The MAK Collection for Occupational Health and Safety

Di(2-ethylhexyl) phthalate (DEHP)

MAK Value Documentation, addendum – Translation of the German version from 2016

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Di(2-ethylhexyl) phthalate (DEHP) / bis (2-ethylhexyl) benzene-1,2-dicarboxylate

MAK Value Documentation

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Abstract

The German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area has re-evaluated the developmental toxicity of di(2-ethylhexyl) phthalate (DEHP) [117-81-7]. In 2014, the maximum concentration at the work place (MAK value) of DEHP has been set to 2 mgm^3 for the inhalable fraction.
In some studies, a correlation between prenatal DEHP exposure and effects on anogenital distance of male newborns, birth weight and pregnancy duration in humans are found, however, the results are not consistent. Teratogenicity is observed in rats only at maternally toxic doses of 1000 mg/kg bw and day and above, in mice at 91 mg/kg bw and day. NOAELs for teratogenicity are 200 mg/kg bw and day for rats and 44 mg/kg body weight and day for mice, the latter corresponding to 29 mg/m^3 after scaling to a concentration at the work place. In contrast to mouse and marmoset the rat is very sensitive to DEHP effects on the male reproductive organs. From a three generation study the Commission deduces a NOAEL for pre- and postnatal developmental toxicity to the male reproductive organs and for fetotoxicity of 46 mg/kg bw and day for rats, corresponding to 54 mg/m^3 after scaling to a concentration at the work place. The reported effects, small testes, reduced absolute weights of testis and epididymis, are considered by the Commission as not being adverse because they do not show correlating consequences for the next generation or dose-response relationship and changes in relative organ weights and histopathological correlates are not observed. After in utero exposure, germ cell organization, mRNA expression and protein level of StAR („steroidogenic acute regulatory protein") in testes are affected at 100 mg/kg bw and day and above. These effects resulted in a NOAEL for prenatal developmental effects of the male reproductive organs of 30 mg/kg bw and day for rats, corresponding to 35 mg/m^3. For mice in prenatal developmental studies a NOAEL for fetotoxicity as well as prenatal developmental toxicity of 48 mg/kg bw and day is derived, corresponding to 32 mg/m^3. Damage to the embryo or foetus is unlikely when the MAK value is observed and DEHP is classified in Pregnancy Risk Group C.

Keywords

di(2-ethylhexyl) phthalate; bis(2-ethylhexyl) phthalate; DEHP; dioctyl phthalate; DOP; 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester; mechanism of action; reproductive toxicity; fertility; developmental toxicity; prenatal toxicity; occupational exposure; maximum workplace concentration; MAK value; toxicity; hazardous substance

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Di(2-ethylhexyl) phthalate (DEHP)

[117-81-7]

Supplement 2016

MAK value (2014)	**2 mg/m³ I (inhalable fraction)**
Peak limitation (2014)	**Category II, excursion factor 2**
Absorption through the skin (2014)	**H**
Sensitization	**–**
Carcinogenicity (2014)	**Category 4**
Prenatal toxicity (2015)	**Pregnancy Risk Group C**
Germ cell mutagenicity	**–**
BAT value	**–**

Reproductive and Developmental Toxicity

Since February 2015, di(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP) and butylbenzyl phthalate (BBP) may only be used after prior authorization (EU 2011).

Documentation was published for DEHP in 2002 (documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009), followed by a supplement in 2015 with new data for the mechanism of action, the effects after inhalation exposure, fertility, genotoxicity and carcinogenicity (supplement "Di(2-ethylhexyl) phthalate (DEHP)" 2015). This supplement is a re-evaluation of the developmental toxicity of the substance.

A large number of reviews are available for the toxicological profile of DEHP: reports by the IARC (2010, 2012), a toxicological evaluation from NICNAS (2008), an EU Risk Assessment Report (EU 2008) and data compiled by the ECHA (2013).

This supplement focuses on reproductive toxicity.

Mechanism of Action

Reproductive toxicity

DEHP has anti-androgenic and oestrogenic effects, but they are not mediated by the corresponding receptors.

DEHP did not compete for the oestrogen receptor (ER) or induce agonistic ER activity in a number of cell lines transfected with the human ER vector (IARC 2012).

Anti-androgenic effects

The production of testosterone in the testes was reduced in the male foetuses of rats given gavage doses of DEHP during gestation of about 300 mg/kg body weight and day and above (Borch et al. 2006; Hannas et al. 2011). However, the testicular production of testosterone was increased in prepubertal rats at 10 mg/kg body weight and day and above after 14-day exposure via the diet from postnatal days 35 to 48 (Akingbemi et al. 2001) and at 5 mg/m^3 and above after 4-week and 8-week inhalation exposure beginning on postnatal day 28 (Kurahashi et al. 2005). Leydig cell steroidogenesis is influenced by the stage of development during exposure, and effects may occur as a result of the modulation of the activity of the enzymes involved in steroidogenesis and the serum LH (luteinizing hormone) levels (Akingbemi et al. 2001).

In the following, only those studies are described which examined the testes of male rats from dams treated during gestation and in vitro tests carried out to clarify the mechanism of action.

In male rats, reduced gene expression and the hormone production of the foetal Leydig cells are the critical steps of the "phthalate mechanism", which causes disruptions in the development of the accessory reproductive organs (Johnson et al. 2012).

Gene expression was impaired in enzymes or receptors involved in steroid synthesis or cholesterol metabolism and transport in rat foetuses with reduced testicular testosterone production (for example Borch et al. 2006; Hannas et al. 2011; Johnson et al. 2012). Reduced mRNA expression was found for *Sr-B1* (scavenger receptor B1 = HDL (high density lipid) receptor), *Star* (steroidogenic acute regulatory protein), *Pbr* (peripheral benzodiazepine receptor) and *P450scc (*side-chain cleavage = CYP11A1 = mitochondrial cholesterol monooxygenase) (Borch et al. 2006; Hannas et al. 2011). Transcription factors were also affected. For example, the gene expression of *Srebp2* (sterol regulatory element-binding protein) was decreased, which is the master regulator of the gene expression of lipid metabolism including the genes for fatty acid, cholesterol and steroid metabolism. Also the binding of CCAAT/enhancer binding protein β to the proximal promotors of the genes for *Cyp11a1, Cyp17a1* and *Star* was inhibited (Johnson et al. 2012).

Mice, however, were resistant to the anti-androgenic effect of DEHP. Effects on steroidogenic gene expression and intratesticular testosterone levels were not observed (Campion et al. 2013).

A study in cultured foetal testicular cells obtained from Sprague Dawley rats on gestation day 14.5 showed that the metabolite monoethylhexyl phthalate (MEHP) directly impaired testosterone production at 1 μM and above by inhibiting the *Cyp17a1* gene and reducing the CYP17A1 protein level. In CYP17A1, not only hydroxylase activity can be found but also 17,20-lyase activity, which is responsible for converting 17α-hydroxyprogesterone to androstenedione. Only the 17,20-lyase activity of the enzyme was inhibited (Chauvigné et al. 2011).

There was a concentration-dependent decrease in testosterone only 24 hours after testis samples obtained from 2 prostate cancer patients had been treated in vitro with DEHP and MEHP (DEHP and MEHP concentrations of 0.01 and 0.1 mM tested). The steroid concentrations of the synthesis pathways for testosterone were determined quantitatively for MEHP after 24 hours (starting from pregnenolone, the following intermediates form up to testosterone via the 2 Δ5 and Δ4 synthesis pathways; Δ5: 17-OH-pregnenolone, dehydroepiandrosterone and androstenedione; Δ4: progesterone, 17-OH-progesterone and androstenedione). All steroid concentrations including that of pregnenolone were reduced compared with the control levels. DEHP and MEHP did not impair the production of insulin-like peptide 3 = insulin-like hormone family 3 (INSL3) or inhibin B in human testis explants (Desdoits-Lethimonier et al. 2012). The gene expression or enzyme activity involved was not determined. However, the reduced concentration of all steroids including that of pregnenolone suggests that steroid synthesis must have already been impaired. The conversion of cholesterol to pregnenolone (catalysed by CYP11A1) may have been affected or even the metabolism or transport of cholesterol; however, this was not investigated.

In immortalized mouse Leydig tumour cells (MLTC-1), MEHP led to the concentration-dependent stimulation of testosterone and progesterone production at 25 μM and above. At 10 μM and above, MEHP up-regulated hormone-sensitive lipase and 3-hydroxy-3-methylglutaryl coenzyme A reductase, which suggests that MEHP increased the amount of cholesterol available for steroidogenesis (Gunnarsson et al. 2008).

Oestrogenic effects

In male Long Evans rats exposed to DEHP for at least 10 weeks from postnatal day 21, the serum testosterone and 17-beta-oestradiol concentrations were increased at 10 mg/kg and day and above. DEHP was thus shown to be indirectly oestrogenic, presumably as a result of the increased aromatase activity induced by LH induction in Leydig cells (Akingbemi et al. 2004).

Reduced testosterone concentrations and histological changes in rat testes correlated with changes in the gene expression of 7 proteins that belong to a pathway network regulated directly or indirectly by oestradiol (Klinefelter et al. 2012). The

authors concluded that the sites of increased aromatase activity should be determined (Veeramachaneni and Klinefelter 2014).

When pregnant mice with an ER-mediated luciferase (luc) reporter gene were given 8 daily DEHP doses of 100 mg/kg body weight and day, the induction of luciferase activity could not be detected in the examined foetal organs or the placenta. Therefore, oestrogenic effects in foetuses are unlikely (ter Veld et al. 2009).

Mechanisms of the effects of DEHP on the testes

The mechanism of action of testicular toxicity induced by DEHP is not yet fully understood. Data suggest that both Sertoli and Leydig cells are targets of the testicular toxicity of DEHP (IARC 2012). In vivo and in vitro studies showed that effects on Sertoli cells resulted in germ cell depletion. Sertoli cells provide not only the physical support but also the proteins that are required for germ cell differentiation/survival and the signal transduction pathways among the cells. In addition, the function of these cells is regulated by the follicle-stimulating hormone (FSH), which is essential for the initiation and maintenance of spermatogenesis. Four different mechanistic hypotheses have been suggested to explain the damage to the testes: zinc-dependent enzyme activity, hormonal status, metabolic interactions and FSH-dependent pathways. Studies demonstrated that DEHP reduces the stimulation of Sertoli cells by FSH. In vitro and in vivo studies revealed that DEHP and MEHP have adverse effects on steroidogenesis in Leydig cells (see above) and on their cell structure (EU 2008). Because of the paracrine relationship between Leydig and Sertoli cells, the dysfunction of Leydig cells probably affects the physiology of adjacent Sertoli cells, and vice versa (EU 2008; Martino-Andrade and Chahoud 2010). Therefore, it is unclear whether the primary change occurs in a specific cell type or in both types of cell at the same time (Martino-Andrade and Chahoud 2010). Comparative studies carried out in foetal and adult Sprague Dawley rats revealed that germ cells in the foetal testis were more sensitive to DEHP. The germ cells are presumably the target in utero because the blood–testis barrier has not yet formed. Sertoli cells are the target cells of DEHP in adult animals because the blood–testis barrier has developed in adult rats (Shirota et al. 2005).

Leydig cells

As described above, Leydig cells are responsible for testicular hormone production (see Section "Anti-androgenic effects").

DEHP and DBP caused cryptorchidism or the underdevelopment of the gubernacular ligaments when high doses of 500 mg/kg body weight and day were given to Sprague Dawley rats (Howdeshell et al. 2007). Gubernacular lesions were induced by DEHP in Wistar rats at 750 mg/kg body weight and day (Wilson et al. 2007).

In mice, Leydig cells produce the Insl3 peptide that binds to the Lgr8 receptor (receptor for Leydig insulin-like peptide) (Kumagai et al. 2002). Male transgenic mice that do not have the *Insl3* gene were found to have bilateral intra-abdominal cryptorchidism (Campion et al. 2013) resulting from abnormal gubernacular de-

velopment (Kumagai et al. 2002). In humans, INSL3 is responsible for the development of the gubernaculum (genito-inguinal ligament) in the developing foetus. The gubernaculum connects the lower poles of the testis and epididymis with the future inguinal canal. The first phase of testicular descent (from the kidney region to the inguinal region) is regulated by INSL3, which initiates a thickening of the gubernaculum by binding to LGR8. In humans, this phase starts in week 25 of gestation (Campion et al. 2013). In male rats, the testicular expression of *Insl3* during gestation is highest between days 17.5 and 19.5 of gestation (Howdeshell et al. 2007). The period of highest sensitivity for phthalate-induced malformations of the male genitals in rats is between days 16 and 18 of gestation (Johnson et al. 2012).

Reduced *Insl3* expression was observed in male Wistar rat foetuses from dams that had been treated with doses of 300 mg DEHP/kg body weight and day from days 7 to 21 of gestation (Borch et al. 2006). This study did not report gross-pathological findings.

Sertoli cells

Sertoli cells are required for seminiferous cord formation (see also Section "Seminiferous cords"; Campion et al. 2013; Martinez-Arguelles et al. 2013; McLaren 1998).

Sertoli cells are the apparent target of the phthalate-induced effects on the seminiferous cords (Johnson et al. 2012). The mechanism is not known. It is assumed that the underlying mechanism is altered cytokinesis (Campion et al. 2013) or altered communication between Sertoli cells and germ cells (Campion et al. 2013; Johnson et al. 2012). In rats, the period of highest sensitivity for phthalate-induced effects on Sertoli cell growth is between days 19 and 21 of gestation (Johnson et al. 2012).

Seminiferous cords

Seminiferous cords are the precursors of the seminiferous tubules that form during puberty. In mammals, the formation of seminiferous cords is regulated by foetal Sertoli cells (Campion et al. 2013; Martinez-Arguelles et al. 2013; McLaren 1998). In utero exposure of rats to phthalates led to changes in the development of the seminiferous cords resulting in focal, dysgenetic cords with intracordal Leydig cells or cords with larger diameters and a large number of multinucleated germ cells in the centres (Johnson et al. 2012). After the exposure of Wistar rats to DEHP, these effects were observed at 100 mg/kg body weight and day and above (Borch et al. 2006).

Effects on the seminiferous cords were observed also in mice that are resistant to DEHP-induced effects on steroidogenesis or testosterone production. This suggests that the effects on seminiferous cords are not mechanistically linked to decreased steroidogenesis or testosterone production (Campion et al. 2013; Johnson et al. 2012).

Unlike Sertoli cells that express the SRY (sex-determining region of Y), foetal Leydig cells do not express either SRY or SOX9 (SRY-box 9). This shows that Leydig cells differentiate as a result of the paracrine activity of Sertoli cells, which is driven mainly by the two signalling molecules DHH (desert hedgehog) and PDGF (platelet-derived growth factor). Sertoli cells, without further involvement of SRY, direct the development of other cell types in the foetal testis, including peritubular myoid cells, endothelial cells and other uncharacterized cells within the interstitial space. These additional cell types support seminiferous cord formation by surrounding Sertoli cells with peritubular myoid cells. They also help form the blood–testis barrier by creating a basal lamina that in conjunction with Sertoli cells encloses germ cells within the seminiferous cords (Campion et al. 2013). SRY or SOX9 with exposure to DEHP were not investigated.

On day 18 of gestation, testes from Fgf9-null (fibroblast growth factor 9) mice developed disorganization of seminiferous cords and disrupted peritubular myoid cell organization. The Fgf9 gene is thus responsible for the proliferation and differentiation of Sertoli cell precursors and supports seminiferous cord formation by initiating the migration of mesonephric cells into the gonads (Campion et al. 2013). There are no studies available for FGF9 and DEHP.

Germ cells

Multinucleated germ cells were observed at 100 mg/kg body weight and day and above in male Wistar rat foetuses from dams that had been given gavage doses of DEHP from days 7 to 21 of gestation (Borch et al. 2006).

In addition to the effect on the seminiferous cords described above, mice were found to have multinucleated germ cells irrespective of the anti-androgenic effect of DEHP (Campion et al. 2013; Johnson et al. 2012).

Theoretically, multinucleated germ cells may result from cell division without cytoplasmic separation or from the collapse of intercellular bridges (Johnson et al. 2012); however, this was not investigated. Multinucleated germ cells formed during gestation persist throughout early postnatal life and may then be eliminated in a p53-dependent manner within 1 or 2 weeks after birth (Johnson et al. 2012). The relationship between the DEHP-induced development of multinucleated germ cells and p53 has not been investigated.

PPAR-mediated effects of DEHP on the offspring

Male and female Sy/129 wild type (murine peroxisome proliferator-activated receptor α; mPparα), Pparα-null knock-out mice (Pparα-null) and mice that express the human PPARα in the liver (hPPARα) were given DEHP doses of 0, 10 to 12, 55 to 64 and 119 to 145 mg/kg body weight and day with the diet for 4 weeks prior to mating. In mPparα mice, the high dose of 119 to 145 mg/kg body weight and day led to a decrease in the number of live foetuses per litter and an increase in the number of resorptions per litter. In hPPARα mice, the number of resorptions per litter was increased after doses of 55 to 64 mg/kg body weight and day and above; in addition,

the average number of foetuses per litter was decreased at the high dose. These effects were not observed in Pparα-null mice. While in pregnant mPparα mice the plasma triglyceride concentration was decreased at the high dose and the hepatic microsomal triglyceride transfer protein was decreased at the medium dose, these effects were not observed in the animals of the other two strains. PPARα expression is required for the foetotoxicity of DEHP; regulation by the microsomal triglyceride transfer protein is of importance in mPparα mice, but not in hPPARα mice (Hayashi et al. 2011). The same research group showed that gavage doses of DEHP of 1950 mg/kg body weight and day for 2 weeks activated PPARα more strongly in mPparα mice than in hPPARα mice, whereas it was vice versa for the constitutive androstane receptor (CAR) (Ito et al. 2012).

An association between Ppar activation and toxic effects on the foetal testes was assumed, but not substantiated in animal studies (Johnson et al. 2012). Male Wistar rat foetuses from dams that had been given gavage doses of DEHP of up to 300 mg/kg body weight and day from days 7 to 21 of gestation were not found to have changes in *Pparα*-mRNA or *Pparγ*-mRNA expression (Borch et al. 2006).

Exposure route-specific effects of DEHP

The 2015 supplement (supplement "Di(2-ethylhexyl) phthalate (DEHP)" 2015) described various receptor-mediated mechanisms that may be involved in exposure route-specific toxicity, including those mediated by PPAR (see documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009) and CAR.

The influence of exposure route-specific metabolism on the systemic toxicity of DEHP is very complex. Both the absence of a first-pass effect in the intestines after ingestion and the species-specific enzyme and receptor capacities are of importance here; therefore, apart from the assumption that exposure to MEHP is lower after inhalation than after ingestion, a conclusive evaluation cannot be made.

Comparison of the effects on different strains and species

In a comparative study in which Sprague Dawley and Wistar rats were given gavage doses of DEHP of 750 mg/kg body weight and day from days 14 to 18 of gestation, there was a higher incidence of epididymal agenesis (partial or complete) in the male foetuses of the Sprague Dawley strain and a reduced incidence of gubernacular lesions (elongation of more than 15 mm or agenesis) compared with the incidences in foetuses of the Wistar strain. The authors assumed that the higher sensitivity of Wistar rats to gubernacular changes or cryptorchidism was due to the lower Insl3 concentrations and less robust gubernacular cords in Wistar rats compared with in Sprague Dawley rats (Wilson et al. 2007). Foetal testosterone production was reduced in both rat strains at 300 mg/kg body weight and day and above and the level of *Insl3* mRNA in the testes was decreased at 500 mg/kg body weight and day and above. The authors concluded from this similar dose–response relationship that the different effects of in utero exposure on epididymal (androgen-dependent)

or gubernacular (Insl3-dependent) differentiation in Sprague Dawley and Wistar rats are probably the result of tissue-specific strain differences in the androgen and Insl3 signalling pathways rather than effects induced by DEHP on foetal testicular testosterone and Insl3 production (Hannas et al. 2011). The reported strain-dependent differences may also be regarded as the result of different androgen signalling requirements in target tissues (Johnson et al. 2012).

Mice were shown to be resistant to the anti-androgenic effect of DEHP. Studies of the differences between rats and mice revealed similar toxicokinetics after the prenatal exposure of mice to equivalent doses of endocrine active phthalates, inducing the same formation of multinucleated germ cells and the same changes to the seminiferous tubules as in rats (Campion et al. 2013). Species-specific toxicodynamics seem to be the reason for the different sensitivity of the rat and mouse foetal Leydig cells; for example, inhibited hormone production in Leydig cells in rats and non-impaired or even increased hormone production in mice. The different sensitivity of the individual species is confirmed by mechanistic studies and molecular signalling pathways (Johnson et al. 2012).

Young adult Sprague Dawley rats are far more sensitive to the testicular toxicity of DEHP after oral administration than adult marmosets (rats: NOAEL (no observed adverse effect level): 3.7 mg/kg body weight after administration with the diet (Poon et al. 1997); marmosets: no effects at 2500 mg/kg body weight with gavage administration (Kurata et al. 1998; Tomonari et al. 2006)). According to the authors, the difference in sensitivity cannot be explained by toxicokinetics alone. Toxicodynamic factors, such as the possible interaction of MEHP with receptor-mediated processes, might be involved in the different reactions of the rodent and primate species (Kessler et al. 2004).

The following aspects have to be taken into consideration when interpreting species-specific divergent differences in phthalate-induced effects: (i) the great differences between mammalian species as regards the organization, structure and biology of the interstitial testis compartment, (ii) the possibly age-related ranges of sensitivity during the foetal and postnatal periods, which vary from species to species and (iii) intrinsic species differences in the timing of testicular development (Albert and Jégou 2014).

Relevance for humans

In male rats, reduced gene expression and the hormone production of foetal Leydig cells are the critical steps of the "phthalate mechanism", which causes disruptions in the development of the accessory reproductive organs (Johnson et al. 2012). Xenograft experiments with foetal testes from rats, mice and humans were carried out in castrate male mice with di-*n*-butyl phthalate (DBP), which has qualitatively similar effects to those of DEHP on the male reproductive organs. When xenografted mice were treated with DBP doses of 500 mg/kg body weight and day from days 4 to 21 of gestation, testicular steroidogenesis was reduced only in mice xenografted with rat testes, whereas multinucleated germ cells were observed in the testis xenografts

from all species (Mitchell et al. 2012). There are no xenograft studies available for DEHP or MEHP. In ex situ organ culture system experiments, 0.1 mM MEHP had no effect on testosterone production in human foetal testes (Lambrot et al. 2009) and induced multinucleated germ cells only in mouse and rat testis cultures, but not in human testis cultures (Habert et al. 2014). It was concluded from these studies that human Leydig cells are resistant to the anti-androgenic effect of phthalates. The molecular pathways that control steroidogenesis in human foetal testes have yet to be clarified (Johnson et al. 2012).

Mice were found to be less sensitive to DEHP-induced effects on the reproductive organs of male foetuses than rats (Johnson et al. 2012; Kay et al. 2014).

These species differences raised concerns about the extrapolation of data from rodents to humans (Habert et al. 2014; Kay et al. 2014).

Rats proved to be the most sensitive animal species for DEHP-induced effects on male reproductive organs. In conclusion, species differences in the toxicokinetics and toxicodynamics of DEHP-induced effects are very complex.

Effects in Humans

Reproductive and developmental toxicity

Fertility

As described in the 2015 supplement supplement "Di(2-ethylhexyl) phthalate (DEHP)" 2015), the studies did not reveal a consistent picture of the relationship between the level of DEHP exposure and adverse effects on male or female fertility in humans.

Developmental toxicity

Evidence of MEHP in biological fluids as a reliable biomarker for exposure to DEHP should be interpreted with caution because also oxidative metabolites are necessary for a full evaluation of the exposure to DEHP (IARC 2012).

A regression analysis yielded a negative correlation between the concentrations of mono-*n*-butyl phthalate, monobenzyl phthalate and monoisobutyl phthalate in maternal urine samples and the anogenital index (weight-adjusted anogenital distance) of male offspring (85 boys, average age of about 1 year; 3–36 months). There was no relationship between MEHP and the anogenital index and there was only a slightly significant relationship for the oxidative metabolites of DEHP, mono-2-ethyl-5-oxo-hexyl phthalate and mono-2-ethyl-5-hydroxyhexyl phthalate. Further studies are required (Swan et al. 2005). The weight-adjusted anogenital distance is a non-validated parameter. The anogenital distance correlates better with body length than with body weight. The phthalate concentrations in the maternal urine were not adjusted for the urinary volume of the bladder. In addition, urinary sam-

ples were taken only once. Altogether, the biological plausibility of this study is questionable (McEwen and Renner 2006).

A follow-up study to Swan et al. (2005) included 140 boys and 153 girls and their mothers. DEHP metabolite concentrations were established in prenatal urine samples from the mothers (Swan et al. 2005). The genitals were examined in the boys and girls; also the size, diameter and length of the penis were recorded in the boys. The anogenital distance was 47.3 ± 7.1 mm in the girls and 70.4 ± 10.8 mm in the boys. In the boys, the anogenital distance correlated with the diameter and length of the penis. The higher the concentrations of MEHP metabolites and of the DEHP metabolites mono-2-ethyl-5-oxo-hexyl phthalate and mono-2-ethyl-5-hydroxyhexyl phthalate in maternal urine during pregnancy, the smaller the penis size and anogenital distance in the boys and the more frequent incomplete testicular descent (Swan 2008).

To clarify the relationship between prenatal exposure to phthalates (DEHP) and changes in the anogenital distance of newborn boys, urine samples from 111 pregnant women were examined for 7 phthalate metabolites by means of high performance liquid chromatography-tandem mass spectrometry (HPLC-TMS). The isoflavone level was determined as a possible cofactor. A multiple regression analysis revealed an inversely significant correlation between the MEHP concentration (log-transformed and pregnancy taken into account) and the anogenital index and a positively significant correlation between smoking in the mother and the anogenital index. No relationship was found for isoflavones (Suzuki et al. 2012). The validity of this study is limited because of uncertainties in determining the anogenital distance and the small number of women examined.

DEHP and MEHP were detected in the cord blood of about 88% of 84 pregnant women. The mean DEHP and MEHP concentrations were 1.19 ± 1.15 µg/ml (95% confidence interval CI: 0.93–1.44; range: 0–4.71) and 0.52 ± 0.61 µg/ml (95% CI: 0.39–0.66; range: 0–2.94), respectively. Newborn babies in which MEHP was detected in cord blood had a lower gestational age compared with those without evidence of MEHP ($p < 0.033$) (Latini et al. 2003).

A Danish-Finnish cohort study carried out from 1997 to 2001 analysed both the concentrations of various phthalates (including MEHP) in breast milk samples collected 1 to 3 months postnatally and sex hormone concentrations in serum samples from 62 male newborn babies with cryptorchidism and 68 male newborn babies without cryptorchidism. The phthalate concentrations in breast milk varied considerably. There was no relationship between the phthalate concentrations in breast milk and the incidence of cryptorchidism. Various tendencies were established between some phthalate levels and the concentrations of sex hormones in serum, but they were not significant (Main et al. 2006).

The concentrations of 10 phthalates were investigated in amniotic fluid samples obtained from 54 pregnant women. The 3 phthalates MEHP, monoethyl phthalate and monobutyl phthalate were quantifiable in the amniotic fluid and their concentrations were lower than in the urine or serum of the normal population (Silva et al. 2004).

A review of the effects of developmental toxicity induced by phthalates reported a relationship between prenatal exposure to phthalates and the duration of pregnancy and birth weights. Some of the results are contradictory. Depending on the inclusion criteria regarding premature birth, the overall results suggest that there may be a relationship between the duration of pregnancy and phthalate exposure. Studies which assessed exposure by means of urinary metabolites, excluded extremely premature infants and carried out further examinations to substantiate effects on birth weights, yielded only a weak association between phthalate exposure and premature births (Kay et al. 2013).

Summary

There is evidence of a relationship between prenatal exposure to DEHP and effects on the anogenital distance in male newborn babies and on birth weights and the duration of pregnancy, although the consistency of these data has yet to be reliably established.

Studies of PVC infusion tubes containing DEHP

Premature infants artificially ventilated with PVC respiratory masks were found to have unusual lung changes at 4 weeks of age. The authors assumed that these were related to exposure to DEHP (WHO 1992).

Extracorporeal membrane oxygenation (body burden similar to intravenous injection) was given to 18 of 28 newborn babies with respiratory disorders. This resulted in DEHP exposures of up to 2 mg/kg body weight over a period of 3 to 10 days (mean DEHP concentration in the plasma: 8 µg/ml). Various clinical parameters of liver, lung and heart function remained unchanged (IARC 2000).

Premature infants and newborn babies who received parenteral nutrition via infusion systems containing PVC or PVC-free infusion systems were compared with regard to the incidence of cholestasis. A retrospective analysis was carried out in 2 groups of 30 and 46 patients before and after changing to the PVC-free system, respectively. The use of tubes containing PVC correlated strongly with the development of cholestasis ($p = 0.0004$), and the incidence of patients with cholestasis dropped from 50% to 13% after changing to PVC-free infusion systems (von Rettberg et al. 2009). DEHP concentrations were not determined.

These studies are not included in the evaluation of developmental toxicity because the exposure to DEHP took place after birth, the exposure data are not available and there may have been further exposure.

Animal Experiments and in vitro Studies

Reproductive and developmental toxicity

Fertility

In the 2015 supplement to DEHP (supplement "Di(2-ethylhexyl) phthalate (DEHP)" 2015), the following conclusions were drawn from the studies of fertility relevant to the evaluation.

The vacuolation of Sertoli cells was observed in adult rats given doses of 38 mg/kg body weight and day and above (Poon et al. 1997), and reduced relative testis weights were reported in mice at doses of 100 mg/kg body weight and above (David et al. 1999, 2000). Fertility was impaired in adult rats of the F0 and F1 generations that received 343 and 391 mg/kg body weight and day and above, respectively, with the diet (NTP 2003, 2005). Fertility was decreased in mice at 200 mg/kg body weight and day and above (Lamb et al. 1987).

Studies with the exposure of juveniles (starting at lactation up to a maximum of 7 or 8 weeks of age) are not relevant to the evaluation of human exposure at the workplace; therefore, the Commission has not included these in the evaluation.

Developmental toxicity

DEHP crosses the placental barrier and was found in the offspring as well as in breast milk after oral exposure to high doses of 2000 mg/kg body weight and day. A large number of studies showed DEHP to be embryotoxic and teratogenic in rats at doses close to those that caused maternal toxicity and in mice at doses that were not toxic to the dams (see documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009; EU 2008; NICNAS 2008).

The documentation for DEHP from 2002 (documentation "Di(2-ethylhexyl) phthalate (DEHP)" 2009) reported malformations (open eyes, exophthalmia, exencephaly, short or no tails, changes in major blood vessels, fused or branched ribs, and fused thoracic vertebral centra) in mice at doses of 91 mg/kg body weight and day and above; these doses were not maternally toxic (Tyl et al. 1988). In rats, toxic effects on development such as reduced body weights and decreased survival were observed only at maternally toxic doses higher than 300 mg/kg body weight and day (NTP 1986; Tyl et al. 1988); malformations were detected at doses higher than 1000 mg/kg body weight and day (Hellwig et al. 1997).

Recent studies that were carried out only with high doses (≥ 300 mg/kg body weight and day) and that yielded no new evidence are not described in detail here (Borch et al. 2005; Dobrzyńska et al. 2012).

Table 1 shows recent developmental toxicity studies that are relevant to the evaluation.

Prenatal exposure

Inhalation

A developmental toxicity study in Wistar rats that were exposed by inhalation (head/nose) to DEHP concentrations of 0, 10, 50 or 300 mg/m^3 from days 6 to 15 of gestation revealed a significantly increased incidence of litters with retardations (at 0, 10, 50 and 300 mg/m^3: 16.7%, 33.3%, 31.3% and 56.3%; $p = 0.05$). The most frequent finding was a dilated renal pelvis. According to the authors, this is a very frequent finding in this rat strain and it was therefore not attributed to the exposure. Maternal toxicity was observed in the animals of the high exposure group in the form of reduced body weight gains on day 21 of gestation. The NOAEC (no observed adverse effect concentration) for toxic effects on prenatal development and maternal toxicity was 50 mg/m^3 (Merkle et al. 1988).

Oral administration

In utero exposure of Sprague Dawley rats to DEHP from days 7 to 18 of gestation induced effects on the testes at 250 mg/kg body weight and day and above; these included an increase in multinucleated germ cells, an increased number of germ cells in the seminiferous cords and hyperplasia of the interstitial cells on day 20 of gestation. The germ cells are the target in utero because the blood–testis barrier has not yet formed. These effects were reversible at doses lower than 500 mg/kg body weight. Sertoli cells are the target cells for DEHP exposure in adult animals because the blood–testis barrier has developed in adult rats. The NOAEL was 125 mg/kg body weight and day for toxic effects on prenatal development in male reproductive organs (Shirota et al. 2005).

When rats were given DEHP from days 7 to 21 of gestation, histopathological effects on the testes (germ cell differentiation and organization) and changes in the gene expression of proteins involved in steroid synthesis and in protein formation were observed at 100 mg/kg body weight and day and above. The NOAEL was 30 mg/kg body weight and day for toxic effects on prenatal development in male reproductive organs (Borch et al. 2006).

Doses of 0.0005 to 500 mg/kg body weight and day given to CD-1 mice with a micropipette from days 1 to 18 of gestation stimulated an increase in foetal and maternal serum testosterone levels. The anogenital distance initially increased up to 0.005 mg/kg body weight and day and then returned to the control value. A quadratic fit was determined because the values of the high dose group did not differ from those of the control group (Do et al. 2012). The results are not regarded as relevant to the evaluation because of the unclear procedure of dosing with a micropipette placed into an animal's mouth and because of the questionable approach of considering changes in the dose–response relationship (quadratic trend), while the linear trend test did not produce significant findings.

Prenatal and postnatal exposure up to the end of lactation

Rats

In a study with Wistar rats given doses of up to 100 mg/kg body weight and day with the diet from day 1 of gestation to the end of lactation, no effects on the reproductive organs or sexual behaviour in male offspring were observed. At 500 mg/kg body weight and day, the litter size was reduced and the number of post-implantation losses was increased in the dams. The epididymal and seminal vesicle weights and the sperm count were reduced in male pups as from puberty. In addition, the sexual behaviour and androgen imprinting were disturbed during development. A NOAEL of 100 mg/kg body weight and day was derived for developmental toxicity in the male reproductive organs and maternal toxicity (Dalsenter et al. 2006).

Female Wistar rats were given gavage doses of DEHP of 0, 0.015, 0.045, 0.135, 0.405 or 1.215 mg/kg body weight and day in the low dose range and 0, 5, 15, 45, 135 or 405 mg/kg body weight and day in the high dose range from days 6 of gestation to day 21 of lactation. Vaginal opening was delayed by about 2 days in female pups at 15 mg/kg body weight and day and above, and a trend was observed at 135 mg/kg body weight and day towards a delay in the age of the first oestrus of about 2 days and liver enlargement. Nipple development and the anogenital distance of the female offspring were not affected (Grande et al. 2006). The observed delays in vaginal opening or the beginning of the first oestrus, which were not dose-dependent, are only of a functional nature and do not persist. It cannot be determined from the document whether the low and high dose groups were treated in parallel or separately. Only 1 control group was used for both tests, however.

On postnatal day 22, the testis weights of the male offspring of this study were significantly increased at doses of 5 to 135 mg/kg body weight and day and thus differed qualitatively from the reduced testis weights at the high dose of DEHP. The histopathological examination revealed changes in the testes at 135 mg/kg body weight and above (multinucleated germ cells on postnatal day (PND) 1; reduced germ cell differentiation in seminiferous tubules on PND 22). In addition, delayed preputial separation was observed at 15 mg/kg body weight and day and above, and nipple retention and a reduced anogenital distance were observed in the animals exposed to the high dose of 405 mg/kg body weight. At 135 mg/kg body weight and day, there were no effects on the anogenital distance (Andrade et al. 2006 a). The observation regarding the treatment groups and control group applies both here and for Grande et al. (2006). The opposing change in testis weights from the low to the high dose is not plausible from a toxicological point of view. The change in anogenital distance observed at 405 mg/kg body weight is only of limited validity. The dose of 135 mg/kg body weight and day is in the dose range that was determined for developmental toxicity in the 2002 documentation (documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009).

Decreases in the absolute adrenal weight and anogenital distance and the first signs of a dysgenesis syndrome of the external genitals were observed in the male offspring of Wistar rats given 10 mg/kg body weight and day from days 7 to 21 of

gestation and from postnatal days 1 to 18. No maternal toxicity was observed up to doses of 900 mg/kg body weight and day (Christiansen et al. 2010). According to OECD Test Guideline 414, the adrenals are weighed on postnatal day 16 and the accuracy depends on the experience of the staff in dealing with the surrounding connective tissue, which can account for up to 10% of the weight. The NOAEL of 10 mg/kg body weight and day derived by the authors for the anogenital distance is questionable because the control values of the two test groups varied considerably (3.68 compared with 3.4 mm) and it can be assumed that the barely significant difference would no longer exist if the values were compared with those for the other control group. In addition, these results are inconsistent with the study of Andrade et al. (2006 a) evaluated above. The anogenital distance is listed as a test parameter in the in utero/lactational protocol (for example EPA OPPTS 870.3800, OECD 416 and OECD 443). This is a very sensitive parameter. A reduction in the anogenital distance in males without there being any change in the females is evidence of an anti-androgenic effect. The biological relevance of the anogenital distance and the relevance of changes are not known (US EPA 2005).

After Sprague Dawley rats were given gavage doses of DEHP from gestation day 8 to postnatal day 17, changes were found in the male offspring at 11 mg/kg body weight and day and above that were referred to as the "phthalate syndrome". This syndrome is the sum of individual effects such as changes in the anogenital distance, testicular lesions, effects on androgen-dependent and INSL3-dependent tissues and nipple retention and is described as a non-monotonic dose–response relationship. The concentration of the MEHP metabolite in the amniotic fluid correlated with the maternal DEHP concentration in the urine or with the DEHP dose. On postnatal day 2, the body weights and the anogenital distance of the male pups were reduced at 300 mg/kg body weight and day and above. In addition, there was a delay in preputial separation and the androgen-dependent absolute organ weights (testes, epididymis, seminal vesicles, prostate gland, Cowper's gland, kidneys and adrenals) were reduced. Maternal toxicity was not observed up to the highest dose of 300 mg/kg body weight and day. The authors derived a LOAEL (lowest observed adverse effect level) of 11 mg/kg body weight and day for developmental toxicity in male reproductive organs; this was the lowest dose tested (Gray et al. 2009). Unlike in studies carried out according to the OECD test guideline, all male offspring were examined in this study to increase the validity of the statistical analysis with the aim of detecting the slightest effects. When the effects were considered individually instead of being combined within the "phthalate syndrome", the first effects detected were non-dose-dependent histopathological changes of the testes observed at 33 mg/kg body weight and day. At 100 mg/kg body weight and day, the incidence of male offspring with malformed coagulating glands was increased and seminal vesicle weights were reduced in a dose-dependent manner. At 300 mg/kg body weight and day, which was the highest dose tested, all the individual effects that were included in the "phthalate syndrome" were significantly increased and were thus in the same dose range as in other studies (see Table 2; Table 3). Litter data for the individual groups were not reported, and all control values for the tested parame-

ters were 0. Historical control data were not included. Therefore, the Commission regards the dose of 33 mg/kg body weight and day as the NOAEL for developmental toxicity in male reproductive organs; it is thus in the same dose range as the NOAEL of 46 mg/kg body weight and day derived from the 3-generation study (see Section "Generation studies"; NTP 2003).

Groups of 10 female Wistar rats were given gavage doses of DEHP of 0, 0.25 or 6.25 mg/kg body weight and day from the day of mating to the end of lactation. Litter parameters, body, kidney and heart weights, kidney function and blood pressure were examined in 5 or 6 offspring per group and sex on the day of birth and at 3, 15 and 21 weeks of age. The body weights of the offspring were reduced on the day of birth at 6.25 mg/kg body weight and day while at 0.25 mg/kg body weight and day the body weights were reduced only at the end of lactation. The relative kidney weights were increased in the male offspring at 6.25 mg/kg body weight and day from the day of birth to week 21, whereas in the females the relative kidney weights decreased in week 15. In both groups, the renal cortex appeared thinner than in the control animals from birth. The number of nephrons was significantly lower at the end of lactation and at 33 weeks of age. Renal protein formation of renin and angiotensin II was reduced on the day of birth and increased at the end of lactation. The mRNA expression of genes involved in renal development (*Foxd1*, *Gdnf*, *Pax2* and *Wnt11*) was decreased at birth, whereas the mRNA expression of other genes (*Bmp4*, *Cdh11*, *Calm1* and *Ywhab*) was increased (Wei et al. 2012). The change in the relative kidney weights can be attributed to the decrease in body weight. Prenatal and postnatal effects were not differentiated. In humans, renal development is complete by week 36 of gestation (Bertram et al. 2011), whereas in rats it is not complete before postnatal weeks 4 to 6 (Zoetis and Hurt 2003). Humans have an average of 1 million nephrons per kidney, but the number varies in individuals from 200 000 to more than 2 million (Bertram et al. 2011). A small number of nephrons is associated with increased sensitivity to the development of high blood pressure and chronic kidney disorders in adulthood together with further factors such as rapid postnatal growth or overfeeding during infancy or childhood (Abitbol and Rodriguez 2009; Bertram et al. 2011; Boubred et al. 2013). In rats, intrauterine growth retardation may induce a reduced number of nephrons at birth, which may not be fully compensated for during the first weeks after birth (Merlet-Bénichou et al. 1994). In this species, prenatal deficits in the number of nephrons resulting from placental insufficiency may be compensated for by increased growth during lactation (Wlodek et al. 2007). There was no correlation between the size of the kidneys and body weights at birth and in adulthood in the two inbred mouse strains C3H and C57Bl6 because the variation of the parameters examined was not higher than the 2-fold variation of the parameters necessary for statistical significance (Murawski et al. 2010). The study of Wei et al. (2012) has not been included in the evaluation because of the unclear relevance of the findings regarding the number of nephrons and its consequence for rats.

Mice

Groups of 7 to 10 female CD-1 mice were fed DEHP doses of 0, 0.05, 5 or 500 mg/kg body weight and day from day 0.5 of gestation to the end of lactation. In the high dose group, there were post-implantation losses of 90%, and no offspring were born. The other doses had no effect on the litter parameters. A total of 30 male and 30 female offspring were examined per group. At sexual maturity, the following findings were recorded at 0.05 mg/kg body weight and day and above: reduced body weights (a 20% to 25% decrease on postnatal day 21), altered gonad weights (testis weights decreased by 13% and ovarian weights increased by 40%), poorer germ cell quality (fewer sperm, viability of the sperm reduced and fewer oocytes that reached the second phase of meiosis) and increased gene expression of gonadotropin subunits in the pituitary gland. In both sexes, DEHP exposure led to changes in oestrogen synthesis, induced dysregulation of the pituitary–gonadal axis and altered the reproductive performance of the treated animals. The authors derived a LOAEL of 0.05 mg/kg body weight and day for developmental toxicity in mice (Pocar et al. 2012). In another study in C3H mice, the authors established a LOAEL of 0.05 mg/kg body weight and day based on increased body weights in the female offspring on postnatal day 21 (Schmidt et al. 2012). As DEHP was administered with the diet until the end of lactation, it is assumed for both studies (Pocar et al. 2012; Schmidt et al. 2012) that the effects were caused by both prenatal exposure and the direct intake of DEHP by the offspring at the end of lactation (around postnatal day 10 and thereafter). A comparison of the studies of Pocar et al. (2012) and Schmidt et al. (2012), who are members of the same research group, revealed contradictory results. Opposite results were obtained for body weights and abdominal fat, albeit in different mouse strains. DEHP was administered with the diet, but no data were reported about the intake or for body weights on postnatal day 1. Furthermore, it is unclear why different numbers of animals were examined per dose group and why not all animals per dose group were examined. The authors did not provide any explanation. The study of Schmidt et al. (2012) consisted of 3 parts. Although the body weights initially differed by 20%, the 3 results were still considered together.

Generation studies

In a 3-generation study with Sprague Dawley rats and continuous mating, the male adult F1, F2 and F3 animals had smaller testes that were outside the range of the historical control values in size after doses of 14 mg/kg body weight and day and above in the diet. At 359/391/359 mg/kg body weight and day and above, increased detachment of the epithelial cells in the epididymis was observed in the adult F0, F1 and F2 animals, and decreased relative and absolute testis weights and testicular atrophy were observed in the adult F1 and F2 animals. In the F1 offspring, the number of foetuses per litter was reduced at this dose. In addition, the anogenital distance in the male offspring of all generations was decreased and the descent of

the testes was delayed at this dose; vaginal opening was delayed in the female offspring. In the F2 offspring, the body weights at birth were reduced and the fertility index was decreased by 55% at 359 mg/kg body weight and above. Unlike in studies carried out according to the OECD Test Guideline, all the male offspring were examined (NTP 2003, 2005). According to the National Toxicology Program (NTP) of the Center for the Evaluation of Risks to Human Reproduction (CERHR), the NOAEL for developmental toxicity in the testes is 4.8 mg/kg body weight and day and the NOAEL for foetotoxicity 46 mg/kg body weight and day (NTP 2006). The results of the 3-generation study were re-evaluated to increase the validity of the statistical analysis with the aim of detecting even the slightest effects. The authors derived a NOAEL of 4.8 mg/kg body weight and day for developmental toxicity in male reproductive organs (Blystone et al. 2010). When the effects were evaluated individually and not combined within the "phthalate syndrome", statistically significant effects on the male reproductive organs were obtained only at 359 mg/kg body weight and day. The Commission does not regard the low incidence of small testes as adverse, especially as no consequences resulted for the following generation. The effects on the absolute weights of epididymis, cauda epididymis and testes in the F2 fathers of the group given 46 mg/kg body weight are not regarded as adverse because there were no changes in the relative organ weights or any histopathological effects. The Commission thus derived a LOAEL of 359 mg/kg body weight and day for developmental toxicity in male reproductive organs and foetotoxicity and a NOAEL of 46 mg/kg body weight and day for both end points.

As a study in CD-1 mice only tested a high dose of 500 mg/kg body weight and day (Doyle et al. 2013), this study does not provide results relevant to developmental toxicity.

The effects on the male reproductive organs of foetuses treated with DEHP in utero and of offspring treated prenatally and postnatally are listed in detail in Table 2 and Table 3, respectively.

Following in utero treatment, an increased incidence of Leydig cell clusters was found in male foetuses at 10 mg/kg body weight and day and above (Klinefelter et al. 2012) and at 50 mg/kg body weight and above (Saillenfait et al. 2013). Leydig cell clusters were found also in the male foetuses of untreated guinea pigs, rats and mice. They are closely associated with the small blood vessels that are centrally located in the angular intertubular areas (Fawcett et al. 1973). Studies showed that although Leydig cell clusters (more than 5 cells) were observed, there was no change in the number of Leydig cells after exposure to DEHP (Ge et al. 2007) or DBP (Mahood et al. 2005; Johnson et al. 2012). As the number of Leydig cells was not increased, the biological relevance of Leydig cell clusters is questionable.

For rats, the NOAEL for developmental toxicity in male reproductive organs was found to be 30 mg/kg body weight and day after prenatal oral exposure (see Table 2; Borch et al. 2006) and 46 mg/kg body weight and day after prenatal and postnatal oral exposure (see Table 3; NTP 2003); this is also the NOAEL for foetotoxicity and fertility.

Table 1 Developmental toxicity studies relevant to the present evaluation with prenatal and prenatal and postnatal exposure to low doses of DEHP

Species	Exposure	Findings	References
prenatal treatment			
inhalation			
rat, Wistar, 25 ♀ per group	**GD 6–15**, 0, 10, 50, 300 mg/m³, 6 hours/day, **examination: GD 20 (n = 20), PND 21 (n = 5)**	**50 mg/m³: NOAEL maternal toxicity, prenatal developmental toxicity**; **300 mg/m³**: dams: body weight gains ↓ (PND 21); foetuses: retardations ↑ (dilated renal pelvis at 0, 10, 50, 300 mg/m³: 16.7%, 33.3%, 31.3%, 56.3%; p = 0.05) (high incidence in historical controls; no other details); pups: no impairment of postnatal development up to PND 21	Merkle et al. 1988
oral			
rat, SD (Crj:CD), 11–12 ♀	**GD 7–18**, 0, 125, 250, 500 mg/kg body weight and day, gavage, **examination: GD 20 (3 ♀ caesarean section, 8–9 ♀ spontaneous birth), offspring: GD 20, PND 4, 7, 14, 21, 35, 70**	**125 mg/kg body weight: NOAEL for developmental toxicity in ♂ reproductive organs**; **250 mg/kg body weight and above**: foetuses: ♀: body weights ↑; ♂: multinucleated germ cells, increase in germ cells in the seminiferous cord, hyperplasia of interstitial cells on GD 20; **500 mg/kg body weight**: foetuses: ♂: body weights ↑, androgen-receptor-positive cells (Leydig cells in the interstitium increased on GD 20), degeneration of germ cells; histological effects on the testes reversible on PND 35, 70; no effects on the number of androgen receptors in the interstitium on PND 35 and 70 and on the number, morphology and function of the sperm on PND 70; no effects on the dams	Shirota et al. 2005

Table 1 (continued)

Species	Exposure	Findings	References
5–6 ♀	**GD 7–18**, 0, 500, 1000 mg/kg body weight and day, gavage, **examination of 8–20 ♂ foetuses on GD 12, 14, 16, 18, 20 and of 6–12 ♂ on PND 49**	**500 mg/kg body weight**: dams: in 2/5 animals, no litter; foetuses: 2 foetuses from 1 litter with malformations; ♂: degeneration of germ cells on GD 16, proliferation of interstitial cells on GD 18 and 20, multinucleated germ cells on GD 20; **1000 mg/kg body weight**: dams: body weight gains ↓; foetuses: body weights decreased on GD 14, 18, mortality increased to 20%–36%, 6 foetuses from 4 litters with malformations (club foot, anal atresia, kinked tail, oedema); ♂: atrophy or dilation of seminiferous tubules on PND 49	
rat, Wistar, 8 ♀	**GD 7–21**, 0, 10, 30, 100, 300, mg/kg body weight and day, in corn oil, gavage, **examination: GD 21**	**30 mg/kg body weight**: **NOAEL for developmental toxicity in ♂ reproductive organs**; **100 mg/kg body weight and above**: number of germ cells ↑, central arrangement of germ cells in the tubules, incidence of multinucleated germ cells, mRNA expression and Star protein formation in the testes ↓; **300 mg/kg body weight**: vacuolation of Sertoli cells, clusters of spindle-shaped Leydig cells, testosterone levels and testosterone production ex vivo in the testes ↓, mRNA expression and protein formation of Sr-B1, Pbr and P450scc in the testes ↓, mRNA expression of Sf-1 and Insl3 in the testes ↓ (examined only at 300 mg/kg body weight); trend towards a dose-dependent decrease in mRNA expression and protein formation of Sr-B1, Star, Pbr and P450scc and protein formation of Ppar γ in the testes; non-significant trend for reduced testosterone levels in the blood; no effects on cholesterol levels in the plasma or mRNA expression of *Ppar α* and *γ* in the testes; no data for litter parameters	Borch et al. 2006

Table 1 (continued)

Species	Exposure	Findings	References
mouse, **CD-1**, **9–20 ♀**	**GD 9–18**, 0, 0.0005, 0.001, 0.005, 0.5, 50, 500 mg/kg body weight and day, oral (micropipetting into the mouth; 30 µl/50 g), **examination of some ♂ foetuses: GD18**	between **0.0005 and 50 mg/kg body weight**: serum testosterone levels ↑; **50 mg/kg body weight and above**: absolute testis weights ↓; serum testosterone levels (pooled) (only ♂ foetuses that were located between 1 ♂ and 1 ♀ foetus in utero) MEHP levels in foetal serum (GD 18) 2.1 ± 0.5, 3.3 ± 0.1, 9.2 ± 1.4, 60.4 ± 6.1, 5918 ± 718, 25767 ± 3304 ng/ml at 0.0005, 0.001, 0.005, 0.5, 50, 500 mg/kg body weight and day; MEHP in maternal serum: 10.6 ± 2.7, 59.8 ± 6.3 mg/ml at 50, 500 mg/kg body weight and day; 1.8 or 2.3 times higher than foetal levels; quadratic fit yielded statistical significance for anogenital distance and serum testosterone levels in foetuses and dams; no effects on dams, litter size, sex ratio, body or testis weights, anogenital distance	Do et al. 2012
prenatal and postnatal treatment			
rat, Wistar, 10–12 ♀	**GD 1–PND 21**, 0, 20, 100, 500 mg/kg body weight and day, in corn oil, gavage, **examination of ♂ offspring (10 per group): PND 15, 21, 33, 65–70, 155–175**	**100 mg/kg body weight: NOAEL for developmental toxicity in ♂ reproductive organs and sexual behaviour in ♂ and maternal toxicity**; **500 mg/kg body weight**: dams: post-implantation losses ↑, litter size ↓; ♂ offspring: prostate and seminal vesicular weights decreased as from puberty, sperm production and sperm count in epididymis decreased as from puberty, change in sexual behaviour (number of ejaculations and number of intromissions up to ejaculation), androgen imprinting disturbed during development; no effects on testis descent or preputial separation, testis and epididymis weights; sperm morphology, plasma testosterone concentrations	Dalsenter et al. 2006

Table 1 (continued)

Species	Exposure	Findings	References
rat, Wistar, 10 ♀	**GD 6–PND 21**, 0, 0.25, 6.25 mg/kg body weight and day, gavage, **examination: PND 0, 21, 95, 147, 231**	**0.25 mg/kg body weight and above**: offspring: systolic blood pressure increased on PND 231, renal cortex thinner, number of nephrons decreased on PND 21 and 231, glomerular size decreased on PND 0–231, glomerular volume increased on PND 0–231, size of Bowman's capsules decreased on PND 0–231, glomerular sclerosis, interstitial fibrosis, fusion of podocytes on PND 231; **0.25 mg/kg body weight**: offspring: body weights decreased on PND 21, diastolic blood pressure increased on PND 231, renin and angiotensin II decreased on PND 0, increased on PND 21; ♂: systolic blood pressure increased on PND 147, Pparα protein formation increased on PND 0 and 21; ♀: Pparα protein formation increased on PND 21; **6.25 mg/kg body weight**: offspring: body weights increased on PND 0, Pparγα protein formation increased on PND 0 and 21; ♂: absolute kidney weights increased on PND 147, relative kidney weights increased on PND 0–147, diastolic blood pressure increased on PND 231; ♀: absolute kidney weights decreased on PND 95, relative kidney weights decreased on PND 95; no effects on heart weights	Wei et al. 2012
rat, Wistar, 11–16 ♀	**GD 6–PND 21**, 0, 0.015, 0.045, 0.135, 0.405, 1.215 mg/kg body weight and day (low dose range) and 0, 5, 15, 45, 135, 405 mg/kg body weight and day, in arachis oil, gavage, **examination of offspring: PND 1, 13, 22, 33**	maternal toxicity and ♀ offspring: **15 mg/kg body weight and above**: delay in vaginal opening (about 2 days); **135 mg/kg body weight and above**: trend towards a delay in the onset of the first oestrus (about 2 days later), absolute liver weights increased on PND 1, liver enlargement; **405 mg/kg body weight: NOAEL maternal toxicity**; no effects on litter parameters, nipple development, anogenital distance	Grande et al. 2006

Table 1 (continued)

Species	Exposure	Findings	References
		♂ offspring: **5 mg/kg body weight and above**: absolute testis weights increased on PND 22 (increased up to 135 mg/kg body weight, at 405 mg/kg body weight decreased); **15 mg/kg body weight and above**: delay in preputial separation; **135 mg/kg body weight and above**: histopathological changes in testes: multinucleated germ cells increased on PND 1 and 22, germ cell differentiation in tubules decreased on PND 22; **405 mg/kg body weight**: nipple retention, anogenital distance decreased on PND 22; no effects on testosterone concentration in testes	Andrade et al. 2006 a
rat, Wistar, 8–16 ♀	**GD 7–21, PND 1–16**, 0, 10, 30, 100, 300, 600, 900 mg/kg body weight and day, in corn oil, gavage, **examination: PND 1, 12, 16**	**10 mg/kg body weight**: foetuses: absolute adrenal weights ↓, signs of dysgenesis syndrome of the external genitals (grade 1); **10 mg/kg body weight and above**: foetuses: anogenital distance ↓ (PND 1); **100 mg/kg body weight**: foetuses: absolute adrenal and testis weights ↓; **300 mg/kg body weight**: foetuses: prostate gland: gene expression of *PbpC3* and *Odc* ↓; **300 mg/kg body weight and above**: foetuses: ♂: body weights ↓, number of nipples ↑ (PND 12), absolute liver weights ↑, number of germ cells ↓, focal hyperplasia of Leydig cells, diameter of seminiferous tubules ↓; **600 mg/kg body weight and above**: foetuses: absolute testis weights ↓; **900 mg/kg body weight: NOAEL for maternal toxicity**; foetuses: ♀: body weights ↓, adrenal weights ↓, foetuses: ♂: prostate gland: gene expression of *PbpC3* and *Odc* ↓; no effects on sex ratio, litter parameters, no histopathological changes in epididymis, prostate gland, seminal vesicles	Christiansen et al. 2010

Table 1 (continued)

Species	Exposure	Findings	References
8–16 ♀	**GD 7–21, PND 1–16**, 0, 3, 10, 30, 100 mg/kg body weight and day, in corn oil, gavage, **examination: PND 1, 12, 16**	**10 mg/kg body weight**: signs of dysgenesis syndrome of the external genitals (grade 1); **10 mg/kg body weight and above**: trend towards a decrease in anogenital distance; **30 mg/kg body weight and above**: prostate gland: gene expression of *PbpC3* and *Odc* ↓; **100 mg/kg body weight**: anogenital distance ↓; **100 mg/kg body weight and above**: signs of dysgenesis syndrome of the external genitals (grade 1); no effects on litter parameters, sex ratio, body weights of offspring or dams, no histopathological changes	
rat, SD, 6–7 ♀	**GD 8–PND 17**, 0, 11, 33, 100, 300 mg/kg body weight, in corn oil, gavage, **examination of ♂ offspring (71–93 per group) on PND 2, 13, 17, (54–76 per group) on PND 35, 63–65**	**11 mg/kg body weight and above**: ♂ offspring: dose-dependent significant increase in number of ♂ with "phthalate syndrome" (changes in anogenital distance, testicular lesions, effects on androgen-dependent and Insl3-dependent tissues, nipple retention) (0%, 11.3%, 11.6%, 12.9%, 51.3% of ♂ offspring at 0, 11, 33, 100, 300 mg/kg body weight and day); evaluation by the Commission: "phthalate syndrome" is not a clearly defined, scientific syndrome; significant differences in individual effects only at much higher doses, control incidences were 0, litter data were not reported; MEHP concentration in amniotic fluid correlated with maternal DEHP concentration in the urine or with DEHP dose, dose-dependent increase in MEHP concentration in amniotic fluid (7.2, 68.4, 168, 748, 2324 ng/ml at 0, 11, 33, 100, 300 mg/kg body weight and day); **33 mg/kg body weight: NOAEL for developmental toxicity in ♂ reproductive organs**, increase in histopathological changes in testes not dose-dependent;	Gray et al. 2009 see also Calafat et al. 2006

Table 1 (continued)

Species	Exposure	Findings	References
		100 mg/kg body weight: ♂ offspring: absolute seminal vesicle weights ↓, number of malformed coagulating glands increased, no histopathological changes in testes; **300 mg/kg body weight**: **NOAEL maternal toxicity**; ♂ offspring: body weights decreased by 7% on PND 2; number of nipples and areolas increased on PND 13, anogenital distance decreased by 50%–60% on PND 2, androgenic effect decreased by 25% on PND 63–65, dose-dependent delay in preputial separation, androgen-dependent absolute organ weights (testes, epididymis, seminal vesicles, prostate gland, Cowper's gland, kidneys, adrenal gland) ↓, sperm count ↓; no effects on litter size, survival index, testosterone or oestradiol in serum;	
	GD 8 to PND 63–65, examination of ♂ offspring (16–20 per group) on PND 35, 63–65	♂ offspring: **100 mg/kg body weight**: absolute liver weights ↑; **300 mg/kg body weight**: dose-dependent delay in preputial separation, androgen-dependent organ weights (penis, epididymis, seminal vesicles, prostate gland, Cowper's gland, kidneys, adrenal gland) ↓, adrenal weights reduced by 15%; no effects on litter size, survival index, testosterone or oestradiol in serum	
mouse, CD-1, 7–10 ♀	**GD 0.5–PND 21**, 0, 0.05, 5, 500 mg/kg body weight and day, diet, **examination of offspring: PND 0, 21, 42**	**0.05 mg/kg body weight and above**: dams: dose-dependent increase in relative liver weights; offspring: body weights ↓ (20%–25% on PND 21 and 6%–14% on PND 42), *Fshr* and *Lhr* mRNA decreased in gonads; only ♀ on PND 42: abdominal adipose tissue ↓, ovarian weights ↑, *Cyp19a1* and *Pgr* mRNA decreased in ovaries, *Lhβ* mRNA decreased in pituitary gland; only ♂ on PND 42: dose-dependent decrease in seminal vesicles, sperm volume decreased by 50%, sperm viability decreased by 20%;	Pocar et al. 2012

Table 1 (continued)

Species	Exposure	Findings	References
		0.05 mg/kg body weight: offspring: only ♂ on PND 42: anogenital distance ↓; **5 mg/kg body weight**: offspring: only ♀ on PND 42: *Cyp17a1* and *Cyp19a1* mRNA decreased in ovaries; only ♂ on PND 42: *Cyp19a1* and *Pgr* mRNA decreased in testes, *Lhβ* and *Fshβ* mRNA increased in pituitary gland; **500 mg/kg body weight**: dams: 90% post-implantation losses: normal development of embryos up to GD 9.5, resorptions as from GD 10.5, only haemorrhagic residues on GD 15.5; offspring: only 1 ♂ offspring, examination not possible; no changes in fertility, survival index or sex ratio in the offspring on PND 0 or any changes in uterus or ovarian weights in the dams	
mouse, C3H/N, 5 or 10 ♀, 3 experiments	**8 weeks**, **(1 week before and 1 week during mating, GD 1–PND 21)**, 0, 0.05, 5, 500 mg/kg body weight and day, in sunflower oil, diet, **examination of offspring: PND 0, 21, 90**	**0.05 mg/kg body weight and above**: dams: F0 animals: body weights, feed consumption and visceral adipose tissue ↑, *leptin* mRNA ↑, *adiponectin* mRNA ↓; superovulated F1 animals: degenerated blastocytes ↑; offspring: body weights (only ♀ on PND 21) and visceral adipose tissue ↑, adipocytes/area ↓, metabolic changes; **0.05 mg/kg body weight**: dams: F0 animals: *Fabp4* mRNA increased in adipose tissue; **500 mg/kg body weight**: dams: 100% abortions, mRNA of *Ppar α* and *γ* increased in liver, Ppar α decreased in adipose tissue, leptin increased in plasma; superovulated F0 animals: degenerated blastocysts ↑; results inconsistent with study of Pocar et al. 2012 from the same research group, lack of data, not relevant to the evaluation	Schmidt et al. 2012

Table 1 (continued)

Species	Exposure	Findings	References
generation studies			
rat, SD, 17 ♂, ♀	**3 generations, continuous mating**, 1.5 (controls), 10, 30, 100, 300, 1000, 7500, 10 000 mg/kg diet (F0: about 0, 0.1, 0.5, 1.4, 4.8, 14, 46, 359, 775 mg/kg body weight and day; F1: 0.09, 0.48, 1.4, 4.9, 14, 48, 391, 543 mg/kg body weight and day; F2: 0.1, 0.47, 1.4, 4.8, 14, 46, 359 mg/kg body weight and day), purity: 98%, diet, **examination: PND 1, 4, 7, 14, 21 and up to week 21**	**14 mg/kg body weight: NOAEL chronic toxicity**; **14 mg/kg body weight and above**: F1 and F2 adults, ♂: small testes, seminal vesicles, prostate gland in 1–3 of 45 F1 or 21 F2 animals (outside the historical control range), not dose-dependent, no histopathological abnormalities, not regarded as adverse by the Commission; **46 mg/kg body weight: NOAEL developmental toxicity in ♂ reproductive organs, fertility and foetotoxicity**; **46 mg/kg body weight and above**: F0 adults, ♀: relative liver weights ↑; F1 adults, ♂/♀: relative and absolute liver weights ↑, minimal to mild liver hypertrophy, dilation and mineralization of renal tubules, sometimes with chronic pyelonephritis; F2 adults, ♂/♀: minimal to mild liver hypertrophy; F2 adults, ♂: absolute weights of epididymis, cauda epididymis and testes ↓; F2 dams: relative liver weights ↑; **359/391 mg/kg body weight and above**: F0 adults, ♂/♀: minimal to mild liver hypertrophy; relative and absolute liver and kidney weights ↑; F0 adults, ♂: exfoliated epithelial cells in the epididymis ↑; F1 adults, ♂/♀: relative and absolute liver weights ↑, cortical vacuolation of adrenal glands ↑; F1 adults, ♂: relative and absolute kidney weights ↑, terminal body weights reduced by 10%; testicular atrophy ↑, sperm parameters (density and number) ↓, relative and absolute weights of epididymis, cauda epididymis and testes ↓, exfoliated epithelial cells in the epididymis ↑; F1 foetuses/offspring: number of foetuses/litter ↓, number of ♂/litter ↓; ♂: anogenital distance ↓; delay in testis descent, preputial separation; ♀: delayed vaginal opening;	NTP 2003, 2005 see also Blystone et al. 2010; NTP 2006

Table 1 (continued)

Species	Exposure	Findings	References
		359/391/359 mg/kg body weight: F2 adults, ♂/♀: relative and absolute liver weights ↑, dilation and mineralization of renal tubules, sometimes with chronic pyelonephritis; F2 adults, ♂: relative and absolute kidney weights ↑, terminal body weights decreased by 14%, testicular atrophy (10/10) ↑, sperm parameters (density and number) ↓, relative weights of epididymis, cauda epididymis and testes ↓, exfoliated epithelial cells in the epididymis ↑; F3 adults, ♂/♀: relative and absolute liver weights ↑; F3 adults, ♂: sperm parameters (density, number) ↓, relative weights of epididymis, testes and absolute prostate weights ↓; F2 foetuses/offspring: body weights ↓ on PND 1–21, fertility index decreased by 55%; ♂: anogenital distance ↓, delay in testis descent, preputial separation; ♀: delayed vaginal opening; F3 foetuses/offspring: ♂: anogenital distance ↓, delay in testis descent and preputial separation; ♀: delayed vaginal opening; **775/543 mg/kg body weight**: F0 adults, ♂/♀: terminal body weights decreased by 6%–21%, cortical vacuolation of adrenal glands ↑; F0 adults, ♂: sperm parameters ↓, weights of epididymis, cauda epididymis and testes ↓, exfoliated epithelial cells in the epididymis ↑, testicular atrophy ↑; F1 adults, ♂/♀: terminal body weights reduced by 6%–21%; F1 adults, ♂: histological examination: atrophy of the seminiferous tubules, no sperms, infertile; F1 adults, ♀: relative and absolute kidney and ovarian weights ↑;	

Table 1 (continued)

Species	Exposure	Findings	References
		F1 foetuses/offspring: body weights decreased on PND 1, 4, 7, 14, 21; no effects on anogenital distance in ♀; teratogenicity not examined further examinations: mating of treated F1 and F2 ♂ (543 and 359 mg/kg body weight, respectively) with untreated ♀: number of implantations ↓, mating, gestation and fertility indices ↓; mating of treated F1 and F2 ♀ (543 and 359 mg/kg body weight) with untreated ♂: anogenital distance decreased in the ♂ offspring, body weights of the offspring of dams of the 359-mg/kg group ↓	
mouse, CD-1, 7–10 ♀	**GD 7–14**, transgenerational, 0, 500 mg/kg body weight and day, gavage, mating of the F1 generation among themselves or with untreated animals (F2, F3, F4), **examination of F1, F2, F3 ♂ offspring: PND 1, 10, 21, 42, 120, F4: PND 360**	**500 mg/kg body weight**: F1: litter size decreased by 17.2% on PND 1, anogenital distance decreased on PND 1, body weights increased on PND 10 (7.81 g compared with 5.63 g in the controls), alopecia (83.4%), additional nipple 16.1%; F2 and F3: delay in onset of puberty (paternal and double-cross lines) of < 1 day, anogenital distance ↓, nipple retention ↑; F1, F2 and F3: (maternal, paternal and double-cross lines): testicular abnormalities on PND 120 (sloughing of germ cells in the middle of the tubules, no lumen, loss of germ cell organization, vacuolation, loss of germ cell stages such as round spermatids, leptotene and pachytene spermatocytes); F4: double-cross mating on PND 120: same effects as in F1, F2 and F3, increase in testicular effects on PND 120; no effects in F2 and F3: on litter parameters or survival, anogenital distance, body weights, alopecia, nipples, testis weights; F0: no effect on survival after birth;	Doyle et al. 2013

Table 1 (continued)

Species	Exposure	Findings	References
	transplantation of spermatogonial stem cells in untreated animals of the F3 generation	F3: fewer sperms in the tubules of transplanted animals, block of spermatogenesis in the stage of undifferentiated spermatogonia, histological examination: testes similar to F1–F3 of the group treated with DEHP	

Fabp4: fatty acid binding protein 4; FSH: follicle-stimulating hormone; GD: gestation day;
Insl3: insulin-like hormone family 3; LH: luteinizing hormone; MEHP: monoethylhexyl phthalate;
Odc: ornithine decarboxylase; PbpC3: prostate binding protein subunit C3;
Pbr: peripheral benzodiazepine receptor; PND: postnatal day;
PPAR: peroxisome proliferator-activated receptor; Pgr: progesterone receptor;
P450scc: side-chain cleavage = CYP11A1; SD: Sprague Dawley;
Sf-1: splicing factor 1 = zinc finger protein 162;
Sr-B1: scavenger-receptor B1 = HDL (high density lipid) receptor;
Star: steroidogenic acute regulatory protein

Summary

The studies that have become available for mice since the 2002 documentation have not been included in the evaluation of developmental toxicity in animals because of inadequate study descriptions, inconsistent results and the analysis of non-validated parameters (Do et al. 2012; Doyle et al. 2013; Pocar et al. 2012; Schmidt et al. 2012). A number of studies in rats are not suitable for the evaluation of developmental toxicity for the same reasons (Andrade et al. 2006 a; Christiansen et al. 2010; Grande et al. 2006; Wei et al. 2012).

Prenatal toxicity studies published since the 2002 documentation (documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009) revealed effects on the testes in rats on day 20 of gestation induced by in utero exposure to DEHP at 250 mg/kg body weight and day and above. Until the blood–testis barrier has developed at 10 weeks of age, DEHP exposure targets the germ cells. In this study, these effects were reversible at doses lower than 500 mg/kg body weight (Shirota et al. 2005). In rats, 30 mg/kg body weight and day is the NOAEL for developmental toxicity in male reproductive organs. At 100 mg/kg body weight and above, effects on germ cell organization and reduced mRNA expression and Star protein formation were observed in the testes (Borch et al. 2006).

The small testes that were observed at 14 mg/kg body weight and day in a 3-generation study in Sprague Dawley rats with continuous mating (NTP 2003) are not regarded as an adverse effect by the Commission because the incidence was low, and there was no evidence of consequences for the following generation or a dose–response relationship. The effects on the absolute weights of the epididymis, cauda epididymis and testes in the F2 fathers of the group that received 46 mg/kg body weight are not regarded as adverse because there were no changes in the relative organ weights or any histopathological effects. The Commission thus derived a LOAEL of 359 mg/kg body weight and day for developmental toxicity in male reproductive organs and for foetotoxicity after prenatal and postnatal exposure and a NOAEL of 46 mg/kg body weight and day for both end points.

Postnatal (after postnatal day 4) and juvenile treatment

Studies after inhalation exposure, oral administration and intravenous injection

The results are not relevant to the evaluation of human exposure at the workplace because exposure started after postnatal day 4 or at the juvenile stage; therefore, the Commission has not included the studies in the evaluation.

Only the results of interest to the evaluation are summarized below.

Rats and monkeys. Inhalation exposure of male, prepubertal Wistar rats to DEHP for 4 or 8 weeks from the age of 28 to 56 days led after 8 weeks to an increase in the plasma testosterone level at 5 mg/m^3 and the seminal vesicle weights at 25 mg/m^3. No other effects were observed. The concentration of 5 mg/m^3 was regarded as the LOAEC (lowest observed adverse effect concentration) for fertility in male

prepubertal rats (Kurahashi et al. 2005). The effects were more pronounced after inhalation than after oral administration (Akingbemi et al. 2001).

Premature vaginal opening, an early first oestrus cycle and increased serum cholesterol, LH and oestradiol levels were observed in young female rats at the age of 42 days after inhalation exposure for 20 days to DEHP concentration of 25 mg/m^3 from days 22 to 42. The NOAEC was 5 mg/m^3 for female prepubertal Wistar rats (Ma et al. 2006).

Male Wistar rats were given gavage doses of DEHP of 1 to 1000 mg/kg body weight and day at the age of 10 to 50 or 50 to 90 days. Androgen-dependent organ weights were decreased in juvenile rats at about 300 mg/kg body weight and above but in adults not until 1000 mg/kg body weight. The immunotoxic parameters were affected at lower doses than the parameters for developmental toxicity. Juvenile rats were generally more sensitive to immunotoxicity than adult rats. This shows that further studies of the developing immune system are required (Tonk et al. 2012).

Juvenile common marmosets (Callithrix jacchus) were given gavage doses of DEHP of 0, 100, 500 or 2500 mg/kg body weight and day for 15 months as from 3 months of age. Effects on the testes were not found (Tomonari et al. 2006).

Mice. In female mice given dermal injections of DEHP of 0, 20 or 40 µg/kg body weight on postnatal days 5, 10, 15 and 20, litter sizes were reduced ($p < 001$) after the animals were mated at 6 weeks of age, and the body weights of the F1 offspring were decreased ($p < 0.01$). The sex ratio was not affected. The number of primordial follicles was found to be considerably depleted in the F1 offspring (Zhang et al. 2013).

Studies of PVC infusion tubes containing DEHP

Studies of PVC infusion tubes containing DEHP are available (Jacobson et al. 1977*;* Kevy and Jacobson 1982*;* Loff et al. 2007*);* they are not described in detail because they are not relevant to exposure at the workplace.

According to IARC (2012), these studies indicate that toxic effects are induced by the DEHP contained in the transfusion tubes, but these associations need further verification. The IARC (2012) reported that similar effects were not observed in rodents, and might have resulted from the route of exposure or may be species-specific.

Table 2 Effects on the male reproductive organs of rats treated with DEHP in utero

Strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
Wistar, 8 ♀	GD 7–21, gavage, 0, 10, 30, 100, 300 mg/kg body weight and day, in corn oil, examination: GD 21	**100 mg/kg body weight: NOAEL**; **300 mg/kg body weight**: level and production ex vivo ↓	not determined	**30 mg/kg body weight: NOAEL**; **100 mg/kg body weight and above**: *Star* ↓	not determined	**30 mg/kg body weight: NOAEL**; **100 mg/kg body weight and above**: germ cells localized centrally in seminiferous tubules, number of germ cells ↑, multinucleated germ cells	Borch et al. 2006
Wistar, 3–6 ♀	GD 14–18, gavage, 0, 100, 300, 500, 625, 750, 875 mg/kg body weight and day, in corn oil, examination: GD 18	**100 mg/kg body weight: NOAEL**; **300 mg/kg body weight and above**: production ex vivo ↓	not determined	**300 mg/kg body weight: NOAEL**; **500 mg/kg body weight and above:** *Star*, *Cyp11a1*, *Insl3* ↓	not determined	not determined	Hannas et al. 2011
Wistar, 5–6 ♀	GD 14–18, gavage, 0, 750 mg/kg body weight and day, in corn oil, examination: PND 2	**750 mg/kg body weight**: production ex vivo ↓	**750 mg/kg body weight**: ↓ on PND 2	**750 mg/kg body weight**: *Insl3* ↓	**750 mg/kg body weight**: testes, epididymis, ventral prostate gland, seminal vesicles, LABC muscle complex ↓	**750 mg/kg body weight**: gubernacular abnormalities ↑	Wilson et al. 2007

Table 2 (continued)

Strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
SD, experiment 1: 5–6 ♀, experiment 2: 11–12 ♀	GD 7–18, gavage, experiment 1: 0, 500, 1000 mg/kg body weight and day, experiment 2: 0, 125, 250, 500 mg/kg body weight and day, in corn oil, examination: experiment 1: GD 12, 14, 16, 18, 20, postnatally after 7 weeks, experiment 2: GD 20, postnatally after 5 and 10 weeks	not determined	not determined	not determined	not determined	**250 mg/kg body weight and above**: multinucleated germ cells, germ cells in seminiferous cords ↑, hyperplasia of interstitial cells GD 20, no longer after 5 and 10 weeks	Shirota et al. 2005
SD, 8–12 ♀	GD 12–19, gavage, experiment 1: 0, 625 mg/kg body weight and day, experiment 2: 0, 50 mg/kg body weight and day, in olive oil, examination: GD 19	**50 mg/kg body weight and above**: production ex vivo ↓	not determined	**50 mg/kg body weight and above**: *Star*, *Sr-B1* ↓	not determined	not determined	Saillenfait et al. 2013

Table 2 (continued)

Strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
SD, 5 ♀, controls: 10 ♀	GD 12–19, gavage, 0, 500 mg/kg body weight and day, in corn oil, examination: GD 19	not determined	**500 mg/kg body weight**: ↓ on GD 19	**500 mg/kg body weight**: *Star*, *Cyp11a1*, *Cyp17a1*, *Sr-B1*, *Insl3* ↓, *3beta-Hsd* ↑	not determined	not determined	Liu et al. 2005
SD, 10 ♀	GD 13–19, gavage, 0, 10, 100 mg/kg body weight and day, in corn oil, examination: GD 19	**100 mg/kg body weight**: production ex vivo ↓	not determined	**100 mg/kg body weight**: *Cyp11a1* ↓	not determined	**10 mg/kg body weight and above**: Leydig cell clusters ↑, questionable biological relevance; **100 mg/kg body weight**: malformed seminiferous cords (precursors of seminiferous tubules), germ cell-like cells in the interstitium rather than in the seminiferous cords, erythrophagosomes in the interstitium	Klinefelter et al. 2012

Table 2 (continued)

Strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
SD, 3–5 ♀	GD 14–PND 0, gavage, 0, 58, 117, 234, 469, 700, 750, 938, 1250 mg/kg body weight and day, in corn oil, examination: GD 19, 20, PND 3, 21, 60	**58 mg/kg body weight: NOAEL**; **117 mg/kg body weight and above**: production ex vivo decreased on GD 20	**938 mg/kg body weight: NOEL**; **1250 mg/kg body weight**: ↓ on PND 60	**234 mg/kg body weight**: *Cyp11a1*, *Cyp17a1* increased on GD 19, lowest dose examined; **469 mg/kg body weight**: *Cyp11a1*, *Cyp17a1* decreased on GD 19	**938 mg/kg body weight and above**: testes ↓	**234 mg/kg body weight and above**: volume of Leydig cells increased on PND 60, lower doses not examined; **938 mg/kg body weight and above**: cryptorchidism	Culty et al. 2008
SD, 3–6 ♀	GD 14–18, gavage, 0, 100, 300, 500, 625, 750, 875 mg/kg body weight and day, in corn oil, examination: GD 18	**100 mg/kg body weight: NOAEL**; **300 mg/kg body weight and above**: production ex vivo ↓	not determined	**300 mg/kg body weight: NOAEL**; **500 mg/kg body weight and above**: *Star*, *Cyp11a1* ↓	not determined	not determined	Hannas et al. 2011

Table 2 (continued)

Strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
SD, 6 ♀	GD 14–18, gavage, 0, 500 mg/kg body weight and day, in corn oil, examination: GD 18 or PND 3	**500 mg/kg body weight**: production ex vivo ↓	**500 mg/kg body weight**: on PND 3	**500 mg/kg body weight**: *Star*, *Insl3* decreased on GD 18	**500 mg/kg body weight**: no statistically significant changes in testes, LABC muscle complex ↓	**500 mg/kg body weight**: testicular malformations ↑ ($p = 0.07$)	Howdeshell et al. 2007
SD, 10 ♀	GD 14–18, gavage, 0, 750 mg/kg body weight and day, in corn oil, examination: GD 18	**750 mg/kg body weight**: production ex vivo ↓	not determined	**750 mg/kg body weight**: *Insl3* ↓	not determined	not determined	Wilson et al. 2004
SD, 5–6 ♀	GD 14–18, gavage, 0, 750 mg/kg body weight and day, in corn oil, examination: PND 2	**750 mg/kg body weight**: production ex vivo ↓	**750 mg/kg body weight**: ↓	**750 mg/kg body weight**: *Insl3* ↓	**750 mg/kg body weight**: testes, epididymis, ventral prostate gland, seminal vesicles, LABC muscle complex ↓	**750 mg/kg body weight**: absence of epididymis ↑	Wilson et al. 2007

Table 2 (continued)

Strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
Long Evans, 6–9 ♀	GD 2–20, gavage, 0, 10, 100, 750 mg/kg body weight and day, in corn oil, examination: GD 21	**10 mg/kg body weight**: production ↑; **100 mg/kg body weight**: no effect; **750 mg/kg body weight**: production ↓	**750 mg/kg body weight**: ↓	**100 mg/kg body weight: NOAEL**; **100 mg/kg body weight and above**: *Cyp11a* ↓	**10 mg/kg body weight: NOAEL**; **100 mg/kg body weight and above**: testes ↓	**10 mg/kg body weight and above**: Leydig cell clusters ↑, questionable biological relevance	Lin et al. 2008
Long Evans, 7 ♀	GD 12–21, gavage, 0, 100 mg/kg body weight and day, in corn oil, examination on PND 21, 35, 90	**100 mg/kg body weight**: basal and LH-stimulated production via isolated Leydig cells decreased only on PND 21	not determined	not determined	**100 mg/kg body weight**: no statistically significant changes in testes or seminal vesicles	**100 mg/kg body weight**: no abnormalities	Akingbemi et al. 2001

* gene expression of enzymes or receptors involved in steroid synthesis or cholesterol metabolism and transport, most sensitive parameter listed in table;
CYP11A1 = mitochondrial cholesterol monooxygenase, catalyses the cleavage of the side chain of cholesterol resulting in pregnenolone;
CYP17A1: catalyses the 17-alpha hydroxylation of pregnenolone and progesterone and the 17,20-lyase reaction, during which 17α-hydroxyprogesterone is converted to androstenedione;
GD: gestation day; 3beta-HSD: 3beta-hydroxysteroid dehydrogenase; Insl3: insulin-like-3 = insulin-like hormone family;
LABC: levator ani-bulbocavernosus; LH: luteinizing hormone; NOEL = no observed effect level; PND: postnatal day; SD: Sprague Dawley rat;
Sr-B1: scavenger-receptor B1 = HDL (high density lipid) receptor; Star: steroidogenic acute regulatory protein

Table 3 Effects on the male reproductive organs of rats and mice treated prenatally and postnatally with DEHP

Species, strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
rat							
Wistar, 8 ♀, controls 16 ♀	GD 7–21 and PND 1–16, gavage, experiment 1: 0, 10, 30, 100, 300, 600, 900 mg/kg body weight and day, experiment 2: 0, 3, 10, 30, 100 mg/kg body weight and day, in corn oil, examination: PND 1, 12, 16	not determined	**experiment 1: 10 mg/kg body weight and above**: ↓ on PND 1; **experiment 2: 100 mg/kg body weight**: ↓ on PND 1; inconsistent results, stereomicroscope	not determined	**300 mg/kg body weight: NOAEL**; **600 mg/kg body weight and above**: testes decreased on PND16	**3 mg/kg body weight and above**: first degree dysgenesis syndrome of the external genitals when the 2 experiments were combined on PND 16, but no change at 30 mg/kg body weight	Christiansen et al. 2010

Table 3 (continued)

Species, strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/ glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
Wistar, 11–16 ♀	GD 6–PND 21, gavage, experiment 1: 0, 0.015, 0.045, 0.135, 0.405, 1.215 mg/kg body weight and day, experiment 2: 0, 5, 15, 45, 135, 405 mg/kg body weight and day, in arachis oil, examination: PND 1, 13, 22, 33, 144	**up to 405 mg/kg body weight**: no statistically significant change in level on PND 1	**135 mg/kg body weight: NOEL**; **405 mg/kg body weight**: ↓ on PND 22, measured manually with Vernier caliper	not determined	**5 mg/kg/body weight and above**: testes increased on PND 22 (up to 135 mg/kg body weight)	**5 mg/kg body weight and above**: cryptorchidism in 1 animal at 5, 135, 405 mg/kg body weight (9–10 animals examined per dose) on PND144, no evaluation in tabular form, number of animals per litter not specified **15 mg/kg body weight and above**: daily sperm production decreased on PND 144; **135 mg/kg body weight and above**: multinucleated germ cells increased on PND 1 and 22, germ cell differentiation in seminiferous tubules decreased on PND 22	Andrade et al. 2006 a, b

Table 3 (continued)

Species, strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/ glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
Wistar, 10–12 ♀	GD 1–PND 21, gavage, 0, 20, 100, 500 mg/kg body weight and day, in corn oil, examination: PND 15, 21, 33, 65–70, 155–175	not determined	not determined	not determined	**500 mg/kg body weight**: ventral prostate gland and seminal vesicles decreased on PND 155–175, testes and epididymis: no statistically significant change	**100 mg/kg body weight: NOAEL**; **500 mg/kg body weight and above**: daily sperm production and sperm count in epididymis decreased as from puberty	Dalsenter et al. 2006
SD, 12–14 ♀	GD 8–PND 17 or GD 8–PND 65, gavage, 0, 11, 33, 100, 300 mg/kg body weight and day, in corn oil, examination: PND 2, 13, 17, 35, 63–65, 7 months	not determined	**100 mg/kg body weight: NOEL**; **300 mg/kg body weight**: ↓ on PND 2	not determined	**100 mg/kg body weight: NOAEL**; **300 mg/kg body weight and above**: testes ↓ (age: 7 months)	**11 mg/kg body weight and above**: total number of all testicular lesions increased on PND 63–65 (7 months), no significant change in individual lesions; **Commission**: **individual effects** yielded a different **NOAEL: 33 mg/kg body weight**	Gray et al. 2009

Table 3 (continued)

Species, strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/ glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
SD, 17 ♀	3 generations, diet, 1.5 (controls), 10, 30, 100, 300, 1000, 7500, 10 000 mg/kg diet (F0: about 0, 0.1, 0.5, 1.4, 4.8, 14, 46, 359, 775 mg/kg body weight and day; F1: 0.09, 0.48, 1.4, 4.9, 14, 48, 391, 543 mg/kg body weight and day; F2: 0.1, 0.47, 1.4, 4.8, 14, 46, 359 mg/kg body weight and day)	not determined	**46/48/46 mg/ kg body weight: NOEL**; **359/391/359 mg/ kg body weight and day and above**: F1, F2, F3: ↓ on PND 1	not determined	**46/48/46 mg/ kg body weight: NOAEL**; **359/391/359 mg/ kg body weight and above**: F1, F2, F3 (adults): absolute and relative right testis and absolute and relative right epididymis ↓	4.8/4.9/4.8 mg/kg body weight: NOAEL (authors) **14/14/14 mg/kg body weight and day**: F1 and F2 (adults): small testes, prostate gland, seminal vesicles in 1–3 animals, outside the historical control range; **Commission**: not adverse, not dose-dependent and no evidence of effects on the following generation; **NOAEL 46/48/46 mg/ kg body weight (Commission)**	NTP 2003, 2005; see also Blystone et al. 2010
SD, 4–5 ♀	GD 14–PND 2, gavage, 0, 750 mg/kg body weight and day, in corn oil, examination: GD 17, 18, 20, PND 2	**750 mg/kg body weight**: production ex vivo decreased on GD 17, 18, 20, PND 2	**750 mg/kg body weight**: ↓ on PND 2	not determined	**750 mg/kg body weight**: testes decreased on GD 20, PND 2; no change on GD 17, 18	**750 mg/kg body weight**: Leydig cell hyperplasia, multinucleated germ cells on GD 20, PND 2	Parks et al. 2000

Table 3 (continued)

Species, strain, number	Treatment	Testosterone	Anogenital distance	Gene expression*	Weights of the male reproductive organs and accessory tissues/ glands	Abnormalities in male reproductive organs determined by gross pathological or histopathological examination	References
Long Evans, 11–13 ♀	GD 12.5–PND 21.5, gavage, 0, 10, 750 mg/kg body weight and day, in corn oil, examination: PND 0, 2, 21, 49	not determined	**10 mg/kg body weight: NOEL**; **750 mg/kg body weight**: ↓ on PND 2	**10 mg/kg body weight and above**: *Star* decreased on PND 0	**up to 750 mg/kg body weight**: no change in testes or prostate gland on PND 49	**10 mg/kg body weight and above**: Leydig cell clusters on PND 49, number of cells per cluster dose-dependent, biological relevance of clusters unclear	Lin et al. 2009
mouse							
Kuming, 10 ♀	GD 12–PND 3, gavage, 0, 100, 200, 500 mg/kg body weight and day, in corn oil, examination: PND 5, 15	not determined	not determined	**100 mg/ kg body weight and above**: *Insl3* decreased on↓ PND 5, 15	not determined	**100 mg/kg body weight and above**: damaged spermatogenic cells, Leydig cells: irregularly shaped nucleus with unclear borders	Song et al. 2008

See Table 2 for abbreviations

Manifesto (reproductive and developmental toxicity)

The critical effects of DEHP are developmental toxicity, liver carcinogenicity and effects on fertility and the lungs and possibly on the larynx in rodents.

Prenatal toxicity. Until 2014, DEHP was classified in Pregnancy Risk Group C with a MAK value of 10 mg/m³.

There is evidence in humans that there may be a relationship between prenatal exposure to DEHP and effects on the anogenital distance in male newborn babies, birth weights and the duration of pregnancy. However, the consistency of these data has yet to be reliably established.

Recent studies of teratogenicity in animals have not become available since the 2002 documentation was published.

In the studies described in the DEHP documentation from 2002 (documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009), toxic effects on development such as decreased body weights and reduced survival were observed in rats only at maternally toxic doses higher than 300 mg/kg body weight and day (NTP 1986; Tyl et al. 1988); malformations were determined at doses higher than 1000 mg/kg body weight and day (Hellwig et al. 1997). The NOAEL for foetotoxicity and teratogenicity in the rat was about 200 mg/kg body weight and day after oral administration (Hellwig et al. 1997). After inhalation, the NOAEC was 50 mg/m³; at 300 mg/m³ retardations were observed in the foetuses together with maternal toxicity (Merkle et al. 1988). Prenatal studies of developmental toxicity in CD-1 mice revealed malformations (open eyes, exophthalmia, exencephaly, short or no tails, major blood vessel malformations, fused or branched ribs and fused thoracic vertebral centra) at doses of 91 mg/kg body weight and day and above and foetotoxicity at 95 mg/kg body weight and day and above. In this species, the NOAELs were 44 mg/kg body weight and day for teratogenicity (Tyl et al. 1988) and 48 mg/kg body weight and day for foetotoxicity (NTP 1988). In the NTP study, concurrent maternal toxicity was observed. In a study with prenatal and postnatal feeding of mice and continuous mating, the number of litters per pair and the number of live offspring were reduced at the LOAEL of 200 mg/kg body weight and day. The NOAEL for foetotoxicity was 20 mg/kg body weight and day (see documentation "Di(2-ethylhexyl)phthalate (DEHP)" 2009; Lamb et al. 1987).

As shown in the Section "Mechanism of Action", rats were particularly sensitive, compared with marmosets, to the effects of DEHP on the male reproductive organs. None of the available studies of toxicity in the male reproductive organs of mice are relevant to the present evaluation.

Recent studies of toxic effects on prenatal development in male reproductive organs revealed effects on the testes in rats exposed to DEHP in utero on day 20 of gestation at levels of 250 mg/kg body weight and day and above. The germ cells are the target of DEHP exposure until the blood–testis barrier has developed at the age of 10 weeks. In this study, these effects were reversible after doses lower than 500 mg/kg body weight (Shirota et al. 2005). At 100 mg/kg body weight and above, effects on germ cell organization and reduced mRNA expression and Star protein formation were observed in the testes. In rats, 30 mg/kg body weight and

day was the NOAEL for developmental toxicity in the male reproductive organs (Borch et al. 2006). The small testes observed at 14 mg/kg body weight and day in a 3-generation study in Sprague Dawley rats with continuous mating (NTP 2003) are not regarded as adverse by the Commission because their incidence was low, and there was no evidence of consequences for the following generation or a dose–response relationship. The effects on the absolute weights of the epididymis, cauda epididymis and testes in the F2 fathers of the group given 46 mg/kg body weight are not regarded as adverse because there were no changes in relative organ weights or any histopathological effects. The Commission thus derived a LOAEL of 359 mg/kg body weight and day for developmental toxicity in male reproductive organs and for foetotoxicity and a NOAEL of 46 mg/kg body weight and day for both end points.

The following toxicokinetic data are used to extrapolate the NOAELs in rats and mice to a concentration in workplace air: the corresponding species-specific correction values for rats and mice determined on the basis of the toxicokinetic data (1:4; 1:7), the oral absorption of 50%, the body weight (70 kg) and the respiratory volume (10 m^3) of the person, and 75% absorption by inhalation. This results in the respective concentrations and divergence from the MAK value of 2 mg/m^3 I listed in Table 4.

Table 4 NOAECs/NOAELs for rats and mice relevant to the evaluation, toxicokinetic extrapolation of the NOAELs to concentrations in air and the resulting divergence from the MAK value of 2 mg/m^3 I

Reference	Species, end point	NOAEC/NOAEL	Toxicokinetic extrapolation[a)]	Divergence from the MAK value of 2 mg/m^3 I
	rat,			
Merkle et al. 1988	prenatal, inhalation	50 mg/m^3		25
Borch et al. 2006	prenatal, oral	30 mg/kg body weight and day: testicular toxicity	35	18
Hellwig et al. 1997		200 mg/kg body weight and day: foetotoxicity and teratogenicity	233	117
NTP 2003	prenatal and postnatal, oral	46 mg/kg body weight and day: testicular toxicity and foetotoxicity	54	27
	mouse,			
Tyl et al. 1988	prenatal, oral	48 mg/kg body weight and day: foetotoxicity	32	16
NTP 1988		44 mg/kg body weight and day: teratogenicity	29	15
Lamb et al. 1987	prenatal and postnatal, oral	20 mg/kg body weight and day: foetotoxicity	13	7

[a)] 70/10 × (1:4 or 1:7) × 0.5 (oral absorption by animals)/0.75 (inhalation absorption by humans)

Classification in Pregnancy Risk Group C is justified taking into account that the MAK value is 7 times lower than the NOAEC calculated for foetotoxicity in mice after continuous prenatal and postnatal feeding (Lamb et al. 1987) and that the NOAEL is 10 times lower than the LOAEL established in this study. The NOAEC for developmental toxicity after inhalation is 25 times higher than the MAK value. Therefore, the margin between the NOAECs calculated for foetotoxicity, developmental toxicity in male reproductive organs and toxic effects on prenatal development and the MAK value of 2 mg/m³ I is sufficiently large, and di-(2-ethylhexyl) phthalate (DEHP) has been classified in Pregnancy Risk Group C.

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