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. Peterson1,2,3

1School of Pharmacy, 2Environmental Toxicology Center, and 3Endocrinology- 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 103–112. 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 littersmates 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 known to 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 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

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: riamoore@ pharmacy.wisc.edu

We thank J. Li for sperm analyses. This research was supported by NIH grants ES 06806 and ES 01332 and by a 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 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 from 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 anorectal pairs (with and without nipple buds) daily beginning on PND 11 and continuing until with no survival on PND 30. 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 CO2 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 musculoskeletal 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 rank 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 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. (None 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 at the high dose 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 between birth and weaning.

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

| Parameter                        | 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 GD 3 to GD 21 for dams that maintained litters throughout this period. 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.

Effects of in utero and lactational DEHP exposure on indices of sexual development are presented in Figure 1. Anogenital distance was 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...
were killed by CO
less reliable than observations made after rats
testis descent in live animals was considered
these testes were very small, assessment of
many DEHP-exposed rats. Because some of
ing, testes from control rats could readily be
and had little if any effect on body weight at
increased time to separation (data not shown)
DEHP exposure slightly but nonsignificantly
indicate that
Preputial separation, an androgen-depen-
dent index of pubertal development, was com-
plete 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 sepa-
ration per litter (Figure 1D) was statistically
significant only at the high dose but is con-
sidered 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 wean-
ing, 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 CO2 overdose. Many testes from
DEHP-exposed rats were in the abdominal cavity at necropsy, often on the
centralateral side, whereas all testes from
control rats were in the scrotum (Figure 2).
Undescended testes were observed at all
doses on PND 21, although the num-
er 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 unde-
scedent 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 pre-
vents 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
testsis/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 mid-
dle 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.

| Organ/age (days) | Maternal DEHP dose (mg/kg/day) |
|------------------|-------------------------------|
|                  | 0                | 375             | 750             |
| Testsis weight (mg) | PND 21 | In adulthood | PND 21 | In adulthood |
| 21 | 247 ± 13 (8)* | 222 ± 17 (7) | 192 ± 15 (8)* | 153 ± 9 (5)* |
| | (0.509 ± 0.017)* | (0.451 ± 0.018)* | (0.435 ± 0.017)* | (0.398 ± 0.028)* |
| 63 | 3.50 ± 65 (8) | 3.96 ± 80 (8) | 2.57 ± 255 (7)* | 2.04 ± 107 (5)* |
| | (1.082 ± 0.020) | (1.069 ± 0.020) | (0.821 ± 0.081)* | (0.696 ± 0.029)* |
| 105 | 3.71 ± 85 (6) | 3.69 ± 136 (7) | 2.624 ± 467 (7) | 1.467 ± 163 (2) |
| | (0.886 ± 0.018) | (0.908 ± 0.040) | (0.645 ± 0.109) | (0.390 ± 0.013) |

Epididymides weight (mg) | PND 21 | In adulthood | PND 21 | In adulthood |
|------------------|-------------------------------|
| 21 | 624 ± 18 (8) | 613 ± 25 (8) | 412 ± 62 (7) | 429 ± 42 (5)* |
| | (0.193 ± 0.005) | (0.186 ± 0.006) | (0.132 ± 0.020)* | (0.148 ± 0.018)* |
| 63 | 1.053 ± 23 (5) | 957 ± 52 (7) | 640 ± 136 (7)* | 581 ± 50 (2) |
| | (0.231 ± 0.005) | (0.236 ± 0.014) | (0.158 ± 0.032)* | (0.155 ± 0.015)* |
| 105 | 27.7 ± 12 (8) | 24.8 ± 13 (7) | 22.7 ± 11.8* | 19.6 ± 4 (5)* |
| | (0.075 ± 0.0026) | (0.0510 ± 0.0022) | (0.0534 ± 0.0049) | (0.0513 ± 0.0037) |
| 63 | 91.1 ± 17 (8) | 88.2 ± 18 (8) | 80.7 ± 2.3 (7) | 75.7 ± 2.7 (3)* |
| | (0.0281 ± 0.0005) | (0.0269 ± 0.0004) | (0.0259 ± 0.0011) | (0.0269 ± 0.0009) |
| 105 | 102.5 ± 15 (8) | 99.1 ± 18 (7) | 88.5 ± 3.7 (7)* | 81.1 ± 3.2 (2)* |
| | (0.0244 ± 0.0003) | (0.0245 ± 0.0010) | (0.0220 ± 0.0010)* | (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.
*Numbers shown are means ± SE, with the number of replicates (litters) in parentheses. %Values shown are organ
weight/ body weight ratio. *Significantly different from control at p < 0.05.
Epididymal sperm number (10⁶ per cauda) 55.5 ± 3.7 46.5 ± 5.1 29.8 ± 8.7* 19.3 ± 7.5*  

Environmental Health Perspectives •

*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.

PND 21 and 63 and 2 on PND 105.

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.

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 (10⁶ per testis) | 34.2 ± 1.5 | 36.5 ± 1.2 | 25.6 ± 4.5 | 24.4 ± 5.4 |
| Epididymal sperm number (10⁶ per cauda) | 55.5 ± 3.7 | 46.5 ± 5.1 | 29.8 ± 8.7* | 19.3 ± 7.5* |

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.

Dorsolateral prostate agenesis was observed in DEHP-exposed rats, but definitive assessments could not be made at necropsy (other than incomplete preputial separation), and results of histological evaluation were inconclusive.

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 F1 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 retraction; 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 prepuberal 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 testses, and hypospasias 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 male 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,
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, as such testicular steroidogenesis is independent of testicular position. Furthermore, only two of the nine DEHP-exposed rats that did not 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 only 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 failure of five of the 16 DEHP-exposed rats to ejaculate in a way that permits detection of some major male reproductive system abnormalities.

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 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 ≤ 8) but which are so unusual among control rats as to be considered biologically significant at 375 mg/kg/day are tests nonsecretant, permanently incomplete preputial separation, and accessory sex organ agenesis.

Although occasional control male rats are 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 endpoints 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
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 M. ychreek 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 concentration 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 potential interspecies 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 normal 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 weights 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.
Articles • Abnormal sexual development in DEHP-exposed male rats

31. Ramirez VM, Sawyer CH. Advancement of puberty in the female rat by estrogen. Endocrinology 76:1159–1168 (1965).

32. Gray LE Jr. Tiered screening and testing strategy for xenoestrogens and antiandrogens. Toxicol Lett 102–103:377–680 (1998).

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. J obling S, Reynolds T, White R, Parker MG, Sumpter J P. 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 J C IV. Reproductive toxicity of three phthalic acid esters and male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. Toxicol Sci 151:143–151 (2000).

39. Wolf CJ, LeBlanc GA, Ostby J S, Gray LE Jr. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 55:132–160 (2000).

40. Sjöberg POJ, Bondesson UG, Sedin E, 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).

41. Parks LG, Ostby J S, Lambright CR, Abbott BD, Klinefelter GR, Barlow N J, 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).