolyte content in overlying water on the availability of EE2 (Figure 4a and 4b). Mechanistically, as EE2 in Sed 1+ Salt exposure was sorbed to the sediment matrix to a greater extent than in Sed 1, the lesser EE2 content in the aqueous phase consequently elicited a lower induction of *vtg1* (Figure 4b). Considering the same sediment characteristics (TOC %, particle size, spiking, sample preparation) for both samples with the sole exception of overlying water quality (Sed 1+Salt; $0.3\% \text{ NaCl}$, $107 \text{ mg CaCO}_3 \text{ L}^{-1}$), this explanation is consistent with studies on the effect of salinity on the higher or lower sorption of EE2 to sediment as shown by Lai et al. [42]. Although, the role of electrolyte content on bioavailability of EE2 can be empirically verified with wide ranges of salinity and hardness levels, this was outside the scope of our study. Moreover, the difference in overlying water was included in our study only to determine variation in biomarker response because E3 salts are generally added in ELS rearing media. While the current trials to assay sediment with *20dpfZF* were first ones of its kind, we did not analyze the dynamics of EE2 content in either compartment. Anyways, in presenting expected responses, the relevancy of *20dpfZF* in sediment bioassay does exist. For further goals, data generated with *20dpfZF* can be employed to study various joint effects of multi-component xenoestrogens.

Choice of suitable developmental stage of zebrafish for evaluation of estrogenicity

Maturity is not the only important life-stage for reproductive success and population maintenance. In support of the relevance of ELS in manifestation of toxicity, age-specific developmental factors, including chorion-permeability and the nutrition by yolk associated with embryonic life as well as post-hatched eleuthero embryos, define important ontogenic stages. There is a common notion that newly post-hatched embryos are the most or equally sensitive life-stage of

oviparous fishes to environmental chemicals [19,37,59]. This said, the rule includes several minor exceptions, which in part directed our study to the early post-yolk stages of zebrafish. While several range-finding exposures with various ages of zebrafish to xenoestrogens were examined at large sub-lethal range, a decision was made to focus on *20dpfZF* based on the survivorship as well as consistency of response levels of genomic regulation associated with the life stage. Furthermore, the combined advantages of an ELS that is both free-swimming and free-feeding (Figure 5b), but also cost-effective in terms of husbandry efforts compared to later or adult stages. The life stage was found to be practical and responsive to different media, including here the exposure to EE2-spiked sediment as a contact assay (Figures 5b and 5c).

Regarding xenoestrogenicity of chemicals, the time frame for ontogenic determination of the phenotype in juvenile life can be considered significant. In zebrafish, an undifferentiated gonochorist, ovary-type gonad in the juvenile hermaphrodite stage (21dpf) is transformed into distinct sex-specific mature gonads (42 dpf), leading to phenotypic sex differentiation [60]. At 20-25 dpf, the fish are developmentally more advanced than embryonic stages. However, the possibility of detecting signals from presumptive females from a pool of samples fish is addressed with normalized gene expression quantified relative to control treatments. With as large a sample size (15-25 fish) as in this study, statistically significant difference of estrogenic gene expression between exposures highlights the reliable detection of a genomic signal. Advancement in existing estrogen-sensitive screening has employed the induction of green fluorescent protein expression in transgenic zebrafish, *cyp19a1b-GFP* [44,45] and *ere-zvtg1:gfp* [61], pointing to non-lethal alternatives of animal testing. Chen et al. [61] showed GFP induction in transgenic zebrafish represented by less than 25% of the exposed fish. Sex differentiation in fish, known to be controlled by various environmental as well as of endogenous (steroidogenic) factors, is supported by the role of sex-steroids on differentiation and phenotypic outcome [62]. More specifically, the modulation of aromatase and vitellogenin, both key players in the onset of endogenous estrogen action, can be directly linked to gonadal sex differentiation from endocrine disruptors [63,64]. The choice of a labile life-stage taking into consideration such biological events, directs to the *20dpfZF* as a suitable ELS model for investigating endocrine modulation.

In conclusion, we documented that the post-embryonic (*20dpfZF*) zebrafish was a potent life-stage using transcripts of *cyp19a1b* and *vtg1* as endpoints to assess xenoestrogenicity of chemicals. The RT-PCR presented analytical tools to evaluate xenoestrogenic modulation in a relatively quick approach. The *20dpfZF* life-stage provided for cost-effective maintenance and experimentation (compared to adult fish), and responded to four model estrogens with varying potencies characterized by corresponding induction patterns. The results of this study recommend the selection of *20dpfZF* as a representative life-stage for use in ongoing and further studies, including exposures to water-sediment interactions and mixtures of environmental chemicals.

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Supplementary material

Use of early juvenile zebrafish *Danio rerio* for in vivo assessment of endocrine modulation by xenoestrogens

Contents:

1. Chemical analyses of water samples
2. Tables S1, S2 and S3
3. Figure S1
4. Tables S1 and S2 were created using Word
5. Table S3 and Figure S1 are TIFF files created using CorelDraw (version 12.0)

1. Chemical analysis of water samples

1.1 Solid-phase extraction (SPE) of water samples

Briefly, Oasis SPE cartridges (Oasis HLB, 3cc, 60 mg) were conditioned with 3 mL methyl-*t*-butyl ether (MTBE), subsequently rinsed with 3 mL methanol and 3 mL ultrapure water. Water samples (1000 mL), containing internal standard (d4-EE2), were acidified to pH 3 before loading onto the cartridges. The extraction was managed as a drop-wise outflow, where after the cartridge was re-equilibrated with 3 mL ultrapure water. The elution solvent, 10% MeOH in MTBE (3×2 mL), was added and allowed to react with the analytes on the sorbents and ultimately eluted into collection tubes. The contents were evaporated to dryness while tubes were immersed in a water bath (ca. 50 °C) under a gentle nitrogen flow. Samples were reconstituted in 200 µL 50:50 (v/v) acetonitrile: 2.5 mM ammonium acetate in water (pH 3).

1.2 GC-MS analysis of EE2

The dried residues were treated (silylated) with 50 µL BSTFA (+1%TMCS) and 50 µL pyridine, incubated at 70 °C for 30 min, where after cooled and evaporated to dryness with nitrogen stream. Finally, hexane (100 µL) was added, followed by transferring the contents to auto sampler vials for the GC-MS, performed with HP 6890 gas chromatograph (Hewlett-Packard, Walbronn, Germany) equipped with HP 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). A HP-5 capillary column (30 m × 0.25 mm × 0.25 µm) was used with helium as the carrier gas by a rate of 0.9 mL min⁻¹. The column temperature was programmed to increase from 120 °C to 190 °C at the rate of 8 °C min⁻¹, 190 °C to 240 °C at 2 °C min⁻¹, 240 °C to 280 °C at 10 °C min⁻¹ (maintained for 8 min), and 280 °C to 290 °C at 10 °C min⁻¹ (maintained for 20 min). The MS was operated in selective ion monitoring mode, and identification was made based on retention time and mass spectra of pure standards.

1.3 LC-MS /MS analysis of BPA, NP and OP

Separation with a reverse phase C18 column (Waters XBridge 3.5 µm, 2.1×100 mm with 3.5 µm, 2.1×10 mm guard column) was performed with Waters Alliance 2795 (MA, USA) LC consisting of tertiary pump, vacuum degasser, auto sampler and column oven. The column temperature was set to 30 °C and that of auto sampler to 20 °C. The injection volume was 20 µL. The determination of target compounds was performed in negative ion electrospray mode (ESI-) and a Quattro Micro triple quadrupole mass spectrometer (MS/MS) (Waters, MA, USA) with

electrospray interface was used as detector. The mobile phases consisted of MeOH (A) and distilled water (B) with a flow of 0.2 mL min⁻¹. The gradient of A was raised to 100% over 10 min, phase proportion maintained for 8 min (10-18 min), and lowered back to 60 % over 2 min. The column was equilibrated for three min (20-23 min) prior to the next injection.

Compound	Fw	Precursor ion m/z	Product ions m/z	Cone voltage (V)	Collision energy (eV)
BPA	228.3	227.1	133.1, 212.0	39	21
d16-BPA(IS)	244.06	241.1	142.1, 223.1	41	28
NP	220.4	219.3	106.0, 118.7	40	34
OP	206.3	205	133	38	27

Table S1: Acquisition parameters of mass spectrometry for target compounds and corresponding internal standard (IS; d16-BPA) used in this study.

Gene	Primer sequences (5'-3')	Size (bp)	Genbank accession
β-actin	Forward-AAGAGCTATGAGCTGCCTGA	108	AF057040.1
	Reverse-ACCGCAAGATTCCATACCCA		
cyp19alb	Forward-TGGACGCATGCATAAGACAG	104	AF226619.1
	Reverse-CACAACCGAATGGCTGGAA		
efla	Forward-AAACATGGGCTGGTTCAAGG	87	AY422992.1
	Reverse-ATGGCATCAAGGGCATCAAG		
cyp11a1	Forward-GCCTGGAAGACTGATAGGATGA	114	AF527755.1
	Reverse-GTTGACACGAGCCACAAAGT		
star	Forward-TGTAAGGGCTGAGAATGG	133	NM_131663.1
	Reverse-TACTCGGTTGATGACAGTT		
vtg1	Forward-GCTTTGCCTATTCCCACATC	136	NM_001044897.2
	Reverse-GCTCTGCTGTAACGGTAGT		
hsd3b1	Forward-AGCCCATCTGCCCATCTTT	120	AY279108.1
	Reverse-CCATGGTCACTAGCTGCCTATT		

Table S2: Primer pair sequences, amplicon sizes, and accession numbers for genes investigated in this study (*β-actin*, beta-actin; *efla*, elongation factor 1a; *vtg1*, vitellogenin isoform 1; *cyp19alb*, brain aromatase; *star*, steroidogenic acute regulatory protein; *cyp11a1*, P450 side chain cleavage; *hsd3b1*, 3-beta-hydroxysteroid dehydrogenase). Note the primer design for VTG1 was custom ordered from Sigma-Aldrich.

EE2(ng L^{-1})			BPA ($\mu\text{g L}^{-1}$)			OP ($\mu\text{g L}^{-1}$)		
Nominal	Measured	% Nominal	Nominal	Measured	% Nominal	Nominal	Measured	% Nominal
5	1.7 ± 0.6	34	100	92 ± 11	92	10	3.9 ± 1.2	39
25	10.8 ± 0.1	43	500	410 ± 40	82	50	20.2 ± 8.0	40
50	28.3 ± 6.4	57	1000	808 ± 307	81	100	45.2 ± 8.7	45

Table S3: Nominal and measured concentrations of EE2, BPA and OP in exposure series I and II. Measured concentrations (mean \pm sd) were determined for exposure samples collected at two time points (days 1 and 4) along the five-day exposure period.

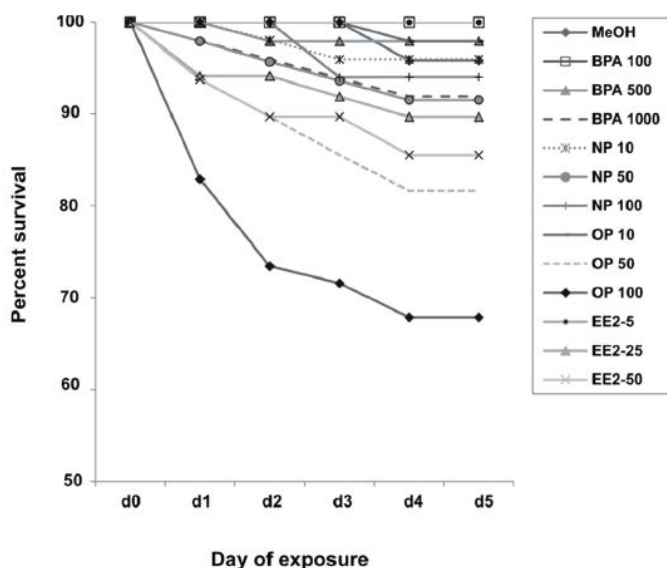


Figure S1: Survival plot of *20dpfZF* exposed to xenoestrogens in Exposure I. The data represent mean of three replicates per experimental and control groups each containing $n = 25$ fish. Note experimental groups exposed to OP (50 and $100 \mu\text{g L}^{-1}$) and EE2 (50 ng L^{-1}) showed mortality (%) at 18, 32 and 15 respectively.