

# Chronic Di-*n*-butyl Phthalate Exposure in Rats Reduces Fertility and Alters Ovarian Function During Pregnancy in Female Long Evans Hooded Rats

Leon Earl Gray Jr.,<sup>1</sup> John Laskey, and Joseph Ostby

Endocrinology Branch, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, ORD, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

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Testis function in fetal and peripubertal male rats is disrupted by subchronic exposure to phthalate esters (PEs). In contrast to the male rat, it is generally held that reproduction in female rats is much less sensitive to phthalate-induced disruption. However, the current study demonstrates that oral administration of dibutyl phthalate (DBP) to female Long Evans (LE) hooded rats from weaning, through puberty, mating, and gestation disrupts pregnancy maintenance at dose levels similar to those that affect testis function in male rats. Administration of 500 and 1000 mg DBP/kg/day, but not 250 mg DBP/kg/day, to female LE rats induced midpregnancy abortions. The percentage of females delivering live pups was reduced by more than 50% at 500 mg/kg/day and by 90% at 1000 mg/kg/day in the absence of overt toxicity, whereas the ages at vaginal opening and first estrus, estrous cyclicity, and mating indices (*N* mated/*N* paired or *N* pregnant/*N* mated) were not significantly affected. On gestational day 13, prior to the stage when litters were being aborted, *ex vivo* ovarian hormone production was significantly decreased by *in vivo* DBP treatment at 500 and 1000 mg/kg/day. These results should be considered when evaluating mechanisms of reproductive toxicity for the PE because it is likely that these reproductive alterations in the female rat arise via a mode of action similar to that operative in male rats.

**Key Words:** dibutyl phthalate; female fertility; pregnancy disruptions; ovarian progesterone.

Phthalate esters (PEs), like di-*n*-butyl phthalate (DBP), diethyl hexyl phthalate (DEHP), and benzyl butyl phthalate, have been shown to induce “antiandrogenic” effects in male rodents when administered during sexual differentiation and puberty. All indications are that PEs are not androgen receptor antagonists; they alter Leydig cell function decreasing fetal and pubertal male rat testosterone synthesis (Agarwal *et al.*, 1986; Gray *et al.*, 1999) and fetal testis insulin-like factor 3 (insl3) hormone mRNA levels at relatively low dosage levels (Wilson *et al.*, 2004). *In utero*, some PEs induce malformations of androgen- and insl3-dependent tissues, whereas pubertal exposure causes a delay in puberty (preputial separation) (Gray *et al.*, 1999) and reduces testis- and androgen-dependent tissue weights. The precise mechanism of action of the PEs on the androgen signaling and insl3 pathways of the male rat has not been determined.

In contrast, it is generally held that the reproductive system of the female rat is much less sensitive to PE toxicity than is the male rat. Lovekamp-Swan and Davis (2003) found that very high acute dosage levels of DEHP (2 g/kg) were required to decrease serum estradiol levels, prolong estrous cycles, and block ovulation in adult, cycling female rats. Studies in which PE-treated female rats were bred with untreated males, the effects of PEs on female rodent fertility have not been consistent. For example, when female Sprague-Dawley (SD) rats were given DBP in the diet at 1.0% for 14 weeks and then mated for 1 week with untreated male rats, DBP did not affect mating, pregnancy, or fertility (Wine *et al.*, 1997). In contrast, results from a preliminary study in our laboratory indicated that fertility of female rats can be dramatically reduced by PE treatment. When 1 g DBP/kg/day was administered chronically by gavage to Long Evans (LE) hooded male and female rats from weaning, through adulthood and mating, fertility was reduced when treated females were mated with untreated male rats. Treated females had difficulty with their pregnancies even though the ages at puberty and estrous cyclicity were normal. Due to the observation of such an unanticipated effect on P0 female rat pregnancy in our laboratory, the current project was conducted to replicate these observations and to try to begin to

<sup>1</sup> To whom correspondence should be addressed at Endocrinology Branch, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, ORD, U.S. Environmental Protection Agency, MD-72, Research Triangle Park, NC 27711. Fax: (919) 541-4017. E-mail: gray.earl@epa.gov.

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determine the target organs affected by DBP in the female rat reproductive system.

The first study was designed to replicate the findings of reduced fertility and fecundity in female rats treated by gavage with 500 mg DBP/kg/day from weaning, through puberty, young adulthood, mating, and three pregnancies. Having replicated the initial observation, a second study exposed LE hooded female rats to 250, 500, and 1000 mg DBP/kg to determine if the reduction in fecundity was associated with alterations of ovarian function *ex vivo* at midpregnancy when the treated dams were losing their litters. These dosage levels were selected because similarly treated LE hooded male rats display testicular lesions and infertility after administration of DBP at 500 and 1000 mg DBP/kg/day, but not 250 mg DBP/kg/day (Gray *et al.*, 1999).

## MATERIALS AND METHODS

Pregnant LE rats (Charles River, Raleigh, NC) were received from Charles River Breeding Laboratory, on day 3 of gestation. Upon receipt, dams were housed individually in clear plastic cages (20 × 25 × 47 cm) with laboratory grade pine shavings as bedding. Animals were maintained on Purina Rat Chow (5001) and tap water *ad libitum*, in a room with a 12:12-h photoperiod and temperature of 20–24°C with a relative humidity of 40–50%. Laboratory grade corn oil was the vehicle chosen to prepare all dosing solutions. On postpartum day 6 the dams were weighed and litters were culled to four males and four females per litter and weighed by sex. On postpartum day 21, pups were weighed, weight ranked by sex, and assigned to treatment groups in a manner that provided each treatment group with equal means and variances in body weight. This project was conducted under an approved National Health and Environmental Effects Research Laboratory Animal Care Project Form.

**Methods for study 1.** At 21 days of age, 20 female rats were assigned to one of two treatment groups in a manner that provided equal means and variances in body weight at weaning. The control group ( $n = 12$ ) was dosed with the vehicle (corn oil) while the other group was dosed with DBP at 500 mg/kg/day ( $n = 8$ ) from 22 days of age for the duration of the study. Females were examined daily for vaginal opening, and the vaginal smears were taken by lavage after vaginal opening for the duration of the study in order to evaluate the effects of DBP on estrous cyclicity.

At 83 days of age, each female was housed with a treated male for 14 days. During the mating period, females were examined daily for the presence of vaginal plugs and/or the presence of sperm in the vagina. The litters born to these females were counted and weighed at birth and 15 days of age, at which time the pups were removed and euthanized with CO<sub>2</sub>. After a 30-day recovery period, the females, now about 150 days of age, were mated for a second time with untreated male rats. The dams began delivering the F1b litters at 170 days of age. The F1b litters were randomly reduced when possible to four males and four females per litter at birth, and pups were euthanized at weaning. Production of an F1c began when the P0 females were mated with untreated males at about 200 days of age. On gestational day (GD) 13, prior to the stage when some of these females had previously aborted their litters, the females were necropsied, the numbers of live and dead fetuses were counted, and serum was taken for progesterone analysis by radioimmunoassay (RIA) (Diagnostic Products Co, Los Angeles, CA, Coat-A-Count Progesterone Kit TKP).

**Methods for study 2.** In the second study, 12–13 weanling female rats were assigned to one of four treatment groups, as above. At 24 days of age, female rats were exposed by gavage exposure with 0, 250, 500, or 1000 mg DBP/kg/day. Rats were dosed 5 days a week until they were about 110 days old

at which age they were dosed 7 days/week, and daily vaginal lavages were taken. On the day a proestrus vaginal smear was detected, a female was placed with an untreated male rat for 24 h. When the male was removed, the female was examined for the presence of sperm or a vaginal plug. F0 dams began delivering the F1a litters at about 140 days of age, and the pups were counted and weighed at 1, 5, and 15 days of age and then euthanized.

After weaning of the F1a pups, P0 dams were remated to control males for 24 h on the day of proestrus. On day 13 of the second pregnancy, females were anesthetized with CO<sub>2</sub> and decapitated and serum was collected for progesterone, testosterone, and estradiol analyses and necropsied. At necropsy, liver, kidney, adrenal, pituitary, and paired ovarian weights were taken. The numbers of live and dead fetuses were counted and hCG-stimulated ovarian P4, E2, and T production over 3.5 h was measured *in vitro* from medium samples from cultured rat ovaries by RIA (Coat-A-Count Kits TKP, TKT, and/or TKE, Diagnostic Products) using the methods of Berman and Laskey (Berman and Laskey, 1993; Laskey *et al.*, 1995; Piasek *et al.*, 2002). Ovaries were trimmed, minced, and incubated in medium 199 with HEPES bicarbonate; trypsin inhibitor and 100 μM hCG were added at 0.5 h. Cultures were centrifuged and supernatants were collected for hormonal analyses and fresh media added at 0.5, 1.5, 2.5, and 3.5 h.

## RESULTS

### Study 1: 0 Versus 500 mg DBP/kg/day

Administration of DBP at 500 mg/kg/day did not affect growth, the age at puberty as determined by the age at vaginal opening, estrous cyclicity from 30 to 80 days of age, or percentages of mated or pregnant females (Table 1). In the DBP group, the numbers of females delivering live F1A and F1B pups were reduced, as compared to controls (Fig. 1), and it was evident from the presence of blood in the vaginal lavages that some of the treated females were pregnant and losing their litters in the middle of pregnancy. When necropsied during midpregnancy of the third mating, the total number of implants was not reduced in the DBP group versus controls, but the percentage of viable fetuses was reduced by DBP treatment, consistent with the reduced live litter size at birth in the first two matings, and DBP-treated females displayed significantly reduced serum progesterone levels (Fig. 2). There were 11, 7, 5, and 2 females in the 0, 250, 500, and 1000 mg DBP/kg/day groups, respectively.

### Results of Study 2

Administration of DBP to the female LE rat did not affect growth, viability, or the ability to mate with a control male. However, for the F1a mating only 42 and 8% of the females were fertile in 500 and 1000 mg DBP groups, respectively, versus 92% of controls (Table 2). The litter sizes from mated females (as determined by sperm or plugs and a leucocytic vaginal lavage of at least 10 days in duration) were reduced on day 1 from 11.4 pups on day 1 in the control group to 1.7 and 0.1 pups in the 500 and 1000 mg DBP/kg/day groups, respectively (Fig. 1). For comparison purposes, data from similarly treated male LE rats mated to untreated females are shown in Table 3 (Gray *et al.*, 1999).

TABLE 1

Effects of Subchronic Oral DBP at 500 mg/kg/day on the Age of Puberty, Estrous Cyclicity, Fertility, and Organ Weights in LE Hooded Female Rats. Shaded Values Differ Significantly From Control Values. For Continuous Variables, the Values Are Means  $\pm$  SEs. "A" Indicates  $p < 0.05$ ; "B" Indicates  $p < 0.01$

Dose of DBP	0	500
Number of P0 females up to F1b	12	8
Age at vaginal opening, days	29.4 $\pm$ 0.5	31.3 $\pm$ 0.4
Estrous cycle length, days (30–80 days of age)	4.9 $\pm$ 0.12	5.2 $\pm$ 0.12
F1a litter size—day 1	12.6 $\pm$ 0.8	7.1 $\pm$ A
F1a litter size—day 15	11.5 $\pm$ 1.1	7.0 $\pm$ B
F1a maternal body weight (g)—day 1	283 $\pm$ 7.2	292 $\pm$ 11
Number F0 with live F1A pups/total mated	11/12	6/8
F1a pup weight (g)—day 1	5.8 $\pm$ 0.2	5.5 $\pm$ 0.3
F1a pup weight (g)—day 15	29.9 $\pm$ 1.1	31.0 $\pm$ 2.5
F1b pup weight—day 1	12.9 $\pm$ 0.8	4.4 $\pm$ 2.0B
P0 maternal body weight—day 1 of F1b	320 $\pm$ 9.0	347 $\pm$ 14.8
Number F0 dams with live F1B pups/total mated	12/12	3/8
F1b pup weight (g)—day 1	6.1 $\pm$ 0.2	5.9 $\pm$ 0.5
F1b pup weight (g)—day 15	35.4 $\pm$ 0.7	29.1 $\pm$ 6.9
Number of F1 mated a third time (for F1C)	6	6
F1c total number of F1C fetuses per female—GD 13	11.7 $\pm$ 2.5	12.5 $\pm$ 1.9
F1c number of dead fetuses per female	2.8 $\pm$ 2.8	3.2 $\pm$ 2.1
P0 maternal body weight at necropsy (g)—GD 13	343 $\pm$ 9.8	353 $\pm$ 18
P0 maternal liver weight (g)	11.4 $\pm$ 0.3	12.1 $\pm$ 0.5
P0 maternal kidney weight (g)	1.03 $\pm$ 0.3	1.17 $\pm$ 0.5
P0 maternal adrenals weight (mg)	61.2 $\pm$ 3.5	59.9 $\pm$ 2.6
P0 maternal ovaries weight (mg)	116 $\pm$ 9.3	113 $\pm$ 13.8
P0 maternal pituitary weight (mg)	14.5 $\pm$ 0.7	14.2 $\pm$ 0.7

In the first mating, every female mated successfully with an untreated male (as indicated by the presence of sperm in the vaginal lavage the day after mating). All but one of the females in the study became pseudopregnant or pregnant (as determined by the presence of leucocytic vaginal lavages for 12–13 days, or longer). One female in the 500 mg DBP/kg/day group mated but displayed persistent estrus throughout most of the study. Many of the 500 and 1000 mg DBP/kg/day-treated females that were pregnant, but did not deliver live pups, displayed a constant leucocytic, pregnancy-like vaginal lavage for 21–29 days in duration and blood was detected in the vagina at or after midpregnancy. These females also gained 30–60 g during pregnancy and some experienced a dramatic weight loss around the expected day of parturition, but no live or dead pups were recovered.

When the females were examined on GD 13 of the second pregnancy, gravid uterine weight was significantly reduced, and the total numbers of fetuses (live plus dead) and live fetuses were reduced in the two highest dose groups; the ovaries of several of the DBP-treated females contained grossly visible hemorrhagic corpora lutea, and serum progesterone values were reduced, but this was statistically significant only at

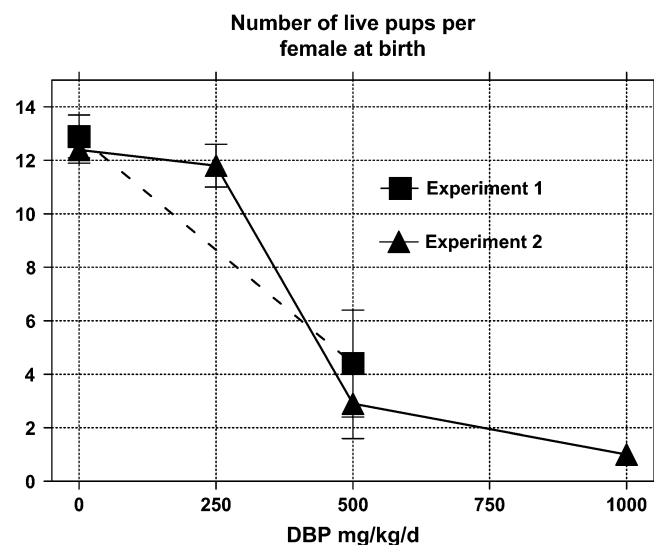


FIG. 1. Subchronic oral DBP treatment from weaning, through puberty, mating, and pregnancy at 250, 500, and 1000 mg/kg/day reduces fertility of LE hooded female rats mated to untreated males. Points are means  $\pm$  SEs.

1000 mg DBP/kg/day (Fig. 2). The weights of the kidneys and liver were only affected by DBP treatment, being increased in the two high-dose groups.

When examined *ex vivo*, ovarian progesterone and estradiol production, using only ovaries from females with live fetuses, was altered by DBP treatment in the current study (Fig. 2). Total (three 1 h intervals pooled) ovarian progesterone production was reduced in the 500- and 1000-mg DBP/kg/day dose groups ( $F = 16.9$  (3,21 df),  $p < 0.001$ ), whereas total estradiol production was increased (Fig. 2). Treated, pregnant females in which all the fetuses were dead had low serum progesterone levels, approaching those seen in nonpregnant females. In nonpregnant females in the 0-, 250-, 500-, and 1000-mg/kg/day groups, the serum progesterone values were below 40 ng/ml. Since many of the serum testosterone and estradiol levels were below the limits of detection (0.2 ng/ml and 20 pg/ml, respectively) in every dose group, the effects of DBP treatment could not be evaluated on pregnant females (data not shown).

## DISCUSSION

In the current investigation, we found that chronic administration of DBP from weaning, through puberty, young adulthood, mating, and pregnancy, had adverse effects on female rat fecundity and fertility in the P0 generation at 500 and 1000 mg/kg/day (Fig. 1). In fact, the effect of DBP at 500 mg/kg/day on fertility in the LE female rat is equivalent to the effect on the fertility of similarly treated LE male rats (Table 3). The two studies herein replicate observations from preliminary studies in our laboratory and clearly demonstrate adverse effects below the 600-mg DBP/kg/day dose proposed by

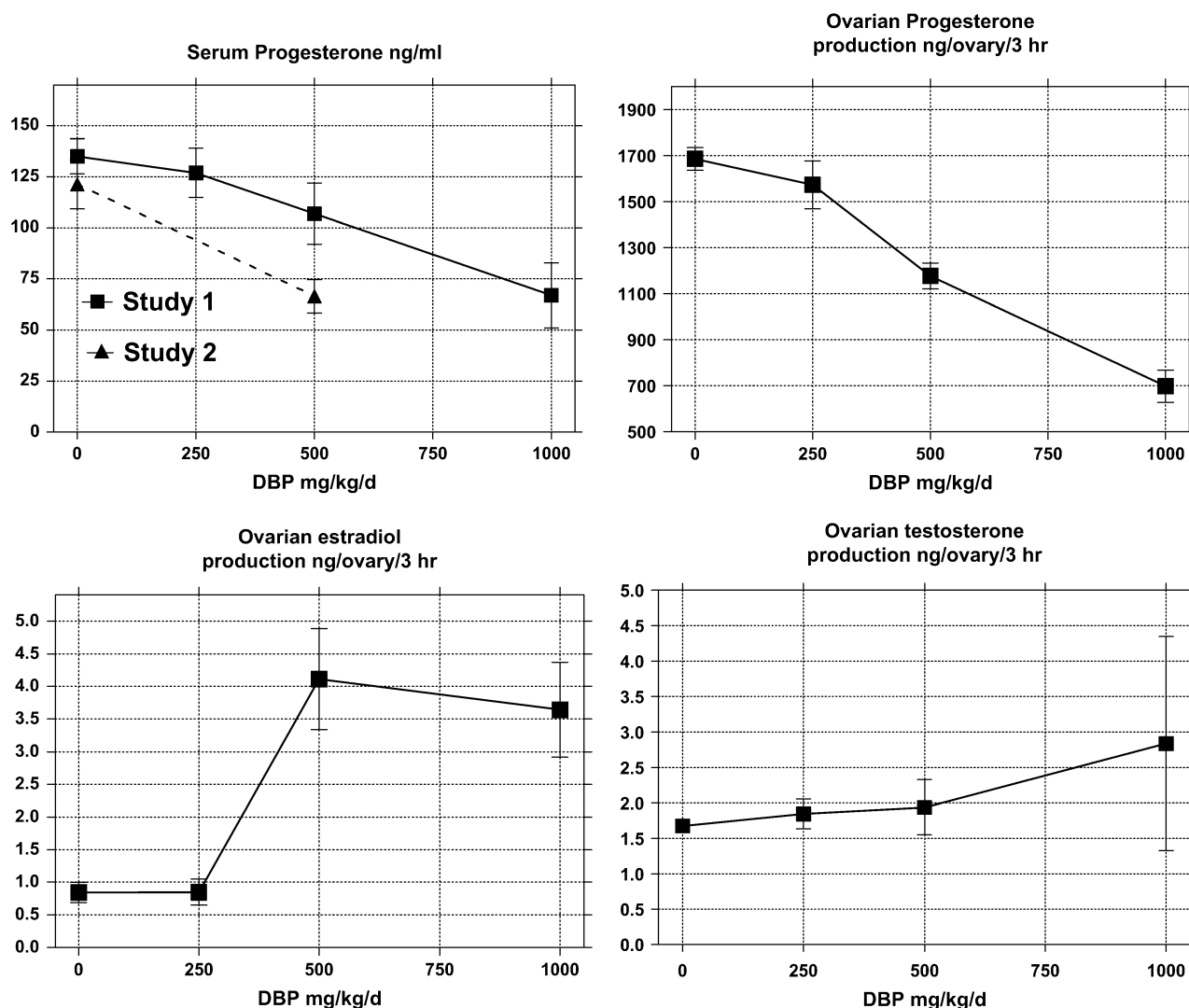


FIG. 2. Effects of chronic DBP gavage treatment from weaning, through puberty, mating, pregnancy, lactation up to day 13 of the second pregnancy on serum and ovarian progesterone and estradiol production (*ex vivo*) in a 3-h minced ovary culture with hCG as per Laskey *et al.* (1995). Testosterone production is not significantly altered. Values on graphs are means  $\pm$  SEs. These data used include only the pregnant females.

McKee *et al.* (2004) as the no observed adverse effect level (NOAEL) for DBP in the female rat. Our ongoing studies indicate that several other PEs that have been shown to inhibit testicular steroidogenesis also induce midpregnancy abortions in the SD rat.

In light of the results reported here, it is noteworthy that phthalate exposures also have been associated with reproductive effects in women and nonhuman primates. In humans, DEHP exposure has been associated with shorter pregnancy duration (Latini *et al.*, 2003) and PE exposures, including DBP, have been strongly associated with the occurrence of endometriosis in women (Reddy *et al.*, 2006). Although it is likely that the doses administered in the current study are far higher than those seen in the general human population, there is a great deal of uncertainty about human exposure levels to individual PEs

and mixtures of PE, which can act additively. Furthermore, PE metabolite levels range in human urine and amniotic fluid over several orders of magnitude, and urinary concentrations of monobutyl phthalate, a DBP metabolite, have been reported as high as 17  $\mu\text{g/ml}$  (Hauser *et al.*, 2004). In the female marmoset, administration of DEHP for 65 weeks throughout puberty increases uterine and ovarian weights, induces enlarged corpora lutea, and elevated serum estrogen levels (Tomonari *et al.*, 2005).

Although midpregnancy is sensitive to disruption by PEs, it is important to note that this is not the most sensitive life stage for PE-induced reproductive toxicity. Clearly, the F1 offspring are more sensitive to the reproductive toxicity of PEs than are either P0 male or female rats due to *in utero* exposures during the differentiation of the reproductive tract. Although 250 mg

TABLE 2

The Effects of Subchronic Oral DBP at 250, 500, and 1000 mg/kg/day on the LE Hooded Female Rat Reproductive Function. Body and Organ Weights are Listed for Only Females That Were Pregnant at Necropsy. For Continuous Variables, the Values Are Means  $\pm$  SEs. "A" or "B" Indicates That the Value Differs Significantly From Control by  $p < 0.05$  or  $p < 0.01$ , Respectively

Dose of DBP (mg/kg/day)	0	250	500	1000
Number of females	13	11	12	13
Body weight (g)	322 $\pm$ 6.1	330 $\pm$ 9.4	338 $\pm$ 11.0	319 $\pm$ 14
Liver weight (g)	12.2 $\pm$ 0.31	12.8 $\pm$ 0.34	12.7 $\pm$ 0.58	14.0 $\pm$ 1.1B
Kidney weight (g)	1.94 $\pm$ 0.05	1.06 $\pm$ 0.02	2.12 $\pm$ 0.09	2.23 $\pm$ 0.12B
Adrenals weight (mg)	55 $\pm$ 1.6	55 $\pm$ 2.7	57 $\pm$ 2.3	57.1 $\pm$ 1.3
Pituitary weight (mg)	10.5 $\pm$ 0.3	11.6 $\pm$ 0.3	12.3 $\pm$ 0.6A	9.7 $\pm$ 0.5
Ovaries weight (mg)	97.8 $\pm$ 4.3	94.9 $\pm$ 3.1	91.9 $\pm$ 3.7	87.1 $\pm$ 4.0
% Ovaries with gross corpora hemorrhagic	0%	0%	10%	25% ( $p > 0.1$ )
% P0 mated (sperm positive—F1a)	100	100	100	100
% P0 (pseudopregnant—F1a)	0	9	0	31 ( $p > 0.1$ )
% P0 constant estrus	0	0	8	0
% P0 pregnant—F1a	100	91	92	69 ( $p > 0.1$ )
% P0 fertile (% females with live pups at birth of the F1A)	92	82	42B	8B
F1a litter size per F0 mated female—day 1	11.4 $\pm$ 1.1	9.6 $\pm$ 1.6	1.7 $\pm$ 0.9B	0.1B ( $n = 1$ )
F1a live pups per F0 female (with any pups)—day 1	12.4 $\pm$ 0.5	11.8 $\pm$ 0.8	2.9 $\pm$ 1.3B	1.0B ( $n = 1$ )
F1a pups per F0 female (with any pups)—day 15	12.3 $\pm$ 0.5	11.1 $\pm$ 0.6	2.0 $\pm$ 1.6B	0B
Maternal body weight (g) after delivery of the F1A	292 $\pm$ 6.3	296 $\pm$ 8.7	288 $\pm$ 14	311
F1a pup weight (g)—day 1	5.9 $\pm$ 0.1	5.7 $\pm$ 0.1	5.9 $\pm$ 0.3	5.4
F1a pup weight (g)—day 5	10.3 $\pm$ 0.3	11.0 $\pm$ 0.4	11.7 $\pm$ 0.5	—
F1a pup weight (g)—day 15	29.7 $\pm$ 0.8	31.2 $\pm$ 1.0	33.1 $\pm$ 4.2	—
F1a pup weight (g)—day 21	43.4 $\pm$ 1.2	46.0 $\pm$ 1.5	49.8 $\pm$ 6.1	—
F1b total number of fetuses—GD 13	14.8 $\pm$ 0.7	14.0 $\pm$ 0.5	8.1 $\pm$ 1.6B	10.0 $\pm$ 1.7A
F1b number of live fetuses—GD 13	13.6 $\pm$ 0.9	13.1 $\pm$ 0.5	5.5 $\pm$ 1.7B	2.0 $\pm$ 1.1B
F1b dead—GD 13	1.3 $\pm$ 0.4	0.9 $\pm$ 0.3	2.6 $\pm$ 1.0	8.0 $\pm$ 1.1B
Gravid uterine weight (g)—GD 13	4.53 $\pm$ 0.37	4.56 $\pm$ 0.17	3.06 $\pm$ 0.5A	3.07 $\pm$ 0.6A

TABLE 3

The Effects of DBP Treatment With 250, 500, and 1000 mg/kg/day in P0 Generation Male LE Hooded Rats, Mated to Untreated Females, for Comparison to the Effects Seen in the P0 Generation Treated Female Rats. These Data Were Discussed in a Review by Gray *et al.* (1999) but not Presented in Full in That Paper. Shaded Values Differ Significantly From Control Values. "A" or "B" Indicates That the Value Differs Significantly From Control by  $p < 0.05$  or  $p < 0.01$ , Respectively

DBP dose and fertility status	0—Fertile	250—Fertile	500—Fertile	500—Infertile	1000—Infertile
Body weight (g)	489 $\pm$ 14	459 $\pm$ 7.2	447 $\pm$ 8.4	468 $\pm$ 15	440 $\pm$ 7.4
Liver weight (g)	24.7 $\pm$ 1.0	21.6 $\pm$ 1.0	23.6 $\pm$ 1.7	24.2 $\pm$ 0.7	25.4 $\pm$ 1.0
Kidney weight (g)	1.75 $\pm$ 0.04	1.65 $\pm$ 0.04	1.83 $\pm$ 0.08	1.74 $\pm$ 0.14	1.84 $\pm$ 0.07
Adrenals weight (mg)	51 $\pm$ 3.3	52.7 $\pm$ 5.1	60 $\pm$ 4.2	45 $\pm$ 7.2	45.7 $\pm$ 2.8
Testis weight (g)	1.86 $\pm$ 0.05	1.77 $\pm$ 0.06	1.58 $\pm$ 0.17A	0.83 $\pm$ 0.41B	0.40 $\pm$ 0.03B
Cauda epididymal weight (mg)	301 $\pm$ 14	282 $\pm$ 7.8	247 $\pm$ 38A	173 $\pm$ 58B	104 $\pm$ 4.4B
Seminal vesicle weight (g)	1.73 $\pm$ 0.07	1.65 $\pm$ 0.08	1.54 $\pm$ 0.12	1.50 $\pm$ 0.25	1.27 $\pm$ 0.09
Cauda epididymal sperm count ( $\times 10^6$ )	148 $\pm$ 11	146 $\pm$ 4.6	118 $\pm$ 25	52 $\pm$ 52B	0.0 $\pm$ 0B
Sperm motility (%)	52 $\pm$ 5.3	65 $\pm$ 3.7	41 $\pm$ 7.8	60 ( $n = 1$ )	—
Testis spermatid head count ( $\times 10^6$ )	197 $\pm$ 8.2	175 $\pm$ 17	164 $\pm$ 31	49 $\pm$ 49B	0.36 $\pm$ 0.36B
Serum LH (ng/ml)	0.475 $\pm$ 0.05	0.350 $\pm$ 0.05	0.508 $\pm$ 0.11	1.26 $\pm$ 0.5B	1.72 $\pm$ 0.23B
Serum FSH (ng/ml)	8.95 $\pm$ 0.4	8.52 $\pm$ 0.5	8.97 $\pm$ 0.6	12.29 $\pm$ 2.8B	14.76 $\pm$ 1.1B
Serum testosterone (ng/ml)	2.8 $\pm$ 0.6	2.44 $\pm$ 0.7	3.01 $\pm$ 0.9	1.04 $\pm$ 0.3	2.26 $\pm$ 0.6
Pituitary weight (mg)	10.7 $\pm$ 0.3	10.2 $\pm$ 0.4	11.0 $\pm$ 0.7	12.3 $\pm$ 0.7	11.3 $\pm$ 0.4
Number fertile/number mated	12/12	7/8	3/8	—	0/8
Number mating (copulatory plugs found)	12/12	8/8	8/8	—	8/8
Age at preputial separation in days (puberty)	39.5 $\pm$ 0.9	42.6 $\pm$ 1.0A	43.4 $\pm$ 1.0B	—	44.4 $\pm$ 0.5B
Body weight at puberty (g)	195 $\pm$ 6.8	213 $\pm$ 9.0	209 $\pm$ 8.4	—	212 $\pm$ 4.7

DBP/kg/day did not reduce fertility in the P0 generation herein, fecundity is reduced with *in utero* and lactational exposure to 250 mg DBP/kg/day. In addition, both male and female offspring from this dose group display low incidences of reproductive and nonreproductive malformations (Gray *et al.*, 1999).

There are several reasons why such dramatic effects on female fertility may not have been reported earlier. First, the effects on pregnancy are not induced with shorter term *in utero* exposure to DBP at 500 mg/kg/day. Females exposed to DBP at 500 mg/kg/day in developmental toxicity and transgenerational studies have normal litter sizes. In addition, in contrast to the robust reductions in testis size and abnormal histology, reduced sex accessory gland size and sperm counts in pubertal males treated with DBP at 500 or 1000 mg/kg/day, treated P0 females only display gross morphological reproductive alterations and reductions in uterine weights during midpregnancy. Even though ovarian progesterone production on GD 13 was significantly compromised, ovarian weight is not altered, the onset of female puberty (ages at vaginal opening and first estrus) was unaffected, and estrous cycles were not disrupted.

We suspect that the effects of PEs on the female rat reproductive function have gone unnoticed in standard multi-generational tests because treated females are usually mated to similarly treated males, and the observed PE-induced infertility is assumed to be primarily male mediated because the effects of DBP on the male reproductive system are so obvious and those in the female rat are not. Hence, female-mediated PE-induced infertility will only be apparent if treated females are mated to untreated males. In this regard, several PEs have been shown to induce female-mediated infertility in the mouse in National Toxicology Program (NTP) studies using the Reproductive Assessment by Continuous Breeding Protocol with crossover mating (Heindel *et al.*, 1989; Lamb *et al.*, 1987). In another NTP study using the same protocol with male and female SD rats, however, Wine *et al.* (1997) did not observe any effect of DBP on fertility, male or female mediated. In addition, there were no effects on testis or sex accessory tissue weights or histology in the P0 generation. The lack of effect in the NTP study in either sex is in stark contrast to the results seen in our protocol, not only for the female but also for the male rat. Although there are several differences between the NTP rat study and our own, we suspect that the duration of treatment prior to mating may have contributed to differences between these two studies with rats. Our dosing begins prior to puberty, a period of life when the male is very sensitive to phthalates, whereas the NTP protocol initiated dosing when the animals were young adults, well beyond the pubertal stage of life (Wine *et al.*, 1997), and our animals were dosed for at least 2 months before being mated, whereas the NTP protocol has a much shorter dosing period prior to the first mating.

Taken together, the data on the effects of DBP on fertility, serum progesterone and *ex vivo* ovarian hormone production, and the presence of hemorrhagic corpora lutea suggest that DBP may be altering pregnancy by inhibiting corpora lutea

function and progesterone synthesis. One could speculate that at 500 and 1000 mg DBP/kg/day, pregnant female rats are not synthesizing sufficient ovarian progesterone levels during midpregnancy to sustain the pregnancy through this critical period. The data of Ema *et al.* (2000) are consistent with this hypothesis. They found that DBP was embryo lethal and interfered with uterine decidualization at 750, 1000, 1250, or 1500 mg/kg given on days 0–8 of pregnancy. Many abortifacients induce pregnancy loss by reducing progesterone levels in the rat. Generally, reducing midpregnancy progesterone levels by half or more is sufficient to terminate pregnancy (Carnathan *et al.*, 1987). For example, treating pregnant rats with a prostaglandin F2alpha analogue ICI 81008, which reduced serum progesterone levels during pregnancy, induced full litter loss in 40% of 300 dams, while 25% had reduced litter sizes and only 35% had normal litters (Warnock and Csapo, 1975). All treated rats with full litter loss had a drastic reduction in midpregnancy plasma progesterone levels, whereas treated animals with only a partly resorbed litter had only a moderate reduction in midpregnancy progesterone levels. On the other hand, it is also possible that midpregnancy fetal mortality results in a decline of fetal-placental hormonal support of ovarian function and progesterone production since the current study shows an association between reduced progesterone levels and fertility but does not establish cause and effect.

It is not surprising that the onset of puberty and estrous cyclicity were not affected in the current study since, unlike the human menstrual cycle, the 4- to 5-day estrous cycle of the rat does not have a functional luteal phase. Only after mating is the corpora lutea function maintained in the rat. For the first 8 to 13 days of pregnancy the corpora lutea are “rescued” by copulation-induced surges of pituitary prolactin and LH. Subsequently, the midpregnancy hypertrophic development of the corpus luteum is dependent on the secretion of hormones by the fetoplacental unit to induce rapid growth and differentiation of the corpora lutea, resulting in a doubling in serum progesterone.

The hypothesis that DBP is altering ovarian steroidogenesis at 250–1000 mg/kg/day is consistent with the proposed mode of action for the effects of DBP and other phthalate diesters on the fetal male reproductive tract. In the developing male rat, phthalates induce “antiandrogenic” effects by reducing testicular testosterone production and tissue levels of testosterone (Mylchreest and Foster, 2000; Mylchreest *et al.*, 1999, 2002; Parks *et al.*, 2000; Wilson *et al.*, 2004). It is possible that the effects of DBP in the corpora lutea of pregnancy also involve disruption of the androgen or *insl3* signaling pathways since these hormones also may contribute to the maintenance of function of the corpus luteum in pregnant rats (Goyeneche *et al.*, 2002; Ivell and Bathgate, 2002).

In summary, exposing LE female rats by gavage to 500 or 1000 mg DBP/kg/day chronically from weaning induces infertility that appears mediated via reductions in ovarian progesterone synthesis and serum progesterone measured at

midpregnancy. The reduction in fecundity of female rats dosed with 500 mg/kg/day is equal to that seen in similarly treated male rats mated to untreated females. The fact that such dramatic effects have not been detected previously for any phthalate, even though they are high production volume chemicals that have been used for decades, can be attributed to several factors. These factors include a focus in the field on the obvious effects of phthalates on the testis of the male rat, a lack of comprehensive multigenerational studies on the phthalates, especially those that include crossover mating of treated females to untreated males, and the fact that assessments of alterations of female rat reproductive physiology are technically more difficult than those used commonly to assess the reproductive system and function in male rat. Future studies on the effects of the phthalates on female rat fertility should examine ovarian expression of the steroidogenic genes as well as *insl3* mRNA levels.

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