TOXICOLOGICAL SCIENCES **125(2)**, 544–557 (2012)
doi:10.1093/toxsci/kfr315
Advance Access publication November 22, 2011

Genomic Biomarkers of Phthalate-Induced Male Reproductive Developmental Toxicity: A Targeted RT-PCR Array Approach for Defining Relative Potency

Bethany R. Hannas,* Christy S. Lambright,* Johnathan Furr,* Nicola Evans,* Paul M. D. Foster,† Earl L. Gray,*,[1] and Vickie S. Wilson*

*Reproductive Toxicology Branch, Toxicology Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711 and †Department of Health and Human Services, National Toxicology Program, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

[1]To whom correspondence should be addressed at Reproductive Toxicology Branch, Toxicology Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Mail Drop-72, Research Triangle Park, NC 27711. Fax: (919) 541-4017. E-mail: gray.earl@epa.gov.

Received September 20, 2011; accepted November 14, 2011

Male rat fetuses exposed to certain phthalate esters (PEs) during sexual differentiation display reproductive tract malformations due to reductions in testosterone (T) production and the expression of steroidogenesis- and INSL3-related genes. In the current study, we used a 96-well real-time PCR array containing key target genes representing sexual determination and differentiation, steroidogenesis, gubernaculum development, and androgen signaling pathways to rank the relative potency of several PEs. We executed dose-response studies with diisobutyl (DIBP), dipentyl (DPeP), dihexyl (DHP), diheptyl (DHeP), diisononyl (DINP), or diisodecyl phthalate (DIDP) and serial dilutions of a mixture of nine phthalates. All phthalates, with the exception of DIDP, reduced fetal testicular T production. Several genes involved in cholesterol transport, androgen synthesis, and *Insl3* also were downregulated in a dose-responsive manner by DIBP, DPeP, DHP, DHeP, DINP, and the 9-PE mixture. Despite speculation of peroxisome proliferator activated receptor (PPAR) involvement in the effects of PEs on the fetal testis, no PPAR-related genes were affected in the fetal testes by exposure to any of the tested PEs. Furthermore, the potent PPARα agonist, Wy-14,643, did not reduce fetal testicular T production following gestational day 14–18 exposure, suggesting that the antiandrogenic activity of PEs is not PPARα mediated. The overall sensitivity of the fetal endpoints (gene expression or T production) for the six phthalates from most to least was *Cyp11b1* > *Star* = *Scarb1* > *Cyp17a1* = T production > *Cyp11a1* = *Hsd3b* = *Insl3* > *Cyp11b2*. The overall potency of the individual phthalates was DPeP > DHP > DIBP ≥ DHeP > DINP. Finally, the observed mixture interaction was adequately modeled by the dose-addition model for most of the affected genes. Together, these data advance our understanding of the collective reproductive toxicity of the PE compounds.

Key Words: **mixture toxicity; antiandrogen; PPAR; phthalate risk assessment.**

Phthalate esters (PEs) are widely used compounds found in a variety of consumer products. Many of the PEs have been extensively characterized as developmental reproductive toxicants. *In utero* exposure to these PEs during the sexual differentiation period of rat development leads to reproductive tract malformations in androgen- and INSL3-dependent tissues (Barlow and Foster, 2003; Foster, 2006; McKinnell *et al.*, 2005; Mylchreest *et al.*, 1999; Parks *et al.*, 2000; Scott *et al.*, 2008; Welsh *et al.*, 2008; Wilson *et al.*, 2004), among other effects. Many studies have focused on elucidation of the mode of action behind this reproductive developmental toxicity and provided detailed histological, morphological, hormone level, and gene and protein expression level data (Andrade *et al.*, 2006; Barlow *et al.*, 2003; Bowman *et al.*, 2005; Howdeshell *et al.*, 2008; Johnson *et al.*, 2007; Lahousse *et al.*, 2006; Mahood *et al.*, 2005; Plummer *et al.*, 2007). Nevertheless, the proximate molecular target of phthalate action in the developing male fetus remains unidentified.

The adult male reproductive tract malformations associated with gestational exposure to phthalates during sexual differentiation include reduced anogenital distance (AGD), retained nipples, undescended testes, and decreased organ weights or agenesis of the epididymis, gubernaculum, glans penis, prostate, seminal vesicles, vas deferens, and testes (Gray *et al.*, 2000; Mylchreest *et al.*, 1998, 1999). Normal

development of these tissues is dependent upon Leydig cell (LC) hormones including androgens and INSL3. Therefore, a causal link has been drawn between the phthalate-induced reduction of testosterone (T) production during the critical developmental programming period and the postnatal malformations (Barlow and Foster, 2003; Hannas *et al*., 2011a; Mylchreest *et al*., 1998, 2000; Parks *et al*., 2000). Available fetal testis gene and protein expression data display effects on steroidogenic enzymes involved in testosterone synthesis including cytochrome P450 (CYP1) side-chain cleavage ($P450_{scc}$), 3β-hydroxysteroid dehydrogenase (3β-HSD), and CYP17α (Barlow *et al*., 2003; Foster *et al*., 1983; Howdeshell *et al*., 2008; Johnson *et al*., 2007; Lehmann *et al*., 2004; Thompson *et al*., 2005). Other related genes affected by *in utero* phthalate exposure include peripheral benzodiazepine receptor (*Bzrap1*), scavenger receptor class B type 1 (*Scarb1*), and steroid acute regulatory protein (*Star*), which are collectively involved in cholesterol uptake and transport into the cell and mitochondria (Gazouli *et al*., 2002; Plummer *et al*., 2007; Thompson *et al*., 2004). Finally, the gene product for insulin-like hormone three (INSL3), a hormone involved in normal testicular descent, is reduced by *in utero* phthalate exposure (Borch *et al*., 2006; McKinnell *et al*., 2005; Wilson *et al*., 2004).

Over the past several decades, there has been an ongoing debate with regards to the role of the peroxisome proliferator activated receptors (PPARs) in phthalate-induced reproductive toxicity. PPARs are nuclear receptors that play roles in regulating cellular differentiation, development, and metabolism, including cholesterol uptake and transport. Some groups have proposed that PEs interfere with testicular development and function or T production by interacting with PPARs (Bhattacharya *et al*., 2005; Boberg *et al*., 2008; Corton and Lapinskas, 2005; Gazouli *et al*., 2002; Plummer *et al*., 2007; Ward *et al*., 1998), potentially by interfering with the regulation of cholesterol transport (Borch *et al*., 2006). This hypothesis is based on data demonstrating that several antiandrogenic phthalate monoester metabolites are also activators of the PPAR receptors (Hurst and Waxman, 2003a,b; Lampen *et al*., 2003; Maloney and Waxman, 1999) and that many PPAR agonists reduce T production *in vitro* using LC cultures from adult rats (Biegel *et al*., 1995; Boberg *et al*., 2008; Borch *et al*., 2006; Gazouli *et al*., 2002; Liu *et al*., 1996). Ward *et al*. (1998) concluded that diethylhexyl phthalate (DEHP)–induced testicular toxicity in mice is PPARα-dependent because PPARα-null mice lacked testicular effects after 16 weeks of feeding on a DEHP-containing diet. These same mice, however, later developed delayed testicular toxicity through a PPARα-independent mechanism. Other groups have hypothesized that PPARβ/δ or PPARγ are potentially involved in phthalate-induced testicular toxicity (Corton and Lapinskas, 2005; Shipley and Waxman, 2004). Little data are available with regards to the expression of PPAR receptors and the potential role of phthalate-induced PPAR activation in the fetal testis during the sexual differentiation period. Therefore, in the current study, we also tested the hypothesis that PPARα activation with the potent agonist, Wy-14,643, during a critical period of sexual differentiation (gestational day, GD 14–18) would reduce fetal testicular T production, as is seen with phthalate exposure. Furthermore, we assessed expression of PPARα, β/δ, and γ, and a few PPAR-target genes in the fetal testes following phthalate exposure during GD 14–18.

To screen additional molecular pathways, which are potentially targeted by phthalates, we designed a custom PCR array containing genes that represent several candidate pathways involved in male sexual determination, differentiation, and development. Specifically, we targeted male testis cell differentiation and signaling, gubernaculum development, steroidogenesis, androgen receptor signaling and other nuclear receptors, Frizzled signaling; PPAR/RXR signaling, neurotropins, and inhibins and activins. Because many antiandrogenic phthalates downregulate expression of *Cyp11a*, *Hsd3b*, *Scarb1*, *Cyp17a*, and *Insl3*, we included these genes on the array as positive controls for the screening of additional less studied phthalates. Therefore, the results reported in the current study include dose-response data demonstrating that several phthalates, which have been shown to reduce fetal testicular testosterone production and also modulate expression levels of candidate target genes following 5-day exposure (GD 14–18).

Exposure data indicate that humans are exposed to multiple phthalates (Silva *et al*., 2004; Wittassek *et al*., 2007). Additionally, phthalate mixture-induced fetal and postnatal reproductive toxicity conforms to dose-additive mathematical models (Hotchkiss *et al*., 2004; Howdeshell *et al*., 2008; Rider *et al*., 2008), indicating that each PE in a mixture will contribute to the overall effect, if the total mixture dose exceeds the no observable adverse effect level. Together, these factors support the need to perform a cumulative risk assessment on these compounds. It has been proposed that dose-addition (DA) modeling is appropriate for use with compounds that all share a similar mechanism of action (Altenburger *et al*., 2000; Silva *et al*., 2002) or target a similar pathway/endpoint (Rider *et al*., 2010), and response addition (RA) is generally used for mixtures with chemicals that act independently or through different mechanisms of action (Greco *et al*., 1992). We previously demonstrated that the effects of a 9-PE mixture were better predicted using a DA model than an RA model for fetal *ex vivo* T production (Hannas *et al*., 2011b). Our current assessment will determine if this same 9-PE mixture similarly affects the fetal testicular genomic endpoints in a dose-additive manner, thereby further supporting cumulative risk assessment of this compound class.

Our previous studies (Hannas *et al*., 2011a,b) rank the potencies of certain antiandrogenic phthalates for reducing *ex vivo* fetal testicular T production when measured on GD 18 following gestational exposure during the sexual differentiation critical programming period of GD 14–18 (Carruthers and

Foster, 2005; Welsh *et al*., 2008). We determined that dipentyl phthalate (DPeP) was the most potent, followed by diisobutyl phthalate (DIBP), and that diisononyl phthalate (DINP) was least potent. In the current study, we built upon these data by determining the dose-response profiles of diheptyl phthalate (DHeP), dihexyl phthalate (DHP), and diisodecyl phthalate (DIDP) for T production. Based on compound structure and postnatal data, we hypothesized the following relative potencies: DPeP > DHP > DIBP ≥ DHeP > DINP and that DIDP would have no effect on T production. Additional objectives of the current study were to, first, determine if the individual phthalates classified as positive for antiandrogenic activity are acting through a similar mechanism by identifying affected genes on the screening PCR array. Second, determine if the individual PE potency for reducing T production correlates with the potency for the reduction of gene expression. Third, use dose-response modeling to predict the ability of a 9-PE mixture to reduce expression of affected genes and test the model predictions.

MATERIALS AND METHODS

Animals. Time-pregnant Sprague Dawley (SD) rats were purchased from Harlan Laboratories (Indianapolis, IN) and shipped on GD 1–2 (GD 0 = sperm positive). All rats were housed individually in 20 × 25 × 47 cm clear polycarbonate cages with laboratory-grade pine shavings for bedding. Environmental conditions were 22°C–23°C, 50–60% humidity, and 14L:10D light cycle (lights off at 9:00 P.M.). Animals were fed NIH 07 breeding diet for rats and were provided municipal drinking water (Durham, NC) *ad libitum*. Water provided to animals in the Environmental Protection Agency (EPA) Reproductive Toxicology Facility is filtered (5 μm), tested monthly for *Pseudomonas*, and tested every 4 months for a subset of pesticides, heavy metals, and other chemical contaminants. The animal use protocol for this study was approved by the National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee, and all studies were conducted at a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Doses and administration of chemicals. Separate experiments were conducted to assess the fetal endocrine responses of rats to DPeP, DIBP, or DINP exposure (as detailed in Hannas *et al*., 2011a,b) and DHeP, DHP, and DIDP. DPeP, DHP, and DHeP have 5-, 6-, and 7-carbon side-chain lengths, respectively. DIBP, DINP, and DIDP are mixtures of various isobutyl, isononyl, and isodecyl esters, respectively. DPeP (CAS 131-18-0, lot # 1431420) and DIBP (CAS 84-69-5; Lot # 07425BJ) were purchased from Sigma. Two separate formulations of DINP (referred to as DINP 1 and DINP 2) were used because they differ in the percent content of different ester side-chain structures, with DINP 1 having more isodecanol than DINP 2 (15–25 vs. 0%, respectively) and less methyl octanols (50–20 vs. 35–40%, respectively), and we therefore wanted to determine if they differed in toxicity to the fetal LC. DINP 1 (CAS 68515-48-0) is manufactured by the "Polygas" process. DINP 2 (CAS 28553-12-0) is *n*-butene based. DINP 2 was a gift from BASF (Badische Anilin und Soda Fabrik; CAS 28553-12-0), and DINP 1 was purchased from Sigma (CAS 68515-48-0). DHeP (CAS 3648-21-3; Lot # 125AG) and DHP (CAS 84-75-3, Lot # 139AG) were obtained from the National Toxicology Program (NTP) at the National Institute of Environmental Health Sciences (NIEHS, Research Triangle Park, North Carolina) where the purity of all tested phthalates was verified at 100% by gas chromatography with flame ionization detection. DIDP (CAS 26761-40-0; Lot # 1379769 23008238) was purchased from Fluka (Buchs, Switzerland).

Pregnant rats were weight ranked and assigned to dosage groups (generally $n = 3$–4) to minimize differences in means and variance among treatment groups. Sample sizes were based on power calculations performed on previous similarly conducted studies which demonstrated 3–4 litters per dosage group with three individual males/litter for T production, and the remaining males' testes pooled for messenger RNA (mRNA) analyses was sufficient for detecting significant dose-related reductions in T production and gene expression.

In the current study, rats were dosed orally on a daily basis during GD 14–18 (covering a critical programming period for male sexual differentiation) with increasing doses of DINP, DHP, DHeP, DIDP, DPeP, or DIBP in separate blocks (one block per PE with approximately 15 pregnant rats per block) except for the two DINP formulations, which were examined in three blocks. The dosage range for each PE was selected based on a combination of preliminary screening of each compound in our laboratory at a single high dosage level, dosage levels used in Howdeshell *et al*. (2008), and postnatal effect studies (Andrade *et al*., 2006; Borch *et al*., 2006; Foster *et al*., 1983; Gray *et al*., 2000; Saillenfait *et al*., 2008, 2009). Dosage levels were as follows: corn oil (vehicle control), 11, 33, 100, or 300 mg DPeP/kg/day; vehicle control, 100, 300, 600, or 900 mg DIBP, DHeP, or DHP/kg/day; and vehicle control, 500, 750, 1000, or 1500 mg DINP or DIDP/kg/day. All phthalates were administered in corn oil (2.5 ml vehicle/kg bw).

An additional study was conducted to determine if gene expression changes in the PPARα pathway were correctly identified with our custom arrays. We dosed pregnant rats with the well-characterized PPARα agonist Wy-14,643 (CAS 50892-23-4, purchased from Cayman Chemical, Ann Arbor, MI). In this block of the study, we also used DIBP (CAS 84-69-5; Lot # 07425BJ; Sigma) as a positive control for changes in testicular gene expression and to directly compare with the effects of Wy-14,643 on the maternal liver. Rats were dosed with 0, 50, 100, or 200 mg Wy-14,643/kg bw/day or 500 mg DiBP/kg bw/day in corn oil from GD 14–18, as detailed above.

Fixed ratio-dilution study with a mixture of 9-PEs. Harlan SD dams were dosed orally on each of GD 14–18 with one of six dilutions (0, 8, 17, 33, 67, and 100% of the top dose of 650 mg/kg/day total phthalate) of a mixture of DEHP, diisoheptyl phthalate (DIHP), DIBP, di-*n*-butyl phthalate (DBP), DHeP, DPeP, DHP, benzyl butyl phthalate (BBP; CAS 85-68-7; Lot No. 03405JH), and dicyclohexyl phthalate (DCHP; CAS 84-61-7; Lot No. 17518JB). The top dose of the mixture consisted of 10 mg DPeP/kg/day and 80 mg/kg/day of each of the other phthalates. The mixture ratio of the phthalates was designed such that each phthalate would contribute equally to the effects of the mixture on fetal testicular *ex vivo* T production, if the phthalates behaved in a dose-additive manner. The treatment groups contained 8, 3, 4, 7, 4, and 3 litters, respectively. ED50 and Hill slope values were determined from dose-response studies with DEHP, DHP, DIBP, DBP, DPeP (Hannas *et al*., 2011a,b, Howdeshell, Lambright, Furr, Evans, Foster, Wilson, and Gray, unpublished data), DHP, and DHeP, whereas BBP and DCHP were assumed equipotent to the mean values for all of the other PEs in the mixture.

Fetal necropsies. Dams were rapidly euthanized by decapitation on GD18, and fetuses were removed, anesthetized via hypothermia on ice, decapitated, and dissected under a Leica MZ6 dissecting microscope (Wetzlar, Germany). For all experiments, a single testis from the first three males identified in a litter were removed and used for *ex vivo* T production (data for DPeP, DIBP, and DINP, previously published in Hannas *et al*., 2011a,b). All remaining testes in each litter were pooled, immediately homogenized in TRI reagent (Sigma, St Louis, MO) on ice using a Kontes pestle homogenizer, and stored at −80°C until used to extract RNA. All necropsies began 1 h following administration of the final maternal dose and were conducted within a 2-h time frame between 08:00 and 10:00 A.M. Eastern Standard Time to avoid any potential confounding effects of fetal growth or time of day on the fetal endpoints.

Ex vivo *fetal testicular T production.* Following removal, fetal testes were immediately transferred individually into a single well on a 24-well plate containing 500 μl M-199 media without phenol red (Hazelton Biologics, Inc., St Lenexa, KS), supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (Hyclone Laboratories, Logan, UT). Testes were incubated for 3 h

at 37°C on a rotating platform. Following incubation, media was removed and stored at −80°C until used for T measurement. The level of T in the media samples was measured by radioimmunoassay according to the manufacturer's instructions (Diagnostic Products Corporation Coat-A-Count kits; Siemens Corp., Los Angeles, CA). The intraassay coefficient of variation was 3.1% (based on variability of the standard curve), and the interassay coefficient of variation was 13.7%. Cross-reactivity of the T antibody with dihydrotestosterone (DHT) was 3.2%. The limit of detection was 0.2 ng/ml for T.

Fetal testis gene expression analysis. RNA was extracted from the fetal testes homogenate as previously described (Wilson *et al.*, 2004) and cleaned to eliminate any potential genomic DNA contamination using Qiagen RNeasy Mini Kit (Valencia, CA) with the on-column DNase treatment step according to the manufacturer's instructions. RNA concentration and purity were determined with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). An additional genomic DNA elimination reaction and complementary DNA (cDNA) synthesis were performed on the RNA samples using the SABiosciences RT^2 First Strand Kit according to the kit instructions. For each individual sample, 300 ng of RNA was added to a single reaction, to be used across a 96-well array plate. The template cDNA was then added to RT^2 SYBR Green qPCR Master Mix (SABiosciences, Fredrick, MD), and 25 µl was added to each well of the plate. The 96-well gene array plate (purchased from SABiosciences) was custom designed to contain 89 individual target genes, 3 housekeeping genes (beta-actin, beta-glucuronidase, and lactate dehydrogenase), an interassay control, a genomic DNA control, a reverse transcription control, and a positive PCR control (genes listed in Fig. 3). To verify interassay consistency in C_T cycling, aliquots of fetal testicular cDNA from males of a corn oil (control)–treated dam were added to a well containing primers for the housekeeping gene beta-2 microglobulin on all plates.

The PCR reaction was run on an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA) using the following cycling parameters: one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Product purity was verified by melting curve analysis. The ΔC_T value for each gene was determined by dividing the gene C_T value by the mean C_T value of three housekeeping genes. The $2^{-\Delta\Delta C}{}_T$ method was used to analyze data and change in gene expression levels were reported as fold change (Tusher *et al.*, 2001) or the ratio of the phthalate treated sample group to the respective control group. The sensitivity, specificity, and reproducibility of the gene array system are discussed in detail by Arikawa *et al.* (2011).

Mixture model predictions. ED50 and Hill slope values derived from the individual phthalate dose-responsive effects on gene expression levels were used to calculate the mixture model response predictions for the 9-PE mixture on genes that were affected significantly by PE exposure. Because dose-response gene data were not available for DEHP, DIHP, DCHP, and BBP in the Harlan SD rat, the ED50 values for these four PEs were assumed equivalent to the average of the ED50s for DBP and DIBP. This assumption was based upon the observation that these PEs all appear to induce malformations at about the same dosage levels in postnatal studies. Predictions were calculated using the DA and RA models for comparison. Hill slope and ED50 values for DBP were obtained from a similarly conducted dose-response study (Howdeshell, Lambright, Furr, Evans, Foster, Wilson, and Gray, unpublished data).

Two separate approaches were taken to compare the DA and RA model predictions to the observed mixture effects. First, the ED50 values derived from the DA and RA model predictions were compared with the ED50 value derived from the observed data by determining if the predicted ED50 fell within the 99% confidence interval (CI) of the observed ED50. Any model prediction ED50 falling outside of the observed ED50 99% CI was considered significantly different from observed. The second approach for comparison was to force fit the observed data (means, SEs, and sample sizes) to the DA or RA models by constraining the top, bottom, Hill slope, and ED50 parameters to that of the DA or RA model parameters. A model that perfectly predicted the observed results would produce an R^2 value for the force fit that would be equal to the R^2 value obtained from the observed data best-fit model. Therefore, the greater the DA or RA predicted models deviated from the observed effects, the greater the R^2 of the force-fit model differed from the best-fit R^2 value.

Data analysis and statistics. Litter mean values were analyzed for T production, based on individual testis incubations. T production data were log10-transformed to correct for heterogeneity of variance and then analyzed with a one-way ANOVA model to detect significant differences in responses to DHeP, DHP, DIDP, or Wy-14,643 using PROC GLM in SAS, version 9.1 (SAS Institute, Cary, NC). *Post hoc t*-tests were performed with the LSMEANS procedure to compare individual doses when a significant overall dose effect was determined by the ANOVA model.

Gene expression was analyzed based on pooled testes samples from each litter. As seen in our previous study (Hannas *et al.*, 2011b) where the two DINP formulations did not differ significantly in their ability to reduce fetal testicular T production, DINP 1 was not more or less potent than DINP 2 in reducing testis gene expression. Therefore, in the current study, litter means are presented by combining the values from both formulations of DINP. Fold change for each gene following exposure to DPeP, DIBP, DINP, DHeP, DHP, DIDP, and Wy-14,643 was considered to be statistically significant different if the F value for the overall ANOVA model was $p < 0.01$, followed by t-test ($p < 0.01$) as above. Effects with a p value between $p < 0.05$ and $p > 0.01$ were considered as "equivocal" changes. Genes with a mean control C_T value > 33 were considered undetected.

Dose-response T production data were converted to percent of control and analyzed using a nonlinear four parameter regression analysis (sigmoidal fit with variable slope using Prism GraphPad 5.01 software; GraphPad Software, Inc., La Jolla, CA). The top and bottom parameters were constrained to 100 and 0% of control, respectively. Dose-response gene expression data were modeled as fold change using the sigmoidal fit with variable slope model, with the top constrained to 1 and the bottom constrained to 0. Potency comparisons between phthalates for individual genes and T production were made using the ED50 values derived from dose-response curves.

RESULTS

Maternal Body Weight and Litter Effects of DHP, DHeP, and DIDP

Maternal body weight gain and fetal mortality were not significantly affected by 5-day (GD 14–18) DHP, DHeP, or DIDP *in utero* exposure at any dose tested (Table 1). However, unlike the power to detect effects on T production and testis gene expression, which have power > 0.90 for the high-dose effects, the statistical power to detect reductions in litter size with an N of three dams per group is less than 0.80. As reported previously, DPeP, DIBP, and DINP, similarly did not induce maternal or fetal overt toxicity as defined by mortality, reduced maternal body weight or reduced litter size at any dose administered following GD 14–18 dosing (Hannas *et al.*, 2011a,b).

Ex vivo *Fetal Testicular T Production Dose-Response for DHP, DHeP, and DIDP*

Dose-response studies were performed to assess the relationship between *in utero* exposure (GD 14–18) to DHeP, DHP, and DIDP and fetal testicular *ex vivo* T production. Both DHeP and DHP reduced T production in a dose-responsive manner, whereas DIDP had no effect (Fig. 1). T Production was reduced significantly at doses of 600 mg DHeP/kg/day and greater and 100 mg DHP/kg/day and greater ($p < 0.05$). The ED50s derived from regression analysis of the dose-response

TABLE 1
Maternal Weight Gain and Fetus Survival Following Gestational Exposure From Day 14–18 to Increasing Doses of DHP, DHeP, DIDP, or Wy-14,643

DHP						
	Control	100	300	600	900	
Weight gain[a]	47.7 ± 6.1	53.2 ± 8.1	38.8 ± 8.9	46.7 ± 4.3	32.5 ± 3.2	
Number of live fetuses	11.7 ± 1.5	14.3 ± 2.2	8.7 ± 3.4	13.0 ± 1.0	7.7 ± 1.8	
Fetal survival[b]	89.7 ± 2.2	97.0 ± 3.0	73.3 ± 26.7	100.0 ± 0.0	53.9 ± 14.2	
DHeP						
	Control	100	300	600	900	
Weight gain	53.2 ± 12.0	34.6 ± 16.9	25.2 ± 2.1	43.8 ± 4.2	56.2 ± 2.8	
Number of live fetuses	12.3 ± 0.3	10.5 ± 1.5	3.7 ± 0.9	12.0 ± 1.5	15.7 ± 1.2	
Fetal survival	97.6 ± 2.4	90.9 ± 9.1	77.8 ± 14.7	91.7 ± 4.8	100.0 ± 0.0	
DIDP						
	Control	100	300	600	900	
Weight gain	48.7 ± 2.5	41.9 ± 1.4	37.2 ± 3.9	38.6 ± 2.0	41.5 ± 1.2	
Number of live fetuses	12.3 ± 2.2	13.7 ± 0.3	12.0 ± 2.1	13.3 ± 0.9	14.0 ± 0.6	
Fetal survival	93.3 ± 6.7	97.8 ± 2.2	100.0 ± 0.0	95.1 ± 2.5	100.0 ± 0.0	
Wy-14,643						
	Control	50 Wy	100 Wy	200 Wy	500 DIBP	
Weight gain	43.2 ± 3.1	55.1 ± 2.7	46.0 ± 3.7	46.0 ± 3.5	48.6 ± 2.8	
Number of live fetuses	12.3 ± 0.3	14.0 ± 1.0	11.0 ± 1.0	11.3 ± 1.2	14.7 ± 0.7	
Fetal survival	100.0 ± 0.0	97.6 ± 2.4	97.0 ± 3.0	100.0 ± 0.0	100.0 ± 0.0	
9-PE Mixture						
	Control	8%	17%	33%	67%	100%
Weight gain	43.8 ± 2.0	51.8 ± 1.3	4.4 ± 6.6	45.6 ± 5.6	36.1 ± 7.0	50.1 ± 5.5
Number of live fetuses	11.0 ± 0.9	13.0 ± 1.0	8.5 ± 2.5	12.7 ± 1.5	8.0 ± 2.6	13.3 ± 0.9
Fetal survival	88.4 ± 6.1	95.2 ± 4.8	77.3 ± 16.0	100.0 ± 0.0	85.0 ± 15.0	95.6 ± 4.4

Note. Values are means ± SEM.
[a]Maternal weight gain = Body weight GD 18 − Body weight GD 14.
[b]Fetal survival (%) = Number of live fetuses/total fetuses.

data for DHP and DHeP were 75.25 and 444.2 mg/kg/day, respectively (Table 2).

Wy-14,643 Maternal Liver Weight and Fetal Endocrine Effects

Overall maternal body weight gain was not affected by the DIBP or Wy-14,643 5-day (GD 14–18) dosing regimen (Table 1). Maternal liver weight was increased in all Wy-14,643 dosage groups but not by DIBP treatment (positive control for fetal endocrine effects; data not shown). DIBP decreased fetal testicular *ex vivo* T production at a dose of 500 mg/kg/day, whereas Wy-14,643 had no effect on T production (Fig. 2).

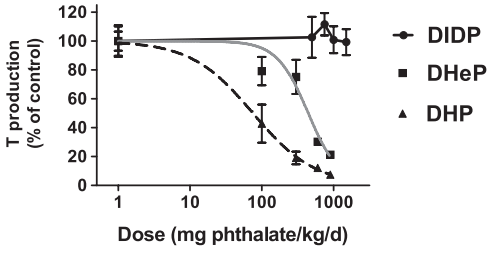


FIG. 1. Fetal testicular *ex vivo* T production following 3-h incubation from SD rats exposed *in utero* to DHeP, DHP, or DIDP during sexual differentiation on GD 14–18. Each data point represents the mean (± SEM) of 2–3 pooled litter values ($n = 2$ for 300 mg DHeP/kg/day group and 100 mg DHP mg/kg/day, $n = 3$ for all other groups).

Gene Array Screening of DHP, DIBP, DHeP, DPeP, DINP, and DIDP Fetal Testes

The effect of DHeP, DIBP, DHP, DPeP, DINP, and DIDP on gene expression following 5-day *in utero* exposure was assessed using a PCR array containing 89 individual candidate target genes (Fig. 3). All tested phthalates with the exception of DIDP were previously ranked (DPeP, DIBP, and DINP in Hannas *et al*., 2011a,b) or ranked in the current study (DHeP and DHP) as positive for antiandrogenic activity based on the ability to reduce *ex vivo* T production in the fetal testes. These PEs consistently reduced gene expression levels of *Cyp11b1*, *Scarb1*, *Star*, *Cyp11a1*, *Cyp17a1*, Insl3, and *Hsd3b* in a dose-responsive manner (Fig. 4, see Fig. 3 for lowest dose at which gene expression level reduction is significant). Comparison of the ED50 values for each of these genes across the positive phthalates demonstrated that DPeP was the most potent phthalate for reducing each gene and DINP was the least potent for most of the affected genes (Table 2). DHP, DHeP, and DIBP all had potencies in between that of DPeP and DINP for the affected genes. We ranked the overall sensitivity of each

TABLE 2
Potency Ranking of the Five Tested "Positive" PEs (first column) and the Nine Fetal Endpoints (first row), Based on the Mean ED50 Following 5-day *in utero* Exposure to Increasing Doses of 1 of 6 Phthalates (GD 14–18)

		1	2	3	4	5	6	7	8	9	
Rank (potency)	Rank (sensitivity)	*Cyp11b1*	*Star*	*Scarb1*	*Cyp17a1*	T production	*Cyp11a1*	*Hsd3b*	*Insl3*	*Cyp11b2*	PE mean
1	DPeP	25.7	43.3	34.8	58.3	45.96	60.9	62.9	73.9	159.1	62.8
2	DHP	88.6	54.1	85.6	118.8	75.3	266.7	185.1	141.6	371.7	154.2
3	DIBP	247.9	294.6	301.7	325.3	304.9	339.1	537.6	392.6		343.0
4	DHeP	183.7	359.6	371.7	405.9	444.2	487.5	655.6	500.8	711	457.8
5	DINP	326.3	596.6	601.8	796.5	852	1148	963	1488	2239	1001.2
	Gene mean	174.4	269.6	279.1	341.0	344.5	460.4	480.8	519.4	870.2	

Note. Shading denotes nongenomic endpoint.

gene endpoint and T production for the six phthalates and determined that the order of sensitivity from most to least was *Cyp11b1* > *Star* = *Scarb1* > *Cyp17a1* = T production > *Cyp11a1* = *Hsd3b* = *Insl3* > *Cyp11b2* (Table 2).

Some mRNA levels for additional genes were occasionally affected by one or two PEs but not consistently affected across all PEs and generally not in a dose-related manner (Fig. 3). Additional research would be required to determine if these "changes" were actually treatment-related or statistical vagaries.

The PPAR-related genes and target genes included on the plate were *Ppara*, *Pparb*, *Pparg*, *Rxra*, *Rxrb*, *Rxrg*, *Acox1*, *Cyp4a1*, *Fabp1*, and *Apoa1*. Of this gene set, DIBP downregulated *Rxrg* at the highest dosage level of 900 mg/kg/day, and DINP downregulated *Acox1* at the highest dose level of 1500 mg/kg/day. *Pparg*, *Cyp4a1*, and *Fabp1* were not detected in the fetal testes, based on a mean control C_T values > 33 (Fig. 3).

The PPARα agonist, Wy-14,643, induced several PPAR-regulated genes, as expected, including *Rxrg*, *Acox1*, *Cyp4a1*, *Fabp1*, *Adh1*, and *Aldh1a1* in the maternal livers following 5-day dosing at 50 mg/kg/day and greater (Fig. 5). Wy-14,643 induced *Rxrb* at 100 mg/kg/day and greater and Rxra at the highest dose of 200 mg/kg/day. *Cyp17a1*, *Tspo*, *Dixdc1*, and *Nr3c1* were also upregulated by this compound, whereas *Tle1* and *Inhba* were downregulated.

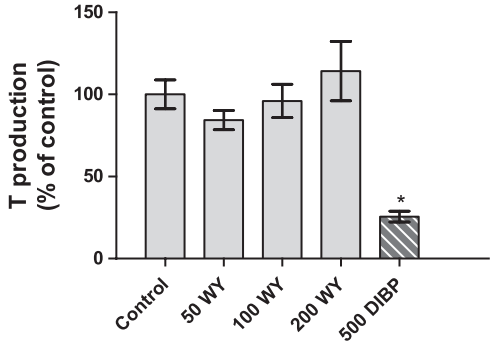


FIG. 2. Fetal testicular *ex vivo* T production following 3-h incubation from SD rats exposed *in utero* to Wy-14,643 during sexual differentiation on GD 14–18. Each data point represents the mean (± SEM) of three pooled litter values. An asterisk (*) denotes statistically different from control ($p < 0.01$).

Phthalate Mixture Gene Expression Results

A dose-response study was conducted using dilutions of the top dose of a 9-PE mixture to determine if response of the affected genes was more accurately predicted by a dose-additive or response-additive model. The mixture significantly reduced expression of (ED50 in mg/kg/day PE mixture shown in parentheses following each gene) *Star* (25.64), *Cyp11a* (34.61), *Hsd3b* (35.7), *Cyp11b2* (77.32), *Cyp17a1* (27.22), *Scarb1* (19.96), *Insl3* (46.23), *Cyp11b1* (14.21), *Lhcr7* (43.65), *Dhcr7* (103.7), *Inha* (75.64), and *Nr5a1* (170.8) in a dose-responsive manner (Fig. 6, data shown for seven of the genes). The mixture also upregulated several genes, including *Sry*, *Sox9*, *Ptgds2*, *Pdgfa*, *Fgf8*, *Inhbb*, *Dvl3*, *Rara*, and *Rarb* at 8 or 17% of the top dose, but these effects were generally not dose related (Fig. 3).

For three of the genes modeled for DA and RA predictions (*Cyp11b1*, *Cyp11a*, and *Cyp17a1*), the DA model was slightly more accurate in predicting the ED50 of the mixture of nine PEs than the RA model. DA provided a better fit to the mixture data than did the RA model with the ED50s being OBS < DA < RA, based upon lack of overlap of the observed data 99% CIs with the model ED50s (Fig. 6). Neither model was superior to the other in predicting the ED50 of the mixture for the following genes: *Scarb1* (the observed ED50 of the mixture was slightly and significantly lower than those predicted by both DA and RA models, with OBS < DA = RA), *Star* (RA = OBS = DA), *Hsd3b* (both model ED50 predictions differed slightly from the observed ED50 with RA < OBS < DA), and *Insl3* (both model predictions were lower than the observed ED50 with RA = DA < OBS). When the observed data were force fit by constraining all four parameters from the DA and RA logistic regression models, the R^2 values for DA exceeded that of the RA fit for two of seven genes (*Cyp11b1* and *Cyp11a*), whereas fitting the observed data to DA and RA model parameters provided similar R^2 values for the other five genes (Fig. 6).

Group	Gene	Refseq #	Cont CT	DHeP	DIBP	DHP	DPeP	DINP	9-PE mix	DIDP
1	Wt1	NM_031534	24.3							
1	Nr5a1	M_00105496	24.2						67%	
1	Lhx9	NM_181367	27.1						17%	
1	Gata4	NM_144730	25.2				300			
1	Emx2	XM_574698	27.4							
1	Lhx1	NM_145880	31.2							
2	Cbx2	XM_221185	27.0							
2	Dmrt1	NM_053706	26.1							
2	Dmrt2	XM_219927	31.4							
2	Sry	NM_012772	31.9						17%	
2	Sox9	XM_343981	26.1	600	900				8%	
2	Zfpm2	XM_235253	27.0							
2	Nr0b1	NM_053317	27.3				33	500		
2	Sox8	XM_220283	29.0							
2/4	Amhr2	NM_030998	27.0		900					
2	Rhox5	NM_022175	27.9							
2	Ptgds2	NM_031644	29.5						33%	
5	**Star**	NM_031558	22.7	600	300	100	11	500	17%	
5	**Cyp11a1**	NM_017286	21.7	600	300	300	11	500	17%	
5	**Cyp11b2**	NM_012538	31.8				100	500	33%	
5	**Hsd3b**	M_00104261	24.1	600	300	100	11	500		
5	**Cyp17a1**	NM_012753	21.3	600	300	100	33	500	17%	
5	Hsd17b3	NM_054007	25.4							
5	Lhcgr	NM_012978	26.0	600					33%	
5	**Scarb1**	NM_031541	22.5	300	300	100	11	500	8%	
5	Mapk3	NM_017347	25.4							
4	**Insl3**	NM_053680	21.7	600	300	100	33	500	33%	
5	Dhcr7	NM_022389	24.5	600			11	500	17%	
5	Tspo	NM_012515	26.8							
5	**Cyp11b1**	NM_012537	28.6	600	300	100	100	500	17%	
3	Dhh	XM_343327	27.5							
3	Pdgfa	NM_012801	28.9						8%	
3	Fgf9	NM_012952	28.3							
3	Fgf8	NM_133286	27.4						8%	
3	Ptch1	XM_345570	26.8	900						
3	Pdgfra	XM_214030	24.3							
3	Fog1	XR_007127	33.4							
3	Smo	NM_012807	26.5							
3	Tgfb1	NM_021578	25.6			900			100%	
4	Rhox10	M_00103758	27.2			600	300			
2	Hoxa2	NM_012581	33.6							
2	Wnt7a	XM_342723	31.6			600				900
10	Ntf3	NM_031073	29.1							
10	Ntrk3	NM_019248	31.7							
11	Inhbb	XM_344130	24.3						8%	
11	Inha	NM_012590	21.9	600		900			33%	
11	Acvr2b	NM_031554	27.7							
11	Inhba	NM_017128	26.5					1000		

FIG. 3. Gene expression changes in fetal testes on GD 18 following 5-day *in utero* exposure to increasing doses of one of six phthalates (GD 14–18). Group number corresponds to listed groups below heat map. Control threshold cycle (C_T) refers to the mean (± SEM) of control litter values across all phthalate blocks ($n = 19$) for comparison of relative expression. Control C_T values ≥ 33 were considered nondetectable and represented by black boxes. Red and yellow boxes indicate downregulation with $p < 0.01$ and $p < 0.05$, respectively, and green boxes represent upregulation of the gene with $p < 0.01$. The number inside the boxes indicates the lowest dose at which a significant change from control was detected. Gene names shown in bold were those that were downregulated by all phthalates that were positive for reducing fetal testicular T production.

DISCUSSION

In the current study, we evaluated the dose-related effects of several PEs on fetal testis gene expression using custom-designed 96-gene real-time (RT) PCR arrays. We compared the sensitivity of the affected genes to T production to identify potential genomic biomarkers of effect and exposure. These data indicate that the PEs which reduce T production act through a similar mode of action in the fetal testis, due to the consistency in reducing expression of a subset of genes involved in steroid transport and synthesis. The order of sensitivity from most to least affected genes was *Cyp11b1* > *Star* = *Scarb1* > *Cyp17a1* = T production > *Cyp11a1* = *Hsd3b* = *Insl3* > *Cyp11b2*. Interestingly, two of these consistently downregulated fetal testis genes, *Cyp11b1* and *Cyp11b2*, code for adrenal enzymes in the adult and are not present in adult LCs. Although several genes involved in androgen synthesis and steroid transport are dramatically downregulated in the fetal testis, genes in the PPARα pathway were not induced by any PE treatment, suggesting that this pathway is not involved in PE-induced fetal testis toxicity.

Group	Gene	Refseq #	Cont C_T	DHeP	DIBP	DHP	DPeP	DINP	9-PE mix	DIDP
8	Sfrp1	XM_224987	24.1							
8	Sfrp2	XM_227314	28.8							
8	Sfrp4	NM_053544	30.3							
8	Sfrp5	XM_219887	33.6							
6	Pcaf	NM_001024252	27.1							
7	Axin1	NM_024405	26.3							
7	Axin2	NM_024355	26.7							
7	Dixdc1	NM_001037654	28.0							
7	Dvl1	NM_031820	26.8							
7	Dvl2	XM_239254	27.2							
7	Dvl3	XM_221304	27.7						8%	
7	Tle1	XM_342851	25.9							
7	Tle2	NM_001039013	26.4							
7	Dkk3	NM_138519	29.9							
7	Dkk1	XM_219804	32.7							
7	RGD1563046	XR_008686	32.2							
6	Sra1	NM_183329	25.1							
6	Ar	NM_012502	28.4							
12	Esr1	NM_012689	27.7			100				
12	Esr2	NM_012754	30.7							
12	Vdr	NM_017058	32.2							
12	Nr4a2	NM_019328	29.3	900						
12	Nr1d1	NM_145775	27.9							
12	Nr3c2	NM_013131	30.3							
12	Nr3c1	NM_012576	26.5							
9	Pparg	NM_013124	35.4							
9	Ppard	NM_013141	28.1							
9	Ppara	NM_013196	28.1							
9	Rxra	NM_012805	25.8							
9	Rxrb	NM_206849	26.2							
9	Rxrg	NM_031765	31.9		900					
9	Rara	NM_031528	26.4						8%	
9	Rarb	XM_223843	29.4						17%	
9	Rarg	XM_217064	27.3							
9	Acox1	NM_017340	26.6					1500		
9	Cyp4a1	NM_175837	36.3							
9	Fabp1	NM_012556	34.6							
9	Apoa1	NM_012738	30.1							
14	Adh1	NM_019286	31.8							
14	Aldh1a1	NM_022407	22.7	900						
13	Pou5f1	NM_001009178	27.7							
15	Actb	NM_031144	19.8							
16	B2m	NM_012512	23.9							
15	Gusb	NM_017015	25.8							
15	Ldha	NM_017025	23.8							
16	RGDC	U26919	36.7							
16	RTC	SA_00104	22.9							
16	PPC	SA_00103	19.5							

1	Sex determination	9	PPAR/RXR signaling
2	Sex differentiation	10	neurotropins
3	Testis cell differentiation/signaling	11	inhibins/activins
4	Gubernaculum dev't/insl3-related	12	Other nuclear receptors
5	Steroidogenesis	13	cell differentiation marker
6	AR/coregulator proteins	14	metabolism
7	Frizzled signaling pathway	15	Housekeeping genes
8	Wnt signaling pathway	16	RT-PCR controls

Down-regulated (p<0.01)
Down-regulated (p<0.05)
Up-regulated (p<0.01)
No change
Undetected (Cont C_T>33)

FIG. 3. Continued

We determined that the order of potency for reducing expression of the affected genes was generally consistent across most of the genes with the order of potency for reducing T production (DPeP > DHP > DIBP ≥ DHeP > DINP; Table 2). We also demonstrated that DIDP was not an active antiandrogenic phthalate, as defined by the lack of an effect on fetal T production.

The relative potency of each phthalate for reducing gene expression was used to conduct a mixture study with nine phthalates in which we found that dose-addition models adequately predicted the mixture effects on the genes (being slightly and statistically superior to RA predictions for two of seven modeled genes and equal to RA predictions for the other five).

We previously evaluated the potencies of DPeP, DINP, DIBP, DEHP, and DIHP for reducing fetal testicular T production (Hannas *et al.*, 2011a,b). DPeP is the most potent phthalate tested to date for reducing T production and inducing postnatal reproductive tract malformations (Hannas *et al.*, 2011a) and testicular toxicity in the pubertal male model (Foster *et al.*, 1980). Based on the results of the current study, DHP is the second most potent for reduction of fetal T production. DHP induces malformations of the reproductive tract in male rat offspring, reduces AGD and induces

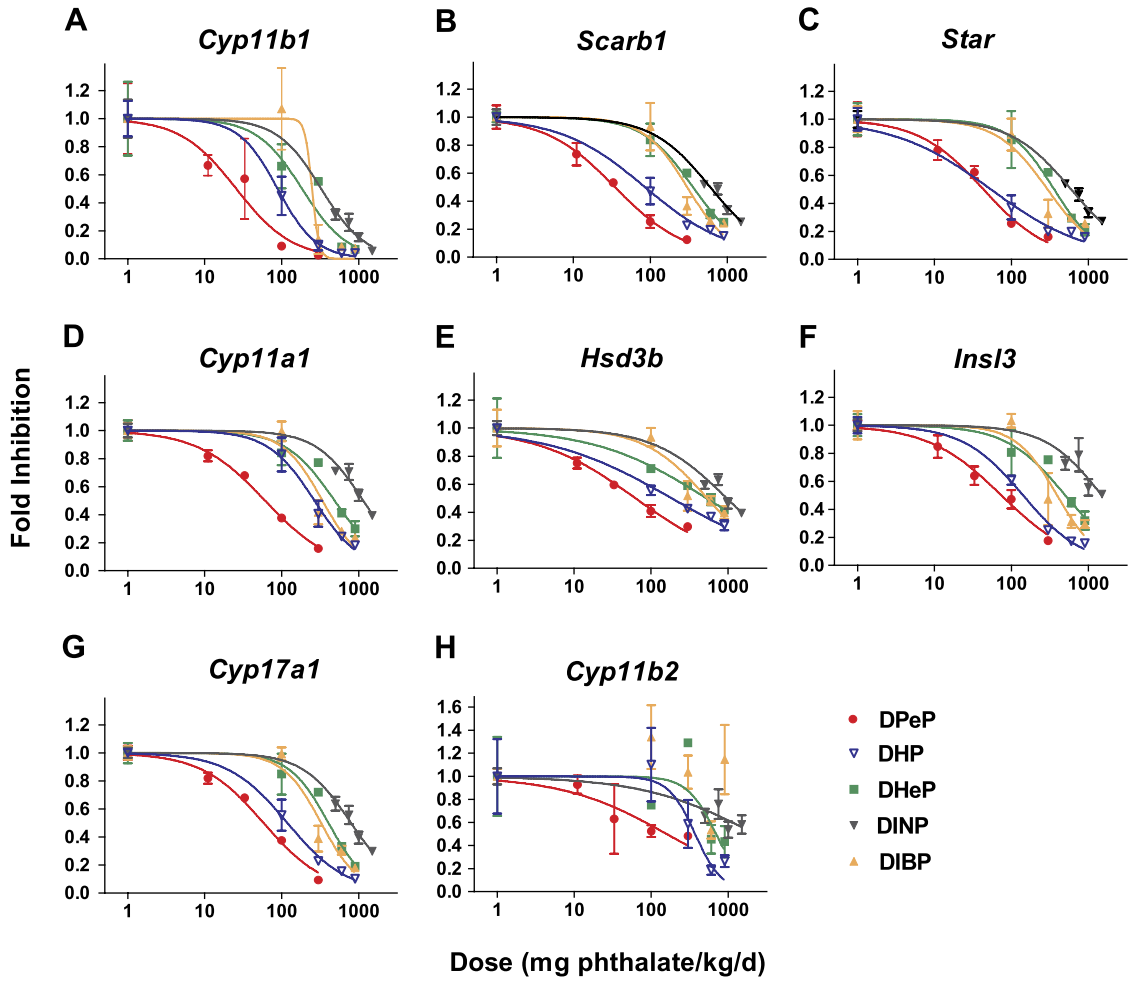


FIG. 4. Dose-response curves for fold inhibition of fetal testis RNA expression levels for (A) *Cyp11b1*, (B) *Scarb1*, (C) *Star*, (D) *Cyp11a1*, (E) *Hsd3b*, (F) *Insl3*, (G) *Cyp17a1*, and (H) *Cyp11b2* on GD 18 following *in utero* exposure (GD 14–18) to DPeP, DIBP, DHP, DHeP, or DINP. Each data point represents the mean (± SEM) of pooled litters (n = 3–4 l). The $2^{-\Delta\Delta C}{}_T$ method was used to analyze data and change in gene expression levels are reported as fold change.

permanent female-like nipples (Saillenfait *et al.*, 2009) at slightly higher dosage levels than does DPeP. When DHP is administered to pregnant SD rats (GD 12–21) at doses of 0, 50, 125, 250, or 500 mg/kg/day, AGD was reduced and areola/nipples were retained (in infants and adults) at 250 and 500 mg DHP/kg/day. In addition, low incidences of severe reproductive malformations were observed in young adult males at 125 and 250 mg DnHP/kg/day, whereas most males were malformed at 500 mg/kg/day. We previously determined that the ED50 for reduction of male AGD on PND 2 by DPeP was 252.3 mg/kg/day (Hannas *et al.*, 2011a) with severe malformations occurring at lower doses (Gray, personal communication). Together, these data indicate that the potency of DHP appears to verge upon that of DPeP for inhibiting differentiation of the reproductive tract of the fetal male rat.

DHeP, which was less potent than both DPeP and DHP for reducing T production and testis gene expression, has one and two additional carbons in the ester side chain compared with DHP and DPeP, respectively. Saillenfait *et al.* (2011) demonstrated that DHeP only reduced AGD in male fetuses exposed *in utero* (GD 6–20) at doses of 500–1000 mg/kg/day administered orally to the dam, whereas testis descent was normal for this stage of development in all dosage groups.

Our observations that DIDP was negative and DINP was the least potent active PE for reducing T production and testis gene expression are consistent with published data on the ability of these PEs to induce the Phthalate Syndrome in male rats. For example, DINP only reduces AGD (Boberg *et al.*, 2011) and induces reproductive tract malformations (Boberg *et al.*, 2011; Gray *et al.*, 2000) at dosage levels of 900 and 750 mg/kg/day, respectively. DIDP, which is a mixture of isomers containing two carbon chains with 9–11 carbons, was inactive in the current fetal screening protocol. This result was expected based on both its structure and the lack of effect on reproductive endpoints examined in two separate two-generation studies (Hushka *et al.*, 2001). In those studies, the male offspring (F1) of Sprague-Dawley rats (F0) administered DIDP in feed from

	DAM LIVERS	
Gene	Control C_T	Reg
Wt1	33.3	
Nr5a1	35.9	
Lhx9	ND	
Gata4	29.7	
Emx2	ND	
Lhx1	32.5	
Cbx2	31.8	
Dmrt1	35.7	
Dmrt2	36.8	
Sry	ND	
Sox9	33.9	
Zfpm2	34.3	
Nr0b1	ND	
Sox8	33.6	
Amhr2	32.9	
Rhox5	31.8	
Ptgds2	28.4	
Star	34.3	
Cyp11a1	36.2	
Cyp11b2	ND	
Hsd3b	ND	
Cyp17a1	29.7	50
Hsd17b3	37.8	
Lhcgr	35.3	
Scarb1	26.3	
Mapk3	28.4	
Insl3	36.3	
Dhcr7	24.6	
Tspo	28.8	50
Cyp11b1	ND	
Dhh	33.1	
Pdgfa	32.3	
Fgf9	35.6	
Fgf8	30.1	
Ptch1	37.5	
Pdgfra	30.0	
Fog1	ND	
Smo	32.7	
Tgfb1	28.7	
Rhox10	ND	
Hoxa2	32.9	
Wnt7a	ND	
Ntf3	30.3	
Ntrk3	ND	
Inhbb	34.5	
Inha	36.6	
Acvr2b	31.7	
Inhba	27.8	200

	DAM LIVERS	
Gene	Control C_T	Reg
Sfrp1	33.2	
Sfrp2	34.9	
Sfrp4	37.3	
Sfrp5	36.8	
Pcaf	27.4	
Axin1	28.7	
Axin2	29.8	
Dixdc1	28.8	100
Dvl1	25.9	
Dvl2	30.3	
Dvl3	31.3	
Tle1	28.5	
Tle2	29.6	
Dkk3	33.8	
Dkk1	ND	
RGD1563046	ND	
Sra1	25.4	
Ar	34.0	
Esr1	27.7	
Esr2	37.1	
Vdr	36.7	
Nr4a2	30.9	
Nr1d1	31.0	
Nr3c2	29.8	
Nr3c1	26.6	100
Pparg	33.9	
Ppard	28.2	
Ppara	26.7	
Rxra	24.4	200
Rxrb	29.4	100
Rxrg	27.6	50
Rara	29.3	
Rarb	27.1	
Rarg	30.0	
Acox1	24.1	50
Cyp4a1	25.7	50
Fabp1	18.8	50
Apoa1	18.8	
Adh1	20.4	50
Aldh1a1	25.2	50
Pou5f1	37.0	
Actb	21.5	
B2m	24.4	
Gusb	26.1	
Ldha	22.7	
RGDC	ND	
RTC	23.6	
PPC	19.4	

Down-regulated (p<0.01)
Down-regulated (p<0.05)
Up-regulated (p<0.01)
No change
Expression undetected (Cont C_T>33)

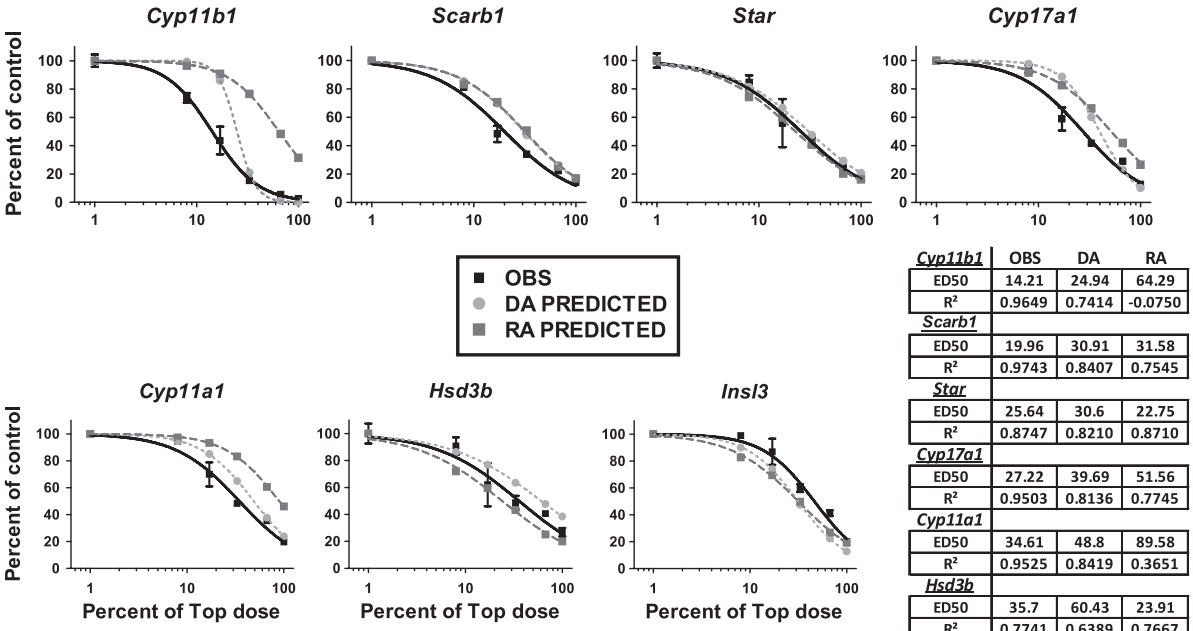
FIG. 5. Gene expression changes in maternal livers and fetal testes on GD 18 following 5-day *in utero* exposure to increasing doses of Wy-14,643 (GD 14–18). Control C_T refers to the mean (± SEM) of control litter values ($n = 3$) for comparison of relative expression. Control C_T values ≥ 33 were considered nondetectable and represented by black boxes. Red and yellow boxes indicate downregulation with $p < 0.01$ and $p < 0.05$, respectively, and green boxes represent upregulation of the gene with $p < 0.01$. The number inside the boxes indicates the lowest dose at which a significant change from control was detected.

10 weeks prior to mating through female lactation, displayed no reproductive abnormalities.

Taken together, these results demonstrate that our fetal T production and gene expression findings are predictive of postnatal androgen- and INSL3-dependent tissue malformations. Numerous studies have demonstrated the ability of PE's to reduce testicular T levels (Howdeshell *et al.*, 2008; Lehmann *et al.*, 2004; Shultz *et al.*, 2001), which occurs through a nonandrogen receptor (AR)–mediated mechanism (Parks *et al.*, 2000). Additionally, expression of *Insl3* is downregulated by antiandrogenic PEs (Howdeshell *et al.*, 2008; Wilson *et al.*, 2004). In the current study, the tested PE's that were classified as positive for reducing fetal testicular T production were all consistent in downregulating gene expression of steroidogenic enzymes, steroid regulatory, and transport proteins (Fig. 7) and *Insl3*. Some of these effects on T production and gene expression were expected based on previous work demonstrating that DBP downregulated many of these genes including *Star*, *Cyp11a1*, *Insl3*, *Scarb1*, *Hsd3b*, and *Cyp17a1* (Barlow *et al.*, 2003; Howdeshell *et al.*, 2008; Johnson *et al.*, 2007; Lahousse *et al.*, 2006; Lehmann *et al.*, 2004; Plummer *et al.*, 2007; Shultz *et al.*, 2001; Wilson *et al.*, 2004). Nevertheless, only a few of these studies have included PEs other than DBP (Howdeshell *et al.*, 2008) or included a sufficient range of doses to be able to determine ED50s or relative potency values for these genomic endpoints. Results of the current study also clearly indicate that all PEs that induce the phthalate syndrome alter fetal LC function via a common endocrine and genomic mode of action and that the overall potency in inducing fetal testis alterations is predictive of their potency to induce reproductive tract malformations.

We derived two important comparisons from the dose-response data generated using the PCR gene array: (1) comparison of the individual phthalate potency for reducing expression of a particular gene and (2) comparison of the sensitivity of different genes to phthalate exposure. In comparing phthalate potency, we previously demonstrated a link between the reduction of fetal T production and early postnatal male reproductive tract malformations following dosing during the sexual differentiation period by determining that the potency of DPeP for the fetal endocrine endpoints was predictive of the postnatal endocrine endpoints (Hannas *et al.*, 2011a). Here, we further demonstrate that phthalate effects on expression of androgen-related genes are linked to reduced T production, based not only on the biological relevancy but also on the congruency in phthalate potency between the endpoints. We thereby further assume that these gene endpoints are also predictive of the postnatal malformations.

The second comparison we made as mentioned above was between the sensitivity of the different affected endpoints to each phthalate. In general, the order of most sensitive to least was consistent for all phthalates. A few of the genomic endpoints ranked more sensitive to PE disruption than T production, including *Cyp11b1*, *Scarb1*, and *Star*, although the difference



Cyp11b1	OBS	DA	RA
ED50	14.21	24.94	64.29
R^2	0.9649	0.7414	-0.0750
Scarb1			
ED50	19.96	30.91	31.58
R^2	0.9743	0.8407	0.7545
Star			
ED50	25.64	30.6	22.75
R^2	0.8747	0.8210	0.8710
Cyp17a1			
ED50	27.22	39.69	51.56
R^2	0.9503	0.8136	0.7745
Cyp11a1			
ED50	34.61	48.8	89.58
R^2	0.9525	0.8419	0.3651
Hsd3b			
ED50	35.7	60.43	23.91
R^2	0.7741	0.6389	0.7667
Insl3			
ED50	46.23	31.36	31.55
R^2	0.9421	0.9409	0.7603

FIG. 6. Gene expression changes in fetal testes on GD 18 following 5-day *in utero* exposure to a dose range of a 9-PE mixture including DEHP, DIHP, DIBP, DBP, BBP, DNCP, DHeP, DHP, and DPeP. The top dose contained a total of 650 mg phthalate/kg/day including 10 mg DPeP/kg/day and 80 mg/kg/day of all other phthalates. Observed (OBS) values are litter means (± SEM) of 3–4 l. RA and DA models are shown for each gene. The ED50 values are derived from the logistic regression model for each fit of the data. The R^2 values describe the percentage of variance described when the OBS data were force fit to each model parameters, using the respective ED50 and Hill slope values for the RA or DA models.

among *Scarb1*, *Star*, and T production was not great. *Cyp17a1* was as sensitive to PE disruption as was T production.

Currently, T production is being considered as a critical endpoint for some phthalate risk assessments. Nevertheless, the findings of this study could potentially support the use of genomic endpoints as the critical effect in future risk assessments, with a few caveats. When considering gene expression as the most sensitive endpoint of phthalate exposure, it is critically important to consider the biological role of the products of the genes. This point is illustrated when taking into account the role of the most sensitive genes detected in our assessment. The "most" sensitive gene detected in the fetal testis, *Cyp11b1*, does not appear to be biologically linked to the postnatal outcomes of concern. CYP11B1, also known as 11β-hydroxylase, is an enzyme responsible for conversion of 11-deoxycortisol to cortisol in the adrenal cortex. It is not expressed in adult testes. On the contrary, recent studies, which examined mouse fetal testes, have demonstrated that a subpopulation of steroidogenic cells that express *Cyp11b1* occur in the fetal LC (Hu *et al*., 2007; Val *et al*., 2006). Hu *et al*. (2007) determined that although *Cyp11b1* gene expression was detected, the protein product was not detected by immunohistochemistry, and enzyme activity was also not detected. The authors of this study suggest that translation of the enzyme product is suppressed to prevent high levels of corticosteroid production in the fetal testes. Although our results demonstrate that message of this gene in the fetal testes is highly susceptible to phthalate exposure, this vulnerability is not likely to translate into the postnatal phthalate effects, and therefore, *Cyp11b1* gene expression would not be a suitable critical effect endpoint in risk assessment. Likewise, *Cyp11b2*, another adrenal enzyme gene detected in the fetal testes, is not likely linked to the postnatal reproductive tract toxicity. In contrast, SR-B1, StAR, and Cyp17A1 are all critical for normal testosterone synthesis. SR-B1 protein facilitates cholesterol uptake into steroidogenic cells, whereas StAR acts as the transport protein for cholesterol across mitochondrial membranes. Cyp17A1 is the steroidogenic enzyme that converts progesterone to the androgen androstenedione, which is then converted to testosterone by another enzyme (Fig. 7). It is reasonable to assume that the vulnerability of these genes is linked to the postnatal reproductive malformations we detect following *in utero* phthalate exposure because the products of these genes feed directly into production of T during the critical period for androgen-dependent tissue development. Furthermore, the ED50 values for the effects of PEs on *Scarb1*, *Star*, and *Cyp17a1* gene expression do not differ greatly from the ED50 value for T production, suggesting that these genomic biomarkers could be considered additional sensitive critical or supportive endpoints for PE risk assessments.

In order to verify that our custom PCR arrays responded appropriately to activation of PPARα, we determined that the

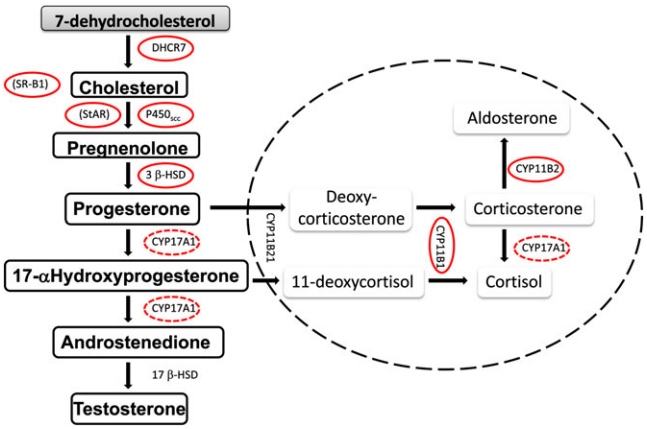


FIG. 7. Steroid biosynthesis pathways of the testes (pathway outside dashed circle) and adrenals (pathway inside dashed circle). Steroidogenesis-related enzymes and transport proteins (noted by parentheses) affected by *in utero* phthalate exposure are circled. Dashed lines around CYP17A1 signify this enzyme acting on multiple steps.

potent PPARα agonist, Wy-14,643, was effective in increasing liver weights of the exposed dams and upregulating genes in the PPARα pathway including: *Rxra*, *Rxrb*, *Rxrg*, *Acox1*, *Cyp4a1*, and *Fabp1*. In contrast, in the same experiment, DIBP had no effect on these genes. Furthermore, we confirmed that Wy-14,643 had no effect on fetal testicular T production, whereas DIBP significantly reduced T production. From this experiment, we conclude that insensitive methodology is not the cause for the lack of phthalate-induced activation of PPARα target genes in the fetal testis.

We did not detect any *Pparg* expression in the fetal testis, also eliminating this as a likely PE pathway for PE-induced reproductive toxicity. Furthermore, *in utero* administration of rosiglitazone, a potent PPARγ agonist, did not reduce fetal AGD, T levels, and *Insl3* or steroidogenic gene expression or induce histopathological changes in the fetal testis, whereas DIBP administration induced all of these affects (Boberg *et al.*, 2008). We did not specifically test the effects of a PPARβ agonist on fetal T production; however, there is currently no evidence to suggest that PPARβ is involved in testicular toxicity. The existing data do not support the hypothesis that activation of PPARα or PPARγ pathways is involved in the effects of PEs on sexual differentiation of the male rat.

Despite lack of effect on the PPARα pathway, the current study provides evidence supporting the hypothesis that phthalate exposure reduces T production by interfering with cholesterol regulation. This mode of action can be inferred based on the consistency of the antiandrogenic phthalates in reducing *Star* and *Scarb1* gene expression as well as the reduction of *Dhcr7* at relatively high doses of DPeP, DHP, and DINP. DHCR7 or 7-dehydrocholesterol is the enzyme, which mediates the final step in cholesterol production. As previously mentioned, SR-B1 and StAR are involved in transport of cholesterol into the cell and mitochondria, respectively, as the precursor to testosterone. Johnson *et al.* (2007) detected reduced *Star* and *Dhcr7* expression following 3 h of *in utero* exposure (GD 19) to 10 or greater and 100 or greater mg DBP/kg, respectively. Plummer *et al.* (2007) also demonstrated downregulation of *Scarb1* and *Star* expression by DBP in a time-dependent fashion between GD 15.5 and 19.5. In light of these collective results, upstream genes in this pathway and earlier time points and/or short phthalate exposure durations during the critical period warrant further investigation.

We previously determined that our 9-PE mixture reduces fetal testicular T production in a dose-additive manner (Hannas *et al.*, 2011b). The impetus behind that study was to provide data to support the recommendation provided to the U.S. EPA by the National Academy of Science National Research Council committee that a cumulative assessment be conducted for antiandrogenic phthalates (National Academy of Sciences, 2008). In the current study, we further assessed fetal testicular samples for gene expression changes to determine if the effects could similarly be modeled using a DA mixture model. The mixture was designed so that each of the nine phthalates would contribute equally in terms of potency for reducing fetal T production if they acted in a dose-additive manner. The top dose was expected to dramatically reduce T production and gene expression if the effects of the nine PEs were dose additive. Based on the data from this mixture study, we can conclude that the DA model adequately predicted the observed values for all seven genes that showed consistent dose-related PE-induced downregulation. The DA model was slightly superior to the RA model for two of seven genes, whereas the models were roughly equivalent in their ability to predict the ED50 of the other five genes.

In conclusion, we used a targeted RT-PCR array approach of toxicity assessment in an attempt to address several of the challenges faced in the human health risk assessment process related to phthalate exposure. Based on the results, we confirmed that the antiandrogenic phthalates we assessed act through a similar mode of toxicity, despite not yet fully understanding the proximate molecular target. We additionally demonstrated that the rank of potency of the individual phthalates largely translates from reduction of T production to the downregulation of gene expression, suggesting that most of the consistently downregulated genes from our array plate are directly linked to the postnatal reproductive tract malformations. Finally, we demonstrated that the targeted genomic response of the fetal testis to a mixture of nine antiandrogenic phthalates was predicted using a DA mathematical model, supporting the notion that a cumulative risk assessment of the phthalates would be most protective of human health as compared with assessments of individual phthalates. Using this targeted gene array approach, we can continue to investigate the behavior of phthalates in male fetuses during a critical period of development by focusing on timing within the sexual

differentiation period and additional target genes/pathways in future assessments.

FUNDING

This project was provided by an Interagency Agreement between the United States Environmental Protection Agency and the NTP at the National Institute of Environmental Health Sciences (IA no. 75-92285501-1) and B.R.H. was funded through a National Academy of Sciences postdoctoral fellowship.

ACKNOWLEDGMENTS

The authors would like to acknowledge Mary Cardon and Hunter Sampson for assistance during necropsies.

REFERENCES

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., and Grimme, L. H. (2000). Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: Mixtures composed of similarly acting chemicals. *Environ. Toxicol. Chem.* **19,** 2341–2347.

Andrade, A. J., Grande, S. W., Talsness, C. E., Grote, K., Golombiewski, A., Sterner-Kock, A., and Chahoud, I. (2006). A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): Effects on androgenic status, developmental landmarks and testicular histology in male offspring rats. *Toxicology* **225,** 64–74.

Arikawa, E., Quelhorst, G., Han, Y., Pan, H., and Yang, J. (2011). RT^2 Profiler^{TM} PCR Arrays: Pathway-focused gene expression profiling with qRT-PCR. *SABiosciences*. Available at: http://www.sabiosciences.com/manuals/pcrarraywhitepaper.pdf. Accessed June 1, 2011.

Barlow, N. J., and Foster, P. M. (2003). Pathogenesis of male reproductive tract lesions from gestation through adulthood following in utero exposure to di(n-butyl) phthalate. *Toxicol. Pathol.* **31,** 397–410.

Barlow, N. J., Phillips, S. L., Wallace, D. G., Sar, M., Gaido, K. W., and Foster, P. M. (2003). Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. *Toxicol. Sci.* **73,** 431–441.

Bhattacharya, N., Dufour, J. M., Vo, M. N., Okita, J., Okita, R., and Kim, K. H. (2005). Differential effects of phthalates on the testis and the liver. *Biol. Reprod.* **72,** 745–754.

Biegel, L. B., Liu, R. C. M., Hurtt, M. E., and Cook, J. C. (1995). Effects of ammonium perfluorooctanoate on Leydig-cell function: In vitro, in vivo, and ex vivo studies. *Toxicol. Appl. Pharmacol.* **134,** 18–25.

Boberg, J., Christiansen, S., Axelstad, M., Kledal, T. S., Vinggaard, A. M., Dalgaard, M., Nellemann, C., and Hass, U. (2011). Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats. *Reprod. Toxicol.* **31,** 200–209.

Boberg, J., Metzdorff, S., Wortziger, R., Axelstad, M., Brokken, L., Vinggaard, A. M., Dalgaard, M., and Nellemann, C. (2008). Impact of diisobutyl phthalate and other PPAR agonists on steroidogenesis and plasma insulin and leptin levels in fetal rats. *Toxicology* **250,** 75–81.

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

Bowman, C. J., Turner, K. J., Sar, M., Barlow, N. J., Gaido, K. W., and Foster, P. M. (2005). Altered gene expression during rat Wolffian duct development following di(n-butyl) phthalate exposure. *Toxicol. Sci.* **86,** 161–174.

Carruthers, C. M., and Foster, P. M. D. (2005). Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. *Birth Defects Res. B Dev. Reprod. Toxicol.* **74,** 277–285.

Corton, J. C., and Lapinskas, P. J. (2005). Peroxisome proliferator-activated receptors: Mediators of phthalate ester-induced effects in the male reproductive tract? *Toxicol. Sci.* **83,** 4–17.

Foster, P. M. (2006). Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int. J. Androl.* **29,** 140– 147; discussion 181–185.

Foster, P. M., Thomas, L. V., Cook, M. W., and Gangolli, S. D. (1980). Study of the testicular effects and changes in zinc excretion produced by some n-alkyl phthalates in the rat. *Toxicol. Appl. Pharmacol.* **54,** 392–398.

Foster, P. M., Thomas, L. V., Cook, M. W., and Walters, D. G. (1983). Effect of di-n-pentyl phthalate treatment on testicular steroidogenic enzymes and cytochrome P-450 in the rat. *Toxicol. Lett.* **15,** 265–271.

Gazouli, M., Yao, Z. X., Boujrad, N., Corton, J. C., Culty, M., and 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 alpha. *Endocrinology* **143,** 2571–2583.

Gray, L. E., Jr, Ostby, J., Furr, J., Price, M., Veeramachaneni, D. N., and Parks, L. (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol. Sci.* **58,** 350–365.

Greco, W., Unkelbach, H.-D., Pöch, G., Sühnel, J., Kundi, M., and Boedeker, W. (1992). Consensus on concepts and terminology for combined action assessment: The Saariselkä agreement. *Arch. Complex Environ. Studies* **4,** 65–69.

Hannas, B. R., Furr, J., Lambright, C. S., Wilson, V. S., Foster, P. M. D., and Gray, L. E. (2011a). Dipentyl phthalate dosing during sexual differentiation disrupts fetal testis function and postnatal development of the male Sprague Dawley rat with greater relative potency than other phthalates. *Toxicol. Sci.* **120,** 184–193.

Hannas, B. R., Lambright, C. S., Furr, J., Howdeshell, K. L., Wilson, V. S., and Gray, L. E., Jr. (2011b). Dose-response assessment of fetal testosterone production and gene expression levels in rat testes following in utero exposure to diethylhexyl phthalate, diisobutyl phthalate, diisoheptyl phthalate, and diisononyl phthalate. *Toxicol. Sci.* **123,** 206–216.

Hotchkiss, A. K., Parks-Saldutti, L. G., Ostby, J. S., Lambright, C., Furr, J., Vandenbergh, J. G. and Gray, L. E., Jr. (2004). A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biol. Reprod.* **71,** 1852–1861.

Howdeshell, K. L., Rider, C. V., Wilson, V. S., and Gray, L. E. (2008). Mechanisms of action of phthalate esters, individually and in combination, to induce abnormal reproductive development in male laboratory rats. *Environ. Res.* **108,** 168–176.

Hu, L., Monteiro, A., Johnston, H., King, P. and O'Shaughnessy, P. J. (2007). Expression of Cyp21a1 and Cyp11b1 in the fetal mouse testis. *Reproduction* **134,** 585–591.

Hurst, C. H., and Waxman, D. J. (2003a). Activation of PPAR alpha and PPAR gamma by environmental phthalate monoesters. *Toxicol. Sci.* **74,** 297–308.

Hurst, C. H., and Waxman, D. J. (2003b). Trans-activation of PXR by phthalate monoesters. *Toxicol. Sci.* **72,** 176.

Hushka, L. J., Waterman, S. J., Keller, L. H., Trimmer, G. W., Freeman, J. J., Ambroso, J. L., Nicolich, M., and McKee, R. H. (2001). Two-generation reproduction studies in rats fed di-isodecyl phthalate. *Reprod. Toxicol.* **15,** 153–169.

Johnson, K. J., Hensley, J. B., Kelso, M. D., Wallace, D. G., and Gaido, K. W. (2007). Mapping gene expression changes in the fetal rat testis following acute dibutyl phthalate exposure defines a complex temporal cascade of responding cell types. *Biol. Reprod.* **77,** 978–989.

Lahousse, S. A., Wallace, D. G., Liu, D. L., Gaido, K. W., and Johnson, K. J. (2006). Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl) phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages. *Toxicol. Sci.* **93,** 369–381.

Lampen, A., Zimnik, S., and Nau, H. (2003). Teratogenic phthalate esters and metabolites activate the nuclear receptors PPARs and induce differentiation of F9 cells. *Toxicol. Appl. Pharmacol.* **188,** 14–23.

Lehmann, K. P., Phillips, S., Sar, M., Foster, P. M., and 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.

Liu, R. C. M., Hahn, C., and Hurtt, M. E. (1996). The direct effect of hepatic peroxisome proliferators on rat Leydig cell function in vitro. *Fundam. Appl. Toxicol.* **30,** 102–108.

Mahood, I. K., Hallmark, N., McKinnell, C., Walker, M., Fisher, J. S., and Sharpe, R. M. (2005). Abnormal Leydig cell aggregation in the fetal testis of rats exposed to di(n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* **146,** 613–623.

Maloney, E. K., and Waxman, D. J. (1999). trans-Activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicol. Appl. Pharmacol.* **161,** 209–218.

McKinnell, C., Sharpe, R. M., Mahood, K., Hallmark, N., Scott, H., Ivell, R., Staub, C., Jegou, B., Haag, F., Koch-Nolte, F., *et al*. (2005). Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by in utero exposure to di(n-butyl) phthalate. *Endocrinology* **146,** 4536–4544.

Mylchreest, E., Cattley, R. C., and Foster, P. M. (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: An antiandrogenic mechanism? *Toxicol. Sci.* **43,** 47–60.

Mylchreest, E., Sar, M., Cattley, R. C., and Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol.* **156,** 81–95.

Mylchreest, E., Wallace, D. G., Cattley, R. C., and Foster, P. M. D. (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol. Sci.* **55,** 143–151.

National Academy of Sciences (2008). *Phthalates and Cumulative Risk Assessment: The Tasks Ahead.* Available at: http://dels.nas.edu/dels/rpt_briefs/phthalates_final.pdf. Accessed May 5, 2010.

Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D., Klinefelter, G. R., Barlow, N. J., and Gray, L. E., Jr. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci.* **58,** 339–349.

Plummer, S., Sharpe, R. M., Hallmark, N., Mahood, I. K., and Elcombe, C. (2007). Time-dependent and compartment-specific effects of *in utero* exposure to di(*n*-butyl) phthalate on gene/protein expression in the fetal rat testis as revealed by transcription profiling and laser capture microdissection. *Toxicol. Sci.* **97,** 520–532.

Rider, C. V., Furr, J., Wilson, V. S. and Gray, L. E., Jr. (2008). A mixture of seven antiandrogens induces reproductive malformations in rats. *Int. J. Androl.* **31,** 249–262.

Rider, C. V., Furr, J. R., Wilson, V. S., and Gray, L. E. (2010). Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. *Int. J. Androl.* **33,** 443–462.

Saillenfait, A. M., Roudol, A. C., Gallissot, F., and Sabate, J. P. (2011). Prenatal developmental toxicity studies of di-n-heptyl and di-n-octyl phthalates in Sprague-Dawley rats. *Reprod. Toxicol.* **32,** 268–276.

Saillenfait, A. M., Sabate, J. P. and Gallissot, F. (2008). Diisobutyl phthalate impairs the androgen-dependent reproductive development of the male rat. *Reprod. Toxicol.* **26,** 107–115.

Saillenfait, A. M., Sabate, J. P., and Gallissot, F. (2009). Effects of in utero exposure to di-n-hexyl phthalate on the reproductive development of the male rat. *Reprod. Toxicol.* **28,** 468–476.

Scott, H. M., Hutchison, G. R., Jobling, M. S., McKinnell, C., Drake, A. J., and Sharpe, R. M. (2008). Relationship between androgen action in the "male programming window," fetal Sertoli cell number, and adult testis size in the rat. *Endocrinology* **149,** 5280–5287.

Shipley, J. M., and Waxman, D. J. (2004). Simultaneous, bidirectional inhibitory crosstalk between PPAR and STAT5b. *Toxicol. Appl. Pharmacol.* **199,** 275–284.

Shultz, V. D., Phillips, S., Sar, M., Foster, P. M. D., and Gaido, K. W. (2001). Altered gene profiles in fetal rat testes after in utero exposure to di(n-butyl) phthalate. *Toxicol. Sci.* **64,** 233–242.

Silva, E., Rajapakse, N., and Kortenkamp, A. (2002). Something from "nothing"—Eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* **36,** 1751–1756.

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.

Thompson, C. J., Ross, S. M., and Gaido, K. W. (2004). Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. *Endocrinology* **145,** 1227–1237.

Thompson, C. J., Ross, S. M., Hensley, J., Liu, K., Heinze, S. C., Young, S. S., and Gaido, K. W. (2005). Differential steroidogenic gene expression in the fetal adrenal gland versus the testis and rapid and dynamic response of the fetal testis to di(n-butyl) phthalate. *Biol. Reprod.* **73,** 908–917.

Tusher, V. G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U.S.A.* **98,** 5116–5121.

Val, P., Jeays-Ward, K. and Swain, A. (2006). Identification of a novel population of adrenal-like cells in the mammalian testis. *Dev. Biol.* **299,** 250–256.

Ward, J. M., Peters, J. M., Perella, C. M., and Gonzalez, F. J. (1998). Receptor and nonreceptor-mediated organ-specific toxicity of di(2-ethylhexyl)phthalate (DEHP) in peroxisome proliferator-activated receptor alpha-null mice. *Toxicol. Pathol.* **26,** 240–246.

Welsh, M., Saunders, P. T., Fisken, M., Scott, H. M., Hutchison, G. R., Smith, L. B., and Sharpe, R. M. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J. Clin. Invest.* **118,** 1479–1490.

Wilson, V. S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., and Gray, L. E., Jr. (2004). Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol. Lett.* **146,** 207–215.

Wittassek, M., Wiesmuller, G. A., Koch, H. M., Eckard, R., Dobler, L., Muller, J., Angerer, J., and Schluter, C. (2007). Internal phthalate exposure over the last two decades—A retrospective human biomonitoring study. *Int. J. Hyg. Environ. Health* **210,** 319–333.