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

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Several phthalate esters have been linked to the Phthalate Syndrome, affecting male reproductive development when administered to pregnant rats during in utero sexual differentiation. The goal of the current study was to enhance understanding of this class of compounds in the Sprague Dawley (SD) fetal rat following exposure on gestational days (GDs) 14–18 by determining the relative potency factors for several phthalates on fetal testes endpoints, the effects of a nine phthalate mixture on fetal testosterone (T) production, and differences in SD and Wistar (W) strain responses of fetal T production and testicular gene expression to di(2-ethylhexyl) phthalate (DEHP). We determined that diisobutyl phthalate (DIBP) and diisoheptyl phthalate (DIHP) reduced fetal testicular T production with similar potency to DEHP, whereas diisononyl phthalate (DINP) was 2.3-fold less potent. DINP was also less potent at reducing STAR and Cyp11a gene expression levels, whereas DIHP was slightly more potent than DEHP. We observed that administration of dilutions of a mixture of nine phthalates (DEHP, DIHP, DIBP, dibutyl-, benzyl butyl-, dicyclohexyl-, diheptyl-, dihexyl-, and dipentyl phthalate) reduced fetal T production in a dose-dependent manner best predicted by dose addition. Finally, we found that the differential effects of in utero DEHP treatment on epididymal and gubernacular differentiation in male SD and W rats (0, 100, 300, 500, 625, 750, or 875 mg DEHP/kg/day) are likely due to tissue-specific strain differences in the androgen and insl3 signaling pathways rather than differential effects of DEHP on fetal testis T and insl3 production.

Key Words: endocrine disruptors; endocrine toxicity; testis; endocrine toxicology; reproductive and developmental toxicity;

Exposure to phthalate esters (PE) is a concern for human health due to both widespread indirect and direct exposures (Blount et al., 2000; Heudorf et al., 2007; Scott et al., 2009; Silva et al., 2004). The developing male reproductive system is one of the most vulnerable targets of phthalate toxicity (Scott et al., 2009; Skakkebaek, 2003; Toppari et al., 1996; Virtanen et al., 2005). Phthalate exposures in rodents produce male reproductive malformations that mirror some of the effects characteristic of the human testicular dysgenesis syndrome (TDS), which is currently of unknown etiology (Fisher, 2003; Mylchreest et al., 1999; Sharpe and Irvine, 2004). It has therefore been proposed that increased environmental exposures to phthalates may contribute to the increased incidences of TDS. For that reason, continued investigation of PE effects on fetal male rat reproductive health could improve understanding of TDS and provide valuable data that may be used in human health risk assessment.

Currently, the cumulative risk assessment of phthalates is being considered by the Consumer Product Safety Commission (CPSC), the U.S. Environmental Protection Agency (U.S. EPA) program offices (U.S. EPA, 2009), and the U.S. EPA National Center for Environmental Assessment with the Office of Research and Development. These actions are supported by the recommendations of the National Academies of Science (NAS) National Research Council committee (NAS, 2008). The recommendation was based on review of data, which provides evidence that (1) humans are exposed simultaneously to mixtures of multiple phthalates (Hauser and Calafat, 2005;
NAS, 2008; Silva \textit{et al.}, 2004) and (2) \textit{in utero} exposure of rats to certain PEs during the period of sex differentiation (gestational days [GDs] 14–18) causes malformations in reproductive tissues of male offspring by reducing critical hormones during this period (Foster, 2006; Gray \textit{et al.}, 2000; Howdeshell \textit{et al.}, 2008).

Cumulative toxicity of anti-androgens, including phthalates, has been best predicted by dose-additive mathematical models (Hotchkiss \textit{et al.}, 2004; Howdeshell \textit{et al.}, 2008; Rider \textit{et al.}, 2008), in which the total mixture toxicity is the sum of the relative dose of each component in the mixture. To predict a dose-additive response, the relative potency of each compound in the mixture must be taken into account. A potency factor is derived from the dose-response profiles of a compound for a particular endpoint. Therefore, a major goal of this study was to obtain dose-response data for the relationship between several individual phthalates and fetal testicular T production and/or gene expression to characterize their relative potency for disrupting fetal testis function, as needed for cumulative risk assessment for this class of compounds.

We compared the dose-related effects of di(2-ethylhexyl) phthalate (DEHP) with other phthalates associated with reproductive toxicity in fetal male Sprague Dawley (SD) rats: diisodecyl phthalate (DIDP), diisobutyl phthalate (DIBP), and diisononyl phthalate (DINP). Of the studied phthalates, DEHP, dibutyl phthalate (DBP), DIBP (Saillenfait \textit{et al.}, 2008), DHP (McKee \textit{et al.}, 2006), DINP (Borch \textit{et al.}, 2004; Gray \textit{et al.}, 2000), DPeP (Hannas \textit{et al.}, 2011), and benzyl butyl phthalate (BBP), all produce similar reproductive alterations in male offspring (Foster \textit{et al.}, 1981; Gray \textit{et al.}, 2000).

Similar to fetal and postnatal effects that have been seen with DEHP or DBP following \textit{in utero} exposures, DIBP decreases fetal rat testicular T production (Borch \textit{et al.}, 2006; Howdeshell \textit{et al.}, 2008), reduces anogenital distance (AGD), and induces testicular histopathological lesions and reproductive tract malformations at similar doses to DEHP (Blystone \textit{et al.}, 2010; Gray \textit{et al.}, 2009; Saillenfait \textit{et al.}, 2008). Therefore, we predicted that DIBP would possess similar dose-response and potency characteristics to DEHP for fetal testicular T production.

We also hypothesized that \textit{in utero} exposure to DIBP reduces testicular T during the fetal period of sexual differentiation in a manner similar to DEHP because it induces reproductive alterations in male rat offspring consistent with the Phthalate Syndrome (reduced AGD, retained nipples and reduced reproductive organ weights, sperm counts and fertility; McKee \textit{et al.}, 2006).

In addition, we expected that DINP would disrupt fetal endocrine function as do the aforementioned phthalates. We hypothesized that DINP would be less potent in reducing fetal testicular T production because administration of a high dose (750 mg DINP/kg/day) during sexual differentiation induces reproductive malformations in male rat offspring, but to a lesser extent than with DEHP exposure (Gray \textit{et al.}, 2000). We additionally hypothesized that DIBP and DINP reduce fetal testicular T production and StAR and Cyp11a gene expression levels with similar potency to DEHP and that DINP would be less potent than DIBP, DHP, and DEHP.

The potency information derived from the dose-response experiments in this study and from previously published studies was used to design a mixture experiment with nine phthalates administered at a fixed ratio over several dilutions of the top dose to determine if they acted jointly (dose additive) or independently (response additive). Based on previous studies of phthalates, we assumed that all tested phthalates would act through a similar mechanism. We therefore hypothesized that simultaneous exposure to multiple antiandrogenic phthalates during GDs 14–18 would decrease fetal testicular T production in a dose-additive fashion.

An additional objective of the first dose-response experiment with DEHP was to determine if fetal testis endocrine function differed among two rat strains (Wistar [W] and SD) that have been shown to display different Phthalate Syndrome profiles. Wilson \textit{et al.} (2007) reported that DEHP administered at 750 mg/kg/day to dams during GDs 14–18 resulted in a higher rate of epididymal lesions (an androgen-dependent tissue) in SD than in W rat offspring, whereas the same exposure caused a higher incidence of gubernacular lesions (an insl3-dependent tissue) in W than SD rat offspring. We hypothesized that the phenotypic differences in epididymal and gubernacular development were due to strain-specific effects of DEHP on fetal testosterone (T) production and Leydig cell insl3 gene expression, respectively.

In the current study, we dosed pregnant rats with increasing concentrations of individual phthalates to obtain dose-response and potency information for fetal testicular T production and gene expression. We also used data from the individual phthalate exposures to test the model predictions for the effect of exposure to the mixture on fetal testicular T production. Finally, the dose-response effect of DEHP on fetal testis gene expression and T production was compared in two rat strains to determine if the strain-specific malformations between SD and W male rat induced by high DEHP doses (Wilson \textit{et al.}, 2007) were related to strain-specific alterations in fetal testis function.

**MATERIALS AND METHODS**

**Animals.** SD and W pregnant rats were purchased from Charles River Laboratories (Raleigh, NC) or Harlan Laboratories (Indianapolis, IN) and shipped on GDs 1–2 (GD 1 = day of sperm plug positive vaginal smear following mating for Charles River and GD 0 = sperm positive for Harlan). All dams were housed individually in 20 × 25 × 47 cm clear polycarbonate cages with heat-treated laboratory-grade pine shavings for bedding. Environmental conditions were 22°C–23°C, 50–60% humidity, and 14:10D light cycle (lights on at 9 P.M.). Animals were fed a pregnancy diet of Purina Rat Chow 5008 for Charles River dams and NIH 07 breeding diet for Harlan dams. All dams were provided municipal drinking water (Durham, NC) ad libitum. Water provided to animals in the EPA Reproductive Toxicology Facility is filtered (5 μm), tested monthly for \textit{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 use and care 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 DEHP, DHP, DIBP, or DINP exposure. In all experiments, dams were weight ranked and assigned to dose groups to minimize differences in means and variance among treatment groups.

In the first experiment, Charles River SD and W timed pregnant rats (3–6 dams per dose group) were dosed daily on GDs 14–18 by oral gavage with vehicle (corn oil) or 100, 300, 500, 625, 750, or 875 mg DEHP/kg/day (Sigma-Aldrich, CAS 117-81-7, lot 094283C). All phthalates were administered in corn oil (2.5 ml vehicle/kg body weight). This 5-day dosing regimen covers the critical programming window for male reproductive development (Welsh et al., 2008).

In a second experiment, Charles River SD dams (4 dams per dose group) were dosed daily on GDs 14–18 by oral gavage with vehicle (corn oil) or 100, 300, 600, or 900 mg DHP/kg/day. The second formulation included daily oral dosing of Harlan SD dams (3 dams per dose group) on GDs 14–18 with corn oil, 100, 300, 600, or 900 mg DINP/kg/day (CAS 84-69-5, lot 07425B).

Finally, in the fourth experiment, Harlan SD timed pregnant rats were orally dosed each day between GDs 14–18 with corn oil (500, 750, 1000, or 1500 mg DIBP/kg/day). Two separate formulations of DINP were tested. The first DINP formulation (CAS 28553-12-0) was a gift from Badische Anilin und Soda Fabrik and was administered to 3–6 dams per dose group across two separate blocks. The second formulation was purchased from Sigma-Aldrich (CAS 68515-48-0, lot 03005TR) and was administered to 3 dams per dose group in a single block. At necropsy on GD 18, there were a total of 37 pregnant dams with 6, 3, 4, and 6 litters treated with 0, 500, 750, 1000, and 1500 mg DINP/kg (CAS 28553-12-0)/kg and 3, 2, 1, 3, and 3 litters treated with 0, 500, 750, 1000, and 1500 mg DINP (CAS 68515-48-0) / kg.

All data used for analysis of DPeP effects were derived from a previous dose-response study in Harlan SD pregnant dams (Hannas et al., 2011). Briefly, dams (3 per dose group) were dosed by oral gavage with 0 (corn oil control), 11, 33, 100, or 300 mg DPeP/kg/day from GDs 14–18. Harlan rats were used in the DPeP, DINP, and DIBP studies to conform to the strain standard used by the National Toxicology Program (NTP) at the National Institute of Environmental Health Sciences.

**Mixture study—a fixed ratio dilution mixture of nine phthalates.** Harlan SD dams were dosed orally on each of GDs 14–18 with the vehicle (0%) or one of five dilutions of a mixture of (1) DEHP, (2) DHP, (3) DINP, (4) DBP, (5) BBP (CAS 85-68-7, lot 03405JH), (6) dicyclohexyl phthalate (DCHP; CAS 84-61-7, lot 17518JB), (7) di(n)heptyl phthalate (D(n)heptylP; CAS 3648-21-3, lot 125AG), (8) di-n-hexyl phthalate (D(n)hexylP; CAS 84-75-3, lot 139AG), and (9) DPeP. The dosage levels were 100, 67, 33, 16.67, and 8.325 (referred to in the text as 100, 67, 33, 17, and 8%) and 0% of the top dose of 650 mg/kg/day total phthalate, including 10 mg DPeP/kg/day and 80 mg/kg/day each of the other phthalates. There were 8, 3, 4, 7, and 4 litters, respectively, in the 0, 8, 17, 33, 17, and 100% treatment groups. The mixture ratio of the phthalates was designed such that each phthalate would contribute equally to the effects of the mixture if the phthalates behaved in a dose-additive manner and our assumptions about the relative potencies of some of the untested phthalates was accurate.

**Fetal necropsies.** Dams were anesthetized with CO2 and euthanized by decapitation on GD 18. Fetuses were removed, anesthetized via hypothermia on ice, and dissected under a Leica MZ6 dissecting microscope (Wetzlar, Germany). For the DEHP, DINP, and DIBP experiments, a single testis from the first three males identified in a litter was removed and immediately transferred individually into 500 µl M-199 media without phenol red (Hazelton Biologics, Inc., St. Lenexa, KS), which was supplemented with 10% dextran-coated charcoal stripped fetal bovine serum (Hyclone Laboratories, Logan, UT) and incubated for 3 h at 37°C as detailed in Wilson et al. (2004). For the DHP study only, fetal testes from all males in a litter were incubated either in the presence or in the absence of 100 mIU/ml human chorionic gonadotropin (hCG; Sigma-Aldrich, C-0434, lot 51K1415). The hCG acts similarly to luteinizing hormone (LH) in stimulating the Leydig cells to synthesize testosterone (Mendelson et al., 1975) and is more stable in testes exposure media. Therefore, we compared the effects of ICG-stimulated versus unstimulated T production in fetal testes to determine if stimulation would enhance the sensitivity for detecting a phthalate-induced decline in T production.

All necropsies were conducted within a 2-h time frame between 8:00 A.M. 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 and gene expression.** Following 3 h of testes incubation, the media was stored at −80°C until used for 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 was 3.2%. The limit of detection was 0.2 ng/ml for T.

Fetal testes from remaining males in the DEHP, DIBP, and DINP experiments were prepared for RNA extraction. Excised testes were immediately transferred to sterile 1.7 ml conical microcentrifuge tubes (pooled by litter) containing ice-cold Tri-Reagent (Sigma-Aldrich, St. Louis, MO). Testes were homogenized on ice using a Kontes pestle homogenizer. RNA was extracted as previously described (Wilson et al., 2004).

**Quantitative PCR analysis of insl3, StAR, and Cyp11a gene expression.** Real-time PCR analysis for insl3, StAR, and Cyp11a was performed as described in Wilson et al. (2004) using primer and probe sequences previously reported by Howdeshell et al. (2007). All three genes were measured in both SD and W rat samples from the DEHP experiment, whereas only Cyp11a and StAR were measured in the samples from the DIBP, DPeP, and DINP experiments. Amplicon lengths for insl3, STAR, and Cyp11a were 61, 88, and 148 bp, respectively.

RNA samples were treated with DNase I (Promega, Madison, WI) to eliminate any potential genomic DNA contamination. Samples were quantitated using Quant-it<sup>™</sup> RiboGreen RNA Reagent and Kit (Molecular Probes, Carlsbad, CA) according to manufacturer’s instructions. Complementary DNA (cDNA) was synthesized from each RNA sample using ImProm-II Reverse Transcriptase and a random hexamer primer (Promega) according to manufacturer’s instructions. Assuming an RNA to cDNA ratio of 1:1, 50 ng of cDNA was added to a 50 µl volume for each gene containing: 1 × PCR buffer, 0.4mM each deoxynucleotide triphosphate, 4 (insl3) or 8 µM (StAR, Cyp11a) of MgCl<sub>2</sub>, 12 pmol each of forward and reverse primer, 1.25 pmol fluorescent probe, 0.5 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) bound to Taq antibody (Life Technologies, Carlsbad, CA), and nuclease-free water (Promega). Each real-time PCR assay included an internal standard curve, which was used to determine absolute starting quantity (SQ) of a specific gene in the cDNA sample. PCR cycling parameters were as follows: initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 15 s, anneal at 56°C for 20 s, and extend at 72°C for 10 s, repeated for 40 cycles, and final extension at 72°C for 10 min. For each experiment, samples and standard for each gene were run in duplicate on a single plate.

**Statistics.** T production was analyzed based on litter mean values obtained from the individual testis incubations. Gene expression was analyzed as measured based upon a single pooled sample from each litter. For the DEHP strain comparison experiments, T production and gene expression starting quantity data were log<sub>10</sub>-transformed to correct for heterogeneity of variance then analyzed by a two-way ANOVA model and assessed for significant differences in responses to DEHP including strain, dose, and a dose by strain interaction using PROC GLM.
in SAS, version 9.1, available at the U.S. EPA (SAS Institute, Cary, NC). Comparisons were made for individual dose treatments between strains using a t-test when a significant interaction or strain effect was determined by the ANOVA model.

Because T production data were obtained following DINP treatment using both CAS number 28553-12-0 and 68515-48-0, these data were analyzed using a two-way ANOVA model to assess differences from different doses, different DINP formulations, and for a dose by DINP formulation interaction using PROC GLM in SAS. An interaction term was considered significant at p < 0.1 and dose or DINP formulation effects in the absence of an interaction were considered significant at p < 0.05. T production data from the studies with DIBP, and DIHP were analyzed as above with a one-way ANOVA (dose as the main effect).

Dose-response percent of control data for T production and messenger RNA (mRNA) levels were analyzed using a nonlinear four parameter regression analysis with the top constrained to 100% and the bottom to 0% of control (sigmoidal fit with variable slope using Prism GraphPad 5.01 software, GraphPad Software, Inc., La Jolla, CA). Differences between dose-response curves among W and SD rats in the DEHP experiment and for ex vivo T production with and without hCG in the DIHP experiment were determined by comparing ED50 values among the phthalates (p < 0.05) using Prism software.

Mixture model predictions. T production response predictions were made for the nine phthalate mixture study using the dose addition (DA; also called concentration addition) model as described by Rider et al. (2009). For comparison, predictions were also calculated using the response addition (RA) model, which has been used historically for mixtures of chemicals that act independently or through different mechanisms of action (Greco et al., 1992). For DA and RA model predictions, the ED50 and Hill slope values derived from the individual phthalate dose-response studies were used to calculate the overall response of the mixture when available. Hill slope and ED50 values for DBP are from a dose-response study conducted under similar conditions as that of DIBP and DIHP in the current study (Howdeshell et al., unpublished data). Similar dose-response studies were also conducted to yield Hill slope and ED50 values for D(heptyl)P and D(hexyl)P (unpublished data). Values for DCHP, DIHP, DEHP, and BBP were assumed to be the average of D(heptyl)P and D(hexyl)P (unpublished data).

The observed effects of the mixture were compared with the DA and RA model predictions using several methods. First, the ED50 values of the DA and RA model predictions were compared with the ED50 value of the observed data using the 99% confidence interval (CI) of the observed results. The model predictions ED50s falling outside of the 99% CI of the observed ED50 were considered to differ significantly from observed. In addition, the observed data (means, SEs, and sample sizes) were force-fit to the DA and RA models by constraining all four model parameters to the DA and RA model parameters. If a DA or RA model perfectly predicted the observed results, the R² value of the forced-fit model would be equal to the R² value obtained with the “best fit” of the observed data. The greater the DA or RA models deviated from the observed effects, the greater the R² of the forced-fit model declined from the “best fit” model R² value.

RESULTS

A Comparison of the Dose Response to DEHP in Two Rat Strains

Maternal body weight gain was significantly decreased at doses of 625 mg DEHP/kg/day and above for both strains (p < 0.02; Fig. 1). Although SD dams were heavier on average than W dams, there was no interaction between strain and treatment effects (p > 0.1), indicating that the body weight decline was similar for the two strains over the 5-day dosing regimen. DEHP did not cause fetal mortality by GD 18 in either rat strain at any of the doses tested.

DEHP reduced ex vivo T production from fetal testes in a dose-responsive manner, with significant reductions observed at 300 mg DEHP/kg/day and higher in both the SD and W strains (Fig. 2a; p < 0.0001; Table 1). T production values in both the control and 100 mg DEHP/kg/day treatment groups in the SD strain were significantly lower than levels in control and 100 mg DEHP/kg/day treatment groups in the W strain (p < 0.01 and p < 0.03, respectively). When the dose-response data for T production was transformed to percent of control, T levels were similar for both strains and were best fit by a single curve with an ED50 of 380 mg/kg (Fig. 2b; p > 0.7). Analysis of T production converted to percent of control value yields ED50 values of 347 and 426 mg DEHP/kg/day for W and SD, respectively, and these values did not differ significantly from one another (> 0.08). We found no strain difference in the response of T production and insl3 mRNA levels to DEHP treatment and therefore conducted all other phthalate dose-response studies in the SD rat strain.

DEHP treatment reduced fetal testis insl3 mRNA expression in both strains (p < 0.0001 for dose effect) in a dose-dependent manner (Fig. 3a). The reduction in insl3 mRNA expression was significant at 625 mg DEHP/kg/day and greater in the SD rat and at 500 mg DEHP/kg/day and greater in the W rat (p < 0.05). Nevertheless, the overall reduction in insl3 mRNA levels did not differ between strains and there was no strain by dose interaction. The ED50 for DEHP effects on insl3 gene copy number was not significantly different for SD and W rats (Fig. 3b). The shared global ED50 for both strains derived from regression analysis was 569 mg DEHP/kg/day for insl3 (534 and 589 mg/kg for W and SD strains, respectively).

The mRNA expression of the androgen synthesis genes StAR and Cyp11a also were significantly reduced at doses of 500 mg DEHP/kg/day and greater in both strains (p < 0.0005 and p < 0.0001, respectively, Figs. 3c and 3e). Overall, DEHP produced a small strain effect on fetal testicular expression levels of StAR and Cyp11a mRNA, with slightly higher levels of each gene in the W strain (p < 0.05 and p < 0.005, respectively). The DEHP dose that produced a 50% reduction in StAR expression levels was not significantly different between the SD and W
strains (p = 0.09, Fig. 3d). Similarly, the DEHP ED50 values did not significantly differ between SD and W rats for Cyp11a gene expression levels (p = 0.78, Fig. 3f). Shared global ED50s for both strains derived from regression analysis were 405 mg DEHP/kg/day for StAR (296 and 443 mg/kg for W and SD strains, respectively) and 569 mg DEHP/kg/day for Cyp11a (555 and 574 mg/kg for W and SD strains, respectively).

**DIHP, DIBP, and DINP Dose-Response Studies**

Treatment with DIHP, DIBP, or DINP did not induce maternal mortality, overt toxicity, or reduce maternal body weight or reduce litter size at any dosage level tested (data not shown).

Testes excised from fetuses of dams administered DIHP were incubated in the presence and absence of hCG to determine if maximal stimulation of T production would enhance the ability to detect a DIHP-induced reduction in androgen synthesis. The addition of hCG significantly increased fetal testicular T production in each dosage group by about 2.3-fold (Fig. 4a; Table 1), indicating that Leydig cell response to an LH-like stimulus was not compromised by DIHP treatment. Despite increasing overall T production in each dosage group, supplemental hCG did not significantly alter the dose-response curve or ED50 of DIHP exposure (Fig. 4b) and therefore did not increase the sensitivity for detecting reductions in T production. T production data transformed to percentage of respective control generated a shared EC50 value of 443 mg DIHP/kg/day for the +hCG and −hCG data sets (467 and 410 mg DIHP/kg/day, respectively).

DIBP significantly reduced fetal testicular T production at doses of 300 mg/kg/day or greater (p < 0.005) and was equipotent to DIHP and DEHP (Fig. 5; Table 1). There was no significant difference in the potency of the dose-response curves of DIBP, DIHP, and DEHP on testicular T production in the SD rat strain and were therefore fit with a single curve (Fig. 5). Curve fit results for these three phthalates resulted in a global ED50 of 373.8 mg phthalate/kg/day.

Both DINP formulations reduced fetal testicular T production similarly in a dose-responsive manner at doses of 500 mg DINP/kg (p < 0.05) or greater (p < 0.01) (Fig. 6; Table 1), and there was no interaction between dose and DINP chemical formulation. Therefore, litter means in subsequent DINP comparisons were calculated by combining the values from all three blocks of DINP dose-response experiments. Curve fit results comparing these two DINP formulations are statistically indistinguishable and resulted in a global ED50 of 852 mg phthalate/kg/day.

Comparison of the global DINP ED50 value with global shared ED50 value for DEHP, DIBP, and DIHP derived from the regression analysis curves revealed that DINP is approximately 2.3-fold less potent for reducing fetal testicular T production than DEHP, DIBP, and DIHP (Fig. 7a). Conversely, results from a previously published study indicate that DPeP is approximately 8-fold more potent than DEHP, DIBP, and DIHP and approximately 18-fold more potent than DINP for reducing fetal T production when administered on GDs 14–18 (Hannas et al., 2011).

**Effects of DIBP and DINP on Gene Expression**

DIBP reduced fetal testis RNA expression levels for StAR at dosage levels of 300 mg/kg/day and greater (p < 0.05) and Cyp11a levels at 100 mg/kg/day and greater (p < 0.05) (Figs. 7b and 7c). The ED50 value for the effects of DIBP on StAR and Cyp11a are 191 and 171 mg/kg/day, respectively.

DINP also reduced StAR and Cyp11a levels, but at higher dosage levels than DIBP and the two formulations did not differ in this regard. There was no interaction between DINP dose and chemical formulation for StAR and Cyp11a (data not shown by CAS), thus mean values for RNA starting quantity values included combined litter values from all three blocks run with both CAS versions of this phthalate. DINP reduced StAR at dosage levels of 1000 and 1500 mg/kg/day (p = 0.005) (Fig. 7b) and DINP reduced Cyp11a expression levels at 1000 and 1500 mg/kg/day (p < 0.001) (Fig. 7c). The ED50 values for the effects of DINP on StAR and Cyp11a are 901 and 1356 mg/kg, respectively.

ED50 comparisons for STAR expression indicate that the order of potency for these phthalates is DPeP > DIBP = DEHP > DINP and DPeP = DIBP > DEHP > DINP for Cyp11a (Figs. 7b and 7c). Based on the individual ED50s for each phthalate, T production was the most sensitive fetal testicular endpoint from the 5-day in utero exposure to DEHP, DINP, and DPeP, whereas Cyp11a expression was most sensitive to DIBP (Table 1; Fig. 7). The effects of these phthalates and the mixture of phthalates on gene expression are being reevaluated using custom designed 96-gene PCR arrays. We
are determining if the PCR arrays improve the precision and accuracy of the gene expression data from the fetal testis after in utero phthalate treatment.

**Nine Phthalate Mixture Study**

A mixture of nine phthalates did not significantly reduce maternal weight gain or fetal viability at any dilution of the top dose of 650 mg/kg/day phthalates (data not shown). The mixture induced significant reductions in fetal testicular T production at doses of 17% and greater \((p < 0.01)\). T production was also reduced at 8% of the top dose, the lowest dose tested by about 15%; however, this effect did not attain statistical significance \((p = 0.15\) using log10 transformed T production and \(p < 0.03\) with untransformed T data).

The logistic fit of the dose and RA models differed from the logistic fit of the observed values for T production, as assessed by sums of squares of \(f\)-test comparing the ED50 and Hill slopes with Prism 5.0 software (Fig. 8). Additionally, the ED50 derived from the logistic curve fit for the DA model of 31% of the top dose did not differ from the ED50 value of 26% of the top dose for the “best fit” to the observed T production data, whereas the RA ED50 prediction of 51% of the top dose was outside of the 99% CI, which ranged from 19.5 to 34.9% of the top dose for the ED50 of the observed data. When the observed data were fit to the DA model the \(R^2\) value was 0.81, which was slightly lower than the \(R^2\) of the best fit model of 0.87 (Fig. 8), whereas the \(R^2\) value for the RA model fit of the observed data was only 0.66.

### TABLE 1

<table>
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<th>DEHP dose</th>
<th>T PROD</th>
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DISCUSSION

Phthalate Syndrome–associated effects in male offspring exposed in utero result from a combination of abnormal Leydig cell aggregation and gonocyte proliferation (Mahood et al., 2005; Mylchreest et al., 2002; Parks et al., 2000), a decrease in insl3 levels (McKinnell et al., 2005; Wilson et al., 2004), disrupted fetal testicular T production (Parks et al., 2000; Scott et al., 2008; Welsh et al., 2008), and associated alterations in genes involved in androgen synthesis (Johnson et al., 2007; Shultz et al., 2001; Thompson et al., 2004). In this study, we focused on characterizing the potency differences and mixture contributions of several phthalates on fetal T production and Cyp11a, StAR, and insl3 RNA gene expression levels.

The study demonstrates that DIBP, DEHP, and DIHP are roughly equipotent in reducing fetal testicular T production and Cyp11a, StAR, and insl3 gene expression similar to the potencies observed for reproductive malformation endpoints. These three phthalates are all more potent than DINP but less potent than DPeP at reducing T production and Cyp11a and StAR gene expression levels. We detected no difference in the ability of two formulations of DINP to significantly reduce T production at higher doses. Although DINP differed quantitatively in potency than the other tested phthalates, it did not differ qualitatively because the pattern of fetal endocrine alterations was identical to that of DIBP, DIHP, DEHP, and DPeP. Finally, we also demonstrated that a mixture of nine phthalates reduced fetal T production in a dose-dependent and dose-additive manner following in utero exposure during sexual differentiation.

Our investigation of DIBP and DIHP demonstrates that these phthalates are as potent as is DEHP in disrupting fetal testicular endocrine function. These results agree with previous T production data from a DIBP dose-response study in which maternal rats were dosed for 10 days (GDs 8–18) including the sexual differentiation period (Howdeshell et al., 2008). The current study confirms that DIBP potency is similar to DEHP for this fetal endpoint when administered for 5 days (GDs 14–18). These data correlate with postnatal male reproductive malformation data demonstrating similar potency of DEHP (Blystone et al., 2010; Gray et al., 2009) and DIBP (Saillenfait et al., 2008) for reducing AGD and inducing male areola/nipple retention. Due to structural similarities, DIBP is commonly used as a substitute compound for DBP. DIBP is extensively used in consumer products and the metabolite, monoisobutyl phthalate has been detected in the blood, saliva, and urine of human adults and children (Silva et al., 2004; Wittassek et al., 2007) at levels between 0.7 and 5.1 ng/ml (Borch et al., 2006; Swan et al., 2005). High usage volume and evidence of human exposures combined with the toxicity/potency results from this study warrants comprehensive analysis of risk for this compound.

DIHP was also identified in the current study as being equipotent to DEHP for reducing fetal testicular T production. The production and use volumes of DIHP are much less than those of DEHP (CERHR, 2000). Sources of exposure to DIHP include occupational exposure during flooring or automobile manufacturing, consumer-use exposure, or exposure via environmental media. In a two-generation reproductive toxicity study, dietary DIHP induced the Phthalate Syndrome in F1
male rat offspring (McKee et al., 2006). The males displayed reduced AGD, increased retention of female-like thoracic nipples, induced testicular abnormalities, reduced weights of the testes and accessory androgen-dependent reproductive organs, and reduced testicular sperm counts. The overall no observable effect level (NOEL) in the reproductive toxicity study was in the range of 64–168 mg/kg/day (gestation-lactation periods) (McKee et al., 2006).

DINP is a widely used, high production volume phthalate with a reported volume of 500 million pounds produced in 2006 alone (U.S. EPA, 2006, 2009). The monocarboxyisooctyl phthalate metabolite of DINP was detected in human urine samples from the 2005–2006 National Health and Nutrition Examination Survey (NHANES) study in 95.2% of samples in levels ranging from 0.7 to 4961 μg/l (Calafat et al., 2011), demonstrating that exposure to DINP is widespread.

Although DINP was less potent in disrupting fetal testis endocrine function than were DPeP, DIHP, DEHP, and DIBP, it did significantly reduce fetal testis testosterone production dosage levels at and above 500 mg DINP/kg/day and reduced testis gene expression. This result is in agreement with results of our study on the postnatal reproductive effects of DINP exposure during sexual differentiation in which DINP was found to be less potent than DEHP (Gray et al., 2000). In the 2000 study, we found that 750 mg DINP/kg induced retained nipples/areolae in 22% of the treated infant male rats versus 87% in 750 mg DEHP/kg exposed males (0% in controls), and reproductive malformations in 7.7% of the DINP dose group (including testicular atrophy, epididymal agenesis, and hemorrhagic testis) versus 84% in the DEHP F1 group (0% in controls). Similarly, Borch et al. (2004) reported “antiandro- genic” effects in male rat offspring exposed to 750 mg DINP/kg in utero (GDs 7–21), including reduced testicular testosterone levels and production (down by about 60%), reduced AGD, and retained nipples.

The congruency between the potency of DINP for inhibiting T production and producing postnatal malformations in androgen-dependent tissues further supports the connection between these two toxicity endpoints. Clearly, DINP is an “endocrine disrupting chemical” but only at very high dosage levels, and it less potent that DPeP, BBP, DBP, DIBP, DIHP, DEHP, and DCHP in this regard.

Our previous phthalate mixture studies (Gray et al., 2006; Hotchkiss et al., 2004, 2008; Howdeshell et al., 2007, 2008; Rider et al., 2008) were included as data sources in the NAS report to EPA recommending a cumulative risk assessment for phthalates (NAS, 2008). The DA result obtained in the current study is consistent with our earlier results and clearly demonstrates that the reduction of fetal T production by a nine phthalate mixture is better predicted by the DA model than RA, suggesting that the tested phthalates affect the same endpoints jointly rather than independently. These data further support

FIG. 4. Fetal testicular ex vivo T production following 3-h incubation in the presence (+) and absence (−) of supplemental (100 mIU/ml) hCG stimulation ± cCG (A) from male offspring exposed in utero during sexual differentiation (GDs 14–18) to DIHP. Values represent means ± SEM of pooled litter values (n = 3–4 litters).

FIG. 5. Testicular ex vivo T production following 3-h incubation from fetuses exposed in utero during sexual differentiation (GD 14–18) to DIBP, DEHP, or DIHP. Values are pooled litter means ± SEM, transformed to percentage of respective control value (n = 3–4 litters).

FIG. 6. T production following 3-h incubation of fetal testes extracted from male offspring exposed in utero during sexual differentiation (GDs 14–18) to DINP CAS number 28553-12-0 or DINP CAS number 68515-48-0. Data points represent the mean ± SEM.
the NAS recommendation for a cumulative risk assessment on antiandrogenic compounds.

The results of the current study also demonstrate that the strain differences among the SD and W rat strains in response to DEHP in utero exposure are not explained by differential reductions in T production and target gene expression. Detection of differing degrees of epididymal agenesis and gubernacular malformations in SD and W male offspring induced through DEHP maternal dosing (Wilson et al., 2007) led to the prediction that strain differences in Leydig cell response to DEHP exposure could be detected and explained through divergent gene changes and T production levels over a range of doses. SD male offspring displayed a high incidence of epididymal agenesis, whereas W males displayed a high incidence of gubernacular malformations after maternal exposure to DEHP at 750 mg/kg/day from GD 14 to 18. Instead, in this study, DEHP decreased fetal testicular T production and insl3 mRNA levels similarly in SD and W strains and induced very minor strain differences in StAR and Cyp11a gene expression.

Based on these results, we conclude that the differential postnatal responses of the strains to DEHP exposure may alternatively be explained by tissue-specific strain differences in hormone signaling coupled with the reductions in tissue T and insl3 levels. Different androgen-dependent fetal tissues utilize cofactors resulting in tissue-specific transcriptional complexes that in turn activate or repress numerous androgen-dependent genes, which also vary from tissue to tissue. One would hypothesize that differentiation of the fetal W rat epididymal tissue requires lower levels of androgens for normal differentiation than does the fetal SD epididymis but higher levels of insl3 mRNA for gubernacular differentiation. One possibility for the observed postnatal difference in gubernacular development (Wilson et al., 2007) is that levels of the insl3 receptor, Rxfp2, or other downstream signaling molecules in the pathway differ between the strains. Gene expression levels for Rxfp2 differ throughout fetal development from GD 16 to 18 (Barthold, 2008; Scott et al., 2008) and potentially to a different extent in SD and W rats. This difference may not be reflected in the ligand (insl3) mRNA expression levels but may explain the previously observed postnatal strain differences in gubernacular development.
In conclusion, we performed this study to characterize the potency of multiple phthalates for decreasing fetal T production and altering gene expression to support the ongoing individual and cumulative phthalate risk assessments ongoing at the CPSC, as mandated by law and at the Integrated Risk Information System (IRIS) program within the U.S. EPA, Office of Research and Development, National Center for Environmental Assessment. These results also provide valuable data required to make predictions about the risk associated with exposure to mixtures of phthalates. With regard to future studies, continued efforts to match postnatal phenotypic with fetal endocrine and genomic outcomes and to characterize mixture toxicity of phthalate exposure will enhance our ability to understand the potential risks posed by individual phthalates and mixtures of phthalates to the human population.

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REFERENCES


