Abnormalities of Sexual Development in Male Rats with *in Utero* and Lactational Exposure to the Antiandrogenic Plasticizer Di(2-ethylhexyl) Phthalate

Robert W. Moore,[1,2] Thomas A. Rudy,[1] Tien-Min Lin,[1] Kinarm Ko,[3] and Richard E. Peterson[1,2,3]

[1]School of Pharmacy, [2]Environmental Toxicology Center, and [3]Endocrinology-Reproductive Physiology Program, University of Wisconsin, Madison, Wisconsin, USA

Several members of the phthalate ester family have antiandrogenic properties, yet little is known about how exposure to these ubiquitous environmental contaminants early in development may affect sexual development. We conducted experiments to determine effects of *in utero* and lactational exposure to the most prevalent phthalate ester, di(2-ethylhexyl) phthalate (DEHP), on male reproductive system development and sexual behavior. Sprague-Dawley rats were dosed with corn oil or DEHP (0, 375, 750, or 1,500 mg/kg/day, *per os*) from gestation day 3 through postnatal day (PND) 21. Dose-related effects on male offspring included reduced anogenital distance, areola and nipple retention, undescended testes, and permanently incomplete preputial separation. Testis, epididymis, glans penis, ventral prostate, dorsolateral prostate, anterior prostate, and seminal vesicle weights were reduced at PND 21, 63, and/or 105–112. Additional dose-related effects included a high incidence of anterior prostate agenesis, a lower incidence of partial or complete ventral prostate agenesis, occasional dorsolateral prostate and seminal vesicle agenesis, reduced sperm counts, and testicular, epididymal, and penile malformations. Many DEHP-exposed males were sexually inactive in the presence of receptive control females, but sexual inactivity did not correlate with abnormal male reproductive organs. These results suggest that *in utero* and lactational DEHP exposure also inhibited sexually dimorphic central nervous system development. No major abnormalities were found in any of eight control litters, but DEHP caused severe male reproductive system toxicity in five of eight litters at 375 mg/kg/day, seven of eight litters at 750 mg/kg/day, and five of five litters at 1,500 mg/kg/day. These results demonstrate that the male reproductive system is far more sensitive to DEHP early in development than when animals are exposed as juveniles or adults. The effects of DEHP on male reproductive organs and sexual behaviors and the lack of significant effects on time to vaginal opening and first estrus in their littermates demonstrate that DEHP (and/or its metabolites) affects development of the male reproductive system primarily by acting as an antiandrogen. The pattern of effects of *in utero* and lactational DEHP exposure differed from patterns caused by other phthalate esters, and the preponderance of anterior prostate agenesis appears to be unique among all chemicals. These results suggest that DEHP acts partly by mechanisms distinct from those of other antiandrogens.
Key words: antiandrogens, di(2-ethylhexyl) phthalate, *in utero* exposure, lactational exposure, male reproductive system development, masculine sexual behaviors, reproductive organ agenesis. *Environ Health Perspect* 109:229–237 (2001). [Online 28 February 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p229-237moore/abstract.html

The development of the male reproductive system is androgen-dependent and is therefore vulnerable to antiandrogens. Among the chemicals humans are routinely exposed to are phthalate esters, several of which have antiandrogenic properties. Phthalate esters are used most commonly as plasticizers. They constitute 10–60% by weight of many plastics because they impart flexibility, transparency, and other desirable physical properties. Because phthalate esters are not covalently bound to the polymers with which they are mixed, they can leach into the foods, beverages, or other materials contained by these plastics. Consequently, because plastics are so commonplace, phthalate esters are ubiquitous in foods and the environment (*1*,*2*).

Many phthalate esters have long been known to be reproductive toxicants when animals are dosed as juveniles or adults, and their teratogenicity is well established (*1*,*3*), yet little has been published on the effects of *in utero* and lactational (or continuous multigenerational) exposure to any phthalate ester on postnatal development of the male or female reproductive systems or sexual differentiation of the central nervous system (CNS).

Of the approximately 20 phthalate esters in common use, di(2-ethylhexyl) phthalate (DEHP) constitutes approximately half the total; 1–4 million tons are produced per year (*1*,*2*). DEHP is used in numerous consumer products, especially those made of flexible polyvinyl chloride. The use of DEHP in teething rings, pacifiers, and toys for young children has largely been discontinued, but DEHP continues to be used in clothing, toys, food containers, and a variety of building, household, and automotive products (*3*,*4*). Typical human exposure is estimated to be 4–30 µg DEHP/kg/day, but some individuals have substantially greater exposure resulting from DEHP-plasticized medical devices such as blood bags, hemodialysis tubing and membranes, autophoresis equipment, and nasogastric feeding tubes (*5*). The average long-term dialysis patient is reported to receive approximately 12 g of DEHP over the course of a year (*6*).

The impetus for our investigations of effects of DEHP was provided by a report that *in utero* and lactational di(*n*-butyl) phthalate exposure disrupts male reproductive system development by what appears to be an antiandrogenic mechanism (*7*). Because DEHP was already known to have antiandrogenic properties, we hypothesized that *in utero* and lactational DEHP exposure would cause similar responses. Effects of DEHP on juvenile or adult rodents include reductions in testis (*8–11*) and epididymis (*12*) weights, severe reductions in sperm production (*12*,*13*), and various pathological effects on the testis (*8*,*10*,*11*,*14–17*). Accessory sex organs are highly androgen-dependent, and reductions in seminal vesicle (*10*,*11*,*18*) and ventral prostate (*10*,*11*,*18*) weights have commonly been observed in DEHP-treated rats. Reductions in serum testosterone concentrations have also been seen (*9*,*10*). In all these studies, males were not exposed until after weaning, when the male reproductive system is far less vulnerable to many toxicants than before weaning, and doses were typically 1,000–2,000 mg DEHP/kg/day. In other studies, *in utero* DEHP exposure (alone) and lactational DEHP exposure (alone) each reduced testis weight and epididymal sperm counts in rats (*19–21*).

The primary information gap when we began this research was that there was not a single publication on the effects of *in utero*

Address correspondence to R.W. Moore, School of Pharmacy, University of Wisconsin, 425 N. Charter Street, Madison, WI 53706 USA. Telephone: (608) 265-2531. Fax: (608) 265-3316. E-mail: rwmoore@pharmacy.wisc.edu

We thank O. Li for sperm analyses.

This research was supported by NIH grants ES 06806 and ES 01332 and by the University of Wisconsin EHS Center for Developmental and Molecular Toxicology.

This article is contribution 331 from the Environmental Toxicology Center, University of Wisconsin, Madison.

Received 15 August 2000; accepted 13 October 2000.

and lactational or continuous multigenerational DEHP exposure on any aspect of postnatal development of the male (or female) reproductive system or sexual differentiation of the CNS in any species, except for a report that fertility was not impaired in male or female rats (*22*). We therefore conducted a dose–response, time course experiment to test the hypothesis that male reproductive system development and sexually dimorphic CNS development in rats are vulnerable to *in utero* and lactational DEHP exposure. While this work was in progress, Arcadi et al. (*23*) reported that low-level *in utero* and lactational DEHP exposure reduces testis weight and alters testicular morphology in rats. And after completing the in-life portion of this research, Gray et al. (*24*) published results of a study in which effects of a single daily maternal dose of DEHP on male reproductive system development in rats were determined.

Methods

Pregnant Sprague-Dawley rats were received from Harlan Sprague Dawley (Madison, WI) on gestation day (GD) 1, the day after they were found to be sperm-positive following overnight mating. Rats were housed individually in suspended plastic cages with heat-treated, chipped aspen bedding and had *ad libitum* access to feed (5012 Rat Diet; PMI Nutrition International, Brentwood, MO) and water. Rooms were kept at 20–21°C; humidity was typically 35–50%; and lights were on from 0600 to 1800 hr. We randomly assigned rats to treatment groups (and randomly reassigned them if necessary) to attain comparable mean body weights in each group. Each dam was dosed orally with tocopherol-stripped corn oil or DEHP (375, 750, 1,500, or 3,000 mg/kg/day) from GD 3 through postnatal day (PND) 21 based on its body weight that day. Corn oil was obtained from ICN Biomedicals (Aurora, OH), and DEHP (99% pure) was purchased from Aldrich (Milwaukee, WI). Rats given 0, 375, or 750 mg/kg/day received 1.53 mL/kg/day corn oil ± DEHP, the volume given to rats dosed with pure DEHP at 1,500 mg/kg/day. Doses were chosen on the basis of those used previously to examine effects of DEHP on the male reproductive system of rats treated as juveniles or adults. All animal procedures were conducted under protocols approved by the University of Wisconsin Research Animal Resources Center.

We conducted the experiment in two blocks, both of which included all treatment groups. Because of excessive toxicity, however, the 3,000 mg DEHP/kg/day dose was not used in the second block. Litters totaled eight each at 0, 375, and 750 mg/kg/day and five at 1,500 mg/kg/day.

To determine the number of pups born to each dam as accurately as possible, we examined cages at frequent intervals during parturition. Dead pups were removed when found and sexed when possible. Pups were toe-clipped so records could be kept on each individual. Litters were normalized to 10 pups each 1–2 days after birth and maintained at 10 by replacing pups that died. When pups had to be added to a litter, they were taken from litters exposed to the same or lower dose of DEHP. Litter independence was maintained because data from pups added to litters are not reported.

We weighed pups on PND 1 (the day after birth), PND 7, and weekly thereafter. We measured anogenital distance using Vernier calipers on PND 1, and we counted areolas (with and without nipple buds) daily beginning on PND 11 and continuing until hair obscured them. These measurements were made on all pups. On PND 21, we removed pups from their mothers and housed them by litter and sex. Dams were necropsied on PND 21–22 and the number of implantation sites was recorded.

Time to vaginal opening was determined by daily inspection of all females starting on PND 24, and time to first estrus was determined by vaginal lavage of two randomly selected females per litter beginning the day of vaginal opening. Time to preputial separation was determined by daily inspection of all males beginning on PND 38. We continued observations until preputial separation was complete or until PND 63, whichever came first, with a final observation at necropsy.

One male per litter was necropsied (when available) on PND 21, 63, and 105; remaining males were necropsied on PND 112. Rats were killed by CO_2 overdose. We recorded testicular position after opening the abdominal cavity. The glans penis, one epididymis, and one testis from rats 63 days of age and older were fixed in neutral-buffered formalin; results will be presented elsewhere. The other testis and epididymis were frozen on dry ice for analysis of daily sperm production and cauda epididymal sperm numbers (*25*). When testis sizes differed noticeably, the smaller testis and corresponding epididymis were fixed and the larger one was frozen. Accessory sex organs were removed on PND 21 under a dissecting microscope by personnel who routinely dissect these organs from neonatal mice. We weighed accessory sex organs without expressing fluid. We counted nipples after shaving the chest and abdomen.

We examined masculine sexual behaviors in males scheduled for necropsy on PND 105. These animals were placed on a reversed light/dark cycle at least 14 days before the test. Each male was allowed to gain sexual experience by spending 30 min with a sexually receptive female followed by 30 min with another, 5–8 days before the test. The females were ovariectomized control adult Sprague-Dawley rats in which sexual receptivity had been induced by subcutaneous injection of 120 µg/kg estradiol benzoate (Sigma Chemical Co., St. Louis, MO) and 5 mg/kg progesterone (Sigma), 48 and 6 hr, respectively, before testing. Steroids were dissolved in corn oil, and these females were also on a reversed light/dark cycle. We conducted tests at least 2 hr into the dark cycle under dim red light on about PND 77. Males were allowed 5 min to habituate to a 60 × 30 × 30 cm glass observation cage with wood shavings before the female was introduced. We recorded or calculated the following male behaviors: number of mounts, number of intromissions, latency to mount, latency to intromission, latency to ejaculation, postejaculatory interval, copulatory rate, and copulatory efficiency (*26,27*). Males were observed for one complete ejaculatory series and the subsequent postejaculatory interval, although observations were discontinued if ejaculation did not occur within 45 min. Females displayed a high degree of sexual receptivity throughout the observations or were replaced by ones that did. All sessions were videotaped for later analysis. Tests were conducted by a person who did not know which treatment group any of the males were from.

We conducted statistical analysis with the litter as the experimental unit. We conducted parametric analyses on untransformed data and on log, square-root, and inverse transforms as well as on ranked data. For data that passed Levene's test for homogeneity of variance and which appeared to be normally distributed, we performed analysis of variance (ANOVA). If a significant effect was found, we used the least significant difference test to determine which group(s) differed from control. We also analyzed data by the Kruskal-Wallis nonparametric ANOVA and by the median test. We used the distribution-free multiple comparison test as the post hoc test for nonparametric analyses. We analyzed body weight data by repeated-measures ANOVA. Incidence data were analyzed by the row × column chi square test, followed by Fisher's Exact test. Significance was set at ($p < 0.05$). Results are presented as means ± SE.

Results

Dose. The first block of the experiment included six sperm-positive females given 3,000 mg DEHP/kg/day. Two had no implantation sites, two were pregnant but miscarried, one gave birth to nine pups that died within hours, and one gave birth to seven pups. Only two of these pups lived

more than a day, and each had severe reproductive system abnormalities (both were males). Consequently, we discontinued the 3,000 mg DEHP/kg/day dose. All results described below are from animals dosed with or exposed to 0, 375, 750, or 1,500 mg DEHP/kg/day.

Effects on dams and litter size. DEHP had no statistically significant effect during pregnancy on the body weight of rats found to have delivered pups (data not shown). However, maternal weight gain subsequent to the start of dosing on GD 3 was significantly reduced on GD 16–20 by the middle and high doses. Weight gain between GD 3 and GD 20 is shown in Table 1. In contrast, DEHP had no significant effect on maternal body weight (data not shown) or weight gain (Table 1) between birth and weaning.

Additional reproductive parameters are shown in Table 1. The mean number of implantation sites per dam appeared to be slightly decreased by DEHP, but this effect was not statistically significant. All rats with implantation sites gave birth to live pups, except one given the middle dose and two given the high dose. The number of pups born per dam was significantly reduced only at the high dose. (One dam given the high dose had no implantation sites but is not included in these calculations because she may not have been pregnant when dosing began.) DEHP appeared to cause dose-related increases in postnatal mortality at the middle and high doses, although effects were not statistically significant when calculated either as postnatal deaths per litter (data not shown) or as percent survival after birth. Nearly all pup deaths occurred within 2 days of birth. The net result of the prenatal and postnatal losses described above is that the number of pups per dam that survived until weaning was significantly reduced at the middle and high doses.

Effects on pup development. In utero and lactational DEHP exposure caused dose-related reductions in body weight. In males exposed to 750 mg/kg/day, the decrease was statistically significant from PND 63 through PND 105 and averaged 6% throughout this time, whereas in males exposed to the high dose the decrease averaged 12% and was statistically significant throughout development. Body weights of female offspring tended to be reduced (by an average of 8% at the high dose), but differences were not statistically significant.

Effects of *in utero* and lactational DEHP exposure on indices of sexual development are presented in Figure 1. Anogenital distance is androgen-dependent and was 54% shorter in control females on PND 1 than in control males (Figure 1A). DEHP exposure caused a dose-related reduction in anogenital distance in males that was statistically significant at the middle and high doses. When normalized to the cube root of body weight [to account for differences in body size (*28*)], anogenital distance in males was still significantly reduced at the middle and high doses (data not shown). In contrast to effects on males, *in utero* and lactational DEHP exposure had no significant effect on anogenital distance in females regardless of whether results were expressed as absolute distance (Figure 1A) or divided by the cube root of body weight (data not shown).

Nipples and/or areolas were present in many DEHP-exposed males, whereas control males had none and females had 12. On PND 14, litter averages for the number of areolas per male were significantly increased at the middle and high doses and averaged 9.7 at the high dose (Figure 1B). The incidence of litters in which one or more males had areolas on PND 14 was statistically significant at all 3 doses (Figure 1C). The number of detectable nipples in adulthood (data not shown) was lower than the number of areolas on PND 14; nevertheless, nipples were found in some males from the low-dose group, most males from the middle dose group, and all males from the high-dose group when necropsies were conducted on

Table 1. Reproductive parameters in rats dosed with DEHP from GD 3 through PND 21.

Parameter	Maternal DEHP dose (mg/kg/day) 0	375	750	1,500
Prenatal weight gain (g)	128 ± 4 (8)	123 ± 7 (8)	99 ± 10 (8)*	87 ± 13 (6)*
Postnatal weight gain (g)	17 ± 3 (8)	24 ± 4 (8)	20 ± 3 (8)	15 ± 4 (5)
Implantation sites per dam	13.5 ± 0.9 (8)	12.1 ± 0.6 (8)	12.2 ± 0.7 (9)	10.5 ± 1.1 (8)
Incidence of parturition	100% (8)	100% (8)	89% (9)	75% (8)
Pups born per dam	12.5 ± 1.0 (8)	11.4 ± 0.8 (8)	9.6 ± 1.3 (8)	7.7 ± 1.4 (6)*
Postnatal pup survival	87 ± 5% (8)	86 ± 4% (8)	74 ± 7% (8)	59 ± 15% (6)
Pups per dam that survived	10.9 ± 1.0 (8)	9.8 ± 0.8 (8)	7.5 ± 1.3 (8)*	5.0 ± 1.3 (6)*

Numbers shown are means ± SE or incidences per dam or litter in each treatment group. The number of replicates (dams or litters) is shown in parentheses. Prenatal weight gain is from GD 3 to GD 20 for dams that delivered one or more living pups, whereas postnatal weight gain is from PND 1 to PND 21 for dams that maintained litters throughout this time. Pups born per dam are for dams known to have given birth to one or more living pups.
*Significantly different from control at $p < 0.05$.

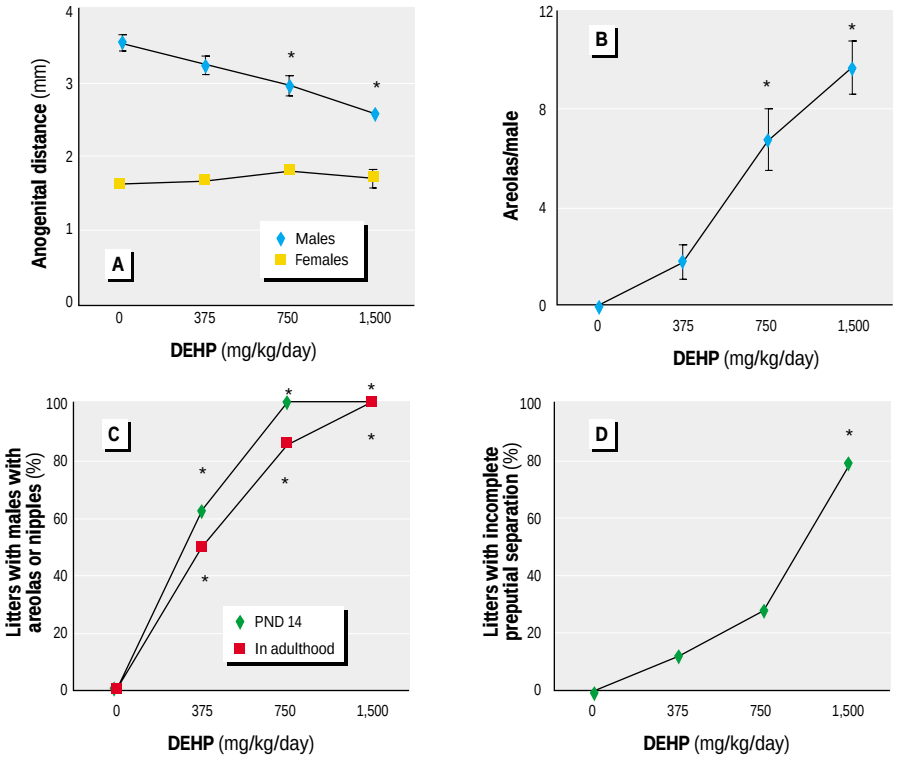

Figure 1. Effects of *in utero* and lactational DEHP exposure on sexual development. Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. (*A*) Anogenital distance on PND 1. (*B*) Areolas per male on PND 14. (*C*) Incidence of litters in which males had areolas on PND 14 or nipples in adulthood. (*D*) Incidence of litters with incomplete preputial separation. Numbers shown are means ± SE of litter averages or are incidences among litters in each treatment group. The number of replicates (litters) was 8 at 0 and 375 mg/kg/day, 7–8 at 750 mg/kg/day, and 5 at 1,500 mg/kg/day.
*Significantly different from control at $p < 0.05$.

PND 63, 105, and 112. Nipple retention per litter in adulthood (Figure 1C) was statistically significant at all DEHP doses tested.

Preputial separation, an androgen-dependent index of pubertal development, was complete in all 34 control males (from 8 litters) at an average of 43 days of age and 196 g body weight. Two of 26 males exposed to the low dose, 3 of 21 males at the middle dose, and 5 of 7 males at the high dose never completed preputial separation. In these rats, the prepuce remained attached to the dorsal surface of the glans penis. In most cases, the penis was otherwise normal in appearance. The incidence of incomplete preputial separation per litter (Figure 1D) was statistically significant only at the high dose but is considered biologically significant at all three doses because this phenomenon is rare in control rats. Among males that completed preputial separation, *in utero* and lactational DEHP exposure slightly but nonsignificantly increased time to separation (data not shown) and had little if any effect on body weight at separation (data not shown).

Effects on male sex organs. After weaning, testes from control rats could readily be detected in the scrotum by palpation, but one or both testes could not be detected in many DEHP-exposed rats. Because some of these testes were very small, assessment of testis descent in live animals was considered less reliable than observations made after rats were killed by CO_2 overdose. Many testes from DEHP-exposed rats were in the abdominal cavity at necropsy, often on the contralateral side, whereas all testes from control rats were in the scrotum (Figure 2). Undescended testes were observed at all three doses on PND 21, although the number per rat was significantly increased only at the highest dose (Figure 2A). However, the incidence of litters with an undescended testis was significantly increased at the 2 highest doses (Figure 2B). In adulthood, the average number of undescended testes per rat was far smaller at each dose than on PND 21 (Figure 2A). Nevertheless, litters in which one or more males had an undescended testis in adulthood were found at each DEHP dose (Figure 2B). These results indicate that *in utero* and lactational DEHP exposure both delays and permanently prevents testis descent. Undescended testes tended to be far smaller than descended testes, but small testes were found in both the descended and undescended positions. On PND 21, most undescended testes were similar in size to their descended partners, but in adulthood only one DEHP-exposed rat (at 750 mg/kg/day) had an undescended testis that was normal size.

Effects of *in utero* and lactational DEHP exposure on testis, epididymis, and glans penis weights are shown in Table 2. Testis weights were reduced to roughly 50% of control values at the high dose at all times examined. On PND 21 the reduction was significant at the 2 highest doses, and testis/body weight ratios were significantly reduced at all 3. On PND 63 both absolute and relative testis weights were significantly reduced at the 2 highest doses. Testis weight data at PND 105 could not be analyzed by parametric statistical procedures because of heterogeneity of variance; reductions in absolute and relative testis weights at this time were not statistically significant.

Epididymis weights were significantly reduced by *in utero* and lactational DEHP exposure at the middle and high doses on PND 63, as were epididymis/body weight ratios. Similar reductions in epididymis weight were seen at PND 105, although the effect was statistically significant only at the middle dose. Visually obvious epididymal abnormalities were seen in one rat at the low dose, five rats (from two litters) at the middle dose, and two rats (from two litters) at the high dose. The most common finding was agenesis of the caput epididymis, though partial or complete absence of the corpus epididymis and a case of epididymal edema were also observed.

Dose-related reductions in glans penis weight were statistically significant at the middle and high doses on PND 21, 63, and 105; however, relative glans penis weight was

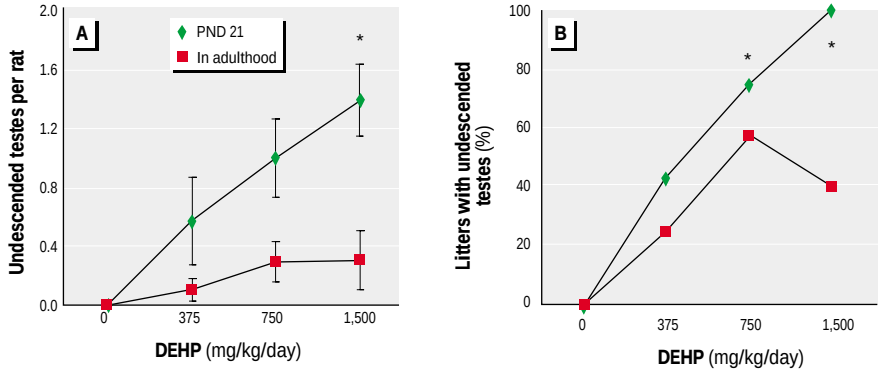

Figure 2. Effects of *in utero* and lactational DEHP exposure on testis descent. Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. (*A*) Number of undescended testes per rat on PND 21 and in adulthood. (*B*) Incidence of litters with undescended testes on PND 21 and in adulthood. Numbers shown are means ± SE or are incidences among litters in each treatment group. The number of replicates (litters) was 8 at 0 mg/kg/day, 7–8 at 375 and 750 mg/kg/day, and 5 at 1,500 mg/kg/day. Results from PND 21 are from one rat per litter necropsied at this time, whereas results from adulthood are litter averages from all rats necropsied as adults.
*Significantly different from control at $p < 0.05$.

Table 2. Effects of *in utero* and lactational DEHP exposure on sex organ weights.

Organ/age (days)	Maternal DEHP dose (mg/kg/day) 0	375	750	1,500
Testes weight (mg)				
21	247 ± 13 (8)[a] (0.509 ± 0.017)[b]	222 ± 17 (7) (0.451 ± 0.018)*	192 ± 15 (8)* (0.435 ± 0.017)*	153 ± 9 (5)* (0.398 ± 0.028)*
63	3,500 ± 65 (8) (1.082 ± 0.020)	3,596 ± 80 (8) (1.095 ± 0.020)	2,571 ± 255 (7)* (0.821 ± 0.081)*	2,048 ± 107 (5)* (0.696 ± 0.029)*
105	3,718 ± 85 (8) (0.886 ± 0.018)	3,689 ± 136 (7) (0.908 ± 0.040)	2,624 ± 467 (7) (0.645 ± 0.109)	1,467 ± 63 (2) (0.390 ± 0.013)
Epididymides weight (mg)				
21	ND	ND	ND	ND
63	624 ± 18 (8) (0.193 ± 0.005)	613 ± 25 (8) (0.186 ± 0.006)	412 ± 62 (7)* (0.132 ± 0.020)*	429 ± 42 (5)* (0.148 ± 0.018)*
105	1,053 ± 25 (8) (0.251 ± 0.005)	957 ± 52 (7) (0.236 ± 0.014)	640 ± 136 (7)* (0.158 ± 0.032)*	581 ± 50 (2) (0.155 ± 0.015%)*
Glans penis weight (mg)				
21	27.7 ± 1.2 (8) (0.0575 ± 0.0026)	24.8 ± 1.3 (7) (0.0510 ± 0.0022)	22.7 ± 1.1 (8)* (0.0534 ± 0.0049)	19.6 ± 0.4 (5)* (0.0513 ± 0.0037)
63	91.1 ± 1.7 (8) (0.0281 ± 0.0005)	88.2 ± 1.8 (8) (0.0269 ± 0.0004)	80.7 ± 2.3 (7)* (0.0259 ± 0.0011)	75.7 ± 2.7 (3)* (0.0269 ± 0.0009)
105	102.5 ± 1.5 (8) (0.0244 ± 0.0003)	99.1 ± 1.8 (7) (0.0245 ± 0.0010)	88.5 ± 3.7 (7)* (0.0220 ± 0.0010)*	81.1 ± 3.2 (2)* (0.0215 ± 0.0006)

ND, not determined. Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. One male per litter was necropsied at each designated time.
[a]Numbers shown are means ± SE, with the number of replicates (litters) in parentheses. [b]Values shown are organ weight:body weight ratio. *Significantly different from control at $p < 0.05$.

significantly reduced only on PND 105 and only by the middle dose. The penis appeared to be structurally abnormal in several DEHP-exposed rats, but definitive assessments could not be made at necropsy (other than incomplete preputial separation), and results of histological evaluation were inconclusive.

Daily sperm production appeared to be inhibited in a dose-related manner on PND 63 by *in utero* and lactational DEHP exposure, both per testis (Table 3) and per gram testis (data not shown), but neither effect was statistically significant. The number of cauda epididymal sperm was significantly reduced at the 2 highest doses (Table 3). These decreases in testicular spermatid and epididymal sperm numbers represent minimum effects of DEHP, because when testis and epididymis sizes differed, the smaller ones were placed in fixative and only the larger ones were frozen for spermatid and sperm counts.

Effects of *in utero* and lactational DEHP exposure on accessory sex organ weights are shown in Figure 3. Ventral prostate weights were significantly reduced by the middle and high doses of DEHP on PND 21 and by the middle dose on PND 105. These effects were also significant when ventral prostate weight was expressed relative to body weight. Ventral prostate weight was also decreased, though not significantly, on PND 63. Reductions in ventral prostate weight were greatest at PND 21, when a 60% decrease was seen at the high dose.

On PND 21, dorsolateral prostate weights were significantly reduced by 80% at the high dose and to a lesser extent at the middle dose. The magnitude of these effects decreased with age, but significant reductions were still seen at the high dose on PND 63 and at the two highest doses on PND 105. The reduction in dorsolateral prostate weight on PND 63 was not statistically significant when calculated relative to body weight, but dorsolateral prostate/body weight ratios were significantly reduced at the middle and high doses at the other two times.

Weights of the anterior prostate were greatly reduced at all times examined: On PND 63 a 75% decrease was observed at the high dose. The reductions were statistically significant at the two highest doses on PND 21 and at all three doses on PND 63 and PND 105. Identical statistical results were obtained when relative anterior prostate weights were calculated.

Dose-related reductions in seminal vesicle weights were observed on PND 21, when weights at the high dose were reduced by 62%. Reductions at the middle and high doses were statistically significant at this time, as were seminal vesicle/body weight ratios at all three doses. Apparent decreases in seminal vesicle weight on PND 63 and PND 105 were not statistically significant.

In utero and lactational DEHP exposure caused agenesis of one or more accessory sex organs in many litters (Table 4). Ventral prostate agenesis (one lobe or both) was dose related and was seen at all 3 doses, whereas dorsolateral prostate agenesis was observed in only one low-dose and one high-dose litter. The most predominant effect of DEHP was anterior prostate agenesis: One or both lobes

Table 3. Effects of *in utero* and lactational DEHP exposure on PND 63 sperm counts.

	Maternal DEHP dose (mg/kg/day)			
	0	375	750	1,500
Daily sperm production (10^6 per testis)	34.2 ± 1.5 (8)	36.5 ± 1.2 (8)	25.6 ± 4.5 (7)	24.4 ± 5.4 (5)
Epididymal sperm number (10^6 per cauda)	55.5 ± 3.7 (8)	46.5 ± 5.1 (7)	29.8 ± 8.7* (7)	19.3 ± 7.5* (5)

Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. One male per litter was necropsied on PND 63. Numbers shown are means ± SE, with the number of replicates (litters) in parentheses.
*Significantly different from control at $p < 0.05$.

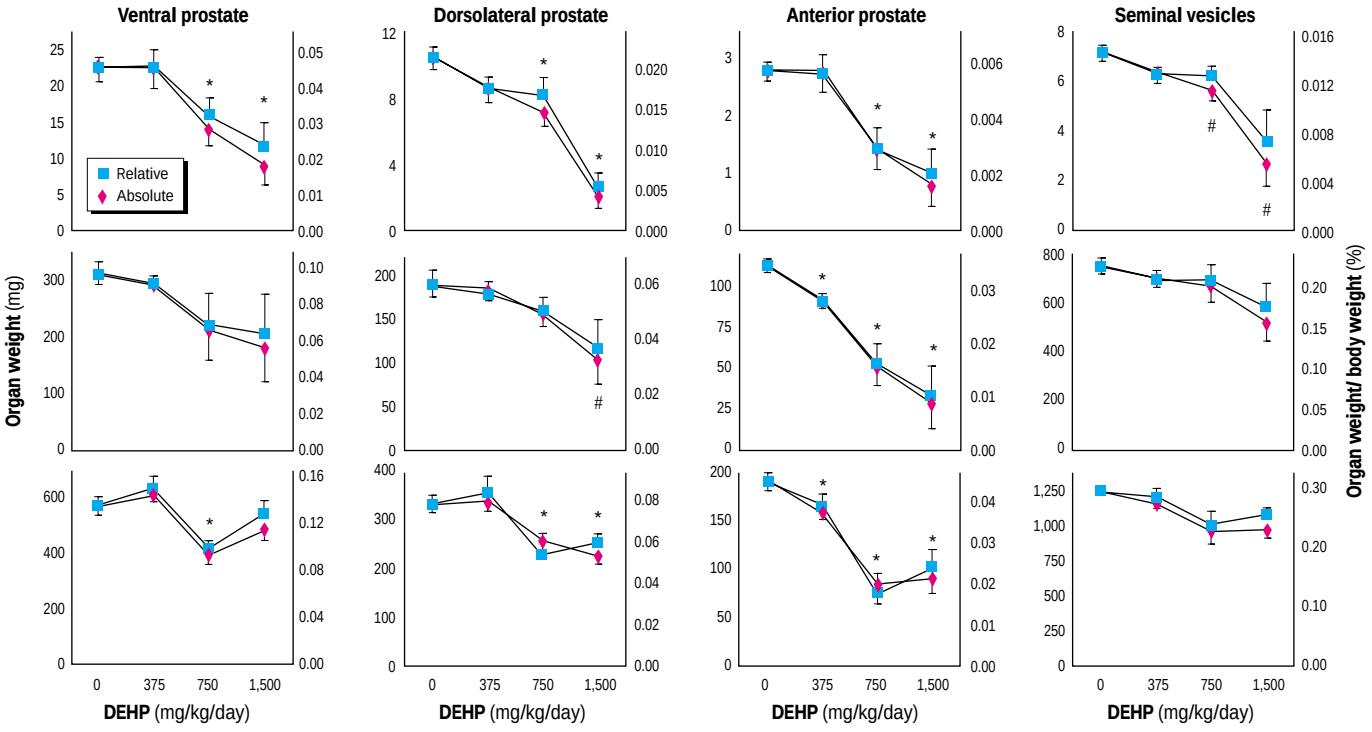

Figure 3. Effects of *in utero* and lactational DEHP exposure on absolute and relative accessory sex organ weights. Top row, PND 21; middle row, PND 63; bottom row, PND 105. Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. One male per litter was necropsied at each designated time. Numbers shown are means ± SE. The number of replicates (litters) was generally 7–8 at 0, 375, and 750 mg/kg/day, whereas at 1,500 mg/kg/day there were 5 replicates on PND 21 and 63 and 2 on PND 105.
*Both absolute and relative organ weights were significantly different from control at $p < 0.05$. #Absolute organ weight was significantly different from control at $p < 0.05$.

were missing from one of eight low-dose litters, five of eight middle dose litters, and four of five high-dose litters. In contrast, seminal vesicle agenesis was seen only at the high dose (two litters). No such abnormalities were seen in any male from any of the eight control litters. Statistical analysis revealed that the only significant effect was anterior prostate agenesis at the middle and high doses; however, the rarity of ventral, dorsolateral, and anterior prostate agenesis in control rats suggests that the absence of these organs at the lowest dose of DEHP was biologically significant. Table 4 also shows the incidence of accessory sex organ agenesis among all pups.

Effects on male sexual behaviors. We examined effects on masculine sexual behaviors by allowing one male per litter to mate with a receptive control female. Males were about 77 days of age when tested. Seven of eight control males displayed typical sexual behaviors and ejaculated within the 45-min observation period (ejaculatory latencies averaged 14 min). In contrast, three of seven low-dose males never mounted, intromitted, or ejaculated; all seven males at the middle dose mounted (although two had extraordinarily long mount latencies), but three never intromitted and four did not ejaculate; and neither rat at the high dose mounted, intromitted, or ejaculated. Due to the small number of animals tested, the only statistically significant effect when exposure groups were analyzed separately was a reduction in the incidence of mounting at the high dose. However, when results from the 2 highest DEHP exposure groups were combined, the reduction in the incidence of ejaculation was statistically significant, and when results from all 16 DEHP-exposed rats were combined, the *p*-value for this effect was 0.0507. No obvious differences appeared between the behaviors of DEHP-exposed rats that were sexually active and those of the sexually active control rats.

Effects on F_1 females. Several observations were made on female offspring besides those described above for body weight and anogenital distance. As shown in Table 5, time to vaginal opening appeared to be reduced (which suggests that DEHP is estrogenic), but time to first estrus appeared to be slightly increased (which suggests that it is not). Neither effect was statistically significant. Body weights at these times were not affected by DEHP, except for a significant reduction at vaginal opening caused by the high dose of DEHP.

Discussion

DEHP causes abnormal sexual development by acting primarily as an antiandrogen. Results of these experiments demonstrate that *in utero* and lactational DEHP exposure can profoundly alter male reproductive system development (including sexual behaviors) in rats. These findings confirm many of the observations made by Gray et al. (*24*) and extend others. Four effects seen in both laboratories (reductions in testis, epididymis, ventral prostate, and seminal vesicle weights) had already been reported in animals given DEHP as juveniles or adults (*8–12,18*), but others (ventral prostate, seminal vesicle, and caput epididymis agenesis; reductions in anogenital distance; areola and nipple retention; reductions in glans penis weight; and penile abnormalities) had not been reported previously. We also observed effects of DEHP not reported by Gray et al. (*24*) or others: dorsolateral and anterior prostate agenesis and weight reductions, undescended testes, permanently incomplete preputial separation, and demasculinized sexual behaviors. Several differences between our observations and those of Gray et al. (*24*) presumably stem from the fact that we examined additional end points, whereas others may stem from the longer dosing period we used. Gray et al. (*24*) observed high incidences of vaginal pouches, hemorrhagic testes, and hypospadias whereas we did not. The reason for these differences is not known.

Nearly every effect we observed is a classic sign of antiandrogenic activity; however, effects of antiandrogens on male reproductive system development are similar in many ways to effects of estrogens (*29*). If DEHP had affected development in males by acting primarily as an estrogen, time to vaginal opening and first estrus should have been reduced in their littermates (*30*). Neither was significantly affected. And most DEHP-exposed males had nipples, which is generally considered to be diagnostic for antiandrogens (*31*). Diethylstilbestrol can also cause nipple retention in males (*32*), but maternal doses orders of magnitude higher than those that shorten time to vaginal opening are needed to cause this effect (*33*). Although DEHP has been reported to be weakly estrogenic (*34*) and to be associated with premature breast development in humans (*35*), we conclude that the effects of DEHP described in this report are due primarily to one or more antiandrogenic mechanisms.

Effects of in utero *and lactational DEHP exposure on masculine sexual behaviors appear to be due to incomplete sexual differentiation of the CNS.* Nine of 16 DEHP-exposed males failed to ejaculate during sexual behavior testing, versus one of eight control males. Eight of these nine had no intromissions, and five failed to mount a single time. If failure to ejaculate had been caused by low circulating testosterone concentrations in adulthood, seminal vesicle weights would have been substantially smaller than normal. Yet seminal vesicles in DEHP-exposed rats that failed to ejaculate averaged 87 ± 3% of the control weight, whereas those in rats that ejaculated averaged 81 ± 9% of control. In addition, circulating testosterone concentrations only one-third of normal are sufficient to fully maintain masculine sexual behaviors in rats (*36*). Clearly,

Table 4. Incidence of sex organ agenesis in rats with *in utero* and lactational DEHP exposure.

	Maternal DEHP dose (mg/kg/day)			
Organ	0	375	750	1,500
Ventral prostate	0/8 (0/42)	1/8 (1/32)	2/8 (5/29)	2/5 (3/12)
Dorsolateral prostate	0/8 (0/42)	1/8 (1/32)	0/8 (0/29)	1/5 (2/12)
Anterior prostate	0/8 (0/42)	1/8 (1/32)	5/8* (9/29)	4/5* (6/12)
Seminal vesicles	0/8 (0/42)	0/8 (0/32)	0/8 (0/29)	2/5 (2/12)

Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. The first pair of numbers show the incidence per litter; the second, in parentheses, show the incidence among all pups.
*Significantly different from control at $p < 0.05$. (Statistical analysis was not performed on results from individual pups because the litter rather than the pup is the experimental unit.)

Table 5. Effects of *in utero* and lactational DEHP exposure on indices of puberty in female rats.

	Maternal DEHP dose (mg/kg/day)			
Index	0	375	750	1,500
Age at vaginal opening (days)	31.1 ± 0.9	29.7 ± 0.9	29.7 ± 1.2	27.2 ± 1.1
Body weight at vaginal opening (g)	94 ± 6	87 ± 6	86 ± 9	64 ± 9*
Age at first estrus (days)	33.5 ± 0.3	33.1 ± 0.4	35.8 ± 2.0	34.4 ± 0.7
Body weight at first estrus (g)	108 ± 4	105 ± 2	116 ± 11	98 ± 2

Dams were orally dosed with DEHP or vehicle from GD 3 through PND 21. Values are means ± SE of the litter means. Age and body weight at vaginal opening were determined for all females per litter, while age and body weight at first estrus were determined for 2 females per litter. There were eight litters with females in the control and low-dose groups, seven at the middle dose, and five at the high dose.
*Significantly different from control at $p < 0.05$.

the lack of ejaculatory behavior in more than half the DEHP-exposed rats cannot be attributed to inadequate circulating testosterone.

It is highly unlikely that undescended testes could account for the lack of ejaculatory behavior, inasmuch as testicular steroidogenesis is independent of testicular position. Furthermore, only two of the nine DEHP-exposed rats that failed to ejaculate had an undescended testis (one each), and one DEHP-exposed rat with an undescended testis had completely normal sexual behaviors.

Incomplete preputial separation could potentially prevent ejaculation but cannot account for failure of five of the 16 DEHP-exposed rats to mount a single time. Furthermore, six of the nine DEHP-exposed rats that did not ejaculate had full preputial separation, and one DEHP-exposed rat that ejaculated had incomplete preputial separation. The glans penis was abnormally small (< 92% of control) in six of nine DEHP-exposed rats that did not ejaculate but also in two of the seven that did (an organ was considered abnormally small if it weighed less than the control mean weight – 2 SDs from the control mean). The reduction in glans penis weight in DEHP-exposed rats that did not ejaculate averaged only 13%. These observations suggest that failure to ejaculate was not caused by smaller penis size. Moreover, a small penis cannot account for the failure of rats to mount. Finally, no DEHP-exposed male that failed to ejaculate had any detectable penile abnormality (other than incomplete preputial separation).

In short, we saw no evidence that abnormal sexual behaviors in DEHP-exposed rats were caused by effects on androgen concentrations in adulthood or by abnormal male reproductive organs. Instead, the most likely explanation is that *in utero* and lactational DEHP exposure causes incomplete sexual differentiation of the CNS. Further research is needed to confirm or reject this hypothesis.

Comparison with standard teratogenicity testing. Although reductions in anogenital distance, epididymal malformations, and accessory sex organ agenesis can be detected before birth, no such effects were reported in any of the 14 published studies on effects of DEHP on fetal morphology (*3*). Classic teratogenicity testing clearly plays an important role in the assessment of potential toxic responses, but studies such as those conducted in our laboratory or by Gray et al. (*24*) demonstrate that teratogenicity testing is not routinely being conducted in a way that permits detection of some major male reproductive system abnormalities.

Sensitivity of male rats to in utero *and lactational DEHP exposure.* Effects of *in utero* and lactational DEHP exposure on male reproductive system development were dose related, and the lowest observed adverse effect level was the lowest dose tested (375 mg/kg/day). Two effects were statistically significant at this dose: reductions in anterior prostate weight and permanent nipple retention. Effects that were not statistically significant within the constraints of this experiment ($n \leq 8$) but which are so unusual among control rats as to be considered biologically significant at 375 mg/kg/day are testis nondescent, permanently incomplete preputial separation, and accessory sex organ agenesis. Although an occasional control male rat is sexually inactive, the total lack of sexual activity by three of seven males suggests that 375 mg DEHP/kg/day can also demasculinize sexual behaviors.

Because no single abnormality was seen in all affected males, we evaluated sensitivity to DEHP in two additional ways. We tabulated the incidence of major male reproductive system defects per litter using data from all necropsies. We found no major abnormalities (defined in the legend to Figure 4) in any of eight control litters, but DEHP caused statistically significant incidences of major reproductive toxicity at each dose tested (Figure 4A). In several cases the same litter had males that appeared normal and others that were severely affected. The cause of this variability is unknown. Because this analysis gave the same weight to litters in which a single male had a single abnormality as it did to litters in which each male had multiple abnormalities, we also evaluated sensitivity to account for differences in the extent to which litters were abnormal. Criteria for this analysis, which were somewhat less stringent than those used above, are stated in the legend to Figure 4. Figure 4B shows that litters exposed to 0, 375, 750, and 1,500 mg DEHP/kg averaged 1, 18, 52, and 73% of the possible abnormalities, respectively, and that the effect of DEHP on the extent to which abnormalities were present was significant at each dose.

Nipple retention was the abnormality most frequently seen in adulthood, and the percentage of males with areolas on PND 14 was even higher. Yet most DEHP-exposed males that had no detectable nipples in adulthood had other abnormalities, as did most DEHP-exposed males without areolas at PND 14. These results demonstrate that multiple male reproductive system end points must be evaluated to detect all rats with abnormalities caused by *in utero* and lactational exposure to DEHP and, presumably, other antiandrogens.

Effects of DEHP resemble but are different from those of other phthalate esters. Although numerous phthalate esters are in wide use, information about their possible effects on sexual development is available for only a few. Single-dose exposure to di(2-methoxyethyl) phthalate (700 mg/kg) caused testicular atrophy and displacement in fetuses (*37*), but no other reproductive system abnormalities were noted. The only effect of continuous dietary exposure to 5.0% di(*n*-octyl) phthalate was a reduction in seminal vesicle weight (*38*), and continuous exposure to di(isononyl) phthalate had no effect on the male reproductive system at 500 mg/kg/day (*39*). Butyl benzyl phthalate has been studied only at doses far smaller than we used; effects

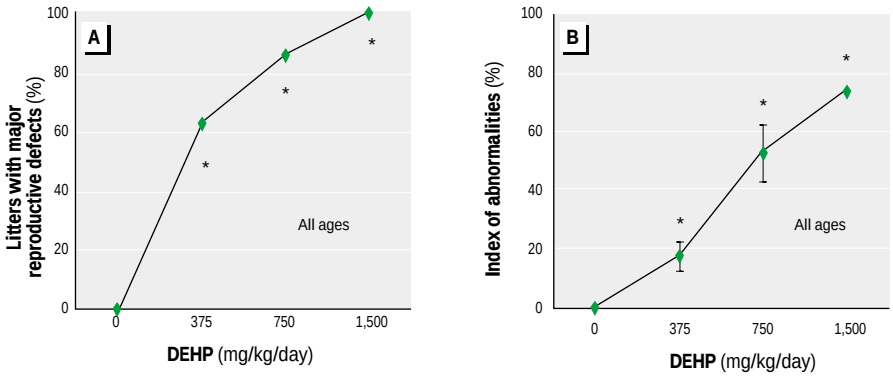

Figure 4. Effects of *in utero* and lactational DEHP exposure on (*A*) the incidence of reproductive deficits per litter and (*B*) the relative number of abnormalities per rat. (*A*) DEHP was considered to have caused major reproductive toxicity in a litter if any male from that litter had one or more of the following: missing or severely malformed sex organs, one testis or epididymis that weighed < 75% of the other, incomplete preputial separation, or an undescended testis in adulthood. (*B*) The index of abnormalities is a score based on how many sex organs were missing, obviously pathological, or abnormally small and, when applicable, whether rats had nipples and/or undescended testes in adulthood, whether preputial separation was permanently incomplete, and whether they failed to ejaculate during sex-behavior testing. Organs were considered to be abnormally small if they weighed less than the mean control weight – 2 SDs from the control mean. Scores were calculated as the percentage of end points (7–13 per rat) in which each male was found to be abnormal. Litter means were then calculated. Each point in (*B*) represents the mean ± SE. The number of replicates (litters) was 8 at 0, 375, and 750 mg/kg/day, and 5 at 1,500 mg/kg/ day.
*Significantly different from control at $p < 0.05$.

were confined to the testis and could not be reproduced consistently (*40–42*). In contrast, development of the male reproductive system in rats is profoundly affected by continuous multigenerational (*43*), *in utero* (*44*), or *in utero* and lactational exposure to di(*n*-butyl) phthalate (*7*,*45*,*46*). Effects appear to be caused by an antiandrogenic mechanism that does not involve direct interaction with androgen receptors (*45*). Our experiments were similar to those of Mylchreest et al. (*7*) in most ways, and many similarities between effects of di(*n*-butyl) phthalate and DEHP were found.

Yet there are several striking differences. Di(*n*-butyl) phthalate caused high incidences of epididymal and seminal vesicle agenesis, whereas DEHP caused low incidences of both, and when DEHP caused epididymal agenesis, it was partial rather than complete. Di(*n*-butyl) phthalate caused ventral prostate agenesis in only one rat and no anterior prostate agenesis, whereas DEHP caused a moderate incidence of ventral prostate agenesis and a high incidence of anterior prostate agenesis. And DEHP prevented completion of preputial separation in several rats, whereas di(*n*-butyl) phthalate did not. The fact that there are substantial differences in the nature of the effects of continuous exposure to DEHP, di(*n*-butyl) phthalate, di(*n*-octyl) phthalate, and di(isononyl) phthalate suggests that these chemicals affect male reproductive system development by somewhat different mechanisms. Additional research is needed to determine which mechanisms are common to all phthalate esters and which are specific to individual members of this family.

We are unaware of any previous report in which the predominant effect of any chemical was agenesis of the anterior prostate. Although many effects of DEHP can potentially be attributed to possible reductions in perinatal androgen concentrations and/or systemic impairment in responsiveness to androgens, the strikingly high incidence of anterior prostate agenesis suggests that DEHP affects this organ by one or more mechanisms unique to the anterior prostate and/or portions of the urogenital sinus and Wolffian ducts that give rise to it. The fact that DEHP exposure was continuous from GD 3 through PND 21 indicates that anterior prostate agenesis is not caused by some unique aspect of the timing of exposure.

The observation that preputial separation was never completed in many DEHP-exposed rats is also highly unusual. Except for Wolf et al.'s study of vinclozolin (*47*), we are unaware of any report that preweaning exposure to any chemical can cause a permanent blockade of preputial separation.

Implications for human health. Most previous experiments on effects of DEHP on the male reproductive system gave juvenile or adult animals 1,000–2,000 mg DEHP/kg/day. Results of our experiments demonstrate that the male reproductive system is substantially more sensitive to DEHP when exposure occurs early in development. In addition, *in utero* and lactational DEHP exposure caused effects (e.g., prostate agenesis) it was inherently incapable of causing if exposure had been delayed until after weaning. Nevertheless, these results do not demonstrate that reproductive system development in the average human male is at risk from DEHP exposure.

Acceptable human exposures to chemicals are typically calculated by reducing no-observed adverse effect levels (NOAELs) from animal experiments 100-fold to account for possible 10-fold differences in inter- and intraspecies variability. Because we did not determine a NOAEL, an additional 10-fold uncertainty factor would typically be used. When these safety factors are considered, the reference dose (acceptable daily intake) for male reproductive system effects in humans would be 375 µg DEHP/kg/day. This is still far higher that typical human exposure to DEHP, which is estimated to be 4–30 µg/kg/day (*5*). Results of our experiments suggest, therefore, that sexually dimorphic development in most humans is unlikely to be affected by DEHP alone (although DEHP could still affect human development in combination with chemicals that act by similar mechanisms). Even adults who receive frequent transfusions from DEHP-plasticized blood bags receive only an additional 36 µg/kg/day (*5*). However, long-term dialysis patients are reported to average 457 µg/kg/day (*5*), and newborn infants undergoing exchange transfusion receive 1,700–4,200 µg/kg (*5*,*48*). Consequently, DEHP exposure by these patients is greater than the acceptable daily intake for abnormal sexual development suggested by our results.

Arcadi et al. (*23*) examined effects of DEHP at substantially lower doses than we used. Rats that consumed 32.5 and 325 µL DEHP per liter of drinking water during pregnancy and lactation (roughly 3–5 and 30–50 mg/kg/day, respectively) gave birth to pups with lower testis wights and altered testicular morphology. Their results imply that the acceptable daily intake for DEHP is only 3 µg/kg/day, which is at the low end of the range of typical human exposure.

Most recently, Gray and colleagues reported that maternal DEHP treatment can greatly reduce testosterone production in fetal and neonatal rats, whereas neither DEHP nor a major metabolite bound to androgen receptors (*49*). Reductions in testosterone synthesis undoubtedly contribute to abnormal male reproductive system development, but this mechanism alone cannot account fully for the pattern of effects we observed.

References and Notes

1. Thomas JA, Thomas MJ. Biological effects of di-(2-ethylhexyl) phthalate and other phthalic acid esters. Crit Rev Toxicol 13:283–317 (1984).
2. Huber WW, Grasl-Kraupp B, Schulte-Hermann R. Hepatocarcinogenic potential of di(2-ethyl hexyl) phthalate in rodents and its implications on human risk. Crit Rev Toxicol 26:365–481 (1996).
3. National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction. CERHR Evaluation of Di(2-ethylhexyl) Phthalate, Intermediate Draft, June 15, 2000. Available: http://cerhr.niehs.nih.gov/news/Draft_Dehp _6_16.pdf [cited 4 August 2000].
4. NTP. Di(2-ethylhexyl) phthalate (CAS No. 117-81-7). In: Report on Carcinogens, 8th ed, Summary 1998. Research Triangle Park, NC:National Toxicology Program, 1998;100–102.
5. Doull J, Cattley R, Elcombe C, Lake BG, Swenberg J, Wilkinson C, Williams G, van Gemert M. A cancer risk assessment of di(2-ethylhexyl)phthalate: application of the new U.S. EPA Risk Assessment Guidelines. Regul Toxicol Pharmacol 29:327–357 (1999).
6. Faouzi MA, Dine T, Gressier B, Kambia K, Luyckx M, Pagniez D, Brunet C, Cazin M, Belabed A, Cazin JC. Exposure of hemodialysis patients to di-2-ethylhexyl phthalate. Int J Pharm 180:113–121 (1999).
7. Mylchreest E, Cattley RC, Foster PMD. Male reproductive tract malformations in rats following gestational and lactational exposure to di(*n*-butyl) phthalate: an antiandrogenic mechanism? Toxicol Sci 43:47–60 (1998).
8. Gray TJ, Butterworth KR, Gaunt IF, Grasso GP, Gangolli SD. Short-term toxicity study of di-(2-ethylhexyl) phthalate in rats. Food Cosmet Toxicol 15:389–399 (1977).
9. Oishi S, Hiraga K. Testicular atrophy induced by phthalic acid esters: effect on testosterone and zinc concentrations. Toxicol Appl Pharmacol 53:35–41 (1980).
10. Oishi S. Reversibility of testicular atrophy induced by di(2-ethylhexyl) phthalate in rats. Environ Res 36:160–169 (1985).
11. Oishi S. Testicular atrophy induced by di(2-ethylhexyl)phthalate: changes in histology, cell specific enzyme activities and zinc concentrations in rat testis. Arch Toxicol 59:290–295 (1986).
12. Siddiqui A, Srivastava SP. Effect of di(2-ethylhexyl)phthalate administration on rat sperm count and on sperm metabolic enzymes. Bull Environ Contam Toxicol 48:115–119 (1992).
13. Parmar D, Srivastava SP, Seth PK. Effect of di(2-ethylhexyl)phthalate (DEHP) on spermatogenesis in adult rats. Toxicology 42:47–55 (1986).
14. Shaffer CB, Carpenter CP, Smyth Jr HF. Acute and subacute toxicity of di(2-ethylhexyl) phthalate with note upon its metabolism. J Ind Hyg Toxicol 27:130–135 (1945).
15. Seth PK, Srivastava SP, Agarwal DK, Chandra SV. Effect of di-2-ethylhexyl phthalate (DEHP) on rat gonads. Environ Res 12:131–138 (1976).
16. Saxena DK, Srivastava SP, Chandra SV, Seth PK. Testicular effects of di-(2-ethylhexyl)phthalate (DEHP): histochemical and histopathological alterations. Ind Health 23:191–198 (1985).
17. Jones HB, Garside DA, Liu R, Roberts JC. The influence of phthalate esters on Leydig cell structure and function *in vitro* and *in vivo*. Exp Mol Pathol 58:179–193 (1993).
18. Gray TJ, Butterworth KR. Testicular atrophy produced by phthalate esters. Arch Toxicol Suppl 4:452–455 (1980).
19. Tandon R, Chowdhary SR, Seth PK, Srivastava SP. Altered development of testis of rat exposed to di(2-ethylhexyl) phthalate (DEHP) during lactation. J Environ Biol 11:345–354 (1990).
20. Tandon R, Seth PK, Srivastava SP. Effect of *in utero* exposure to di(2-ethylhexyl)phthalate on rat testes. Indian J Exp Biol 29:1044–1046 (1991).
21. Hellwig J, Freudenberger H, Jäckh R. Differential prenatal toxicity of branched phthalate esters in rats. Food Chem Toxicol 35:501–512 (1997).
22. Carpenter CP, Weil CS, Smyth HF Jr. Chronic oral toxicity

of di(2-ethylhexyl) phthalate for rats, guinea pigs, and dogs. AMA Arch Ind Hyg Occup Med 8:219–226 (1953).
23. Arcadi FA, Costa C, Imperatore C, Marchese A, Rapisarda A, Salemi M, Trimarchi GR, Costa G. Oral toxicity of bis(2-ethylhexyl) phthalate during pregnancy and suckling in the Long-Evans rat. Food Chem Toxicol 36:963–970 (1998).
24. Gray LE Jr, Wolf C, Lambright C, Mann P, Price M, Cooper RL, Ostby J. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p*'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 15:94–118 (1999).
25. Blazak WF, Treinen KA, Juniewicz PE. Application of testicular sperm head counts in the assessment of male reproductive toxicity. In: Male Reproductive Toxicology, Vol 3A (Chapin RE, Heindel JJ, eds). New York: Academic Press, 1993;86–94.
26. Booth JE. Sexual behavior of neonatally castrated rats injected during infancy with oestrogen and dihydrotestosterone. J Endocrinol 72:135–141 (1977).
27. Dewsbury DA. Description of sexual behavior in research on hormone-behavior interactions. In: Endocrine Control of Sexual Behavior (Beyer C, ed). New York:Raven Press, 1979;3–32.
28. Gallavan RH Jr, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 13:383–390 (1999).
29. Gray LE Jr, Kelce WR, Wiese T, Tyl R, Gaido K, Cook J, Klinefelter G, Desaulniers D, Wilson E, Zacharewski T, et al. Endocrine Screening Methods Workshop Report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reprod Toxicol 11:719–750 (1997).
30. Ramirez VD, Sawyer CH. Advancement of puberty in the female rat by estrogen. Endocrinology 76:1158–1168 (1965).
31. Gray LE Jr. Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicol Lett 102–103:677–680 (1998).
32. Boylan ES. Morphological and functional consequences of prenatal exposure to diethylstilbestrol in the rat. Biol Reprod 19:854–863 (1978).
33. Rothschild TC, Calhoon, RE, Boylan ES. Effects of diethylstilbestrol exposure *in utero* on the genital tracts of female ACI rats. Exp Mol Pathol 48:59–76 (1988).
34. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect 103:582–587 (1995).
35. Raloff J. Girls may face risks from phthalates. Sci News 158:165 (2000).
36. Damassa DA, Smith ER, Tennent B, Davidson JM. The relationship between circulating testosterone levels and male sexual behavior in rats. Horm Behav 8:275–286 (1977).
37. Campbell J, Holt D, Webb M. Dimethoxyethylphthalate metabolism: teratogenicity of the diester and its metabolites in the pregnant rat. J Appl Toxicol 4:35–41 (1984).
38. Heindel JJ, Gulati DK, Mounce RC, Russell SR, Lamb JC IV. Reproductive toxicity of three phthalic acid esters in a continuous breeding protocol. Fundam Appl Toxicol 12:508–518 (1989).
39. Waterman SJ, Keller LH, Trimmer GW, Freeman JJ, Nikiforov AI, Harris SB, Nicolich MJ, McKee RH. Two-generation reproduction study in rats given di-isononyl phthalate in the diet. Reprod Toxicol 14:21–36 (2000).
40. Ashby J, Tinwell H, Lefevre PA, Odum J, Paton D, Millward SW, Tittensor S, Brooks AN. Normal sexual development of rats exposed to butyl benzyl phthalate from conception to weaning. Regul Toxicol Pharmacol 26:102–118 (1997).
41. Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ Health Perspect 103:1136–1143 (1995).
42. Sharpe RM, Turner KJ, Sumpter JP. Endocrine disruptors and testis development [Letter]. Environ Health Perspect 106:A220–A221 (1998).
43. Wine RN, Li L-H, Barnes LH, Gulati DK, Chapin RE. Reproductive toxicity of di-*n*-butyl phthalate in a continuous breeding protocol in Sprague-Dawley rats. Environ Health Perspect 105:102–107 (1997).
44. Ema M, Miyawaki E, Kawashima K. Further evaluation of developmental toxicity of di-*n*-butyl phthalate following administration during late pregnancy in rats. Toxicol Lett 98:87–93 (1998).
45. Mylchreest E, Sar M, Cattley RC, Foster PMD. Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. Toxicol Appl Pharmacol 156:81–95 (1999).
46. Mylchreest E, Wallace DG, Cattley RC, Foster PMD. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. Toxicol Sci 55:143–151 (2000).
47. Wolf CJ, LeBlanc GA, Ostby JS, Gray LE Jr. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 55:152–161 (2000).
48. Sjöberg POJ, Bondesson UG, Sedin EG, Gustafsson JP. Exposure of newborn infants to plasticizers. Plasma levels of di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate during exchange transfusion. Transfusion 25:424–428 (1985).
49. Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE Jr. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol Sci 58:339–349 (2000).