Phthalates modulate steroid 5-reductase transcripts in the Western clawed frog embryo

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Abstract

Phthalates are used worldwide in the manufacturing of plastics, added to cosmetic products, personal care products, pharmaceuticals, medical devices, and paints; and are widely detected in soil, surface water, and organism tissues. Phthalate esters have been previously shown to interfere with the endocrine system in vertebrates. However, few studies have investigated the effects of phthalates on testosterone-converting enzymes that affect hormone levels and reproduction. In the present study, we exposed the Western clawed frog (*Silurana tropicalis*) to 0.1, 1, and 10 μM diethylhexyl phthalate (DEHP), dibutyl phthalate (DBP), and diethyl phthalate (DEP) during early amphibian embryonic development. Additional DBP exposures were conducted *ex vivo* using mature frog testes. Malformations and mRNA levels of genes associated to reproduction and oxidative stress were evaluated. 0.1 μM DEHP, DBP, and DEP induced an array of malformations, including incomplete gut coiling, edemas, and eye malformations. Moreover, all three phthalates increased the expression of androgen-related genes, such as steroid-5α-reductase 1, 2, 3, steroid-5β-reductase, and androgen receptor at concentrations ranging from 0.1 to 10 μM depending on the phthalate and gene. Data suggest that the phthalate esters tested are teratogens to the amphibian embryo and that these phthalates exhibit an androgenic activity in amphibians.

Keywords: Androgen disruption; DHEP; DBP; DEP; Srd5α1; Srd5α2; Srd5α3; Srd5β.

1. Introduction

Phthalates are used worldwide in the manufacturing of plastics (Daniels, 2009). Leaching due to the non-covalent bonding of phthalates to polymers leads to the introduction of phthalates into our ecosystems. Phthalates are also used as additives in various cosmetic products, medical devices, personal care products, pharmaceuticals, and paints (reviewed in Magdouli et al., 2013). Due to their wide use, phthalates are commonly detected in soil, surface water, and organism tissues (Bauer and Herrmann, 1997; Blair et al., 2009). Diethylhexyl phthalate (DEHP) is one of the most used plasticizers, and was detected in various environmental compartments (reviewed in Magdouli et al., 2013). For example, DEHP was detected at concentrations ranging between 0.01 and 25 μg/L in rivers in Japan (Suzuki et al., 2001; Yuwatini et al., 2006) and reported in the influent of a wastewater treatment plant in France at concentrations up to 44 μg/L (Dargnat et al., 2009). Dibutyl phthalate (DBP) and diethyl phthalate (DEP) are two other plasticizers that have been widely detected in waters. These phthalate esters were detected in the Tama River in Japan at concentrations ranging from 0.088 to 0.54 μg/L DBP and 0.004 to 0.31 μg/L DEP (Suzuki et al., 2001). The False Creek in Vancouver, BC, Canada also showed concentrations in seawater of ~ 0.1 μg/L DEP (Blair et al., 2009).

Phthalate esters have been shown to interfere with vertebrate development on different levels. The main mechanism of action behind phthalate induced transcriptional changes has been reported to be the peroxisome proliferation-activated receptors (PPARs, Gazouli et al., 2002). In addition, heat shock proteins have been shown to be modulated after phthalate exposure in different species and are attributed to early warning signs of cellular stress (reviewed in Gupta et al., 2010). Cellular oxidative stress is caused by the presence of reactive oxygen species, which can lead to DNA damage in cells and result in abnormalities. For example, proteins such as

75 glutathione transferase, glutathione peroxidase, and heat shock protein 70 have been previously
76 reported to be altered when exposed to the phthalate DEHP (reviewed in Mathieu-Denoncourt et
77 al., 2015a).

78 In addition, research has shown that phthalates can have androgenic and/or anti-androgenic
79 properties and can adversely affect development and reproduction of male vertebrates (Latini et
80 al., 2006; Kay et al., 2014). For example, feminization of gonads by exposure to DBP was found
81 in juvenile Murray rainbowfish (Bhatia et al., 2015). In addition, disrupted spermatogenesis was
82 observed in the African clawed frog after DBP exposure (Lee and Veeramachaneni, 2005). The
83 mechanism of action by which phthalates mediate their action is still not completely understood
84 (Mathieu-Denoncourt et al., 2015a). Previous research suggested that phthalates interfere with
85 hormone synthesis by modulating the expression of sex steroid-related genes (Wong and Gill,
86 2002; Lehmann et al., 2004; Thompson et al., 2004). For example, decreased mRNA and protein
87 levels of StAR have been observed in rat testis after DEHP exposure (Borch et al., 2006). StAR
88 is responsible to transport cholesterol to the inner mitochondria in order to synthesize steroids,
89 including androgens. Decreased expression levels of *star* have also been correlated with reduced
90 levels of the androgen testosterone (T) (Borch et al., 2006), suggesting that T metabolism could
91 be directly affected by phthalate exposure.

92 Testosterone is converted to the potent androgen 5α-dihydrotestosterone (5α-DHT) by
93 steroid-5α-reductase (Srd5α) and to 5β-dihydrotestosterone (5β-DHT) by steroid-5β-reductase
94 (Srd5β). Few mammalian studies have investigated if phthalates are capable of modulating Srd5α
95 and Srd5β. Exposure to mono-ethylhexyl phthalate (MEHP) decreased Srd5α protein levels in a
96 primary cell culture of immature rat Leydig cells (Svechnikov et al., 2008). Similarly, exposure
97 to DBP in rats significantly decreased Srd5α2 protein in the proximal penis (Kim et al., 2010). In

98 contrast, a concentration dependent increase of Srd5α activity was detected in testis after DEHP
99 exposure of pubertal rat (Kim et al., 2003). These studies show that phthalates can modulate
100 Srd5α. However, no studies have addressed the effects of phthalates on Srd5β expression.

101 Srd5 are involved in vital biological functions (reviewed in Langlois et al., 2010a) and their
102 dysregulation leads to a variety of diseases in humans, in particular in the male reproductive
103 system and liver (reviewed in Azzouni et al., 2012). Thus, there is a need to determine how
104 phthalates with androgenic and/or anti-androgenic properties affect Srd5α and Srd5β in lower
105 vertebrates, such as amphibians.

106 The overall objective of the present study was to understand the effect of the three phthalates
107 DEHP, DBP, and DEP in the Western clawed frog (*Silurana tropicalis*). Specifically, we
108 exposed the Western clawed frog to DEHP, DBP, and DEP during early embryonic development
109 and investigated malformations and mRNA levels of genes involved in oxidative stress and
110 reproduction. As the frog embryos responded to DBP, we further chemically-challenged mature
111 frog testes *ex vivo* in order to analyze if DBP could interfere with normal testis regulation in
112 males. Thus, this study presents novel insights in regards to interactions between phthalates and
113 *srd5* during two critical periods of *S. tropicalis.*

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2. Materials and Methods

2.1 Experimental design

Maintenance of male and female *S. tropicalis* occurred in dechlorinated and aerated water at the Queen's University Animal Care facilities (Kingston, ON, Canada) in accordance with guidelines of the Institution's animal care protocols and the Canadian Council on Animal Care. Animals were kept in a 12:12 h light:dark cycle with a water temperature of 26 ± 1 °C.

In vivo exposure was executed by exposing eggs of *S. tropicalis* to phthalates. Breeding procedure was performed as described in Langlois et al. (2010b). Briefly, eggs were collected and kept in Frog Embryo Teratogenesis Assay-Xenopus (FETAX) solution consisting of 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \cdot 2H_2O$, 30 mg KCl, 15 mg $CaCl_2$/L, and 0.04 ppm gentamycin sulphate. The fertilized eggs were dejellied using 2% (w/v) L-cysteine. 200 embryos (divided in 5 jars) were exposed to 0.1, 1, and 10 µM DEHP (Sigma, Oakville, ON, Canada), DBP (Sigma, Oakville, ON, Canada), or DEP (Sigma, Oakville, ON, Canada) once the eggs reached Nieuwkoop and Faber (NF) stage 11 (Nieuwkoop, 1994). NF 11 embryos were also exposed to two negative controls: a water only control and a solvent control (0.05% Ethanol) and to a positive control to test alteration of *srd5* mRNA levels; finasteride (100 µM; a known Srd5 inhibitor; Langlois et al., 2010c). The FETAX solution was kept at 26 °C and changed every 24 h. Daily water change also ensured a steady source of phthalate exposure, as phthalates are known to degrade over time. Dead embryos were discarded once a day and recorded. Embryos were sampled in pools (n = 10) at stage NF 46 (after an exposure time of 72 h) and flash frozen on dry ice until RNA isolation.

The *ex vivo* assay used has been previously optimized for frogs and described in Bissegger et al. (2014). Briefly, six male adult frogs were anesthetized in 0.1% MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma, Oakville, ON, Canada). Testes were carefully dissected from each animal. Each testis (n = 6 per treatment group, one testis per frog was used as a control sample and the other testis was exposed to DBP) was weighed (to correct for steroid production) and placed in a separate 1.5 mL centrifuge tube filled with 500 µL ice cold Lebovitz (L-15 media, Sigma, Oakville, ON, Canada) containing 10 mM HEPES, 50 µg/mL gentamicin (Fisher Scientific, Ottawa, ON, Canada) and 2% synthetic serum replacement (Sigma, Oakville, ON, Canada) at pH 7.4. Once all animals were dissected, the testes were transferred into designated wells in a 24-well plate containing 500 µL ice cold L-15 media. Prior to the start of the incubation, the media in each well of the 24-well plate was replaced by 500 µL of L-15 media containing 0.05% ethanol (control samples) or L-15 media containing 10 µM of DBP. The 24-well plates were incubated for 6 h at 26 °C using an orbital shaker at 100 rpm. After 6 h, the organs were snap-frozen on dry ice and stored at -80 °C for subsequent RNA isolation. The media of each sample was also collected and stored at -80 °C for steroid analysis.

2.2 Malformation analysis

After the embryonic exposure, a subset (n = 46 – 103) of randomly collected animals at NF 46 was fixed in 10% formalin for each treatment in order to conduct morphological analysis. Malformation analysis was performed based on the Atlas of Abnormalities (Bantle et al., 1998). A Nikon SMZ18 microscope (Nikon, Mississauga, ON, Canada) was used to observe malformations in eyes (reduction in size, asymmetric formation, incomplete separation from the brain and cyclops), tails (shortening and flexure), hearts (failure to coil in an 'S' shape), guts

(failure to coil), gills (shredded appearance), and head and face (reduction in size and unusual shape).

2.3 Analysis of phthalate concentration

Phthalate concentrations present in the water during the embryo exposure were measured using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) optimized for DEHP, DBP, and DEP. Chromatographic separation was performed using an Accela 600 LC system (Thermo Scientific, Waltham, MA, USA) with a Zorbax HDHR Eclipse plus C18 column combined with C18 Eclipse plus (12.5 X 2.1 mm ID., 1.8 μm) guard column (Agilent Technologies, Santa Clara, CA, USA) and using a gradient of two mobile phases. Initial mobile phase conditions consisted of 2 mM ammonium formate in 0.1% formic acid buffer and 0.1% formic acid in methanol ran at a ratio of 60:40, respectively. From 1 to 6 min, the gradient was changed gradually to 1:99 followed by a hold at 1:99 for 4.75 min. The initial buffer composition was then held for 4.25 min until the next sample was started. The flow rate was 0.3 mL/min. Ten μL of the sample or its dilution were injected using an autosampler kept at 4 °C. As an internal standard in each sample, dimethyl-d6 phthalate (CDN Isotopes, Pointe-Claire, QC, Canada) was used at a concentration of 1 mg/L. MS detection was performed using a high resolution Orbitrap detector with a pneumatic assisted heated electrospray ion (HESI) source set to 3500V. Capillary and vaporization temperatures were 250 °C and 350 °C, respectively. Helium was used as a collision gas at a pressure of 2.5 mTor. Data was analyzed using the Thermo Xcalibur software (Thermo Scientific, Waltham, MA, USA). The limits of detection were 0.95 μg/L for DEHP, 1.93 μg/L for DBP, and 1.12 μg/L for DEP.

182 **2.4 RNA isolation and cDNA synthesis**

183 Total RNA from embryo (NF 46) samples was isolated with the E.Z.N.A Total RNA kit II
184 (VWR, Mississauga, ON, Canada). Sample homogenization and disruption was carried out by a
185 Mixer Mill MM400 (Retsch, Newtown, PA, USA) at 20 MHz for 1 min. In contrast, total RNA
186 from testes was isolated using the Trizol reagent (Life Technologies Inc., Burlington, ON,
187 Canada) because of higher tissue weight. Sample homogenization and disruption was done using
188 a sonicator (Fisher Scientific, Toronto, ON, Canada). RNA was resuspended or eluted in 20 µL
189 nuclease free water for both embryo and testes samples and the concentration and quality was
190 assessed using the NanoDrop-2000 spectrophotometer (Fisher Scientific, Toronto, ON, Canada).
191 Residual genomic DNA was eliminated by the TURBO DNA-free kit (Life Technologies Inc.,
192 Burlington, ON, Canada). Total cDNA from embryo samples was obtained from 1 µg RNA and
193 0.5 µg random primers utilizing the GoScript reverse transcriptase (Fisher Scientific, Toronto,
194 ON, Canada). In contrast, cDNA from testes samples was obtained from 0.5 µg RNA using the
195 QuantiTect Reverse Transcription Kit (Qiagen, Toronto, ON, Canada).

196 **2.5 Real-time RT-PCR**

197 The expression levels of genes associated with reproduction (*srd5α1*, *srd5α2*, *srd5α3*, *srd5β,*
198 *star,* aromatase *(cyp19),* estrogen receptor *(erα),* androgen receptor *(ar)*); oxidative stress
199 (glutathione transferase *(gst),* glutathione peroxidase *(gpx),* heat shock protein 70 *(hsp70)*); and a
200 nuclear receptor protein regulating DNA transcription (peroxisome proliferator activated
201 receptor γ *(pparγ)*) were determined relative to the reference gene ornithine decarboxylase (*odc*)
202 by real-time RT-PCR using the SYBR Green detection system. All primers except *gst* and *gpx*
203 were previously designed and optimized (Langlois et al. 2010c; Soriano et al., 2013; Mathieu-

204 Denoncourt et al., 2015b). Primers for *gst* and *gpx* were designed and optimized in the present
205 study and gene products have been confirmed by sequencing. Assay conditions are presented in
206 Table 1. Each sample was diluted 1:80 (embryo samples) or 1:40 (testes samples) and analyzed
207 in duplicate using the GoTaq qPCR Master Mix (Fisher Scientific, Toronto, ON, Canada) with
208 the optimized concentration of forward and reverse primer (0.1 to 0.65 μM) on a CFX96
209 Touch™ real-time RT-PCR machine (Bio Rad, Mississauga, ON, Canada). The program used to
210 run all samples included an enzyme activation step at 95 °C for 2 min followed by 40 cycles with
211 95 °C for 15 sec and 58 or 62 °C (depending on target gene) for 1 min. After the amplification
212 phase, a dissociation curve was established in order to ensure the presence of a single amplicon.
213 Reaction efficiencies were 100 ± 10 % with an $R^2 > 0.990$ and calculated by the CFX Manager
214 Software (Bio Rad, Mississauga, ON, Canada).

215 In each assay, a standard curve (0.048 to 50 ng), a no template control, and a no reverse
216 transcription control (to ensure the absence of genomic DNA in the samples) were run with the
217 samples. The standard curve was generated by pooling equal amounts of the treated and control
218 samples and was then serially diluted 1:4 to obtain concentrations from 50 to 0.048 ng. The
219 standard curve was then used to interpolate and calculate the mRNA level of target and reference
220 gene in each sample. The mRNA level of each target gene was calculated relative to the
221 reference gene *odc*.

222 **2.6 Sex steroid measurement**

223 For the *ex vivo* exposure, concentrations of T and 5α-DHT excreted from the testes into
224 the media were measured using commercially available enzyme-linked immunosorbent assays
225 (T: Cedarlane, Burlington, ON, Canada; 5α-DHT: Diagnostics Biochem Canada, Dorchester,

ON, Canada). Media samples were thawed on ice and diluted in the immunoassay buffer. The immunoassay protocol was followed as described by the manufacturer. All samples were measured in duplicate. The absorbance of samples after the designated incubation time of 1 h for 5α-DHT and 2 h for T were measured using a TECAN Infinit M1000 PRO microplate absorbance reader (TECAN, Männedorf, Switzerland) at 415 nm for T and at 450 nm for 5α-DHT. The limit of detection according to the manufacturer was 6 pg/mL for T and 5α-DHT. Hormone concentrations were normalized to tissue weight.

2.7 Statistical analysis

All statistical analysis except malformation analysis was performed using the software GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Differences of phthalate concentrations between 0 and 24 h, and differences between phthalate treatment concentrations were analyzed using an unpaired t-test. Gene expression results of the embryo samples were analyzed after removing outliers using one-way analysis of variance (ANOVA) with a subsequent post-hoc test (Dunnett's) to determine significant differences between treatments. Gene expression results of the testes exposed to DBP *ex vivo* were analyzed using an unpaired t-test. The effect of DBP exposure on hormone concentration in *ex vivo* exposed testis was also analyzed using an unpaired t-test. Analysis of malformation data was performed using the software XLSTAT (2014.4.06, Addinsoft^{TM}). Differences in malformation frequencies were determined using chi-square tests on contingency tables followed by a post-hoc test based on adjusted residuals for each malformation type and phthalate compound. Significant differences were reported when $p < 0.05$.

3. Results

3.1 Water analysis

LC-HRMS analysis showed that all water and solvent control samples were below the detection limit for DEHP, DBP, and DEP. Average measured concentrations (before exposure) were as follows: 138.6 µg/L, 618.9 µg/L, and 2932.3 µg/L for DEHP; <LOD, 60.6 µg/L, and 2064.6 µg/L for DBP; and 11.3 µg/L, 85.4 µg/L, and 1278.0 µg/L for DEP (Table 2). DBP significantly degraded over the 24 h exposure time (85.4 µg/L: $p = 0.042$; 1278.0 µg/L: $p = 0.017$). The measured concentrations are lower than the nominal concentrations, which may be due to losses associated with adsorption to glassware and material used during sampling and sample preparation.

3.2 Mortality and malformation of embryos

Survival rates of both, the water and solvent controls were above 90%, as recommended by the American Society for Testing and Materials (2004; Table 3). Embryos exposed to DEHP, DBP, and DEP did not result in a significantly higher mortality rate than control embryos. However, phthalate exposure increased malformation rates. Exposure to DEHP, DBP, and DEP increased both, incomplete gut coiling (21.2%, 25.5%, and 31%; respectively) and eye malformations (17.3%, 19.1%, and 51.7%; respectively). Moreover, DEHP and DEP increased tail abnormalities (21.2% and 44.8%; respectively), and DEP increased the occurrence of edemas (13.8%).

3.3 Gene expression of larvae exposed to phthalates

Exposure to phthalate esters modulated androgen-related mRNA levels. Exposure to 685 µg/L DEHP increased the expression of *srd5β* and *ar* (ANOVA: *srd5β*: 1.4-fold increase, p = 0.0057; *ar*: 1.4-fold increase, p = 0.0228, Figures 1 and 2). Frogs exposed to DEHP at a concentration 10X higher (i.e., 2,932 µg/L) responded with increased mRNA levels of all four *srd5* genes (ANOVA: *srd5α1*: 1.4-fold increase, p = 0.0001; *srd5α2*: 1.7-fold increase, p = 0.0003; *srd5α3*: 1.5-fold increase, p = 0.0010; *srd5β*: 1.8-fold increase, p = 0.0001). Interestingly, DBP resulted in significant changes at the lowest (below the detection limit of 2 µg/L) and highest (2,065 µg/L) concentrations only. Low DBP stimulated *srd5β* transcription *(*ANOVA: 1.4-fold increase, p = 0.0302) and high DBP increased the mRNA level of *srd5α1, srd5α2, srd5α3, srd5β,* and *ar* (ANOVA: *srd5α1*: 1.3-fold increase, p = 0.0046; *srd5α2*: 1.6-fold increase, p = 0.0029; *srd5α3*: 1.4-fold increase, p = 0.0178; *srd5β*: 1.6-fold increase, p = 0.0001; *ar*: 1.4-old increase, p = 0.0287). Exposure to DEP resulted in a change of expression of *srd5α1* (ANOVA: 1278 µg/L: 1.3-fold increase, p = 0.0094) and *srd5β* (ANOVA: 85 µg/L: 1.5-fold increase, p = 0.0042). The three phthalates did not affect the transcript levels for *cyp19*, *erα, star, gst, gpx,* or *pparγ*. As expected, finasteride exposure significantly decreased the mRNA level of *srd5α2* (ANOVA: 2-fold decrease, p = 0.01). In addition to androgen related gene expression changes, *hsp70* resulted in significantly changed mRNA levels for <2 µg/L DBP (ANOVA: 1.5-fold decrease, p = 0.01) and 11 µg/L DEP (ANOVA: 1.6-fold decrease, p = 0.014, Figure 3).

3.4 Frog testes exposed to DBP *ex vivo*

The total amount of T and 5α-DHT was measured in the media of control and DBP-exposed testes tissues. Interestingly, neither the secreted concentration of T or 5α-DHT into the media was significantly altered after DBP exposure ($p > 0.05$; Figure 4). In addition, the mRNA levels of *srd5α1, srd5α2, srd5α3,* and *srd5β* were also not significantly modified after DBP exposure (Figure 5).

4. Discussion

Both, phthalate esters and their metabolites have been linked to endocrine disruption in wildlife (Mathieu-Denoncourt et al., 2015b). Amphibians, in particular, are at a high risk due to their external egg development and permeable skin, which allows chemical penetration. One of the phthalates, DBP, used in the present study showed significant degradation after 24 h, suggesting that embryos have been also exposed to its major metabolite mono-n-butyl phthalate (MBP; Silva et al., 2007). The contribution of DBP and its metabolites are thus confounded when investigating the interference with the amphibian development and reproductive system in the current study. As we were interested in sub-lethal effects, such as endocrine disruption, we first established that the phthalate concentrations chosen did not affect the survival rate. Similarly, previous studies have shown that exposure up to 1,000 µg/L DBP did not result in decreased mortality of fathead minnow embryos (Mankidy et al., 2013), while exposure to 5,000, 10,000, and 15,000 µg/L DBP significantly decreased the survivability of *Xenopus laevis* larvae (Lee et al., 2005). Likewise, higher DEP concentrations as the ones used in the present study (e.g., 10,000 µg/L and above), decreased survival rate of fathead minnow and zebrafish embryos

310 (Kim et al., 2015; Mankidy et al., 2013). Similar to the findings in our study, 10 µg/L DEHP did
311 not result in significantly decreased mortality in Japanese medaka (Chikae et al., 2004).

312 In the present study, exposure to the lowest concentrations of DEHP, DBP, and DEP
313 augmented the occurrence of malformations in *S. tropicalis*, in particular the presence of
314 incomplete gut coiling, tail abnormalities, and eye malformations. Previous studies have shown
315 that endocrine disrupting chemicals don't always follow a normal dose response curve where the
316 observable effects increase with increasing dose (reviewed in Vandenberg et al., 2012). Thus, the
317 present finding of a higher effect at low doses is not unexpected for phthalates. Other studies
318 have also observed that phthalate exposures to similar concentrations as used in this study
319 resulted in malformed animals. For example, exposure to DBP and DEP at concentrations
320 exceeding 500 µg/L significantly increased malformations, including abnormal gut coiling,
321 cardiac abnormalities, and malformed faces, eyes, and brains in *X. laevis* (Bantle et al., 1999;
322 Lee et al., 2005; Gardner et al., 2016). Furthermore, *S. tropicalis* exposed to dimethyl phthalate
323 (DMP) and dicyclohexyl phthalate (DCHP) also resulted in malformations, such as edemas,
324 improperly developed hearts, tail abnormalities, improperly coiled guts, and/or absent gills
325 (Mathieu-Denoncourt et al., 2016).

326 Malformations of the tail and gut are often attributed to cellular stress. Heat shock proteins
327 are known as early warning signs of cellular stress and have been previously shown to be
328 induced after phthalate exposure in different species (reviewed in Gupta et al., 2010). In the
329 present study, the lowest DBP and DEP concentrations significantly decreased mRNA levels of
330 *hsp70*. In contrary, 1000 µg/L butyl benzyl phthalate (BBP) and DEHP induced *hsp70* mRNA
331 levels in *Chironomus riparius* larvae (Planelló et al., 2011) and exposure of *S. tropicalis* larvae
332 to DCHP also increased *hsp70* mRNA levels (Mathieu-Denoncourt et al., 2016). Cellular

oxidative stress is caused by the presence of reactive oxygen species, which can lead to DNA damage in cells. Cells can metabolize reactive oxygen species by producing antioxidant enzymes, including glutathione transferase and glutathione peroxidase that transform reactive oxygen species to less reactive compounds such as oxygen. In the present study, the expression of *gst* and *gpx* did not change suggesting that the developmental abnormalities are not likely the result of oxidative stress. However, in the current study, protein activity was not measured and it is known that mRNA levels and protein activities do not always correlate (Koussounadis et al., 2015). Therefore, it is possible that proteins involved in oxidative stress changed in activity level but no change in mRNA level was detected after phthalate exposure.

In addition to inducing malformations, phthalates are known to alter the endocrine system in mammalian species. Previous studies have suggested that phthalates can interfere with steroidogenesis and affect both the female and male reproductive systems. Multiple regulating pathways involved in the maintenance of steroid homeostasis have been shown to be affected by phthalates (reviewed in Mathieu-Denoncourt et al., 2015a). In order to examine the effects of the studied phthalates in amphibians, a series of endocrine related targets were analyzed. First, the expression of a gene involved in cholesterol transport (e.g., *star*) was analyzed as previous reports that demonstrated that phthalates interfere with this critical step in steroid synthesis (reviewed in Mathieu-Denoncourt et al., 2015a). In the present study, none of the phthalates of interest modulated *star* transcription, reinforcing the point that each phthalate presents unique molecular mechanisms of action. In addition, previous literature has also demonstrated that the synthesis and signaling of the female sex steroids were affected by phthalate treatments. For example, mRNA levels of *cyp19*, the enzyme responsible to aromatize T to estradiol, decreased in rodent cell lines when treated with MEHP and DEHP (Lovekamp and Davis, 2001; Gupta et

356 al., 2010). In contrast, in amphibians, exposure to DCHP during embryogenesis in the Western
357 Clawed frog increased *cyp19* mRNA level; however, DMP and its metabolite, MMP did not alter
358 *cyp19* expression in the same species (Mathieu-Denoncourt et al., 2016). The later study is
359 similar to the data found in this study as none of DEHP, DBP, or DEP modulated the expression
360 of estrogen-related genes, such as *cyp19,* and *erα*.

361 Androgen synthesis is also known to be altered by phthalate exposure in mammalian species.
362 Generally, it is recognized that phthalates do not exert their action through *ar* (reviewed in
363 Rouiller-Fabre et al., 2015). As an example, juvenile and adult liver tissues exposed to MMP did
364 not alter transcript levels of *ar* (Mathieu-Denoncourt et al., 2015a). Similarly, *ar* expression was
365 not changed following exposure to 10,000 μg/L DEP and 1,000 μg/L DBP in fathead minnow
366 embryos (Mankidy et al., 2013). In contrast, our data revealed that exposure to 10 μM DEHP and
367 DBP increased *ar* transcription. Agonists of AR have been shown to induce transcriptional
368 changes (Li et al., 2017), suggesting that DEHP and DBP can act as agonists of AR. In addition
369 to the measured increase in *ar* mRNA level, *srd5* expression was also augmented in the frog
370 larvae. All three phthalates increased *srd5α1* transcripts. In addition, *srd5α2* and *srd5α3*
371 expression levels were also increased by DEHP and DBP exposures. Similarly in mammalian
372 species, DEHP amplified the activity of SRD5α in the pubertal rat testes (Kim et al., 2003). An
373 increase of *srd5α* could lead to a higher than normal conversion of T to 5α-DHT. However, other
374 studies demonstrated that phthalate exposure decreases SRD5α activity. For example, a decrease
375 in Srd5α2 activity was demonstrated after DBP exposure *in vitro* in gonad microsomal
376 homogenates isolated from the common carp (Thibaut and Porte, 2004). Moreover, prenatal
377 exposure to DBP in rats significantly decreased Srd5α2 protein expression in the proximal penis
378 (Kim et al., 2010). In contrast, MMP, DMP, and DCHP did not alter *srd5α2* mRNA level in *S.*

379 *tropicalis* (Mathieu-Denoncourt et al., 2016). These results suggest that the chemical nature of
380 the phthalates as well as the developmental stage or tissue are important as to how the specific
381 compound interferes with the level of certain genes/proteins.

382 Interestingly, not only *srd5α* isoforms, but also the *srd5β* transcript level increased after
383 DEHP, DBP, and DEP treatments. Srd5β is known to be involved in clearing excess steroids in
384 bird brains (Steimer and Hutchison, 1981). The observation that *srd5β* increased after phthalate
385 treatment may suggest a disturbance of the normal balance of sex steroids. However, no previous
386 studies have assessed the effects of phthalates on *srd5β* expression and limited studies have
387 examined this gene. Treatments with methyltrienolone and atrazine have been shown to alter
388 *srd5β* levels in human prostate cells and frog liver, respectively (Bolton et al., 2007; Langlois et
389 al., 2010a). Our results provide evidence that phthalates can interfere with *srd5β* and perhaps
390 result in adverse reproductive effects. Since *srd5β* is involved in many other biological functions,
391 including bile acid synthesis and erythropoiesis, other adverse effects may be observed. To test
392 this hypothesis, exposure of phthalates to animals throughout development and sexual
393 differentiation would be required. Taken together, these findings suggest that phthalates exert
394 their action through different mechanisms depending on species and tissues and affect androgen
395 synthesis in various ways.

396 As androgen-related genes were altered by DBP during frog early development, further
397 investigations were pursued in DBP-exposed testes of adult males due to a high androgen
398 synthesis in gonads. However, DBP *ex vivo* exposure did not alter the T or 5α-DHT steroid
399 levels nor did it alter *srd5α* expression in frog testes. Previous studies had demonstrated that
400 phthalates, including DEHP, DBP, DEP, MEP, monobutyl phthalate, dipentyl phthalate (DPeP),
401 monopentyl phthalate (MPeP), benzylbutyl phthalate (BzBP), mono-n-octyl phthalate (MnOP),

and MEHP decreased T levels in mammalian species (reviewed in Mathieu-Denoncourt et al., 2015a). As an example, DBP exposure of prenatal male rats resulted in significant decreased of T levels, which was accompanied by a decreased expression of *Srd5α2* in testicular tissues (Jiang et al., 2016). Similarly, primary cultures of rat Leydig cells exposed to MEHP decreased Srd5α activity in immature, but not in adult Leydig cells (Svechnikov et al., 2008). The lack of any observed effect in our study may be due to the fact that the *ex vivo* exposure was stopped after 6h, which may have been too short of an exposure time to see any changes at the transcriptional level. Nevertheless, our embryonic data suggest that phthalates can induce transcriptional changes of genes associated with reproduction.

This study demonstrated that DEHP, DEP, and DBP interfere with normal frog development by inducing an array of malformations to the developing animals. Exposure to these three phthalates also increased the expression of androgen-related genes, in particular the four *srd5* during amphibian embryogenesis, which suggests that DEHP, DEP, and DBP have an androgenic activity in the amphibian embryo. Thus, this data shows evidence that certain phthalates act via the srd5 signaling pathway. Furthermore, this finding also supports previous studies suggesting that phthalate esters induce adverse effects in vertebrates by altering important biological functions, including a hormonal imbalance.

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References

American Society for Testing and Materials, 2004. Standard guide for conducting the frog embryo teratogenesis assay—Xenopus (FETAX).

Bantle, J.A., Dumont, J., Finch, R.A., Linder, G., Fort, D.J., 1998. Atlas of abnormalities: a guide for the performance of FETAX, second edition. ed. Printing services, Oklahoma State University.

Bantle, J.A., Finch, R.A., Fort, D.J., Stover, E.L., Hull, M., Kumsher-King, M., Gaudet-Hull, A.M., 1999. Phase III interlaboratory study of FETAX part 3. FETAX validation using 12 compounds with and without an exogenous metabolic activation system. J. Appl. Toxicol. 19, 447–472.

Bauer, M.J., Herrmann, R., 1997. Estimation of the environmental contamination by phthalic acid esters leaching from household wastes. Sci. Total Environ. 208, 49–57.

Bhatia, H., Kumar, A., Chapman, J.C., McLaughlin, M.J., 2015. Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (Melanotaenia fluviatilis). J. Appl. Toxicol. 35, 806–816.

Bissegger, S., Martyniuk, C.J., Langlois, V.S., 2014. Transcriptomic profiling in Silurana tropicalis testes exposed to finasteride. Gen. Comp. Endocrinol. 203, 137–145. doi:10.1016/j.ygcen.2014.01.018

Blair, J.D., Ikonomou, M.G., Kelly, B.C., Surridge, B., Gobas, F.A.P.C., 2009. Ultra-Trace Determination of Phthalate Ester Metabolites in Seawater, Sediments, and Biota from an Urbanized Marine Inlet by LC/ESI-MS/MS. Environ. Sci. Technol. 43, 6262–6268.

Bolton, E.C., So, A.Y., Chaivorapol, C., Haqq, C.M., Li, H., Yamamoto, K.R., 2007. Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev. 21, 2005–2017.

Borch, J., Metzdorff, S.B., Vinggaard, A.M., Brokken, L., Dalgaard, M., 2006a. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223, 144–155.

Borch, J., Metzdorff, S.B., Vinggaard, A.M., Brokken, L., Dalgaard, M., 2006b. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223, 144–155.

Chikae, M., Hatano, Y., Ikeda, R., Morita, Y., Hasan, Q., Tamiya, E., 2004. Effects of bis(2-ethylhexyl) phthalate and benzo[a]pyrene on the embryos of Japanese medaka (Oryzias latipes). Environ. Toxicol. Pharmacol. 16, 141–145.

Daniels, P.H., 2009. A brief overview of theories of PVC plasticization and methods used to evaluate PVC-plasticizer interaction. J. Vinyl Addit. Technol. 15, 219–223.

Dargnat, C., Teil, M.-J., Chevreuil, M., Blanchard, M., 2009. Phthalate removal throughout wastewater treatment plant. Sci. Total Environ. 407, 1235–1244.

Gardner, S.T., Wood, A.T., Lester, R., Onkst, P.E., Burnham, N., Perygin, D.H., Rayburn, J., 2016. Assessing differences in toxicity and teratogenicity of three phthalates, Diethyl phthalate, Di-n-propyl phthalate, and Di-n-butyl phthalate, using Xenopus laevis embryos. J. Toxicol. Environ. Health A 79, 71–82.

Gazouli, M., Yao, Z.X., Boujrad, N., Corton, J.C., Culty, M., Papadopoulos, V., 2002. Effect of

peroxisome proliferators on leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport, and steroidogenesis: role of the peroxisome proliferator-activator receptor a. Endocrinol. 143, 2571–2583.

Gupta, R.K., Singh, J.M., Leslie, T.C., Meachum, S., Flaws, J.A., Yao, H.H.-C., 2010. Di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate inhibit growth and reduce estradiol levels of antral follicles in vitro. Toxicol. Appl. Pharmacol. 242, 224–230.

Gupta, S.C., Sharma, A., Mishra, M., Mishra, R.K., Chowdhuri, D.K., 2010. Heat shock proteins in toxicology: How close and how far? Life Sci. 86, 377–384.

Jiang, J.-T., Zhong, C., Zhu, Y.-P., Xu, D.-L., Wood, K., Sun, W., Li, E.-H., Liu, Z.-H., Zhao, W., Ruan, Y., Xia, S.-J., n.d. Prenatal exposure to di-n-butyl phthalate (DBP) differentially alters androgen cascade in undeformed versus hypospadiac male rat offspring. Reprod. Toxicol.

Kay, V.R., Bloom, M.S., Foster, W.G., 2014. Reproductive and developmental effects of phthalate diesters in males. Crit. Rev. Toxicol. 44, 467–498.

Kay, V.R., Chambers, C., Foster, W.G., 2013. Reproductive and developmental effects of phthalate diesters in females. Crit. Rev. Toxicol. 43, 200–219.

Kim, H.-S., Saito, K., Ishizuka, M., Kazusaka, A., Fujita, S., 2003. Short period exposure to di-(2-ethylhexyl) phthalate regulates testosterone metabolism in testis of prepubertal rats. Arch. Toxicol. 77, 446–451.

Kim, S.-M., Yoo, J.-A., Baek, J.-M., Cho, K.-H., 2015. Diethyl phthalate exposure is associated with embryonic toxicity, fatty liver changes, and hypolipidemia via impairment of lipoprotein functions. Toxicol. In Vitro 30, 383–393.

Kim, T.S., Jung, K.K., Kim, S.S., Kang, I.H., Baek, J.H., Nam, H.-S., Hong, S.-K., Lee, B.M., Hong, J.T., Oh, K.W., Kim, H.S., Han, S.Y., Kang, T.S., 2010. Effects of in utero exposure to DI(n-Butyl) phthalate on development of male reproductive tracts in Sprague-Dawley rats. J. Toxicol. Environ. Health A 73, 1544–1559.

Koussounadis, A., Langdon, S.P., Um, I.H., Harrison, D.J., Smith, V.A., 2015. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system, Sci Rep. 5, 10775.

Langlois, V.S., Zhang, D., Cooke, G.M., Trudeau, V.L., 2010a. Evolution of steroid-5α-reductases and comparison of their function with 5β-reductase. Gen. Comp. Endocrinol. 166, 489–497.

Langlois, V.S., Carew, A.C., Pauli, B.D., Wade, M.G., Cooke, G.M., Trudeau, V.L., 2010b. Low Levels of the Herbicide Atrazine Alter Sex Ratios and Reduce Metamorphic Success in Rana pipiens Tadpoles Raised in Outdoor Mesocosms. Environ. Health Perspect. 118, 552–557.

Langlois, V.S., Duarte-Guterman, P., Ing, S., Pauli, B.D., Cooke, G.M., Trudeau, V.L., 2010c. Fadrozole and finasteride exposures modulate sex steroid- and thyroid hormone-related gene expression in Silurana (Xenopus) tropicalis early larval development. Gen. Comp. Endocrinol. 166, 417–427.

Latini, G., Del Vecchio, A., Massaro, M., Verrotti, A., De Felice, C., 2006. Phthalate exposure and male infertility. Toxicology 226, 90–98.

Lee, S.K., Owens, G.A., Veeramachaneni, D.N.R., 2005. Exposure to Low Concentrations of Di-n-butyl Phthalate During Embryogenesis Reduces Survivability and Impairs Development of Xenopus Laevis Frogs. J. Toxicol. Environ. Health A 68, 763–772.

Lee, S.K., Veeramachaneni, D.N.R., 2005. Subchronic Exposure to Low Concentrations of Di-n-Butyl Phthalate Disrupts Spermatogenesis in Xenopus laevis Frogs. Toxicol. Sci. 84, 394–407.

Lehmann, K.P., Phillips, S., Sar, M., Foster, P.M.D., Gaido, K.W., 2004. Dose-Dependent Alterations in Gene Expression and Testosterone Synthesis in the Fetal Testes of Male Rats Exposed to Di (n-butyl) phthalate. Toxicol. Sci. 81, 60–68.

Li, J., Chang, J., Li, W., Guo, B., Li, J., Wang, H., 2017. Disruption of sex-hormone levels and steroidogenic-related gene expression on Mongolia Racerunner (Eremias argus) after exposure to triadimefon and its enantiomers. Chemosphere 171, 554-563.

Lovekamp, T.N., Davis, B.J., 2001. Mono-(2-ethylhexyl) Phthalate Suppresses Aromatase Transcript Levels and Estradiol Production in Cultured Rat Granulosa Cells. Toxicol. Appl. Pharmacol. 172, 217–224.

Magdouli, S., Daghrir, R., Brar, S.K., Drogui, P., Tyagi, R.D., 2013. Di 2-ethylhexylphtalate in the aquatic and terrestrial environment: A critical review. J. Environ. Manage. 127, 36–49.

Mankidy, R., Wiseman, S., Ma, H., Giesy, J.P., 2013. Biological impact of phthalates. Toxicol. Lett. 217, 50–58.

Mathieu-Denoncourt, J., Wallace, S.J., de Solla, S.R., Langlois, V.S., 2015a. Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. Gen. Comp. Endocrinol. 219, 74–88.

Mathieu-Denoncourt, J., de Solla, S.R., Langlois, V.S., 2015b. Chronic exposures to monomethyl phthalate in Western clawed frogs. Gen. Comp. Endocrinol., Disruption of the thyroid and sex steroid hormone systems and their crosstalk in aquatic wildlife 219, 53–63.

Mathieu-Denoncourt, J., Martyniuk, C.J., Loughery, J.R., Yargeau, V., de Solla, S.R., Langlois, V.S., 2016. Lethal and sublethal effects of phthalate diesters in Silurana tropicalis larvae. Environ. Toxicol. Chem. 35, 2511-2522.

Nieuwkoop, P.D., 1994. Normal Table of Xenopus Laevis (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg Till the End of Metamorphosis. Garland Pub.

Planelló, R., Herrero, O., Martínez-Guitarte, J.L., Morcillo, G., 2011. Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of Chironomus riparius based on gene expression assays related to the endocrine system, the stress response and ribosomes. Aquat. Toxicol. 105, 62–70.

Rouiller-Fabre, V., Guerquin, M.J., N'Tumba-Byn, T., Muczynski, V., Moison, D., Tourpin, S., Messiaen, S., Habert, R., Livera, G., 2015. Nuclear Receptors and Endocrine Disruptors in Fetal and Neonatal Testes: A Gapped Landscape. Front. Endocrinol. 6.

Silva, M.J., Samandar, E., Reidy, J.A., Hauser, R., Needham, L.L., Calafat, A.M., 2007, Metabolite profiles of di-n-butyl phthalate in humans and rats. Environ. Sci. Technol. 41, 7576-7580.

552 Soriano, J.J., Mathieu-Denoncourt, J., Norman, G., Solla, S.R. de, Langlois, V.S., 2013. Toxicity
553 of the azo dyes Acid Red 97 and Bismarck Brown Y to Western clawed frog (Silurana
554 tropicalis). Environ. Sci. Pollut. Res. 21, 3582–3591.
555 Steimer, T., Hutchison, J.B., 1981. Metabolic control of the behavioural action of androgens in
556 the dove brain: Testosterone inactivation by 5β-reduction. Brain Res. 209, 189–204.
557 Suzuki, T., Yaguchi, K., Suzuki, S., Suga, T., 2001. Monitoring of Phthalic Acid Monoesters in
558 River Water by Solid-Phase Extraction and GC-MS Determination. Environ. Sci.
559 Technol. 35, 3757–3763.
560 Svechnikov, K., Svechnikova, I., Söder, O., 2008. Inhibitory effects of mono-ethylhexyl
561 phthalate on steroidogenesis in immature and adult rat Leydig cells in vitro. Reprod.
562 Toxicol. 25, 485–490.
563 Thibaut, R., Porte, C., 2004. Effects of endocrine disrupters on sex steroid synthesis and
564 metabolism pathways in fish. J. Steroid Biochem. Mol. Biol. 92, 485–494.
565 Thompson, C.J., Ross, S.M., Gaido, K.W., 2004. Di(n-Butyl) Phthalate Impairs Cholesterol
566 Transport and Steroidogenesis in the Fetal Rat Testis through a Rapid and Reversible
567 Mechanism. Endocrinology 145, 1227–1237.
568 Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.-H., Shioda,
569 T., Soto, A.M, vom Saal, F.S., Welshons, W.V., Zoeller, R.S., Myers J.P., 2012.
570 Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic
571 Dose Responses. Endocrine Reviews, 33, 378–455.
572 Wong, J.S., Gill, S.S., 2002. Gene Expression Changes Induced in Mouse Liver by Di(2-
573 ethylhexyl) Phthalate. Toxicol. Appl. Pharmacol. 185, 180–196.
574 Yuwatini, E., Hata, N., Taguchi, S., 2006. Behavior of di(2-ethylhexyl) phthalate discharged
575 from domestic waste water into aquatic environment. J. Environ. Monit. 8, 191–196.
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579 **Table 1** qPCR primers and assay conditions of *gpx* and *gst* genes for *S. tropicalis*.

Target gene	Primer direction	Sequence (5' - 3')	Annealing temperature (°C)	Primer (nM)
gst	Forward	ATTGCGTGGGAGATGAGGTG	60	350
	Reverse	ATTGTGGGATAGGGGGCAAG		
gpx	Forward	CGAACCCAACTTCCCCTTGT	60	350
	Reverse	TAGGATACGGAAGTTGCCCC		

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583 **Table 2** Di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), and diethyl phthalate (DEP) measured in
584 the water of exposed embryos at 0 h and after 24 h. Average concentrations are bold. Legend: Ctr, Control; LOD,
585 limit of detection; Stdev, standard deviation.

Nominal Concentration (µg/L)		**Measured Concentration (µg/L)**	**Stdev (µg/L)**
Water Ctr	0 h	<LOD	0.0
	24 h	<LOD	0.0
Solvent Ctr	0 h	<LOD	0.0
	24 h	<LOD	0.0
39 DEHP	0 h	247.1	285.1
	24 h	30.0	14.0
	Average	**138.6**	**216.2**
390 DEHP	0 h	552.9	453.6
	24 h	684.8	183.3
	Average	**618.9*[a]**	**317.8**
3900 DEHP	0 h	4304.8	3864.5
	24 h	1559.9	1082.3
	Average	**2932.3*[a]**	**2950.0**
28 DBP	0 h	<LOD	0.0
	24 h	<LOD	0.0
	Average	**<LOD**	**0.0**
280 DBP	0 h	121.2	44.5
	24 h	<LOD*[b]	0.0
	Average	**60.6*[a]**	**72.2**
2800 DBP	0 h	3033.4	533.1
	24 h	1095.8*[b]	173.9
	Average	**2064.6*[a]**	**1119.0**
22 DEP	0 h	13.0	2.3
	24 h	9.7	0.2
	Average	**11.3**	**2.3**
220 DEP	0 h	86.4	6.6
	24 h	84.4	1.3
	Average	**85.4*[a]**	**4.3**
2200 DEP	0 h	1291.3	125.4
	24 h	1264.6	129.6
	Average	**1278.0*[a]**	**114.9**

586 *[a] indicates a significant concentration difference to the previous lower concentration
587 *[b] indicates significant degradation between 0 h and 24 h of exposure
588

589 **Table 3** Effects of di(2-ethylhexyl) phthalate (DEHP), di-n-butyl phthalate (DBP), and diethyl phthalate (DEP)
590 spiked water on mortality and malformation of *S. tropicalis* larvae at exposure completion (Nieuwkoop and Faber
591 stage 46). The mortality data are expressed as percent mean ± SD (%) and the malformation results are expressed as
592 a percentage (%) of malformed animals to the total number of animals analyzed. Asterisks indicate statistically
593 significant differences between treatments (DEHP, DBP, and DEP) and solvent (0.05% ethanol) control.

Treatments (µg/L)	Mortality (%)	Average malformation (%)	Malformations observed (%)				
			Tail	Edema	Eye	Heart	Gut
Water Ctrl	4.5 ± 2.1	9.5 ± 5.1	12.8	2.3	12.8	0.0	11.6
Solvent Ctrl	5.0 ± 4.0	7.0 ± 2.2	8.7	6.8	4.9	0.0	9.7
39 DEHP	4.4 ± 3.1	16.5 ± 6.4*	21.2*	7.7	17.3	0.0	21.2*
390 DEHP	3.8 ± 3.2	8.2 ± 4.5	14.3*	4.1	6.1	0.0	10.2
3,900 DEHP	5.6 ± 2.4	12.3 ± 9.6	21.3*	2.1	21.3*	0.0	8.5
28 DBP	3.8 ±3.2	14.5 ± 4.0*	14.9	2.1	19.1*	0.0	25.5*
280 DBP	8.8 ± 6.0	5.2 ± 4.4	10.0	0.0	8.0	0.0	8.0
2,800 DBP	5 ± 2.0	6.1 ± 3.2	4.3	0.0	8.7	0.0	15.2
22 DEP	8.1 ± 4.3	33.8 ± 8.1*	44.8*	13.8	51.7*	0.0	31.0*
220 DEP	4.4 ±2.4	18.6 ± 5.5*	30.5*	8.5	28.8*	1.7	10.2
2,200 DEP	8.1 ±3.8	12.3 ± 8.2	21.2*	1.9	13.5*	0.0	7.7

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595

596

Figure Captions

Figure 1 mRNA levels of A) *srd5α1*, B) *srd5α2*, C) *srd5α3*, and D) *srd5β* in frog embryos following exposure to FIN, DEHP, DBP, and DEP. Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4-8; $p < 0.05$). Legend: DBP, dibutyl phthalate; di-2-ethylhexyl phthalate DEHP, diethyl hexyl phthalate; DEP, diethyl phthalate; FIN, finasteride; SC, Solvent Control; WC, Water Control; *, significant different from control at $p < 0.05$.

Figure 2 mRNA levels of A) *ar*, B) *erα*, C) *cyp19*, and D) *star* in frog embryos following exposure to FIN, DEHP, DBP, and DEP. Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4-8; $p < 0.05$). Legend: DBP, dibutyl phthalate; di-2-ethylhexyl phthalate DEHP, diethyl hexyl phthalate; DEP, diethyl phthalate; FIN, finasteride; SC, Solvent Control; WC, Water Control; *, significant different from control at $p < 0.05$.

Figure 3 mRNA levels of A) *hsp70*, B) *gst*, C) *gpx*, and D) *pparγ* in frog embryos following exposure to FIN, DEHP, DBP, and DEP. Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using one-way analysis of variance (n = 4-8; $p < 0.05$). Legend: DBP, dibutyl phthalate; di-2-ethylhexyl phthalate DEHP, diethyl hexyl phthalate; DEP, diethyl phthalate; FIN, finasteride; SC, Solvent Control; WC, Water Control; *, significant different from control at $p < 0.05$.

Figure 4 Concentration of testosterone and 5α-dihydrotestosterone in the media after *ex vivo* exposure of testes to dibutyl phthalate (DBP). Bars represent the mean + STD. Data were analyzed using a two tailed t-test (n = 6). Legend: DBP, dibutyl phthalate; SC, Solvent Control.

Figure 5 mRNA levels of A) *srd5α1*, B) *srd5α2*, C) *srd5α3*, and D) *srd5β* in frog testes after *ex vivo* exposure to 10 μM dibutyl phthalate (DBP). Data are expressed relative to the reference gene *odc*. Bars represent the mean + SEM. Data were analyzed using a two tailed t-test (n = 4). Legend: DBP, dibutyl phthalate; SC, Solvent Control.

624

Fig 1

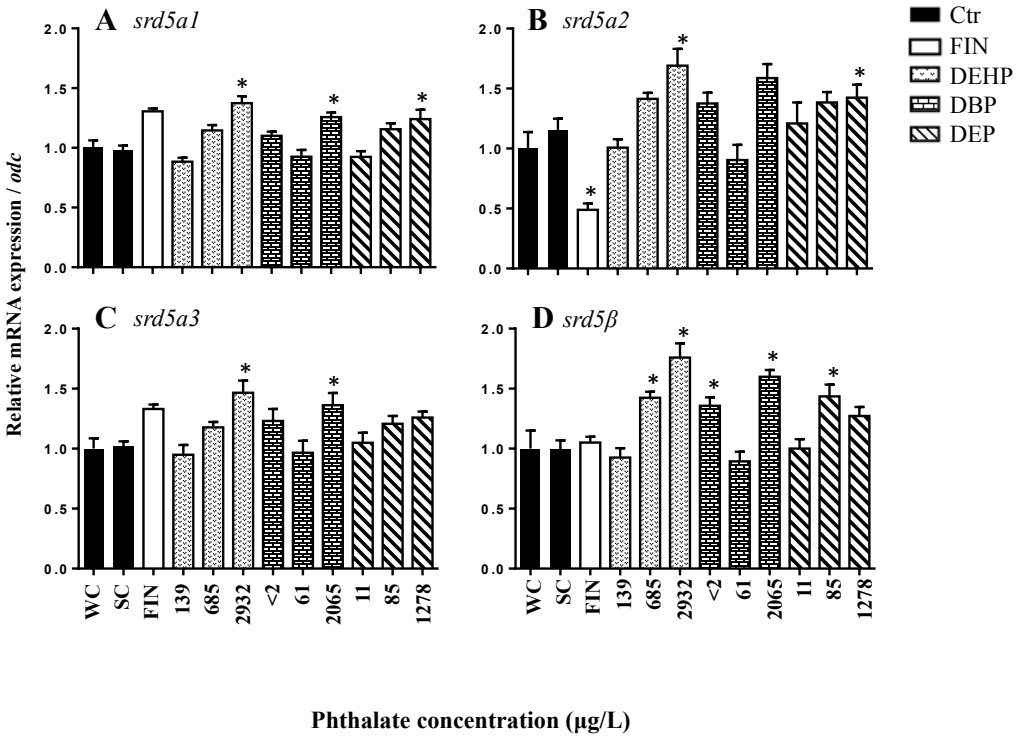

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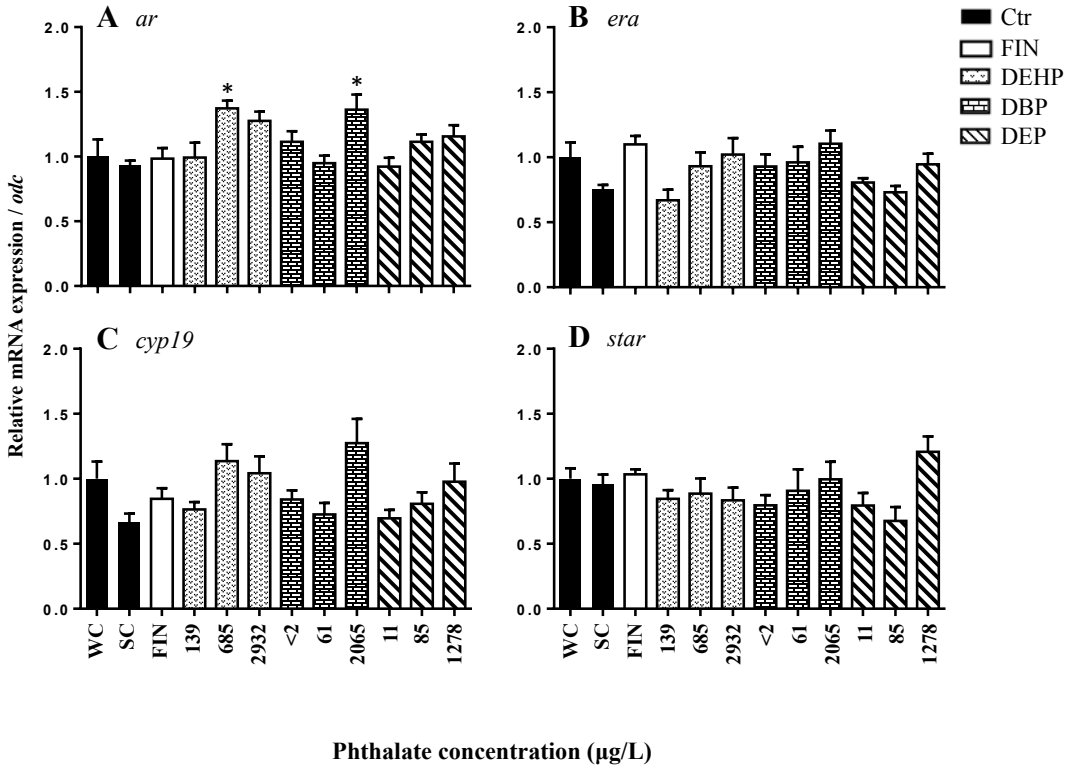
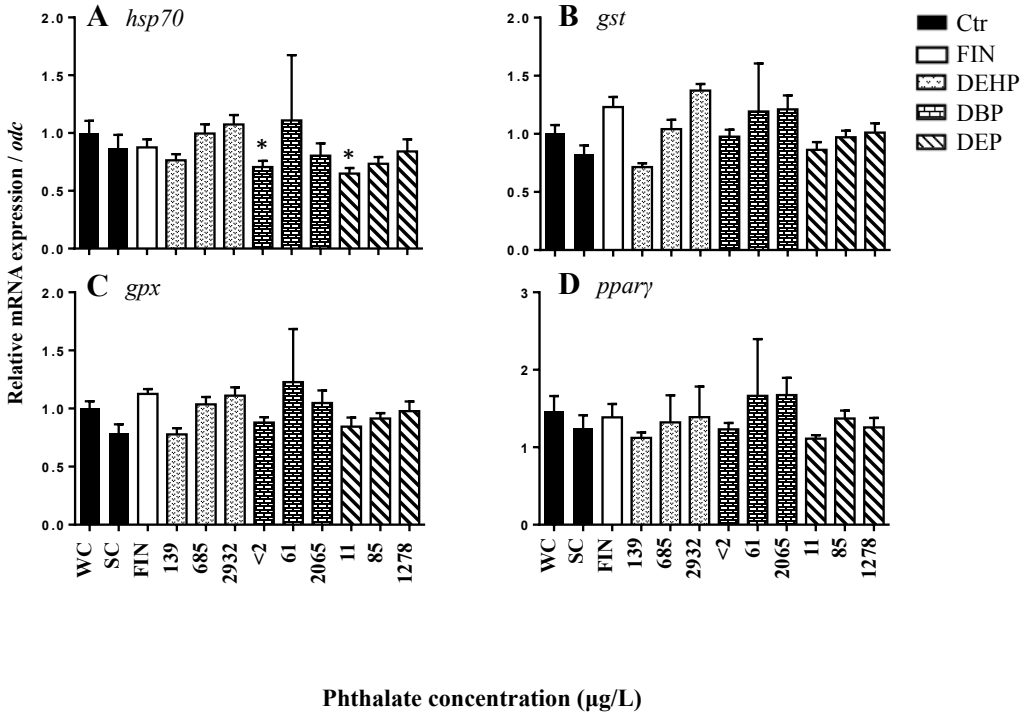
Fig 2


A ar
B era
C cyp19
D star
Ctr
FIN
DEHP
DBP
DEP
Relative mRNA expression / adc
2.0
1.5
1.0
0.5
0.0
*
*
WC
SC
FIN
139
685
2932
<2
61
2065
11
85
1278
Phthalate concentration (µg/L)

627

628

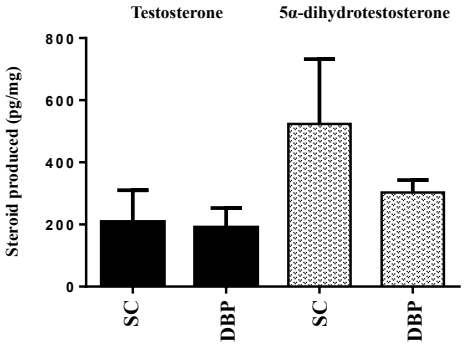
Fig 3


A hsp70
B gst
C gpx
D pparγ
Relative mRNA expression / odc
Ctr
FIN
DEHP
DBP
DEP
WC
SC
FIN
139
685
2932
<2
61
2065
11
85
1278
Phthalate concentration (µg/L)

629

630

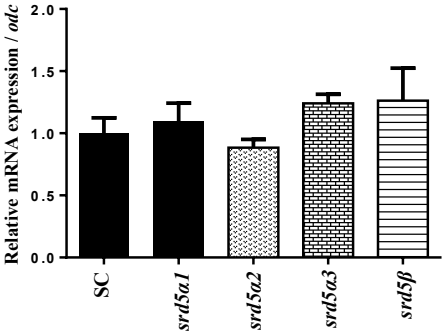
Fig 4


Testosterone
5α-dihydrotestosterone
Steroid produced (pg/mg)
800
600
400
200
0
SC
DBP
SC
DBP

631

632

Fig 5


Relative mRNA expression / odc
2.0
1.5
1.0
0.5
0.0
SC
srd5a1
srd5a2
srd5a3
srd5β

633