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Transgenerational Effects of Di (2-Ethylhexyl) Phthalate in the Male CRL:CD(SD) Rat: Added Value of Assessing Multiple Offspring per Litter

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In the rat, some phthalates alter sexual differentiation at relatively low dosage levels by altering fetal Leydig cell development and hormone synthesis, thereby inducing abnormalities of the testis, gubernacular ligaments, epididymis, and other androgen-dependent tissues. In order to define the dose-response relationship between di(2-ethylhexyl) phthalate (DEHP) and the Phthalate Syndrome of reproductive alterations in F1 male rats, Sprague-Dawley (SD) rat dams were dosed by gavage from gestational day 8 to day 17 of lactation with 0, 11, 33, 100, or 300 mg/kg/day DEHP (71–93 males per dose from 12 to 14 litters per dose). Some of the male offspring continued to be exposed to DEHP via gavage from 18 days of age to necropsy at 63–65 days of age (PUB cohort; 16–20/dose). Remaining males were not exposed after postnatal day 17 (*in utero*-lactational [IUL] cohort) and were necropsied after reaching full maturity. Anogenital distance, sperm counts and reproductive organ weights were reduced in F1 males in the 300 mg/kg/day group and they displayed retained nipples. In the IUL cohort, seminal vesicle weight also was reduced at 100 mg/kg/day. In contrast, serum testosterone and estradiol levels were unaffected in either the PUB or IUL cohorts at necropsy. A significant percentage of F1 males displayed one or more Phthalate Syndrome lesions at 11 mg/kg/day DEHP and above. We were able to detect effects in the lower dose groups only because we examined all the males in each litter rather than only one male per litter. Power calculations demonstrate how using multiple males versus one male/litter enhances the detection of the effects of DEHP. The results at 11 mg/kg/day confirm those reported from a National Toxicology Program multigenerational study which reported no observed adverse effect levels-lowest observed adverse effect levels of 5 and 10 mg/kg/day DEHP, respectively, via the diet.

Key Words: **di(2-ethylhexyl) phthalate; sexual differentiation; phthalate syndrome; dose response.**

Phthalate esters (PEs) are high production volume chemicals used in a variety of consumer products including polyvinyl chloride plastics, toys, personal care products, cosmetics and pharmaceuticals. Among the PEs, di(2-ethylhexyl) phthalate (DEHP) is the most abundant, with about four million tons of di-octyl phthalates being produced in 1997. Several PEs, including DEHP, are reproductive toxicants in animals with effects being seen in rats (Parks *et al*., 2000), mice (Oishi, 1993; Song *et al*., 2006; Tyl *et al*., 1988), hamsters (Gray *et al*., 1982), guinea pigs (Gray *et al*., 1982), rabbits (Higuchi *et al*., 2003), ferrets (Lake *et al*., 1976), and pubertal-age nonhuman primates (Tomonari *et al*., 2006). Within the last few years, it has become apparent that PEs alter sexual differentiation of the male rat at dosage levels below those that affect adult male rats (Mylchreest *et al*., 1998; Sjoberg *et al*., 1986). When examined during sexual differentiation, these PEs (DEHP, di(*n*)butyl phthalate [DBP], di-isobutyl, benzyl butyl phthalate [BBP], and di-isononyl phthalate) reduce testis testosterone production and insulin-like three peptide hormone mRNA (Borch *et al*., 2004; Wilson *et al*., 2004), which results in the "Phthalate Syndrome" (Fisher *et al*., 2003; Foster *et al*., 2001; Gray *et al*., 2000), a suite of malformations of the testis, gubernacular ligaments, testis descent, the epididymides and other androgen-dependent tissues. Because the rodent Phthalate Syndrome bears similarity to the human Testicular Dysgenesis Syndrome, a concept that poor semen quality, testis cancer, undescended testis, and hypospadias are symptoms of a single disorder in men (Skakkebaek, 2002; Wohlfahrt-Veje *et al*., 2009), concerns have arisen about the potential effects of PEs on human reproductive development (Fisher *et al*., 2003; Lottrup *et al*., 2006). Although there are several studies describing the effects of DEHP and other PEs on fetal testis endocrine function, few comprehensive and robust transgenerational studies of DEHP have been published.

The current study was designed to address this data gap by exposing dams during pregnancy and lactation to relatively low levels of DEHP by gavage, followed by a thorough examination of a sufficient number of adult offspring to be able to detect low incidence of males with the Phthalate Syndrome. To this end, female Sprague-Dawley (SD) rats were dosed by

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gavage from gestational day (GD) 8 to day 17 of lactation with 0, 11, 33, 100, or 300 mg/kg/day DEHP. The SD rat strain was selected because they are more sensitive to some PE-induced reproductive lesions than are Wistar (Wilson *et al*., 2007) or Long Evans Hooded rats (Howdeshell *et al*. 2007; Noriega et al., submitted).

The study was conducted in two blocks. DEHP exposure was continued in approximately half the male offspring in each litter in the first block, being dosed by gavage from 18 to 63–65 days of age with DEHP (n = 16–20 males per dose) and necropsied at 63–65 days of age (pubertal [PUB] cohort). The rest of the developmentally exposed males were not dosed after day 17 and were necropsied at full maturity, referred to herein as the *in utero*-lactational (IUL) cohort. The study examined 71–93 males per dose group (n = 12–14 litters per treatment) including those from the two (IUL and PUB) cohorts.

In every male, anogenital distance (AGD) was measured at birth, retained areolae/nipples were counted at 13 days of age, the age at puberty was determined, and reproductive tissues were examined for malformations and weighed at necropsy. The tissues examined at necropsy included the testes, epididymides, seminal vesicles plus coagulating glands, glans penis, Cowper's glands, ventral prostate, levator ani plus bulbocavernosus muscles, retained nipples, and gubernacular cord lengths. In addition, in the IUL cohort both testes (seven sections per testis) and epididymides were examined for histopathological lesions in every F1 male.

In a parallel study, pregnant rats in metabolism cages were dosed with DEHP, as above, maternal urine collected and the dams were necropsied at the end of sexual differentiation on GD 18 and amniotic fluid collected for monoethyl hexyl phthalate (MEHP) analyses (Calafat *et al*., 2006), so that we could correlate the fetal exposures to MEHP with the incidence of reproductive tract malformations seen herein.

MATERIALS AND METHODS

Animals. Timed-pregnant SD (CR:CD(SD)IGSBR) rats (approximately 90 days of age) were purchased from Charles River Laboratories (Raleigh, NC). Dams were delivered to EPA facilities on gestational day (GD) 2; the day sperm plug positive was considered GD1. Animals were housed in 20 × 25 × 47 cm clear polycarbonate cages with laboratory-grade heat-treated pine shavings (heat-treated to remove resins; Northeastern Products, Warrensburg, NY). Animals were maintained on a 14:10 light/dark photoperiod (lights off at 1100 h) at 20–24°C and 40–50% relative humidity. Dams were fed *ad libitum* with Purina Rat Chow 5008, and weanling and adult rats were fed Purina Rat Chow 5001. Animals had 24 h access to filtered (5 μm) Durham, NC, municipal drinking water. 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 a protocol approved by the National Health and Environmental Effects Research Laboratories Institutional Animal Care and Use Committee.

Doses and experimental design. In each of the two blocks, there were six to seven pregnant females per dose group per block (detailed samples sizes in Table 1). Pregnant dams were assigned to treatment groups on GD 8 in a manner that provided similar mean (± SE) body weight per group prior to dosing. Laboratory-grade corn oil (8001-30-7, lot # 89H0149) and DEHP (CAS # 117-81-7, lot # 106H3487. listed purity 99.1%) were purchased from Sigma (St Louis, MO). Dosing solutions stored in glass bottles in dark with stirring bar removed (to avoid contact with plastic). Dams were dosed daily with 0 (vehicle control), 11, 33, 100, or 300 mg/kg/day DEHP from GD 8 until day 17 of lactation (sperm positive = GD 1). Pregnant and lactating dams and half of their weanling males in the first block were dosed daily by oral gavage with DEHP or the vehicle (corn oil) on a mg/kg/day basis at a rate of 2.5 ml vehicle per kg body weight. The number of dams per group is listed in Table 1.

In the first block, two to three randomly selected male offspring per litter were dosed directly from postnatal day (PND) 18 until necropsy at 63–65 days of age (pubertal [PUB] cohort, see Table 2 for sample sizes). The remaining

TABLE 1

DEHP Administration from GD 8 to 17 of Lactation Had No Effect on Maternal Weight or Weight Gain during Dosing or Litter Size at 2 days of Age, but Reduced AGD and Body Weight in 2-Day-Old Male Rat Pups and Induced Female-Like Areolas/Nipples in 13-Day-Old Male Rats

Dose of DEHP mg/kg/d	0	11	33	100	300
Number of dams	13	13	14	14	13
GD 8 weight (g)	269 ± 2.5	271 ± 3.4	266 ± 3.2	268 ± 3.3	269 ± 4.8
GD 22 weight (g)	399 ± 8.5	403 ± 7.2	395 ± 6.0	401 ± 7.4	398 ± 6.0
Pregnancy weight gain (g) (GD 8–22)	130 ± 7.5	132 ± 7.4	129 ± 4.6	132 ± 5.5	129 ± 5.6
Birth weight (g)	306 ± 4.1	307 ± 3.8	307 ± 5.7	302 ± 5.8	299 ± 5.5
Weight at day 17 of lactation	324 ± 2.4	333 ± 4.8	332 ± 5.1	331 ± 5.8	333 ± 5.6
AGD (mm) in females	1.34 ± 0.04	1.26 ± 0.02	1.29 ± 0.03	1.36 ± 0.02	1.31 ± 0.03
AGD (mm) in males	3.25 ± 0.11	3.21 ± 0.05	3.17 ± 0.09	3.17 ± 0.05	2.74 ± 0.08**
Day 2 weight (g) females	6.95 ± 0.17	7.04 ± 0.12	7.10 ± 0.13	7.27 ± 0.11	6.74 ± 0.08, down 3%
Day 2 weight (g) males	7.51 ± 0.17	7.44 ± 0.11	7.42 ± 0.15	7.64 ± 0.12	6.98 ± 0.10**, down 7%
Day 2 litter size	14.1 ± 0.69	14.5 ± 0.54	12.9 ± 0.81	13.5 ± 0.44	14.1 ± 0.29
Percent of males with areolae	11% ± 5.5	21% ± 8.9	10% ± 4.7	16% ± 6.7	55% ± 10.1**
Number of areolae per male out of 12	0.7 ± 0.4	0.8 ± 0.3	0.3 ± 0.1	0.7 ± 0.3	2.9 ± 0.6**

Note. Pup values are litter means ± SE. Pup data were analyzed using a PROC MIXED model that accounted for the clustering of pups within litters. Values are means ± SE, *$p < 0.05$, **$p < 0.01$.

TABLE 2
Necropsy Data from Male F1 SD Rats in the PUB Cohort Necropsied at 64 Days of Age

Dose of DEHP mg/kg/d	0	11	33	100	300
Number males (litters)	20 (7)	16 (6)	19 (7)	17 (7)	20 (7)
Body weight (g)	371 ± 17	385 ± 7.5	373 ± 10.1	388 ± 12.7	356 ± 9.0
Glans penis (mg)	102 ± 3.2	103 ± 2.8	96.5 ± 3.3	103 ± 4.9	90.4 ± 3.8
Ventral prostate (mg)	361 ± 19	362 ± 21	374 ± 16	365 ± 15	303 ± 14**
Seminal Vesicle (mg)	1015 ± 47	1104 ± 27	1078 ± 46	1063 ± 47	836 ± 54**
Levator ani-bulbocavernosus (mg)	913 ± 47	972 ± 27	900 ± 30	926 ± 23	756 ± 26**
Cowper's glands (mg)	94.5 ± 3.7	91.2 ± 4.9	86.2 ± 4.5	88.3 ± 5.1	76.1 ± 3.8**
Epididymides (mg)	667 ± 17	674 ± 25	685 ± 15	659 ± 21	530 ± 22**
Whole epididymal sperm count × 10^6	92.3 ± 4.5	84.4 ± 3.4	91.6 ± 2.8	84.2 ± 4.1	53.3 ± 6.5**
Paired testes (mg)	3019 ± 72	2971 ± 139	3068 ± 77	3186 ± 78	2797 ± 93
Adrenals (mg)	50.6 ± 2.7	48.3 ± 2.4	47.2 ± 3.0	47.0 ± 2.1	42.7 ± 1.3 a
Liver (g)	16.6 ± 0.72	18.3 ± 0.63	18.0 ± 0.81	20.3 ± 0.78**	19.8 ± 0.86**
Kidneys (mg)	2936 ± 123	3066 ± 96	2956 ± 72	2945 ± 113	2755 ± 93*
Age at puberty (PPS)	45.7 ± 0.64	47.3 ± 1.6	47.6 ± 0.77	47.1 ± 0.98	49.1 ± 0.7 a
Weight at puberty	233 ± 7.3	251 ± 12.0	251 ± 11.4	252 ± 9.7	251 ± 6.3
Weight at 18 days	34.7 ± 1.3	33.5 ± 0.8	36.0 ± 0.9	37.3 ± 1.0	34.6 ± 0.9
Body weight gain	338 ± 9.2	349 ± 6.0	334 ± 6.1	351 ± 8.1	324 ± 5.5
Testosterone (ng/ml)	2.13 ± 0.28	2.81 ± 0.48	1.99 ± 0.25	2.16 ± 0.28	1.75 ± 0.19
Estradiol (pg/ml)	72.4 ± 23.1	48.0 ± 9.4	48.9 ± 13.6	37.0 ± 4.9	56.0 ± 20.0

Note. DEHP was administered from gestational day 8 to day 17 of lactation to the dam and then directly to the male offspring up to necropsy. Values are litter-based means ± SE. Data were analyzed using a PROC MIXED model that accounted for the clustering of pups within litters. *$p < 0.05$, **$p < 0.01$, a=$p < 0.05$ Dunnett's test of PROC MIXED analysis.

male litter mates in block 1 were not dosed after maternal dosing ended on PND 17. Rats were weaned at 28 days of age and housed two to three per cage with similarly treated littermates for the duration of the study. In block 2, none of the male offspring were dosed by gavage after PND17 with DEHP. The samples sizes for the IUL cohort is shown in Table 3. For the IUL males, the exposure is only via the dam (transplacental and from the milk). In all blocks, the rats were weighed daily during the dosing period to administer the dose per kilogram body weight and to observe the health of the dams.

TABLE 3
DEHP Administration from GD 8 to 17 of Lactation to the Dam Reduces Androgen-Dependent Organ Weights in F1 Male SD Rat Offspring from the IUL Cohort Necropsied as Adults

Dose of DEHP mg/kg/d	0	11	33	100	300
Number males (litters)	63(13)	55(12)	67(14)	76(14)	54(13)
Body weight (g)	607 ± 14	664 ± 17 a	637 ± 18	634 ± 16	616 ± 15
Glans penis (mg)	102 ± 1.9	102 ± 2.2	100 ± 1.5	100 ± 1.9	93.0 ± 1.6**
Ventral prostate (mg)	794 ± 35	781 ± 40	819 ± 21	734 ± 20	691 ± 33*
Seminal vesicle (mg)	2107 ± 66	2031 ± 68	2045 ± 47	1999 ± 62*	1720 ± 46**
Levator ani-bulbocavernosus (mg)	1309 ± 32	1368 ± 40	1352 ± 19	1319 ± 34	1162 ± 33**
Cowper's glands (mg)	205 ± 11	194 ± 13	205 ± 11	198 ± 13	169 ± 5.4**
Epididymis (mg)	659 ± 11	637 ± 16	655 ± 8.1	630 ± 18	550 ± 48**
Testis (mg)	1797 ± 25	1767 ± 57	1841 ± 27	1800 ± 46	1660 ± 75[a]
Adrenals (mg)	45.0 ± 2.0	44.4 ± 3.4	46.8 ± 1.3	46.5 ± 1.9	44.9 ± 2.1
Liver (g)	19.0 ± 0.64	21.1 ± 0.63	20.1 ± 0.70	20.6 ± 0.8	19.2 ± 0.62
Kidney (mg)	1979 ± 57	2035 ± 39	1965 ± 58	1975 ± 48	1780 ± 42**
Number of nipples/male	0 ± 0	0.08 ± 0.08	0 ± 0	0.15 ± 0.12	1.22 ± 0.41**
Serum testosterone (ng/ml)	1.51 ± 0.21	1.29 ± 0.16	1.32 ± 0.16	1.36 ± 0.10	1.22 ± 0.16
Amniotic fluid[b] MEHP (ng/ml) (no. litters/ no. pups)	7.2 ± 2.2 (2/24)	68.4 ± 17 (2/26)	168 ± 24 (2/28)	748 ± 236 (2/26)	2324 ± 430 (2/32)

Note. MEHP, monoethyl hexyl phthalate. Values are litter-based means and standard errors. Data were analyzed using a PROC MIXED model that accounted for the clustering of pups within litters. *$p < 0.05$, **$p < 0.01$ with body weight as a covariate; a = $p < 0.05$ only without body weight as a covariate.

[a]$p < 0.05$ by Fisher Exact test of the number individuals with abnormal testis weights.

[b]Data from Calafat *et al*. (2006).

Neonatal and infant male rat offspring. AGD and body weight were measured in all male and female pups on PND 2 (birth day = PND 1) Measurements were made in a manner where the observer was blind to the maternal treatment. Pups were examined using a Leica MZ6 dissecting scope (Wetzlar, Germany). Skin between the phallus and tail-base was extended maximally and AGD measurements were made to the nearest 0.1 mm using a 10-mm ocular reticule. Scope magnification at 15× (1.5 × 10) was calibrated using a 1-mm stage micrometer with 0.01-mm divisions.

On PND 13, areola/nipple numbers and location were noted for each (male and female) pup in a blinded fashion. Male offspring were weaned on PND 24 and housed in unisexual groups of two to three litter mates per cage.

Preputial separation. In the PUB and IUL cohorts, male offspring were examined daily for the onset of preputial separation (PPS), an androgen-dependent landmark of puberty in the male rat, from PND 35 until complete separation. The age at PPS and the body weight at PPS were recorded for each male.

Necropsy of the PUB and IUL cohorts. All F1 males were retained and examined thoroughly (AGD, nipples, PPS, organ weights, histopathology of testes and epididymides, and malformations was determined in every male) in this study. At necropsy of the PUB cohort at 63–65 days of age, there were 20 males from seven litters, 16 males from 6 litters, 19 males from seven litters, 17 males from seven litters and 20 males from seven litters in the 0, 11, 33, 100, and 300 mg/kg/day DEHP dose groups, respectively for a total of 92 males. A total of 315 F1 males from the IUL cohort were necropsied at about 7 months of age (see Table 3 for sample sizes). Animals were anesthetized with CO_2 prior to decapitation after which blood was collected for radioimmunoassay (RIA) analyses for serum testosterone and estradiol levels. Testosterone levels were measured by RIA using Coat-a-Count kits according to manufacturer's protocols (Diagnostic Products Corporation, Los Angeles, CA; Coat-a-Count Kit Total Testosterone Manual, #PITKTT-4, 2005-03-18). The intra-assay coefficient of variation (CV) was 3.1%, based upon the variability of the standard curve and the interassay CV was 13.7%. Cross reactivity with DHT was 3.2%. The limits of detection of the RIAs were 0.2 ng/ml testosterone. Estradiol was determined by RIA using kits according to manufacturer's protocol (Diagnostic Systems Laboratories (Webster, TX), #DSL-39100). The kit has a minimum detection limit of 0.6 pg/ml and the intra-assay precision is reported to be a CV = < 4%.

We examined each male for internal and external reproductive malformations, including agenesis or hypoplasia of the gubernacular ligaments, cauda, corpus and caput epididymides, vas deferens, seminal vesicles, coagulating glands, ventral prostate, Cowper's glands, cranial suspensory ligaments, testes, descent of the testes and retained female reproductive tract tissues, the presence of retained nipples (after carefully shaving the ventral surface), hypospadias, cleft phallus, and vaginal pouch. Organ weights included individual testes and epididymides for all males, and ventral prostate, seminal vesicle with coagulating glands and fluids, paired adrenals, both kidneys, liver, paired Cowper's glands, glans penis (if not malformed) and levator ani and bulbocavernosus muscle complex for most males.

Both testes and epididymides (IUL cohorts) were preserved in Bouin's fixative and placed in 70% ethanol after 24 h. In the PUB cohort, one epididymis from each male was examined for histological lesions while the other whole epididymis was processed for enumeration of sperm numbers as per Gray *et al.* (1997). Malformed epididymides were not included in the sperm count analyses. All histopathological examinations of the testes and epididymides were conducted by board-certified veterinary pathologists at Veritas (block 1) and Experimental Pathology Laboratories, Inc (Research Triangle Park, NC) (block 2). Tissues were sectioned at 4–6 μm and stained using hematoxylin and eosin. In addition, we also evaluated the tissues from the pubertal cohort of block 1. Because a number of effects were seen in the testes of treated males in the lower dosage groups and because we were concerned that subtle histopathological alterations in the testis might go undetected from evaluation of a single cross section, we resubmitted the testis tissue blocks from all the males in the IUL cohort to Experimental Pathology Laboratories for further processing. Because each tissue block contained two transverse sections of each testis, this represents a total of over 1250 blocks (two per testis, four per rat). Three hemotoxylin and eosin stained slides were prepared for microscopic examination of each block resulting in almost 3800 sections of rat testes, which were evaluated for lesions.

Statistical analyses. Data were analyzed using PROC MIXED from SAS version 9.1 (Statistical Analyzing Systems, Inc., Cary, NC). The use of mixed effects ANOVA allows the introduction of random litter effects and corrects for the statistical nonindependence of pup measurements from the same litter. Analysis of organ weight data included body weight at necropsy as a covariate. Statistically significant effects F values were further examined using the LSMEANS (two-tailed t-test) procedure to compare groups. In cases where the F value was not significant, treated-group means were compared with control means using a Dunnett's test.

Treatment means and standard errors presented in the tables were calculated using PROC MEANS using litter based rather than individual means. Individual data rather than litter mean data also are presented because using litter-based values can obscure severe effects if they only occur in a small percentage of the treated animals (i.e., individual testis weights).

In the PUB cohort, data were analyzed using PROC MIXED, as above. Age a puberty and weight at puberty (PPS) were analyzed using body weight at 44 days of age, approximately the mean age at puberty, as a covariate whereas organ weight data were analyzed using body weight at necropsy as a covariate.

Malformations of the reproductive tract of males from the PUB and IUL cohorts indicative of alterations of androgen and insl3-dependent tissues and histopathological lesions of the testes or epididymides were examined to determine if treated males displayed lesions consistent with the Phthalate Syndrome. The percentage of affected males per dose group was analyzed by Chi square and then each DEHP-treated group was compared with the control group by Chi-square analyses). This analysis was repeated with and without the higher dose group (0, 11, 33 and 100 but not 300 mg/kg/day) to determine if the frequencies differed among the low dose groups and control. Effects included for this analysis were (1) morphology and histology of the testes (fluid filled and flaccid, atrophic, undescended or displaying seminiferous tubular atrophy), (2) epididymidal agenesis or hypoplasia (similar to, but less severe than aplasia) of the caput, corpus and/or the cauda (confirmed histologically, not including tissues only reduced in size) and epididymal granuloma, (3) malformed seminal vesicles or coagulating glands (agenesis of both horns or a single horn or coagulating glands detached completely from the seminal vesicles, not including tissues only reduced in size), (5) permanent nipples at adult necropsy (not including areolae without nipples), (6) gubernacular agenesis or elongation (greater than 15 mm), (6) agenesis of the ventral prostate (not including tissues only reduced in size), (7) cleft phallus with hypospadias, (8) vaginal pouch, and (9) retained cranial suspensory ligaments attaching the undescended testis to the kidney. We did not include small but normally formed tissues in this analysis because it is possible such changes arose from hormonal alterations in adult life rather than being of developmental origin.

Power calculations. The data also were analyzed using PROC MIXED to obtain estimates of components of variation (for litters versus pups within litters) that make up the overall variability of the means, in order to assess the implications for statistical power of using data from multiple pups per litter. Following this analysis, we calculated power to detect the treatment effects as described by Raudenbush and Xiao-Feng (2001). The objective of this was to determine the proportion of the overall error variance due to litter-to-litter variability versus the proportion accounted for by the pups-within-litters. This retrospective analysis can be useful for designing optimal sampling strategies for future transgenerational and multigenerational studies. In short, this analysis allows one to determine how much the statistical power of a study is enhanced by examining several pups from the same litter rather than using only one pup/per sex/litter.

Because the pups in the DEHP-treated litters do *not* respond identically, the more variable the pups are within litter the more power is enhanced, and hence the standard error of the mean is reduced, by examining multiple pups from the same litter. For simplicity, we employed Cox's Ratio as a general rule to determine how many pups per litter could be sampled to enhance the power of the study. This is clearly described as "Cox's rule of thumb" (Bergerud, 1995) (http://www.for.gov.bc.ca/hre/biopamph/pamp50.pdf).

Intraclass correlation coefficients (ICCs) also were calculated to describe the degree of similarity of pups within litters for the multiple endpoints measured in the current study (ICC = (Variance due to pups within litters)/(Variance of pups within litters + Variance of litters). Power calculations also were calculated for categorical effects based upon the numbers of malformed versus unaffected males per dose group using SigmaStat 3.1 software (San Jose, CA). These results were also discussed in a review by Hotchkiss *et al*, (2008).

RESULTS

Maternal Effects

Daily treatment with DEHP from GD 8 until day 17 of lactation did not adversely affect the dams during pregnancy or lactation. Maternal viability and body weights were similar among all the treatment groups (Table 1).

Neonatal and Lactational Effects

Daily treatment with DEHP at dosage levels up to 300 mg/kg/day did not affect litter size or pup viability (Table 1), and body weight of the male rat pups was reduced by 7% at 2 days of age ($p < 0.01$). AGD at 2 days of age was significantly shortened by 15% in male but not female pups in the 300 mg/kg/day DEHP dose group. Because females have AGD values about 50–60% shorter than control males, 300 mg/kg/day DEHP inhibited the masculinizing action of androgens on this male trait by about 25% of maximum.

When examined as infants at 13 days of age, males in this dose group also displayed female-like areolae/nipples (Table 1) an androgen-dependent trait that develops during prenatal life, which in contrast to AGD is not at all influenced by even the most profound effects on the size of the animal.

Postweaning Effects in the PUB Cohort

(Maternal *in utero* and lactational treatment followed by direct DEHP treatment of the males by gavage from PND 18 to necropsy at 63–65 days of age.)

DEHP treatment did not affect growth or viability of the male offspring in any dose group from weaning until 65 days of age (Table 2). However the age at puberty, as indicated by the completion of PPS, was delayed in a dose-related manner, being significant only at 300 mg/kg/day DEHP by Dunnett's test (Fig. 1). Because these males were growing normally, the males in this group attained puberty at a heavier body weight (Table 2) but this effect was not statistically significant using litter-based values. All androgen-dependent organ weights except the glans penis were significantly reduced in the 300 mg/kg/day DEHP treatment group (Table 2). The reproductive tract malformations and testis and epididymal histological lesions in the PUB cohort are presented together with the IUL cohort. Liver weights were significantly increased at 100 and 300 mg/kg/day DEHP and paired adrenal weights were reduced at 300 mg/kg/day (Table 2). Serum testosterone and estradiol levels were not significantly affected at by DEHP treatment at any dose level.

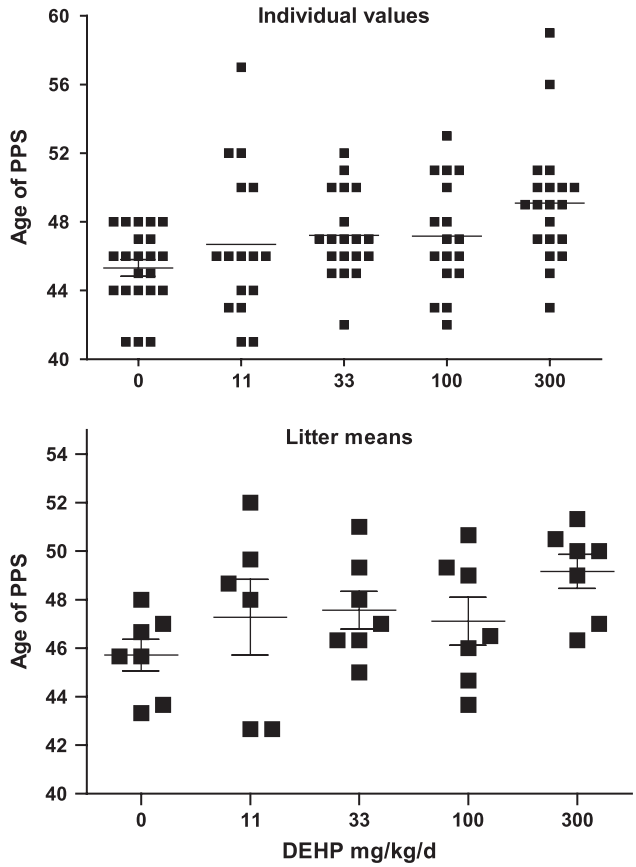

FIG. 1. Individual (top) and litter mean (bottom) values for the age at PPS in males in the PUB cohort. DEHP was administered from GD 8 to 17 of lactation to the dam and then directly to the male offspring up to necropsy at 64 days of age. Age at PPS was significantly delayed in the 300 mg/kg/day DEHP group (Dunnett's test).

Postweaning Effects in the IUL Cohort

(Maternal in utero and lactational treatment only with no direct exposure to the offspring)

The age at PPS was not significantly delayed in the IUL cohort (data not shown). DEHP treatment during gestation and lactation reduced seminal vesicle weight at 100 and 300 mg/kg/day. Glans penis, ventral prostate, LABC, Cowper's glands, epididymal, testis, and kidney weights were reduced at 300 mg/kg/day, a dose that did not affect body weight at necropsy (Table 3). In addition, F1 males in the high dose group displayed a low number of retained female-like nipples.

Individual right and left testis and epididymal weights were reduced in some males at 11–300 mg/kg/day DEHP dose range (Fig. 2). It is evident from the graphs of these data that a significant percentage of the testes, and epididymides from DEHP-exposed males were below the range of the control values, some being dramatically affected. On rare occasion, we also find testes that are much larger than normal due to excessive fluid accumulation in males with epididymal malformations. Examination of the graph of the adult testis

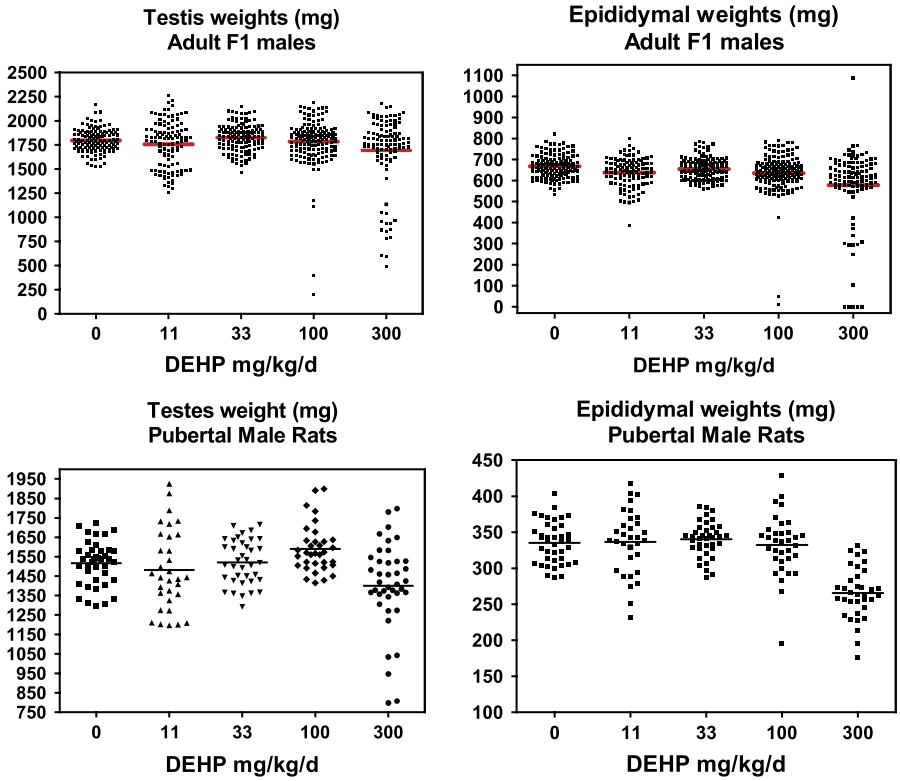

FIG. 2. Individual testis (left) and epididymal (right) weights for males in the IUL (top panels) and PUB (bottom panels) cohorts. DEHP was administered from GD 8 to 17 of lactation to the dam in the IUL cohort, whereas males in the PUB cohort were dosed by gavage from 18 to 64 days of age. The IUL cohort was necropsied at full maturity and the PUB cohort was necropsied at about 64 days of age. Litter mean epididymal weights, but not testis weights, were significantly reduced in the 300 mg/kg/day DEHP group in both IUL and PUB cohorts. Individual testis weights were significantly reduced in the IUL cohort in the 300 mg/kg/day DEHP group by Fisher Exact test.

weight data clearly indicates that several testes ($n = 15/108$ males in the 300 mg/kg/day group and 4/152 males in the 100 mg/kg/day group) were more than five standard deviations below the control mean. In addition, in the 11 mg/kg/day group 28/108 testes were lighter than the smallest control testis. This classification of "abnormal" is supported by the histopathological examinations of the testes. Clearly, a litter-based analysis of the testis weight data, which are only marginally statistically significant with an overall p-value for the F-value of 0.08, does not accurately represent the severity of the effect of DEHP on the testes of some of the offspring. This lack of statistical significance for testes weights results from at least two factors: (1) DEHP-treated testis weight data inflate the variance in the ANOVA model, being much more variable than control testes, and (2) a single severely affected testis in a litter can be obscured in the litter mean.

Treatment with DEHP produced nonsignificant, inverted U-shaped dose responses on necropsy body and liver weights with the males from the 11 mg/kg/day dose group being heavier than control males (Table 3). These effects, however, were not significant by PROC MIXED ANOVA or Dunnett's test, but the contrast between the control and low dose group was significant by t-test. As these were not a priori hypotheses, these results require independent replication before they can be considered to be more than random variations around the control mean. However, the fact that the males in the 11 mg/kg/day dose group were larger, albeit of questionable statistical significance, has a significant effect on the data analysis if organ weight to body weight adjustments are made using relative organ weights and, in some cases, with analysis of covariance. Although relative organ weights are often used to analyze reproductive organ weight in toxicological studies, we believe that this method is generally not valid because it assumes that a linear relationship exists between all organ weights and body weight and the intercept of the relationship goes through zero, assumptions that are rarely met. In addition, analysis of covariance assumes that the covariate does not covary with the organ weight. For, example, if one examines the relative organ weight data, W-shaped dose-response curves are displayed by some of the reproductive organ weights, with some organs being significantly reduced at 11 and 300 mg/kg/day but not 33 or 100. The effects at 11 mg/kg/day likely results from the larger weights of the males in this dose group, whereas the effects at 300 mg/kg/day are also significant when the unadjusted data are analyzed. Analysis of the unadjusted organ weight data indicates that all of the androgen-dependent

tissue weights are smaller in males from the 300 mg/kg/day DEHP dose group.

Phthalate Syndrome Analysis

For this analysis, reproductive tract malformations and histological testicular and epididymal lesions were combined to discriminate affected from unaffected males. The "Phthalate Syndrome" analysis is based upon effects reported for DEHP and other reproductive toxicant phthalates, and includes both androgen- and insl3-dependent tissues as these are all affected by PE-induced reduction in fetal Leydig cell hormone production as described earlier (Gray and Foster, 2003). Testicular lesions also are included in syndrome as the testis is the primary target of in utero PE treatment. Males in the lower DEHP dose groups (11–100 mg/kg/day) displayed the following lesions and were considered as "affected"; retained nipples, fluid-filled flaccid testes (Fig. 3), hypoplastic (incompletely developed, similar to aplasia, but less severe) or malformed epididymides (Fig. 3), epididymal granuloma with small testis, testicular seminiferous tubular degeneration (both moderate and mild severity were noted; Fig. 3), malformed seminal vesicles or coagulating glands, and true hermaphroditism, in one male with uterine tissue and an ovotestis (Fig. 4). Permanent nipples are not seen in control males. Only two males, one in the 100 mg/kg/day dose group and the other in the 300 mg/kg/day dose group had abnormal gubernacular ligaments, a tissue that differentiates under the influence of the peptide hormone insulin-like 3 (insl3) and androgens.

Using this definition of the "Phthalate Syndrome", there was a statistically significant increase in the percentage of affected male rats in the 11, 33, 100, and 300 mg/kg/day DEHP groups (Fig. 5, Table 4), as analyzed by Chi-square analyses. In addition, a comparison of the controls to the 11, 33, and 100 mg/kg/day groups without the high dose group in the data set demonstrated that DEHP induced a significant increase in reproductive tract abnormalities in the lower range, not just at 300 mg/kg/day.

Analysis of Components of Variation: Litter versus pup within litter

Components of variance (inter- and intralitter variances) were estimated using PROC MIXED for all the reproductive measures in the IUL and PUB cohorts. We used the components of variance to calculate Cox's Ratios and, ICC (intralitter correlation coefficients) as per (Bergerud, 1995) and power curves for means from the control and a DEHP-treated

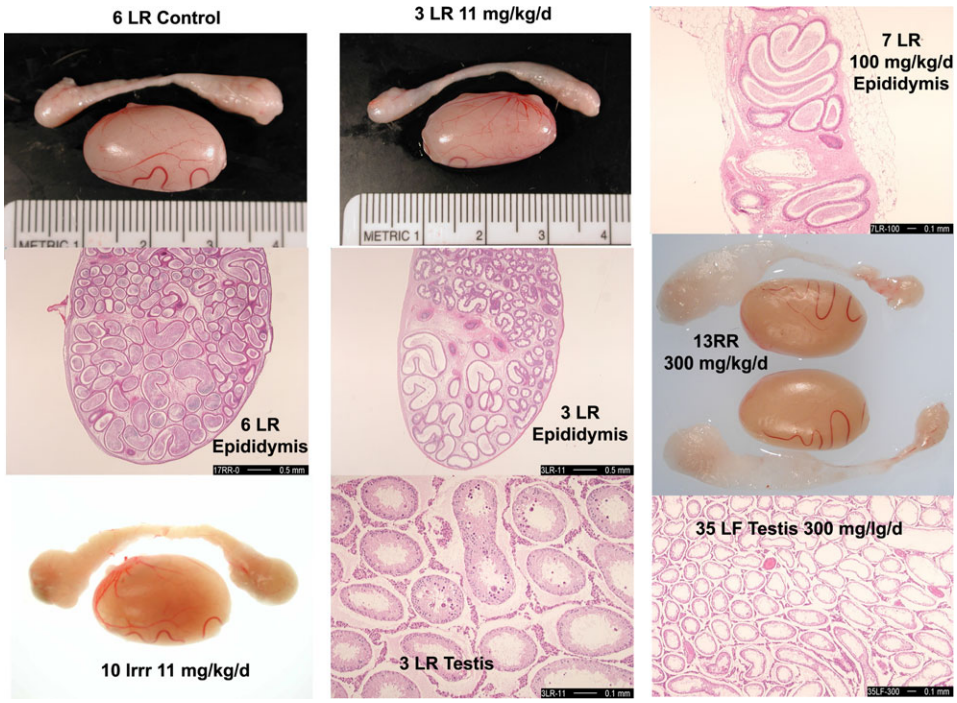

FIG. 3. Testis and epididymis of a control male rat (top row, left column: ID 6LR, PUB cohort). A photomicrograph of a cross section of the control epididymis (middle row, far left column: ID 6LR). Malformed testis and epididymis from a male exposed to 11 mg/kg/day DEHP (bottom row, left column; ID 10 LRRR, IUL cohort). Slightly underdeveloped, testis and epididymis from a male exposed to 11 mg/kg/day DEHP (top row, middle column; ID 3LR, PUB cohort). A photomicrograph of a cross section of the epididymis of 3LR with hypospermatogenic tubules (middle row and middle column). A photomicrograph of a cross section of the testis of 3LR showing severe tubular atrophy (bottom row, middle column). A photomicrograph of a cross section of the epididymis of a male exposed to 100 mg/kg/day DEHP (ID 7LR, PUB cohort) with hypospermatogenic tubules (top row, right column). Malformed testes and epididymides from a male exposed to 300 mg/kg/day DEHP (middle row, right column; ID 13RR, PUB cohort). A photomicrograph of a cross section of the testis of a male exposed to 300 mg/kg/day DEHP with severe hypospermatogenesis (bottom row, right column; ID 35LF, PUB cohort).

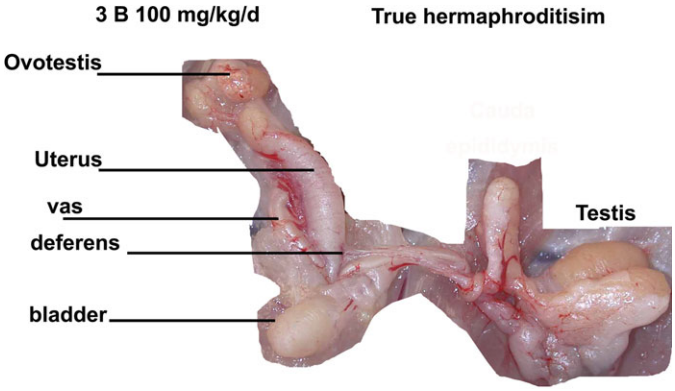

FIG. 4. Internal reproductive tract of a true hermaphrodite exposed to 100 mg/kg/day DEHP. The right undescended gonad is an ovotestis with attached uterine tube and vas deferens. The left testis also is undescended but did not contain ovarian tissue. Both gubernacular ligaments were absent (top panel: ID 3B, IUL cohort). A male displaying hypospadias and a vaginal pouch (middle panel: ID 3B, IUL cohort). Malformed seminal vesicles from two males exposed to 300 mg/kg/day DEHP (bottom panel: IDs 9LF, IUL cohort and 35LR, PUB cohort).

group. The power curves compare the probability of detecting significant treatment effects for one to five male pups per litter from 10, 15, or 20 litters. Cox's Ratio (Cox's ratio = 4 × (variation due to pups within litters/variation due to litters)) ranged from 4.1 to > 50 for reproductive endpoints, indicating that there is utility in examining the reproductive tracts of 4 or more male pups per litter. In contrast, Cox's Ratio was consistently smaller for body weights during pubertal development ranging from 2 to 2.6 Table 5 (previously reviewed by Hotchkiss *et al.* (2008). Power curves for these endpoints are presented in Figures 6 and 7 (also reviewed by Hotchkiss *et al.*, 2008).

It also is evident that histopathological examination on only 10 animals per dose group is underpowered and will fail to detect as statistically significant alterations unless a high percentage (> 50%) of the offspring are affected (Fig. 8; Hotchkiss *et al.*, 2008).

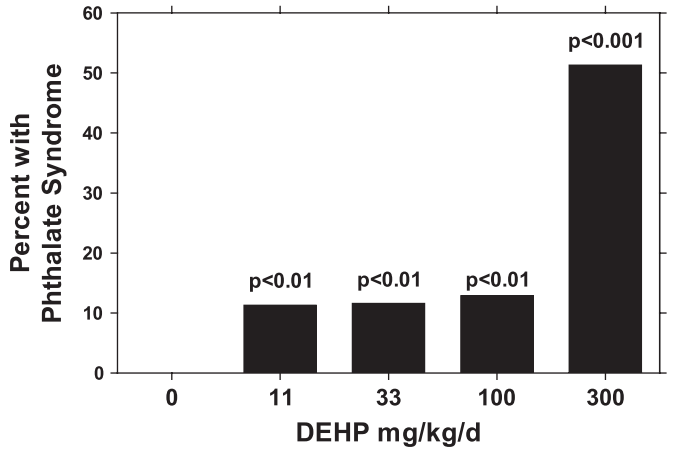

FIG. 5. Percentage of F1 males displaying any Phthalate Syndrome trait. All dose groups are significantly different from control by Fisher Exact tests.

DISCUSSION

PEs such as DEHP that are widely used in a variety of consumer products have been implicated in adverse developmental reproductive effects in humans including a shortened AGD index in boys (Swan *et al.*, 2005) and girls (Huang *et al.*, 2009; Lottrup *et al.*, 2006; Scott *et al.*, 2008; Sharpe, 2005), altered serum hormone levels (Main *et al.*, 2006) and shortened pregnancy (Latini *et al.*, 2003). In the rat, PEs alter sexual differentiation by altering fetal Leydig cell development and hormone synthesis which in turn, results in a "Phthalate Syndrome" which includes abnormalities of the testis, gubernacular ligaments, epididymis and other androgen-dependent tissues. Administration of PEs during development also has been shown to alter reproductive development in rabbits (Higuchi *et al.*, 2003) and primates (Hallmark *et al.*, 2007) in an antiandrogenic manner. Furthermore, neonatal administration of DEHP by either IV or oral routes permanently affects testis sperm production in the rat (Cammack *et al.*, 2003; Dostal *et al.*, 1988).

In spite of the potential effects of phthalates in humans and decades of commercial use, robust studies including relatively low dosage levels with developmental exposure, a thorough examination of all the sensitive endpoints, and an adequate number of adult offspring have not been published for many PEs. In the current study, SD rats were dosed by gavage with DEHP from GD 8 to day 17 of lactation with 0, 11, 33, 100, or 300 mg/kg/day. Statistically significant, developmentally induced adverse effects were seen at all dosage levels. It is noteworthy that the dose-response seen in the current study is remarkably similar to that reported from a National Toxicology Program multigenerational study, which reported a dietary no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) of 5 and 10 mg/kg/day DEHP, respectively in a multigenerational study of DEHP (Foster *et al.*, 2006).

Although there are several other published reports on the transgenerational effects of in utero DEHP exposure (Andrade *et al.*, 2006a, b; Arcadi *et al.*, 1998; Borch *et al.*, 2004, 2005, 2006; Grande *et al.*, 2006, 2007; Jarfelt *et al.*, 2005; Shirota *et al.*, 2005), the current study along with an National Toxicology Program (NTP) multigenerational study (CERHR, 2003) are the only studies that provide a comprehensive assessment of the phthalate syndrome in a large enough number of male offspring to detect adverse reproductive effects at low dose levels. The NOAEL found here is consistent with the NOAEL identified by the NTP CERHR Panel in its review of the NTP study and, for this reason, we support the level of concern that they expressed about the potential effects of DEHP on humans (http://cerhr.niehs.nih.gov/chemicals/dehp/DEHP-Monograph.pdf). The NTP panel found that "There is sufficient evidence in male rats to conclude that DEHP causes

TABLE 4
Summary Table of Gross and Histological Reproductive Lesions Induced by Exposure to DEHP *In Utero* and during Lactation (IUL Cohort) or *In Utero*, during Lactation and from 18 to 63–65 Days of Age (PUB Cohort)

Dose of DEHP mg/kg/d	0	11	33	100	300
PUB cohort 1	0/20	2/16	0/19	2/17	7/20
IUL cohort 1	0/23	3/25	6/31	5/25	17/23
Pooled 1	0/43 pups	5/41	6/50	7/42	24/43
IUL 2	0/40	3/30	4/36	5/51	14/31
Pooled 1 and 2, % affected	0/83	8/71, 11.3%	10/86, 11.6%	12/93, 12.9%	38/74, 51.3%
Chi-square test block 1 and 2	—	8.0, $p < 0.005$	8.6, $p < 0.003$	9.2, $p < 0.002$	55.1, $p < 0.001$

Note. Malformations included testis and epididymal agenesis, fluid-filled, flaccid testes, epididymal granulomas, epididymal epithelial thickening, absent or malformed sex accessory and coagulating gland tissues, prostate pathology, retained nipples (but not areolas), atrophic seminiferous tubules, vacuolated Sertoli cells, minimal hemorrhagic testis, elongated gubernacular ligament (> 10 mm).

Overall Chi square = 43.4, $p < 0.001$.

Chi square (without the high dose group) = 11.1, $p < 0.011$.

Chi square (with only 0, 11 and 33 mg/kg/day dose groups) = 10.3, $p < 0.006$.

reproductive toxicity when exposure is by oral gavage or in feed at 10–113 mg/kg/day for exposures that included gestational and/or peripubertal periods." The critical effects included small reproductive organ size (14–23 mg/kg/day and focal testicular tubular atrophy (113 mg/kg/day), effects also seen in the current study at similar dosage levels.

We also provide unique information on the statistical power derived from examining multiple pups per litter; information that can be used in the future to design more efficient multigenerational protocols; ones that use fewer animals overall, but have statistical power equal to or that exceeds the current protocols that examine one F1 pup/sex/litter after

TABLE 5
Calculation of Cox's Ratios and Intralitter Correlation Coefficients from PROC MIXED Analyses

Variable	Litter Variance	Pups (residual) Variance	TRT F value p value	Cox's ratio (TRT/litter) × 4	ICC Litter/(Litter + Pups)
AGD day 2 (IUL + PUB)	0.05503	0.06889	5.4, 0.0003	5.007	0.444
IUL cohort data					
Epididymal weight	5021	5167	3.5, 0.009	4.116	0.493
Liver weight	4.95	6.22	1.6, 0.17	5.031	0.443
Body weight	2579	3481	1.6, 0.16	5.399	0.426
Kidney weight	23,537	32,670	3.6, 0.008	5.552	0.419
Cowper's gland weight	1129	1694	1.8, 0.13	6.002	0.400
Testis weight	21,506	32,376	1.7, 0.14	6.022	0.399
LABC weight	10,417	15,736	5.92, 0.0002	6.042	0.398
Glans penis weight	31.2	56.5	3.4, 0.01	7.244	0.356
Seminal vesicle weight	25,980	71,543	6.72, 0.0001	11.015	0.266
Ventral prostate	5346	23,258	3.05, 0.02	17.402	0.187
Pubertal necropsy					
Body weight day 18	14.225	7.131	0.80	2.005	0.666
Body weight day 44	348.2	201.02	0.50	2.309	0.634
Body weight necat necropsy	753.87	492.14	0.40	2.611	0.605
Glans penis weight	44.4	70.85	1.63, 0.18	6.383	0.385
Epididymal weight	839.64	1708.64	13.6, 0.0001	8.140	0.329
Testis weight	23,297	61,044	0.22	10.481	0.276
LABC weight	2666	7417.5	6.0, 0.0004	11.129	0.264
Seminal vesicle weight	4925	14,445	4.8, 0.0022	11.732	0.254
Adrenal weight	8.214	34	2.1, 0.093	16.719	0.193
Age at PPS	1.391	8.0744	1.97, 0.12	23.219	0.147
Body weight at PPS	107.00	720.00	1.48, 0.22	26.916	0.129
Cowper's gland weight	14.9954	189.57	2.94, 0.03	50.568	0.073

Note. Hotchkiss *et al*., 2008.

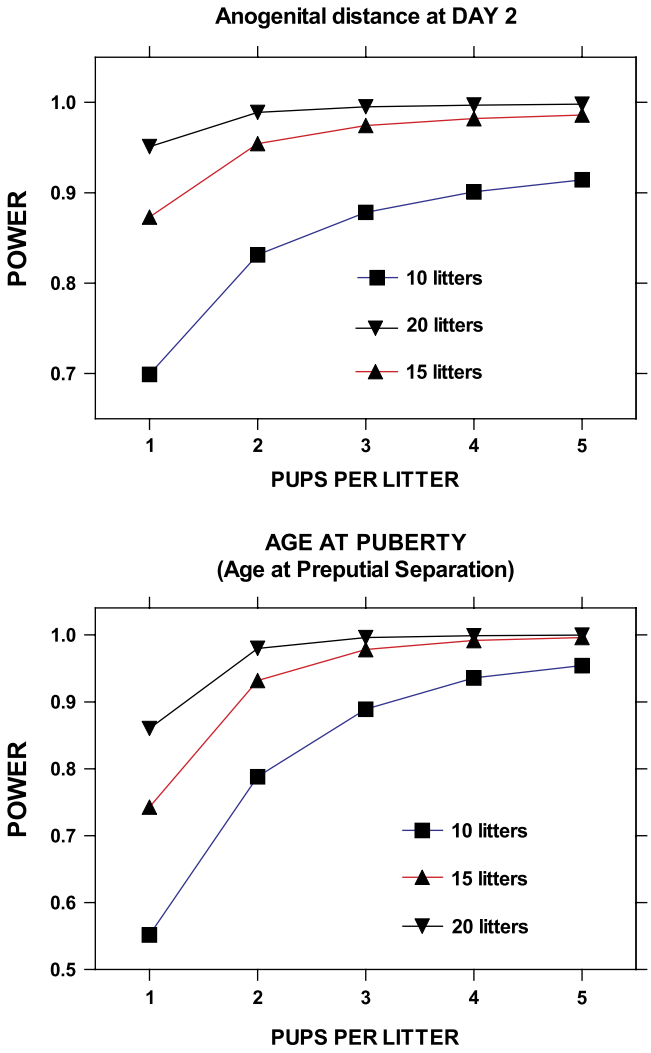

FIG. 6. Power curves for AGD (all pups) and age at puberty (PUB cohort) based upon 10, 15, or 20 litters per dose group (control and the group treated with DEHP at 300 mg/kg/day) using one to five pups per litter. Increasing the numbers of F1 pups examined from one to five pups per litter increases the statistical power of a study to detect the effects of DEHP on the reproductive tract of the male offspring. For example, examining two male from 15 litters provides statistical power that equals or exceeds that attained using one pup from 20 litters.

weaning. According to Cox's Rule Of Thumb (Bergerud, 1995), there is little or no increase in power when the number of subsamples (pups in this case) is greater than Cox's Ratio (Cox's ratio = 4 × (variation due to pups within litters/variation due to litters)). Power curves flatten out when the number of pups/litter sampled is equal to the Ratio, indicating that the most efficient design would not sample more pups per litter beyond this Ratio. When the components of variance were estimated using PROC MIXED for all the reproductive measures taken at necropsy, it was evident that the component of variation for pups was generally several-fold greater than the component of variation for litters. The Cox's Ratios and ICC (intralitter correlation coefficients) obtained from these analysis are shown in Table 5. Clearly, even in the most conservative case, sampling all the male pups in each litter would enhance the power of a transgenerational reproduction study like the current study because the litters rarely have six or more male pups per litter. If the objective was to use fewer litters per dose group, reducing total animal use, sampling three pups from 15 litters or three to four pups from ten litters/dose would generally provide about the same statistical power as one pup from 20 litters (Supplemental Figures). For example, power curves were calculated for testis and epididymal weights from the control and high dose group for one to five pups per litter and 10, 15, and 20 (standard sample size) litters per dose (Figs. 6 and 7). It is evident from the testis weight figure that three to four pups sampled from 10 litters provides about the same statistical power to detect the effects of DEHP (at 100 or 300 mg/kg/day) as does the standard method of sampling only one male from 20 litters.

The Phthalate Syndrome of malformations was the most sensitive index of the reproductive toxicity of DEHP being displayed by 11.3%, 11.6%, 12.9%, and 51.3% of the males in the 11, 33, 100, and 300 mg/kg/day dose groups, respectively (all $p < 0.05$). In the 300 mg/kg/day group, shorter AGD (15%) and retained areolae/nipples were seen in 55% of the male pups. We were only able to detect these lesions as statistically significant because (1) we examined every endpoint in 71–93 males from 12 to 14 litters per dose group; (2) we examined paired testes and epididymides for histopathological lesions in every male; and (3) we compared all the numbers of "affected" animals with any lesion from the Phthalate Syndrome with the control group, rather than rely upon a single endpoint to detect a LOAEL (Table 6). An example of how the statistical power improves with examination of more males per dose is shown in Figures 6–8 (also reviewed by Hotchkiss *et al.*, 2008). Similarly, Foster *et al.* (2006) noted that adding extra males to the histopathological evaluation of DEHP-exposed F1 males lowered the NOAEL by two orders of magnitude.

In a parallel study (Calafat *et al.*, 2006), MEHP concentrations (including free and glucuronidated) were measured in maternal urine before and during dosing and in amniotic fluid on GD 18 from each fetus (n = two litters per dose group with 24, 26, 28, 26, and 32 individual fetuses (male and female) in the 0, 11, 33, 100, and 300 mg/kg/day dose groups, respectively). The purpose of the MEHP study was to correlate fetal and maternal exposure levels with the effects reported here. We euthanized two DEHP-treated dams per group and collected amniotic fluid from each pup for analysis of MEHP levels on GD 18. Free MEHP amniotic fluid AF levels ranged from 68 to 2924 ng/ml in the 11 to 300 mg/kg/day groups (Table 3 shows means ± SEs based upon litter means MEHP values from Calafat *et al.*, 2006). Our reanalysis of the data indicated that free MEHP amniotic fluid levels varied considerably among pups within litters with an overall CV of 39% and free MEHP concentrations varied significantly from litter to litter for the 100 ($p < 0.0001$) and 300 ($p < 0.001$) mg/kg/day dose groups

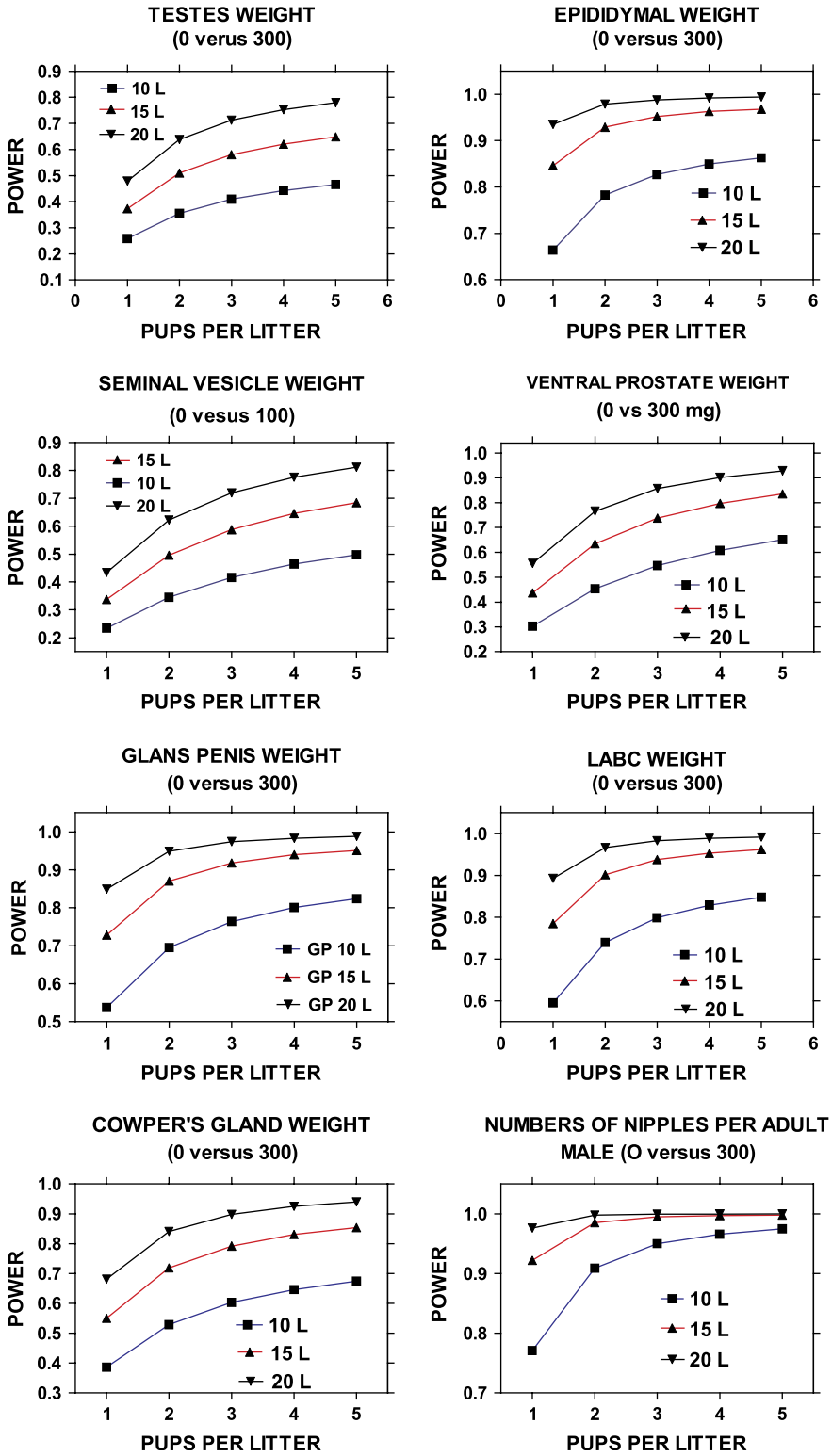

FIG. 7. Power curves for testis weight (Control vs. 300 mg/kg/day), epididymal weight (Control vs. 300), seminal vesicle weight (Control vs. 100), ventral prostate weight (Control vs. 300), glans penis weight (Control vs. 300), Levator ani bulbocavernosus (LABC) weight (Control vs. 300), Cowper's glands weight (Control vs. 300) and the numbers of permanent nipples in adult IUL cohort male SD rat offspring. Calculations were based upon 10, 15, or 20 litters per dose group using one to five pups per litter. Increasing the numbers of F1 pups examined from one to five pups per litter increases the statistical power of a study to detect the effects of DEHP on the reproductive tract of the male offspring. For example, examining two male from 15 litters provides statistical power that generally exceeds that attained using one pup from 20 litters.

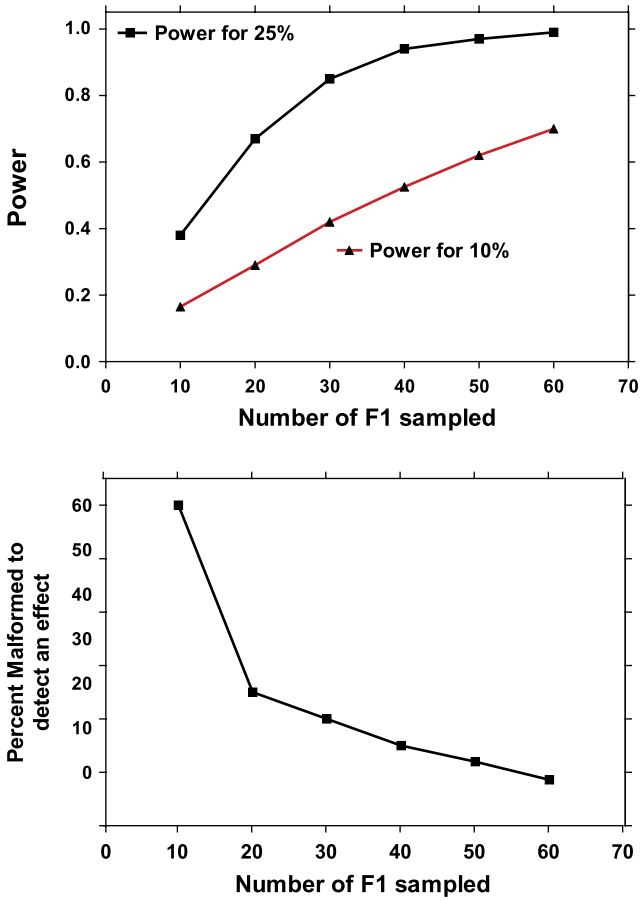

FIG. 8. Top panel: Power calculations (SigmaStat 3.1) of the statistical power to detect malformations of histopathological lesions in 10 and 25% of the F1 male rat offspring. Using only 10 males per dose group, as is often done in standard multigenerational guideline studies, the statistical power to detect lesions in 25% of the males is less than 50%, for example. Bottom panel: Calculation of the minimum % of male rat offspring that can be detected as statistically significantly over control using 10–60 offspring per dose group (using Fisher exact tests on SigmaStat 3.1). Note that 60% or more of the offspring need to be malformed before statistical significance ($p < 0.05$) is attained using 10 males per dose group, the minimum required in many test guidelines.

TABLE 6
Incidences of Specific Reproductive Tract Lesions in F1 Male Rat Offspring in the IUL and PUB Cohorts after DEHP Exposure during Development

Dose of DEHP (mg/kg/day)	0.0	11.0	33.0	100.0	300.0
Permanent nipples%	0.0	1.4	0.0	2.2	27.0[a]
Malformed seminal vesicle %	0.0	0.0	0.0	1.1	5.4
Gross testis abnormality %	0.0	5.6	0.0	1.1	17.6[a]
Testis histopathology %	0.0	4.2	11.6[a]	4.3	24.3[a]
Gross epididymal abnormality %	0.0	1.4	0.0	1.1	20.3[a]
Epididymal histopathology %	0.0	1.4	0.0	2.2	14.9[a]
Severe malformation of glans penis %	0.0	0.0	0.0	1.1	1.4
Ovotestis/uterus present %	0.0	0.0	0.0	1.1	0.0
Coagulating gland malformed %	0.0	2.8	0.0	6.5[a]	6.8[a]
Hypospadias %	0.0	0.0	0.0	1.1	1.4
Vaginal pouch %	0.0	0.0	0.0	1.1	1.4
Cranial suspensory ligament—testis %	0.0	0.0	0.0	0.0	0.0
Abnormal gubernaculum %	0.0	0.0	0.0	1.1	1.4
Vas defens agenesis%	0.0	0.0	0.0	1.1	0.0
Total affected with % with phthalate syndrome	0.0	11.3[a]	11.6[a]	12.9[a]	51.3[a]

Note. None of these effects would reach the level of statistical significance using the sample sizes recommended in standard multigenerational tests which only requires 20/dose group for organ weights and gross observations and 10/sex/group for histopathological assessments.

[a]Indicates that the % is significantly higher than control by Chi-square analysis.

but not for the lower dose groups. Taken together, our results and those of Calafat *et al.* (2006) indicate that fetal levels of MEHP as low as 68 ng/ml may be sufficient to induce malformations in about 10% of the animals. For comparison, MEHP levels as high as 2.8 ng/ml were seen in human amniotic fluid in a study of 54 women (Silva *et al.*, 2004). Thus, the level of MEHP associated with a low incidence of male reproductive tract malformations in F1 male rats is about 25-fold above the highest concentration seen amniotic fluid of pregnant women. These women also had detectable levels of monobutyl phthalate (mBP), the active metabolite of di-*n*-butyl phthalate, and the maximum levels were only sixfold below the levels that are associated with subtle reproductive effects in male rat offspring (Mylchreest *et al.*, 2000) (1400 ng mBP/ml in rat amniotic fluid versus a maximum value of 264 ng/ml in human amniotic fluid). Because DEHP and DBP both disrupt sexual differentiation via a common mode of action one might expect that they would act in a cumulative manner in humans as they do in rats (Gray *et al.*, 2006; Hotchkiss *et al.*, 2004; Howdeshell *et al.*, 2007).

In the pubertal cohort, DEHP delayed puberty (1.6 and 3.5 days at 100 and 300 mg/kg/day, respectively); whereas, serum testosterone and estradiol were unaffected. In the IUL cohort, when DEHP was not administered after weaning, puberty was not significantly delayed. In general, the other effects of DEHP were similar in the PUB and IUL cohorts with one exception, the exception being the weight of the adrenal glands. Direct exposure to DEHP at 300 mg/kg/d induced a 15% decrease in adrenal weight whereas adrenal weight was unaffected in males in the IUL cohort that there not directly exposed to DEHP.

To date, we have conducted three experiments that we have conducted in the male rat with pubertal DEHP exposures. In all three studies, we have found that oral administration of DEHP at doses ranging from 300 to 900 mg/kg/day results in delays in PPS, an index of puberty in the male rat. Similar pubertal delays have been reported for DBP and BBP (Gray *et al.*, 1999; Nagao *et al.*, 2000). In contrast to the model proposed by Akingbemi *et al.* (2004) that low doses of DEHP would accelerate puberty in boys and girls due to increased levels of serum gonadal steroids, administration of lower doses of DEHP (10 and 100 mg/kg/day) did not accelerate puberty in the male rat or elevate serum testosterone or estradiol levels. In fact, we found that 100 mg/kg/day DEHP and above were

associated with reduced, rather than increased, ex vivo testis testosterone production in the peripubertal male rat (Noriega et al., in prep.). Similar to our studies with rats, higher DBP and DEHP metabolite levels in the urine have been shown to be associated with lower serum levels of free testosterone in workers (Pan *et al*., 2006) and in the marmoset, peripubertal DEHP exposure results in a delayed onset in detectable serum testosterone levels (unpublished data available in the public docket from (Tomonari *et al*., 2006) study presented to NTP CERHR evaluation of DEHP).

In summary, results of the current study are consistent with the NOAEL identified in the NTP study or 4.8 mg/kg/day (Foster *et al*., 2006) and used in the European Union risk assessment for DEHP (EuropeanCommission, 2008). All of the adverse effects in the current study increased in severity with increasing dose; that is, none of the effects displayed a significant nonmonotonic dose response. Furthermore, detection of the Phthalate Syndrome in a low percentage of F1 males in the current study and the NTP study were possible only because both studies thoroughly examined more than one male per litter. As the power calculations demonstrate, in multigenerational studies there would be little loss, if any, in statistical power to detect treatment effects if the total numbers of litters per dose group was reduced from 20 to 10 or 15 litters per treatment but more males per litter were examined.

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