# Reproductive Toxicity of Di-*n*-butylphthalate in a Continuous Breeding Protocol in Sprague-Dawley Rats

**Robert N. Wine,[1] Ling-Hong Li,[1] Leta Hommel Barnes,[2] Dushyant K. Gulati,[2] and Robert E. Chapin[1]**

[1]National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 USA;
[2]Environmental Health Research and Testing, Lexington, KY 40503 USA

The phthalate ester di-*n*-butylphthalate (DBP) is used extensively in the manufacture of plastics; its reproductive toxicity was tested in rats by the National Toxicology Program's Reproductive Assessment by Continuous Breeding protocol. Levels of 0.1, 0.5, and 1.0% DBP in the diet were selected, and this dosing design yielded average daily DBP intakes of 52, 256, and 509 mg/kg for males and 80, 385, and 794 mg/kg for females, respectively. DBP consumption by $F_0$ rats reduced the total number of live pups per litter in all treated groups by 8–17% and live pup weights in the 0.5% and 1.0% dose groups by <13%. In tests to determine the affected sex, the number of offspring was unchanged, but the weights of pups from treated females were significantly decreased and offspring from treated males were unchanged. At necropsy, high-dose $F_0$ females had a 14% reduction in body weight, and both sexes had ≈10–15% increased kidney and liver to body weight ratios compared to controls. Sperm parameters and estrous cyclicity were not affected. In the $F_1$ mating trial, indices of mating, pregnancy, and fertility in the 1.0% dose group were all sharply decreased (one live litter was delivered out of 20 cohabited pairs), concomitant with a 13% decrease in dam body weight. Live $F_2$ pup weights were 6–8% lower in all dose groups. $F_1$ necropsy results revealed that epididymal sperm counts and testicular spermatid head counts were significantly decreased in the 1.0% dose group. Histopathologic investigation showed that 8 of 10 $F_1$ males consuming 1.0% DBP had degenerated seminiferous tubules and 5 of 10 had underdeveloped or otherwise defective epididymides. No ovarian or uterine lesions were observed. In conclusion, this study showed that DBP is a reproductive/developmental toxicant in Sprague-Dawley rats exposed both as adults and during development; it also indicates that the adverse reproductive/developmental effects of DBP on the second generation were greater than on the first generation. *Key words*: continuous breeding, developmental defects, di-*n*-butylphthalate, rat, reproductive toxicity. *Environ Health Perspect* 105:102–107 (1997)

The esters of *o*-phthalic acid are employed extensively in the manufacture of plastics where they are used to impart flexibility. Since they are not covalently linked to the plastic polymer, they can leach from the matrix, providing opportunity for widespread exposure [see Thomas and Thomas (*1*) for review]. The phthalate ester di-*n*-butylphthalate (DBP) is used in the manufacture of consumer products as diverse as nail polish, insect repellents, and denture base material (*2–4*). DBP has recently been found to have clinical applications as well, selectively eliminating tumor cells from bone marrow (*5,6*).

DBP has been previously characterized as a developmental and reproductive toxicant in the rat (*7–12*). Comparable oral doses of DBP given to juvenile and adult male rats produces a testicular lesion characterized by early sloughing of germ cells, vacuolization of the Sertoli cell cytoplasm, and testicular atrophy (*7,10*). It has also been shown that the testicular toxicity of phthalates is age dependent, with immature animals being more sensitive than mature animals (*13,14*). However, the reproductive toxicity following exposure during development has not been addressed. Although no data are available specifically on the transfer of DBP across the placenta, studies with other phthalate esters show that these compounds readily cross the placenta of rats (*15–17*) and that exposure of lactating dams to another phthalate, di (2-ethylhexyl) phthalate (DEHP), could lead to DEHP and mono (2-ethylhexyl) phthalate (MEHP) exposure in the suckling rat pups. Because DBP is lipid soluble, as are DEHP and MEHP, one might predict a certain degree of lactational transfer of DBP.

Recently, Jobling et al. (*18*) reported that DBP could reduce the binding of estrogen to the estrogen receptor and stimulate transcriptional activity These authors called for further *in vivo* data before any firm conclusions as to the estrogenicity of DBP could be drawn. Review of archived chemical studies generated with the National Toxicology Program's Reproductive Assessment by Continuous Breeding (RACB) protocol revealed an earlier study that examined the reproductive toxicity of DBP in Sprague-Dawley rats. If the findings of Jobling et al. (*18*) are correct and DBP can act as an estrogen, then one would expect to see greater reproductive effects in second generation animals (*19*) under the RACB protocol. This is expected in the RACB design because the $F_0$ rats are exposed only as adults, the $F_1$ rats are born to mothers that are treated during gestation and lactation, and the $F_1$ animals themselves are treated during maturation to sexual maturity and through mating. The results of this DBP RACB study are presented below; the results show that structural defects are seen in the second generation rats that were not found in the first.

## Methods

***General.*** This study was conducted using the National Toxicology Program's RACB protocol for which the detailed methods have been previously described (*20,21*). Briefly, the protocol is composed of four segments, or tasks. Task 1 is a 14-day range-finding study that is used to set three dose levels used in Task 2. Task 2 is the 14-week continuous breeding phase, generating up to five litters per pair. Task 3 consists of crossover matings between treated and control $F_0$ animals to determine the affected sex, and Task 4 assesses the fertility of the last litter ($F_1$) born during continuous breeding (Task 2).

***Chemical.*** DBP was obtained from Chem Central via Midwest Research Institute (Kansas City, MO, Lot/Batch # L-121 1-83/02). The purity of DBP used in preparation of the feed formulations was greater than 99% as determined by gas chromatography.

***Dosed feed formulations.*** DBP was blended into powdered NIH-07 (Zeigler Bros., Gardeners, PA) feed on a weight-to-

Address correspondence to R.E. Chapin, National Institute of Environmental Health Sciences, P.O. Box 12233, Mail Drop A2-02, Research Triangle Park, NC 27709 USA.

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weight basis. Animals in the control group received undosed NIH-07. Each dose level was independently formulated and prepared at least every 3 weeks and stored frozen. Dosage formulation studies indicated that DBP blended with feed at a concentration of 0.5 mg/g was stable for 3 weeks when sealed at -20°C and for 7 days when open to air and light at room temperature. Thus, dosed feed was stored in the dark and refrigerated and was changed off the cages every week.

***Animals.*** VAF Crl:CD BR outbred Sprague-Dawley albino rats were purchased from Charles River Breeding Laboratories (Portage, MI). During quarantine, representative animals were sacrificed and their sera evaluated for antibodies against nine rodent viruses and *Mycoplasma pulmonis* (Charles River Professional Services, Wilmington, MA) All sera were negative for viral antibodies. Animals were quarantined separately for approximately 3 weeks for Task 1 and 2 weeks for Task 2; rats were 70 days of age at the start of both Tasks 1 and 2. Histopathologic evaluations were performed on representative animals during quarantine to check for signs of infectious disease.

Male and female rats were housed two per cage by sex during quarantine and the 1-week pre-mating period on SaniChip bedding (P.J. Murphy Forest Products) in solid bottom polycarbonate cages with stainless steel wire-bar lids. Deionized water and rodent meal feed (NIH-07 diet; Zeigler Bros.) were provided *ad libitum*.The rats were subsequently housed as breeding pairs or individually at 23 ± 2°C with a 14:10-hr light:dark cycle. All housing and procedures were performed in accordance with the NIEHS guidelines for the humane use of animals in research.

***Study design.*** DBP was administered via feed to CD Sprague-Dawley rats. In Task 1, the dose-finding segment of the study, doses of 0.0 (control), 0.1, 0.5, 1.0, 1.5, and 2.0% (w/w) DBP were tested using eight animals of each sex per group. Endpoints for Task 1 were clinical signs of toxicity, body weight, and food consumption.

In an optimal study, the highest dose during Task 2 should be set so as not to depress weight gain by more than 10% or result in greater than 10% mortality. If a compound is a reproductive toxicant, the middle dose should elicit reproductive effects with little or no systemic toxicity in evidence, and the lowest dose should provide a no-effect level. Based on the results of the Task 1 pilot (data not shown), concentrations of 0.1, 0.5, and 1.0% DBP in the feed were selected for Task 2.

Task 2, the continuous breeding phase, used a control group consisting of 40 male/female breeding pairs and three dose groups with 20 breeding pairs per group. The endpoints for Task 2 were clinical signs of toxicity, parental body weight and average consumption of feed during representative weeks, fertility (the proportion of cohabited pairs producing any live pups), the number of litters per pair, the number of live pups per litter, the proportion of pups born alive, the sex ratio of live pups, and the pup body weights immediately after birth. The pups in the first four litters were removed after birth, counted, weighed, sexed, and killed without necropsy. The last litter, born during the holding period following the continuous breeding phase, was reared by the dam until weaning (day 21 after birth); at this time, treatment of the $F_1$ animals was initiated by the same route and at the same concentrations as consumed by their parents in Task 2. These animals were used for assessment of second generation fertility in Task 4.

During Task 2, if an effect on fertility was detected, a 1-week crossover mating trial, Task 3, was performed to determine the affected sex. In this study, this was performed after the last litter of Task 2 was weaned. The mating trial consisted of three groups of 20 pairs each: control males × control females, control males × 1% females, and 1% males × control females. Endpoints for Task 3 were the same as Task 2, with the addition of checking for the presence of a vaginal copulatory plug or sperm. After the litter in Task 3 had been delivered, evaluated, and disposed of, the $F_0$ females were subject to vaginal lavage for 12 days to evaluate estrous cyclicity; the control and high dose $F_0$ adults were then killed by $CO_2$ asphyxiation and subjected to necropsy. Necropsy endpoints were organ weights and histopathology, body weight, epididymal sperm motility (counted visually), sperm morphology, and sperm count. For histopathological evaluation, selected organs were examined after fixation in 10% neutral buffered formalin or Bouin's fixative (ovaries) and embedded in glycol methacrylate (testis) or paraffin. Sections were stained with periodic acid–Schiff (PAS) and hematoxylin (testis) or hematoxylin and eosin according to standard procedures.

Task 4 assessed the growth and fertility of $F_1$ animals. The last litter from Task 2 was reared, weaned, and held until mating at postnatal day 88 ± 10. During maturation, siblings were housed by sex at a maximum of two per cage and received the same chemical treatment as their parents. Upon reaching sexual maturity, 20 each nonsibling $F_1$ males and females within the same treatment groups were housed in pairs for 7 days and then housed individually until delivery of a litter. The endpoints for the Task 4 mating trial was the same as in Task 2, with the addition of checking for the presence of a copulatory plug and vaginal sperm. At the end of Task 4, $F_1$ animals in all dose groups were killed by $CO_2$ asphyxiation and subjected to necropsy with the same endpoints as in the Task 3 necropsy.

***Statistics.*** When data were expressed as a proportion, such as the fertility, mating, and pregnancy indices, the Cochran-Armitage test was used to test for a dose-related trend (*22*). Each dose group was compared to the control group with a $\chi 2$ test (*23*). In Task 3, where the animals were cross-mated and dose groups did not represent increasing dose levels, a $\chi 2$ test for homogeneity was used to test for an overall difference in fertility among groups for pairwise comparisons.

The number of litters and the number of live pups per litter were computed on a per-fertile-pair basis and treatment group means were determined. The proportion of live pups was defined as the number of pups born alive divided by the total number of pups produced by each pair. The sex ratio was expressed as the proportion of male pups born alive divided by the total number of pups produced by each pair. In Task 2, dose group means for these and other parameters were compared to the control group with Shirley's test (*24,25*). When a trend was present, Jonckheere's trend test (*26*) was used, otherwise Dunn's test (*27*) was applied. In Task 3, parameters were tested for overall differences using the Kruskal-Wallis test (*28*), and multiple comparisons were made using Dunn's test.

To remove the potential effect of the number of pups per litter on the average pup weight, an analysis of covariance (ANCOVA) was performed (*29*) using the average litter size, including live and dead pups, as the covariate to produce an adjusted live pup weight. Least squares estimates of dose group means, adjusted for litter size, were computed and tested for overall equality using an F-test and pairwise equality using Dunnett's test (*30*). To evaluate potential sex differences, these analyses were performed on males, females, and both sexes combined.

Absolute body and organ weights and organ weights adjusted for body weight were analyzed by Shirley's or Dunn's test, while dose-related trends were identified by Wilcoxon's test in Task 3 or Jonckheere's test in Task 4.

Vaginal cytology data were analyzed using a multivariate analysis of variance (ANOVA) (*31*) to test for the simultaneous equality of measurement across dose levels.

Before running the ANOVA, an arcsine transformation was performed to bring the data into close conformance with normality assumptions.

Group means, standard deviations, and standard errors were calculated for all data and for each sex. Jonckheere's test was performed to look for increasing or decreasing trends. Significance among dose groups was then calculated using Shirley's test if a trend was evident or Dunn's test if no trend was evident.

## Results

***Continuous breeding (Task 2).*** Representative dosage formulations from Task 2 were 99–106% of the target concentrations. The average daily feed consumption was similar for control and DBP-dosed animals. During the first 14 days of Task 2, body weight gain was significantly lower only in the high-dose females (2%), compared with a 7% increase for control females. This resulted in significantly reduced body weights after each litter and at necropsy for these high-dose females. During the course of the Task 2 continuous breeding phase, body weights were within 10% of the controls except for high-dose females at week 17 (the end of cohabitation) when they were 11% less than the controls. Over the duration of continuous breeding, there were no clinical signs of toxicity noted during the twice-daily health surveillance.

Based on body weights and food consumption, the average calculated daily intakes of DBP for the 0.1, 0.5, and 1.0% dose groups were 52, 256, and 509 mg/kg body weight for males and 80, 385, and 794 mg/kg for females, respectively.

DBP treatment did not affect fertility or the average number of litters per pair, but it did dose-dependently reduce the number of lives pups per litter (Table 1). In addition, live pup weights (both absolute and adjusted for litter size) were significantly decreased in the middle and high dose groups. The cumulative-days-to-litter values were similar for control and treated animals.

Dam weights at delivery were significantly decreased at each litter of the high dose group (by ≤10%). During nursing of the final litter, dam weights were significantly decreased at postnatal day 21 in the low dose group (by 7%), at postnatal days 14 and 21 in the middle dose group (by ≈6%), and at all time points in the high dose group ( by ≈10%; data not shown).

***Crossover mating (Task 3).*** Since adverse effects on reproduction were observed in $F_0$ rats, the crossover mating (Task 3) was performed on $F_0$ animals to determine the affected sex. For Task 3, control animals were randomly paired with other control or high dose (1.0%) animals for 7 days. Among the three groups (control male × control female, 1% male × control female, and control male × 1% female), there was no overall difference with respect to mating, pregnancy, or fertility indices (Table 2). Live pup weight adjusted for litter size was significantly reduced in the control male × 1% female group. Treated females weighed ≈11% less than controls during Task 3 and consumed ≈12% less feed. There were no significant clinical signs of toxicity noted during Task 3. Although two females died, one in the control group and the other in the high dose group, neither of these deaths were attributed to DBP exposure.

At the end of Task 3 (4 weeks after cohabitation), male body weights were unchanged, but high dose females weighed ≈14% less than controls. At necropsy of the controls and high-dose rats, organ-to-body weight ratios for the liver and kidneys were significantly increased in both sexes in the high dose group (Table 3). The gross structure of the reproductive system was unchanged by DBP consumption, and sperm studies showed that sperm concentration, motility, percent abnormal forms, and testicular spermatid head counts were not affected by 1% DBP consumption. DBP caused no apparent changes in average estrous cycle length (control length 4.20 ± 0.07 days; $n$ = 33) or progression through the stages of the cycle (data not shown).

***Second generation fertility (Task 4).*** The final Task 2 litters of all dose groups were reared and exposed to the same concentrations of DBP between weaning and necropsy as their parents were. Before weaning, $F_1$ pups were counted and weighed on postnatal days 0, 4, 7, 14, and 21. Survival was not affected, but live male and female pup weights in the 1.0% dose group were significantly (10–15%) lower than controls at postnatal days 0, 14, and 21.

During rearing (at ≈postnatal day 46), three $F_1$ males in the high dose group were found to have small and malformed prepuces and/or penises and were without palpable testes. Just prior to mating (postnatal day ≈80), one of these males had palpable testes. No such abnormalities were noted for the control males.

At 88 (±10) days of age, $F_1$ rats were cohabited for 1 week. Feed consumption values during the week of cohabitation were ≈12% lower in the 1% DBP dose group compared to the control group. Mating, pregnancy, and fertility indices in the 1.0% group were significantly lower: only 1 litter was born to 20 breeding pairs in the high dose group, compared with 19 litters to 20 breeding pairs in the control group (Table 4). While there was no change in the numbers of live pups per litter, absolute and adjusted live $F_2$ pup weights were ≈6–9% lower in all treatment groups. The average number of days to lit-

**Table 1.** Reproductive performance of first generation breeding pairs

| Reproductive parameter | Dose group[a] 0% | 0.1% | 0.5% | 1% | Trend |
|---|---|---|---|---|---|
| Average litters per pair | 4.8 ± 0.1 (40) | 5.0 ± 0.1 (20) | 4.9 ± 0.1 (19) | 4.9 ± 0.1 (20) | $p = 0.322$ |
| Live pups per litter | 12.9 ± 0.2 (40) | 11.9 ± 0.3 (20)* | 11.0 ± 0.5 (19)* | 10.7 ± 0.4 (20)* | $p < 0.001$ |
| Absolute live pup wt (g) | 5.96 ± 0.06 (40) | 5.99 ± 0.06 (20) | 5.74 ± 0.07 (19)* | 5.38 ± 0.10 (20)* | $p < 0.001$ |
| Adjusted live pup wt (g) | 6.04 ± 0.06 (40) | 5.99 ± 0.08 (20) | 5.66 ± 0.08 (19)* | 5.30 ± 0.08 (20)* | $p < 0.001$ |

[a]Data are mean ± SE (number of breeding pairs).
*$p < 0.05$ when dosed groups are compared to controls.

**Table 2.** Mating trial to determine affected sex: fertility and reproductive performance

| Reproductive parameter | Treatment group[a] Control male × control female | 1% Male × control female | Control male × 1% female | Overall difference |
|---|---|---|---|---|
| Mating index[b] | 16/19 (84%) | 19/20 (95%) | 16/19 (84%) | $p = 0.487$ |
| Pregnancy index[c] | 12/18 (67%) | 18/20 (90%) | 15/19 (79%) | $p = 0.212$ |
| Fertility index[d] | 12/15 (80%) | 18/19 (95%) | 15/16 (94%) | $p = 0.303$ |
| Live pups per litter | 12.7 ± 1.3 (12) | 13.4 ± 0.6 (18) | 12.9 ± 0.7 (15) | $p = 0.934$ |
| Adjusted live pup wt (g) | 5.96 ± 0.16 (12) | 6.16 ± 0.12 (18) | 5.28 ± 0.14 (15)*# | $p < 0.001$ |

[a]Data are mean ± SE (number of breeding pairs).
[b]Number of females with plug/number of cohabiting pairs (% with plug).
[c]Number of fertile pairs/number of cohabiting pairs (% pregnant).
[d]Number of fertile pairs/number of females with plug (% fertile).
*$p<0.05$ when dosed groups are compared to controls.
#Treated groups differ at $p<0.05$.

ter and average dam weight at delivery were not affected by DBP consumption.

At the end of Task 4, all $F_1$ animals were weighed, killed, and necropsied. The high dose males and females weighed ≈8–14 % less than their controls (Table 5). Body weights were unchanged in the middle and lower dose groups. For males, only the 1% treatment group showed a significant increase in liver weight, while kidney weight was increased at both the middle and high dose levels. $F_1$ females showed no significant change in liver or kidney weights. $F_1$ ventral prostate, seminal vesicle/coagulating gland, and right testis weights all were decreased in the 1.0% treatment group, while average ovary weights remained unchanged (Table 5).

Epididymal sperm counts and testicular spermatid head counts were both reduced in the high dose group (Table 5). Epididymal sperm concentration values (expressed as 1,000 sperm/mg caudal tissue) in high dose rats were 51% those of control animals. Likewise, both the total spermatid heads per testis, a measure of testicular output, and total spermatid head per gram of testis, a measure of spermatogenic efficiency, were decreased in 1.0% DBP treated males.

Histopathologic examination of selected organs was performed on 10 representative males from the control, 0.5%, and 1.0% groups and is summarized in Table 6. Three males (of 10 total) in the 0.5% dose group showed degeneration of seminiferous tubules, compared with 1 control, and 8 males in the 1.0% group. In addition, 7 of the 10 males examined in the 1.0% DBP treatment group demonstrated apparent interstitial cell hyperplasia. Finally, half of the rats (5 of 10) observed in the high dose group had either underdeveloped or otherwise defective epididymides.

## Discussion

The results from the present RACB study indicate that DBP is a developmental toxicant in that live pup weights (absolute and adjusted) were significantly decreased in both the 0.5% and 1.0% dose groups in Task 2 (Table 1) and in control males × 1.0% dosed females of Task 3 (Table 2). Decreased fetal weight is considered an early indicator of developmental toxicity (*32*) and has been observed in Wistar rats treated with 600 mg DBP/kg by gavage on gestation days 0–21 (*33*). Similar results were observed by Reel and Lawton (*34*) in an earlier DBP RACB study using Swiss mice. Although general toxicity of DBP (a significant increase in liver and kidney weights in both males and females and

**Table 3.** Mating trial to determine affected sex: necropsy outcome

| | Dose group | | |
|---|---|---|---|
| Organ | 0% | 1% | Difference |
| Male | | | |
| Absolute body wt | 683.6 ± 7.6 (40)[a] | 656.8 ± 15.0 (20) | $p<0.154$ |
| Adjusted liver wt | 37.6 ± 0.63 (40) | 43.4 ± 1.1 (20)* | $p<0.001$ |
| Adjusted kidney wt | 6.6 ± 0.11 (40) | 7.3 ± 0.18 (20)* | $p=0.004$ |
| Female | | | |
| Absolute body wt | 379.0 ± 9.6 (38) | 326.4 ± 6.5 (19)* | $p<0.001$ |
| Adjusted liver wt | 34.0 ± 0.62 (38) | 38.9 ± 0.69 (19)* | $p<0.001$ |
| Adjusted kidney wt | 7.0 ± 0.10 (38) | 7.6 ± 0.14 (19)* | $p=0.002$ |

[a]Mean ratio (mg/g body weight) ± SE (number of animals).
*$p<0.05$ when dosed groups are compared to controls (Wilcoxon test).

**Table 4.** Mating, fertility, and reproductive performance of second generation breeding pairs

| | Dose group[a] | | | | |
|---|---|---|---|---|---|
| Reproductive parameter | 0% | 0.1% | 0.5% | 1% | Trend |
| Mating index[b] | 20/20 (100%) | 19/20 (95%) | 18/20 (90%) | 6/20 (30%)* | $p<0.001$ |
| Pregnancy index[c] | 19/20 (95%) | 17/20 (85%) | 17/20 (85%) | 1/20 (5%)* | $p<0.001$ |
| Fertility index[d] | 19/20 (95%) | 17/19 (89%) | 17/18 (94%) | 1/6 (17%)* | $p<0.001$ |
| Live pups per litter | 14.0 ± 0.8 (19) | 15.5 ± 0.4 (17) | 12.8 ± 0.8 (17) | 13.0# | $p=0.233$ |
| Absolute live pup wt (g) | 5.97 ± 0.11 (19) | 5.60 ± 0.09 (17)* | 5.60 ± 0.09 (17)* | 5.00# | $p=0.020$ |
| Adjusted live pup wt (g) | 5.98 ± 0.08 (19) | 5.69 ± 0.09 (17)* | 5.50 ± 0.09 (17)* | — | $p<0.001$ |

[a]Data are mean ± SE (number of breeding pairs).
[b]Number of females with plug/number of cohabiting pairs (% with plug).
[c]Number of fertile pairs/number of cohabiting pairs (% pregnant).
[d]Number of fertile pairs/number of females with plug (% fertile).
*$p<0.05$ when dosed groups are compared to controls.
#Treated groups differ at $p<0.05$.

**Table 5.** Second generation male and female necropsy organ weights

| | Dose group[a] | | | | |
|---|---|---|---|---|---|
| Organ | 0% | 0.1% | 0.5% | 1% | Trend |
| Male | | | | | |
| Body (g) | 506.0 ± 8.6 (20) | 508.8 ± 14.2 (20) | 496.8 ± 11.8 (20) | 466.7 ± 9.8 (20)* | $p = 0.008$ |
| Liver | 40.7 ± 0.89 (20) | 38.9 ± 0.68 (20) | 40.2 ± 0.95 (20) | 47.4 ± 0.73 (20)* | $p<0.001$ |
| Kidneys | 7.7 ± 0.11 (20) | 7.9 ± 0.14 (20) | 8.2 ± 0.10 (20)* | 8.2 ± 0.14 (20)* | $p = 0.006$ |
| Prostate | 1.7 ± 0.07 (20) | 1.6 ± 0.13 (20) | 1.5 ± 0.07 (20) | 1.3 ± 0.11 (20)* | $p = 0.022$ |
| Seminal vesicles | 5.0 ± 0.18 (20) | 5.1 ± 0.23 (20) | 5.0 ± 0.17 (20) | 3.9 ± 0.30 (20)* | $p = 0.003$ |
| Right testis (mg)[b] | 1774.6 ± 67.0 (20) | 1767.4 ± 47.8 (20) | 1810.7 ± 105 (20) | 1087.9 ± 125 (20)* | $p = 0.008$ |
| Epididymal sperm parmeters | | | | | |
| Number | 574.9 ± 37.6 (19) | 575.8 ± 22.2 (20) | 547.5 ± 36.9 (19) | 295.1 ± 84.5 (18) | $p = 0.017$ |
| Percent motile[c] | 71.0 ± 1.6 (19) | 72.0 ± 2.0 (20) | 69.8 ± 1.5 (20) | 72.4 ± 3.2 (9) | $p = 0.770$ |
| Percent abnormal[d] | 1.0 ± 0.21 (18) | 0.97 ± 0.12 (20) | 1.0 ± 0.14 (19) | 0.94 ± 0.12 (7) | $p = 0.470$ |
| Total spermatid heads per testis[e] ($\times 10^7$) | 14.52 ± 0.95 (20) | 16.84 ± 0.61 (20) | 15.26 ± 0.99 (20) | 6.69 ± 1.73 (20)# | — |
| Total spermatid heads per g testis[f] ($\times 10^7$) | 8.29 ± 0.58 (20) | 9.64 ± 0.41 (20) | 8.29 ± 0.54 (20) | 4.37 ± 0.98 (20)# | — |
| Female | | | | | |
| Body (g) | 323.0 ± 5.1 (20) | 311.6 ± 9.3 (20) | 318.6 ± 8.6 (20) | 281.2 ± 8.6 (20)* | $p<0.001$ |
| Liver | 37.0 ± 0.82 (20) | 36.3 ± 0.77 (20) | 37.4 ± 0.96 (20) | 37.9 ± 0.62 (20) | $p = 0.275$ |
| Kidneys | 7.4 ± 0.11 (20) | 7.7 ± 0.14 (20) | 7.8 ± 0.26 (20) | 7.8 ± 0.14 (20) | $p = 0.128$ |
| Right ovary | 0.16 ± 0.01 (20) | 0.19 ± 0.01 (20) | 0.18 ± 0.01 (20) | 0.15 ± 0.01 (19) | $p = 0.250$ |

[a]Mean ratio (mg/g body weight) ± SE (number of animals).
[b]Not adjusted for body weight.
[c]Dose group means and SEs are computed only from samples with at least 20 sperm.
[d]Dose group means and SEs are computed only from samples with at least 100 sperm.
[e]$X(10{,}000)Y$; ($X$, average number of spermatid heads per tertiary square; $Y$, dilution factor).
[f]Total spermatid heads per testis/testicular weight in grams.
#Significantly different ($p<0.05$) from the control group.
*$p<0.05$ when dosed groups are compared to controls.

**Table 6.** Summary of incidence of histopathologic lesions (Task 4)

| | Treatment group | | |
|---|---|---|---|
| | Control | 0.5% | 1.0% |
| Liver (total examined) | (10) | (10) | (10) |
| Hepatocellular degeneration | 3[a] | 1 | — |
| Focal hepatitis | 6 | 1 | 4 |
| Testis (total examined) | (10)[b] | (10) | (10) |
| Degeneration of seminiferous tubules | 1 | 3 | 8 |
| Interstitial cell hyperplasia | 1 | 1 | 7 |
| Granuloma | 1 | — | — |
| Epididymis (total examined) | (10) | (10) | (10) |
| Degeneration of epithelial cells | 1 | 2 | 1 |
| Interstitial epididymitis | 1 | 1 | 1 |
| Focal granuloma | — | — | 1 |
| Underdeveloped epididymis | — | — | 5 |
| Presence of fluid/ degenerated cells | 1 | 1 | 2 |
| Apparent sperm content reduction | 1 | 1 | 3 |
| Seminal vesicles (total examined) | (10) | (10) | (10) |
| Vesticulitis | — | — | 1 |
| Inspissated secretion | — | — | 1 |

[a]Number of animals exhibiting lesion.
[b]One animal had variable loss of spermatogonia, spermatocytes, and spermatids and had an apparent increased number of Sertoli and intersititial cells.

reduction in body weight in females) was observed in the 1.0% DBP group, there was no difference in the body weights between the control and 0.5% dose groups. This shows that pup weight was reduced in the absence of a change in maternal weight at 0.5% DBP.

In the litters produced by the $F_1$ adults (Task 4), decreased live pup weights (absolute and adjusted) were observed in all the dose groups (including the 0.1% dose group). This was seen in the absence of obvious general toxicity (Table 5). These results suggest that the animals exposed during development were much more sensitive to the developmental toxicity of DBP than were animals exposed only as adults. Furthermore, severe structural defects in the male reproductive system were observed in the 0.5% and 1.0% dose groups (results in Task 4 and Table 6). The structural defects included small and malformed prepuces and/or penises, underdeveloped or otherwise defective epididymides, and degenerate seminiferous tubules. From these data alone, we cannot deduce whether the effects represent developmental toxicity alone, if developmental exposure renders the animals more susceptible to the adverse effects of DBP during adult exposure, or both, but if DBP is an estrogenic compound (*18*), the former may be more likely.

The statistically significant decreases in the number of live pups per litter seen in all $F_0$ dose groups during continuous breeding (Task 2, Table 2) were not seen in the Task 3 crossover or in the second generation (Tables 2 and 4, respectively), despite being of equivalent absolute magnitude (i.e., ≈two pups per litter). Earlier analyses (*35*) suggest that this is due in part to the differences in statistical sensitivity: in Task 2, up to five litters were generated, while Tasks 3 and 4 generated only one litter. The multiple litters in Task 2 yield a test that is statistically much more powerful than the single litters of the Task 3 or 4 mating trials. Based on this, one might predict that the nonsignificant difference between the controls and middle dose animals in Task 4 (14 vs. 12.8) would become statistically significant if five litters were produced.

While the reproductive toxicity of DBP was relatively mild in the $F_0$ rats (Table 1, Table 2), the reproductive functions of $F_1$ rats were severely damaged. First, all the indices (mating, pregnancy, and fertility index) in $F_1$ 1.0% dose group were significantly reduced because only one litter of live young was delivered (Table 4). Second, while the weight of the right ovary of $F_1$ females in all the dose groups remained normal, the weights of ventral prostate, seminal vesicles/coagulating gland, and right testis in $F_1$ males of the 1.0% dose group were markedly reduced. This may indicate that the $F_1$ males are more sensitive to DBP than $F_1$ females, although the ovary is notoriously insensitive as an index of female reproductive toxicity (*35,36*). Third, histologic examination of the reproductive systems of $F_1$ males showed degeneration in seminiferous tubules together with significantly fewer spermatids in testes (Table 5, 6). While the numbers of epididymal sperm were reduced at the high dose, morphology and visually estimated motility were normal in all groups; the high dose animals were making fewer sperm, although these sperm appeared normal. In contrast to the males, female rats in both the $F_0$ and $F_1$ generations seemed to be relatively resistant to the reproductive toxicity of DBP in terms of numbers of live young and necropsy endpoints. But in the $F_0$ generation, females appeared to be more sensitive to the systemic toxicity of DBP because the body weight of $F_0$ females in the 1.0% dose group was significantly decreased while the males at that concentration showed no detectable change.

Consistent with previous reports on other phthalates (*13*), the present data indicate that effects of DBP exposure are greater in animals exposed from conception than those exposed as adults only. Because the $F_1$ rats were possibly exposed during gestation and throughout nursing and were dosed during maturation until and through mating, we cannot specify which period of development (e.g., which developmental trimester, nursing, puberty, etc.) is more sensitive to the effects of DBP. However, the male reproductive structural defects suggest that the period of organogenesis and perhaps hormonal imprinting for these tissues, in the prenatal and perinatal periods, is the most critical time. The mechanisms underlying this developmental toxicity are still unknown. In the past, most of the toxicity studies on phthalates have focused on their adverse effects on dosed juvenile and adult animals, rather than on pups of dosed dams. Nevertheless, these early studies have led to the widespread view that phthalates affect the testis primarily at the Sertoli cells (*14,37,38*). Indeed, in neonatal rat testes, significant decreases in testis weights and Sertoli cell numbers after five daily oral doses of 1000 mg/kg DEHP have been reported by Dostal et al. (*39*).

Jobling et al. (*18*) found that DBP interacted weakly with the estrogen receptor in a variety of constructs. If DBP demonstrated such an interaction *in vivo*, one would expect to find altered reproductive development (*19*). The present study found such alterations: degenerate seminiferous tubules with decreased sperm counts, apparent interstitial cell hyperplasia, and structural defects in epididymides and penises of 1.0% DBP-treated $F_1$ rats when they reached maturity. These mirror similar effects seen in rats and mice exposed perinatally to the strong estrogen agonist diethylstilbestrol (DES) (*40,41*) and are consistent with the possibility that the effects seen in the present study were mediated through an interaction with the estrogen receptor system. Interestingly, there are also reports showing that some phthalates have hormone-disrupting effects in male adult rats (*42–44*).

In conclusion, the results from the present RACB study indicate that the developmental and reproductive toxicities of DBP are more prominent in animals exposed during development and maturation than in animals exposed as adults only. This is prompting an investigation of the period of sensitivity for more of the phthalates.

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