Ovarian Metabolism of an Environmentally Relevant Phthalate Mixture

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ABSTRACT

Phthalates are synthetic chemicals with widespread human exposure due to their use as additives in consumer products. Phthalate diesters are hydrolyzed in the environment and in the body to monoesters that may be more toxic than the parent compounds. This study tested the hypothesis that adult mouse antral follicles, but not neonatal ovaries, are able to metabolize an environmentally relevant mixture of phthalates. Whole neonatal ovaries and isolated adult antral follicles from CD-1 mice were cultured in media treated with vehicle control or 0.1–10 µg/ml of a mixture composed of 35% diethyl phthalate (DEP), 21% di(2-ethylhexyl) phthalate (DEHP), 15% dibutyl phthalate (DBP), 15% diisononyl phthalate (DiNP), 8% diisobutyl phthalate (DiBP), and 5% benzylbutyl phthalate (BzBP). After 4 days of culture, media were subjected to high-performance liquid chromatography tandem mass spectrometry to measure the amounts of diester phthalates and monoester metabolites. Ovaries and follicles were collected to measure the gene and protein expression of the enzymes required for phthalate metabolism. Monoester metabolites for all phthalates except DiNP were detected in the media for both culture types at most doses. The long-chain phthalates (BzBP, DEHP, and DiNP) were metabolized less than the short-chain phthalates (DEP, DBP, and DiBP) compared with respective controls. Expression of metabolizing enzymes was observed for all treatment groups in both culture types. These data indicate that mouse ovaries are capable of metabolizing low doses of phthalates and suggest that metabolic capacity differs for follicles at different stages of development.

Key words: ovary; follicles; phthalates; metabolism.

Phthalates are a class of chemicals used widely in consumer products as plasticizers, solvents, and additives. They are esters of *ortho*-phthalic acid with hydrocarbon side chains of varying lengths and branching structures (Figure 1). Their structural variety creates a wide range of properties, making them industrially useful and leading to their ubiquity in consumer products (Schettler *et al.*, 2006). Short-chain phthalates are excellent solvents for carrying fragrances and are typically found in personal care products (Craig and Ziv-Gal, 2018; Koniecki *et al.*, 2011). Long-chain phthalates are generally used as plasticizers and can be found in products containing polyvinyl chloride (PVC), including children's toys, vinyl flooring, blood bags, and medical tubing (Heudorf *et al.*, 2007).

Humans are widely exposed to phthalates through inhalation and dermal contact as well as through ingestion of

phthalate-contaminated food (Heudorf *et al.*, 2007; Serrano *et al.*, 2014). Phthalate metabolites have been measured in 99% of urine samples from the general population (Silva *et al.*, 2004) and have been detected in serum, sweat, breastmilk, and ovarian follicular fluid (Du *et al.*, 2016; Genuis *et al.*, 2012; Högberg *et al.*, 2008; Krotz *et al.*, 2012). In urine from the general population, individual phthalate metabolites typically average 0.01–1 ng/ml (Center for Disease Control and Prevention, 2018), but exposure from medical devices and medications can result in urine concentrations up to the micrograms per milliliters range (Calafat *et al.*, 2004; Hauser *et al.*, 2004). Phthalates are associated with many negative health outcomes in reproduction and development, including adverse pregnancy outcomes, precocious puberty, and endometriosis (reviewed in Kay *et al.*, 2013) as well

Figure 1. Phthalates used in this study. The phthalate mixture is composed of the 6 diester phthalates on the left, arranged top to bottom from smallest to largest by molecular weight. Their primary monoester metabolites are shown on the right side of the arrows. Benzylbutyl phthalate is cleaved primarily to MBzP, but some MBP may also be formed. Note: DiNP is a mixture of isomers. One representative isomer is shown.

as impaired male reproductive function (reviewed in [Albert and Jegou, 2014](#)).

Most studies on the effects of phthalates have focused on single compounds, the most well-studied of which is di(2-ethylhexyl) phthalate (DEHP). However, humans are constantly exposed to mixtures of phthalates, making mixture studies of vital importance for modeling exposure. Only a few studies have been performed on mixtures of phthalates in female reproduction. Female rats exposed *in utero* to a mixture of 5 phthalate diesters exhibited reproductive tract malformations ([Hannas et al., 2013](#)). Perinatal exposure in rats to a mixture of environmental chemicals, including phthalates, resulted in irregular estrous cycles, reduced ovary weights, and reduced follicle numbers ([Johansson et al., 2016](#)). Human placental trophoblast progenitor cells cultured with 4 phthalate metabolites exhibited altered PPAR γ expression and human chorionic gonatropin levels ([Adibi et al., 2017](#)). Previously, we have cultured mouse adult antral follicles with a mixture of 6 phthalates

and found alterations in gene expression as well as decreased hormone levels and increased oocyte fragmentation ([Zhou and Flaws, 2016](#)). *In vivo* studies with the same mixture revealed multigenerational and transgenerational disruption of female fertility as well as altered organ weights, reduced anogenital distance, and induced cystic ovaries ([Zhou et al., 2017a,b](#)).

It is especially important to study the metabolism of phthalates in the body because evidence suggests that monoester metabolites, not the diesters found in consumer products, are causing the toxic effects of phthalates ([Davis et al., 1994](#); [Hannon et al., 2015a,b](#); [Lovekamp and Davis, 2001](#); [Lovekamp-Swan and Davis, 2003](#); [Wang et al., 2012](#)). The diesters are hydrolyzed easily to monoesters both in the human body and in the environment ([Staples et al., 1997](#)). Phthalates are short-lived in biological systems, but their ubiquity in consumer products means that exposure is constant and unavoidable ([Teuten et al., 2009](#)). The half-lives of phthalates in the body are on the order of days, with a significant proportion of metabolism to the

Figure 2. Scheme of the metabolism of phthalates. Parent diester phthalates are easily cleaved to monoester metabolites during phase I metabolism and may also be modified via oxidation, hydroxylation, and/or conjugation to increase hydrophilicity and facilitate excretion (Committee on the Health Risks of Phthalates, 2008; Frederiksen *et al.*, 2007; Rusyn *et al.*, 2006) (Figure 2). Hydrolysis to the monoester is catalyzed by lipases primarily found in the saliva and gut; however, intravenous medical exposure bypasses oral metabolism and can lead to higher concentrations of diester reaching other organs before further detoxification in the liver (Albro, 1986; Albro and Thomas, 1973). Therefore, it is important to understand the capacity of target organs to bioactivate phthalates.

monoester occurring within hours (reviewed in Frederiksen *et al.*, 2007). Short-chain phthalates are typically excreted without further modification, but long-chain phthalates require additional oxidation, hydrolysis, and/or conjugation to increase hydrophilicity and facilitate excretion (Committee on the Health Risks of Phthalates, 2008; Frederiksen *et al.*, 2007; Rusyn *et al.*, 2006) (Figure 2). Hydrolysis to the monoester is catalyzed by lipases primarily found in the saliva and gut; however, intravenous medical exposure bypasses oral metabolism and can lead to higher concentrations of diester reaching other organs before further detoxification in the liver (Albro, 1986; Albro and Thomas, 1973). Therefore, it is important to understand the capacity of target organs to bioactivate phthalates.

If the ovary can metabolize phthalate diesters into more hazardous monoesters, then the ovary may be exposed to higher concentrations of toxic phthalates than what is measured by biomonitoring. Thus, in this study, we measured the ability of mouse ovaries at different stages of development to metabolize a mixture of phthalates and identified metabolizing enzymes that are expressed in the ovary. We employed 2 culture systems to compare metabolism at different stages of follicular development: Whole neonatal ovaries, which are composed of primordial and primary follicles undergoing natural follicle development (Eppig and O'Brien, 1996), and isolated adult antral follicles. We tested the hypothesis that the more developed adult antral follicles metabolize phthalates to a greater extent than the primordial and primary follicles contained in the neonatal ovaries.

MATERIALS AND METHODS

Chemicals. The phthalate mixture used in this study contains 35% diethyl phthalate (DEP), 15% dibutyl phthalate (DBP), 8% diisobutyl phthalate (DiBP), 5% benzylbutyl phthalate (BzBP), 21% DEHP, and 15% diisononyl phthalate (DiNP) by weight. The composition of the mixture was derived from measured metabolites in the urine of pregnant women in Illinois (IKIDS study; Yazdy *et al.*, 2018). Diethyl phthalate, DBP, DiBP, BzBP, DEHP, and DiNP were purchased from Sigma-Aldrich (St Louis, Missouri). A stock solution of the pure phthalate mixture was prepared as previously described (Zhou and Flaws, 2016) and stored at -20°C before use. Mixture treatment solutions of 0.13, 1.33, and 13.3 mg/

ml were prepared by diluting the phthalate stock in dimethyl sulfoxide (DMSO) such that the same volume of mixture and vehicle could be added per milliliters of culture media. The final working concentrations of phthalate mixture in media were 0.1, 1, and $10\mu\text{g/ml}$. Each working concentration contained the same volume of chemical and vehicle solution ($0.75\mu\text{l}$ of phthalate mixture in DMSO solution per ml of culture media). The actual doses of each phthalate in the media before culture were verified by mass spectrometry (Supplementary Figure 1).

Mass spectrometry analysis of culture media measured the concentrations of all 6 mixture components as well as selected primary monoester metabolites. Standards for monoethyl phthalate (MEP), monobutyl phthalates (MBP), monoisobutyl phthalates (MiBP), monobenzyl phthalates (MBzP), monoethylhexyl phthalates (MEHP), and monoisononyl phthalates (MiNP) were purchased from Toronto Research Chemicals (Canada).

Animals. Adult CD-1 mice were purchased from Charles River Laboratories (Wilmington, Massachusetts) and allowed to acclimate to the facility before use. Breeding pairs were housed at the University of Illinois at Urbana-Champaign, Veterinary Medicine Animal Facility. Pregnant females were housed separately and allowed to give birth. Postnatal day (PND) 4 female pups were euthanized for neonatal ovary culture as described below. The remaining female pups were weaned at 21 days and housed in groups until euthanization for antral follicle culture as described below. Food (Harlan Teklad 8604) and water were provided for *ad libitum* consumption. Temperature was maintained at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, and animals were subjected to 12-h light-dark cycles. The Institutional Animal Use and Care Committee at the University of Illinois at Urbana-Champaign approved all procedures involving animal care, euthanasia, and tissue collection.

Neonatal ovary culture. Whole neonatal ovaries were chosen for these experiments because they are composed largely of primordial and primary follicles and thus, provide information on the metabolic capacity of primordial and primary follicles (Eppig and O'Brien, 1996). Whole ovaries were collected from CD-1 pups at PND4 and cultured as previously described (Hannon *et al.*, 2015a,b). Briefly, ovaries were removed from pups and cleaned with watchmaker's forceps using a light microscope. Ovaries were cultured in pairs in 4-well plates

(Thermo Fisher Scientific, Waltham, Massachusetts). Each ovary pair was placed in a drop of media on a 0.4 μ M Millicell culture plate insert (Millipore, Billerica, Massachusetts) floating on 500 μ L of media. The supplemented media contained Dulbecco-modified Eagle medium/Ham F12 minus phenol red medium (Life Technologies, Grand Island, New York) with 1 mg/ml bovine serum albumin (Sigma-Aldrich), 1 mg/ml Albumax (ThermoFisher), 50 μ g/ml ascorbic acid (Sigma-Aldrich), 5 units/ml penicillin plus 5 μ g/ml streptomycin (Thermo Fisher), and 27.5 μ g/ml transferrin (Sigma-Aldrich) per well. Media were dosed with 0.75 μ L/ml of the appropriate phthalate mixture stock in DMSO (see above) before plating. Ovaries were cultured for 4 days until PND8 in an incubator at 37°C supplying 5% CO₂. Media were not changed for the duration of the experiment to mirror the antral follicle culture conditions (see below) for accurate comparison. The drop of media on the Millicell insert was refreshed daily. Following culture, media were collected for metabolism analysis and ovaries were designated for reverse transcription quantitative polymerase chain reaction (qPCR) analysis ($n=3$ with 3 ovaries per n from 2 to 3 individuals each) or Western blotting ($n=3$ with 6 ovaries per n) as described below.

In vitro antral follicle culture. In these experiments, antral follicles were isolated from whole ovaries to test the metabolic capacity of antral follicles. It was necessary to isolate antral follicles from adult ovaries because the adult ovary contains many follicle types, as well as corpora lutea and interstitial cells. Whole ovaries were collected from 32 to 42 day old CD-1 mice and cultured as previously described (Zhou and Flaws, 2016). Briefly, antral follicles from 220 to 400 μ M were removed from the ovary with watchmaker's forceps and placed individually in 96-well culture plates in 150 μ L media. Supplemented media contained α -MEM with 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium, Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 100 mg/ml streptomycin (Sigma-Aldrich), 5 IU/ml recombinant follicle-stimulating hormone (Dr A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, California), and 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia). The appropriate phthalate mixture stock in DMSO (0.75 μ L/ml) was added to the media before plating. Each treatment group (0.1, 1, or 10 μ g/ml or DMSO vehicle only) was plated on a separate 96-well plate to reduce airborne contamination of treatment groups with volatile phthalates. For each plate, 12 follicles were plated in treated media and 3 follicles were plated in control media to identify between-plate differences. In addition, 4 control wells per plate contained treated media with no tissue to observe environmental breakdown of the phthalate mixture. Follicles were cultured for 4 days in an incubator at 37°C supplying 5% CO₂. Media were not changed for the duration of the experiment. Following culture, media were collected for metabolism analysis and follicles were collected for qPCR ($n=4$ with 12 follicles per n) or Western blotting ($n=3$ with 50 follicles per n).

Analysis of metabolites in culture media. All 6 parent compounds were measured as well as their respective primary monoester metabolites MEP, MBP, MiBP, MBzP, MEHP, and MiNP. Media samples were stored at -80°C until analysis and were injected directly into the instrument. Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, Massachusetts) in the Metabolomics Lab of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent

Technologies, Santa Clara, California) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on a Phenomenex (Torrance, California) Gemini C6-phenyl column (2 $\times$ 100 mm, 3 μ m) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.25 ml/min. The linear gradient was as follows: 0–1 min, 70% A; 10–15 min, 50% A; 20–27.5 min, 10% A; and 28–34 min, 70% A. The autosampler was set at 10°C. The injection volume was 5 μ L. Mass spectra were acquired under both positive electrospray ionization (ESI) with the ion spray voltage of 5000 V and negative ESI with the ion spray voltage of -4500 V. The source temperature was 500°C. The curtain gas, ion source gas 1, and ion source gas 2 were 32, 50, and 65 psi, respectively. Multiple reaction monitoring was used for quantitation. Transitions monitored are listed in [Supplementary Table 1](#).

Gene expression analysis. Metabolizing enzymes for gene expression analysis were chosen based on literature reports of enzymes responsible for the metabolism of DEHP (Albro and Thomas, 1973; Ito et al., 2005). qPCR analysis for gene expression was performed on collected ovaries ($n=3$ with 3 ovaries per n) and follicles ($n=4$ with 12 follicle per n) from each culture type. Ovaries and follicles were snap frozen and stored at -80°C until RNA extraction. RNA was isolated using an RNeasy Micro kit (Qiagen Inc., Valencia, California) following the manufacturer's instructions. RNA was eluted in 14 μ L of RNase-free water and the concentration was determined using a NanoDrop ($\lambda = 260/280$ nm; ND 1000; Nanodrop Technologies Inc., Wilmington, Delaware). Total RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) using the iScript RT kit (Bio-Rad Laboratories Inc., Hercules, California) according to the manufacturer's protocol. Analysis of qPCR was conducted using the C and FX96 Real-Time PCR Detection System (Bio-Rad Laboratories) and accompanying CFX Manager Software according to the manufacturer's protocol. The machine quantifies the amount of PCR product generated by measuring SsoFastEvaGreen dye (Bio-Rad Laboratories) that fluoresces when bound to double-stranded DNA. Specific qPCR primers (Integrated DNA Technologies, Coralville, Iowa) for the genes investigated (18S ribosomal RNA [Rn 18S], alcohol dehydrogenase 1 [Adh1], aldehyde dehydrogenase family 1, subfamily A1 [Aldh1a1], isoamyl acetate-hydrolyzing esterase 1 homolog [Iah1], lipoprotein lipase [Lpl], uridine diphosphate glucuronosyltransferase 1 family, polypeptide A6A [Udg1a6a], uridine diphosphate glucuronosyltransferase family 1 member A1 [Udg1a1], cytochrome P450, family 3, subfamily a, polypeptide 11 [Cyp3a11]) are listed in [Supplementary Table 2](#). The PCR reactions were performed with 1.67 ng cDNA, forward and reverse primers (7.5 pmol each), and SsoFast EvaGreen Supermix for a reaction volume of 10 μ L. The qPCR program consisted of an enzyme activation step (95°C for 1 min), an amplification and quantification program (36 cycles of 95°C for 10 s, 60°C for 10 s, single fluorescence reading), a step of 72°C for 5 min, a melt curve (65°C–95°C heating, 0.5°C/s with continuous fluorescence readings), and a final step at 72°C for 5 min per the manufacturer's protocol. All gene expression data were normalized to the housekeeper Rn18S. Relative fold changes were calculated as the ratio to DMSO group level and were analyzed using the Pfaffl model for relative quantification of real-time PCR data (Pfaffl, 2001).

Protein expression analysis. Western blotting for protein expression was performed on collected ovaries ($n=3$ with 6 ovaries

per n) and follicles ($n = 3$ with 50 follicle per n) from each culture type for the vehicle and 1 $\mu\text{g}/\text{ml}$ dose phthalate mixture. Ovaries and follicles were snap frozen and stored at -80°C until use. Samples were ground with mortar and pestle in Tissue Protein Extraction Reagent (ThermoFisher) and centrifuged at $10\,000\times g$ for 5 min. Protein content of the supernatant was determined using Pierce BCA Protein Assay Kit (ThermoFisher) according to the manufacturer's instructions. Protein samples (10 μg) were mixed with NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Thermo Fisher) and heated at 70°C for 10 min. The samples were loaded on a NuPAGE 4%–12% bis-tris electrophoresis gel and transferred to an Invitrolon 0.45 μm PVDF membrane (ThermoFisher). The membrane was blocked for 1 h in 5% nonfat dried milk in Tris-buffered saline with 0.1% Tween 20 (T-TBS) and incubated with LPL monoclonal antibody (Santa Cruz sc-373759, 1:5000) in 5% milk in T-TBS overnight at 4°C . The membrane was incubated with secondary antimouse antibody (Santa Cruz sc-516102, 1:5000) in T-TBS for 1 h and imaged with FluorChem R FR0109 system (ProteinSimple, San Jose, California). The membrane was stripped for 15 min in Restore Plus Western Blot Stripping Buffer (ThermoFisher) at room temperature and reprobed with antibodies against ALDH1A1 (Abcam Ab52492, 1:2000) and GAPDH (EnCor, MCA-1D4, 1:1000). Relative protein levels were analyzed using National Institutes of Health ImageJ. Expression was normalized to GAPDH.

Statistical analysis. All analyses were performed using IBM SPSS Statistics 25 software (SPSS Inc., Chicago, Illinois). Data were expressed as means $\pm$ standard error bars. For mass spectrometry analysis, missing values were input as the limit of detection for a conservative calculation of significance. Outliers were removed by the Grubb's test using GraphPad outlier calculator software (GraphPad Software Inc., La Jolla, California). Data were continuous and assessed for normality by Shapiro-Wilk analysis. Normal data were compared using independent samples t -tests. Non-normal data sets were analyzed using Kruskal-Wallis followed by Mann-Whitney U nonparametric 2-independent sample tests. Statistical significance was assigned at $p \leq .05$, but $p < .1$ was considered to exhibit a trend toward significance.

RESULTS

Diester Phthalates in Neonatal Ovary Culture Media

To determine the metabolism of phthalates by primordial and primary follicles, PND4 mouse ovaries (which largely contain primordial and primary follicles) were cultured in either vehicle (DMSO) or 1 of the 3 phthalate mixture doses (0.1, 1, or 10 $\mu\text{g}/\text{ml}$). After 4 days of culture, media samples were analyzed by LC-MS/MS. To ensure that the measured phthalate metabolism was performed by tissue and not due to environmental breakdown, the concentration measured in the culture media was compared with the concentration measured in media from control wells that contained no ovaries, but otherwise experienced the same conditions. Concentrations of the remaining parent diester phthalate mixture components DEP, DBP, DiBP, BzBP, DEHP, and DiNP were measured after culture. A reduction in the concentration of diester phthalates remaining in the experimental wells compared with the control wells supports the occurrence of metabolism. The concentrations of diester phthalates in the media after culture were significantly less ($p \leq .05$) at the 1 and 10 $\mu\text{g}/\text{ml}$ doses for DBP, DiBP, and BzBP compared with controls. No DiNP was detected (Figs. 3 and 4).

Monoester Phthalates in Neonatal Ovary Culture Media

Concentrations of the phthalate monoesters MEP, MBP, MiBP, MBzP, MEHP, and MiNP were measured after 4 days of culture. The presence of more monoester phthalate in the experimental wells compared with the control wells supports the occurrence of metabolism. The monoester phthalates MEP, MBP, MiBP, and MBzP were measured at all doses in statistically significant amounts ($p \leq .05$) compared with control media (Figs. 3 and 4). Monoethylhexyl phthalates measured at the 1 and 10 $\mu\text{g}/\text{ml}$ doses showed a statistically significant increase compared with controls ($p \leq .05$). No MiNP was detected for any of the doses (Figure 4).

Diester Phthalates in Antral Follicle Culture Media

To determine the metabolism of phthalates by antral follicles, isolated adult mouse antral follicles were cultured individually with the same phthalate mixture treatments as the neonatal ovary cultures (above) for the same amount of time. The mass spectrometry analyses of di- and monoesters in culture media samples and control media were the same. Significant decreases in the amount of diester in samples with follicles compared with control was observed for DBP at the 1 and 10 $\mu\text{g}/\text{ml}$ doses, DiBP at the 1 $\mu\text{g}/\text{ml}$ dose, and BzBP at the 10 $\mu\text{g}/\text{ml}$ dose (Figs. 5 and 6).

Monoester Phthalates in Antral Follicle Culture Media

Concentrations of the monoester phthalate metabolites were measured after culture for both experimental and control wells. MEP, MBP, and MiBP were detected at all doses, whereas MBzP and MEHP were detected at the 1 and 10 $\mu\text{g}/\text{ml}$ doses. Monoisobutyl phthalates was not detected (Figs. 5 and 6).

Comparison of Metabolism by Structure

To compare the capacity of each culture type to metabolize the different sizes of phthalates, the ratio of summed short-chain (Σ_{short} = MEP, MBP, and MiBP) and long-chain (Σ_{long} = MBzP, MEHP, and MiNP) monoester phthalates were compared for each culture type (Table 1). Neonatal ovaries (ie, primordial and primary follicles) had much higher ratios of short- to long-chain phthalates ($\Sigma_{\text{short}}:\Sigma_{\text{long}}$), indicating that primordial and primary follicles are more effective than antral follicles at metabolizing short-chain phthalates. Antral follicles had more equitable ratios of $\Sigma_{\text{short}}:\Sigma_{\text{long}}$, but also metabolized short-chain phthalates to a greater extent than long-chain phthalates.

Metabolic Enzyme Gene Expression in Neonatal Ovaries

To investigate which enzymes may be performing the observed metabolism, qPCR for gene expression of selected enzymes was performed on the neonatal ovaries and antral follicles after culture for all treatment groups. Expression was observed for *Lpl*, *Aldh1a1*, *Iah1*, *Adh1*, *Ugt1a6a*, and *Ugt1a1* (Figure 7). Expression was unaffected by treatment, except for increased expression of *Aldh1a1* at 1 $\mu\text{g}/\text{ml}$ of the phthalate mixture compared with control ($p = .02$). Expression of *Lpl* was higher than expression of the other enzymes (Figure 9). No expression was observed for *Cyp3a11*.

Metabolic Enzyme Gene Expression in Antral Follicles

qPCR of antral follicles after culture showed expression of *Lpl*, *Aldh1a1*, *Iah1*, *Adh1*, *Ugt1a6a*, and *Ugt1a1* (Figure 8). Expression was unaffected by treatment, except for increased expression of *Lpl* at 1 $\mu\text{g}/\text{ml}$ of the phthalate mixture compared with control ($p = .03$). Expression of *Aldh1a1* was higher than expression of

Figure 3. Short-chain phthalates detected by LC-MS/MS in neonatal ovary culture media following 4 days of culture in DMSO or 0.1–10 µg/ml phthalate mixture. Graphs represent means $\pm$ SEM from 5–6 separate experiments. Asterisks (*) indicate significant differences in the treatment (gray bars) from the control (black bars) ($p \leq .05$).

the other enzymes (Figure 9). No expression was observed for Cyp3a11.

Metabolic Enzyme Protein Expression in Neonatal Ovaries and Antral Follicles

Protein expression for LPL and ALDH1A1 was measured by Western blots after culture with vehicle or 1 µg/ml mixture. Expression of both enzymes was observed (Figure 10). No significant differences were observed between treatment groups. In DMSO treated neonatal ovaries, expression of LPL was significantly higher than ALDH1A1 ($p = .01$). In DMSO treated antral follicles, expression of ALDH1A1 was borderline significantly higher than LPL ($p = .07$).

DISCUSSION

In this study, we utilized 2 mouse ovarian culture systems to measure the ability of the ovary to metabolize phthalates at difference stages of folliculogenesis. We cultured whole neonatal ovaries, which are composed of primordial and primary follicles, to study the early stages of folliculogenesis. The second culture system consisted of isolated antral follicles from adult mice. Previously, we have shown that both DEHP and MEHP inhibited steroidogenesis and induced atresia in antral follicle culture (Hannon *et al.*, 2015a,b). However, in neonatal ovary culture, MEHP, but not DEHP, increased follicle recruitment and altered factors in the phosphatidylinositol 3-kinase (PI3K)-signaling pathway, which regulates primordial follicle recruitment (Hannon *et al.*, 2015a,b). The differential response to DEHP in the 2 culture systems led us to hypothesize that DEHP may be

Figure 4. Long-chain phthalates detected by LC-MS/MS in neonatal ovary culture media following 4 days of culture in DMSO or 0.1–10 µg/ml phthalate mixture. Graphs represent means $\pm$ SEM from 5–6 separate experiments. Asterisks (*) indicate significant differences in the treatment (gray bars) from the control (black bars) ($p \leq .05$). No DiNP or MiNP was detected.

transformed to the active metabolite MEHP in antral follicles, but not in neonatal ovaries. In the current study, we used a mixture of phthalates rather than just DEHP to better represent human environmental exposures. Our main finding is that phthalates are metabolized by both neonatal ovaries (ie, primordial and primary follicles) and antral follicles and that the ability to metabolize phthalates with different structures changes during folliculogenesis.

Phthalates are easily hydrolyzed in the environment. Thus, to ensure that the observed metabolism was attributable to ovarian tissue rather than photolysis, each experiment contained control wells with no tissue. Some phthalate metabolites were detected in the control samples after culture, with short-chain phthalates detected most frequently (Figs. 3–6). We measured phthalate levels in untreated media before and after culture to monitor contamination from laboratory materials and did not find any significant phthalate contamination (data not shown).

In our analyses, the 6 phthalates were divided into 2 groups: Short-chain (ie, low molecular weight: DEP, DBP, and DiBP) and long-chain (ie, high molecular weight: BzBP, DEHP, and DiNP). These groupings are roughly representative of commercial use, with short-chain phthalates used predominantly in personal care products and long-chain phthalates used as plasticizers. We found that almost all phthalates were metabolized to some extent in each culture type and that, in general, short-chain phthalates were more effectively metabolized than long-chain phthalates (Figs. 3–6). For all treatments in both culture types, more short-chain phthalates were metabolized than long-chain phthalates. However, neonatal ovaries had much higher ratios of $\sum \text{short} : \sum \text{long}$ metabolites than antral follicles, indicating

that primordial and primary follicles have a much stronger preference for metabolizing short-chain phthalates compared with antral follicles. The increased ability of antral follicles to metabolize long-chain phthalates is consistent with our previous finding that DEHP was more active in antral follicles than neonatal ovaries (Hannon et al., 2015a,b).

Previously, Hansen et al. reported the metabolism of DEP, DBP, and DEHP (assessed individually) in human thyroid cell cultures (Hansen et al., 2016). Diethyl phthalate was found to be metabolized more than the long-chain phthalates, whereas DEHP was metabolized to MEHP the least. The general trend of less metabolism with increasing molecular weight is consistent with our results, except for DEP. We found that approximately 10% of the DEP measured in the control was metabolized to MEP in both neonatal ovaries and antral follicles, whereas approximately 50% of the DBP was metabolized to MBP (Figs. 3 and 5). The decreased metabolism of DEP, the smallest phthalate tested, may be due to the tissue type, mixture effects, or the concentration; DEP was the largest component of the mixture and the smallest by mass and therefore had a significantly higher dose by molarity than any other phthalate in the mixture. Further studies should compare metabolism by individual phthalates compared with the mixture to determine whether the mixture interferes with metabolism.

For many of our measurements of parent phthalates and metabolites following culture, the amount of parent phthalates that remained in the media summed with the metabolites detected (accounting for hydrolysis of 1 side chain) does not account for all the phthalates in each dose of the mixture. The concentrations of each phthalate that each culture received were measured in the prepared media taken before the culture

Figure 5. Short-chain phthalates detected by LC-MS/MS in antral follicle culture media following 4 days of culture in DMSO or 0.1–10 µg/ml phthalate mixture. Graphs represent means $\pm$ SEM from 4 separate experiments ($n = 12$ follicles/treatment/experiment). Asterisks (*) indicate significant differences in the treatment (gray bars) from the control (black bars) ($p \leq .05$); (^) indicates borderline significance compared with control ($p \leq .10$).

began. We attribute the differences from the calculated dose to be due to the difficulty preparing the initial mixture stock from pure phthalates, which are viscous oils (Supplementary Figure 1). However, this does not account for all the lost material (Supplementary Figs. 2 and 3). One explanation for the differences may be phthalates adsorbing onto the surface of the culture plate during incubation. This effect should be the same for experimental and control samples and should minimally impact metabolism. The differences in recovery between experimental and control samples that are not accounted for by metabolites may be due to the partitioning of phthalates into the tissue during culture. The greatest phthalate loss was observed for the heaviest phthalates, DEHP and DiNP, which are also the most lipophilic phthalates. When used as a plasticizer in PVC blood bags, DEHP has been long known to partition into red blood cells, providing a stabilizing effect and increasing the shelf life

of the blood by incorporating into the plasma membrane (Rock *et al.*, 1984). Future studies will incorporate both extraction of phthalates from tissue following culture and washing the culture plate with an organic solvent to attempt to recover all phthalates.

Another potential source of loss of material, especially of the long-chain phthalates, is metabolism into molecules that were not detected during targeted mass spectrometry analysis. The larger the phthalate, the more metabolites are possible; over 15 metabolites of DEHP have been measured in humans (Frederiksen *et al.*, 2007). The primary monoester metabolites were selected as the target metabolites after preliminary studies with DEHP in which MEHP, mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) were measured, but only MEHP was found above the limit of

Figure 6. Long-chain phthalates detected by LC-MS/MS in antral follicle culture media following 4 days of culture in DMSO or 0.1–10 µg/ml phthalate mixture. Graphs represent means $\pm$ SEM from 4 separate experiments ($n = 12$ follicles/treatment/experiment). Asterisks (*) indicate significant differences in the treatment (gray bars) from the control (black bars) ($p \leq .05$). No MiNP was detected.

Table 1. Ratios of the Sums of Short-Chain Phthalates to Long-Chain Phthalates for Each Treatment Group in Neonatal Ovaries and Antral Follicles

Phthalate Mixture Treatment	Σ short : Σ long	
	Neonatal Ovaries	Antral Follicles
DMSO	N/A	N/A
0.1	89:1	5:1
1	15:1	5:1
10	15:1	6:1

detection (data not shown). However, other metabolites may be present and may account for the apparent low metabolism of DEHP. Diisononyl phthalate is commercially available as

mixtures of isomers containing at least 15 isomers, resulting in an even more complex (and largely unknown) metabolite profile. Numerous metabolites of DiNP, including MiNP, have been detected in human and animal urine samples (Saravanabhavan and Murray, 2012). We chose to detect MiNP because a standard was commercially available for mass spectrometry analysis. However, recovery of DiNP was low and no MiNP was measured, suggesting that DiNP may be easily environmentally hydrolyzed, but we did not choose the correct metabolites to measure. Due to the complex nature of DiNP, further studies will need to be carefully designed to incorporate additional metabolites such as isomers of MiNP.

We measured gene expression and corresponding protein levels of metabolizing enzymes following culture to identify which enzymes may be performing this metabolism. We selected enzymes identified in previous studies as responsible

Figure 7. Gene expression of metabolizing enzymes in neonatal ovary culture media following 4 days of culture in DMSO or 0.1–10 µg/ml phthalate mixture. Ovaries were collected and subjected to qPCR analysis relative to *Rn18s*. Graphs represent means $\pm$ SEM from 3 separate experiments ($n = 3$ ovaries/treatment/experiment). Asterisk (*) indicates significant difference in the treatment compared with control ($p = .02$).

for the metabolism of DEHP (Ito et al., 2005). Hydrolysis from the diester to the monoester is performed primarily by lipases, including LPL (Albro and Thomas, 1973). Additionally, LPL has been measured in mouse and human ovarian tissue (Holmes et al., 2011). As we found predominantly monoester metabolites in culture, we expected to find significant expression of LPL. We also examined oxidation enzymes (ALDH1A1 and ADH1), an esterase to represent hydrolysis to phthalic acid (IAH1), glucuronidation enzymes (UGT1A6A and UGT1A1), and a biotransformation enzyme found in the liver (CYP3A11). Gene expression was observed for all enzymes except *Cyp3a11* and was independent of treatment, with the exception of statistically significant increases in *Aldh1a1* at 1 µg/ml for neonatal ovaries and *Lpl* at 1 µg/ml in antral follicles (Figs. 7 and 8). When we compared rates of expression of each of the metabolizing enzymes in the DMSO treatment groups, we found that *Lpl* was most

highly expressed in the neonatal ovaries and *Aldh1a1* was most highly expressed in antral follicles (Figure 9). However, gene expression does not necessarily correlate with protein expression, so we also measured protein expression of LPL and ALDH1A1 (Figure 10). Both enzymes were detected in both tissue types, with greater expression of LPL in neonatal ovaries than antral follicles and vice versa for ALDH1A1 (Figure 10), consistent with the gene expression results in Figure 9.

The increased presence of ALDH1A1 in antral follicles compared with neonatal ovaries is interesting for several reasons. Further modification beyond the initial hydrolysis to the monoester is more important for more complex long-chain phthalates. These phthalates are metabolized preferentially by antral follicles. Although the products of ALDH1A1 metabolism were not detected in this study, the increased presence of this enzyme in antral follicles is consistent with our finding that antral

Figure 8. Gene expression of metabolizing enzymes in antral follicle culture media following 4 days of culture in DMSO or 0.1–10 µg/ml phthalate mixture. Follicles were collected and subjected to qPCR analysis relative to Rn18s. Graphs represent means $\pm$ SEM from 4 separate experiments ($n = 12$ follicles/treatment/experiment). Asterisk (*) indicates significant difference in the treatment compared with control ($p = .03$).

follicles are more capable of metabolizing more complex phthalates. Neonatal ovaries are more able to break down smaller, less complex phthalates into monoesters that can be excreted directly. Additionally, secondary oxidations of DEHP and DiNP metabolites performed by ALDH1A1 may account for some of the missing phthalates that were not detected as diester or monoester. Gene expression of glucuronidation enzymes was minimal, suggesting that conjugation is not a likely metabolism pathway in the ovary.

In conclusion, our study shows that the mouse ovary can metabolize a mixture of phthalates, with varying metabolizing capacity at different stages of folliculogenesis. Phthalates have been detected in human ovarian follicular fluid at concentrations roughly corresponding to serum levels (Krotz et al., 2012), indicating that significant concentrations reach the ovary.

Although substantial metabolism of phthalates occurs in the saliva, gut, and liver before reaching the ovary, higher initial doses of phthalates likely result in greater concentrations of diester reaching other tissues, including the ovary (Albro, 1986; Albro and Thomas, 1973; Genuis et al., 2012). Intravenous exposure from blood transfusion and IV lines is most likely the primary source of diester in the ovary (Kevy and Jacobson, 1982). If human ovaries are able to metabolize phthalates, then traditional analysis of phthalate exposure via blood and urine may be underestimating the exposure of the ovary to toxic metabolites.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

Figure 9. Relative gene expression for the 6 investigated metabolizing genes for neonatal ovaries (A) and antral follicles (B) for DMSO vehicle control groups following 4 days of culture. Expression of UGT1a1 is set to 1.

Figure 10. Protein expression of metabolizing enzymes in culture media following 4 days of culture in DMSO or 1 µg/ml phthalate mixture. Ovaries and follicles were collected and subjected to Western blotting for expression of LPL, ALDH1A1, and GAPDH. No statistical differences were observed between treatment groups. Graph compares relative expression between DMSO treatment groups in both tissues. Graph represent means $\pm$ SEM from 3 separate experiments ($n = 6$ ovaries/treatment/experiment or 50 follicles/treatment/experiment).

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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REFERENCES

Adibi, J. J., Zhao, Y., Zhan, L. V., Kapidzic, M., Larocque, N., Koistinen, H., Huhtaniemi, I. T., and Stenman, U. H. (2017). An investigation of the single and combined phthalate

metabolite effects on human chorionic gonadotropin expression in placental cells. *Environ. Health Perspect.* 125, 1–12.

Albert, O., and Jegou, B. (2014). A critical assessment of the endocrine susceptibility of the human testis to phthalates from fetal life to adulthood. *Hum. Reprod. Update* 20, 231–249.

Albro, P. W. (1986). Absorption, metabolism, and excretion of di(2-ethylhexyl) phthalate by rats and mice. *Environ. Health Perspect.* 65, 293–298.

Albro, P. W., and Thomas, R. O. (1973). Enzymatic hydrolysis of di-(2-ethylhexyl) phthalate by lipases. *Biochim. Biophys. Acta* 360, 380–390.

Calafat, A. M., Needham, L. L., Silva, M. J., and Lambert, G. (2004). Exposure to di-(2-ethylhexyl) phthalate among premature neonates in a neonatal intensive care unit. *Pediatrics* 113, e429.

Center for Disease Control and Prevention (2018). Fourth Report on Human Exposure to Environmental Chemicals, Updated Tables. Atlanta, GA. U.S. Department of Health and Human Services. https://www.cdc.gov/exposurereport/pdf/FourthReport_UpdatedTables_Volume1_Mar2018.pdf

Craig, Z. R., and Ziv-Gal, A. (2018). Pretty good or pretty bad? The ovary and chemicals in personal care products. *Toxicol. Sci.* 162, 349–360.

- Davis, B. J., Weaver, R., Gaines, L. J., and Heindel, J. J. (1994). Mono-(2-ethylhexyl) phthalate suppresses estradiol production independent of FSH-cAMP stimulation in rat granulosa cells. *Toxicol. Appl. Pharmacol.* **128**, 224–228.
- Du, Y. Y., Fang, Y. L., Wang, Y. X., Zeng, Q., Guo, N., Zhao, H., and Li, Y. F. (2016). Follicular fluid and urinary concentrations of phthalate metabolites among infertile women and associations with in vitro fertilization parameters. *Reprod. Toxicol.* **61**, 142–150.
- Eppig, J. J., and O'Brien, M. J. (1996). Development in vitro of mouse oocytes from primordial follicles. *Biol. Reprod.* **54**, 197–207.
- Frederiksen, H., Skakkebaek, N. E., and Andersson, A.-M. (2007). Metabolism of phthalates in humans. *Mol. Nutr. Food Res.* **51**, 899–911.
- Genuis, S. J., Beesoon, S., Lobo, R. A., and Birkholz, D. (2012). Human elimination of phthalate compounds: Blood, urine, and sweat (BUS) study. *Sci. World J.* **2012**, 1–10.
- Hannas, B. R., Howdeshell, K. L., Furr, J., and Earl Gray, L. (2013). In utero phthalate effects in the female rat: A model for MRKH syndrome. *Toxicol. Lett.* **223**, 315–321.
- Hannon, P. R., Brannick, K. E., Wang, W., and Flaws, J. A. (2015). Mono(2-ethylhexyl) phthalate accelerates early folliculogenesis and inhibits steroidogenesis in cultured mouse whole ovaries and antral follicles. *Biol. Reprod.* **92**, 120–120.
- Hannon, P. R., Brannick, K. E., Wang, W., Gupta, R. K., and Flaws, J. A. (2015). Di(2-ethylhexyl) phthalate inhibits antral follicle growth, induces atresia, and inhibits steroid hormone production in cultured mouse antral follicles. *Toxicol. Appl. Pharmacol.* **284**, 42–53.
- Hansen, J. F., Brorson, M. M., Boas, M., Frederiksen, H., Nielsen, C. H., Lindström, E. S., Hofman-Bang, J., Hartoft-Nielsen, M.-L., Frisch, T., Main, K. M., et al. (2016). Phthalates are metabolised by primary thyroid cell cultures but have limited influence on selected thyroid cell functions in vitro. *PLoS One* **11**, e0151192.
- Hauser, R., Duty, S., Godfrey-Bailey, L., and Calafat, A. M. (2004). Medications as a source of human exposure to phthalates. *Environ. Health Perspect.* **112**, 751–753.
- Heudorf, U., Mersch-Sundermann, V., and Angerer, J. (2007). Phthalates: Toxicology and exposure. *Int. J. Hyg. Environ. Health* **210**, 623–634.
- Högberg, J., Hanberg, A., Berglund, M., Skerfving, S., Remberger, M., Calafat, A. M., Filipsson, A. F., Jansson, B., Johansson, N., Appelgren, M., et al. (2008). Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. *Environ. Health Perspect.* **116**, 334–339.
- Holmes, R. S., Vandeberg, J. L., and Cox, L. A. (2011). Comparative studies of vertebrate lipoprotein lipase: A key enzyme of very low density lipoprotein metabolism. *Comp. Biochem. Physiol. Part D Genomics Proteomics* **6**, 224–234.
- Ito, Y., Yokota, H., Wang, R., Yamanoshita, O., Ichihara, G., Wang, H., Kurata, Y., Takagi, K., and Nakajima, T. (2005). Species differences in the metabolism of di(2-ethylhexyl) phthalate (DEHP) in several organs of mice, rats, and marmosets. *Arch. Toxicol.* **79**, 147–154.
- Johansson, H. K. L., Jacobsen, P. R., Hass, U., Svingen, T., Vinggaard, A. M., Isling, L. K., Axelstad, M., Christiansen, S., and Boberg, J. (2016). Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging. *Reprod. Toxicol.* **61**, 186–194.
- Kay, V. R., Chambers, C., and Foster, W. G. (2013). Reproductive and developmental effects of phthalate diesters in females. *Crit. Rev. Toxicol.* **43**, 200–219.
- Kevy, S. V., and Jacobson, M. S. (1982). Hepatic effects of a phthalate ester plasticizer leached from poly(vinyl chloride) blood bags following transfusion. *Environ. Health Perspect.* **45**, 57–64.
- Koniecki, D., Wang, R., Moody, R. P., and Zhu, J. (2011). Phthalates in cosmetic and personal care products: Concentrations and possible dermal exposure. *Environ. Res.* **111**, 329–336.
- Krotz, S. P., Carson, S. A., Tomey, C., and Buster, J. E. (2012). Phthalates and bisphenol do not accumulate in human follicular fluid. *J. Assist. Reprod. Genet.* **29**, 773–777.
- Lovekamp-Swan, T., and Davis, B. J. (2003). Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ. Health Perspect.* **111**, 139–145.
- Lovekamp, T. N., and 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.
- National Academy of Sciences, Committee on the Health Risks of Phthalates (2008). *Phthalates and Cumulative Risk Assessment: The task Ahead*. National Academies Press Washington, DC. <http://www.nap.edu/catalog/12528.html>.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
- Rock, G., Tocchi, M., Ganz, P. R., and Tackaberry, E. S. (1984). Incorporation of plasticizer into red cells during storage. *Transfusion* **24**, 493–498.
- Rusyn, I., Peters, J. M., and Cunningham, M. L. (2006). Effects of DEHP in the liver: Modes of action and species-specific differences. *Crit. Rev. Toxicol.* **36**, 459–479.
- Saravanabhavan, G., and Murray, J. (2012). Human biological monitoring of diisononyl phthalate and diisodecyl phthalate: A review. *J. Environ. Public Health* **2012**, 1.
- Schettler, T., Skakkebaek, N. E., De Kretser, D., and Leffers, H. (2006). Human exposure to phthalates via consumer products. *Int. J. Androl.* **29**, 134–139.
- Serrano, S. E., Braun, J., Trasande, L., Dills, R., and Sathyanarayana, S. (2014). Phthalates and diet: A review of the food monitoring and epidemiology data. *Environ. Heal. A Glob. Access Sci. Source* **13**, 43.
- Silva, M. J., Barr, D. B., Reidy, J. A., Malek, N. A., Hodge, C. C., Caudill, S. P., Brock, J. W., Needham, L. L., and Calafat, A. M. (2004). Urinary levels of seven phthalate metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. *Environ. Health Perspect.* **112**, 331–338.
- Staples, C. A., Peterson, D. R., Parkerton, T. F., and Adams, W. J. (1997). The environmental fate of phthalate esters: A literature review. *Chemosphere* **35**, 667–749.
- Teuten, E. L., Saquing, J. M., Knappe, D. R. U., Barlaz, M. A., Jonsson, S., Björn, A., Rowland, S. J., Thompson, R. C., Galloway, T. S., Yamashita, R., et al. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philos. Trans. R. Soc. B Biol. Sci.* **364**, 2027–2045.
- Wang, W., Craig, Z. R., Basavarajappa, M. S., Hafner, K. S., and Flaws, J. A. (2012). Mono-(2-ethylhexyl) phthalate induces oxidative stress and inhibits growth of mouse ovarian antral follicles. *Biol. Reprod.* **87**, 1–10.

- Yazdy, M. M., Coull, B. A., Gardiner, J. C., Aguiar, A., Calafat, A. M., Ye, X., Schantz, S. L., and Korrick, S. A. (2018). A possible approach to improving the reproducibility of urinary concentrations of phthalate metabolites and phenols during pregnancy. *J. Expo. Sci. Environ. Epidemiol.* **28**, 448–460.
- Zhou, C. and Flaws, J.A. (2017) Effects of an environmentally relevant phthalate mixture on cultured mouse antral follicles. *Toxicol. Sci.* **156**, 217–229.
- Zhou, C., Gao, L., and Flaws, J. A. (2017). Exposure to an environmentally relevant phthalate mixture causes transgenerational effects on female reproduction in mice. *Endocrinology* **158**, 1739–1754.
- Zhou, C., Gao, L., and Flaws, J. A. (2017). Prenatal exposure to an environmentally relevant phthalate mixture disrupts reproduction in F1 female mice. *Toxicol. Appl. Pharmacol.* **318**, 49–57.