Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at Community level of

Di-n-hexyl phthalate (DnHP)

ECHA/RAC/CLH-O-0000001541-83-03/A1

EC number: 201-559-5
CAS number: 84-75-3

Adopted

13 September 2011

CONTENTS

PART A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	**Di-*n*-hexyl phthalate**
EC number:	201-559-5
CAS number:	84-75-3
Annex VI Index number:	-
Degree of purity:	generally >97% based on information found in MSDS
Impurities:	no information available

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation	**Directive 67/548/EEC (Dangerous Substances Directive; DSD)**
Current entry in Annex VI, CLP Regulation	-	-
Current proposal for consideration by RAC	Repr. 1B – H 360FD	Repr. Cat. 2; R61 Repr. Cat. 2; R60
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Repr. 1B – H 360FD	Repr. Cat. 2; R61 Repr. Cat. 2; R60

1.3 Proposed harmonised classification and labelling based on CLP Regulation and/or DSD criteria

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None		None	Not evaluated
2.2.	Flammable gases	None		None	Not evaluated
2.3.	Flammable aerosols	None		None	Not evaluated
2.4.	Oxidising gases	None		None	Not evaluated
2.5.	Gases under pressure	None		None	Not evaluated
2.6.	Flammable liquids	None		None	Not evaluated
2.7.	Flammable solids	None		None	Not evaluated
2.8.	Self-reactive substances and mixtures	None		None	Not evaluated
2.9.	Pyrophoric liquids	None		None	Not evaluated
2.10.	Pyrophoric solids	None		None	Not evaluated
2.11.	Self-heating substances and mixtures	None		None	Not evaluated
2.12.	Substances and mixtures which in contact with water emit flammable gases	None		None	Not evaluated
2.13.	Oxidising liquids	None		None	Not evaluated
2.14.	Oxidising solids	None		None	Not evaluated
2.15.	Organic peroxides	None		None	Not evaluated
2.16.	Substance and mixtures corrosive to metals	None		None	Not evaluated
3.1.	Acute toxicity - oral	None		None	Not evaluated
	Acute toxicity - dermal	None		None	Not evaluated
	Acute toxicity - inhalation	None		None	Not evaluated
3.2.	Skin corrosion / irritation	None		None	Not evaluated
3.3.	Serious eye damage / eye irritation	None		None	Not evaluated
3.4.	Respiratory sensitisation	None		None	Not evaluated
3.4.	Skin sensitisation	None		None	Not evaluated
3.5.	Germ cell mutagenicity	None		None	Not evaluated
3.6.	Carcinogenicity	None		None	Not evaluated
3.7.	Reproductive toxicity	Repr. 1B – H 360FD			
3.8.	STOT –single exposure	None		None	Not evaluated
3.9.	STOT – repeated exposure	None		None	Not evaluated
3.10.	Aspiration hazard	None		None	Not evaluated
4.1.	Hazardous to the aquatic environment	None		None	Not evaluated
5.1.	Hazardous to the ozone layer	None		None	Not evaluated

[1)] Including specific concentration limits (SCLs) and M-factors

[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling:

Pictogram: GHS08
Signal word: Danger
Hazard statements: H360FD: May damage fertility or the unborn child

Proposed notes assigned to an entry: None

Table 4: Proposed classification according to DSD

Hazardous property	Proposed classification	Proposed SCLs	Current classification [1)]	Reason for no classification [2)]
Explosiveness	None		None	Not evaluated
Oxidising properties	None		None	Not evaluated
Flammability	None		None	Not evaluated
Other physico-chemical properties *[Add rows when relevant]*	None		None	Not evaluated
Thermal stability	None		None	Not evaluated
Acute toxicity	None		None	Not evaluated
Acute toxicity – irreversible damage after single exposure	None		None	Not evaluated
Repeated dose toxicity	None		None	Not evaluated
Irritation / Corrosion	None		None	Not evaluated
Sensitisation	None		None	Not evaluated
Carcinogenicity	None		None	Not evaluated
Mutagenicity – Genetic toxicity	None		None	Not evaluated
Toxicity to reproduction – fertility	Repr. Cat. 2; R60		None	
Toxicity to reproduction – development	Repr. Cat. 2; R61		None	
Toxicity to reproduction – breastfed babies. Effects on or via lactation	None		None	Not evaluated
Environment	None		None	Not evaluated

[1)] Including SCLs
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling:

Indication of danger: T
R-phrases: R60/R61
S-phrases: S(1/2)-45-53

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

No previous harmonised classification and labelling.

2.2 Short summary of the scientific justification for the CLH proposal

Studies available show irrefutable effects of DnHP treatment on development in rodents. It induces embryo-mortality (decrease in litter and pups production) in absence of maternal body weight modification in mice and rats by oral route. Embryo-toxicity has been described in rats with numerous malformations, delayed ossification and increased incidence of skeletal variants in rats together with specific testicular toxicity. Regarding fertility, it impacts male reproductive system in mice and rats, primarily in treated animals and in the offspring treated during gestation. Testicular toxicity observed after *in utero* exposure is based on toxic insult and modification of the morphology of the Sertoli cells. Finally, the similarity of these effects with those described with other phtalates also support that these effects are an intrinsic property of this compound.

2.3 Current harmonised classification and labelling

No current harmonised classification in Annex VI of CLP.

2.4 Current self-classification and labelling

No information

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

DnHP has a CMR property (reproductive toxicity). Harmonised classification and labelling for CMR and respiratory sensitisation is a Community-wide action under article 36 of CLP. Repeated dose toxicity data are presented for information as they may provide relevant data for assessment of reproductive toxicity but no classification is discussed and proposed for this endpoint.

PART B.

SCIENTIFIC EVALUATION OF THE DATA

*Please note that this Background Document supporting the RAC opinion has been prepared on the basis of the submitted CLH report. According to the "**RAC Working Procedure on Processing of Dossiers for Harmonised Classification and Labelling (May, 2010**)" the dossier submitter has integrated the comments received during the public consultation where relevant. For transparency, the information provided by the dossier submitter in the revised CHL report has not been modified. The RAC assessment of the relevant information has been included in separated sections through the document.*

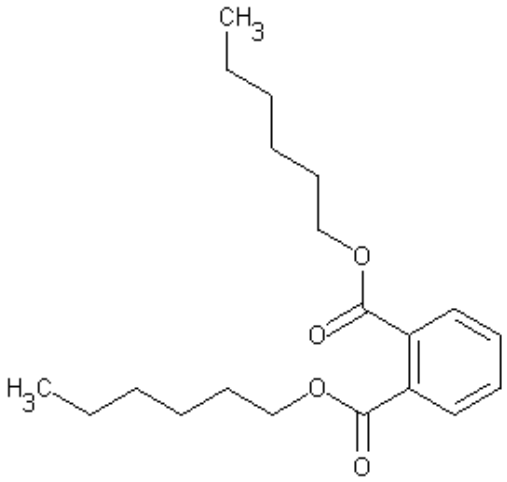
1 IDENTITY OF THE SUBSTANCE

1.1. Name and other identifiers of the substance

Table 5: Substance identity

EC number:	201-559-5
EC name:	dihexyl phthalate
CAS number (EC inventory):	
CAS number:	84-75-3
CAS name:	1,2-Benzenedicarboxylic acid, dihexyl ester
IUPAC name:	dihexyl phthalate
CLP Annex VI Index number:	none
Molecular formula:	C20H30O4
Molecular weight range:	334.46 g/mol

Structural formula:



1.2. Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Di-*n*-hexyl phthalate		>97%	
No data concerning other the constituent of di-*n*-hexyl phthalate are available.			

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
No data concerning the impurities of di-*n*-hexyl phthalate are available.		<3%	

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
No data concerning the additives of di-*n*-hexyl phthalate are available.				

1.2.1. Composition of test material

1.3. Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Clear, oily liquid with a slightly aromatic odour.	NICNAS 2008	Not available
Melting/freezing point	-27.4°C	NICNAS 2008	Not available
Boiling point	350°C	NICNAS 2008	Not available
Relative density	1.011 at 20°C	NICNAS 2008	Not available
Vapour pressure	$6.67x10^{-7}$ kPa at 25°C	NICNAS 2008	Not available
Surface tension	No data		
Water solubility	0.05 mg/l at 25°C	NICNAS 2008	Not available
Partition coefficient n-octanol/water	6.30	NICNAS 2008	Not available
Flash point	192°C	OSHA 2001	Not available
Flammability	No data		
Explosive properties	No data		
Self-ignition temperature	>500°C	OSHA 2001	Not available
Oxidising properties	No data		
Granulometry	Not relevant (liquid)		
Stability in organic solvents and identity of relevant degradation products	No data		
Dissociation constant	No data		
Viscosity	25 MPa.s at 25°C	Eastman, 2002	Not available

2 MANUFACTURE AND USES

2.1. Manufacture

Not relevant for this dossier.

2.2. Identified uses

DnHP is a plasticizer used in the manufacture of polyvinyl chloride (PVC) and other plastics.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated in this dossier.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

4.1.1.1 Absorption

Dermal absorption of DnHP has been studied in the rat along with a series of phthalates (Elsisi A.E. *et al.*, 1989). Hair from a skin area (1.3 cm in diameter) on the back of male F344 rats was clipped, 157 µmol/kg of 14C-labeled phthalate diester was applied in a dose of, and the area of application was covered with a perforated cap. The radioactive isotope was synthesized using 14C-radiolabeled phtalic acid (uniformly labelled on the ring) and the appropriate alcohol. The rats were restrained and housed for 7 days in a metabolic cage that allowed separate collection of urine and feces. Urine and feces were collected every 24 hours, and the amount of 14C excreted was taken as an index of the percutaneous absorption. At 24 hours, diethyl phthalate showed the greatest excretion (26%). As the length of the alkyl side chain increased, the amount of 14C excreted in the first 24 hours decreased significantly. The cumulative percentage dose excreted in 7 days was greatest for diethyl, dibutyl, and diisobutyl phthalate, about 50-60% of the applied 14C; and intermediate (20-40%) for dimethyl, benzyl butyl, and dihexyl phthalate (DnHP excretion was approximately 18%). Urine was the major route of excretion of all phthalate diesters except for diisodecyl phthalate. This compound was poorly absorbed and showed almost no urinary excretion. After 7 days, the percentage dose for each phthalate that remained in the body was minimal and showed no specific tissue distribution. Most of the unexcreted dose remained in the area of application. These data show that the structure of the phthalate diester determines the degree of dermal absorption. Absorption maximized with diethyl phthalate and then decreased significantly as the alkyl side chain length increased. Urine was the principal route of excretion, and there was no evidence of accumulation in any tissue that was examined. Deisenger et al. (Deisinger P.J. *et al.*, 1998) in studies with 14C-labeled Di(2-ethylhexyl) phthalate (DEHP) showed that absorption through skin from plasticized PVC film was significantly lower than that found from exposure to liquid DEHP when the plasticized PVC film used was in good shape and let in place for a short period (24 hours).

4.1.1.2 Biotransformation

No information is available on biotransformation of DnHP. However, as other phthalates are converted to monoesters and alcohol and rapidly excreted, it is anticipated that DnHP would behave in the same way (NTP-CERHR, 2003) resulting in monohexyl phthalate (MnHP) and n-hexanol. N-hexanol is described by one of its producer as harmful if swallowed or inhalated. It could cause irritation to skin, eyes and respiratory tract and affect the central nervous system. Distribution

Dermally absorbed DnHP was widely distributed throughout the body with no tissue containing >0.6% of the applied dose 7 days after application. There was no evidence for accumulation in any tissue (Elsisi A.E. *et al.*, 1989).

4.1.1.3 Excretion

The major route of excretion of dermally absorbed DnHP was via the urine. Its rate of excretion was much less than that of the shorter chain analogs, probably due to a slower dermal uptake process (Elsisi A.E. *et al.*, 1989).

4.1.1.4 Side Chain-associated Toxicokinetics

n-Hexanol and mono (2-ethylhexyl) *phthalate* (MnHP) are probable metabolites of DnHP. Hexanol is oxidized to the fatty acid and metabolized by the fatty acid oxidation pathway (Mann A.H. *et al.*, 1985). Phtalate esters are generally well absorbed in the gastrointestinal tract. When orally ingested, dialkyl phthalates are probably absorbed from the gut primarily in the form of their monoester hydrolysis product (Kluwe W.M., 1982). Results from a series of animal studies suggest that the monoester metabolite (generally the major metabolite) of the parent compound in responsible for adverse reproductive and developmental effects of phthalates (European Commission, 2004).

4.1.2 Human information

None

4.1.3 Summary and discussion on toxicokinetics

By dermal route, 18% of the applied dose of DnHP is absorbed and excreted after 24 hr, mainly in urine. No oral or inhalation toxicokinetic data have been reported for DnHP. However, as other phthalates are converted to monoesters and alcohol and rapidly excreted, it is anticipated that DnHP would behave in the same way (NTP-CERHR, 2003).

4.2 Acute toxicity

Not evaluated in this dossier.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated in this dossier.

4.4 Irritation

Not evaluated in this dossier.

4.5 Corrosivity

Not evaluated in this dossier.

4.6 Sensitisation

Not evaluated in this dossier.

4.7 Repeated dose toxicity

Table 10: Summary table of relevant repeated dose toxicity studies

Method	Results	Remarks	Reference
Subacute study of 21 days in 4-week-old Wistar rats (3/ treated group, 6/ control group) fed a diet +/- 20,000 ppm DnHP	Effects on liver (fatty change and centrilobular necrosis) DnHP treatment had no effect on testes weight or the gross appearance of testes, kidney, or pancreas.	OECD 407 but: - 21 days instead of 28 but 2 intermediary schedules necropsy - 1 dose instead of 3 - few animals/ timepoint	(Mann A.H. *et al.*, 1985)
Subacute study of 21 days in male Wistar albino rats receiving control diet or diet containing 2% w/w DnHP corresponding approximately to 2000 mg/kg/day. Groups of six control rats and four treated rats killed 3, 10, and 21 days after initial offering of the diet.	Increased activity in the thyroid gland accompanied by electron microscopic changes in response to treatment. Fat accumulation within the liver in response to treatment.	OECD 407 but: - 21 days instead of 28 but 2 intermediary schedules necropsy - 1 dose instead of 3 - few animals/ timepoint	(Hinton R.H. *et al.*, 1986)

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

Systemic effects following DnHP treatment for 3, 10, or 21 days were examined in 4-week-old Wistar rats (Mann A.H. *et al.*, 1985). The effects were compared to those produced by approximately equal concentrations of di-n-octyl phtalate (DnOP), another straight-chain phthalate, and DEHP, a branched-chain phthalate (Mann A.H. *et al.*, 1985). A group of 12 male rats was fed a diet containing 20,000 ppm DnHP (4 animals killed/ timepoint) and a control group of 18 rats was fed the basal diet (6 animals killed/ timepoint). Using actual food intake levels and rat body weights on the day of sacrifice, a DnHP dose of 1,824 mg/kg bw/day was calculated. DnHP treatment did not cause a change in body weight gain or food intake levels. DnHP treatment had no effect on testes weight or the gross appearance of testes, kidney, or pancreas (Mann A.H. *et al.*, 1985). However, liver weight was significantly increased following 21 days of DnHP treatment, with histology and chemistry changes observed at all 3 assessment times. Centrilobular necrosis and loss of glycogen were first observed at 3 days and centrilobular fatty accumulation was observed at 10 days of treatment. The effects became more pronounced with increasing duration of treatment. Examination by electron microscopy revealed proliferation and dilation of smooth endoplasmic reticuli and shortening of the microvilli in bile canaliculi at 3 days, the presence of lipid droplets of fat around central veins within hepatocytes at 10 days, and possibly a small increase in lysosomes and peroxisomes at 3 and 21 days, respectively. The activity of the peroxisomal proliferation marker, cyanide-insensitive palmitoyl CoA oxidase, was significantly increased at levels approximately 2-fold greater than controls in rats only after 10 days of treatment. There was no change in total catalase activity, but catalase activity in the particulate fraction was significantly increased at 10 and 21 days of treatment. A significant decrease in glucose-6-phosphate activity at 21 days of treatment was the only other effect on liver enzymes. Biochemical evidence of peroxisome proliferation (cyanide-insensitive palmitoyl CoA oxidation) occurred earlier with DEHP treatment (after 3 days of treatment) and was approximately 7-fold higher than it was following DnHP or DnOP treatment. Although DEHP was a stronger inducer of peroxisome

proliferation, DnHP and DnOP also induced peroxisome proliferation following longer treatment periods.

In another study, male Wistar albino rats were divided into four groups which received control diet or diet containing 2% w/w of DEHP, DnHP, or DnOP corresponding approximately to 2000 mg/kg/day. Groups of six control rats and four rats from each experimental group were killed 3, 10, and 21 days after initial offering of the diet. Liver histopathology, enzyme activity, and peroxisome proliferation were examined. Levels of thyroid hormones in serum and thyroid histopathology were also examined (Hinton R.H. *et al.*, 1986) and showed an increased activity in the thyroid gland, accompanied by a lowering of plasma T4 but with plasma T3 remaining close to control values. The short-term thyroid changes observed in response to DnOP, DnHP, and DEHP treatment emphasize that the risk of thyroidal changes is not restricted to compounds or to species which show proliferation of peroxisomes. Electron microscopic changes indicative of thyroid hyperactivity (increased lysosomal numbers and size, enlarged Golgi apparatus, and mitochondrial damage) were also observed (Hinton R.H. *et al.*, 1986). Thyroid effects are similar for all three phthalates. DEHP treatment resulted in a more pronounced increase in liver weight and in increased mitotic activity. Less fat was accumulated following treatment with DEHP in the midzonal and periportal zones compared to DnHP or DnOP treatment, with which accumulation was more pronounced and occurred rather in the centrilobular region.

4.7.1.2 Repeated dose toxicity: inhalation

No inhalation data have been reported for DnHP.

4.7.1.3 Repeated dose toxicity: dermal

No dermal data have been reported for DnHP.

4.7.1.4 Repeated dose toxicity: other routes

No data.

4.7.2 Human information

No human data.

4.7.3 Other relevant information

No other relevant information.

4.7.4 Summary and discussion of repeated dose toxicity

Repeated dose toxicity data are presented for information as they may provide relevant data for assessment of reproductive toxicity. No classification is discussed and proposed for the repeated dose toxicity endpoint.

DnHP induces hepatic effects typical of peroxisome proliferation and thyroid effects in rats exposed to 1800/2000 mg/kg/d DnHP through diet for up to 21 days. No testicular effect was observed under these experimental conditions.

4.8 STOT

Not evaluated in this dossier.

4.9 Germ cell mutagenicity (Mutagenicity)

Not evaluated in this dossier.

4.10 Carcinogenicity

Not evaluated in this dossier.

4.11 Toxicity for reproduction

Table 11: Summary table of relevant reproductive toxicity studies

Method	Results	Remarks	Reference
CD1-mice (16–19 pairs of males and females) fed with 0.0, 0.3, 0.6, and 1.2% of DnHP corresponding to 0, 430, 880, or 1,870 mg/kg or 0, 380, 800, or 1,670 mg/kg bw/day depending on the estimation7 days prior to and during 98-day cohabitation period.	Continuous breeding phase: Dose-related decrease in the proportion of pairs able to produce even a single litter (respectively 100%, 82%, 5% and 0%). Dose related decrease of number of pups alive. Pup weight adjusted for litter size was unchanged by treatment. Adverse effects observed at levels below those exhibiting large effects on body weight. Crossover mating trial: High-dose males and control females: significant decrease in detected matings and only 1 of 18 treated males sired a litter. High-dose females and control males: no decrease in copulatory plugs, but none of the females became pregnant. Only control and high-dose groups were necropsied. In females, no histological effects were seen in reproductive organs. In male, extensive atrophy of the seminiferous epithelium with mature sperm markedly diminished in the epididymis was observed in line with decrease in sperm number (7% of control) and motility (22% of control) parameters and decreases in the relative weights of the epididymis (-28%), testis (-70%), and seminal vesicle (-	Standard "Reproductive Assessment by Continuous Breeding" (RACB) protocol consists of four related tasks. These tasks include: Task 1, dose-finding; Task 2, continuous breeding phase; Task 3, identification of the affected sex, and Task 4, offspring assessment. Reliability 2 as the protocol was not standardised and validated internationally.	Lamb et al. 1987

	25%).		
12 pubertal male Sprague Dawley rats (4-weeks-old) fed with normal diet or supplemented with a single dose level of DnHP (2.4 g/kg bw/day) in corn oil for 4 days	Testis weight and testicular zinc content decreased in absence of body weight effects (65% and 50% of controls respectively). Seminiferous tubular atrophy with the majority of tubules showing few spermatogonia and Sertoli cells, but normal Leydig cell morphology.	Reliability 2, considered as supportive evidence	(Foster et al. 1980)
Time-mated CD-1 mice (48-50 dams/group), oral by gavage with 9,900 mg/kg bw/day (undiluted chemical, 10 mL/kg/day) or corn oil for 8days (dose-range finding study) or 7 days (screening assay: GD 6-13)	No live litters (0/34) to be examined. One exposed dam died. Body weight changes in dams could not be evaluated due to complete litter loss.	Chernoff-Kavlock screening assay Reliability 2, considered as supportive evidence	(Hardin B.D. *et al.*, 1987)
Pregnant Sprague-Dawley rats (8 to 12/group), dosed by oral gavage with 0 (olive oil), 250, 500, and 750 mg/kg/day from GD 6 to GD 20	Dose-related developmental toxic effects, including marked embryo mortality at 750 mg/kg/day (60% dead foetuses compared to controls), and presence of malformations and significant decreases in foetal weight at 500 and 750 mg/kg/day (-10% and -20% compared to controls respectively). Significant delay of ossification and increase in the incidence of skeletal variants also appeared at 250 mg/kg/day. Dose-related decrease in the anogenital distance of male foetuses at all doses (100%, 93%, 80% and 65% of controls respectively), and there was a significant increase in the incidence of male foetuses with undescended testis at 500 (11% of the animal examined) and 750 mg/kg/day (41%) of DnHP. DnHP showed clear embryolethality and teratogenicity. There was evidence that DnHP could alter the development of the male reproductive system after *in utero* exposure in absence of maternal toxicity.	Consistent with OECD guideline 414 Reliability: 1	(Saillenfait A.M. *et al.*, 2009a)
Pregnant Sprague-Dawley rats), dosed by oral gavage with 0, 50, 125, 250, or 500 DnHP/kg/day and 500 mg DEHP/kg/day from GD 12 to GD 20	No significant effect of DnHP on maternal body weight gain and pup weights during lactation. In absence of maternal toxicity,	No guideline but well conducted study Reliability: 2 due to the number of	(Saillenfait A.M. *et al.*, 2009b)

	decreased number of live pups on postnatal day (PND) 1 at all doses (99%±3, 95.6%±7.9, 91.3%±14.8, 85.4%±26.7 & 76.4%±38.4 respectively). Male offspring displayed reduced AGD on PND 1 at 125 mg DnHP/kg/day and above, and areola/nipple retention before weaning and at adulthood at 250 and 500 mg DnHP/kg/day. At necropsy on PND 70-78 or PND 111-120, severe malformations of the reproductive tract observed in young adult males at 125 mg DnHP/kg/day and higher doses + seminiferous tubule degeneration at the two high doses. Prenatal exposure to DnHP caused permanent and dose-related alterations of the male rat reproductive development, with a similar profile as DEHP.	animals< number required in guideline studies.	

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Lamb (Lamb J.C. et al., 1987)

The reproductive toxic potential of DnHP was evaluated in both sexes of Swiss CD1-mice following a RACB protocol (Task 1, dose-finding; Task 2, continuous breeding phase; Task 3, identification of the affected sex, and Task 4, offspring assessment.). During task 2, groups of 16–19 pairs of males and females received DnHP (purity ≈98%) for 7 days prior to and during 98-day cohabitation period. Previous study results (NIOSH, unpublished data) were used to determine the dietary amount of 0.0, 0.3, 0.6, and 1.2% in feed. Morrissey et al. (Morrissey R.E. *et al.,* 1989) estimated intake levels of 0, 430, 880, or 1,870 mg/kg whereas NTP report mentioned that Chapin and Sloane (Chapin R.E. et Sloane R.A., 1997) estimated intake levels of 0, 380, 800, or 1,670 mg/kg bw/day (information not found in the publication). In the control group, 3 males died, 3 females died in the 0.3% group, 1 female died in the 0.6% group and 4 females died in the 1.2% group. Food consumption was not altered by the presence of DnHP. Mean body weights for the male and female were significantly ($p<0.05$) reduced at necropsy and body weight gain was decreased in a dose-related manner in by DnHP in diet (data not shown). Exposure to DnHP during the continuous breeding phase resulted in a dose-related decrease in the proportion of pairs able to produce even a single litter (Table 12). There were no live pups at the high dose and one pair able to deliver four litters of 6.5 pups in average at the middle dose (Table 12). At the low dose, there was a significant reduction in the mean number of litters per pair (3.4, vs 4.9 for the controls). Also at the low dose, the number of live pups per litter was reduced from 12.3 (controls) to 3.4, the

proportion born alive was reduced by 14%. Pup weight adjusted for litter size was unchanged by treatment. These effects occurred in the absence of an effect on postpartum dam body weights. Adverse effects were observed at levels below those exhibiting large effects on body weight.

Table 12: Fertility and reproductive performance of mating pairs (F_0 generation) during continuous breeding.

% DnHP in diet	Control	0,3	0,6	1,2
No. Fertile/ No. Cohabited (%)[a]	37/37 (100)	14/19 (74)*	1 /19 (5)**	0 /16 (0)**
Litter/pair (n)[b]	4,89 ± 0,05 (37)	3,43 ± 0,34 (14) **	4 (1)	-
Live pups/ litter (n)	12,29 ± 0,4 (37)	3,43 ± 0,48 (14) **	6,5 (1)	-
Proportion of pups born alive (n)	0,99 (37)	0,84 ± 0,03 (14) **	0,79 (1)	-
Live pup weight (n)	1,6 ± 0,01 (37)	1,77 ± 0,05 (14) **	1,65 (1)	-

[a] pairs were considered fertile if they produced one or more litters.

[b] Values are mean ± SE

**** Significantly different from control (p< 0.01)**

*** Significantly different from control (p< 0.05)**

These significant reproductive effects prompted the determination of the affected sex by performing a crossover mating trial using the control and 1.2% DnHP-treated mice. Each group had 17 to 20 pairs of mice. The mating index (proportion of pairs showing copulatory plugs) in groups with 2 control partners, with a DnHP treated male, or a DnHP-treated female, was 90, 56*, and 88% (* indicates significantly different from controls), showing that the treated females were cycling and could be receptive, and that mating capability was reduced in the group of treated males (Table 13). However, no treated females bore any litters, and only 1 of 18 treated males sired a litter (Table 13). Effectively, both sexes were infertile at this level of DnHP exposure. After the litters from the crossover were examined and discarded, the Fo adults from the control and 1.2% DnHP groups were the only ones to be necropsied.

Body weight in the high dose males was 10% less than controls, and absolute testis weight was 70% less (Table 14). Body-weight-adjusted liver and prostate weights were increased by 34 and 9%, respectively, and adjusted weights of kidney, epididymis, and seminal vesicles were reduced by 9, 23, and 18%, respectively; as was adrenal glands. Epididymal sperm concentration was reduced by 93%, and motility was reduced by 78%. There were extensive atrophy of the seminiferous tubules in the 1.2% DnHP treated males was described. The tubules were lined primarily by Sertoli cells and lacked evidence of normal spermatogenesis.

Body weight of DnHP-exposed Fo females was 6% less than controls, while adjusted weights of liver was increased by 32% and adjusted kidney weights were decreased by 6%. Not surprisingly, epididymal sperm concentration was reduced by 93%, and motility was reduced by 80%. No treatment-related histopathological differences were noted in the reproductive organs of females (i.e. ovaries, uterus, vagina).

No second generation was produced as adverse effects were already seen in the first generation. These data clearly demonstrate that DnHP is a reproductive toxicant in mice. The relative sensitivity of the liver and the reproductive system cannot be judged from these data, but the reproductive effects occurred in the absence of large changes (at the top dose) or any changes (low and middle doses) in body weight.

Table 13: Mating trial of F_0 pairs to determine the affected sex.

	F_o pairings		
	Control male x control female	1,2% DnHP male x control female	Control male X 1,2% DnHP female
No. With copulatory plugs/ No. Cohabited (%)	18 /20 (90)	10 /18 (56)*	15 /17 (88)
No. fertile [a] / No. Cohabited (%)	17 /20 (85)	1 /18 (6)**	0 /17 (0)**
Live pups/ litter (n) [b]	9,41 ± 0,92 (85)	14 (1)	-
Proportion of pups born alive (n)	0,94 ± 0,04 (17)	1 (1)	-
Live pup weight in g (n)	1,64 ± 0,04 (17)	1,58 (1)	-

[a] A pair was judged fertile if it produced a litter of one or more live or dead pups.

[b] Values are mean ± SE

**** Significantly different from control (p< 0.01)**

*** Significantly different from control (p< 0.05)**

Table 14: F_0 male body weights, organ weights, and sperm parameters at necropsy.

	% di-n-hexyl phtalate in diet	
	Control	1,2
Body weight (g)	40,33 ± 0,68 (37)[b]	36,18 ± 0,58 (18)**
Liver (g)	2,14 ± 0,05 (37)	2,74 ± 0,08 (18)**
Kidneys and adrenals (g)	0,86 ± 0,02 (36)	0,75 ± 0,03 (18)**
Left testis and epididymis (mg)	206 ± 5 (37)	93 ± 10 (18)**
Right testis (mg)	140 ± 3 (37)	42 ± 7 (18)**
Right epididymis (mg)	58 ± 2 (37)	42 ± 2 (18)**
Prostate (mg)	56 ± 5 (37)	46 ± 8 (18)
Seminal vesicles (mg)	499 ± 15 (37)	376 ± 21 (17)**
% motile sperm	68.61 ± 3,89 (37)	14,83 ± 6,66 (6) [a] **
Sperm concentration (No. Sperm x 10^3/ mg caudal tissue)	357 ± 25 (35)	25 ± 22 (18)**
% abnormal sperm [c]	10.97 ± 0,87 (34)	8,02 ± 1,05 (18) [d] **

[a] Only 6 of 18 males had sufficient sperm counts for determining motility.

[b] Values are mean ± SE

[c] Tailless sperm were not included in the determination of abnormal sperm.

[d] Only 3 of 18 males had sufficient sperm counts for determining abnormal and tailless sperm.

**** Significantly different from control (p< 0.01)**

*** Significantly different from control (p< 0.05)**

Table 15: F_0 female body and organ weights at necropsy.

	% di-n-hexyl phtalate in diet

	Control	1,2
Body weight (g)	37,06 ± 0,6 (38)[a]	34,94 ± 0,57 (17)*
Liver weight (g)	2,19 ± 0,05 (38)	2,7 ± 0,11 (17)**
Kidneys and adrenals weight (g)	0,64 ± 0,01 (38)	0,57 ± 0,02 (17)**

[a] **Values are mean ± SE.**

**** Significantly different from control (p< 0.01)**

*** Significantly different from control (p< 0.05)**

Within the main study, significant effects occurred at the lowest dose level with clear adverse effects seen in the absence of any body weight effects (The number of litter per pair and number of live pups/litter was reduced in the 380 mg/kg bw/day group (n= 3 versus 12 in control group)). In the crossover mating trial, fertility of male and female were affected. Body and relative kidney/adrenal weights were significantly decreased and liver to body weight ratio was significantly increased in both males and females of the high-dose group, but histological changes were not noted.

Foster (Foster P.M. et al., 1980)

In a short-term study (Foster P.M. et al., 1980) which employed a single dose level of DnHP (2.4 g/kg bw/day, purity>99%) given by gavage in corn oil to a group of 12 pubertal male Sprague Dawley rats (4-weeks-old) for 4 days, marked effects on testis weight (65% of control value) and on testicular zinc content were noted (Table 16) in the absence of body weight effects. Histologic examination of formalin-preserved testes revealed a marked seminiferous tubular atrophy with the majority of tubules showing few spermatogonia and Sertoli cells, but normal Leydig cell morphology.

Table 16: Effect (% of control) of DnHP treatment on relative testis weight and testicular zinc content in immature rats.

	Relative testis weight	Zn-65 content/ 100 mg testis	Total Zn-65 content
Corn Oil	100 ± 2,1	100 ± 2,9	100 ± 4,5
DnHP, 2,4 g/kg/day	64,8 ± 3,0*	80,7 ± 3,8*	49,7 ± 3,1*

*p<0.001 (Student's t test for unpaired data) relative to control.

4.11.1.2 Human information

Numerous studies evaluating the link between phthalate exposure and various impacts on human fertility are published. Some studies have suggested a possible association between exposure to low molecular weight classified phthalates and effects on human fertility. In particular these studies have looked at DEHP which is used in medical device applications. However, within this abundant literature, none is dealing with DnHP on its own or within a mixture.

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

Hardin (Hardin B.D. et al., 1987)

Developmental toxicity DnHP was evaluated in the Chernoff-Kavlock screening assay (Hardin B.D. *et al.*, 1987). Time-mated CD-1 mice (48-50 dams/group) were gavaged on GD 6-13 with 9,900 mg/kg bw/day DnHP (undiluted chemical, purity not specified, 10 mL/kg/day) or corn oil. Dose was selected based on the outcome of a dose-finding study where non pregnant female mice were orally dosed for 8 consecutive days. In the main study and according to the standard protocol, dams were allowed to litter. As soon as possible after delivery, the number of liveborn pups and total litter weight were recorded. With DnHP, there were no live litters (0/34) to be examined. One exposed dam died. Body weight changes in dams were not presented as the authors considered that it could not be evaluated due to complete litter loss.

Table 17: Findings

	Dose (mg/kg/d)	No. Dead/ treated	Weight changes (g)	Viable litters	Liveborn per litter	Percentage of survival	Birth weight (g per pup)	Weight gain (g per pup)
Di(n-hexyl) phtalate	9900b	1/48	NA	0/34*	0,00	NA	NA	NA
Control	Corn oil	0/50	10,5 ± 2,2	34/38	11,5 ± 1,7	99,1 ± 4	1,6 ± 0,1	0,7 ± 0,2

Saillenfait (Saillenfait A.M. et al., 2009a)

Twenty to twenty-five pregnant Sprague-Dawley rats were exposed to DnHP at doses of 0 (olive oil), 250, 500, and 750 mg/kg/day, by gavage, on Gestational Days (GD) 6 through 20. DnHP identity and purity (> 98 %) were confirmed by gas chromatography mass spectrometry (GC/MS) and infrared spectroscopy. Dosing solutions were formulated in olive oil as vehicle and were stable for up to 14 days. Concurrent control groups received the vehicle under the same conditions. The dosage levels of the definitive study were based on the findings of preliminary studies in which pregnant rats (8 to 12/group) were given 250, 500, or 750 mg/kg/day of DnHP. This study was consistent with OECD guideline 414 except that the age of the dams is not given in the publication. However, the weight of the dams when supplied was consistent with Sprague-Dawley females aged 8 to 9 weeks, which is in agreement with OECD 414 requirement to use young adult animals.

No dam died before the scheduled necropsy and no treatment-related clinical findings were noted. There were no effects on maternal body weight and food consumption at 250 and 500 mg/kg/day. Except from the GD 9-12 interval, maternal body weight gain was significantly decreased during the treatment period at 750 mg/kg day (Table 18).

Table 18: Maternal findings

	DnHP (mg/kg/day)			
	0	**250**	**500**	**750**
No. dead/treated	0/25	0/25	0/25	0/24
Body weight on GD 0 (g)	221±11 [a]	219±11	221±11	222±14
Body weight change (g)				
GD 0-6	32±6	32±5	33±7	30±6
GD 6-9	14±4	13±5	12±7	8±5 **
GD 9-12	19±4	17±5	16±5	15±6
GD 12-15	22±5	21±5	20±4	15±5 **
GD 15-18	42±8	42±8	37±7	19±12 **

GD 18-21	53±11	48±7	47±10	23±16 **
GD 6-21	149±23	141±20	132±23	80±33 **
Gravid uterine weight	106±22	99±17	90±20	36±31 **
Corrected weight gain [b]	43±10	42±10	43±12	44±9
Food consumption (g/day)				
GD 0-6	23±2	23±2	23±2	22±2
GD 6-9	22±2	22±2	22±3	20±2 **
GD 9-12	23±2	23±3	23±3	21±2 *
GD 12-15	24±3	23±2	24±3	22±3 *
GD 15-18	27±3	26±3	26±3	23±3 **
GD 18-21	25±2	24±2	25±3	21±3 **
GD 6-21	24±2	24±2	24±3	21±2 **

[a] Values are expressed as means ± SD.

[b] Body weight gain during GD 6-21 minus gravid uterine weight.

* and ** denote significant differences from the vehicle control, $p<0.05$ and $p<0.01$, respectively.

Food consumption was significantly reduced throughout the treatment period at the high dose. Whatever doses, there was no effect on body weight change on GD 6-21 corrected for the gravid uterus.

In the additional liver investigations, there was a slight but significant increase in serum ASAT activity levels at 750 mg/kg/day (data not shown). There was no statistically significant effect on other serum parameters. Liver weight (absolute, relative to body weight on GD 21 or relative to body weight on GD 21 minus uterine weight) was significantly higher than the concurrent control at all doses. DnHP induced a slight increase in the hepatic activity of cyanide-insensitive palmitoyl-CoA oxidation (1.5 to 2.1 fold, compared to the control). No treatment-related histological lesions were seen in the liver (data not shown).

DnHP treated group (750 mg/kg/day) displayed a high incidence of post-implantation loss, with approximately two thirds of implants resorption (Table 19). The number of litters with resorptions was significantly increased. There was no significant differences in the number of foetal death (no more than one per litter) and in the foetal sex ratio. Foetal body weight (all, males, and females) was significantly decreased in a dose-related manner and was significantly lower than control at the two high doses (about 9 and 18-19 % less at 500 and 750 mg/kg/day, respectively). Significant and dose-related decrease in the AGD of the male foetuses was seen at all doses. Differences were also significant when foetal body weight was used as covariate for analysis, or after normalizing with the cubic root of body weight. AGD was 7.6, 20.3, and 35.2 % below the concurrent control value at 250, 500 and 750 mg/kg/day, respectively. AGD is a sensitive marker of masculinization of external genitalia in rats, and is controlled by 5α-dihydrotestosterone. Shorter AGD has been described in male offspring prenatally exposed to antiandrogenic agents (e.g. DEHP and DBP) during the period of male sexual differentiation (i.e. approximately last half of gestation) (Ema M. *et al.*, 2003; Foster P.M. *et al.*, 2001). No effect on female AGD was observed.

Table 19: Gestational parameters

	DnHP (mg/kg/day)			
	0	**250**	**500**	**750**
All litters [a]	25	24	21	20

No. implantation sites per litter	14.8±3.0 b	14.1±3.0	14.8±2.4	12.4±4.6
%post-implantation loss /litter [c]	6.0±9.7	6.5±7.8	16.6±18.8	66.6±29.2 **
No. litters with dead foetus	1	0	1	2
% dead foetuses per litter	0.3±1.5	0.0±0.0	0.3±1.3	0.7±2.4
No. litters with resorptions	11	12	13	19 **
% resorptions per litter	5.7±9.8	6.5±7.8	16.3±18.6	65.9±29.3 **
Live litters [d]	25	24	21	17
No. live foetuses per litter	14.0±3.3	13.1±2.6	12.2±3.0	5.7±4.7 **
% male foetuses per litter	50.2±17.6	51.3±15.6	57.2±11.6	52.8±30.1
Foetal body weight (g)				
All foetuses	5.74±0.28	5.60±0.37	5.24±0.34 **	4.63±0.72 **
Male foetuses	5.89±0.30	5.75±0.41	5.35±0.36 **	4.80±0.72 **
Female foetuses	5.60±0.28	5.43±0.34	5.11±0.41 **	4.61±0.56 **
AGD (mm)				
Male foetuses	3.01±0.12	2.78±0.19 **	2.40±0.20 **	1.95±0.24 **
Female foetuses	1.20±0.06	1.19±0.04	1.16±0.06	1.11±0.08 **
AGD/body weight $^{1/3}$				
Male foetuses	1.67±0.07	1.55±0.09 **	1.37±0.09 **	1.15±0.10 **
Female foetuses	0.67±0.03	0.68±0.03	0.68±0.04	0.67±0.05

[a] Includes all pregnant females at euthanization.

[b] Values are expressed as means ± SD.

[c] Resorptions plus dead foetuses.

[d] Includes all animals with live foetuses at euthanization.

** *denotes significant differences from the vehicle control, p<0.01.*

As shown in Table 20, a number of external, internal, and skeletal malformations only occurred in the DnHP treated groups, at the intermediate and high doses. They were considered treatment-related, and mainly consisted of cleft palate, eye defects (microphthalmia, anophthalmia), and alterations of the axial skeleton. These included absence of ribs, vertebral archs and/or centra, and fusion of sternebrae, vertebral archs and/or centra. Most often, foetuses exhibited more than one malformation. Two cases of central nervous malformations were also observed: a foetus from the 500 mg/kg/day group showed exencephaly and protruding tongue, and one foetus of the 750 mg/kg/day group had spina bifida with multiple skeletal malformations and abnormal anus. Diaphragmatic hernia was present in all groups, although with a somewhat higher incidence at 750 mg/kg/day. The total numbers of foetuses and litters displaying external, visceral, or skeletal malformations were significantly elevated at 750 mg/kg/day. The number of foetuses with any malformations was also significantly higher than controls at 500 mg/kg/day.

Table 20: Foetal malformations

	Dose (mg/kg/day)			
	0	250	500	750
Total No. fetuses (litters) examined [a]				
External	351 (25)	315 (24)	256 (21)	97 (17)
Visceral	176 (25)	158 (24)	128 (21)	49 (15)
Skeletal	175 (25)	157 (24)	128 (21)	48 (14)
External malformations				
Edema	0	0	1 (1)	1 (1)
Ablepharia (bilateral)	0	0	1 (1)	0
Cleft palate	0	0	0	4 (2)
Protruding tongue	0	0	1 (1)	0
Exencephaly	0	0	1 (1)	0
Spina bibida	0	0	0	1 (1)
Omphalocele	0	0	0	1 (1)
Anus, abnormal shape and small	0	0	0	1 (1)
No. (%) fetuses with external malformations	0	0	2 (0.8)	7 (7.2) **
No. (%) litters with external malformations	0	0	2 (9.5)	5 (33.3) *
Mean % fetuses with external malformations per litter	0.0±0.0 [b]	0.0±0.0	1.48±4.89	12.05±26.67
Soft tissue malformations				
Eye, absent or small	0	0	2 (1)	8 (7) #
Eye, absent (uni or bilateral)	0	0	2 (1)	7 (6)
Eye, small (unilateral)	0	0	0	1 (1)
Diaphragmatic hernia	1 (1)	2 (2)	1 (1)	7 (5)
Kidney, persistent mesonephrotic tissue	0	0	0	1 (1)
No. (%) fetuses with visceral malformations	1 (0.6)	2 (1.3)	3 (2.3)	14 (28.6) **
No. (%) litters with visceral malformations	1 (4.0)	2 (8.3)	2 (9.5)	9 (60.0) **
Mean % fetuses with visceral malformations per litter	0.50±2.50	1.43±4.91	5.44±21.89	33.22±36.98 ##
Skeletal malformations				
Sternebrae, fused	0	0	0	5 (5)
Rib malformation (any)	0	0	2 (2)	4 (4)
Rib, absent (n=1, 2, or 5)	0	0	1 (1)	4 (4)
Ribs, fused	0	0	1 (1)	1 (1)
Vertebra malformation (any)	1 (1)	0	4 (3)	11 (9) ##
Cervical vertebral archs fused	0	0	0	3 (3)
Thoracic or lumbar vertebral arch, absent (n=1, 2, or 5)	0	0	1 (1)	4 (4)
Thoracic vertebral archs, fused	0	0	1 (1)	3 (3)
Thoracic or lumbar vertebral arch(s) and centrum, fused	0	0	0	5 (4)
Thoracic vertebral centra, fused	0	0	1 (1)	1 (1)
Thoracic vertebral centrum, absent (n=1, 2, or 6)	0	0	1 (1)	2 (2)
Thoracic or lumbar vertebral centrum, hemicentric	1 (1)	0	2 (1)	3 (3)
Thoracic vertebral centra, misaligned	0	0	1 (1)	3 (3)
Sacral archs and centrum, fused	0	0	0	1 (1)
No. (%) fetuses with skeletal malformations	1 (0.6)	0	4 (3.1)	12 (25.0) **
No. (%) litters with skeletal malformations	1 (4.0)	0	3 (14.3)	9 (64.3) **
Mean % fetuses with skeletal malformations per litter	0.57±2.86	0.0±0.0	4.38±14.78	39.76±39.38 ##
No. (%) fetuses with any malformations	2 (0.6)	2 (0.6)	8 (3.1) *	28 (28.9) **
No. (%) litters with any malformations	2 (8.0)	2 (8.3)	4 (19.0)	12 (70.6) **
Mean % fetuses with any malformations per litter	0.57±1.79	0.69±2.40	5.27±17.57	41.53±40.67 ##

[a] The incidence of individual defect is presented as number of fetuses (number of litters).

[b] Mean ± SD

* and ** denote significant differences from control , p<0.05 and p<0.01, respectively (Fisher's test).

and ## denote significant differences from control, p<0.05 and p<0.01, respectively (Mann-Whitney test).

A common external variation consisting in club foot was seen sporadically, at single occurrences, including in the control group (Table 21). Undescended testes (unilateral or bilateral) attributed to DnHP were observed in 2.6 % of the male foetuses submitted to soft tissue examination in the low dose group, 19.5 % of the foetuses in the mid dose group, and in 83.3 % of the male foetuses in the high dose group.

Table 21: Principal foetal variations

	Dose (mg/kg/day)			
	0	250	500	750
Total No. fetuses (litters) examined [a]				
External	351 (25)	315 (24)	256 (21)	97 (17)
Visceral	176 (25)	158 (24)	128 (21)	49 (15)
Skeletal	175 (25)	157 (24)	128 (21)	48 (14)
External variations				
Club foot	1 (1)	1 (1)	0	1 (1)
Soft tissue variations				
Dilated renal pelvis	0	0	1 (1)	1 (1)
Distended ureter	0	1 (1)	2 (2)	4 (3)
Ovaries, malpositioned	0	0	0	1 (1)
Testis, malpositioned (uni or bilateral) [b]	0	2 (2)	15 (9) #	20 (11) ##
severe	0	0	6 (4)	13 (9) ##
moderate	0	2 (2)	9 (7)	7 (5)
Skeletal variations				
Frontals and parietals, incomplete ossification (moderate)	0	0	0	1 (1)
Supraoccipital, bipartite ossification	0	0	0	1 (1)
Hyoid, incomplete ossification	0	0	10 (6)	12 (9) ##
Sternebrae fused, first and second only	0	0	4 (3)	4 (4)
Sternebra ossification,				
bipartite, incomplete, or absent (one to three, mostly 5th)	3 (2)	2 (2)	15 (10) #	22 (11) ##
misaligned	1 (1)	0	0	0
misshapen (more than two)	1 (1)	0	1 (1)	5 (5)
Cervical rib(s)	5 (3)	14 (8)	30 (13) ##	16 (8) ##
14th rib(s), supernumerary, any	33 (15)	96 (23) ##	117 (21)##	46 (13) ##
14th rib(s), supernumerary, long	0	1 (1)	5 (5)	7 (6)
Thoracic or lumbar vertebral centra, incomplete ossification	12 (8)	9 (9)	12 (8)	11 (8)
27 presacral vertebrae	0	0	1 (1)	1 (1)
No. of ossification centers				
Metacarpals	3.99±0.03 [c]	4.00±0.00	4.00±0.00	4.00±0.00
Forelimb proximal phalanges	3.90±0.21	3.78±0.35	3.25±0.62 §§	2.27±0.94 §§
Metatarsals	4.97±0.08	5.00±0.00	4.98±0.07	5.00±0.00
Hindlimb proximal phalanges	3.04±0.71	2.02±1.21 §§	0.59±0.74 §§	0.15±0.41 §§
Caudal vertebral centra	6.67±0.64	6.97±0.67	6.93±0.80	7.63±1.29 §§

[a] The incidence of individual defect is presented as number of fetuses (number of litters).
[b] Number of litters with male fetuses : 24, 23, 21, and 13 at 0, 250, 500, and 750 mg/kg/day, respectively.
[c] Mean ± SD.
and ## denote significant differences from control, $p<0.05$ and $p<0.01$, respectively (Mann-Whitney test).
§§ denotes significant differences from control, $p<0.01$ (Dunnett's test).

All testes of control foetuses were located in the inguinal region, near the bladder neck. At 750 mg/kg/day, about one third of the testes (right and left) was still in the upper half of the abdominal cavity. This finding may also be considered as a fertility endpoint. There was no significant difference in the incidence of other soft tissue variations. Skeletal examination revealed increased incidences of several variations: sternebral anomalies and cervical ribs were significantly elevated at 500 and 750 mg/kg/day, and poorly ossified hyoid at 750 mg/kg/day. The incidence of 14th supernumerary ribs (mostly short), was also significantly greater than control at all doses, and showed dose-response dependency (19, 61, 91, and 96 % of the foetuses at 0, 250, 500, and 750 mg/kg/day, respectively). Significantly delayed ossification was noted in the hindlimb proximal phalanges at doses ≥250 mg/kg/day, and in the forelimb phalanges at 500 and 750 mg/kg/day. Delayed ossification occurred in doses in which foetus body weight were significantly decreased. The elevated number of caudal ossification centers at the high dose was not considered of toxicological significance.

Thus, prenatal exposure to DnHP was mostly associated with malformations of the same systems (i.e. eye, palate, axial skeleton) like the ones obtained after exposure to other phthalates such

as DBP, butyl benzyl phthalate or DEHP. Nevertheless, there were rare or no malformations of the cardiovascular system and of the kidney in the current study in Sprague-Dawley rats. A high incidence of resorptions was found at 750 mg DnHP/kg/day, and it is possible that severely affected embryos may not have survived.

Saillenfait b (Saillenfait A.M. et al., 2009b)

This study aims to evaluate the effects of in utero exposure to DnHP on the reproductive development of the male rat and should therefore also be considered for the fertility endpoint. Pregnant Sprague-Dawley rats were administered DnHP or DEHP, by gavage on gestation Days 12 to 21, at doses of 0, 50, 125, 250, or 500 DnHP/kg/day and 500 mg DEHP/kg/day. Doses of DnHP were based on the results of a prenatal study (Saillenfait A.M. *et al.*, 2009a). The females were allowed to litter and all male pups were raised to adulthood. At euthanization (postnatal week 10-12, PNW), the external genitalia of all males was examined. Two or three animals per control litter and all DnHP and DEHP males exposed in utero were observed internally for gross abnormalities of the reproductive organs. Organs were not weighed. The testing conditions of the main study were based on OECD guideline 414. However, the protocol bared differences due to the aim of the study that focused on effects of in utero exposure to DnHP on the reproductive development of the male rat: Nine to twelve females were used instead of the 20 advised and their age was not given in the publication. However, the weight of the dams when supplied was consistent with Sprague-Dawley females aged 8 to 9 weeks, which is in agreement with OECD 414 requirement to use young adult animals. Four doses were used instead of the 3 required in the guideline and dosing occurred on GD 12 (instead of day 1 of implantation) to GD 21, which is a sensitive period of male reproductive tract differentiation in rats. The females were allowed to litter and all male pups were raised to adulthood. The day parturition was completed was designated PND 0. Litters were examined as soon as possible after birth to determine the number of viable and stillborn pups. Pups were uniquely identified and anogenital distance (AGD) of males was measured on PND 1 using a dissecting microscope fitted with an micrometer eyepiece (accuracy 0.08 mm). Litters were culled to 10 pups on PND 4, retaining as many males as possible. Litters with less than 7 pups were not maintained. Discarded pups were euthanized by a subcutaneous injection of sodium pentobarbital. Internal examination of pups culled on PND 4 indicated that all had been sexed correctly. On PND 12-14, all pups were examined for the presence of areola and/or nipples on the ventral surface of the thorax. No distinction was made between an areola and a nipple. At weaning on PND 21, all males from all litters were retained for further postnatal assessments, as detailed below. Unselected animals were euthanized and submitted to an internal examination to confirm sex. Individual pup body weight was recorded on PND 1, 4, 7, 14 and 21 and then at weekly intervals, until euthanization. After weaning, the nursing dams were euthanized, their uteri were stained with 10 % ammonium sulfide, and the number of implantation sites was recorded. All males were examined for preputial separation (PPS), beginning on PND 40 until the prepuce was completely retracted from the glans penis. Individual body weights were recorded at acquisition. Young adult males were necropsied on PND 70-78 (~ PNW 10-11, all litters, three males in each litter whenever possible) or on PND 111-120 (~ PNW 16-17, the remaining males in each litter). They were killed by carbon dioxide asphyxiation, shaved, and examined for the presence of areolas and/or nipples on the ventral surface of the thorax, for gross abnormalities of external and internal genitalia, and for position of testes. On PND 70-78, the following organs of each male from the control and DnHP groups were weighed: liver, kidneys, testes, epididymides, seminal vesicles (with the coagulating glands and seminal fluid), and prostate. On both PND 70-78 and PND 111-120, histopathology was conducted on testes and epididymides of all control and DnHP animals. For information, a

preliminary study was also conducted in which groups of 9 to 12 pregnant rats were administered 625 mg/kg/day of DEHP, or 0, 500 or 625 mg/kg/day of DnHP, on GD 12 to GD 21.

Results of the preliminary study

The results of the preliminary study are summarized in the table below (Table 22).

Table 22: Preliminary study. Findings summary after treatments in (mg/kg/day)

	DnHP			DEHP
	0	500	625	625
Neonatal observations				
No. Females pregnant	12	10	12	9
No. Litters delivered	12	10	12	9
Parturition GD 23	0	4	1	0
Post-implantation loss per litter (%)	3.1±5.0 [a]	5.7±3.5	15.6±16.5 ##	5.2±6.1
Proportion of live pups PND1 (%) [b]	98.3±3.1	80.3±18.4 ##	84.3±22.5 #	91.8±6.0 #
Pup survival PND 1-4 (%)	98.9±2.5	87.9±31.2	88.2±28.1	98.1±3.7
Pup survival PND 4-21 (%)	88.3±11.1	97.1±6.0	89.8±12.6	94.3±6.9
Pup weight (g)				
PND 1	6.48±0.24	6.24±0.87	5.90±0.67 ##	5.91±0.45 ##
PND 4	8.8±0.5	9.1±0.9	8.3±1.5	8.5±0.6
PND 7	12.1±0.7	12.8±1.1	11.2±2.3	11.8±1.1
PND 14	23.4±1.2	25.1±2.2	22.5±4.2	23.1±2.2
PND 21	37.0±2.3	38.3±5.2	35.5±5.8	35.4±3.9
Main reproductive tract malformations				
No. Adult males/Litters evaluated (%)	75/12	57/9	44/11	54/9
Small penis	0	4/2 (7.0) [c]	16/7 (36.4)	8/3 (14.8)
Cleft prepuce	0	6/3 (10.5)	18/7 (40.9)	8/3 (14.8)
Hypospadias	0	17/7 (29.8)	28/11 (63.6)	20/6 (37.0)
Cleft phallus with exposed os penis	0	6/3 (10.5)	20/8 (45.5)	14/5 (25.9)
Vaginal pouch	0	3/1 (5.3)	12/6 (27.3)	8/3 (14.8)
Undescended testis	0 [d]	20/8 (35.1)	32/11 (72.7)	30/9 (55.6)

[a] Litter mean ± SD.

[b] Mean % live pups PND 1/ pups delivered.

[c] (No. males affected/total males evaluated)X100.

[d] Internal examination of 26 animals/12 litters in the control group.

[# and ##] significantly different from control group, $p<0.05$ and $p<0.01$, respectively (Mann-Whitney test).

Prenatal exposure to 625 mg DnHP/kg/day resulted in significant increase in the incidence of post-implantation loss per litter. Length of gestation was 23 days in several DnHP dams, but 21 or 22 days in all control and DEHP animals. All dams delivered live litters, but the proportion of newborns alive on PND 1 was significantly reduced at 500 and 625 mg DnHP/kg/day. In both DnHP groups, all pups from one litter did not survive to PND 4. Pup weight was slightly lower than control at 625 mg/kg/day from birth to weaning ($p<0.01$ on PND 1). Severe malformations of the external genitalia were observed in mature DnHP animals, including hypospadias, cleft phallus associated with exposed os penis, cleft prepuce, and vaginal pouch. Internal examination of the treated males revealed a high incidence of undescended testis in both DnHP groups. Seminal vesicles and/or prostate were absent in one male at 500 mg DnHP/kg/day and in three males at 625 mg DnHP/kg/day. The size of the testis and/or epididymis was highly reduced in several DnHP animals. A testis was absent in two and one animals at 500 and 625 mg DnHP/kg/day, respectively. Thus, 500 mg DnHP/kg/day was the high dose selected for the main study.

In utero exposure to 625 mg DEHP/kg/day resulted in a decrease in the proportion and weight of live pups on PND1. Reproductive tract alterations were seen in the male offspring at adulthood. They mainly consisted of undescended testis, hypospadias, and cleft phallus with exposed os penis. In the main study, DEHP was administered at the same level as DnHP for comparative purpose.

Maternal and Reproductive Data

There was no maternal mortality during the gestation period, nor significant difference in maternal weight gain between groups (Table 23). Exposure to 500 mg DnHP/kg/day slightly increased the time to parturition and one dam died three days after having delivered stillborn pups on GD 24. A second dam in the same high dose group gave birth to a litter of dead pups. In addition, all neonates from two 250 mg DnHP/kg/day litters and one 500 mg DnHP/kg/day litter were found dead or were cannibalised during the first days after delivery. Consequently, the proportion of live pups on PND 1 and the number of live pups on PND 1 were reduced in the 250 and 500 mg DnHP/kg/day groups, although not significantly. At the two highest doses, the viability of the offspring was decreased during the lactation period, but the effect was not statistically significant. The mean percent of pups surviving on PND 4-21 was lower than control at 125 mg DnHP/kg/day, although not significantly. This decrease was attributed to a litter with a poor postculling survival (10 %).

Table 23: Reproductive parameters in rats treated with DnHP or DEHP during late gestation (GD12-21)

	Treatment (mg/kg/day)					
	DnHP					DEHP
	0	50	125	250	500	500
No. Females pregnant	9	11	10	13	11	12
Maternal weight gain (g)						
GD 0-12	63±13 [a]	57±8	58±13	56±11	61±13	63±15
GD 12-21	88±12	87±10	93±13	87±16	88±16	85±16
PND 1-21	36±17	28±30	47±11	37±10	45±16	37±10
Gestation length (days)	21.7 ± 0.5	21.8 ± 0.4	22.1 ± 0.3	22.1 ± 0.3	22.4 ± 0.7	21.9 ± 0.3
Parturition GD 23 or 24	0	0	1	1	3	0
No. Dams littering	9	11	10	13	11	12
No. Litters with live pups PND 4	9	11	10	11	8	12
Post-implantation loss per litter (%)	6.8±13.8	6.8±10.9	3.7±6.0	7.4±15.8	7.4±7.0	6.6±6.1
Proportion of live pups PND 1 (%) [b]	99.0±3.0	95.6±7.9	91.3±14.8	85.4±26.7	76.4±38.4	87.2±14.8 ##
No. live pups per litter PND 1 [c]	11.8±3.4	13.8±2.3	13.5±2.1	10.9±4.5	10.5±5.4	11.8±3.2
Pup survival PND 1-4 (%) (preculling)	97.5±3.8	100.0±0.0	97.1±7.1	91.0±28.7	88.0±33.1	98.3±3.1
Pup survival PND 4-21 (%) (postculling)	90.0±13.2	82.7±19.5	74.6±27.8	63.0±18.9	71.3±25.9	73.5±24.0
Pup weight PND 1 (g)						
Males	6.49±0.74	6.32±0.65	6.68±0.47	6.93±0.96	6.48±0.67	6.40±0.57
Females	6.16±0.74	5.94±0.54	6.15±0.55	6.35±0.76	5.93±0.59	6.02±0.53
Male AGD PND 1 (mm)	2.45±0.16	2.39±0.12	2.28±0.19	2.17±0.12 **	2.02±0.16 **	2.01±0.08 **
Male AGD PND 1/Body weight $^{1/3}$ [d]	1.32±0.08	1.30±0.04	1.21±0.10 *	1.14±0.07 **	1.08±0.08 **	1.08±0.05 **

[a] Litter mean ± SD.
[b] Mean % live pups PND 1/ pups delivered.
[c] Means at 250 and 500 mg DnHP/kg/day when the litters with no live pups were removed from the analysis : 11.8±3.2 and 12.8±1.9, respectively.
[d] Ratio of AGD to the cube root of body weight.
* and **, significantly different from control group, $p<0.05$ and $p<0.01$, respectively (Dunnett's test).
significantly different from control group, $p<0.01$ (Mann-Whitney test).

Offspring Data

Whatever doses, DnHP did not alter the male and female pup weights on PND 1 and during the lactation period (Table 23 and Table 24).

Table 24: Effect of in utero exposure to DnHP or DEHP on body weight of offspring during lactation

	Treatment (mg/kg/day)					
	DnHP					DEHP
	0	50	125	250	500	500
Males						
PND 4 a	8.6±1.3 [b]	8.4±1.6	8.8±1.0	8.7±0.9	8.7±0.7	9.0±1.3
PND 7	12.8±1.5	12.4±2.3	13.5±1.7	12.8±2.0	12.7±1.4	13.2±2.1
PND 14	27.0±3.2	27.8±5.0	28.4±4.3	30.4±5.1	29.4±4.2	29.7±4.2
PND 21	44.1±6.4	43.3±8.6	47.8±10.2	49.7±7.8	47.8±5.8	50.2±6.4
Females						
PND 4 a	8.4±1.3	8.4±1.3	8.4±0.8	8.4±1.0	7.9±1.0	8.7±1.2
PND 7	12.5±1.5	12.9±1.5	12.7±1.7	12.3±1.8	11.4±1.9	13.1±2.3
PND 14	27.2±3.1	27.5±2.3	30.0±3.9	29.4±3.7	27.5±3.2	29.0±3.8
PND 21	43.9±4.8	42.4±7.7	48.2±6.0	48.8±4.9	43.9±4.9	48.5±6.9

[a] After culling. [b] Litter mean ± SD (8-11 litters).

There was a significant and dose-related decrease in the AGD of PND 1 males at 250 and 500 mg DnHP/kg/day (11 and 18 %, respectively), while the 7 % reduction elicited by males exposed to 125 mg DnHP/kg/day was not statistically significant (Table 23). The 125, 250, and 500 mg DnHP/kg/day-treated groups were significantly different from control when AGD was normalized to the cubic root of body weight, or when body weight was used as covariate ($p<0.05$). Prenatal treatment with 250 or 500 mg DnHP/kg/day resulted in an increased incidence of males with thoracic areolas and/or nipples on PND 12-14 and at adult necropsy (Figure 1).

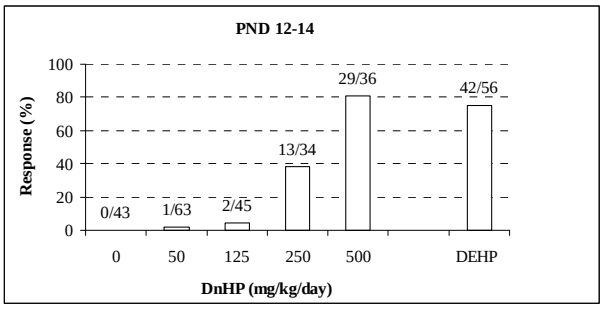


Figure 1: Incidence of males with thoracic areolas and/or nipples at 12-14 days of age. The number of affected males to total examined males is indicated above each bar. There were 8 to 11 litters per groups.

At adulthood, the average numbers of thoracic areolae/nipples per affected male were 0, 2.29, and 2.86, at 0, 250 and 500 mg DnHP/kg/day, respectively. In most cases, no thoracic areolae and/or nipples were observed in male offspring at 50 and 125 mg DnHP/kg/day: They were found on only three pups on PND 12-14 (1 out of 63 pups, and 2 out of 45 pups, at 50 and 125 mg DnHP/kg/day, respectively), and a single male from the 125 mg DnHP group displayed permanent areolae (two) at adulthood. The age at the onset of PPS were unaffected by DnHP treatment (Table 25). However, 500 mg DnHP/kg/day-exposed males achieved PPS later than the control animals, although not significantly. There was no treatment-related effect on male offspring body weight after weaning.

Table 25: Effect of in utero exposure to DnHP or DEHP (mg/kg/day) on preputial separation (PPS) in male offspring

	DnHP					DEHP
	0	50	125	250	500	500
No. Males/Litters evaluated	42/9	61/11	41/9	29/10	27/8	44/11
Age at PPS onset (days) [a,]	44.0±1.8 [b]	44.1±2.3	42.6±1.6	44.2±1.7	44.7±1.4	42.9±1.7
Body weight at PPS onset (g)	189±15	184±12	190±8	208±16 *	197±11	191±11
Age at PPS completed (days) [a]	46.8±2.3	47.4±2.7	45.8±1.9	47.2±3.8	49.4±2.9	47.0±3.5
Body weight at PPS completed (g)	209±17	209±9	214±21	231±31 *	232±14 *	222±22

[a] PPS was not evaluated in males with hypospadias or small penis (i.e. 3/2, 7/3, and 10/5 males/litters at 250 mg DnHP/kg/day, 500 mg DnHP/kg/day, and 500 mg DEHP/kg/day, respectively).

[b] Litter mean ± SD.

* significantly different from control group, $p<0.05$ (Mann-Whitney test).

Prenatal exposure to DnHP resulted in marked malformations of the external and internal genitalia in mature male offspring at 125 mg DnHP/kg/day and higher doses (Table 26). In the 125 mg DnHP/kg/day group, one animal displayed an ectopic testis, and another an absent testis (unilateral). Hypospadias was present in respectively, 9 and 21 % of the males from the 250 and 500 mg DnHP/kg/day groups. Few of the most severely affected animals showed exposed os penis, and concurrent cleft prepuce. Approximately 6 and 38 % of the adult animals exhibited undescended testis at 250 and 500 mg DnHP/kg/day, respectively. Whatever doses, non scrotal testes were located in the inguinal region, none was intra-abdominal. There was a 12 % incidence of animals displaying unilaterally absent or hypoplastic (less than 20 % of control weight) testis at 500 mg DnHP/kg/day. Crossed vasa deferentia (18 %) and single cases of reduced size of the penis, vaginal pouch, or partial agenesis of the vas deferens (unilateral) were only observed at this highest dose. Enlarged testes (more than 150 % of control weight) were present occasionally at 125 mg DnHP/kg/day and above. With the exception of two rats (from two different litters) displaying a firm and tan ventral prostate at 500 mg DnHP/kg/day, seminal vesicles and prostate appeared grossly normal. In addition to the reproductive organs, gross lesion of the kidney (brown, organ size reduced) was noted in one animal from the 250 mg DnHP/kg/day dose group.

Table 26: Main reproductive tract malformations in adult male rats following *in utero* exposure to DnHP or DEHP

	Treatment (mg/kg/day)					
	DnHP					DEHP
	0	50	125	250	500	500
No. Males/Litters evaluated (%)	42/9	61/11	41/9	32/10	34/8	54/11
Cleft prepuce	0	0	0	0	2/2 (5.9) [a]	2/1 (3.7)
Hypospadias	0	0	0	3/2 (9.4)	7/3 (20.6)	8/4 (14.8)
Cleft phallus with exposed os penis	0	0	0	1/1 (3.1)	4/3 (11.8)	2/1 (3.7)
Undescended testis (uni or bilateral)	0	0	1/1 (2.4)	2/2 (6.3)	13/5 (38.2)	16/7 (29.6)
Undescended testis (bilateral)	0	0	0	0	4/3 (11.8)	3/2 (5.6)
Enlarged testis [b]	0	0	3/3 (7.3)	4/4 (12.5)	1/1 (2.9)	1/1 (1.9)
Absent or markedly underdeveloped testis [c, d]	0	0	1/1 (2.4)	0	4/3 (11.8) [d]	6/5 (11.1)
Crossed vasa deferentia	0	0	0	0	6/4 (17.6)	5/5 (9.3)
Malformed epididymis	0	0	0	1/1 (3.1) [e]	1/1 (2.9) [e]	3/2 (5.6) [f]
Absent seminal vesicles and prostate	0	0	0	0	0	2/2 (3.7)

[a] (No. males affected/total males evaluated)X100.

[b] More than 150 % of the control weight. All were scrotal.

[c] Less than 20 % of the control weight. Undescended testes are not included.

[d] All were unilateral, except from one animal at 500 mg DnHP/kg/day, which showed absence of both testis and associated epididymis.

[e] Small head (unilateral).

[f] Long and thin body (unilateral).

At adult necropsy on PND 70-78, the body weights and liver and kidneys weights were comparable across groups (Table 27). Undescended testes were markedly decreased in size, with weights ranging from 0.09 to 1.19 g. Most of them (84.6 %) weighed less than half of control testis weight. With the exception of two rats in the high dose group, the epididymides of undescended testes were much smaller than those of descended testes (approximately 32 to 73 % lower than control weight). There was no significant changes in the mean weights of descended testes, although testes weighing less than one gram were observed in six males from three different litters at 250 mg DnHP/kg/day, and in two males from two different litters at 500 mg DnHP/kg/day (uni- or bilaterally). The weight of epididymides, excluding those of undescended testes, was prone to be slightly reduced at 250 and 500 mg DnHP/kg/day. However, it did not follow a dose-response relationships, and a statistically significant difference was only observed in the left epididymis weight adjusted for body weight, in the 250 mg DnHP/kg/day dose group. The weight of the seminal vesicles was slightly decreased at 125 mg DnHP/kg/day and higher doses, and was significantly different from the concurrent control at 250 mg DnHP/kg/day (relative) and 500 mg DnHP/kg/day (absolute, relative, or with body weight as covariate). Prostate weight tended to be lower in the 500 mg DnHP/kg/day group, with significant difference in the relative weight or when body weight was used as covariate.

Table 27: Absolute and relative organ weights of PND 70-78 male rats following *in utero* exposure to DnHP

	DnHP Treatment (mg/kg/day)				
	0	50	125	250	500
No. males/litters	26/9	32/11	25/9	23/10	22/8
Body weight (g)	366±40 a	357±29	369±18	390±21	358±38
Absolute organ weight (g)					
Liver	18.76±2.67	18.46±1.98	18.91±1.46	20.28±1.89	19.41±2.50
Kidneys (paired)	3.16±0.44	3.53±1.31	3.18±0.20	3.22±0.29	3.03±0.45
Right testis b, c	1.81±0.16	1.79±0.17	1.86±0.10 (23/9)	1.77±0.28 (22/10)	1.91±0.50 (15/7)
Right epididymis b	0.44±0.05	0.42±0.05	0.43±0.02 (23/9)	0.41±0.07 (22/10)	0.41±0.06 (16/7)
Left testis b, d	1.78±0.17	1.84±0.14	1.95±0.22	1.93±0.59 (22/9)	1.72±0.27 (17/8)
Left epididymis b	0.43±0.05	0.41±0.06	0.42±0.03	0.39±0.08 (22/9)	0.40±0.04 (17/8)
Seminal vesicles	1.27±0.15	1.23±0.21	1.18±0.12	1.20±0.14	1.03±0.14 **
Prostate	0.70±0.08	0.69±0.10	0.69±0.06	0.68±0.08	0.58±0.11

Relative organ weight (g/100 g body weight)

Liver	5.12±0.29	5.17±0.29	5.13±0.19	5.19±0.27	5.41±0.38
Kidneys (paired)	0.86±0.07	0.99±0.38	0.86±0.05	0.83±0.06	0.85±0.12
Right testis	0.50±0.03	0.50±0.40	0.50±0.04	0.45±0.07	0.51±0.09
Right epididymis	0.12±0.01	0.12±0.01	0.12±0.01	0.10±0.02	0.11±0.02
Left testis	0.49±0.03	0.52±0.03	0.53±0.06	0.49±0.15	0.47±0.06
Left epididymis	0.12±0.01	0.12±0.01	0.11±0.01	0.10±0.02 *	0.11±0.01
Seminal vesicles	0.35±0.05	0.35±0.06	0.32±0.04	0.31±0.03 *	0.29±0.02 *
Prostate	0.19±0.02	0.19±0.03	0.19±0.02	0.18±0.02	0.16±0.02 *

[a] Values are litter mean ± SD.

[b] Malformed reproductive organs are not included, i.e. at 125 mg DnHP/kg/ day: 1 male/1 litter with absent right testis and inguinal right epididymis (0.20 g), and 1 male/1 litter with undescended right testis (0.67 g) and epididymis (0.29 g) ; at 250 mg DnHP/kg/day: 1 male/1 litter with undescended right testis (0.55 g) and epididymis (0.23 g), and 1 male/1 litter with undescended left testis (0.58 g) and epididymis (0.19 g) ; at 500 mg DnHP/kg/day: 1 male/1 litter with hypoplastic right testis, 1 male/1 litter with absent left testis and inguinal left epididymis (0.11 g), 6 males/3 litters with undescended right testis (mean: 0.42 ± 0.22 g) and epididymis (mean: 0.21 ± 0.06 g), and 4 males/4 litters with undescended left testis (mean 0.79 ± 0.34 g) and epididymis (mean: 0.33 ± 0.11 g).

[c] When enlarged right testes are excluded, the mean is 1.84±0.38 at 500 mg DnHP/kg/day ; not significantly different from control. No enlarged right testis was seen in the control and 50, 125 and 250 mg DnHP/kg/day groups.

[d] When enlarged left testes are excluded, the means are 1.83±0.15, 1.62±0.41 and 1.72±0.27, at 125, 250 and 500 mg DnHP/kg/day, respectively ; not significantly different from control. No enlarged left testis was seen in the control and 50 mg DnHP/kg/day groups.

* and **, significantly different from control group, $p<0.05$ and $p<0.01$, respectively (Mann-Whitney test).

Histological examination revealed similar testicular and epididymal changes in the animals necropsied on PND 70-78 or PND 111 120. Degeneration of the seminiferous tubules was seen in all groups, including controls (Table 28). Nevertheless, the incidence and severity of the lesions were much higher in the 250 and 500 mg DnHP/kg/day dose groups. Thus, intense degeneration (i.e. severity grade 4 or 5) up to complete atrophy was seen in 0, 6.6 (4/61 animals ; 3/11 litters), 2.4 (1/41 animal ; 1/9 litter), 25.0 (8/32 animals ; 4/10 litters), and 51.5 % (17/33 animals ; 7/8 litters) of the males at 0, 50, 125, 250, and 500 mg DnHP/kg/day, respectively. It correlated with oligospermia or azoospermia in the corresponding epididymides, and sloughed cells were frequently noted in the epididymal lumen. Marked degeneration of the tubules was noted in all undescended or hypoplasic descended testes, but was also present in descended testes. A few males displayed interstitial cell hyperplasia at the two highest doses. Almost all the enlarged testes showed dilated tubular lumen. No changes were noted in their associated epididymides. At 50 mg DnHP/kg/day, the moderate to severe seminiferous tubule degeneration observed in five animals (70-78 or 111-120-day-old) from three different litters was questionable, but it was not clearly dose-related. It was recorded in one control animal as well, and in only one 125 mg/kg/day exposed animal, which testis was undescended.

Late gestational exposure to 500 mg DEHP/kg/day resulted in a reduction of the proportion of live pups at birth and in alterations in the development of the reproductive tract of the male offspring. This was evidenced by a shorter anogenital distance in male pups on PND 1, an increased incidence of males with areolas and/or nipples before weaning and at adult stage, and by marked malformations, including hypospadias, absent and/or hypoplasic testis, and undescended testis (inguinal). The spectrum and incidence of the developmental reproductive effects of 500 mg/kg/day of DnHP and DEHP were roughly similar.

Table 28: Histopathologic lesions in the testis and epididymis of adult male rats following *in utero* exposure to DnHP [a]

	DnHP (mg/kg/day)				
	0	50	125	250	500
Number of males/litters examined	42/9	61/11	41/9	32/10	33/8
Epididymides					
. Oligospermia	1	3	0	2	3
. Azoospermia	0	2	2	7	16
. Sloughed cells	3	2	5	8	13
. Decreased size of tubular lumen	1	2	2	8	16
Testes					
. Tubular degeneration-atrophy/hypoplasia [b]					
. grade 1	1	0	2	1	1
. grade 2	0	0	0	1	0
. grade 3	1	1	0	1	1
. grade 4	0	3	0	1	1
. grade 5	0	1	1	7	16
. Tubular necrosis	0	0	0	0	5
. Interstitial cell hyperplasia	0	0	0	1	6

[a] Results are expressed as the number of males affected. The lesions appeared unilaterally or bilaterally.

[b] Only the highest severity of tubular degeneration-atrophy/hypoplasia was mentioned when the lesion was bilateral.

Degeneration was estimated based on the approximate percentage of affected seminiferous tubules: less than 5 % (grade 1), 5-25 % (grade 2), 26-45 % (grade 3), 46-85 % (grade 4), 86-100 % (grade 5).

These results showed that prenatal exposure to DnHP caused permanent and dose-related alterations of the male rat reproductive development, with a similar profile as DEHP. In this study, oral administration of DnHP to pregnant Sprague-Dawley rats during late gestation (GD 12-21) had profound and permanent adverse effects on the development of the male reproductive system.

4.11.2.2 Human information

Numerous studies evaluating the link between phthalate exposure and various impacts on human development are published. However, within this abundant literature, none is dealing with DnHP on its own or within a mixture.

4.11.3 Other relevant information

4.11.3.1 Mode of action

DnHP has been studied in an in vitro assay in order determine the mechanism of testicular toxicity. Incubation of rat Sertoli and germ cell cultures with 1, 10, or 100 µM DnHP resulted in a dose-related detachment of germ cells from the Sertoli cell monolayer (Gray T.J. et Gangolli S.D., 1986). The detached germ cells were viable and structurally normal, but changes were observed in the morphology of the Sertoli cells. The findings suggest that germ cell loss following in vivo exposure to DnHP is a secondary effect resulting from toxic insult to Sertoli cells.

In order to be informative for fertility classification, the dose at which this mode of action occurs with DnHP is compared to the dose at which it occurs for classified phthalates in the (Q)SAR paragraph.

4.11.3.2 Endocrine disruptor property

DnHP showed weak estrogenic activity when assessed by competitive ligand-binding to ER and no activity when assessed by gene expression in some *in vitro* assays at high concentrations, and it was negative in vivo in the uterotrophic and vaginal cornification assays (Harris C.A. *et al.*, 1997; Okubo T. *et al.*, 2003; Zacharewski T.R. *et al.*, 1998). DnHP had an anti-androgenic activity for human androgen receptors *in vitro* (Takeuchi S. *et al.*, 2005), but it was not active in the Hershberger assay (Yamasaki K. *et al.*, 2004). It seems that antiandrogenic effects observed after exposure to phthalates are not mediated by interaction with androgen receptor, but rather by inhibition of testosterone production (Mylchreest E. *et al.*, 1999).

4.11.3.3 (Q)SAR, category approach

Table 29: List of a few phthalates presented in this paragraph

Name	Acronym	Structural formula	CAS No.	Classification	
Di-n-butyl phthalate	DBP	C6H4[COO(CH2)3CH3]2	84-74-2	Cat.3;R62	Cat.2;R61
Di(2-ethylhexyl) phthalate	DEHP	C6H4[COOCH2CH(C2H5)(CH2)3CH3]2	117-81-7	Cat.2;R60	Cat.2;R61
Diethyl phthalate	DEP	C6H4(COOC2H5)2	84-66-2		
Di-n-hexyl phthalate	DnHP	C6H4[COO(CH2)5CH3]2	84-75-3		
Di(n-octyl) phthalate	DnOP	C6H4[COO(CH2)7CH3]2	117-84-0		
Di-n-pentyl phthalate	DNPP	C6H4[COO(CH2)4CH3]2	131-18-0	Cat.2;R60	Cat.2;R61
Di-n-propyl phthalate	DPP	C6H4[COO(CH2)2CH3]2	131-16-8		

Analyses of existing data for structure-activity relationships (SAR) of phthalates have shown that *ortho*-phthalates with a linear portion of three to six carbons in the alkyl chains (e.g. DBP, DEHP) produced similar reproductive effects in experimental animals. They are embryotoxic and teratogenic, and exert severe and permanent alterations of the male reproductive tract in rats when administered *in utero*, during sexual differentiation. Within phthalate esters, the most potent reproductive toxicants are the di-n-hexyl and n-diethylhexyl phthalates (Table 30). As the side chain is lengthened to 8 carbons (di-octyl-) or shortened to 2 carbons (di-ethyl-), reproductive toxicity is lost even at doses which cause body weight loss over the period of continuous breeding study. As the length of the n-alkyl substitution is shortened from 6 (di-n-hexyl) to 5 (di-n-pentyl-), to 4 (di-n-butyl-), to 3 (di-n-propyl-), there is a reduction in the potency of reproductive effects.

Results obtained with DnHP, which has a linear side chain of six carbons, are consistent with this assumption. Thus, DnHP showed a similar profile of embryonic and foetal effects, in the same range of doses. Comparatively, prenatal exposure to Dicyclohexy Phthalate (DCHP), which has a ring side chain of six carbons, resulted in few developmental changes (i.e. reduced foetal growth). Moreover, its effects on the development of the male reproductive system were much less pronounced than those of the transitional C4-C6 phthalates. The major metabolite of butyl benzyl phthalate, monobenzyl phthalate has a benzyl side chain. In contrast to DCHP, it showed developmental toxic effects comparable to those of the transitional phthalates in prenatal studies in rats (Ema M. *et al.*, 2003). This suggests that the length of the side chain is not the sole structural element that affects the biological effects of phthalic esters. Additional experimental data are still

needed to better assess the SAR of this large chemical family, especially for compounds at the C4-C6 boundaries and/or with uncommon structural features.

Table 30: Reproductive performance in CD-1 mice during the continuous breeding phase. (Morrissey R.E. *et al.*, 1989)

	DBP			**DEHP**			**DEP**			**DnHP**			**DnOP**			**DnPP**			**DPP**		
Dose level (% in feed or	**0,03**	**0,3**	**1**	**0,01**	**0,1**	**0,3**	**0,25**	**1,25**	**2,5**	**0,3**	**0,6**	**1,2**	**1,25**	**2,5**	**5**	**0,5**	**1,25**	**2,5**	**1,25**	**2,5**	**5**
Fertility index	_	_	↓	_	↓	↓	_	_	_	↓	↓	↓	_	_	_	↓	↓	↓	_	_	↓
Mean No. litters per	_	_	↓	_	↓	NF	_	_	_	↓	*	NF	_	_	_	↓	NF	NF	_	_	NF
Mean No. Live pups per	_	_	↓	_	↓	*	↑	↑	_	↓	*	*	_	_	_	↓	NF	NF	_	↓	NF
Mean No. live male pups per	_	_	↓	_	↓	*	↑	_	_	↓	*	*	_	_	_	↓	NF	NF	_	↓	NF
Mean No. live female pups	_	_	↓	_	↓	*	↑	↑	_	↓	*	*	_	_	_	↓	NF	NF	_	↓	NF
Proportion of pups born	_	_	↓	_	↓	*	_	_	_	↓	*	*	_	_	_	_	NF	NF	_	↓	NF
Sex of pups born alive [b]	_	_	↑	_	↑	*	_	_	_	↓	*	*	_	_	_	_	NF	NF	_	_	NF
Mean live pup weight per	_	_	_	_	_	NF	_	_	_	↑	*	NF	_	_	_	_	NF	NF	_	_	NF
Mean live male pup	_	_	_	_	_	*	_	_	_	↑	*	*	_	_	_	_	NF	NF	_	_	NF
Mean live female pup	_	_	_	_	↑	*	_	_	_	↑	*	*	_	_	_	_	NF	NF	_	_	NF
Adjusted mean live pup	_	_	_	_	_	*	_	_	_	_	*	*	_	_	_	*	*	*	_	↓	NF
Adj. Mean live male pup	_	_	↓	_	↓	*	_	_	_	_	*	*	_	_	_	*	*	*	_	↓	NF
Adj. Mean live female pup weight	_	_	_	_	_	*	_	_	_	_	*	*	_	_	_	*	*	*	_	↓	NF
Estimated daily dose (g/kg bw)	0,04	0,42	1,41	0,01	0,13	0,41	0,46	2,44	4,4	0,43	0,88	1,87	1,82	3,62	7,46	0,76	2,16	4,65	1,91	4,07	8,63

Reproductive performance in CD-1 mice during the crossover mating trial

	DBP		**DEHP**			**DnHP**			**DnPP**		**DPP**	
Dose level (% in feed or	**1**		**0,3**			**1,2**			**2,5**		**5**	
Sex	F	M	F	M		F	M		F	M	F	M
Mating index	_	_	_	_		_	↓		_	↓	_	_
Fertility	↓	_	↓	↓		↓	↓		↓	↓	↓	_
Mean No. Live pups per	↓	_	NF	_		NF	*		NF	NF	NF	↓
Mean No. Live male	↓	_	*	_		*	*		NF	NF	NF	_
Mean No. Live female	↓	_	*	_		*	*		NF	NF	NF	_
Proportion of pups born	↓	_	*	↓		*	*		NF	NF	NF	_
Sex of pups born alive	_	_	*	_		*	*		NF	NF	NF	_
Mean Live pup weight	↓	_	*	_		*	_		NF	NF	NF	_
Mean Live male pup	_	_	_	_		*	*		NF	NF	NF	_

Mean Live female pup	_	↓		_	_			*	*			N F	N F		N F	_		
Adjusted mean live pup	↓	_		_	_			*	*			N F	N F		N F	_		
Adjusted mean live male pup	↓	_		_	_			*	*			N F	N F		N F	_		
Adjusted mean live female pup	↓	_		_	_			*	*			N F	N F		N F	_		
Estimated daily dose	1,3 2			0,4 4				1,8 2				5			9,8 2			

[a] A pair is judged fertile when it produced one or more litters. A litter was defined as one or more live or dead pups. Fertility index = (No. fertile/ No. cohabited) x 100

[b] Sex ratio of pups born alive = No. males/ total no. live pups.

Overall response at low, mid and high dose are presented as a significant increase (↑), decrease (↓) or non significant difference (-) between treated and controm groups. Statistical significance indicated a $p<0.05$.

NF: no fertile pair.

* insufficient data for statistical analysis.

In another study, nine phthalates were evaluated in a Chernoff-Kavlock screening assay and the authors concluded that dramatically positive results were seen with the diesters having intermediate chain lengths: n-butyl, i11 butyl, and n-hexyl. The shorter (methyl and ethyl) and longer (n-octyl and i-decyl) diesters were generally negative, although litter size and neonatal weight gain were both reduced in the di (n-octyl) phthalate group relative to its concurrent control" (Hardin B.D. *et al.*, 1987).

Table 31: Test results (mean ± SD; NA= not available) by experimental laboratory and block (Hardin B.D. *et al.*, 1987)

		Maternal response variables			**Maternal response variables**			
	Dose (mg/kg/d)	**No. Dead/ treated**	**Weight changes (g)**	**Viable litters**	**Liveborn per**	**Percentage of survival**	**Birth weight (g per pup)**	**Weight gain (g per pup)**
Di(i-decyl) phtalate	9 650,00	0/50	± 4	31/34	9,8 ± 2,4	99,5 ± 3,7	1,6 ± 0,1	0,6 ± 0,1
DEHP	9 860,00	0/48	) ± 1	2/32*	6,50	95,50	1,50	0,50
2-ethyl-l-hexanol	1 525,00	17/49	) ± 3,2*	11/20*	6,8 ± 3,4*	73,45 ± 32,2*	1,4 ± 0,2*	0,3 ± 0,2*
Control	Corn oil	0/50	± 2,5	33/34	9,9 ± 2,4	98,2 ± 8,8	1,6 ± 0,1	0,6 ± 0,1
Mono(2-ethylhexyl) phtalate	545,00	6/49	) ± 1,7	2/33*	9,00	83,80	1,70	0,80
DnHP	9900[a]	1/48	A	0/34*	0,00	NA	NA	NA
DnOP	9780 [a]	0/50	) ± 2,4*	39/40*	10,2 ± 2,8*	97,2 ± 16*	1,7 ± 0,1*	0,6 ± 0,1*
Control	Corn oil	0/50	,5 ± 2,2	34/38	11,5 ± 1,7	99,1 ± 4	1,6 ± 0,1	0,7 ± 0,2

[a]Undiluted chemical , 10 mL/ kg/ d ; 5 mL/kg/d in blo 5-A.

*p<0.05 relative to concurrent control.

The malformation profile of males prenatally exposed to 500 mg DnHP/kg/day closely resembles the profile observed with males exposed *in utero* to DEHP at the same dose, and was consistent with the effects of several other potent phthalates previously reported in rats after prenatal exposure. DnHP produced alterations in testosterone- and dihydrotestosterone-dependent

endpoints, e.g. nipples/areolae retention, AGD, and hypospadias (i.e. external genitalia). This suggests that DnHP has an anti-androgenic activity. The adverse effects of DBP and DEHP on male rat reproductive development have been partly attributed to the disruption of androgen synthesis, but not to receptor binding mechanisms (Foster P.M. *et al.,* 2001).

In a short-term study (Foster P.M. *et al.*, 1980) which employed a single dose level of dimethyl phthalate, DEP, DPP, DNPP, DnHP, or DnOP of 7.2 mmol/kg/day (equivalent of 2 g/kg bw/day of DBP) given by gavage in corn oil to a group of 12 pubertal male Sprague Dawley rats (4-weeks-old) for 4 days, it was shown that DNPP and DnHP produced testicular atrophy similar to that reported for DBP (Foster P.M. *et al.*, 1980).

Finally, data provided by Gray (Gray T.J. et Gangolli S.D., 1986) from different publications are displayed in table 32 .

Table 32: Effect of some phthalate monoesters on germ cell detachment in rat testicular cell cultures.

Phtalate monoester	**Testicular toxicity in vivob**	**No. Of germ cells detached (as % of control) at various monoester concentrations (µM)a**						**Classification for fertility**
		1	10	100	1000	3000	10000	
DEHP	+	208*	213*	384*	c	c	c	Cat.2;R60
DBP	+	c	113	143$	165$	228*	c	Cat.3;R62
DNPP	+	111	147	206*	276*	c	c	Cat.2;R60
DnHP	+	132$	199*	231*	c	c	c	

[a] Values are means for four culture dishes after a 24-hr treatment period and expressed as a percentage of the number of germ cells detaching from corresponding control cultures

[b] Data of Foster et al. (Foster P.M. *et al.*, 1980; Foster P.M. *et al.*, 2001) and unpublished data

$ Significantly different from control, $p<0.01$ (Student's t-test)

* Significantly different from control, $p<0.001$ (Student's t-test)

c Not dertermined

This table allows comparing the potency of these various phthalates in terms of germ cell detachment in rat testicular cell cultures and their classification. When comparing those phthalates, DnHP appears to impact rat testicular cell culture with less potency than DEHP but with more potency than DnPP, both classified Cat.2; R60 for fertility.

In a more recent publication (Fabjan E. *et al.*, 2006), a category of phthalates for reproductive effects such as reduction of fertility of both sexes, effects on reproductive organs (particularly in males, and more pronounced if exposure occurred during sexual development), and teratogenic effects is proposed. This category includes DEHP, DBP and BBP that are classified as reproductive toxicants for fertility and development. It is also proposed to include DnHP by read-across and also DPP and DIHP (Fabjan E. *et al.*, 2006).

4.11.4 Summary and discussion of reproductive toxicity

DnHP is a potent developmental and reproductive toxicant in rats and mice, and the male reproductive system is a target of its toxicity.

Regarding its impact on fertility, a dose-related decrease in the proportion of pairs able to produce litter together with a decrease of pups alive has been observed in mice (Lamb J.C. *et al.*, 1987). These effects were observed from the low dose 430 mg/kg onward in absence of parental toxicity. At this dose, no significant effect on embryolethality was observed in developmental toxicity studies (Saillenfait 2009). Taken together, these studies allow saying that the effects observed in the study of Lamb et al. can clearly be attributed to an alteration of fertility. With higher dose of DnHP (1670-1870 mg/kg) in mice, it was demonstrated that male fertility was highly impaired (Lamb *et* al. 1987). Spermatic parameters together with testicular histopathology are impacted by DnHP in mice (Lamb et al. 1987). Testicular effects were also observed in rats further to a 4-day exposure by gavage (Foster 1980). Although doses were particularly high (2400 mg/kg/d) it contributes to show that rats are although sensitive to testicular effects of DnHP. Additional evidence that male reproductive system affected by DnHP is brought by *in vitro* data with identification that Sertoli cells are a target of toxic effect of DnHP. At the doses (1670-1870 mg/kg) used in the Lamb et al. study (Lamb et al. 1987), females were not able to produce litter but it may have been caused either by an adverse effect on female fertility or by a high rate of post-implantation loss. No treatment-related microscopic lesions were detected in the ovaries, uterus, or vagina of the female mice with high dose of DnHP.

Developmental toxicity of DnHP was shown in rats where marked embryo mortality was consistently observed at 750 mg/kg/day (exposure on GD 6-20) (Saillenfait A.M. *et al.*, 2009a) and at 625 mg/kg/day in the preliminary study of Saillenfait et al. (exposure on GD 12-20) (Saillenfait A.M. *et al.*, 2009b).

Dose-related developmental toxic effects where observed as delay of ossification and increase in the incidence of skeletal variants (e.g. supernumerary lumbar ribs) that appeared at 250 mg/kg/day onward, presence of malformations and significant decreases in foetal weight at 500 and 750 mg/kg/day (Saillenfait, Gallissot, and Sabaté 2009).

DnHP induced a significant and dose-related decrease in the anogenital distance of male foetuses at all doses (Saillenfait, Gallissot, and Sabaté 2009;Saillenfait, Sabaté, and Gallissot 2009), and there was a significant increase in the incidence of male fetuses with undescended testis at 500 and 750 mg/kg/day of DnHP. Malformations of the male reproductive tract were still present at adult age and accompanied by histological effects on epidydimes and testes. Prenatal exposure to DnHP therefore caused permanent and dose-related developmental toxicity on the male reproductive tract (Saillenfait, Gallissot, and Sabaté 2009;Saillenfait, Sabaté, and Gallissot 2009). This is in line with impairment of fertility in males as identified in the fertility studies considering that *in utero* development is a sensitive window for the reproductive system and that toxicity seems to trigger Sertoli cells in particular (Foster et al. 2001;Gray and Gangolli 1986).

These effects occurred in absence of maternal toxicity.

The reproductive and developmental effects of phthalates are dependent on their chemical structure. Based on the similarity in the toxicity profiles of DEHP, DBP, and BBP, it is generally assumed that ortho-phthalic esters with a linear portion of four to six carbons in their alkyl side chains will produce developmental and reproductive toxic effects in rodents. DnHP has a straight backbone chain of six carbons. The results on DnHP presented in this report are in agreement with the reproductive toxicity profile of phthalates.

Table 33: Summary of main studies investigating developmental toxicity of DnHP.

Species	Route	Dose /Conc.	Study type and reliability	Exposure period : number of days during pregnancy	Observations and remarks	Ref.
CD1-mice (16–19 pairs of males and females)	In diet	0.0, 0.3, 0.6, and 1.2% corresponding to 0, 430, 880, or 1,870 mg/kg or 0, 380, 800, or 1,670 mg/kg bw/day depending on the estimation.	Standard RACB protocol	7 days prior to and during 98-day cohabitation period	During the continuous breeding phase, a few animals died. Food consumption was not altered by the presence of DnHP. They were a dose-related decrease in body weight gain by DnHP in diet. A dose-related decrease in the proportion of pairs able to produce even a single litter was observed from the low dose. There were no live pups at the high dose and a dose related decrease of number of pups alive. Pup weight adjusted for litter size was unchanged by treatment. These effects occurred in the absence of an effect on postpartum dam body weights. Adverse effects were observed at levels below those exhibiting large effects on body weight. A crossover mating trial was performed between the high-dose males and control females. There was a significant decrease in detected matings (56%) compared to controls (90%), and only 1 of 18 treated males sired a litter. When the high-dose females were mated with control males, there was no decrease in copulatory plugs, but none of the females became pregnant. Only the control and high-dose DnHP groups were necropsied. No histological effects were seen in reproductive organs in females whereas in male, extensive atrophy of the seminiferous epithetlium with mature sperm markedly diminished in the epididymis was observed. Sperm assessment showed a significant decrease in sperm number (7% of control) and motility (22% of control) parameters. There were significant decreases in the relative weights of the epididymis, testis, and seminal vesicle. No treatment-related microscopic lesions were detected in the ovaries, uterus, or vagina of the female mice althought uterine weight significantly decreased (31%). A second generation was not evaluated.	Lamb et al. 1987
12 pubertal male	Gavage	Single dose level of DnHP (2.4		4 days	Testis weight diminished of 65% / control value	(Foster P.M.

Sprague Dawley rats (4-weeks-old)		g/kg bw/day) in corn oil			Testicular zinc content decreased in absence of body weight effects. Histologic examination of formalin-preserved testes revealed a marked seminiferous tubular atrophy with the majority of tubules showing few spermatogonia and Sertoli cells, but normal Leydig cell morphology.	*et al.*, 1980)
Time-mated CD-1 mice (48-50 dams/group)	Gavage	9,900 mg/kg bw/day (undiluted chemical, 10 mL/kg/day) or corn oil	Chernoff-Kavlock screening assay	Dose-finding study: 8d Screening assay: GD 6-13	In the main study with DnHP, there were no live litters (0/34) to be examined. One exposed dam died. Body weight changes in dams could not be evaluated due to complete litter loss.	(Hardin B.D. *et al.*, 1987)
Pregnant Sprague-Dawley rats (8 to 12/group)	Gavage	0 (olive oil), 250, 500, and 750 mg/kg/day	Consistent with OECD guideline 414 Reliability : 1	GD 6-20	Maternal food consumption and body weight gain were significantly reduced at 750 mg/kg/day of DnHP but corrected maternal weight was not impacted. DnHP caused dose-related developmental toxic effects, including marked embryo mortality at 750 mg/kg/day, and presence of malformations (mainly cleft palate, eye defects, and axial skeleton abnormalities) and significant decreases in foetal weight at 500 and 750 mg/kg/day. Significant delay of ossification and increase in the incidence of skeletal variants (e.g. supernumerary lumbar ribs) also appeared at 250 mg/kg/day. DnHP induced a significant and dose-related decrease in the anogenital distance of male foetuses at all doses, and there was a significant increase in the incidence of male foetuses with undescended testis at 500 and 750 mg/kg/day of DnHP. In conclusion, DnHP showed clear embryotoxicity and teratogenicity in absence of maternal toxicity. There was evidence that DnHP could alter the development of the male reproductive system after *in utero* exposure.	(Saillenfait A.M. *et al.*, 2009a)
Pregnant Sprague-Dawley rats	Gavage	0, 50, 125, 250, or 500 DnHP/kg/day and 500 mg DEHP/kg/day	Reliability : 2	GD 12-21	DnHP had no significant effect on maternal body weight gain and pup weights during lactation. The proportion of live pups on postnatal day 1 was slightly, but not significantly, lower than control at 250 and 500 mg DnHP/kg/day. Male offspring displayed reduced anogenital distance on postnatal day 1 (PND) at 125 mg DnHP/kg/day and above, and areola/nipple retention before weaning and at adulthood at 250 and 500 mg DnHP/kg/day. At necropsy on PND 70-78 or PND 111-120, severe malformations of the reproductive tract were observed in young adult males at 125 mg DnHP/kg/day and higher doses.	(Saillenfait A.M. *et al.*, 2009b)

					They mainly consisted of hypospadias, underdeveloped testis, and undescended testis. Additionally, histopathological examination revealed seminiferous tubule degeneration at the two high doses. Prenatal exposure to DnHP caused permanent and dose-related alterations of the male rat reproductive development, with a similar profile as DEHP, those in absence of maternal toxicity.	

4.11.5 Comparison with criteria

Rationale for classification in Repr. 1B:

The CLP criteria for classification in Repr. 1B are as follows:

*"The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide **clear evidence** of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is **considered not to be a secondary non-specific consequence of other toxic effects**. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate."*

Overall, based on animal studies:

- DnHP induces embryo-mortality (decrease in litter and pups production) in absence of maternal body weight modification in mice and rats by oral route.
- Embryo-toxicity has been described in rats with reduced pup body weight, delayed ossification and increased incidence of skeletal variants.
- Teratogenicity has been described in rats with numerous malformations at higher doses.
- DnHP impacts the male reproductive system with impairment of fertility after repeated exposure in mice in adults and permanent alteration of testis, epididymis and seminal vesicle weight and histopathology in rats exposed *in utero*.

It is concluded that the data provided in the report provide **clear evidence** of teratogenic and foeto-toxic effects of DnHP. They also provide **clear evidence** of an adverse effect on male sexual function and fertility. Moreover, there is no mechanistic evidence that could lead to think that these effects are not relevant for human.

Moreover:

- DnHP was shown to have a weak estrogenic activity and contradictory results regarding its potential anti-androgenic activity *in vitro*. *In vivo* results tend to prove no direct anti-androgenic activity. However, data from closely related phthalates point out an effect on testosterone production rather than direct effect on androgen receptor.
- Mechanism of testicular toxicity and germ cell loss following exposure to DnHP *in vivo* seems to involve a toxic insult and modification of the morphology of the Sertoli cells, as observed *in vitro*. Comparison of the doses for which this toxic effect is

observed allows comparing the potency of DnHP with other phthalates classified for fertility.

– The similarity of effects between different phtalates also support that these effects are an intrinsic property of these compounds depending on their structure.

The effects on development were identified in the Saillenfait studies where no significant maternal toxicity was observed and on fertility, they occur from the low dose in mice in Lamb 1987 where no significant parental toxicity was observed. **Therefore, they cannot be considered to be secondary to other toxic effects.**

A classification **Repr. 1B –H360FD** is therefore warranted (Repr. Cat. 2; R60/R61 according to Directive 67/548/EEC). As no data available by inhalation or dermal route, it is proposed not to specify route of exposure in the hazard statement.

Classification in Repr 1A is not appropriate as it should be based on human data and no human data specific of DnHP are available.

Classification in Repr 2 is not appropriate as all the developmental studies available on DnHP are considered reliable. They provide all clear-cut results regarding developmental effects. Considering the whole database (on DnHP and of the phthalate class) in a weight of evidence approach, the level of evidence is considered as clear evidence and not as some evidence.

4.11.6 Conclusions on classification and labelling

A classification **Repr. 1B –H360FD** is proposed (Repr. Cat. 2; R60/R61 according to Directive 67/548/EEC) with no specific route of exposure added.

Opinion of RAC

The dossier submitter has prepared a thorough analysis of the available data, and RAC supports that the data warrant classification for reproductive toxicity, including effects on sexual function and fertility as well as developmental toxicity.

Regarding effects of DnHPon fertility, a dose-related decrease in the proportion of pairs able to produce litter (100-74-5-0% fertility in the pairs exposed to 0 – (380-430) – (800-880) – (1670-1870) mg/kg/day for 7 days prior to and during the 98-day cohabituation period) together with a decreased number of live pups per litter and proportion of pups born alive have been observed in a continuous breeding study in mice (Lamb J.C. *et al.*, 1987). These effects were observed from the low dose 370-430 mg/kg onward in absence of parental toxicity. Only control and high dose animals were necropsied, no histological findings were observed in reproductive organs in females, whereas findings in the treated males (1670-1870 mg/kg/day) included severe effects on testis weight, epididymal sperm concentration and motility, and extensive atrophy of the seminiferous tubules, providing a plausible basis for the decreased mating index in the mouse study. There were no live pups at the high dose, one litter with four pups at middle dose, and at the low dose a significant reduction of number of litters per pair (3.4 vs 4.9) and the number of live pups per litter (3.4 vs 12.3). The mating index in crossover mating trials showed that treated females were cycling

and could be receptive and that mating capability was reduced in the group of treated males. Some systemic effects (decreased mean body weight (6-10%), increased liver weight (32-34%), and decreased kidney weight (6-9%)) were described in both sexes at the high dose but the reproductive effects occurred also at the low and middle doses.

Testicular effects were also observed in adult rats exposed during gestation. At a dose of 250 mg/kg/day, intense degeneration or complete athrophy of the seminiferous tubules were noted in 25% of the males (Saillenfait *et al.*, 2009b), correlating with oligospermia and azoospermia in the corresponding epididymes. Some of these cases (6%) were explained by occurring in undescended testis. The testicular toxicity will result in a decreased fertility, but the potency in rats has not been assessed in a 2-generation study. Developmental toxicity of DnHP was shown in rats where marked embryo mortality was consistently observed at 750 mg/kg/day (exposure on GD 6-20) with high incidence of post implantation loss (Saillenfait *et al.*, 2009a) and at 625 mg/kg/day in the preliminary study of Saillenfait et al. (exposure on GD 12-20) (Saillenfait *et al.*, 2009b). DnHP also decreased the number of live pups per litter as from exposure levels of 370-430 mg/kg/day in a continuous breeding study in mice (Lamb et al, 1987).

Dose-related developmental effects were observed, such as delay of ossification and an increase in the incidence of skeletal variants (e.g. supernumerary lumbar ribs) that appeared at 250 mg/kg/day onward, as well as presence of malformations (cleft palates and eye defects) and significant decreases in foetal weight at 500 and 750 mg/kg/day (Saillenfait et al 2009a). These effects occurred in absence of maternal toxicity.

DnHP induced a significant and dose-related decrease in the anogenital distance and increased incidence of thoracic aerolas and/or nipples of male rat foetuses at all doses (Saillenfait et al, 2009a;Saillenfait et al 2009b), and there was a significant increase in the incidence of male fetuses with undescended testis at 500 and 750 mg/kg/day of DnHP. Malformations of the male reproductive tract were still present at adult age and were accompanied by histological effects on epidydimes and testes. Prenatal exposure to DnHP therefore caused permanent and dose-related developmental toxicity on the male reproductive tract (Saillenfait et al, 2009a; Saillenfait et al, 2009b). This is in line with impairment of fertility in males as identified in the fertility studies considering that *in utero* development is a sensitive window for the reproductive system and that the toxicity seems to affect Sertoli cells in particular (Foster et al. 2001;Gray and Gangolli 1986).

The information from experimental studies on DnHP is sufficient in itself as supporting the proposal for classification for effects on sexual function and fertility and development. A read across analysis based on other C4-C6 phthalates is considered as supportive information, as the effects and dose-effect relationships for DnHP is compatible with those for the other phthalates. Effects of phthalates on fertility and development are dependent on their chemical structure. It is generally acknowledged that ortho-phthalic esters with a linear portion of four to six carbons in their alkyl side chains (e.g., DEHP, DBP, and BBP) will produce developmental and reproductive toxic effects in rodents. DnHP has a straight backbone chain of six carbons, and based on the similarity in structure and toxicity profile with those of DEHP, DBP, and BBP, the reproductive toxicity observed by DnHP is supported by read across arguments. The read across is particularly relevant for DEHP-DnHP, which have very similar chemical structures and similar toxicological profiles. The proposed classification for DnHP is thus identical to the current classification for DEHP.

Six member states have commented the proposal. They all support classification for developmental toxicity, and five support classification for effects on fertility. One member state has questioned whether the evidence for effects on sexual function and fertility is sufficient for classification. RAC is of the opinion that the severe testicular toxicity observed in rats at doses well below the limit dose is a sufficient basis for classification. The testicular toxicity, observed in developmental toxicity studies, will result in a decreased fertility, although this has not been studied in a 2-generation study. Classification for effects on sexual function and fertility is also supported by a decreased mating index in mice and read across from similar phthalates with C4-C6 carbon chains, which are presently classified for effects on sexual function and fertility.

Overall, based on animal studies:

DnHP dose-dependently reduces the fertility of adult mice in a continuous breeding study

– Teratogenicity has been described in rats with numerous malformations at higher doses. Specifically, DnHP impacts the male reproductive system with permanent alteration of testis, epididymis and seminal vesicle weight and histopathology in rats exposed *in utero*, likely leading to impairment of fertility after repeated exposure;

DnHP induces embryo-mortality (decrease in litter and pups production) in absence of maternal body weight modification in mice and rats by oral route.

Embryo-toxicity has been described in rats with reduced pup body weight, delayed ossification and increased incidence of skeletal variants.

It is concluded that the data provided in the report provide **clear evidence** of teratogenic and foeto-toxic effects of DnHP. They also provide **clear evidence** of an adverse effect on male sexual function and fertility. Moreover, there is no mechanistic evidence that could lead to think that these effects are not relevant for human.

When comparing the data with the criteria, RAC is of the opinion that there is clear evidence of effects both on fertility and development supporting CLP classification in Repr. Cat. 1B. Repr Cat. 1A is not appropriate considering the lack of human data. Repr Cat. 2 is not appropriate as the database gives clear evidence and not only 'some evidence' as required for Cat. 2. The corresponding classification resulting according to DSD is Repr. Cat. 2; R 60-61.

4.12 Other effects

Not evaluated in this dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not evaluated in this dossier

6 OTHER INFORMATION

Information considered in this report was collected by a literature search last updated on June 2010.

DnHP was planned for registration on November 30th, 2010 (however, it is still not registered at the end of February 2011). Therefore, we performed a consultation by emailing concerned registrants in order to require the existing data they would like to be considered within our classification proposal. The ECPI was contacted and answered that none of their french members was producing this substance and probably no European neither. If imported, ECPI said that DnHP would be imported at low tonnage.

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