Cumulative Effects of Dibutyl Phthalate and Diethylhexyl Phthalate on Male Rat Reproductive Tract Development: Altered Fetal Steroid Hormones and Genes

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Exposure to plasticizers di(n-butyl) phthalate (DBP) and diethylhexyl phthalate (DEHP) during sexual differentiation causes male reproductive tract malformations in rats and rabbits. In the fetal male rat, these two phthalate esters decrease testosterone (T) production and insulin-like peptide 3 (insl3) gene expression, a hormone critical for gubernacular ligament development. We hypothesized that coadministered DBP and DEHP would act in a cumulative dose-additive fashion to induce reproductive malformations, inhibit fetal steroid hormone production, and suppress the expression of insl3 and genes responsible for steroid production. Pregnant Sprague Dawley rats were gavaged on gestation days (GD) 14–18 with vehicle control, 500 mg/kg DBP, 500 mg/kg DEHP, or a combination of DBP and DEHP (500 mg/kg each chemical; DBP + DEHP); the dose of each individual phthalate was one-half of the effective dose predicted to cause a 50% incidence of epididymal agenesis. In experiment one, adult male offspring were necropsied, and reproductive malformations and androgen-dependent organ weights were recorded. In experiment two, GD18 testes were incubated for time PCR. The DBP + DEHP dose increased the incidence of many reproductive malformations by ≥ 50%, including epididymal agenesis, and reduced androgen-dependent organ weights in cumulative, dose-additive manner. Fetal T and expression of insl3 and cyp11a were cumulatively decreased by the DBP + DEHP dose. These data indicate that individual phthalates with a similar mechanism of action, but with different active metabolites (mono-butyl phthalate versus monoethylhexyl phthalate), can elicit dose-additive effects when administered as a mixture.

Phthalate esters are a high-production volume group of chemicals used to impart flexibility to polyvinyl plastics and other materials, including medical dialysis tubing and intravenous bags, pharmaceuticals, cosmetics, and personal care products, and plastic food wrap. Phthalates and/or their metabolites can leach from such products and have been detected in the environment (Kolpin et al., 2002) and in the saliva and urine of children and adults (Blount et al., 2000; Silva et al., 2004a, 2005). Infants may be exposed to phthalates in the womb via maternal circulation (Latini et al., 2003; Silva et al., 2004b), breastfeeding (Mortensen et al., 2005), or via medical devices in neonatal intensive care units (Green et al., 2005).

Prenatal exposure to some phthalate esters during the period of sexual differentiation inhibits male reproductive development in laboratory rodents. In utero exposure to either dibutyl phthalate (DBP) or diethylhexyl phthalate (DEHP) leads to an increased incidence of reproductive malformations and reduced androgen-dependent organ weights in adult male rats (Gray et al., 2000), which indicate a suppression of androgen and insulin-like peptide 3 (insl3), a hormone responsible for gubernacular ligament development (Ivell and Bathgate, 2002; McKinnell et al., 2005; Wilson et al., 2004; Zimmermann et al., 1999). Unlike some antiandrogens, phthalate esters do not interact with the androgen receptor at physiological concentrations (Gray et al., 2006b; Parks et al., 2000). In utero exposure to either DBP or DEHP (750 mg/kg/day) reduces fetal testosterone (T) synthesis (Parks et al., 2000) and inhibits insl3 mRNA expression. Therefore, DBP and DEHP share a similar mode of action of suppressing the testicular androgen synthesis and insl3 expression in fetal rats, which results in adverse effects on the male rat reproductive tract.

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While risk assessments have traditionally been done on a chemical by chemical basis, the Food Quality Protection Act of 1996 requires the United States Environmental Protection Agency (US Congress, 1996) to evaluate the cumulative risk of chemicals which share a similar mechanism of action. However, few experiments to date have tested the effects of prenatal exposure to combinations of antiandrogenic chemicals (Gray et al., 2006b; Hotchkiss et al., 2004). Recent reports from health advocacy groups have emphasized the need for testing combinations of phthalates to better assess the health risks of known human exposure to multiple sources of these chemicals (DiGangi et al., 2002; Purvis and Gibson, 2005), while other organizations have responded that this approach is not scientifically grounded (Stanley, 2002).

In the current study, we hypothesized that prenatal exposure of male rats to a mixture of two phthalates with the same mechanism of action, but different active metabolites, would act in a cumulative, dose-additive fashion to (1) increase the frequency of reproductive malformations, (2) decrease androgen-dependent organ weights, and (3) inhibit fetal testicular steroid hormone production and gene expression profiles. To address this hypothesis, we studied male rats prenatally exposed to DBP (active metabolite = monobutyl phthalate [MBP]; Tanaka et al., 1978; Williams and Blanchfield, 1975) or DEHP (active metabolite = monoethylhexyl phthalate [MEHP]; Albro and Lavenhar, 1989) or a combination of both chemicals during the fetal period of sexual differentiation (gestation days [GD] 14–18). We predicted that an oral dose of 500 mg/kg/day of DBP or DEHP to the rat dam during GD14–18 would be approximately half of the total effective dose which produces a 50% incidence (ED50) of epididymal agenesis. Our prediction was based on a study from our laboratory which observed 0% incidence of epididymal and testicular malformations in Long Evans rats treated with 500 mg/kg/day DBP during 4 days of sexual differentiation (Gray et al., 1999). Epididymal agenesis is one of the most common reproductive malformations associated with prenatal phthalate exposure in laboratory rats (Gray et al., 2000; Mylchreest et al., 2000). In addition, we compared the observed effects for each reproductive end point of the phthalate mixture dose to predictions of dose and response addition, which were based on a preliminary dose-response study which administered DBP or DEHP at several dosage levels during sexual differentiation (Gray, unpublished data). We emphasize that the objective of the current study was not to establish a low adverse effects level (LOAEL) for subtle end points like anogenital distance (AGD) or nipple retention but rather to observe whether well-characterized doses of DBP and DEHP (each of which at sub- or at near threshold levels for causing epididymal malformations) would work in a dose-additive fashion such that the phthalate mixture would be the ED50 for this malformation. Since we suspect that the reproductive effects in the male offspring result from abnormal Leydig cell migration (Mahood et al., 2005) and delayed onset of expression of the genes for the steroidogenic enzymes and insl3 peptide hormone (Thompson et al., 2004), we predicted that these endocrine measures would also be cumulatively affected by the mixture of DBP with DEHP. To test for cumulative effects of phthalate exposure on fetal hormone synthesis, rat dams were euthanized on GD18, and fetal rat testes were collected for an assessment of: (1) T production and (2) expression levels of insl3 and genes related to steroid production and sexual differentiation.

MATERIALS AND METHODS

General Methods

Animals. Adult female Sprague Dawley (SD) rats (Charles River, Raleigh, NC) were mated by the supplier and shipped on GD2. Mating was confirmed by sperm presence in vaginal smears (day of sperm plug positive = GD1). Animals were housed individually in 20 × 25 × 47 cm clear polycarbonate cages with laboratory-grade heat-treated pine shavings (Northeastern Products, Warrensburg NY) with a 14:10 light/dark photoperiod (lights off at 1100 h) at 20–24°C. Pregnant and lactating females were fed Purina Rat Chow 5008, and weaning and adult rats were fed Purina Rat Chow 5001 ad libitum. Animals were provided access to filtered (5 μm filter) municipal drinking water (Durham, NC) ad libitum. Water was tested monthly for Pseudomonas and every 4 months for a suite of chemicals including pesticides and heavy metals. The current study was conducted under protocols approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee.

Doses and administration of chemicals. Two separate experiments were conducted. In each experiment, pregnant rat dams were assigned to treatment groups on GD14 in a manner that provided similar mean (± SE) body weight per treatment group prior to dosing. Laboratory-grade corn oil (CAS 8001-30-7, lot #89H0149), DBP (CAS 201-557-4, purity = 99%, lot 81K0429), and DEHP (CAS 204-211-0, purity = 99%, lot 101K3696) were purchased from Sigma (St Louis, MO). Animals were gavaged from GD14 through GD18 with 0 (vehicle control), DBP (500 mg/kg/day), DEHP (500 mg/kg/day), or a combination of DBP and DEHP (DBP + DEHP; 500 mg/kg/day each) dissolved in corn oil. The doses were delivered in 2.5 μl corn oil per gram body weight. The rat dams were weighed daily during the dosing period to administer the dose per kilogram body weight and to observe the health of the dams.

Developmental Study

Neonatal and pubertal data. Control and treated dams (n = 6 litters per treatment) were allowed to deliver naturally. At postnatal day 3 (PND3; day of birth = PND1), individual pup weights were recorded. AGD was measured using a dissecting scope at ×15 with an ocular micrometer as per Hotchkiss et al. (2004); the observer measuring AGD was blinded as to the pup’s treatment group. The AGD was defined as the distance between the base of the genital papilla and the rostral end of the anal opening. At PND14, male offspring were weighed and examined for presence or absence of areolae or nipples. Male rat pups do not normally retain areolae since their higher levels of endogenous androgens, relative to females, cause regression of the nipple anlagen in utero. At PND22, pups were weaned and males were weighed and housed two siblings per cage. The rat dams were euthanized after weaning the pups, and the number of uterine implants was recorded.

Necropsy. Males were necropsied when they reached at least 7 months old following CO2 anesthesia and decapitation. Blood was collected for determination of serum T and males were necropsied. The ventral surface of each male was shaved and examined for abnormalities, including the number and location of retained nipples, and hypospadias. The animals were examined...
internally for additional reproductive malformations, including epididymal agenesis, gubernacular malformations (e.g., agenesis and elongated under-developed of gubernacular ligaments), testicular malformations (e.g., testicular atrophy, cryptorchid testes, fluid-filled testes), and vas deferens, prostatic, and seminal vesicular agenesis. Gubernacular underdevelopment is characterized by thread-like gubernacular cords measuring longer than the normal length of 11 mm. Gubernacular ligaments measuring > 11–14 mm were considered mildly underdeveloped (but were scored as normal), while gubernacular ligaments measuring > 14 mm in length were considered markedly underdeveloped and were counted as gubernacular malformations. Organ weights were recorded for the following: glans penis, ventral prostate, seminal vesicles, testes, epididymides, levator ani/bulbocavernous (LABC) muscle, Cowper’s glands, kidneys, and liver. The incidence of total percent malformed males was calculated by adding together the number of males that had any occurrence of the following malformations at necropsy: nipple retention, hypospadias, vas deferens agenesis, ventral prostate agenesis, seminal vesicle agenesis, testes malformations, epididymal agenesis, and gubernacular agenesis/hypoplasia.

Testes were preserved in Bouin’s solution for 24 h then transferred into 70% ethanol until histological examination. Three transverse sections of the testes (through the rete, middle, and caudal regions) were stained with hematoxylin and eosin to observe any histopathology to validate any effects that were observed during necropsy as well as to detect any testicular lesions that were not evident at necropsy.

**Serum T radioimmunoassay.** At necropsy, a blood sample was collected for each animal. The blood was allowed to clot at 4°C for a minimum of 30 min in Vacutainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ), then centrifuged at 1000 x g for 15 min at 4°C. The serum was stored in Eppendorf tubes at −80°C until assayed by radioimmunoassay (RIA). T was measured in 50 μl sera by RIA using Coat-a-Count kits according to manufacturer’s protocols (Diagnostic Products Corporation, Los Angeles, CA). The level of detection of the RIA was 0.2 ng/μl T. Data are presented as litter means (± SE).

**Fetal Endocrine and Gene Expression Study**

This study consisted of three experimental blocks with two dams per treatment for a total number of six control dams, six DBP-treated dams, six DEHP-treated dams, and six dams receiving the combination dose of DBP and DEHP (DBP + DEHP). A total of three male fetuses per litter were evaluated for hormone production, and fetal testes were pooled by litter for the gene expression end points.

**Fetal necropy.** On the morning of GD18, the rat dams were anesthetized with CO2 and killed by decapitation. Fetuses were immediately removed, anesthetized, and killed on ice, and testes were removed under a dissecting microscope. Testes from the first three males were immediately transferred to TRI-reagent reaction as per manufacturer’s instructions. Prior to qrtPCR analysis, RNA samples (0.5 µg/reaction) were digested with DNase I and synthesized into cDNAs in a 20.9 µl reverse transcriptase reaction as per manufacturer’s instructions. The reverse transcriptase reaction began with 500 ng DNase I (11 µl volume) and 1 µl random hexamer primers (0.5 µg/µl) heat denatured at 70°C for 5 min and then chilled on ice. The remaining ingredients were added to each reaction: 4 µl 5X Improm buffer, 2.4 µl 25mM MgCl2, 1 µl 25mM deoxynucleoside triphosphate (dNTP), 0.5 µl Rnasin RNAse inhibitor, and 1 µl Improm reverse transcriptase enzyme. The reaction was annealed at 25°C for 5 min, first strand extended at 42°C for 60 min, heat inactivated at 70°C for 15 min, and cooled to 4°C. Finally, reactions were diluted to 20 ng/µl cDNA with the addition of 28.6 µl DEPC-treated dH2O; the RNA to cDNA ratio was considered 1:1.

The qrtPCR was performed on a BioRad iCycler real-time detection system (Hercules, CA). Primer sets and primer-specific, dual-labeled fluorescent probes (5’-Fam-labeled and 3’-Black Hole Quencher 1) specific to rat, or mouse and rat, were synthesized by Integrated DNA Technologies (Coralville, IA; Table 1). The qrtPCRs were carried out in a 50-µl volume containing 5 µl cDNA sample (50 ng/reaction), 1× PCR buffer, 0.4 mM each dNTP, 8 or 16 µl of 25mM MgCl2 (insl3, 8 µl; other primers, 16 µl), 12 pmol reverse primer, 12 pmol forward primer, 1.25 pmol fluorescent probe, 0.5 U Platinum Taq DNA Polymerase (Invitrogen) with Taq antibody added, and DEPC-treated dH2O. An internal standard curve was run in each assay with serial dilutions ranging from 1 x 10^3 to 1 x 10^7 copies per reaction so that an absolute determination of the starting quantity (SQ) of the cDNA sample could be determined. The cDNA standards were made by PCR, purified by phenol extraction and isopropanol precipitation, and quantified by Ribogreen quantitation reagent as stated above. Real-time PCR cycling conditions were an initial heat denaturation step of 95°C for 3 min, followed by multiple cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 10 s. Reactions were completed with a final annealing step at 72°C for 10 min. For each real-time PCR assay, all samples and standards were run in duplicate on a single plate. In cases where the data were collected in two separate real- time PCR runs, we assigned the samples to the two runs with all treatment groups equally distributed on each PCR plate to avoid confounding the effects of treatment with the effects of a different PCR plate.

**Statistics.** The data were analyzed using two-way ANOVA on the general linear measures procedures from the Statistical Analysis Systems (SAS, Inc., Cary, NC). Post hoc comparisons were made using the Least Squared Means

### TABLE 1

| Primer and Probe Sequences for Real-Time Quantitative RT-PCR Analyses |
|------------------|------------------|
| **Gene**          | **Primer and probe set** |
| insl3            | Forward 5'-TG GCC AAC CAC AC CTT GTG-3' |
|                  | Reverse 5'-ACC CAA AA GGT CTT GCT GGG-3' |
|                  | Probe 5'-ACC CGT C TGT CTCT ACT GCT GTC-3' |
| cyp11a           | Forward 5'-GGG ACT TAA AGC GGA AAG CGA-3' |
|                  | Reverse 5'-AT GTT CTT GAAG GGC AGC TTG-3' |
|                  | Probe 5'-AGT A C TGT GTC TCT TAT AGC CTG TCT GGG-3' |
| sf-1             | Forward 5'-TG GCC T GTG C TGA A ATG-3' |
|                  | Reverse 5'-G GCG CCA ATC TG T GCT TT C-3' |
|                  | Probe 5'-CA A GAG A AGC C GGC G C TTT AGA-3' |
| StaR             | Forward 5'-AGA AGG A AA GCG AAC GAG AGA-3' |
|                  | Reverse 5'-TCT CCA T A C GTC ATG-3' |
|                  | Probe 5'-TAG ACC AG C C A C G G G A C ACT CT AGA A-3' |
procedure on SAS, which is appropriate for \textit{a priori} hypotheses. We expected treatments to reduce organ weights and AGD in male offspring but increase androgen- and insl3-dependent tissue malformation rates. For analysis of treatment effects, litter means were used as the sample size versus the number of animals. Differences were considered significant at $p \leq 0.05$. The data of percent retention rate of areola/nipples were arcsine transformed prior to statistical analysis to normalize the data. If organs were absent at the time of necropsy, their weight was recorded as 0 g (e.g., some phthalate-treated males lacked entire epididymides). Individual organ weight data were examined statistically by two-way ANOVA and Fisher's exact test or chi-square analyses (Sigma Stat, Systat Software, San Jose, CA) because it was evident from the scatter plot graphs that the litter means analysis could obscure severe effects when the effects were limited to a small percentage of the male offspring. As reproductive organ weights are not correlated with body weight, the organ weights of the current study were not adjusted by body weight. Histopathological observations of the testes were statistically analyzed using the Fisher's exact test (Sigma Stat).

The data of fetal testicular T and P4 production were analyzed using litter means generated by pooling the individual data within a litter. The gene expression data were analyzed for the effects of treatment and PCR plate (since the samples were divided between two PCR plates per primer set) as well as the interaction between the treatment and PCR plate. The RNA from incubated versus nonincubated testes were run on two separate PCR plates for all genes except sf-1. For sf-1, RNA from both incubated and non-incubated testes were run on the same plate and incubation type was compared. If there was no interaction between PCR plate and treatment, the PCR results were pooled per litter. The significance values of the gene expression data reflect the \textit{post hoc} comparisons of the log-transformed SQ of each gene.

\textbf{Estimation of dose versus response addition.} The frequency of reproductive malformations and the percent reduction in reproductive tissue weights were used to determine whether the DBP + DEHP mixture was exerting dose versus response additive effects as described by Rider and LeBlanc (2005). Necropsy data from a preliminary dose-response study of the individual phthalates DBP and DEHP (250–1000 mg/kg/day from GD14–18 in SD rats; Gray, unpublished data) were fitted to the following logistic equation (formula 1): 

\[ \frac{1}{1 + \left( \frac{\text{Dose}}{\text{ED50}} \right)} \]

where $R$ is the response, $D$ is the daily dose, $p$ is the slope of the curve, and ED50 is the dose resulting in a 50% effect (formula 1).

Dose addition, referred to as concentration addition in Rider and LeBlanc (2005), is predicted to occur if two chemicals work through the same mechanism of action. In order to account for interexperimental variability, we calibrated the previously determined dose-response curves using the data from the current study. The formula for the ED50 (shown below; formula 2) is simply the logistic equation mentioned above solved for the ED50:

\[ \text{ED50} = \left[ \left(1 - R \right) \left( \frac{D'}{R} \right) \right]^\frac{1}{p} \]

where $R$ is the response, $D$ is the daily dose in the current study, and $p$ is the slope of the curve for DBP or DEHP derived from the preliminary dose-response studies. A dose-addition model was used to calculate the DBP + DEHP mixture response using the ED50 for the current study and the previously determined slopes for the individual phthalates with the following formula (formula 3):

\[ R = \frac{1}{1 + \left( \sum_{i=1}^{n} \frac{D_i}{\text{ED50}_i} \right)^p} \]

where $R$ is the response of the mixture, $D_i$ is the dose of individual chemical $i$ in the mixture, ED50$_i$ is the dose of chemical causing a 50% response, and $p$ is the average slope associated with the two phthalates in this mixture.

Response addition is expected when two chemicals in the mixture operate via different mechanisms of action and was calculated using the following equation (formula 4):

\[ R = 1 - \prod_{i=1}^{n} (1 - R_i) \]

where $R$ represents the response to the mixture and $R_i$ is the response to individual chemical $i$ in the mixture. Statistical differences between dose or response addition estimates and the observed DBP + DEHP mixture values were determined using Fisher’s exact test (Sigma Stat) for the percent incidence data, such as percent incidence of reproductive malformations and areola/nipple retention (expressed as % of 12 possible areola/nipple). Statistical differences between dose or response addition estimates and observed DBP + DEHP mixture effects were determined by 99% confidence mean limits (Statistical Analysis Systems) for the continuous data, such as percent reductions in AGD at PND3 and percent reduction in adult organ weights, fetal hormone production, and fetal gene expression relative to controls. The dose and response addition estimates were made using the individual means, not the litter means, for the effects of DBP or DEHP administered individually from both the preliminary dose response and the current DBP + DEHP mixture studies for all the postnatal end points. Response addition estimates for reductions in fetal hormone production and gene expression were estimated based on litter means. Dose addition for the fetal end points could not be calculated because these end points were not evaluated in the preliminary dose-response study. Finally, observed effects that statistically exceeded both response and dose additions were classified as synergy (i.e., greater than additive effects).

\textbf{RESULTS}

\textbf{Maternal and Pregnancy Data}

There were no treatment differences in the mean body weights of dams at culmination of treatment (GD18; Table 2), and all dams appeared healthy. However, the maternal weight gain, defined as the difference between GD14 and GD18 body weight, was reduced by 11.5 g in DEHP dams ($p < 0.05$) and by 20.8 g in DBP + DEHP dams ($p < 0.001$) relative to controls. Fetal/neonatal mortality, which we defined as the difference between the number of uterine implantations and the number of live pups on PND3, was significantly increased in the DBP + DEHP–treated litters relative to controls ($p < 0.005$; Table 2). The average number of live pups per litter (litter size) was smaller ($p < 0.001$) with seven pups per DBP + DEHP litter versus 13 pups per control litter. The litter sizes of DBP and DEHP-exposed dams were not significantly different from controls (Table 2). Of the six DBP + DEHP dams, one dam died due to an accident during dosing. Another DBP + DEHP dam had 1 live pup and 10 dead pups at PND3 and no pups at weaning on PND22.

\textbf{Neonatal and Infant Data}

Body weight on PND3 was unaffected by treatment in both male and female pups (Table 2). AGD was significantly
Maternal Body Weight and Litter Characteristics After Prenatal Exposure to Control (Corn Oil), DBP, DEHP, or DBP + DEHP (500 mg/kg/day per Individual Chemical) from GD14–18

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DBP</th>
<th>DEHP</th>
<th>DBP + DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters on PND3</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Maternal body weight at GD14</td>
<td>280.7 ± 7.97</td>
<td>296.8 ± 2.91</td>
<td>292.5 ± 4.80</td>
<td>293.6 ± 4.74</td>
</tr>
<tr>
<td>Maternal body weight at GD18</td>
<td>319.8 ± 11.92</td>
<td>328.2 ± 2.41</td>
<td>320.2 ± 5.70</td>
<td>312.0 ± 6.80</td>
</tr>
<tr>
<td>Maternal body weight gain (g)</td>
<td>39.2 ± 5.06</td>
<td>31.3 ± 1.17</td>
<td>27.7 ± 1.94</td>
<td>18.4 ± 5.65</td>
</tr>
<tr>
<td>No. of implantation scars</td>
<td>13.5 ± 1.06</td>
<td>15.3 ± 0.49</td>
<td>14.2 ± 0.79</td>
<td>14.8 ± 0.66</td>
</tr>
<tr>
<td>Litter size at PND1 (pups)</td>
<td>12.5 ± 0.76</td>
<td>13.7 ± 0.42</td>
<td>13.7 ± 0.84</td>
<td>7.4 ± 2.50</td>
</tr>
<tr>
<td>Fetal and neonatal mortality</td>
<td>1.0 ± 0.68</td>
<td>1.67 ± 0.61</td>
<td>0.5 ± 0.34</td>
<td>7.4 ± 3.08</td>
</tr>
<tr>
<td>Male F1 body weight at PND3 (g)</td>
<td>8.86 ± 0.18</td>
<td>8.80 ± 0.26</td>
<td>8.98 ± 0.35</td>
<td>8.11 ± 0.93</td>
</tr>
<tr>
<td>Female F1 body weight at PND3 (g)</td>
<td>8.53 ± 0.10</td>
<td>8.31 ± 0.11</td>
<td>8.54 ± 0.13</td>
<td>8.80 ± 0.19</td>
</tr>
</tbody>
</table>

Indicates value differs from control by *p* < 0.05. Values are litter mean ± SEM.

Maternal body weight gain defined as the difference between body weight on GD18 and GD14.

Fetal mortality is defined as the difference between observed implantation number and number of pups on PND3; day of birth = PND1.

Necropsy Data

While the male body weight in adulthood did not reach overall significance by *F*-statistic on ANOVA (*F* = 2.04, *p* = 0.1444), adult male body weight was significantly reduced (*p* < 0.05) by the DBP + DEHP dose relative to controls as determined by individual post hoc *t*-test analyses of litter means compared to control values. Body weight was not affected by either DBP or DEHP treatment alone (Table 3). The number of permanent nipples per adult male was significantly higher (*p* < 0.005) in the DBP + DEHP treatment relative to controls and tended to be higher in the DEHP treatment (*p* = 0.06; Fig. 1c). Likewise, the percent of adult males with nipples was increased by prenatal phthalate exposure relative to control males with the most dramatic effects seen in the DBP + DEHP males. The percent of adult males with nipples was 21.8 ± 13.4% for DBP (not significant), 41.3 ± 16.7% for DEHP (*p* < 0.05), and 82.5 ± 7% for DBP + DEHP (*p* < 0.0005) versus 0% nipple retention in the controls.

The combination dose of DBP + DEHP significantly increased the incidence of penile abnormalities (Fig. 2). Hypospadias occurred in 43.3% of DBP + DEHP males, compared to the absence of this effect in controls. In contrast, the DEHP-treated males exhibited only a low frequency of hypospadias (1.9% of males), and the DBP-treated males exhibited no genital abnormalities.

Phthalate treatment induced several internal malformations with the greatest incidence of malformations occurring with DBP + DEHP exposure. Treatment with DBP + DEHP significantly increased the incidence of testicular malformations by 74.2% (*p* < 0.0005), epididymal agenesis by 65.9% (*p* < 0.001), and gubernacular malformations by 61.5% (*p* < 0.005; Fig. 2). In contrast, neither the DBP nor DEHP individual chemical treatments caused a significant increase in the incidence of these reproductive malformations (Fig. 2). Of the DBP + DEHP males with gubernacular malformations, 25% of the males lacked one or both gubernacular ligaments, and 35% of the males had one or both gubernacular ligaments measuring greater than double the normal length of 11 mm with a range of 26.5–57.5 mm gubernacular length (Figs. 3a and 3b). The absence of both gubernacular ligaments in one DBP + DEHP male resulted in crossed testes, in which the testes descended into the contralateral scrotal sacs and the right and left spermatic arteries as well as vas deferens crossed over one another (Fig. 3c). Seminal vesicle abnormalities (agenesis and small lobes) occurred most frequently in DBP + DEHP–treated males (63.1%; *p* < 0.001), 11.1% (NS) of the DEHP males, and were not found in the control or DBP-treated males. Agenesis of the vas deferens was induced by all phthalate treatments reaching statistical significance (*p* < 0.001) only in the DBP + DEHP–treated males relative to controls (Fig. 2).
There were no gross, morphological ventral prostate malformations noted for any treatment group (data not shown).

The male reproductive organ weights were significantly decreased by the DBP + DEHP combination treatment (Table 3). The DBP + DEHP treatment significantly (p < 0.005) reduced glans penis weight by 8.3% relative to controls. Ventral prostate weight was reduced by 26.6% (p < 0.05), and paired seminal vesicle weight was reduced by 54% (p < 0.005) in the DBP + DEHP group versus control values. The DBP + DEHP treatment also significantly reduced paired testes weight by 35.8% (p < 0.0005) and paired epididymal weight by 58% (p < 0.0005) relative to control values. Prenatal exposure to DBP or DEHP alone did not significantly reduce the litter means of androgen-dependent reproductive organ weights with the exception of the LABC. The LABC muscle was one of the most sensitive tissues to individual phthalate doses, and it demonstrated a cumulative weight reduction with the combination dose. The paired Cowper’s gland weight was not affected by phthalate exposure (control, 227.58 ± 22.56 mg; DBP, 220.38 ± 15.76 mg; DEHP, 230.97 ± 11.09 mg; and DBP + DEHP, 202.89 ± 22.59 mg).

While the analysis of litter means indicated significant organ weight reductions principally with the combination dose, a scatter plot analysis of individual organ weights indicated that there was a sensitive subpopulation of males that were significantly affected by either DBP or DEHP alone (Figs. 4A–D). For example, using a two-way ANOVA, the means of individual epididymal and testis weights, paired seminal vesicle, and LABC weights were significantly reduced by the DEHP and DBP + DEHP doses (Figs. 4A and 4C). The DBP treatment significantly reduced LABC weights (p < 0.05 as in the litter means analysis) and tended (p = 0.07) to decrease seminal vesicle weights. Similar results were calculated by Fisher’s exact test (LABC and seminal vesicles) or chi-square analysis (testes and epididymides) for the individual organ’s weights in Figure 4 with two exceptions: the individual epididymal weights tended (p = 0.07) to be decreased by the DBP treatment and the seminal vesicle weights were only significantly decreased in the DBP + DEHP treatment group.

Histopathological analysis revealed that all phthalate treatments caused testicular lesions with the greatest percentage of males affected in the DBP + DEHP group. No testicular lesions were observed in any of the control testes. Testicular degeneration was observed in 14% of the DBP-treated males (p < 0.05), 33% of the DEHP-treated males (p < 0.005), and 75% of the DBP + DEHP–treated males (p < 0.001). Interstitial cell hyperplasia was observed in all phthalate groups (DBP, 8% and DEHP, 12%) but only reached significance in the testes of the DBP + DEHP–treated males (45%; p < 0.001).

Fetal Testis Steroid Hormone Production

Fetal testicular T production (ng T produced/testis/3 h) was significantly reduced in all three treatment groups versus control with a 29% decrease for DBP (p < 0.01), a 52% reduction for DEHP (p < 0.0001), and a 74% reduction for DBP + DEHP in combination (p < 0.0001) (Fig. 5a). The reduced T production in the DBP + DEHP group was also
significantly different from either the DBP ($p < 0.01$) or the DEHP ($p < 0.05$) exposure groups. Fetal testicular P4 production (ng P4 produced/testis/3 h) was significantly reduced only in the DBP + DEHP combination dose ($p < 0.05$) with a 32% reduction in P4 relative to control values (Fig. 5b). The DEHP exposure tended ($p = 0.07$) to reduce fetal testicular P4 levels with a 27% reduction in the DEHP treatment group relative to control values. In contrast to phthalate-induced changes in fetal testicular hormone production, there were no treatment effects in adult T sera levels in prenatally exposed male SD rats (data not shown).

**Fetal Testis Gene Expression**

The expression of *insl3* mRNA was significantly reduced in the treatment groups containing DEHP with a 47% reduction in mRNA for DEHP ($p < 0.01$) and a 66% reduction in mRNA for DBP + DEHP in combination ($p < 0.005$) relative to control values (Fig. 6a). The expression of *StAR* was significantly reduced by all phthalate exposures. The expression of *StAR* was decreased by DEHP alone or DBP + DEHP in combination with a 75% reduction in mRNA for DEHP ($p < 0.05$) and an 81% reduction for DBP + DEHP ($p < 0.05$) relative to control values, while DBP reduced *StAR* by only 49% ($p = 0.05$) compared to controls (Fig. 6b). However, only the DBP + DEHP treatment reduced the *cyp11a* expression ($p < 0.0005$) resulting in a 58% reduction relative to control values (Fig. 6c).

Estimation of Response Addition versus Dose Addition

The dose-addition model most accurately estimated toxicity to the developmental and adult end points analyzed (Table 4). The DBP + DEHP–induced reduction in AGD at PND3 was accurately estimated by dose addition. The effects of DBP + DEHP on areolae retention at PND14 and nipple retention in adulthood (expressed as percent of 12 possible areolae/nipples) were best estimated by both dose and response addition. Response addition consistently underestimated the observed effects in the DBP + DEHP–treated males for the percent incidence of reproductive malformations and percent reduction of androgen-dependent organ weights (Table 4). In contrast, dose addition was the best fit for the DBP + DEHP–induced incidence of epididymal agenesis and the percent reduction of

**TABLE 3**

Adult Male Rat Body Weight and Reproductive Tissue Weights After Prenatal Exposure to Corn Oil (Vehicle Control), DBP, DEHP, or DBP + DEHP (500 mg/kg/day per Individual Chemical) on GD14–18

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control</th>
<th>DBP</th>
<th>DEHP</th>
<th>DBP + DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>713.4 ± 14.0</td>
<td>713.2 ± 28.9</td>
<td>705.3 ± 26.1</td>
<td>633.7 ± 23.2</td>
</tr>
<tr>
<td>Glans penis (mg)</td>
<td>125.37 ± 1.77</td>
<td>124.65 ± 2.00</td>
<td>124.65 ± 1.58</td>
<td>114.93 ± 2.12</td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>741.95 ± 71.32</td>
<td>703.52 ± 55.98</td>
<td>728.63 ± 37.33</td>
<td>544.52 ± 43.37</td>
</tr>
<tr>
<td>Seminal vesicles (mg)</td>
<td>2139.56 ± 107.92</td>
<td>1919.08 ± 130.84</td>
<td>1950.79 ± 181.23</td>
<td>984.64 ± 203.53</td>
</tr>
<tr>
<td>Paired testes (mg)</td>
<td>3729.26 ± 137.58</td>
<td>3653.86 ± 209.46</td>
<td>3422.75 ± 245.27</td>
<td>2395.09 ± 191.69</td>
</tr>
<tr>
<td>Paired epididymes (mg)</td>
<td>1354.14 ± 49.70</td>
<td>1309.96 ± 64.46</td>
<td>1094.25 ± 154.91</td>
<td>567.58 ± 186.82</td>
</tr>
<tr>
<td>LABC (mg)</td>
<td>1415.09 ± 35.81</td>
<td>1267.57 ± 66.92</td>
<td>1245.37 ± 55.52</td>
<td>884.42 ± 70.09</td>
</tr>
</tbody>
</table>

*Indicates value differs from control by $p < 0.0005$.

Indicates value differs from control by $p < 0.05$.

Indicates value differs from control by $p = 0.07$.

Indicates value differs from control by $p = 0.0001$. 

$\text{a} n = 6$ litters per treatment, except DBP + DEHP with a total of four litters. Total number of males per treatment means were: control, $n = 25$; DBP, $n = 36–37$; DEHP, $n = 41–42$, and DBP + DEHP, $n = 19–20$. Values are litter mean ± SEM.

$\text{b} n = 4$ litters. Values are litter mean ± SEM. Control males did not exhibit reproductive abnormalities.
weights of the epididymides, testes, LABC as well as vas deferens (DBP + DEHP = 70.0%, response addition = 23.5%, dose addition = 77.0%). While dose addition overestimated the effect of the DBP + DEHP dose for testicular malformations, dose addition (100%) appeared to be a better model than response addition (36.3%) with the observed effect of 75% incidence more closely resembling dose addition (Table 4). The DBP + DEHP induction of three reproductive malformations (hypospadias, gubernacular agenesis/hypoplasia, and seminal vesicle malformations) actually exceeded the response or dose-addition model predictions indicating that DBP + DEHP mixture had a statistically significant synergistic effect in inducing these malformations.

While we could not model dose addition for all end points, we assessed the ability of the response addition model to accurately predict the response to the DBP + DEHP mixture.
using the USEPA definition of synergy as significantly greater than additive (USEPA, 2002). We are currently planning an expanded study with several levels of the DBP + DEHP mixture to improve our modeling of the effects of this phthalate mixture on the reproductive tract tissues and to further examine the nature of the interaction of these two phthalates on the end points that responded synergistically in the current study. Taken together, the data indicate that DBP and DEHP primarily interacted in a dose-additive manner in this study as predicted based upon their common mechanism of toxicity during sexual differentiation.

The potential for DBP and DEHP to produce cumulative, dose-additive effects in rodents in utero raises concerns for the effects of phthalate ester mixtures in the human fetus during sexual differentiation since human amniotic fluids may contain this mixture. Silva et al. (2004b) reported that metabolites MBP and MEHP were detected in 93 and 24%, respectively, of 54 human amniotic fluid samples tested. Although the median levels of the DBP and DEHP metabolites were very low, 2% of the human amniotic fluid samples had MBP and MEHP levels that differed by only five- and 24-fold, respectively, from the levels in rat amniotic fluid (Calafat et al., 2006) from dams treated with oral dosage levels near their LOAELs (Mylchreest et al., 2000; Gray, unpublished data). Strikingly, the margin of exposure in amniotic fluid samples between effective doses in rats and the maximum levels in human amniotic fluid is well below 100-fold. In addition, high MBP levels (ppm) have been detected in humans using pharmaceuticals containing DBP (Hauser et al., 2004).

Based upon the fact that the androgen synthesis pathway is highly conserved among rats and humans, and prenatal exposure to phthalates has demonstrable effects on rat sexual differentiation, some public health advocacy groups have raised concerns that exposure to multiple phthalates could result in adverse effects on human reproductive development (DiGangi et al., 2002; Purvis and Gibson, 2005). Indeed, Swan et al. (2005) recently reported an association between higher maternal blood levels of phthalate metabolites during pregnancy in women and shorter anogenital index (body weight–adjusted AGD) in their male offspring, suggesting that the maternal phthalate load suppressed androgen levels in utero. The hypothesis of this human epidemiological study was based, in part, upon the phthalate metabolites acting in a cumulative fashion.

A second objective of the current study, beyond determining if the phthalate ester mixture acted in a dose-additive fashion,
was to determine if the hormones and genes that are causally related to these lesions were also more affected by the DBP + DEHP treatment versus DBP or DEHP alone. We are presently unable to make dose additivity predictions since we have not collected individual dose-response data for the fetal end points; however, the results of the current study indicate that cumulative effects were seen in fetal testicular T production and the expression of insl3 and Cyp11a.

Prenatal DBP + DEHP exposure of male rats resulted in cumulative inhibition of insl3 expression, which likely caused a cumulative increase in the incidence of gubernacular malformations. The most unique malformation observed in the phthalate-treated males was cryptorchidism, where the gubernacular cords were either underdeveloped or absent, which often impaired the normal descent of testis into the scrotal sac. Cryptorchidism and/or the general underdevelopment of the gubernacular cords is a common “high-dose” malformation noted in phthalate studies from our laboratory (Gray et al., 2000) and others (Foster, 2006; McKinnell et al., 2005; Tyl et al., 2004); this malformation is not seen with other classes of antiandrogenic chemicals. The peptide hormone insl3 is secreted by the Leydig cells and is responsible for normal gubernacular development, which facilitates the first phase of testicular descent (from the kidney area to the inguinal region) in the developing male fetus (Zimmermann et al., 1999). In the male rat, testicular expression of insl3 is highest during late fetal development (GD17.5–19.5), after which time expression subsides until it is high again during puberty and adulthood (McKinnell et al., 2005). The inhibition of fetal testicular insl3 gene expression by phthalate exposure was first reported by our research group (Wilson et al., 2004) and has since been confirmed by others (Lehmann et al., 2004; McKinnell et al., 2005). As the assay becomes more available, we plan to measure the levels of insl3 peptide hormone production and testicular tissue content to better characterize the effect of the phthalate esters on Leydig cell production of this hormone. It is possible that the production of insl3 peptide
hormone may be more sensitive to phthalate exposure than \textit{insl3} mRNA expression.

The inhibition of fetal testicular steroid hormone production and associated gene expression due to prenatal phthalate exposure likely induced the reproductive malformations and the decrease in androgen-dependent tissue weights in adult prenatally exposed male rats. While fetal testicular T production was suppressed by all phthalate treatments, fetal testicular P4 production was suppressed only by the DBP + DEHP dose indicating that the phthalates were impacting a point in the steroidogenic pathway further upstream of the 3 beta-hydroxysteroid dehydrogenase enzyme. Thus, we measured the expression of the side-chain cleavage enzyme gene \textit{cyp11a}, a rate-limiting enzyme responsible for the conversion of cholesterol to pregnenolone, and \textit{StAR}, a gene encoding the protein responsible for shuttling cholesterol into the mitochondria to begin the steroidogenic pathway. Exposure to DBP (500 mg/kg/day) from GD11–18 is known to inhibit fetal expression of \textit{cyp11a} and \textit{StAR} (Barlow et al., 2003; Lehmann et al., 2004). However, \textit{cyp11a} expression was only significantly suppressed by the DBP + DEHP dose with our 5-day dosing regime. While \textit{StAR} expression was significantly suppressed by DBP and DEHP treatments, the magnitude of the suppression of \textit{StAR} expression by the DBP + DEHP treatment was similar to DEHP, suggesting that \textit{StAR} was maximally inhibited by the 500 mg/kg/day DEHP dose and that further suppression of expression with the DBP + DEHP mixture was not possible. Similar to Thompson et al. (2004), we did not observe phthalate ester effects on the expression of \textit{sf-1}, a known regulator of \textit{StAR} and \textit{cyp11a} genes (Parker, 1998). However, \textit{sf-1} is essential for normal testis differentiation from an indifferent gonad, which occurs earlier in fetal life than the stage of development assessed in the current study.

Maternal body weight gain and postimplantation embryo/neonatal loss also displayed cumulative responses to DBP + DEHP treatment, likely due to the high amount of total phthalate esters administered to the dam. Reductions in maternal body weight gain relative to control dams have been observed with daily dosing of > 500 mg DBP/kg/day regimes during pregnancy of (Mylchreest et al., 1999) and DEHP (Gray et al., 2000; Jarfert et al., 2005; Parks et al., 2000). The postimplantation embryo/neonatal loss may have been due, in part, to phthalate-induced suppression of P4 in the rat dams in mid-late pregnancy (Gray et al., 2006a).

In conclusion, the dose-additive effects of prenatal exposure on the male rat reproductive system demonstrated by our current study and the striking association of multiple phthalate metabolites on the anogenital index in human infants

<table>
<thead>
<tr>
<th>Androgen- and Ins13-dependent alterations</th>
<th>Observed effects</th>
<th>Model predictions for DBP + DEHP</th>
<th>Best model (interaction, if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD on PND3</td>
<td>DBP 9.2</td>
<td>DEHP 9.8</td>
<td>DBP + DEHP 27.8 ± 5.5</td>
</tr>
<tr>
<td>Areolae retention (% of 12 possible)</td>
<td>22.0</td>
<td>25.4</td>
<td>66.4</td>
</tr>
<tr>
<td>Hypospadias</td>
<td>0</td>
<td>2.4</td>
<td>25</td>
</tr>
<tr>
<td>Epididymal agenesis</td>
<td>5.4</td>
<td>28.6</td>
<td>70</td>
</tr>
<tr>
<td>Vas deferens agenesis</td>
<td>2.7</td>
<td>21.4</td>
<td>70</td>
</tr>
<tr>
<td>Testis malformations</td>
<td>10.8</td>
<td>28.6</td>
<td>75</td>
</tr>
<tr>
<td>Gubernacular agenesis/hypoplasia</td>
<td>27</td>
<td>4.8</td>
<td>75</td>
</tr>
<tr>
<td>Seminal vesicle malformations</td>
<td>0</td>
<td>14.3</td>
<td>65</td>
</tr>
<tr>
<td>Nipple retention (% of 12 possible)</td>
<td>7.4</td>
<td>17.3</td>
<td>31.7</td>
</tr>
<tr>
<td>Total malformed males</td>
<td>31.7</td>
<td>45.5</td>
<td>100</td>
</tr>
<tr>
<td>Paired epididymal weight</td>
<td>3.2</td>
<td>22.8</td>
<td>57 ± 23.5</td>
</tr>
<tr>
<td>Paired testis weight</td>
<td>1.3</td>
<td>10</td>
<td>34.6 ± 13.3</td>
</tr>
<tr>
<td>Seminal vesicle weight</td>
<td>11.5</td>
<td>12.6</td>
<td>56.4 ± 21.1</td>
</tr>
<tr>
<td>LABC weight</td>
<td>9.6</td>
<td>14.3</td>
<td>32.5 ± 7.1</td>
</tr>
<tr>
<td>Glans penis weight</td>
<td>0.1</td>
<td>0.4</td>
<td>8.5 ± 8.4</td>
</tr>
<tr>
<td>Ventral prostate weight</td>
<td>4.5</td>
<td>4.4</td>
<td>24.7 ± 19.3</td>
</tr>
</tbody>
</table>

\begin{table}
\centering
\caption{Summary of Observed Effects in Male Rats Prenatally Exposed to DBP, DEHP, or DBP + DEHP (500 mg/kg/day per Individual Chemical) versus Predictions of Response Addition (RA) and Dose Addition (DA) for the DBP + DEHP Group. Values for Malformations and Areola/Nipple Retention Are Expressed as the Percentage of Animals Affected, whereas AGD and Organ Weights Are Shown as Percent Reduction from Controls ± 99% Confidence Limits. The Entries in Bold Represent The Best Model}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Androgen- and Ins13-dependent alterations & Observed effects & Model predictions for DBP + DEHP & Best model (interaction, if any) \\
\hline
AGD on PND3                             & DBP 9.2          & DEHP 9.8                         & DBP + DEHP 27.8 ± 5.5           & RA 18.1 DA 31 \textit{DA}                      \\
Areolae retention (% of 12 possible)    & 22.0             & 25.4                             & 66.4                            & RA 41.8 DA 100 \textit{RA and DA}              \\
Hypospadias                             & 0                & 2.4                              & 25                              & RA 2.4 DA 0 \textit{Observed > RA or DA} (synergy) \\
Epididymal agenesis                     & 5.4              & 28.6                             & 70                              & RA 32.4 DA 87 \textit{DA}                      \\
Vas deferens agenesis                   & 2.7              & 21.4                             & 70                              & RA 23.5 DA 77 \textit{DA}                      \\
Testis malformations                    & 10.8             & 28.6                             & 75                              & RA 36.3 DA 100 \textit{DA > observed > RA} (synergy) \\
Gubernacular agenesis/hypoplasia        & 27               & 4.8                              & 75                              & RA 30.5 DA 27 \textit{DA > observed > RA} (synergy) \\
Seminal vesicle malformations           & 0                & 14.3                             & 65                              & RA 14.3 DA 11 \textit{DA > observed > RA} (synergy) \\
Nipple retention (% of 12 possible)     & 7.4              & 17.3                             & 31.7                            & RA 23.4 DA 29 \textit{DA > observed > RA} (synergy) \\
Total malformed males                   & 31.7             & 45.5                             & 100                             & RA 62.8 DA 100 \textit{DA > observed > RA} (synergy) \\
Paired epididymal weight                & 3.2              & 22.8                             & 57 ± 23.5                       & RA 25.3 DA 80 \textit{DA > observed > RA} (synergy) \\
Paired testis weight                    & 1.3              & 10                               & 34.6 ± 13.3                     & RA 10.8 DA 27 \textit{DA > observed > RA} (synergy) \\
Seminal vesicle weight                  & 11.5             & 12.6                             & 56.4 ± 21.1                     & RA 22.7 DA 100 \textit{DA > observed > RA} (synergy) \\
LABC weight                             & 9.6              & 14.3                             & 32.5 ± 7.1                      & RA 22.5 DA 33 \textit{DA > observed > RA} (synergy) \\
Glans penis weight                      & 0.1              & 0.4                              & 8.5 ± 8.4                       & RA 0.5 DA 0 \textit{DA > observed > RA} (synergy) \\
Ventral prostate weight                 & 4.5              & 4.4                              & 24.7 ± 19.3                     & RA 8.7 DA 12 \textit{DA > observed > RA} (synergy) \\
\end{tabular}
\end{table}
emphasize the need for more research to assess the cumulative effects of phthalates, and mixtures of other antiandrogens, in combination with each other. It is noteworthy that the dose–addition model, which assumes that the chemicals are acting via a common mechanism of action, was predictive of the majority of the androgen-dependent end points measured in the DBP + DEHP–exposed male offspring. As hormone-dependent development in rats shares considerable evolutionary conservation to humans, the cumulative increase in reproductive malformations and decreased reproductive organ weights due to prenatal exposure to the DBP + DEHP mixture is a factor that warrants consideration in future risk assessments on phthalate esters as well as other chemicals known to impact the T pathway. Future experiments are needed to address how closely fetal testicular hormone production and gene expression changes are associated with, and thus predictive of, reproductive malformations in adulthood.

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