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A dose–response study following *in utero* and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): Effects on androgenic status, developmental landmarks and testicular histology in male offspring rats

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Abstract

An extensive dose–response study following *in utero* and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP) was conducted. A wide range of low and high DEHP doses were tested. Reproductive effects were evaluated on male offspring rats. Female Wistar rats were treated daily with DEHP and peanut oil by gavage from gestation day 6 to lactation day 21 at doses of 0.015, 0.045, 0.135, 0.405 and 1.215 mg DEHP/kg body weight (bw)/day (low doses) and at 5, 15, 45, 135 and 405 mg DEHP/kg bw/day (high doses). Nipple retention and reduced anogenital distance, both sensitive markers of anti-androgenic effects during development, were only seen in males exposed to the highest dose (405 mg/kg/day). Delayed preputial separation was observed in animals exposed to 15 mg DEHP/kg/day and higher doses. Histopathological examination of the testis on postnatal days (PNDs) 1 and 22 revealed changes at 135 and 405 mg DEHP/kg/day. The most prominent finding on PND 1 was the presence of bi- and multinucleated gonocytes. On PND 22 signs of reduced germ cell differentiation in seminiferous tubules of exposed animals were observed. Testis weight on PND 22 was significantly increased at 5, 15, 45 and 135 mg/kg/day, an effect that qualitatively differs from exposure to higher doses. The current results show that DEHP acts as an anti-androgen at a high dose exposure (405 mg/kg/day). However, these results also indicate that other subtle developmental effects occur at lower DEHP doses.

Keywords: Di-(2-ethylhexyl) phthalate (DEHP); Male offspring rats; Dose–response; Development; Endocrine disruptors

1. Introduction

Several chemicals present in the environment have the potential to interfere with the normal function of the endocrine system. While there is good agreement that these compounds may induce reproductive, developmental and behavioral changes at high doses in experimental animals, discussion still persists whether low (human and environmentally relevant) doses may also contribute to the induction of disorders in humans and wildlife (Daston et al., 2003; WHO, 2002).

Phthalate esters have recently attracted special attention of the scientific community, regulatory agencies and

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the general public as a consequence of their high production volume, widespread use and possible endocrine-related effects (Gray et al., 2000; Kavlock et al., 2002; Moore et al., 2001; Mylchreest et al., 2002). They are economically important chemicals that are added to plastic products of polyvinyl chloride (PVC) to impart flexibility and durability (ATSDR, 2002; Shea, 2003). Phthalates can easily leach out to contaminate the external environment because they are not covalently bound to the plastic matrix or to other chemicals in formulations (Bosnir et al., 2003; Petersen and Breindahl, 2000). Recent biomonitoring studies in the USA and Europe have detected unexpectedly high levels of phthalate metabolites (monoesters) in the urine of the general population (Koch et al., 2003, 2004; Silva et al., 2004). Di-(2-ethylhexyl) phthalate (DEHP) is currently the most commonly used phthalate accounting for approximately 50% of the market for PVC plasticizers in the European Union countries (SCMPMD, 2002). The median daily intake of DEHP in the German general population was recently estimated at 13.8 μg/kg/day (Koch et al., 2003). The major route of exposure is the ingestion of contaminated food and water. DEHP is able to cross the placenta and pass into breast milk, and, therefore, exposure during gestational and lactational periods is of particular concern (Dostal et al., 1987; Latini et al., 2003; Shea, 2003).

Phthalates are known reproductive toxicants in mammals. However, it was only recently that transgeneration studies demonstrated the ability of these compounds to disrupt the sexual differentiation of the male fetus (Gray et al., 1999, 2000; Mylchreest et al., 1999, 2000). The spectrum of effects induced by perinatal exposure to active phthalates like di(butyl) phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP) are believed to arise from disruption of Sertoli and Leydig cell functions in the developing testis (Fisher et al., 2003; Sharpe, 2001). When exposed to high doses, male offspring rats display reduced anogenital distance, retained nipples, undescended testis, hypospadias, small accessory sex glands and epididymal and testicular abnormalities (Gray et al., 2000; Moore et al., 2001; Mylchreest et al., 1999). A number of these reproductive tract anomalies are characteristic of disturbances in androgen-dependent development. In addition, disturbance of normal gonocyte development resulting in multinucleated cells and subsequent changes in testicular function (e.g., reduced sperm production) have been associated with abnormal Sertoli cell function and proliferation (Dostal et al., 1988; Li et al., 2000; Fisher et al., 2003).

Even though developing organisms are considered particularly vulnerable to phthalate exposure, there are still significant gaps in the dose–response data of DEHP during the pre- and early postnatal periods. For instance, there is currently no published data on the effects of developmental exposure to DEHP at low (human relevant) levels of exposure. The present work is part of a comprehensive dose–response study aimed to evaluate the effects of DEHP on the reproductive development of male and female rat offspring exposed *in utero* and during lactation. We used two wide ranges of doses, which included dose levels relevant for human exposure as well as high doses typically used in toxicological studies to address the question whether qualitative differences in response occur between the low and high dose regions. In a recent study, we investigated the effects of DEHP on sexual development of females, where the main finding was a significant delay in the onset of puberty (age at vaginal opening) at doses as low as 15 mg DEHP/kg body weight (bw)/day (Grande et al., 2006). Here, we evaluated the sexual development of male offspring from birth until puberty including the investigation of androgen sensitive endpoints, sexual developmental landmarks and testicular histology.

2. Materials and methods

2.1. Animals, dose selection and treatment

Animal husbandry was described in detail by Grande et al. (2006). The experimental protocol was approved by the Office for Work and Health Protection and Technical Safety of the State of Berlin in accordance with the German National Animal Protection Law (Tierschutzgesetz BGBI. I S. 1105, 1998). Wistar rat dams were administered DEHP (Sigma–Aldrich Chemie GmbH, Schnelldorf, Germany, lot no. S11126-334) or peanut oil (Bombastus-Werke AG, Freital, Germany) by daily gavage from day 6 of gestation (mating = day 0) to day 21 of lactation. The dosing volume used was 5.0 ml/kg body weight. Two wide ranges of doses, low and high, were used. The low and the high doses were respectively 0.015, 0.045, 0.135, 0.405 and 1.215 mg DEHP/kg bw/day, and 5, 15, 45, 135 and 405 mg DEHP/kg bw/day. One group of animals received only peanut oil and served as vehicle control. A total number of 11–16 rat dams (litters) per group were used (Table 1). The low-dose range was selected starting from a dose (0.015 mg/kg/day) similar to the estimated median daily intake of the general German population (0.0138 mg/kg/day) reported by Koch et al. (2003). Four additional doses were calculated by applying a space factor of three between doses. The high-dose range was chosen starting from 5 mg/kg/day and with a space factor of three, so that the highest level (405 mg/kg/day) would be a dose known to induce reproductive adverse effects in male offspring rats without causing overt maternal toxicity (Moore et al., 2001). Concentration of all doses was verified by gas chromatography/mass spectrometry (Hewlett Packard 5860/5971A, Palo Alto, CA).

Table 1
Organ weights of rat offspring exposed to DEHP during pregnancy and lactation

Parameter	Control	Maternal DEHP dose (mg/kg/day)									
		Low dose					High dose				
		0.015	0.045	0.135	0.405	1.215	5	15	45	135	405
Total number of litters	16	11	13	13	15	16	13	12	11	14	12
PND 1											
No. of pups (no. of litters)	21 (16)	17 (11)	19 (12)	19 (12)	20 (13)	26 (16)	13 (11)	15 (11)	15 (10)	17 (12)	16 (10)
Body weight (g)	6.2 ± 0.14	6.2 ± 0.17	6.8 ± 0.20*	6.3 ± 0.12	6.5 ± 0.17	7.0 ± 0.13*	6.7 ± 0.24*	6.6 ± 0.12	6.6 ± 0.15	6.3 ± 0.15	6.2 ± 0.17
Liver weight (mg)	265 ± 7.2	259 ± 4.5	271 ± 5.5	258 ± 6.1	259 ± 7.9	281 ± 4.5	288 ± 10.7	263 ± 6.6	273 ± 9.1	290 ± 6.4*	299 ± 6.1*
Brain weight (mg)	247 ± 5.3	245 ± 5.0	255 ± 6.0	252 ± 4.3	252 ± 5.9	265 ± 3.5	264 ± 6.7	262 ± 4.2	260 ± 5.6	259 ± 3.6*	247 ± 4.8
PND 22											
No. of pups (no. of litters)	18 (13)	13 (8)	12 (9)	18 (11)	18 (11)	20 (14)	11 (8)	15 (10)	12 (9)	18 (11)	15 (8)
Body weight (g)	50.6 ± 0.8	49.0 ± 1.4	48.2 ± 1.9	47.9 ± 1.5	48.0 ± 1.3	52.9 ± 0.9	49.3 ± 1.9	48.2 ± 1.6	51.0 ± 1.8	51.2 ± 1.1	46.3 ± 2.1
Liver weight (g)	1.92 ± 0.05	1.93 ± 0.10	1.95 ± 0.11	1.87 ± 0.04	1.89 ± 0.07	2.13 ± 0.06	1.96 ± 0.09	1.90 ± 0.07	2.00 ± 0.09	1.97 ± 0.08	1.76 ± 0.10[a]
Brain weight (g)	1.43 ± 0.02	1.45 ± 0.02	1.45 ± 0.02	1.39 ± 0.02	1.43 ± 0.01	1.44 ± 0.01	1.41 ± 0.02	1.39 ± 0.01	1.44 ± 0.01	1.44 ± 0.01	1.36 ± 0.03[a]
Epididymis weight (mg)	17.4 ± 0.66	18.7 ± 0.46	16.3 ± 0.97	17.4 ± 0.54	17.5 ± 0.61[b]	18.6 ± 0.56	17.2 ± 0.56	18.3 ± 0.51	18.7 ± 0.85	19.0 ± 0.58	17.4 ± 0.68[a]

Values are presented as means ± S.E. In the statistical analysis of all data, the litter was included in the mixed model as a random, nested factor (within treatment). Body weight was used as a covariate in the analysis of organ weights.

[a] $N = 13$ (7).

[b] $N = 17$ (11).

* Significantly different from control group ($p < 0.05$).

Because of the large number of animals involved, the study was conducted in two blocks, each comprising approximately half of the total number of dams per dose. Maternal weight was monitored daily throughout pregnancy and lactation. Dams were killed by decapitation and their pups weaned on postnatal day (PND) 22 (PND 22).

The low DEHP doses used are considered relevant to human exposure because they range from the estimated daily intake of the general population to doses that may occur in particular exposure scenarios, such as those of critically ill patients undergoing medical treatments and neonates in intensive care units (Koch et al., 2003, 2004, 2006; Latini, 2005; Silva et al., 2004). In a pilot study conducted by Koch et al. (2006), the daily DEHP intake of 45 neonates, who were treated with various medical procedures, was calculated. The median and 95th percentile was 0.042 and 1.780 mg DEHP/kg bw/day, respectively, and the maximum calculated intake was 2.3 mg DEHP/kg bw/day.

2.2. Offspring data

On PND 1 (day of delivery) the number of live and dead pups, weight, sex and signs of general toxicity were recorded. At this age, one or two male pups per litter were randomly selected and necropsied (litters with less then three male pups were not used at this age). The right testis was removed and stored at −80 °C for later analysis of intratesticular testosterone concentration. The left testis of six pups per group (from different litters) was removed and immediately placed in Bouin's fixative solution for 1 h. Brain and liver weights of all animals were recorded as well. On PND 13 all male pups were examined for the presence of nipples/areolas (dark focal areas lacking hair with or without nipple bud) by a single investigator unaware of treatment groups. On PND 22 (weaning), one to three male pups per litter were randomly selected for measurement of the anogenital distance (AGD) and necropsy (litters with less then three male pups were not used at this age). The AGD was measured with a manual caliper by a single investigator in a blinded manner. Following the AGD measurements, pups were killed by decapitation and the brain, liver, testis and epididymis were excised and weighed. Testicular position was recorded after opening the abdominal cavity. The left testis of six pups per group (from different litters) was removed and immediately placed in Bouin's fixative solution for 24 h.

The age at testis descent and preputial separation were examined as landmarks of male sexual development. Beginning on PND 15 pups were daily examined for the age at testis descent by scrotum palpation. The age at preputial separation was monitored daily by manual retraction of the prepuce starting on PND 33 and the external genitalia were examined for malformations.

2.3. Intratesticular testosterone

The effects of DEHP on testicular testosterone levels were investigated in male offspring on PND 1. Testes from individual newborn rats were homogenized with 100 μl of phosphate buffered saline containing 0.1% gelatine (PBS-gel) and extracted three times with a total volume of 1.5 ml of diethyl ether (Mylchreest et al., 2002; Parks et al., 2000). The ether fraction was poured off into clean tubes and the three extractions pooled and evaporated under nitrogen. Extracts were resuspended in 500 μl of PBS-gel and analyzed by radioimmunoassay (RIA) with a commercially available kit (Diagnostic Products Corporation, Los Angeles, USA). Two hundred and fifty microliters of each standard of the kit were extracted as described above except that the extracts were resuspended in 250 μl of PBS-gel. Intra- and inter-assay coefficients of variation were 7.5 and 12%, respectively.

2.4. Testicular histology

After fixation in Bouin's solution, testes collected on PNDs 1 and 22 were transferred to 70% ethanol, embedded in paraffin and cut at 5 and 3 μm, respectively. Sections were stained with hematoxilin and eosin and evaluated by light microscopy. Histopathological evaluation was performed by an experienced investigator blind to treatment group. In addition, the diameter of seminiferous tubules on PND 22 was measured in 15 round tubular sections per animal ($n = 6$ rats/group) at 100× magnification using the Scion image software version 1.62 (Scion Corporation, Frederick, MD).

2.5. Statistical analysis

Statistical analysis was conducted using either SPSS 12.1 (SPSS Inc., Chicago, IL) or SAS 9.1 (SAS Institute Inc., Cary, NC). Normality and homogeneity of variances were evaluated prior to data analysis. We used a linear mixed model (proc mixed) with treatment as a main effect and litter as a random factor (nested for treatment) to adjust for litter effects. In addition, block was included as an additional fixed factor in the model, because the experiment was conducted in two sets. Organ weights and anogenital distance were analyzed with body weight as a covariate. When an overall treatment effect (F-test) was observed, post hoc comparisons were performed using least square means (LSMEANS). The proportion of animals and litters with nipple retention was analyzed by Fisher's exact test and the proportion of animals with complete preputial separation and descended testes was analyzed by chi-square. Differences were considered to be statistically significant at a probability level of 5% ($p < 0.05$).

3. Results

3.1. Maternal data

Maternal and reproductive outcome data were presented elsewhere (Grande et al., 2006). Briefly, DEHP had no significant effect on maternal weight gain, litter size, sex ratio and number of viable pups at any dose tested. Moreover, no signs of general toxicity were detected in dams and offspring.

3.2. Offspring data

In the males selected for necropsy on PND 1, we observed a significant increase in liver weight at 135 and 405 mg DEHP/kg/day (Table 1). Body weight was slightly increased in the 0.045-, 1.215- and 5-mg/kg/day groups (Table 1). Brain weight was similar in all groups with exception of an increase observed at 135 mg/kg/day. On PND 22, no effects were seen in body, liver and brain weight (Table 1). Testis weight at this age was significantly increased in the 5-, 15-, 45-, and 135-mg/kg/day doses, but not in the 405 mg/kg/day group, which showed a trend towards reduction (not statistically significant) (Fig. 1). Moreover, increased testis weight was of borderline significance at 0.405 and 1.215 mg/kg/day ($p = 0.05$ and $p = 0.06$, respectively; LSMEANS). The diameter of seminiferous tubules measured on PND 22 followed the same pattern observed for testis weight. However, no significant differences were detected (overall treatment effect: F-test = 1.91; $p = 0.07$) (Fig. 1). Undescended testis was not observed at any dose tested. No changes were observed in epididymis weight as well (Table 1).

Anogenital distance (AGD) measured on PND 22 was significantly reduced in animals exposed to the highest dose (405 mg/kg/day) and slightly increased in the 0.015 mg/kg/day group (Fig. 2). Nipple retention in males on PND 13 was not observed in controls and in any treatment group with the exception of animals exposed to 405 mg DEHP/kg/day. Presence of nipples was sig-

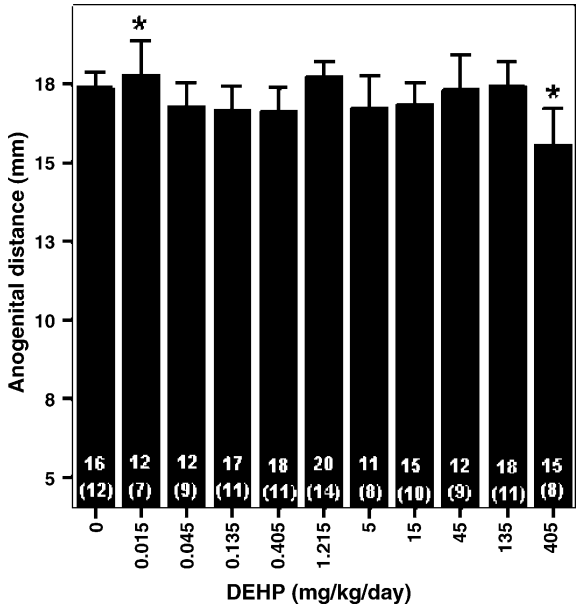

Fig. 2. Anogenital distance of male offspring rats (PND 22) exposed *in utero* and during lactation to peanut oil (vehicle control) or DEHP. Bars indicate mean ± S.E. Statistical analysis was performed with body weight as a covariate. The number of pups is indicated in each bar and the number of litters is in parenthesis. In the statistical analysis, the litter was included in the mixed model as a random, nested factor (within treatment). * Significantly different from control group ($p < 0.05$).

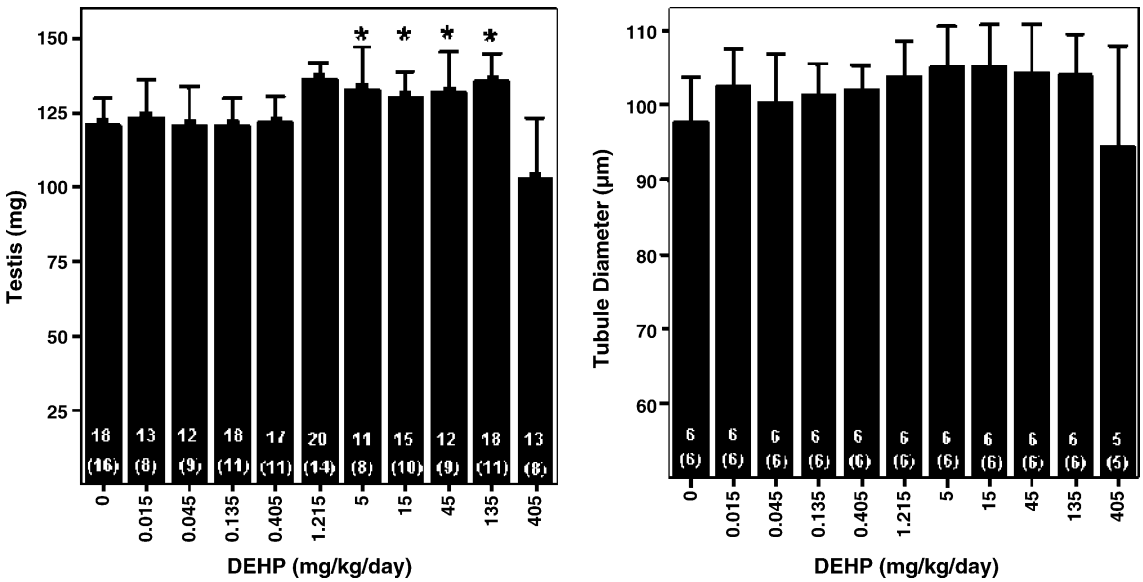

Fig. 1. Effects of *in utero* and lactational DEHP exposure on testis weight and seminiferous tubule diameter of male offspring rats on PND 22. Bars indicate mean ± S.E. Testis weight was analyzed with body weight as a covariate. The number of pups is indicated in each bar and the number of litters is in parenthesis. In the statistical analysis of testis weight, the litter was included in the mixed model as a random, nested factor (within treatment). For tubular diameter, 5–6 animals from different litters were used. * Significantly different from control group ($p < 0.05$).

nificant at this dose when analyzed both as incidence in individual animals and as incidence in litters (Table 2).

The testicular testosterone content measured in neonatal testis of DEHP exposed rats was not significantly different from controls at any dose tested (Table 2).

3.3. Testicular histology

Examination of testes by light microscopy revealed histopathological alterations on PNDs 1 and 22 in the two highest dose groups (135 and 405 mg/kg/day) (Table 3). The most prominent finding on PND 1 was the presence of bi- and multinucleated (enlarged) gonocytes, which frequently showed signs of degeneration (Fig. 3B). This was most evident in the 405 mg/kg/day group, where it was present in all testes examined and the incidence ranged from sporadic degenerating multinucleated cells in one animal to one or more multinucleated cells in most seminiferous cords (three animals). In the 135 mg/kg/day group, such cells occurred in all but one animal, but to a much lesser degree compared to 405 mg/kg/day. At both doses there was occasional acute interstitial hemorrhage and loosening of connective tissue as well. No similar changes were seen in lower doses or controls, with the exception of one binuclear gonocyte in one animal of the 5 mg/kg/day group. On PND 22 we observed signs of reduced germ cell differentiation in seminiferous tubules of exposed animals. In opposition to normal tubules, which displayed stratified multiple layered germ cells in various stages of differentiation, affected tubules showed only one or two layers of fairly homogeneous cells, most likely gonocytes accompanied by Sertoli cells (Fig. 3D). This change was seen in three out of five animals of the 405 mg/kg/day group, varying from singly affected tubules (one animal) to foci of several tubules (two animals). In one animal this was accompanied by focal hyperemia. In addition, affected tubules often showed reduced to absent tubular lumen and reduced diameter. A slight reduction of germ cell layers and increased homogeneity of cells were seen in another animal of the 405 mg/kg/day group and in three animals of the 135 mg/kg/day group. It is important to note, however, that even in animals that showed the most prominent changes, the majority of the tubules appeared not to be affected.

3.4. Age at testis descent and preputial separation

We analyzed testis descent and preputial separation by chi-square because these sexual developmental landmarks are categorical variables. No significant changes

Table 2
Effects of developmental exposure to DEHP on intratesticular testosterone (PND 1), nipple retention (PND 13) and body weight at preputial separation in male offspring rats

Parameter	Control	Maternal DEHP dose (mg/kg/day): low dose				
		0.015	0.045	0.135	0.405	1.215
Intratesticular testosterone (ng/testis)	1.59 ± 0.50 (18/15)	0.91 ± 0.34 (12/10)	2.00 ± 0.56 (11/11)	1.34 ± 0.44 (13/11)	1.38 ± 0.36 (15/13)	1.57 ± 0.43 (18/16)
Animals with nipples/total animals	0/60	0/45	0/46	0/54	0/58	0/63
Litters with nipples/total litters	0/16	0/11	0/13	0/13	0/15	0/16
Body weight at preputial separation (g)	135 ± 1.2 (42/16)	130 ± 1.9 (32/11)	135 ± 1.8 (34/13)	125 ± 1.7* (36/13)	125 ± 2.0* (40/15)	130 ± 1.1 (43/16)
Parameter	Control	Maternal DEHP dose (mg/kg/day): high dose				
		5	15	45	135	405
Intratesticular testosterone (ng/testis)	1.59 ± 0.50 (18/15)	1.23 ± 0.31 (12/11)	1.64 ± 0.26 (13/11)	1.74 ± 0.55 (12/11)	1.15 ± 0.37 (13/11)	1.32 ± 0.40 (10/9)
Animals with nipples/total animals	0/60	0/42	0/50	0/41	0/56	13/41*
Litters with nipples/total litters	0/16	0/13	0/12	0/11	0/14	5/12*
Body weight at preputial separation (g)	135 ± 1.2 (42/16)	133 ± 1.8 (31/13)	134 ± 1.7 (35/12)	130 ± 1.4 (29/11)	132 ± 1.4 (38/14)	123 ± 2.3* (27/12)[a]

Intratesticular testosterone and body weight at preputial separation are presented as means ± S.E. In the statistical analysis of all data, the litter was included in the mixed model as a random, nested factor (within treatment). Values in parentheses indicate (number of animals/number of litters) used. Nipple retention in males was analyzed both as incidence in individual pups and as incidence in litters.

[a] Body weight of one animal in this group was not recorded.

* Significantly different from control group ($p < 0.05$).

Table 3
Histopathological alterations in testes of newborn and weaning rats exposed *in utero* and during lactation to DEHP

Parameter	Control					Maternal DEHP dose														
						0.015–45 mg/kg/day					135 mg/kg/day					405 mg/kg/day				
Grade	−	(+)	+	++	+++	−	(+)	+	++	+++	−	(+)	+	++	+++	−	(+)	+	++	+++
PND 1																				
Presence of bi- and multinucleated gonocytes	6	0	0	0	0	6	0	0	0	0	1	3	2	0	0	0	1	0	2	3
Elevated rate of degenerated gonocytes	6	0	0	0	0	6	0	0	0	0	2	0	4	0	0	0	0	4	2	0
Loose connective tissue in the interstitium	6	0	0	0	0	6	0	0	0	0	4	2	0	0	0	1	3	2	0	0
PND 22																				
Signs of reduced germ cell differentiation	6	0	0	0	0	6	0	0	0	0	3	3	0	0	0	1	1	1	1	1
Focal hyperemia	6	0	0	0	0	6	0	0	0	0	6	0	0	0	0	4	0	1	0	0

Six animals per dose level and age from different litters were examined (five animals in the 45 mg/kg/day group on PND 1 and in the 405 mg/kg/day group on PND 22). Histopathological changes were detected only at the two highest dose levels tested (one binuclear gonocyte was detected in one animal of the 5 mg/kg/day group). The table shows the number of affected animals in each category: −, not observed; (+), very slight; +, slight; ++, moderate; +++, severe.

in the age at testis descent were detected at any dose tested (Fig. 4). However, a significant delay in the age at preputial separation was observed in the 15-, 45-, 135- and 405-mg/kg/day groups (Fig. 4). Body weight at preputial separation was mostly unchanged and significant differences (decreased body weight) were only detected at 0.135, 0.405 and 405 mg/kg/day (Table 2). We did not observe any malformations of the external genitalia (e.g., hypospadias) or incomplete preputial separation at any dose tested.

4. Discussion

In the current study we evaluated the effects of *in utero* and lactational exposure to low (human relevant) and high doses of DEHP on sexual development of male offspring rats. Delayed onset of puberty (preputial separation) and testicular changes were the most sensitive endpoints affected. We observed a significant delay in the age at preputial separation in the groups exposed to 15, 45, 135 and 405 mg DEHP/kg/day. Previously, delayed preputial separation was reported in rats exposed *in utero* to di-butyl phthalate (DBP) at 100, 250 and 500 mg/kg/day (Mylchreest et al., 1999). Notwithstanding, this result was not confirmed in a subsequent study by the same group (Mylchreest et al., 2000). In relation to DEHP, prior studies did not observe any significant change in this endpoint after *in utero* (Gray et al., 1999, 2000) or *in utero* and lactational (Moore et al., 2001) exposure to high doses (375–750 mg/kg/day). However, a recent unpublished multigeneration continuous breeding study (as reviewed by NTP-CERHR (2005)) presented results that are in agreement to those of our investigation. In this study, the age at preputial separation was significantly delayed by approximately four days at 7500 ppm DEHP (estimated intake of 392–592 mg/kg/day) and by 11 days at 10 000 ppm (543–775 mg/kg/day) in F_1 animals (NTP-CERHR, 2005). In our results, the observed delay in the age at preputial separation ($\geq$15 mg DEHP/kg/day) seems to be unrelated to changes in the androgenic status, as other androgen-dependent endpoints (nipple retention and AGD) were not affected at these doses, with the exception of the highest dose group (405 mg/kg/day). Interestingly, when female littermates were evaluated for vaginal opening a significant delay was observed at the same doses causing delayed preputial separation in males (Grande et al., 2006). This finding was also observed in the multigeneration study reviewed by NTP-CERHR (2005).

Testes of newborn and weaning rats revealed histopathological changes at 135 and 405 mg/kg/day. The main effect observed on PND 1 was the presence of bi- and multinucleated (enlarged) gonocytes presenting signs of degeneration. This effect was previously described in fetal and neonatal testes of rats exposed to DBP (Fisher et al., 2003; Mylchreest et al., 2002) and DEHP (Gray et al., 2000; Li et al., 2000; Parks et al., 2000) and is believed to be associated with abnormal Sertoli cell function and disruption of Sertoli-germ cells

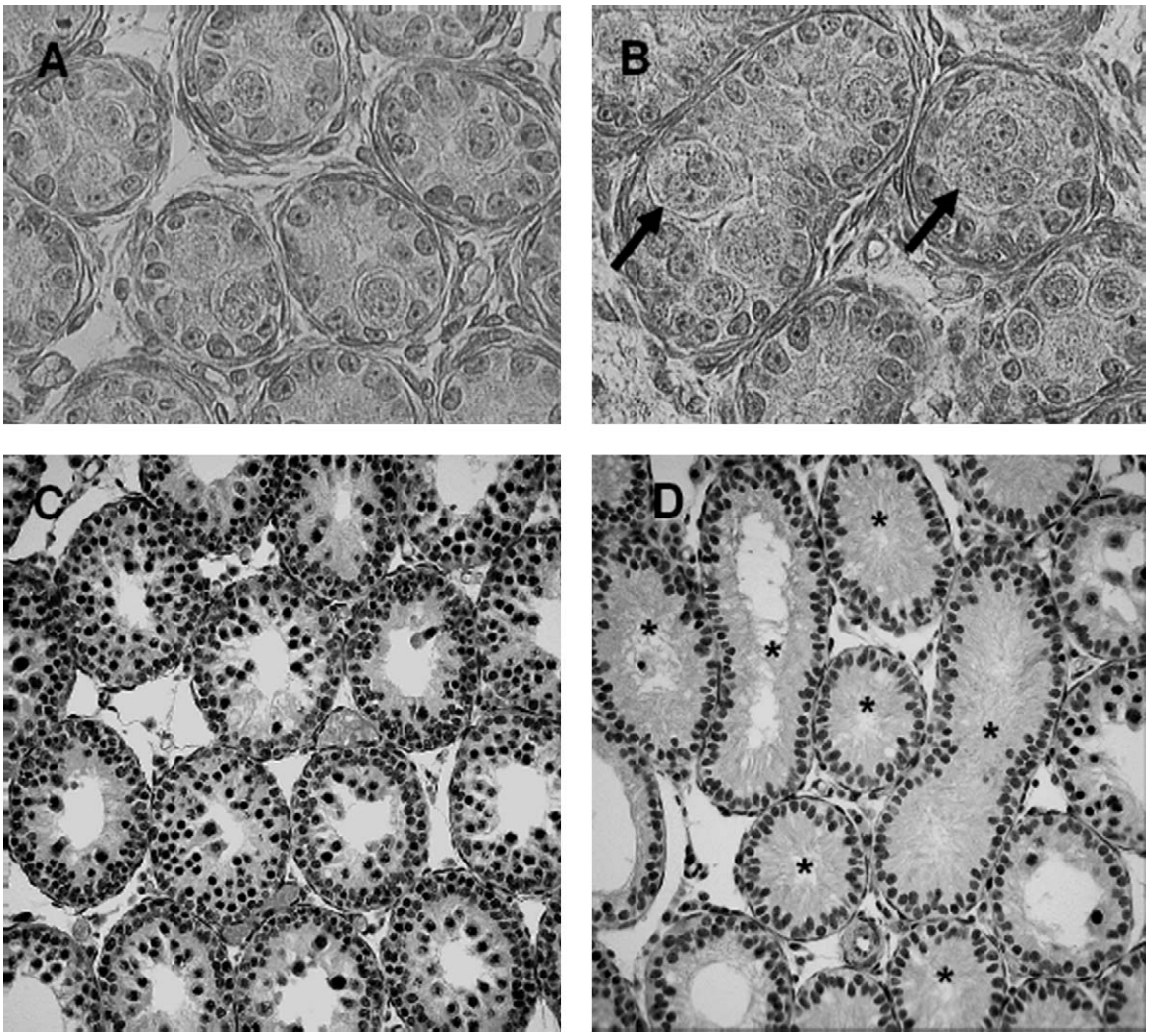

Fig. 3. Photomicrographs of testicular sections of rat offspring of dams exposed to peanut oil (A, C) or 405 mg DEHP/kg/day (B, D) during gestation and lactation. (A) Testicular section of a control rat on PND 1. Note the presence of gonocytes with single nucleus. (B) Testicular section of a treated animal on PND 1 containing multinucleated (enlarged) gonocytes (arrows). (C) Testicular section of a control rat on PND 22 showing tubules with multiple layered germ cells in various stages of differentiation. (D) Testicular section of a treated animal showing tubules with reduced germ cell differentiation (asterisks). Note that tubular lumen is reduced or absent in affected tubules. 400× magnification (A, B); 200× magnification (C, D).

interactions. Such changes could also be responsible for the signs of reduced germ cell differentiation detected on PND 22. The observed reduction/absence of tubular lumen in affected tubules provides further evidence of abnormal Sertoli cell function, as lumen formation is directly related to apical fluid secretion by Sertoli cells (Russell et al., 1989). Surprisingly, testis weight on PND 22 was significantly increased at 5, 15, 45 and 135 mg/kg/day and was also increased with borderline significance at 0.405 and 1.215 mg/kg/day. Although not statistically significant, a reduction in testis weight was observed in the 405 mg/kg/day group which is in agreement with other reports at high DEHP doses (Gray et al., 2000; Moore et al., 2001). Moreover, the diameter of seminiferous tubules followed the same pattern observed for testis weight. However, no significant differences were noted. Even though we did not find any histopathological alterations associated with increased testis weight (e.g., tumors, edema, and inflammation), it was a consistent change detected in at least four out of 10 doses tested. It is important to note that the effect on testis weight at the lower doses is qualitatively different from observations normally seen at high doses, where affected animals display reduced testis weight and tubular atrophy (Gray et al., 2000; Moore et al., 2001). In the *in utero* and lactational study conducted by Moore et al. (2001),

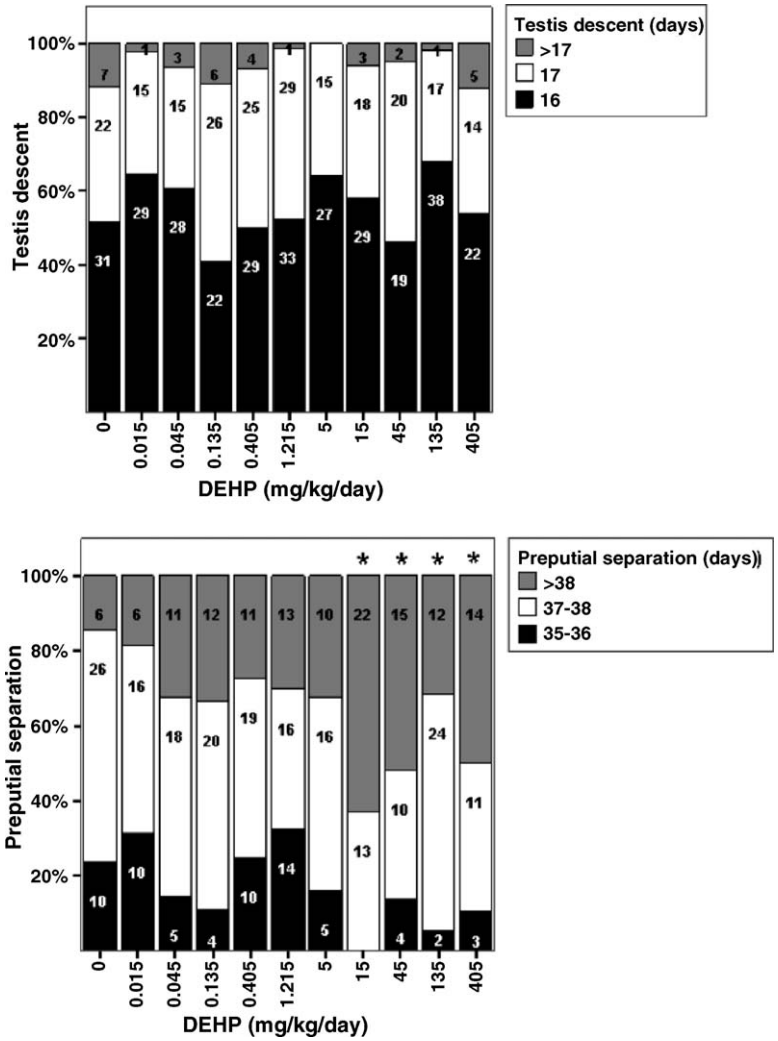

Fig. 4. Effects of DEHP on age at testis descent and preputial separation in male rats exposed *in utero* and during lactation. Bars indicate the percentage of animals displaying descended testis and complete preputial separation in the assigned categories. * Statistically different from control $p < 0.05$ (chi-square). Preputial separation and testis descent were investigated in all pups from all litters. Number of pups is indicated inside bars.

testis weight on PND 21 was significantly reduced at 375, 750 and 1500 mg/kg/day, but lower doses were not investigated. Our results indicate a non-monotonic (biphasic) response for testis weight and illustrate the importance of evaluating a wide range of doses in descriptive animal toxicity tests, where the shape of the dose–response curve is determined by complex toxicokinetic and toxicodynamic processes (Conolly and Lutz, 2004).

Reduced anogenital distance and nipple retention, both sensitive indicators of anti-androgenic effects during development, were only observed in males exposed at the highest DEHP dose (405 mg/kg/day). We also found a significant increase in AGD at 0.015 mg/kg/day, which is probably unrelated to treatment as no similar changes were observed at any other low dose tested. However, we cannot rule out the possibility that this effect occurs at even lower doses which were not evaluated. In the *in utero* and lactational study conducted by Moore et al. (2001), the lowest observed adverse effect levels (LOAEL) for nipple retention and reduced AGD were 375 and 750 mg/kg/day, respectively, giving a LOAEL of 375 mg/kg/day for the study. A recent work by Swan et al. (2005) provided the first evidence of an association between altered anogenital distance in human infants and exposure to phthalates. In a regression analysis, the authors described a negative relationship between concentration of phthalate metabolites in maternal urine (including DEHP oxidative metabolites) and the anogenital index (weight-adjusted AGD) in male infants. These results indicate that humans may be more sensitive to

prenatal phthalate exposure than rodents, although AGD results in larger human populations are necessary to confirm this suspicion.

It has been previously demonstrated that *in utero* exposure to high doses of DEHP (300–750 mg/kg/day) and other phthalates significantly decreases the testicular testosterone content in the fetal testis (Borch et al., 2004, 2006; Lehmann et al., 2004; Mylchreest et al., 2002; Parks et al., 2000). In the present study, however, no changes were detected in the levels of intratesticular testosterone of newborn rats (PND 1), although anti-androgenic effects (nipple retention and reduced anogenital distance) were observed at the highest dose (405 mg/kg/day). It is important to note, however, that in male rats, testosterone surges markedly during late gestation and again following parturition (Baum et al., 1988; Ward et al., 2003). The prenatal surge starts approximately on gestation day 16 and persists for approximately 3–4 days reaching a peak on days 18–19 of gestation (Ward et al., 2003). In contrast, the postnatal peak is much briefer, occurring during the first few hours after birth. Thus, it is possible that the DEHP-induced decrease in testosterone production occurs primarily during late gestation. On the other hand, the large but brief testosterone surge after birth may add significant variability to the data and impair the detection of small changes in testosterone levels in newborn rats (Baum et al., 1988).

To the best of our knowledge, this is the first comprehensive dose–response study for the effects of DEHP on male reproductive development that incorporated a wide range of low (human relevant) and high doses. A main advantage in using such a wide spectrum of doses is that we were able to characterize qualitative differences in the dose–response data and distinguish consistent changes across doses (e.g., our results from testis weight) from effects that may occur by chance. Overall, our results confirm previous observations that DEHP acts as an anti-androgen at high doses but also indicate that other subtle developmental effects (e.g., delayed preputial separation and increased testis weight) occur at lower doses. Histopathological alterations of the testis were evident at both time points investigated, but limited to the two highest dose groups tested (135 and 405 mg/kg/day). We also observed a significant increase in testis weight on PND 22 in animals exposed to 5, 15, 45 and 135 mg/kg/day, an effect that qualitatively differs from higher dose exposures. The no observed adverse effect level (NOAEL) for the endpoints evaluated up to puberty was 1.215 mg DEHP/kg bw/day, which is approximately four times lower than the NOAEL of 5 mg DEHP/kg bw/day used by the European Union (EFSA, 2005). This is a cause of concern because according to recent studies (Koch et al., 2006; Koo and Lee, 2005) the current level of DEHP exposure in some individuals of the general population already exceeds the USA reference dose (0.020 mg DEHP/kg bw/day) and EU tolerable daily intake (0.050 mg DEHP/kg bw/day: derived from the currently accepted NOAEL of 5 mg/kg/day) values. In addition, particular concern exists for critically ill patients, including neonates in intensive care units, who may be exposed to significantly higher levels of DEHP during medical procedures (Latini, 2005). Moreover, what is urgently needed is additional information of the effects of developmental exposure to DEHP and other phthalates on human reproduction.

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