

Exposure to Di(2-ethyl-hexyl) phthalate (DEHP) *in Utero* and during Lactation Causes Long-Term Pituitary-Gonadal Axis Disruption in Male and Female Mouse Offspring

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The present study examined the effects in mice of exposure to di(2-ethyl-hexyl) phthalate (DEHP) throughout pregnancy and lactation on the development and function of the pituitary-gonadal axis in male and female offspring once they have attained adulthood. Groups of two to three dams were exposed with the diet from gestational d 0.5 until the end of lactation, at 0, 0.05, 5, and 500 mg DEHP/kg · d. The experiment was repeated three times (total: seven to 10 dams per treatment). The 500-mg dose caused complete pregnancy failure, whereas exposure to doses of 0.05 and 5 mg did not affect pregnancy and litter size. In total, about 30 male and 30 female offspring per group were analyzed. Offspring of the DEHP-treated groups, compared with controls, at sexual maturity showed: 1) lower body weight (decrease 20–25%, $P < 0.001$); 2) altered gonad weight (testes were ~13% lighter and ovaries ~40% heavier; $P < 0.001$); 3) poor germ cell quality (semen was ~50% less concentrated and 20% less viable, and ~10% fewer oocytes reached MII stage, $P < 0.001$); 4) significant lower expression of steroidogenesis and gonadotropin-receptor genes in the gonads; and 5) up-regulated gonadotropin subunit gene expression in the pituitary. In conclusion, our findings suggest that, in maternally exposed male and female mice, DEHP acts on multiple pathways involved in maintaining steroid homeostasis. Specifically, *in utero* and lactational DEHP exposure may alter estrogen synthesis in both sexes. This, in turn, induces dysregulation of pituitary-gonadal feedback and alters the reproductive performance of exposed animals. (*Endocrinology* 153: 937–948, 2012)

Phthalates (phthalic acid esters) are plasticizers that are added to polymers, especially polyvinyl chloride, to impart softness and flexibility. They are widely used in the manufacture of a wide range of consumer goods such as medical devices, clothing, packaging, food containers, personal-care products, and children's toys (1). The most commonly used phthalate is di(2-ethylhexyl) phthalate (DEHP) with a production of 1 to 4 million tons per year, which makes it one of the most widespread environmental contaminants worldwide (2, 3). Phthalates do not form strong molecular linkages with the polymer so they can

diffuse throughout the matrix and leach into the environment (4, 5). As a result, the general population is widely and continuously exposed to these compounds through ingestion, inhalation, or skin absorption. They therefore pose significant public health concerns, on account of their endocrine-disrupting activity (6, 7).

The reproductive system is particularly susceptible to the endocrine-disrupting activity of phthalates. In rats these effects include reduction in fertility (8), litter size/viability (9, 10), sperm density and motility (11), and biochemical and morphological alterations of male and fe-

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Abbreviations: AGD, Ano-genital distance; COC, cumulus-oocyte complexes; DEHP, di(2-ethyl-hexyl) phthalate; dpc, days postcoitum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin; PND, postnatal day; PPAR, peroxisome proliferator-activated receptor.

male gonads (8). Furthermore, phthalates can cross the placental barrier and also pass into breast milk, with a significant risk of damage for the developing fetus and newborn (12, 13). Unfortunately, many of the reproductive abnormalities resulting from developmental exposure only become apparent after puberty (long-latency effect), and this is a strong obstacle to the development of a cause and effect relationship.

To date, clear evidence of DEHP reproductive toxicity in maternally exposed adult male animals has been reported (14–17), whereas little is known about the effects of pre- and perinatal exposure to DEHP on females (18). The mechanisms underlying the phthalates' reproductive toxicity is not yet fully understood, but morphological and functional alterations of the reproductive system in animal models suggest phthalate-mediated alterations to steroid hormone-dependent processes in both males and females (19–24).

The close correlation between gonadal steroidogenesis and pituitary gonadotropins is well documented, and fertility depends on precise hormonal regulation of this axis. However, it is still not clear how pre- and perinatal exposure to DEHP influences the pituitary-gonadal axis as regards the regulation of steroid-gonadotropin cross talk.

The aim of the present study was to evaluate, in mice given DEHP throughout pregnancy and lactation, the effects on pituitary-gonadal function at the morphological and molecular levels in male and female offspring once they reached adult age. Dams were exposed until the end of lactation to cover the complete period of reproductive system development in the mouse, which is largely postnatal, whereas in other mammals, including human, reproductive organ development is completed *in utero*. DEHP dosages and the administration with food were chosen for their relevance to human exposure.

Materials and Methods

Animals and treatments

Virgin female 5-wk-old CD-1 mice were purchased from Charles River (Calco, Italy) and allowed to acclimatize for 2 wk. They were housed in the animal facilities of the Department of Animal Pathology and Health, Faculty of Veterinary Medicine, University of Milan, under controlled conditions (23 ± 1 C, 12-h light, 12-h dark cycle). Standard pellet food (4RF21, Charles River) and tap water were available *ad libitum*.

Groups of two or three females were mated with one male and inspected daily for a mating plug. The day of the vaginal plug detection [0.5 d postcoitum (dpc)] each female was housed individually in type II cages with stainless steel covers and hardwood shavings as bedding. From this moment (0.5 dpc) through lactation until weaning [postnatal d 21 (PND) 21], dams were given diet containing DEHP or vehicle. DEHP (Sigma-Aldrich,

Hamburg, Germany) was diluted in commercial sunflower oil and used for preparing treated chow in a specialized company (Altromin, Lage, Germany). The amount of DEHP added to the chow to obtain the desired mg/kg · d doses (0, 0.05, 5, and 500 mg DEHP/kg · d) was calculated based on the mean daily food intake of CD1 mice, which was calculated by a preliminary study in the same physiological conditions and confirmed by the literature (25). Therefore, the chow was dosed by the concentrations of 0.2857, 28.57, and 2857.0 mg/kg food to ensure a mean daily intake of 0.05, 5, and 500 mg/kg · d, respectively, for the three experimental groups. Each batch of diet was tested before use in an accredited laboratory (SGS Laboratory GmbH, Hamburg, Germany).

Two to three pregnant mice were randomly assigned among the groups, and the experiment was replicated at least three times (total seven to 10 dams per treatment). The dose range was selected considering as reference value an amount close to the estimated daily intake of the general population (0.058 mg/kg · d) as reported by Kavlok *et al.* (1). Because of the scant data on mice, the highest dose was based on data reported for rats. Therefore, the two highest doses were calculated by applying a factor of 100 so that the largest (500 mg/kg · d) was known to induce reproductive adverse effects in rat offspring without causing overt maternal toxicity (26). Dams and lactating offspring were examined daily for clinical signs of toxicity.

On PND 21 dams were euthanized by CO₂ inhalation, and organs were collected. Variables including litter size, sex ratio, pup weight, and the number of viable pups were recorded. The liver, ovaries, and uterus were removed, weighed, and snap-frozen in liquid nitrogen for later analysis. To count postimplantation losses, an additional group of 15 dams per dose was exposed to DEHP from dpc 0.5 and killed at specific times during pregnancy (dpc 9.5, 10.5, 11.5, 13.5, and 15.5). Gross fetal and placental morphology was compared between groups.

On PND 21 all pups were grouped according to gender, and body weight was recorded. Males and females from each litter were housed in groups for another 3 wk (up to PND 42). Standard pellet food (Charles River 4RF21) and tap water were available *ad libitum*.

On PND 42, at least three animals of each sex per litter were randomly selected for measurements of body weight, ano-genital distance (AGD), and autopsy. A total number of 85 male and 87 female offspring was evaluated (25–35 per each treatment group).

Mice were euthanized by CO₂ inhalation followed by cervical dislocation, and ano-genital distance (defined as the distance between the center of the anus and the base of the genital bud) was measured using manual calipers by a single investigator. The animals were handled carefully to avoid variation in the measurements due to stretching of the perineal region.

Male external genitalia were examined for malformations, and testicular position was recorded after opening the abdominal cavity. Pituitaries and reproductive organs of both sexes were removed and weighed, and the mean weight was used in subsequent analyses. All organs significantly correlated with body weight were adjusted for body weight. Organs were then snap-frozen in liquid nitrogen for later analysis.

Care and experimental procedures with mice were in accordance with accepted standard of human animal care following Italian national regulations and were approved by the University of Milan ethics committee.

Sperm collection and dead-live ratio

Sperm was obtained from the cauda epididymes of adult offspring. Both cauda were dissected out from the body and transferred into 500 μl of previously equilibrated Whittingham medium (37 C at 5% CO_2 in air). Sperm was passively released into the culture medium by puncturing the cauda three to four times with a 27G needle. Some of the samples were diluted (1:100) with water, and a sperm count was done in a Neubauer chamber. Other samples were diluted (1:20) with 0.9% NaCl, and stained by a modified Kovács-Foote method (27). Briefly, one drop of dilute sample was mixed on a microscope slide with one drop of iso-osmotic 0.2% Trypan blue (Sigma T-8154; Sigma-Aldrich, St. Louis, MO) and smeared with the edge of another slide. The slides were vertically air dried then fixed for 2 min with fixative solution (86 ml 1 N HCl plus 14 ml 37% formaldehyde solution and 0.2 g neutral red (Fluka, 72210), and rinsed with tap and distilled water. Finally, the slides were dried in air, and covered with Eukitt (Fluka, 03989) and a coverslip.

Stained smears were examined by light microscopy at $\times 400$ magnification. The status of the head and tail of at least 100 spermatozoa was classified in each smear. Sperm with white or pale pink heads (intact plasma membrane) were classified as alive, and sperm with black to dark-purple heads (damaged membrane) were classified as dead.

In vitro oocyte maturation

Maturation-competent cumulus-oocyte complexes (COC) were collected from adult offspring injected with 3.5 IU Folligon [pregnant mare serum gonadotropin (PMSG), Intervet International, Boxmeer, The Netherlands] before oocyte collection, and matured *in vitro*. Briefly, COC were collected in M2 medium by gently puncturing visible antral follicles on the ovary surface with a 30.5-gauge needle. Germinal vesicle-stage oocytes with an intact vestment of cumulus cells were collected and pooled from at least two mice per group. Maturation was in microdrops (200 μl , 20–30 COC per drop) of bicarbonate-buffered α -MEM supplemented with 10% (vol/vol) fetal calf serum, 1 mM glutamine, 10 IU/ml PMSG (Folligon, Intervet International) and 5 IU/ml human chorionic gonadotropin (hCG, Chorulon, Intervet International), 50 $\mu\text{g}/\text{ml}$ streptomycin, and 75 $\mu\text{g}/\text{ml}$ penicillin G and cultured at 37 C in 5% CO_2 in air.

Maturation was evaluated after 14–15 h. Oocytes with diffuse or slightly condensed chromatin or with clumped or strongly condensed chromatin with or without metaphase plate but no polar body were classified as not matured (germinal vesicle and metaphase I). Oocytes with a metaphase plate and a polar body were considered mature MII oocytes. Oocytes with no visible chromatin or with fragmented cytoplasm and/or abnormal chromatin patterns were considered degenerated. Experiments were replicated at least four times, with a minimum of five mice per treatment selected from different litters.

In vitro fertilization and embryo culture

Oocytes or sperm from untreated mice were used for assessing fertilization, and developmental capacity of male and female gametes was derived from maternally exposed adult offspring.

Briefly, females were superovulated by ip injection of 3.5 IU Folligon (PMSG, Intervet International), followed 48 h later by an ip injection of 5 IU Chorulon (hCG, Intervet). Spermatozoa were collected as described above and capacitated for 60 min in

Whittingham medium (37 C at 5% CO_2 in air). COC were recovered 14 h after hCG from oviducts in M2 medium (Sigma-Aldrich). After rinsing in Whittingham medium, COC were inseminated with 2×10^6 capacitated spermatozoa. Putative fertilized eggs (6 h after insemination) were then transferred to 250- μl drops of M16 medium (Sigma-Aldrich) covered with paraffin oil and incubated at 37 C at 5% CO_2 in air for further 96 h. Cleavage and blastocyst rate were assessed at 24 h and 96 h after insemination, respectively.

RNA isolation and RT-PCR

Total RNA was isolated from one testis or ovary of all autopsied mice using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was checked for integrity and DNA contamination using a UV spectrophotometer and 1.3% agarose gel electrophoresis. Total RNA (1 μg) extracted from each sample was used to synthesize the cDNA using a SuperScript kit (Invitrogen). The reverse transcription reaction was carried out at 42 C for 1 h, and terminated by heating at 94 C for 2 min.

Polyadenylated [poly(A)+] RNA from pituitaries was extracted using a Dynabeads mRNA DIRECT kit (Deutsche Dynal, Hamburg, Germany). Briefly, single pituitaries were lysed for 10 min at room temperature in 200 μl lysis buffer [100 mmol Tris-HCl (pH 8), 500 mmol LiCl, 10 mmol EDTA, 1% (wt/vol) sodium dodecyl sulfate, and 5 mmol dithiothreitol]. After lysis, 10 μl prewashed Dynabeads-oligo(deoxythymidine) were pipetted into the tube, and poly(A)+ RNA binding to oligo(deoxythymidine) was allowed for 5 min at room temperature. The beads were then separated with a Dynal MPC-E magnetic separator and washed twice with 50 μl washing buffer A [10 mmol Tris-HCl (pH 8), 0.15 mmol LiCl, 1 mmol EDTA, and 0.1% (wt/vol) sodium dodecyl sulfate] and three times with 50 μl washing buffer B [10 mmol Tris-HCl (pH 8.0), 0.15 mmol LiCl, and 1 mmol EDTA]. Poly(A)+ RNA were then eluted from the beads by incubation in 11 μl diethylpyrocarbonate-treated sterile water at 65 C for 2 min. Aliquots were immediately used for reverse transcription with the PCR Core Kit (PerkinElmer Corp., Wellesley, MA), using 2.5 μmol random hexamers to obtain the widest array of cDNA. The reverse transcription reaction was carried out in a final volume of 20 μl at 25 C for 10 min and 42 C for 1 h, followed by a denaturation step at 99 C for 5 min and immediate cooling on ice.

Table 1 lists the primers and PCR conditions for the genes analyzed. Transcripts were selected because of their direct or indirect involvement in the pituitary-gonadal cross talk. For each set of primers, the optimal cycle number at which the transcript was amplified exponentially was established by running a linear cycle series and the number of PCR cycles was kept within this range. Approximately 1 μl cDNA per sample was used for amplification. The cDNA fragments were generated by initial denaturation at 94 C for 3 min. The PCR products were separated by electrophoresis on 1.3% agarose gel and detected under UV light. To normalize signals from different RNA samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were coamplified as an internal standard. Quantitative expression was analyzed with Quantity One software using the software's Volume Analysis Report (Bio-Rad Laboratories, Inc., Hercules, CA).

TABLE 1. List of primers and PCR conditions

Gene	Accession no.	Primers	Annealing C	Product size
<i>lhr</i>	NM013582	F: TCTACCTGCTGCTCATTCGCTC R: AAGGCAGCTGAGATGGCAAAG	57	553
<i>fshr</i>	NM013523	F: ATGTGTAACCTCGCCTTTGCTG R: AACATACAGCTGCGACAAAGGG	57	393
<i>star</i>	BC082283	F: GAAGGAAAGCCAGCAGGAGAAC R: CTGCGATAGGACCTGGTTGATG	58	496
<i>cyp17a1</i>	AY594330	F: ACGGTGGGAGACATCTTTGGG R: CCTTCGGGATGGCAAACCTCTC	57	283
<i>cyp19a1</i>	NM007810	F: CCTCTGGATACTCTGCGACGAG R: CGAATGGTGGAGTTTGTGTGG	56	508
<i>lh&β</i>	NM008497	F: CATCACCTTCACCACCAGCATC R: GAGGTCACAGGCCATTTGGTTG	60	259
<i>fsh&β</i>	NM008045	F: CTGCCATAGCTGTGAATTGACC R: CACAGCCAGGCAATCTTACG	55	203
<i>pgr</i>	NM008829	F: GATGAGCCTGATGGTGTTTGGC R: GGGCAACTGGGCAGCAATAAC	57	490
<i>gapdh</i>	NM008084	F: TCACCATCTTCCAGGAGCG R: CTGCTTCACCACCTTCTTGA	57	572

F, Forward; R, reverse.

Electrophoresis and immunoblot analysis

Protein from ovaries and testes was extracted using RIPA buffer with added proteinase and phosphatase cocktail (catalog nos. P 2714 and P5726, respectively). The lysates were mixed 1:1 with 2 \times Laemmli sample buffer and heated to 90 C for 5 min and then centrifuged at 13,000 rpm for 2 min. Immunoblot analysis was done as described previously (28). Cyp19a1 was detected using a goat polyclonal anti-Cyp19 antibody (SC-1425; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The secondary antibody used to detect the Cyp19a1-primary antibody complex was horseradish peroxidase-conjugated bovine antigoat IgG (SC-2378; Santa Cruz Biotechnology). Proteins on the membranes were visualized using the WestPico ECL detection system (Pierce Chemical Co., Rockford, IL). After the initial analysis, the membranes were washed in a stripping buffer (2% sodium dodecyl sulfate, 100 mM β -mercaptoethanol, 50 mM Tris, pH 6.8) to remove bound antibodies and reprobed with a monoclonal anti- β -actin antibody (catalog no. A1978). The secondary antibody for detection of the β -actin-primary antibody complex

was horseradish peroxidase-conjugated goat antimouse IgG (Pierce Chemical Co.). Protein content was analyzed in each blot (from three different experiments) using the Volume Analysis Report of Quantity One software (Bio-Rad).

Statistical analysis

All data were analyzed using GraphPad Prism software 5.03 (GraphPad Software, San Diego CA). Differences between the means for litter size, AGD, organ weight, semen parameters, and gene expression were tested by the D'Agostino and Pearson normality test to confirm Gaussian distribution and then examined by one-way ANOVA, with statistical significance at $P \leq 0.05$. When ANOVA gave a significant P value, treatments were compared using Bonferroni's test in the *post hoc* analysis. Data for *in vitro* oocyte maturation and embryo development were analyzed by binary logistic regression. Controls were taken as the reference group. Experiments were replicated at least three times, and each replicate was fitted as a factor. The log-likelihood ratio

TABLE 2. Reproductive outcome and organ weights of dams treated with DEHP throughout pregnancy and lactation

Parameter	DEHP			
	0 mg/kg \cdot d	0.05 mg/kg \cdot d	5 mg/kg \cdot d	500 mg/kg \cdot d
No. of dams	10	7	7	10
Pregnancy at term (%)	10/10 (100) ^a	6/7 (85.7) ^a	7/7 (100) ^a	1/10 (10) ^b
Abortion/miscarriage	0/10 ^a	1/7 ^a	0/7 ^a	9/10 ^b
Litter size	12.9 \pm 0.7	15.0 \pm 1.5	10.6 \pm 1.5	— ^c
Sex ratio (% F:M)	43.3: 56.7	48.7: 51.3	50.9: 49.1	—
Viability index (%)	98.5 \pm 1.5	98.3 \pm 1.4	100.0 \pm 0.0	—
Liver weight (% of BW)	8.5 \pm 0.2 ^a	9.4 \pm 1.2 ^{a,b}	10.0 \pm 0.8 ^b	—
Ovary weight (% of BW)	0.019 \pm 0.02	0.018 \pm 0.02	0.021 \pm 0.02	—
Uterus weight (% of BW)	0.48 \pm 0.05	0.53 \pm 0.07	0.55 \pm 0.09	—

Values are means \pm SEM. Viability index: (number of pups at weaning/number of pups alive on PND 2) \times 100. BW, Body weight; F, female; M, male.

^{a, b} Different superscripts indicate statistical differences for $P \leq 0.05$. ^c Of the 10 pregnancies, only one reached term and a single male pup was born (500 mg/kg \cdot d group), and this pup was excluded from the analysis of the parameters reported in the table.

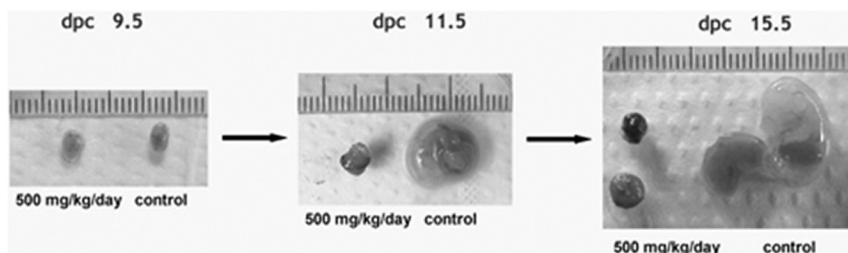


FIG. 1. Morphological abnormalities of 500 mg DEHP/kg · d-treated fetuses and extra-embryonic tissues taken on dpc 9.5, 11.5, and 15.5, compared with no exposure to DEHP at the same times.

statistic was used to detect between-treatment differences with dosage set as an explanatory variable. Significance was set at $P < 0.05$.

Results

DEHP disturbs maternal reproductive outcome

Exposure to 500 mg/kg · d dramatically increased post-implantation losses (Table 2), with only one of 10 females able to deliver. Autopsy at specific times during pregnancy indicated that embryonic vesicles appeared macroscopically normal until dpc 9.5. Between dpc 10.5 and 11.5, resorption started and fetuses and fetal envelopes rapidly degenerated. By dpc 15.5, only hemorrhagic remnants could be seen in the uterine cavity (Fig. 1). There were no signs of maternal toxicity and by dpc 19.5 (predicted time of delivery), the uterus had recovered almost completely although sometimes implantation sites were still evident.

In the 5 mg/kg · d group litters were slightly smaller than controls. However, variability in litter sizes among dams meant that the percentage of postimplantation losses did not correlate significantly with the reduction in mean litter size. There were no differences in mean litter size and postimplantation losses in the 0.05 mg/kg · d group compared with control.

The sex ratio and viability index of offspring were unaffected by treatment, and there were no adverse clinical findings in the newborn pups. DEHP-treated dams had a dose-dependent increase in mean liver weight compared with control. DEHP did not affect the dams' ovary and uterus weights.

DEHP affects morphological and reproductive indices in offspring

Morphological indices (PND 21–42)

Pre- and perinatal treatment with 0.05 and 5 mg/kg · d DEHP significantly reduced the body weight of female and male pups at PND 21 and 42 (Table 3). At weaning (3 wk of age), both male and female DEHP-treated offspring were 20–25% lighter than control animals, the males in

the 5 mg/kg · d group being most affected. The weight difference persisted up to 6 wk of age, when treated offspring still weighed between 6% and 14% less than control animals of the same sex. Abdominal fat weight in females was significantly lower than in controls, with respectively 41% and 30% reductions in the 0.05 mg/kg · d and 5 mg/kg · d groups, but these differences between doses were not significant. No significant differences were seen in male adiposity (Table 3).

Reproductive indices (PND 42):

Table 4 shows results for male and female offspring. In males, the 5 mg/kg · d and 0.05 mg/kg · d DEHP doses significantly reduced testis and seminal vesicle weight. Seminal vesicles in both groups were 20–25% lighter, and testes from the 0.05 mg/kg · d group weighed approximately 13% less. In female pups, ovarian weight was significantly higher than controls in the 0.05 and the 5 mg/kg · d groups, with increases of about 45% and 35%. Uterus weights were unaffected, and AGD was unaffected by DEHP at any dose, in males and females.

DEHP affects *in vitro* oocyte maturation and developmental competence in adult female offspring

A total of 524 oocytes was used for analysis of nuclear maturation. After isolation (0 h) most of the oocytes

TABLE 3. Morphological indices in male and female offspring (PND 21–42)

	DEHP		
	0 mg/kg · d	0.05 mg/kg · d	5 mg/kg · d
Male offspring			
No. of animals	33	24	28
Body weight PND 21 (g)	9.9 ± 0.3 ^a	8.1 ± 0.3 ^b	7.5 ± 0.4 ^c
Body weight PND 42 (g)	32.9 ± 0.4 ^a	28.2 ± 0.8 ^b	30.0 ± 0.7 ^b
Abdominal fat (% of BW)	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Female offspring			
No. of animals	33	27	27
Body weight PND 21 (g)	9.5 ± 0.5 ^a	7.2 ± 0.6 ^b	7.8 ± 0.2 ^b
Body weight PND 42 (g)	30.8 ± 0.6 ^a	28.9 ± 0.4 ^b	27.6 ± 0.6 ^b
Abdominal fat (% of BW)	2.4 ± 0.2 ^a	1.4 ± 0.3 ^b	1.7 ± 0.1 ^b

Values are means ± SEM. BW, Body weight. ^{a, b, c} Different superscripts indicate statistical differences for $P \leq 0.05$.

TABLE 4. Reproductive indices in male and female offspring (PND 42)

	DEHP		
	0 mg/kg · d	0.05 mg/kg · d	5 mg/kg · d
Male offspring			
No. of animals examined	33	24	28
AGD [cm/BW ^{1/3}]	0.530 ± 0.004	0.520 ± 0.005	0.520 ± 0.007
Testis weight (g)	0.086 ± 0.002 ^a	0.075 ± 0.003 ^b	0.089 ± 0.003 ^a
Seminal vesicles weight (g)	0.179 ± 0.007 ^a	0.143 ± 0.008 ^b	0.132 ± 0.008 ^b
Female offspring			
No. of animals examined	33	27	27
AGD [cm/BW (1/3)]	0.23 ± 0.004	0.23 ± 0.005	0.22 ± 0.007
Ovary weight (g)	0.0063 ± 0.0002 ^a	0.0083 ± 0.0002 ^c	0.0071 ± 0.0002 ^b
Uterus weight (g)	0.16 ± 0.01	0.13 ± 0.01	0.14 ± 0.01

Values are means ± SEM. BW, Body weight. ^{a, b, c} Different superscripts indicate statistical differences for $P \leq 0.05$.

(>99%) were at the germinal vesicle nuclear stage, independently of treatment. Table 5 shows the *in vitro* maturation of oocytes from maternally treated female offspring. After 14 h of culture, about 10% fewer oocytes reached MII stage in the 0.05 and 5 mg/kg · d groups compared with controls ($P < 0.001$). In addition, in both treatment groups the percentage of degenerated oocytes was almost double that of controls. The percentages of immature oocytes were not significantly different between the groups, independently of treatment.

Developmental competence of female gametes was tested on a total of 909 oocytes. The oocytes of the 0.05 mg/kg · d group produced embryos with a significantly reduced capacity to complete the first mitotic division and to subsequently reach the blastocyst stage compared with both controls and the 5 mg/kg · d group ($P \leq 0.0001$; Table 6).

DEHP affects semen characteristics and *in vitro* developmental capacity in adult male offspring

Figure 2 shows semen characteristics of 6-wk-old pups exposed to DEHP during gestation and lactation. Sperm concentration and viability, intended as membrane integrity, were significantly depressed by exposure to DEHP. Compared with controls, semen from treated animals was about 50% less concentrated (sperm count – DEHP 0: $5.9 \times 10^6 \text{ ml}^{-1} \pm 0.8$; 0.05: $2.8 \times 10^6 \text{ ml}^{-1} \pm 0.2$; 5: $2.9 \times 10^6 \text{ ml}^{-1} \pm 0.4$), and nearly 20% less viable (viable sperm – DEHP 0: $71.3 \pm 2.2\%$; 0.05: $56.7 \pm 5.3\%$; 5: $57.1 \pm$

3.5%). DEHP exposure compromised sperm developmental capacity but not its fertilization capacity. In tests using oocytes (a total of 404) from untreated females and *in vitro* fertilization protocols, the sperm from both the 0.05 and the 5 mg/kg · d groups resulted in zygotes with the same ability to complete first mitotic division, but with a significantly reduced capacity to reach the blastocyst stage, compared with controls ($P \leq 0.05$; Table 6).

DEHP-induced alterations in gene expression profiles of adult offspring gonads and pituitaries

Expression of steroidogenesis-related genes in the gonads

There was significant down-regulation of *cyp19a1* in the ovaries (0.05 and 5 mg/kg · d; Fig. 3A) and testes (5 mg/kg · d group; Fig. 3B). In ovaries, 5 mg/kg · d DEHP lowered the expression level of the *cyp17a1* transcript (Fig. 3A). Gene expression for *cyp19a1* was confirmed by immunoblot analysis (Fig. 3C). The progesterone receptor (*pgr*) transcript was significantly down-regulated in testes (5 mg/kg · d) and ovaries (0.05 and 5 mg/kg · d) (Fig. 3D). Finally, the mRNA levels for gonadotropin receptors, *fshr* and *lhr*, in male and female gonads were significantly down-regulated in treated animals at all doses (Fig. 4A).

Expression of gonadotropin mRNA in the pituitary

There was a dose-dependent increase in the expression of *lhβ* mRNA in treated females, whereas the expression

TABLE 5. Effect of DEHP exposure on meiotic oocyte maturation in adult female offspring

DEHP dose (mg/kg · d)	Oocytes/mouse	Immature (%)	Mature (%)	Degenerated (%)
0	35.2 ± 3.1	3.88 ± 0.6	88.0 ± 1.0 ^a	8.2 ± 1.18 ^a
0.05	36.8 ± 2.3	2.1 ± 0.5	79.8 ± 1.2 ^b	18.2 ± 0.8 ^b
5	32.8 ± 5.1	2.1 ± 0.8	80.0 ± 1.5 ^b	17.9 ± 2.2 ^b

Values are mean ± SEM. ^{a, b} Different superscripts within the same column indicate statistical differences for $P \leq 0.05$.

TABLE 6. Effect of pre- and perinatal DEHP exposure on developmental capacity of gametes from adult male and female offspring

	DEHP		
	0 mg/kg · d	0.05 mg/kg · d	5 mg/kg · d
Males			
No. of oocytes	162	138	140
Cleavage rate (%)	65.2 ± 9.3	65.0 ± 10.2	47.4 ± 18.3
Blastocyst rate (%)	43.9 ± 11.5 ^a	13.50 ± 6.5 ^b	4.4 ± 0.8 ^b
Females			
No. of oocytes	301	298	310
Cleavage rate (%)	59.63 ± 4.5 ^a	34.48 ± 4.2 ^b	63.61 ± 4.6 ^a
Blastocyst rate (%)	42.46 ± 5.6 ^a	9.03 ± 2.4 ^b	48.17 ± 3.6 ^a

Values are mean ± SEM. ^{a, b} Different superscripts within the same column indicate statistical differences for $P \leq 0.05$.

of *fshβ* mRNA did not differ between groups (Fig. 4B). The *lhβ* mRNA expression in control mice was respectively 2 and 3.5 times lower than in the 0.05 mg/kg · d and 5 mg/kg · d animals. In males, the pituitary expression of both *lhβ* and *fshβ* mRNA was significantly up-regulated only in the 5 mg/kg · d group (1.3 and 1.5 times control levels).

Discussion

To our knowledge this is the first study showing that pre- and perinatal exposure of mice to DEHP doses in the range

of the estimated general human exposure (1, 6, 29–31) induced permanent molecular and morphological pituitary-gonadal alterations, which may explain the reproductive deficiencies in both male and female offspring when they reach adult age. DEHP exposure of pregnant and lactating dams resulted in their offspring, at sexual maturity, having: 1) lower body weight; 2) altered gonad weight (lighter testis and heavier ovary); 3) poor germ cell quality; 4) low expression of steroidogenesis and gonadotropin-receptor genes in the gonads; and 5) up-regulated gonadotropin subunit gene expression in the pituitary.

In addition to these effects on the offspring's reproductive health, we observed a dramatic acute consequence of DEHP treatment on pregnant dams: complete pregnancy failure with the highest dose (500 mg/kg · d). These data are in agreement with a recent paper by Gray *et al.* (32) reporting midgestation abortions in rats upon oral exposure to high doses (500 and 1000 mg/kg · d) of phthalates. Furthermore, chronic occupational exposure to high levels of phthalates was associated with low pregnancy rates and high rates of miscarriage in factory workers (33).

In the present study, examination of conceptuses in the dams given 500 mg/kg · d showed that on dcp 10.5 embryos and their extraembryonic envelopes started to degenerate, and embryos from all treated dams were no longer viable by dpc 11.5. Vascular development in the postimplantation mouse embryo and placentation essentially begins on dpc 6.5; therefore the embryonic circulation is fully functional by dpc 12.5 (34). DEHP activates the peroxisome proliferator-activated receptors (PPAR), a family of transcription factors recently implicated in the inhibition of proliferation and differentiation of endothelial cells *in vitro* and impaired neovascularization *in vivo* (35–38). It is therefore possible that DEHP exposure in pregnant mice affects placental vascularization through activation of PPAR, leading to total pregnancy failure at high doses (500 mg/kg · d).

The low body weight of the offspring exposed to DEHP during the pre- and perinatal period agrees with previously

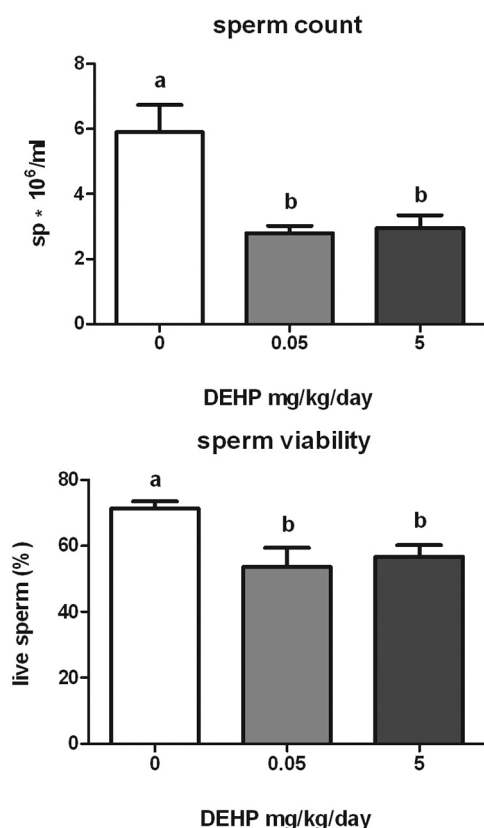


FIG. 2. Counts and viability of caudal epididymal sperm from mice treated *in utero* and during lactation with DEHP. Different superscripts denote significant differences between columns ($P < 0.05$).

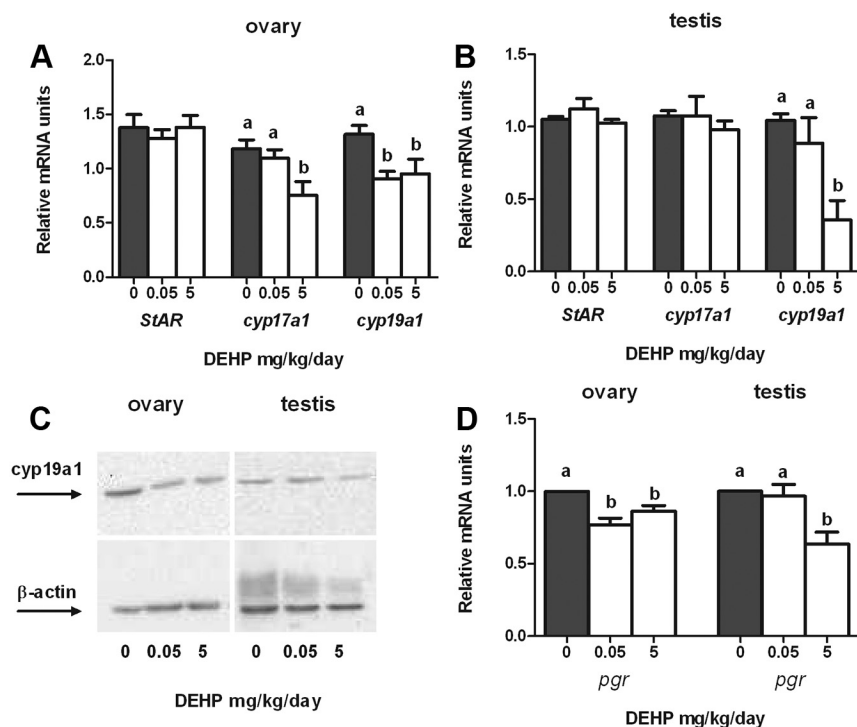


FIG. 3. A, B, and D, Effect of DEHP on the mRNA levels of target genes in the gonads of female and male mouse offspring on PND 42. Quantitative analysis of *StAR*, *cyp17a1*, *cyp19a1*, and *pgr* mRNA in the gonads exposed to DEHP during pregnancy and throughout lactation. Each column represents the mean \pm SEM of at least three separate experiments. The mRNA normalized to the endogenous reference (*gapdh*) was analyzed by RT-PCR using specific primers as described in *Materials and Methods*. Different superscripts denote significant differences between columns ($P \leq 0.05$). C, Representative immunoblot analysis of *cyp19a1* and β -actin protein from total ovary and testis lysate of adult female and male mouse offspring on PND 42.

published results. For example, mouse fetuses exposed to DEHP during gestation from dpc 0 through dpc 17 had significantly reduced body weight (39). Rats exposed to di-n-butyl phthalate during pregnancy also had low birth weight and reduced weight gain (40). Furthermore, a recent nested case-control study in humans found a close correlation with high phthalate levels in umbilical vein blood in low-birth-weight cases compared with normal-weight newborns (41).

It is noteworthy to remember that alteration in birth weight and body weight gain has been often linked with altered onset of puberty in the offspring (42, 43). Furthermore, several reports suggested that prepubertal exposure to a variety of environmental chemicals, including DEHP (for review, see Ref. 44), can hasten or delay the onset of puberty in several animal models. Therefore, despite the present study specifically aimed to analyze the effects of maternal exposure to DEHP on the functionality of pituitary-gonad axis in the offspring at adult age, further analyses would be necessary to further clarify the effects of maternal DEHP exposure on the pubertal development and add further proof of the deleterious effects of DEHP

on maturation of the pituitary-gonadal axis in the exposed progeny.

The significant weight changes of the testis and ovary in adult offspring after exposure to DEHP *in utero* and during lactation are in agreement with reports describing changes in gonad weight when DEHP was orally administered at doses similar to what we used (2, 45–49). The novelty of the present study is that the morphological changes were related to gonadal function in male and female mice. As far as we know, this is the first study showing that *in vivo* pre- and perinatal exposure to DEHP concomitantly increases ovarian weight and impairs oocyte maturation competence, reducing oocytes' ability to complete the first meiotic division, hence increasing the percentage of gametes that eventually degenerate. Furthermore, matured oocytes showed reduced developmental capacity compared with the unexposed counterpart. Recent *in vitro* studies have found impairment of meiotic maturation and developmental competence in oocytes directly exposed in culture to either DEHP or mono-(2-ethylhexyl) phthalate (50, 51), thus supporting our observations upon *in vivo* treatment. It is noteworthy to notice that, in the present study, major adverse effects were observed in the lowest investigated dose (0.05 mg/kg · d), suggesting nonmonotonic response curves and low-dose effects. This result is in agreement with recent studies reporting that treatment of rat dams with active phthalates may result in nonlinear, mainly U-shaped, dose-response curves effects in the offspring (23, 52, 53).

The mechanisms underlying phthalates' influence on oocyte quality is not yet fully understood. It is known that these compounds may disrupt ovarian estrogen biosynthesis pathways through a PPAR-mediated mechanism (21), and lower estradiol secretion from granulosa cells may be responsible for the impaired oocyte quality (54). There is evidence that *in vitro* exposure to phthalates suppresses *cyp19a1* transcript levels and reduces 17 β -estradiol production in rat (20) and human (55) granulosa cells, and that both DEHP and mono-(2-ethylhexyl) phthalate reduce 17 β -estradiol production and *cyp19a1* transcript levels, inhibiting the growth of cultured whole antral follicles from mice (56).

The results of the present study may suggest that adverse effects observed in oocyte quality may be related to

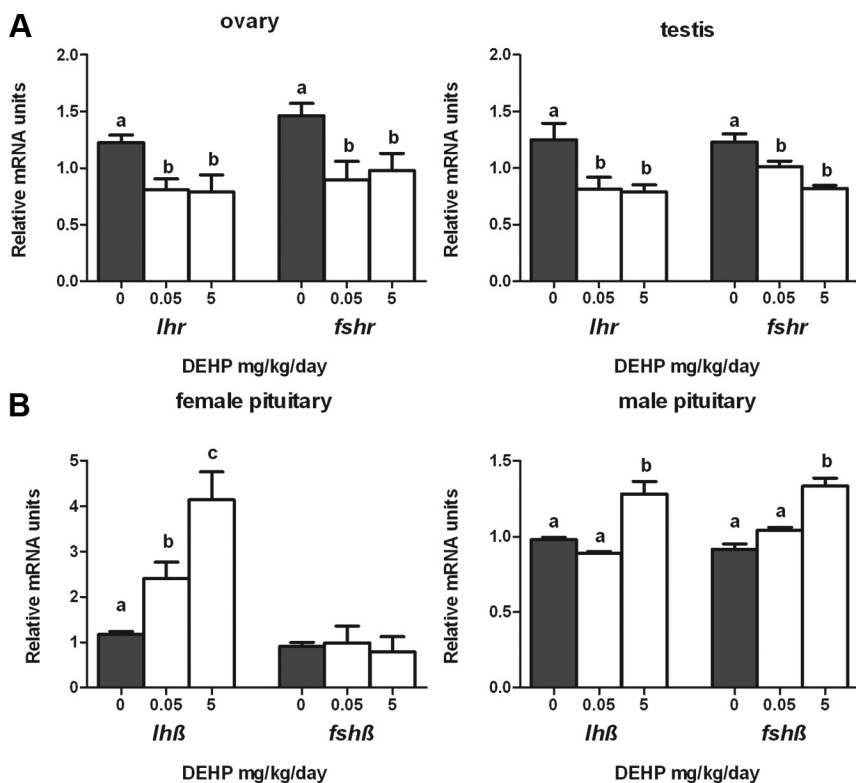


FIG. 4. Effect of DEHP on the mRNA levels of target genes on PND 42 in the pituitaries and gonads of female and male mouse offspring exposed to DEHP during pregnancy and throughout lactation. **A**, Quantitative analysis of *lhr* and *fshr* mRNA in the gonads from male and female adult offspring. **B**, Quantitative analysis of *lhβ* and *fshβ* mRNA in the pituitaries from male and female adult offspring. Each column represents the mean \pm SEM of at least three separate experiments. The mRNA normalized to the endogenous reference (*gapdh*) was analyzed by RT-PCR using specific primers as described in *Materials and Methods*. Different superscripts denote significant differences between columns ($P < 0.05$).

dysregulated steroid synthesis. In fact, together with a sub-optimal oocyte competence we found significant down-regulation of *Cyp17a1* and *Cyp19a1* gene expression in ovaries, suggesting a persistent alteration of the estrogen synthesis pathway. This is paralleled by the significantly low expression, even at the smallest DEHP dose, of the *pr* gene, a known estrogen target gene.

Interestingly, the cause-effect relationship between DEHP-induced altered expression of key transcripts involved in estrogen biosynthesis and low reproductive performance of female mice observed here may also apply to male offspring from the same litter. In fact, in testes of treated male offspring we observed decreases of *cyp19a1* and *pr* expression, with low sperm count, poor sperm viability, and reduced developmental competence. It is therefore conceivable that male mice also, exposed to phthalates pre- and perinatally, may have long-lasting altered estrogen biosynthesis in the testis that, in turn, results in disturbances of reproductive performance in adulthood.

This conclusion is supported by several recent reports. Reduced AGD and impaired testicular descent have been

observed in boys of mothers exposed to high levels of phthalates during pregnancy, which is consistent with the disruption of androgen-dependent development (16). In male rats, *in utero* exposure to phthalate upon oral administration of 500 mg/kg \cdot d to pregnant dams inhibits fetal testosterone synthesis (17). In men, impaired aromatase activity due to defective *cyp19a1* is related to low sperm concentration and motility (57) and disturbance of acrosome formation (58, 59), and also by evidence of a strong inverse association between estradiol levels and sperm DNA damage (60). It is noteworthy to notice that phthalate-induced sperm damage has been related to poorer embryo development and lower pregnancy rates among partners of men undergoing assisted reproductive treatments (61, 62), which nicely parallels the observation of the present study indicating that sperm derived from treated male offspring have reduced developmental capacity *in vitro*.

Estrogen deficiency or insensitivity in man might also result in the accumulation of fluid in efferent ductules and subsequent atrophy of the testis (63).

These data are in agreement with our findings of low testis weight in exposed males, further supporting an abnormality in the regulation of estradiol synthesis.

Although we did not measure circulating steroid hormones, poor gamete quality from exposed animals, together with the down-regulation of *cyp19a1* and *pgr* expression in adult offspring of both sexes, may suggest low serum estrogen levels, which, in turn, may affect the hypothalamus-pituitary-gonadal negative feedback mechanism. We therefore hypothesized that the reproductive health deficits in male and female mice exposed to DEHP *in utero* and during lactation, may be caused by long-lasting damage to the entire pituitary-gonadal axis. This hypothesis is further supported by the up-regulated expression levels of mRNA for gonadotropin β subunits in pituitaries of both male and female treated offspring, which may reflect attenuated negative feedback by estradiol on the pituitary. The increase in gonadotropin transcript levels observed in the present study, which, with the exception of the *lh* in the female pituitary, was relatively small, may not unequivocally produce increased serum gonadotropin levels. However, several lines of evidence

support this hypothesis. In fact, in line with our observations, it has been reported that in adult rats direct exposure by oral gavage to phthalates (1–500 mg/kg · d) enhanced the pituitary capacity to secrete LH and/or, resulting in high gonadotropin serum levels (2, 64, 65). Furthermore, a dysregulation in gonadotropin secretion is also suggested by the observed down-regulation of *fshr* and *lhr* mRNA in the testes and ovaries of DEHP-treated animals. In fact, like other polypeptide hormone receptors, gonadotropin receptors undergo down-regulation at mRNA and protein levels in response to ligands (66–69).

In conclusion, our findings suggest that in maternally exposed male and female mice DEHP acts on multiple pathways involved in maintaining steroid homeostasis. In particular, results may suggest that exposure to the action of phthalates contributes to disruption of estrogen biosynthesis pathways in both male and female gonads and leads to imbalance of pituitary-gonadal cross talk. This endocrine interference during critical windows of reproductive development would impair gonad function and gamete quality when the offspring reaches adulthood.

Nonetheless, DEHP might have altered offspring reproduction by affecting other pituitary gonadal cross talk mechanisms, such as the activin/inhibin pathway. The equilibrium between activin and inhibin is a well known physiological system regulating gonadal function and gamete quality, including *cyp19a1* expression (70, 71). Furthermore, developmental exposure to phthalate has been shown to affect inhibin expression in the rat testis (72). To expand our knowledge on the molecular mechanisms underlying the DEHP-induced effects observed in the present study, further investigation is necessary.

The doses we employed were within the range of environmental exposure levels in humans. Obviously, mouse data, due to the known species differences in metabolism and sensitivity to exogenous chemicals, must be assessed very carefully before being extrapolated to the human. However, because pathways leading to ovarian hormone production are similar in rodents and humans and phthalates can cross the placenta in both species, our observations of the inhibitory effect of DEHP on estrogen production and, in turn, on reproductive performance, may give reason for concern.

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