Substance Name: Diisobutyl phthalate (DIBP)

EC Number: 201-553-2

CAS Number: 84-69-5

SUPPORT DOCUMENT TO THE OPINION OF THE MEMBER STATE COMMITTEE FOR IDENTIFICATION OF

DIISOBUTYL PHTHALATE (DIBP)

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES WHICH CAUSE PROBABLE SERIOUS EFFECTS TO HUMAN HEALTH AND THE ENVIRONMENT WHICH GIVE RISE TO AN EQUIVALENT LEVEL OF CONCERN TO THOSE OF CMR[1] AND PBT/vPvB[2] SUBSTANCES

Adopted on 11 December 2014

[1] CMR means carcinogenic, mutagenic or toxic for reproduction

[2] PBT means persistent, bioaccumulative and toxic; vPvB means very persistent and very bioaccumulative

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Substance Name(s): Diisobutyl phthalate (DIBP)

EC Number(s): 201-553-2

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- Diisobutyl phthalate (DIBP) should be identified as a substance of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of REACH Regulation.

Summary of how the substance meets the criteria set out in Article 57 of the REACH Regulation

Diisobutyl phthalate (DIBP) should be identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to human health and the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

DIBP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway. The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, effects on spermatogenesis and testicular changes including weight changes and changes in Leydig an Sertoli cells.

In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction has an even higher potential for causing long term negative effect at the population level for such taxa.

Adverse effects caused by exposure to DIBP has not been observed in non-mammalian wildlife as no fish, amphibian or invertebrate studies including endocrine relevant endpoints have been found for DIBP. However, cross-species extrapolation for hazard identification of endocrine disruptive properties seems relevant, e.g. between rodents and fish, since the key molecular initiating events (in this case inhibition of enzymes involved in steroid synthesis) are similar between species (even though apical responses vary across phyla and some differences in sensitivity to adverse effects have been observed). In addition, read across between structural analogues for hazard identification of the endocrine disruptive properties of DIBP from other phthalates with similar main metabolites, such as DEHP and DBP where experimental data in fish and rodents provide evidence of probable serious effects to wildlife species, seems appropriate until further investigations are available on the effects of DIBP on non-mammalian wildlife. In conclusion, when available information is evaluated, DIBP can be considered an endocrine disruptor for both the environment and for human health as it fulfils the WHO/IPCS definition of an endocrine disruptor and the recommendations from the European Commission's Endocrine Disrupters Expert Advisory Group for a substance to be identified as an endocrine disruptor.

DIBP is considered as a substance giving rise to an equivalent level of concern because scientific evidence shows that exposure during sensitive time windows of development may cause irreversible developmental programming effects leading to severe effects on development and reproduction, regarded as particularly serious in relation to human health

and wildlife species, also because these adverse effects may first manifest themselves in later life stages as a consequence of exposure during early life stages. Adverse effects on development and reproduction are in addition generally regarded as endpoints of concern, and as such frequently used for regulatory hazard and risk assessment both for human health and for environmental species.

Registration dossiers submitted for the substance: Yes

JUSTIFICATION

1 Identity of the substance and physical and chemical properties

1.1 Name and other identifiers of the substance

Table 1: Substance identity

EC number:	201-553-2
EC name:	Diisobutyl phthalate
CAS number (in the EC inventory):	84-69-5
CAS number:	84-69-5
CAS name:	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
IUPAC name:	Diisobutyl phthalate
Index number in Annex VI of the CLP Regulation	607-623-00-2
Molecular formula:	$C_{16}H_{22}O_4$
Molecular weight range:	278.34 g/mol
Synonyms:	DIBP

Structural formula:

1.2 Composition of the substance

Name: DIBP

Description: The substance is a mono constituent substance (typical concentration of diisobutyl phthalate 80-100%(w/w)).

1.3 Physico-chemical properties

Table 2: Overview of physicochemical properties

Property	Value	IUCLID section	REACH ref Annex, §
Physical state at 20°C and 101.3 kPa	Colourless, clear mostly odourless viscous liquid	3.1	VII, 7.1
Melting/freezing point	-37C	3.2	VII, 7.2
Boiling point	320C	3.3	VII, 7.3
Vapour pressure	0.01 Pa at 20C	3.6	VII, 7.5
Water solubility	20 mg/l at 20C	3.8	VII, 7.7
Partition coefficient n-octanol/water (log value)	logPow: 4.11	3.7	VII, 7.8
Dissociation constant	--	3.21	XI, 7.16

2 HARMONISED CLASSIFICATION AND LABELLING

DIBP is listed in Regulation (EC) No 1272/2008 as follows:

Classification and labelling of DIBP according to Annex VI, Table 3.1 of Regulation (EC) No 1272/2008

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling		Specific Conc. Limits, M-factors
				Hazard Class and Category Code(s)	Hazard Statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	
607-623-00-2	Diisobutyl phthalate	201-553-2	84-69-5	Repr. 1B	H360Df	GHS08 Dgr	H360Df	Repr. 1B; H360Df: C ≥ 25 % Repr. 2; H361f: 5 % ≤ C < 25 %

Classification and labelling of DIBP according to Annex VI, Table 3.2 of Regulation (EC) No 1272/2008 (The list of harmonized classification and labelling of hazardous substances from Annex I to Directive 67/548/EEC)

Index No	International Chemical Identification	EC No	CAS No	Classification	Labelling	Concentration limits
607-623-00-2	Diisobutyl phthalate	201-553-2	84-69-5	Repr. Cat. 2; R61 Repr. Cat. 3; R62	T R: 61-62 S: 53-45	Repr. Cat. 2; R61: C ≥ 25 % Repr. Cat. 3; R62: C ≥ 5 %

3 ENVIRONMENTAL FATE PROPERTIES

The environmental fate properties of DIBP was not discussed by the ECHA Member State Committee in the "Support document for the identification of diisobutyl phthalate as a substance of very high concern because of its CMR properties" (ECHA 2009) and no EU Risk Assessment Report exists for DIBP. Information about degradation, distribution and bioaccumulation has been obtained from the registration dossier available at ECHAs homepage in August 2014 (ECHA 2014) and from the EU RAR on DBP as these provide best possible estimates for DIBP (EU RAR 2004).

Infomation on environmental fate properties, in particular persistency and bioaccumulation is included as background information. Fate related properties are not required for identification of SVHCs with endocrine disruptive properties according to Article 57(f).

3.1 Degradation:

In a non-GLP, non-guideline study the second order hydrolysis rate constant of diisobutyl phthalate was found to be 0.0014 ± 0.0002 M-1.s-1. In conclusion, the second order hydrolysis rate constant of diisobutyl phthalate was found to be 0.0014 ± 0.0002 M-1.s-1. (ECHA 2014).

In an OECD 301D guideline-following study diisobutyl phthalate was found to fully mineralize (66 -70% ThOD) within 28 days. It is concluded that the substance is readily biodegradable. (ECHA 2014).

The use of BIOWIN is appropriate for diisobutyl phthalate as this compound falls within the applicability domain of the model. The results indicate that diisobutyl phthalate is rapidly degradable and not expected to be persistent. (ECHA 2014).

"The contribution of hydrolysis to the overall environmental degradation of phthalate esters, including DBP, is expected to be low. Photo-oxidation by OH radicals contributes to the elimination of DBP from the atmosphere. An atmospheric half-life of about 1.8 days has been estimated for the photo-oxidation reaction. The metabolic pathway of aerobic and anaerobic biodegradation of phthalates can be summarised as follows. First the di-ester is hydrolysed into the mono-ester by esterases with low substrate specificity. Subsequently the mono-ester is converted into phthalic acid. There is ample evidence that DBP is ready biodegradable under aerobic conditions. The same literature sources indicate that biodegradation of DBP is much slower in the anaerobic environment, e.g. sediments or deeper soil or groundwater layers." Citation from EU RAR (DBP) 2004. In support an atmospheric half-life for DIBP of 1,2 days has been calculated for indirect photolysis atmospheric oxidation (25 deg C) by the AoPWin v1.92 model.

3.2 Distribution

The Henry's law constant of diisobutyl phthlate is according to (ECHA 2014) 2.48 Pa.m3/mol-1.Henry Win (ver. 3.20) estimates 0.065-0.12 Pa.m3/mol^{-1}

The Koc of diisobutyl phthalate was calculated from Log Kow 4.11 using the TGD calculation of Koc = 0.49 . Log Kow + 1.05. . The log Kow used to calculate this log Koc is that reported for diisobutyl phthalate. In conclusion, diisobutyl phthalate has a Log Koc of 3.06. (ECHA 2014). KOCWIN v2.00 estimates a log Koc of 2.91-3.07.

The Henry's law constant indicates that DIBP will only slowly volatilize from surface waters, i.e. virtually all of the DiBP will remain in the water phase at equilibrium. The octanol/water partition coefficient (Kow) of DBP is high and consequently the equilibrium between water and

organic carbon in soil or sediment will be very much in favour of the soil or sediment. Despite its low volatility, In the air DIBP will be transported and removed by both wet and dry deposition.

3.3 Bioaccumulation:

The BCF of diisobutyl phthalte was calculated to be 622. The log Kow of 4.11 used to calculate this BCF is that reported for diisobutyl phthalate. (ECHA 2014). BCFWin (ver. 3.01) estimates for DIBP a BCF of 239, a half-life in fish of 0.06 d and a BCF and BAF (using the Arnot-Gobas method, upper trophic level) of only 26. All estimations are based on the reported log Kow of 4.11 (ECHA 2014).The estimated BCFs, the low estimated BAF and the low estimated half-life in fish indicates that diisobutyl phthalate is not likely to bioaccumulate significantly in the lipids of fish.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

The toxicokinetics of DIBP was not discussed by the ECHA Member State Committee in the "Support document for the identification of diisobutyl phthalate as a substance of very high concern because of its CMR properties" (ECHA 2009) and no EU Risk Assessment Report exists for DIBP.

In the opinion of RAC when evaluating the Danish restriction proposal for 4 phthalates in 2011, they wrote: "For DIBP it is assumed it has the same absorption fractions as DBP, given the similarities between these two phthalates". Absorption fractions for DBP/DIBP are 100% for oral and inhalatory absorption and 10% for dermal absorption (ECHA 2012).

For the metabolism, distribution and elimination relating to human health, relevant excerpts from the EU RAR on DBP are provided below as these will provide the best estimates for DIBP (EU RAR 2004).

Human health

"Dibutylphthalate is rapidly absorbed and excreted after oral administration as was demonstrated in studies in laboratory animals. Up to more than 90% of oral doses given to rats or hamsters was excreted in urine within 24-48 hours. Fecal excretion is low (1.0-8.2%). Also in man oral absorption of DBP takes place. After dermal exposure of rats to DBP ca. 60% of the dose was excreted in urine within 7 days. In feces ca. 12% of the dose was found. An *in vitro* study revealed slower absorption of DBP by the human skin (2.40 µg/cm2/hr) than by the rat skin (93.35 µg/cm2/hr). Data on absorption after exposure by inhalation are not available. A substantial fraction of DBP is initially excreted in the bile and subsequently enters the enterohepatic circulation. No significant accumulation in tissues was observed in laboratory animals after oral as well as dermal exposure; limited inhalation data revealed an indication for some accumulation in tissues. The major part of DBP is hydrolysed to mono-n-butyl phthalate (MBP) and the corresponding alcohol prior to absorption by the small intestines, but hydrolysis can also occur in liver and kidneys. The metabolites that occur in urine are MBP, MBP-glucuronide, various ω- and ω-1- oxidation products of MBP (more polar ketones, carboxylates) and a small amount of free phthalic acid. Species differences in the excretion of MBP and its glucuronide were observed; rats excreted a larger proportion unconjugated MBP in urine than hamsters. There are no data on biotransformation after dermal exposure and exposure by inhalation. Transplacental transfer of DBP and its metabolites was demonstrated in an oral study with 14C-labelled DBP in rats. Radioactivity in embryonic tissues contained

less than 0.12-0.15% of the administered dose. MBP accounted for most of the radioactivity in maternal plasma, placenta and embryo. Unchanged DBP was found in only small amounts. No accumulation of radioactivity was seen in maternal or embryonic tissues."

4.2 Other effects: Endocrine disruption

4.2.1 General approach – Human Health

Criteria on how to assess whether or not a substance has endocrine disrupting properties and/or is an endocrine disruptor are currently being developed in the European Union. The timeline for the finalization of the process is not currently known.

The basis for the criteria is envisaged to be the widely accepted definition of an endocrine disruptor by the WHO/IPCS (WHO/International Programme on Chemical Safety 2002):

An endocrine disruptor is an exogenous substance or mixture that

1) alters function(s) of the endocrine system and

2) consequently causes

3) adverse health effects in an intact organism, or its progeny, or (sub)populations.

The European Commission's Endocrine Disrupters Expert Advisory group agreed in 2013 "that the elements for identification of an endocrine disrupter were demonstration of an adverse effect for which there was convincing evidence of a biologically plausible causal link to an endocrine disrupting mode of action and for which disruption of the endocrine system was not a secondary consequence of other non-endocrine-mediated systemic toxicity. Relevance of the data to humans should be assumed in the absence of appropriate data demonstrating non-relevance." (JRC 2013)

As it is assumed in this report that a substance should fulfil the recommendations from the European Commission's Endocrine Disrupters Expert Advisory group outlined above in order to be identified as an endocrine disruptor, available information is assessed based on the following topics:

1) Adverse health effects
2) Endocrine mode of action
3) Plausible link between adverse effects and endocrine mode of action
4) Human relevance

The most marked adverse effects of DIBP have been described for male reproductive system and most work performed to elucidate the mode of action of DIBP has been carried out in experimental tests studying developing male rats. The following discussion therefore focuses on adverse effects on male reproduction induced by inhibition of steroid synthesis in fetal testis. DIBP may also have other endocrine disrupting modes of action. Although data on these modes of action are sparse, data on estrogenic action and thyroid disruption will be discussed briefly here to give a complete overview of the possible endocrine disrupting modes of action of DIBP.

4.2.2 Adverse health effects – Analysis of available information from rodent studies

a) Background

DIBP is classified as a substance toxic to reproduction (Repr. 1B, H360Df) based on evidence of adverse effects on the reproductive organs in adult and developing rodents. The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia of which almost all are considered adverse (OECD 2008). The evidence of reproductive toxicity on male reproductive organs was described in the "Support document for identification of diisobutyl phthalate as a substance of very high concern because of its CMR properties" (SVHC support document) with the following introduction (citations in *italics*) (ECHA 2009):

Repeated dose toxicity: oral

In toxicity studies with repeated oral application the male reproductive system was identified as one and most important target organ of toxicity for DIBP. Available early feeding studies in experimental animals revealed reproductive effects in adult males (e.g., decreased testes weight and reduced sperm production in rats) at relatively high oral doses. Sub-acute studies with rats and mice, and sub-chronic studies with rats and dogs are available. Although these studies were not comparable to guideline studies and not in conformity with GLP they point clearly out the critical organs of toxicity for DIBP – the male sex organs. The distinctions in study design compared to published guidelines include e.g., no precise data on strain, number and sex of the used animals; in some studies only animals of one sex were tested. Similar studies with the correspondent monoester, mono-iso-butyl phthalate (MIBP) to which DIBP is hydrolysed are also available and summarised below. MIBP is likely to be the major metabolite of DIBP (Mentlein and Butte, 1989).

Effects on fertility

Whereas no fertility studies (one-, two- or multigeneration studies) could be identified in the current toxicological data base for DIBP, adverse effects on male reproductive organs (testicular toxicity) and on spermatogenesis had been observed during repeat dose toxicity studies with DIBP and with MIBP at relatively high dosages (c.f. 5.6.1 Repeated dose toxicity: oral), indicating that the monoester MIBP to which DIBP is hydrolysed by human and rat hepatic esterases (Mentlein and Butte, 1989) should be considered an active metabolite.

Several rodent studies on DIBP included in the SVHC document demonstrated adverse reproductive effects in repeated dose studies (Hodge 1954; Oishi and Hiraga 1980a, 1980b, 1980c, 1980d; Foster et al 1981), whereas no one-, two- or multigeneration studies had been performed. The study summaries listed in Annex 1 are taken from the SVHC support document for DIBP (ECHA 2009). These studies are considered reliable (i.e. in most cases with a Klimisch score 1 or 2).Furthermore, the SVHC support document (ECHA 2009) highlighted that the monoester metabolite MIBP is considered the active metabolite of DIBP. The reproductive toxicity of DIBP is further substantiated by studies carried out after the publication of the SVHC document (see below).

Two studies included in the SVHC support document from 2009 (ECHA, 2009) found reduced anogenital distance (AGD) (Borch et al. 2006; Saillenfait et al. 2008) and one study found increased nipple retention in male pups (Saillenfait et al. 2008). AGD and nipple retention in male pups are generally known to be androgen dependent endpoints and decreased AGD and increased nipple retention are associated with an anti-androgenic mode of action (Bowman et al. 2003; Imperato-McGinley et al. 1985; Imperato-McGinley et al. 1986). Thus the findings by Borch et al. (2006) and Saillenfait et al. (2008) strengthen the hypothesis of DIBP as an endocrine disruptor. Evidence of reproductive toxicity and a discussion of endocrine activity were presented in the SVHC support document from 2009 (ECHA 2009) (*in italics*):

"*DIBP was found to adversely affect the reproductive organs in adult males in experimental studies which may affect their fertility. DIBP was also found to be a developmental toxicant. The results of these evaluations are reflected in the classification as Repr. Cat 3; R62 and Repr. Cat 2; R 61 according to directive 67/548/EEC. Any generation or fertility studies are not available in the toxicological data base for DIBP. However, adverse effects on male*

reproductive organs (testicular toxicity) and on spermatogenesis had been induced in studies with young adult male rats and mice after repeated oral administration of DIBP at relatively high dosages. Similar effects were also revealed after treatment with MIBP, the major monoester metabolite of DIBP." (…) "*The structures affected by in utero exposure to DIBP are indicative of an antiandrogenic mode of action. In particular, the development of dihydrotestosterone-regulated tissues (e.g. areolas/nipples, external genitalia including AGD and hypospadias) were severely affected. DIBP had also marked effects on the final inguinoscrotal descent, which is known to require androgens. The changes in the androgen-dependent endpoints induced by DIBP treatment are congruent with the findings of lowered fetal levels of testosterone and changes in the expression of several genes in the cholesterol uptake, transport and testicular testosterone biosynthesis in other studies. Although phthalates do not act as classical antiandrogenic chemicals by binding to the androgen receptor, they obviously have the same effects of blocking androgen-action at the target tissue and therefore may be considered as acting antiandrogenic*" (ECHA 2009).

The reproductive toxicity of DIBP was thus evaluated to be likely induced via an endocrine disrupting mode of action, i.e. interference with steroid hormone synthesis. This conclusion is further substantiated by studies carried out after the publication of the SVHC support document from 2009.

b) Adverse effects indicative of endocrine disruption

Although no one-, two- or multi-generation studies have been performed, some reproductive toxicity studies have been published for DIBP. Some of these were included in the SVHC support document from 2009 and a few additional studies have been published recently. These reproductive studies are summarized in table 3 below showing adverse effects and effects showing an *in vivo* endocrine mode of action, including the key studies from the SVHC document (*in italics*), the more recent reproductive studies and one additional acute exposure study.

The studies included in table 3 are generally evaluated as reliable (Klimisch score 1 or 2). Overall, the dataset is evaluated as very reliable due to the consistency of the findings with regards to both the adverse effects and the mode of action.

Table 3 Summary of studies *in vivo* showing adverse effects and/or showing an *in vivo* endocrine mode-of action. Study summaries in italics are included in the SVHC support document for DIBP from 2009 (ECHA 2009).

Pregnant rats, gavage GD 7 to 19 or 21	*Mated female Wistar rats (n=8/group) were gavaged from GD 7 until GD 19 or until GD 20/21 with either vehicle (corn oil) or 600 mg/kg bw/d of DIBP (purity 99%), when they were sacrificed and their male offspring evaluated. At sacrifice on GD 19 five dams from the control and six dams from the treated group provided litters and at sacrifice on GD 20/21 six dams from the treated group provided litters. Anogenital distance (AGD) was measured in all fetuses, fetuses were decapitated and their trunk blood collected, and from males testes removed for histopathology and for immunohistochemistry, for measurement of testosterone production ex vivo, respectively measurement of testosterone content. Administration of DIBP resulted in statistically significant reduction in AGD in male pups (and increased AGD in female pups) at GD 20/21 together with 10 % reduction in bodyweights of male and female fetuses and in a significant reduction in testicular testosterone production and testicular testosterone content in the male offspring at GD 20/21. Histopathological investigations revealed testes pathology as seen with other phthalates, in particular clustering of small Leydig cells on GD19 or GD20/21 and vacuolisation of Sertoli cells on GD 20/21. Immunohistochemistry revealed reduced staining for StAR and P450scc, indicative for reduced expression of these two proteins and thereby reduced capacity of the testicular steroid synthesis. Further results from the study by Borch et al., 2006 were reported by Boberg et al. (2008), who quantified levels of insulin,*	Borch et al., 2006 and Boberg et al., 2008

	leptin, MCP1, IL-1B, PAI-active, IL6, and TNFα in pooled samples of plasma. In addition, livers, adrenals and testes tissue from the male fetuses and ovaries from the females had been used for gene expression (mRNA expression) analysis and for steroid hormone measurements (estradiol, testosterone). Treatment with DIBP had resulted at GD 21 in statistically significant reduction of protein levels of insulin and of leptin, whereas no alterations were seen in plasma levels of MCP1, IL-1B, PAI-1 active, IL6 or TNFα. Gene expression analysis on genes involved in steroid synthesis revealed reduced testicular mRNA levels of SR-B1, StAR, P450c17, P450scc and Insl-3 at GD 19 and GD21. In addition testicular SF-1 mRNA levels were reduced on GD 19, whereas no alterations were seen for testicular mRNA levels of aromatase or PBR. In the ovaries of DIBP treated animals an increase in mRNA levels of aromatase was revealed at GD 21. Gene expression analysis on PPARα and on PPARγ revealed significantly reduced mRNA levels of PPARα in livers and testes of DIBP exposed males at GD 19 but not at GD 21. PPARγ mRNA levels were very low in both testes as well as livers and appeared unaltered by DIBP treatment. In the ovaries of DIBP treated animals no alterations were seen in the expression of ERα, ERβ, PPARα, or PPARγ. Besides reductions in mRNA levels there were also indications for reduced protein levels of P450c17 and of PPARγ in the Leydig cells of DIBP treated animals at GD 19 and GD21 (evidenced from reduced immunostaining intensity).	
Pregnant rats, gavage GD 14 to 18	Pregnant Sprague-Dawley rats were exposed to 0, 100, 300, 600, or 900 mg/kg bw/day of DIBP from GD 14 to 18 by gavage (n=3 dams per group). Testicular testosterone production ex vivo was assessed by incubation of testes of 18 day old fetuses for 3 hours and testosterone measurement in the media. Dose-related decreases in testosterone production was seen for DIBP and the other tested phthalates (DEHP and DIHP (diisohexyl phthalate)) from 300 mg/kg bw/day and above (NOAEL 100 mg/kg bw/day), and for DINP from 500 mg/kg bw/day.	Hannas et al., 2011
Pregnant rats, gavage GD 14 to 18	Pregnant Sprague-Dawley rats were exposed to 0, 100, 300, 600, or 900 mg/kg bw/day of DIBP from GD 14 to 18 by gavage (n=3-4 dams per group). In the same study as described by Hannas et al., 2011, gene expression studies were performed in testes at GD 18. A number of steroidogenesis-related genes were downregulated at 300 mg/kg of DINP and above and also by other phthalates examined (dihexyl-, diheptyl-, dipentyl-, and diisononyl phthalate), but not by diisodecyl phthalate. DIBP downregulated the expression of: StAR, Cyp11a1, HSD3b, Cyp17a1, Scarb1, Insl3, Cyp11b1 and Rxrg, and upregulated the expression of Amhr2 and Sox9. These data were applied for potency ranking of phthalates and it was concluded that several phthalates including DIBP affected the same pathways.	Hannas et al., 2012
Pregnant rats, gavage GD 8 to 18	*In a further study on Sprague-Dawley rats, which was designed to provide dose-response information on the effects of a series of individual phthalates on fetal testosterone production and on the use of the data obtained for the prediction of effects of phthalate mixtures on fetal testosterone production, DIBP was administered to pregnant animals (5-8 animals per treatment group) by gavage at doses of 0 (corn oil), 100, 300, 600, and 900 mg/kg bw/d on GD 8-18 (Howdeshell et al., 2008). Maternal body weights were taken on GD 8 and on GD 18 at sacrifice, when the uterus was removed and the number of fetuses (live and dead) and resorptions were counted and recorded. The total number of implantations was calculated by adding together the number of live and dead fetuses with the total number of resorptions. Fetal mortality was calculated by adding together the number of resorptions and dead fetuses then divided by the total number of implantations. Testes from three males/dose group (2 replicate determinations in individual testes) in were used for investigation of ex vivo testis testosterone production. Maternal body weight gain was reduced from 73 g in controls to 48 and 43 g in the 600 and 900 mg/kg/d dose group. DIBP-induced complete litter loss in 1/5 dams at 900 mg/kg/d, and induced greater than 50% resorptions*	Howdeshell et al., 2008

	in 2/5 dams at 900 mg/kg/d and in 1/5 dams at 600 mg/kg/d resulting in increased percentages of fetal mortality of 17% at 600 mg/kg/d and of 59% at 900 mg/kg/d as compared to 1.3% in the controls. It is reported, that many of the testes collected from DiBP fetuses at dosages of 600 and 900 mg/kg/d were smaller, mucinilagous, and/or located higher in the abdominal cavity. The functional assay on testes ability for hormone production revealed that fetal testicular testosterone production was statistically significantly ($p<0.001$) reduced at dosages of 300 mg/kg/d or higher. The overall results indicated that DIBP (as well as DBP and BBP) was of equivalent potency to DEHP at reducing fetal testosterone production. Dosage levels reducing fetal testosterone production were about one-half to one-third of that required to increase fetal mortality, indicating changes in fetal testicular testosterone production to be a sensitive parameter. Based on statistically significantly lower fetal testosterone production at 300 mg/kg/d a NOAEL of 100 mg/kg bw/d can be derived from this study. BASF, 2003: *A further guideline according prenatal toxicity study on Wistar rats (BASF, 2003; cited in Saillenfait and Laudet-Hesbert, 2005) with dietary administration is indicated in the data base, for which the study report is not available to the rapporteur. It is reported that a decrease in fetal weights and an increase in skeletal variations was observed in rats that had ingested 942 mg/kg DIBP with their diet during pregnancy.*	
Pregnant rats, gavage GD 6 to 20	*In a dose-range finding study on Sprague-Dawley rats, DIBP was administered to pregnant animals (10-14 animals per treatment group) by gavage at doses of 0 (olive oil), 250, 500, 750 and 1000 mg/kg bw/d (Saillenfait et al., 2005) on GD 6-20. Maternal body weights and clinical signs were recorded. Dams were euthanised on GD 21, and the uterine contents were evaluated for number of implantations, resorptions, fetal deaths, and live fetuses. All live fetuses were submitted to external examination and to internal gross examination of the reproductive tract. Maternal body weight gain was transiently depressed on GD 6-9 at the two higher dose levels. However, the weight gains during GD 6-21 corrected for uterine weight were comparable across groups. A marked increase in the number of resorptions of 38% and of 61% was observed at the 750 and 1000 mg/kg bw/d dose level. A dose-related reduction in fetal body weight was observed amounting to 21% at 1000 mg/kg. Gross internal examination of the reproductive tract revealed undescended testes in 56% and 70% of the male fetuses at 750 and 1000 mg/kg. No further visceral or skeletal examinations were conducted.*	Saillenfait et al., 2005
Pregnant rats, gavage GD 6 to 20	*In a further guideline according prenatal toxicity study on Sprague-Dawley rats, DIBP was administered to pregnant animals (23-24 animals per treatment group) by gavage at doses of 0 (olive oil), 250, 500, 750 and 1000 mg/kg bw/d on GD 6-20 (Saillenfait et al., 2006). Endpoints included in addition were determination of the degree of transabdominal testicular migration (TTM). There were no maternal deaths. Signs of transient maternal toxicity were observed, as evidenced by reduction in body weight gain, at the beginning of treatment (GD 6-9) at 500 mg/kg bw/d and higher doses, however, overall weight gain corrected for gravid uterus was not different from controls at the end of gestation. No changes could be observed for maternal food consumption, pregnancy rate or number of implantations. The incidences of resorptions were statistically significantly increased to 28% at 750 mg/kg bw/d and to 59% at 1000 mg/kg bw/d. Mean fetal body weight was statistically significantly reduced at 500 mg/kg/d and higher doses amounting to a decrease of 24% -26% at 1000 mg/kg/d in comparison to controls. The incidence of total external malformations (neural tube closure defects, anophthalmia) and of total visceral malformations (urinary tract and vascular defects) was statistically increased at 750 and 1000 mg/kg bw/d. Skeletal evaluations revealed malformations primarily of the axial column with the incidences of fused sternebrae statistically significantly*	Saillenfait et al., 2006

	increased at 750 and 1000 mg/kg bw/d and variations (delayed ossification and supernumerary ribs) at 750 and 1000 mg/kg bw/d with supernumerary ribs in 95% of the fetuses of the 1000 mg/kg group. Visceral variations involved mainly the urinary tract with statistically significantly increased incidences of ureter variations in the 1000 mg/kg group and the male reproductive system. Unilateral or bilateral undescended testes occurred at 500 mg/kg/d and was significantly increased at 750 mg/kg/d (in 30/55 male fetuses and in 16/20 litters) and at 1000 mg/kg bw/d (in 30/34 male fetuses and in 16/17 litters). In addition the degree of transabdominal descent was significantly impaired at 500 mg/kg/d with about two third of the testes located in the upper half of the abdominal cavity at the 1000 mg/kg dose group. Thus, it appeared that alterations of the male reproductive system occurred at lower doses than those producing structural malformations/variations and embryotoxicity. No evidence of embryo or fetal effects was found at the 250 mg/kg dose level. Therefore, a NOAEL/developmental toxicity of 250 mg/kg/d can be derived from the study.	
Pregnant rats, gavage GD 12 to 21	*In a study on Sprague-Dawley rats, which was performed to determine whether in utero exposure to DIBP would induce permanent and dose-responsive alterations of male reproductive development, DIBP was administered to pregnant animals (11-13 animals per treatment group) by gavage at doses of 0 (olive oil), 125, 250, 500, and 650 mg/kg bw/d on GD 12-21 (Saillenfait et al., 2008). Doses were based on an unpublished preliminary study in which 625 mg DIBP/(kg day) on GD 12-21 caused reproductive tract malformations in male offspring and had no effects on litter size or pup survival. Litters of the definite study were examined as soon as possible after birth to determine the number of viable and stillborn pups. Pup body weights were recorded on PND 1, 4, 7, 14 and 21. AGD was measured on PND1 and litters culled to 10 pups on PND 4. All pups were examined for the presence of areola and/or nipples on the ventral surface of the thorax on PND 12-14. At weaning on PND 21 three to four male pups from each litter were randomly selected and retained and unselected pups sacrificed and submitted to internal examination. After weaning the dams were sacrificed and the number of implantations recorded from their uteri. All retained males were examined for preputial separation (PPS) and individual body weights recorded at acquisition. Adult males were necropsied on PND 76-86 (two males in each litter) or on PND 111-122 (the remaining males in each litter). They were 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. Testes, epididymides, seminal vesicles (with the coagulating glands and seminal fluid), and prostate were weighed. Histopathology was conducted on testes and epididymides of all DIBP animals necropsied on PND 76-88. No differences in maternal body weight gain were observed between the controls and the treatment groups. All dams delivered live pups. Post-DIBP implantation loss, litter size, sex ratio, and pup survival to PND 4 and PND 21 were unaffected by treatment. AGD measured on PND 1 was dose-dependently significantly reduced in male pups from 250 mg DIBP/(kg day) to the higher doses with or without adjustment for body weight. The decrease amounted to 11% at 250 mg DIBP/(kg day) and 22% at 625 mg DIBP/(kg day), compared to controls. AGD of females was not affected at any dose. Pup body weight at PND 1 of both sexes was statistically significantly decreased at 625 mg DIBP/(kg day), and remained lower in comparison to controls in the male pups at weaning. During the post weaning period mean body weights of the offspring were lower than controls at 500 and 625 mg DIBP/(kg day) (6-8% and 10- 12%, respectively). On PND 12-14 or at adult necropsy retained areolas and/or nipples were apparent in males at 250 mg DIBP/(kg day) and their incidence increased with dose. No such effects were observed in animals from vehicle controls or the 125 mg DIBP/(kg day) treated*	Saillenfait et al., 2008

	group. Acquisition of PPS was delayed by approximately 4 days at 500 mg DIBP/(kg day). Evaluation of PPS was precluded in half of the males at the high dose by presence of hypospadias. Mature males displayed severe malformations (hypospadias with exposed os penis in the more severely affected animals, and non-scrotal testis) at the two high doses. Non-descended testes were always located in the inguinal or supra-inguinal area; none were in the intra-abdominal position. Markedly underdeveloped (less than 10% of control weight) or absent testes and/or epididymes were seen in 2%, 16% (7 males from 5 litters), and 13% (5 males from 4 litters) of the animals in the 250, 500 and 625 mg /(kg day) dose groups. At sacrifice (PND 76-86, resp. PND 111-122) organ weights of the testes, epididymes, seminal vesicles and prostate were significantly reduced (with or without body weight as covariate) at 500 and 625 mg DIBP/(kg day). These reductions amounted to 39-59% for the testes and the epididymes, and 28-33% for the seminal vesicles and the prostate. Histological examinations revealed testicular damage in all DIBP treated groups with moderate or severe degeneration of seminiferous tubules (including Sertoli cell only tubules). The lesions were uni- or bilateral and associated with oligospermia or total azoospermia in the corresponding epididymides. Based on these observations a NOAEL/developmental toxicity could not be determined. Therefore, a LOAEL/developmental toxicity of 125 mg DIBP/kg bw/day can be derived from this study.	
Male rats and mice, gavage PND 21 only and PND 21 to 28	In an acute exposure study, males were exposed to DIBP at 100, 300, 500, 800, or 1,000 mg/kg in corn oil by oral gavage, and sacrificed the next day. In a subchronic exposure experiment, males were daily given DiBP at concentrations of 100, 300, 500, 800, or 1,000 mg/kg for 7 consecutive days by oral gavage, and sacrificed the day after the last administration. In a recovery experiment, male SD rats were once given 1,000 mg/kg of DIBP, and were sacrificed 1 to 8 days after administration. In all experiments, testes were excised and prepared for histological examination and staining for markers of apoptosis (TUNEL staining) and cytoskeletal changes (vimentin). In the acute exposure experiment, testis weights were not altered by DIBP in rats or mice, but the number of TUNEL positive (apoptotic) cells was increased at doses of 500 mg/kg and above in rats, but not in mice. In the subchronic exposure experiment testis weights were reduced in rats at doses of 500 mg/kg and above, and at the top dose of 1000 mg/kg in mice. The number of TUNEL positive cells were increased at doses of 500 mg/kg and above in rats, but not in mice. In the recovery experiment reduced testis weights were seen at 2 and 5 days after last dosing, but not at 6 or 8 days after dosing. The number of TUNEL positive cells was increased at 1, 2 and 5 days after last dosing, but not at 6 or 8 days after dosing. Dysorganization of the vimentin cytoskeleton was seen in DIBP exposed rats exposed for 7 days. The authors concluded that DiBP causes a significant increase of TUNEL-positive spermatogenic cells and disorder of vimentin filaments in Sertoli cells in rats and that DiBP shows species-specific toxicity.	Zhu et al., 2010

In summary, several studies have demonstrated adverse reproductive effects of DIBP and the metabolite MIBP. Repeated dose studies on DIBP and MIBP in rodents and dogs showed effects on testes (weight and histology) and spermatogenesis (Hodge 1954; Oishi and Hiraga 1980a, 1980b, 1980c; Foster et al 1981; Zhu et al 2010). Developmental studies on DIBP showed adverse reproductive effects in males including decreased ano-genital distance, increased nipple retention, testicular changes, chryptorchidism, hypospadias, delayed preputial separation and weight changes in male reproductive organs (Borch et al. 2006; Boberg et al. 2008; Howdeshell et al. 2008; Saillenfait et al. 2005; Saillenfait et al. 2006; Saillenfait et al. 2008). It is well-known that this type of effects can be induced via endocrine disrupting modes of action. Chemicals acting as androgen receptor antagonists can induce comparable effects (Wolf et al., 1999), but in the case of DIBP it is highly plausible that interference with steroid hormone synthesis in fetal testis in responsible for the anti-androgenic effects.

In conclusion, several rodent studies have demonstrated adverse effects in intact organisms on male reproductive development and adult male reproductive organs.

4.2.3 Endocrine mode of action

The studies in table 3 showed adverse effects of DIBP and/or an endocrine mode of action *in vivo*. Mode of action is defined as effects on organ/tissue/organism/physiological level. The *in vivo* mode of action data from rodent studies showed effects of DIBP on steroidogenesis, e.g. decreased testosterone production, further substantiated by mechanistic *in* vivo data showing down regulation of steroidogenesis-related genes and reduced expression of proteins related to steroid synthesis (Borch et al. 2006 and Boberg et al.2008, Hannas et al. 2011 and 2012, Howdeshell et al. 2008). Mechanistic data / data on mechanism of action are defined as effects at the cellular/sub-cellular/organelle/biochemical level (genes, receptors, enzymes etc). It is important to note that the initial events at the molecular level for DEHP and related phthalates are not known, but that there is strong weight of evidence for an anti-androgen mode of action related to decreased fetal testosterone production. These results show an endocrine mode of action of DIBP *in vivo* (Hannas et al. 2011 and 2012).

Decreased fetal testosterone production is considered a key event in a cascade leading to adverse effects in the male reproductive system, as visualized by NRC, 2008. The reduction in testosterone production will decrease the activity of the androgen receptor in target tissues and interfere with androgen-mediated development. This will in turn lead to reproductive tract malformations including effects on anogenital distance, nipple retention, reproductive system malformations and reduced semen quality (Fig. 1).

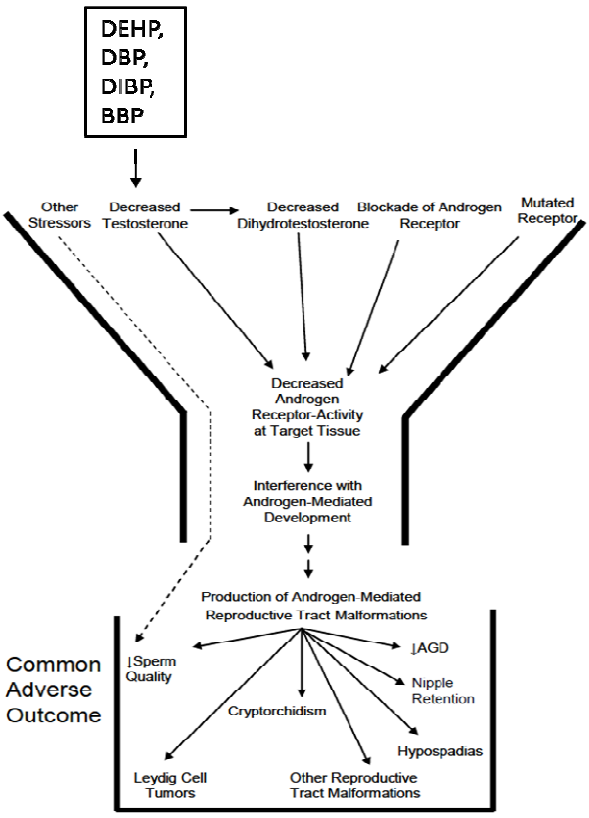

Fig.1. Modified from NRC, 2008.

This was further substantiated by *in vitro* studies. In the SVHC support document from 2009, ECHA reports "other relevant information" (citation in *italics*):

Mode of action – in vitro assays

DIBP was negative for oestrogenic activity in a yeast two-hybrid assay (Nishihara et al., 2000) and showed extremely weak oestrogenic activity in a recombinant yeast assay and in cell proliferation assays with MCF-7 and ZR-75 cells (Harris et al., 1997). In a commercial Ligand Screening Assay DIBP (up to 10-5M) had no binding affinity for the oestrogen receptor α or β in vitro (Toda et al., 2004). In a reporter gene assay DIBP was found to induce oestrogen receptor hERα-mediated oestrogenic activity (at 10-5M) and possess antiandrogenic activity in vitro but showed no activity towards hERβ in CHO-K1 cells (Takeuchi et al., 2005).

DIBP was found to interact with the androgen receptor *in vitro* showing anti-androgenic activity (Takeuchi et al. 2005). In contrast, the *in vitro* studies showed no or very weak interactions of DIBP with estrogen receptors (Nishihara et al., 2000; Harris et al., 1997; Toda et al., 2004; Takeuchi et al., 2005). These studies support the anti-androgenic activity of DIBP.

Phthalates are absorbed as monoesters and/or rapidly metabolized to monoesters and monoesters are transported across the placenta and reach the fetus (David 2006). Thus, it is the metabolites of these phthalate diesters that are endocrine disrupting and mainly effects of metabolites such as MIBP are relevant. Thus, the adverse effects of DIBP are considered to be primarily related to changes in steroidogenesis.

In conclusion, several rodent studies have demonstrated an endocrine mode of action *in vivo* which is substantiated by mechanistic data from *in vivo* and *in vitro* studies. Several of the studies showed decreased testosterone levels and decreased steroidogenic activity, indicating an anti-androgenic mode of action of DIBP. It is biologically highly plausible that the suggested anti-androgenic mode of action give rise to the adverse reproductive effects on the male reproductive system of DIBP and MIBP reported in the previous section.

4.2.4 Plausible link between adverse effects and endocrine mode of action

Altered steroidogenesis is related to adverse effects in males as well as females, and the adverse effects of DIBP on male reproductive system can be attributed to decreased testosterone levels, i.e. an anti-androgenic mode of action. Investigation of toxicological effects of DIBP in rat studies have provided convincing evidence that exposure can cause effects on the developing endocrine system as well as irreversible adverse reproductive effects. Anogenital distance and nipple retention in male pups are some of the adverse effects observed and are generally known to be androgen dependant, and decreases in anogenital distance and increases in nipple retention in males is associated with an anti-androgenic mode of action (Bowman et al. 2003; Wolf et al., 1999; Imperato-McGinley et al. 1985; Imperato-McGinley et al. 1986). Targeted studies on phthalate mode of action showed changes in steroidogenesis, including reduced testosterone production and down-regulation of genes involved in steroid synthesis (Hannas et al., 2011, Howdeshell et al., 2008; Parks et al., 2000; Wilson et al., 2004). Reduced testosterone production can in turn impair androgen signalling in androgen sensitive target organs during sensitive periods of development. Based on these findings it is highly biologically plausible that the observed adverse effects are linked to the endocrine disrupting mode of action of DIBP and the metabolite MIBP.

4.2.5 Further work substantiating the plausible link between adverse effects and endocrine mode of action

In addition to the above studies showing an endocrine disrupting mode of action of DIBP, a review paper by David, 2006, describes alternative cascades of events that could lead to the adverse health effects observed for DEHP, DBP and BBP. As both the observed *in vivo* adverse effects of DIBP are similar to those observed for DEHP, DBP and BBP and also the observed *in vivo* anti-androgenic mode of action, supported by similar mechanistic *in vivo* data, of DIBP is

similar to that observed for DEHP, DBP and BBP, it is considered biologically plausible that the mechanisms of action described by David, 2006, also are relevant for DIBP.

Path A describes how altered gene expression for cholesterol transport and steroidogenesis in Leydig cells (Lehmann et al., 2004, Schultz et al., 2001, Barlow et al., 2003, Lee et al., 2004; Liu et al., 2005) can lead to decreased cholesterol transport (Schultz et al., 2001, Gazouli et al., 2002, Barlow et al., 2003) and subsequent decreased T synthesis (Bell et al., 1978, Foster et al., 1983, Parks et al., 2000, Akingbemi et al., 2001; Zhu et al., 2005). In turn, this can lead to the adverse health effects of hypospadias and underdeveloped secondary sex organs (Wine et al., 1997, Mylchreest et al., 1998, 1999, 2000, Gray et al., 1999, 2000, Parks et al., 2000).

Path B describes how altered gene expression of insl3 protein in Leydig cells (Lehmann et al., 2004; Liu et al., 2005) can lead to decreased levels of insl3 (Wilson et al., 2004; Liu et al., 2005) and failure of gubernacular ligament to develop (Nef and Parada, 1999). In turn, this can lead to the adverse health effect of cryptorchidism (Gray et al., 1999, 2000, Parks et al., 2000).

Path C describes effects on Sertoli cells and gonocytes including presence of multinucleated gonocytes in the seminiferous tubules. Influences on Sertoli cells are not clear but include decreased expression of cyclin D2 in neonatal Sertoli cells, decreased gene expression for cell junctions, decrease in Sertoli cell proliferation, interference with cytoskeleton, decreased intercellular communication, and inhibition of gap junctional intercellular communication (Liu et al., 2005, Li and Kim, 2003, Li et al., 1998, 2000, Kleymenova et al. 2005, Yu et al., 2005, Kang et al 2002). Additionally, decreased T production in Leydig cells may lead to inhibition of Sertoli cell numbers (Atanassova et al., 2005). Gonocyte effects may be related to Sertoli cell changes, but this has not been clarified.

Investigation of toxicological effects of these phthalates in rat studies have provided clear evidence that exposure can cause changes in the developing endocrine system as well as permanent adverse reproductive effects. Although several target genes involved in the development and function of fetal Leydig cells, germ cells and Sertoli cells have been identified so far, the mechanism by which phthalates alter the expression of these genes is currently unknown. The three pathways described above provide biological plausibility that a causal relationship exists between exposures and the observed altered function of the developing endocrine system and the adverse reproductive effects observed in rodent studies.

Overall, it is highly bioligcally plausible that the described adverse effects on the male reproductive system are induced through an endocrine disrupting mode of action mainly related to altered steroidogenesis following exposure to DIBP.

4.2.6 Human relevance

Human relevance of the experimental data will be addressed also using read across to other phthalates when relevant, as data on human relevance of the effects of DIBP are sparse. This approach is considered justified, as many similarities have been found between phthalate esters containing a straight-chain backbone of approximately 4-6 carbons. DEHP is branched with straight C6 backbones. DBP is linear with straight C4 backbones. BBP has one benzyl side chain and a straight C4 backbone. DBP and BBP share the same metabolite, mono-butyl phthalate. DIBP is the branched isoform of DBP with straight C3 backbones. For these phthalates many similarities also have been found between (1) adverse effects in endocrine related organs, (2) in *in vivo* endocrine modes of action and (3) a plausible link between the adverse effects and the modes of action.

For example, several studies have shown similar adverse effects and endocrine mode of action for phthalates containing a straight chain backbone of 3-7 carbons. Adverse effects on reproductive organs, genital development and nipple retention were observed in males exposed to DEHP, BBP, DINP or DBP (Gray et al. 1999; Gray et al. 2000). Moreover, DEHP,

DBP, BBP, DPP, DIBP and DINP reduced testosterone production, indicating an anti-androgenic mode of action of these phthalates (Borch et al. 2004; Howdeshell et al. 2008; Liu et al. 2005). As the adverse effects of the phthalates plausibly are linked to their anti-androgenic mode of action, a read-across between phthalates is considered relevant, for example when evaluating human relevance.

Due to recent studies showing differences in male reproductive effects of these phthalates between different species (rats, mice and marmosets), the issue of human relevance has been debated. Current knowledge indicates that phthalate induced effects on fetal testosterone production are not consistently found in mice, marmoset or human testis (ex vivo), but that changes in germ cell development can be induced by phthalates in different species.

Several studies are indicative of species differences in the reproductive effects of phthalates. In a study by Tomonari et al. (2006), no reproductive effects were seen in male marmosets (n=5-6 per dose group) exposed to DEHP by oral gavage at 100, 500 and 2500 mg/kg bw/day from 3 months of age until sexual maturity (18 months). Similarly, no reproductive effects were seen in a study by Kurata et al., 1998, in which male marmosets (n=4 per dose group) were dosed with 100, 500 and 2500 mg/kg bw/day of DEHP during 12-15 months of age. However, in another study on 4-day-old marmosets (5 co-twins and 4 non-twins, total n=14) treated for 14 days with 500 mg/kg bw/day of MBP, an increased Leydig cell volume was observed (Hallmark et al., 2007). A second study from the same authors revealed suppressed blood testosterone levels in male marmosets (n = 9) exposed at 2-7 days of age to a single dose of 500 mg/kg bw/day of MBP (measurement 5h after dose). In 4 day old co-twin marmosets (5 co-twins, n=10) were exposed to MBP neonatally during 14 days, and no effects on germ cell number or differentiation were apparent (McKinnell et al., 2009). It has been argued that the critical programming window for reproductive effects in marmosets is exposure during week 7 to 15 of gestation, but MBP did not alter the male reproductive system in the one study using this exposure period (McKinnell et al., 2009). In that study, no effects on testicular morphology, reproductive tract, testosterone levels at birth, germ cell number nor germ cell proliferation were observed in male offspring (n=6) of pregnant marmosets exposed to 500 mg/kg bw/day MBP from GD 49-105 (McKinnell et al., 2009). However, unusual clusters of undifferentiated germ cells were found in two of six males examined at birth, and the biological significance of this observation is unclear. Overall, data from marmoset studies are weakened by a low number of animals, and results appear to depend on the timing of exposure.

In mice it has proved difficult to find comparable effects of phthalates on testosterone production to those seen in rats. A study in fetal mice exposed to DBP did reveal changes in several immediate genes, but no decreases were observed in testosterone levels or in genes related to cholesterol homeostasis or steroidogenesis as would be expected for rats (Gaido et al., 2007). The study in fetal DBP-exposed mice showing no influence on steroidogenesis did reveal comparable changes in germ cells to those seen in fetal rats, i.e. increased seminiferous cord diameter, and increased numbers of multinucleated gonocytes (Gaido et al., 2007). *In vitro* studies on cultured rat, but not human, fetal testes have shown the ability of phthalates to reduce testosterone production, indicating species differences in sensitivity to the testosterone suppressing effect of phthalates (Hallmark et al., 2007; Lambrot et al., 2009, Chauvigné et al., 2009). In these *in vitro* studies human testis samples were from first or second trimester fetuses, but it is not clear whether these ages correspond to the sensitive window for phthalate exposure in rats (Lambrot et al., 2009, Hallmark et al., 2007). Data from *in vitro* studies are not consistent, as an vitro study on adult human testes has shown that exposure to DEHP and MEHP impaired testosterone production, and that the measured concentrations of phthalate metabolites in the incubated testes were as low as the phthalate metabolite levels measured in humans (Desdoits-Lethimonier et al., 2012).

In contrast to the possible differences seen between species regarding phthalate-induced changes in testosterone production, there appears to be similarities between rats, mice, marmosets and humans regarding influence of phthalate exposure on germ cell proliferation and differentiation. *In vitro* studies on phthalate exposure of fetal testis tissue have been able

to show comparable changes in germ cells whether using testes from rats, mice or humans (Lambrot et al., 2009, Lehraiki et al., 2009, Chauvigné et al., 2009, Habert et al., 2009). This clearly supports that reproductive effects of phthalates are relevant to humans.

Another experimental model has been applied for species comparisons, i.e. transplantation of testicular tissue from fetal rats or humans to a (transgenic) castrated mouse. A study using this model was able to demonstrate a testosterone inhibiting effect of DBP when using rat fetal testis explants, but not when using human fetal testis explants (Mitchell et al., 2012). However, there were several differences in study design between the fetal rat testis graft and the fetal human testis graft study, including duration of grafting before exposure and timing of exposure and age of the testis explant at the time of exposure. In the fetal human graft study, mice were supplied with hCG to promote testosterone production, whereas no LH (luteinizing hormone) stimulation was necessary for the rat graft to produce testosterone, and absolute testosterone levels therefore greatly differed in the two experimental setups (Mitchell et al., 2012). The differences in study design between the fetal rat testis graft study and the fetal human testis graft study thus complicate conclusions, and no firm conclusions regarding human relevance can be made on the basis of this study.

Another recent study comparing phthalate effects on rat, mouse and human testis in xenotransplant studies revealed similar effects as those described by Mitchell et al 2012 (Heger et al., 2012). Fetal testis xenotransplant studies revealed that effects on steroidogenic gene expression and ex vivo testosterone production were only seen with fetal rat testis, whereas multinuclear gonocytes were seen with rat, mouse and human fetal testis tissue (Heger et al 2012). Another study on fetal human testis xenografts showed that DBP did not affect testosterone levels or weights of androgen-sensitive host organs, whereas a CYP17A1 inhibitor, abiraterone acetate, did (Spade et al., 2013). DBP increased the number of multinucleated germ cells and altered the expression of oxidative stress response genes and actin cytoskeleton genes (Spade et al., 2013). These gene expression changes may reflect possible mechanistic targets that are suggested as subjects for further studies. Changes in the seminiferous chords may be important to germ cell development and may be related to persistent effects on testes as seen in the testicular dysgenesis syndrome (Toppari et al., 2010).

Human epidemiological studies are difficult to interpret due to the effects being delayed relative to the time of exposure. Interestingly, a study comparing phthalate exposure in mother's milk and testosterone levels in their infant sons revealed correlations between exposure to certain phthalate monoesters and the ratio of LH to testosterone (Main et al., 2006). This is in good agreement with the marmoset study showing that neonatal phthalate exposure impaired testosterone production and induced testicular effects characteristic for high LH levels (Hallmark et al., 2007), and may indicate that the neonatal period may be a sensitive window of exposure for humans/primates. As described by Welsh et al., 2008, testosterone levels peak in late gestation in rats, but earlier (week 14-18) in humans, and this coincides with important periods of differentiation of reproductive organs. However, reproductive development continues postnatally in humans and may also be sensitive to exposure to endocrine disrupting compounds during early development (den Hond and Schoeters, 2006, Jacobson-Dickman and Lee, 2009).

In a recent review, data on phthalate toxicity to the fetal rat testis were compared with data from studies using mice or human testicular tissue (Johnson et al., 2012). The overall conclusions were that species-specific differences in testicular response following in utero phthalate exposure between mice and rats were observed, and that the response of human fetal testis to phthalate exposure may be more comparable to the response of a mouse than a rat. This review recognized two different pathways of phthalate effect on the fetal testes, namely a) suppression of steroidogenic gene expression and suppressed testosterone secretion and b) increase in multinucleated gonocyte number. A better understanding of molecular mechanisms responsible for the differences in sensitivity or resistance to developmental phthalate exposure and more insight into the molecular pathways controlling steroidogenesis in the human fetal testis is warranted. In relation to risk assessment Johnson et al. (2012)

conclude that "molecular mechanistic understanding will be needed for risk assessment to progress beyond the default protective assumption that humans respond similarly to the most sensitive species".

A recent publication provides a critical assessment of in vivo and in vitro studies exploring phthalate effects in humans (Albert and Jegou 2014). This paper highlights the variation among species in the window of susceptibility to the effects of phthalates and variation among species in timing of the development of the testis. Another conclusion of this literature study is that the indications of species differences found in e.g xenografting studies have methodological limitations and that "Caution before concluding that phthalates are innocuous in the human feta testis should be kept until these issues have been addressed" (Albert and Jegou 2014).

In their assessment of this Background document to the Opinion on the Annex XV dossier proposing restrictions on four phthalates (ECHA 2012), ECHA's Risk Assessment Committee (RAC) concluded regarding human relevance of reproductive effects of these four phthalates: "For marmosets, however, limited data are available for in utero, peri- and neonatal exposure. There is no study with exposure during the entire life cycle such as the multigeneration studies in rats. In fact, there is only one developmental toxicity study (using a single high dose of MBP) with a period of exposure that covers the sensitive window for the programming of the male reproductive system, demonstrating some effects on the testes of neonatal marmosets of which the toxicological significance is unclear. This, combined with the relatively low number of (non-inbred) animals tested in the marmoset studies, makes it difficult to compare the results with those found in (inbred) rats. All in all, RAC concluded that there is too much uncertainty in the data available to allow a conclusion on humans being less, equally or more sensitive than rats and thus suggested not to deviate from the default interspecies factor of 10" .

Overall, there are clear indications of species differences in metabolism (Kurata 2012a, 2012b) and possibly in effects on fetal steroidogenesis, but there are also important differences in timing and duration of exposure in the experimental studies showing these species differences. Thus, the current knowledge on species differences is not sufficient to disregard the human relevance of phthalate effects. There are clear indications that changes in germ cell development can be induced by phthalates in several species including rats, mice, marmosets and xenotransplanted human fetal testis tissue. The implications or importance of these germ cell changes on long term effects on male reproduction are not fully elucidated, but it is evident from the current knowledge on the human testicular dysgenesis syndrome that early changes in the seminiferous chords may be important to germ cell development and related to persistent effects on testes (Toppari et al., 2010).

In conclusion, DIBP was classified as toxic to reproduction based on the evidence of adverse effects on the reproductive organs in rats, which are attributed to an anti-androgenic mode of action. It is assumed that these effects are relevant to humans, as no data demonstrate non-relevance.

4.2.7 Summary – Human health

Based on the definition of endocrine disrupters by WHO/IPCS in 2002 and the recommendation from the Endocrine Disrupter Expert Advisory Group in 2013, the following four topics are covered to clarify how DEHP fulfills the definition of being an endocrine disrupter:

1) Adverse health effects
2) Mode of action
3) Causality / plausible link between adverse effects and mode of action
4) Human relevance of experimental data

The SVHC support document from 2009 (ECHA 2009) acknowledges that "Although phthalates do not act as classical antiandrogenic chemicals by binding to the androgen receptor, they

obviously have the same effects of blocking androgen-action at the target tissue and therefore may be considered as acting antiandrogenic" and that based on the studies available at the time "The structures affected by *in utero* exposure to DIBP are indicative of an antiandrogenic mode of action". These studies available in 2009 and more recent studies confirm this hypothesis.

Rodent studies on DIBP and the metabolite MIBP have demonstrated adverse reproductive effects in male reproductive organs, such as testicular changes, chryptorchidism, hypospadias, decreased spermatogenesis and decreased ano-genital distance and nipple retention, and it is considered as highly plausible that these effects are induced by an endocrine mode of action of DIBP. Further, studies also showed decreased levels of testosterone and other effects on steroidogenesis such as e.g. down-regulation of steroidogenesis-related genes, confirming an endocrine disrupting mode of action of DIBP. There is convincing evidence of a biologically plausible link between the adverse effects observed in males and the anti-androgenic mode of action of DIBP and MIBP.

The anti-androgenic related effects of DIBP that are suspected to be relevant in humans are congenital malformations of the male reproductive organs, reduced semen quality and reduced male reproductive hormone levels. It has been hypothesized that these disorders may comprise a testicular dysgenesis syndrome with a common origin in fetal life. Testicular cancer may also be part of this syndrome.

In conclusion, DIBP is classified as toxic to reproduction based on evidence of adverse effects on the reproductive organs in adult and developing male rodents, and these adverse effects are attributed to the anti-androgenic mode of action of DIBP. Thus, DIBP is considered as an endocrine disrupter that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism and its progeny.

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Other effects – Endocrine Disruption

5.1.1 General approach - Environment

To clarify how DIBP fulfil the definition of being endocrine disrupters, the topics described in chapter 4 will be covered in relation to the environment.

Endocrine disruptive effects in mammalian species are analysed in chapter 4 and will not be repeated here. It is important to emphasize that the results on mammalian species are generally considered of direct relevance for mammalian wildlife, especially to wildlife species with low reproductive output including top predators, primates and other larger mammals (including endangered species), because any negative effect on development or reproduction has a high likelihood of leading to serious effects at the population level for such species. In addition, in relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular regulatory relevance because such effects are likely to manifest themselves at the population level.

However, cross-species extrapolation seems relevant, even if apical responses vary across phyla, since the key molecular initiating events (in this case inhibition of enzymes involved in steroid synthesis) are similar between species even though sensitivity to adverse effects have been observed, e.g. between rats and fish (Ankley and Gray (2013)). In addition to cross species extrapolation, as mentioned above in the start of section 4.2.6., DIBP has a strong structural similarity of main features of the molecule to other phthalates (such as DBP and DEHP) with a more extensive experimental data, including data in fish, concerning endocrine

activity and adverse effects. Hence read across for hazard identification of the endocrine disruptive properties between DIBP and DBP and DEHP seems appropriate.

As described for human health, in this report it is assumed that a substance should fulfil the recommendations from the European Commission's Endocrine Disrupters Expert Advisory group in order to be identified as an endocrine disruptor, and the available information is assessed based on the following topics:

1) Adverse effects

2) Endocrine mode of action

3) Plausible link between adverse effects and endocrine mode of action

For considering endocrine disrupting effects in the environment, data from both terrestrial and aquatic species should be analysed. This is in conformity with the agreement of the European Commission's Endocrine Disrupters Expert Advisory group that "In relation to ecotoxicology, data on all species, including mammalian data generated to assess human toxicity, are generally considered relevant for the assessment of effects on ecosystems. In addition, since ecotoxicological assessment relates to impact at the population level rather than the individual level, relevance is applied in the context of identified adverse effects being relevant for the population" (JRC 2013).

Hence the fourth issue that should be considered as regards endocrine disrupters in relation to the environment is – not as for human health, human relevance – but rather environmental relevance, i.e. whether the adverse effects observed are also likely to cause effects at the population level.

Generally in regulatory ecotoxicology effects on survival, growth, but in particular development and reproduction are considered relevant endpoints for effects on populations and as such these endpoints are used to derive regulatory hazard and risk assessment decisions. It is noted that effects after longer time exposure relating to development and reproduction are generally preferred types of data for such decision.

Hence, the reproductive effects of DIBP on mammals are of regulatory relevance for the environment.

5.1.2 Effects in the aquatic compartment (including sediment)

5.1.2.1 Fish and amphibians

No studies including relevant endocrine endpoints could be found for DIBP. Only one 96 h acute toxicity tests have been performed with fish. A study where fathead minnow (*Pimephales promelas*) were exposed for 96 h and a LC50 value of 0.9 mg/l was reported (Geiger et al. (1985).

5.1.2.2 Aquatic invertebrates

5.1.2.2.1 Long-term toxicity to aquatic invertebrates

No long term tests could be found

5.1.3 Adverse environmental effects related to endocrine disruption

No experimental data are available for DIBP to conclude on non-mammalian adverse effects. However read across for hazard identification of the endocrine disruptive properties from DBP and DEHP seems appropriate

5.1.4 Endocrine mode of action

No experimental data are available for DIBP. However read across for hazard identification of the endocrine disruptive properties from DBP and DEHP seems appropriate.

5.1.5 Plausible link between adverse effects and endocrine mode of action

No experimental data on DIBP available. However read across for hazard identification of the endocrine disruptive properties from DBP and DEHP seems appropriate.

5.1.6 Summary - Environment

Mammals: The 3 first topics for identifying endocrine disrupters including the severity of the observed adverse effects of DIBP on rodents (impact on development and reproduction) as presented in chapter 4 are generally accepted as endpoints of concern for mammalian wildlife and as such accepted for reaching conclusions in regulatory hazard (and risk) assessment. Furthermore developmental and reproductive effects such as those of DIBP are particular concern in relation to mammalian wildlife including top predator species, primates and large mammals (inclusive endangered species), where the described reproductive effects are expected to cause serious effects at population level because of a natural low reproductive output of such taxa.

Fish and amphibians: No experimental studies including relevant endocrine endpoints could be found for DIBP. Only one 96 h acute toxicity tests have been performed with fish. A study where fathead minnow (*Pimephales promelas*) were exposed for 96 h and a LC50 value of 0.9 mg/l was reported (Geiger et al. (1985).

However, as mentioned above, cross-species extrapolation for hazard identification of endocrine disruptive properties, seems relevant, e.g. between rodents and fish, even though apical responses vary across phyla, since the key molecular initiating events (in this case inhibition of enzymes involved in steroid synthesis) are similar between species even though some differences in sensitivity to adverse effects have been observed, e.g. between rodents and fish (Ankley and Gray (2013)).

In addition read across between structural analogs for hazard identification of the endocrine disruptive properties of DIBP from other phthalates, such as DEHP and in particular DBP, with sufficient experimental data in fish and rodents supports that DIBP has endocrine properties for which there is evidence of probable serious effects to wildlife species.

6 CONCLUSIONS ON THE SVHC PROPERTIES

6.1 Conclusion on fulfilment of WHO definition of endocrine disruptor

A summary of the findings in chapters 4 and 5 are compared with the definition of an endocrine disrupter as given by WHO/IPCS, and as further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group (JRC 2013) on elements for identification of an endocrine disrupter.

According to the widely accepted definition of an endocrine disruptor by the WHO/IPCS (WHO/International Programme on Chemical Safety 2002), an "*endocrine disruptor is an exogenous substance or mixture that*

1) alters function(s) of the endocrine system and 2) consequently causes 3) adverse health effects in an intact organism, or its progeny, or (sub)populations."

This has been further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group that has recommended that for a substance to be identified as an endocrine disruptor, available information should be assessed as regards the following topics:

1) Adverse effects
2) Endocrine mode of action
3) Plausible link between adverse effects and endocrine mode of action
4) Human relevance (for human health only)

In relation to effects on wildlife (the environment) the above mentioned topic 4) human relevance should be replaced with "environmental relevance" (see section "5.1.1 General approach – Environment").

Re 1)

The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, effects on spermatogenesis and testicular changes including weight changes and changes in Leydig an Sertoli cells. DIBP causes adverse –and serious – reproductive toxicity effects in rodents and a harmonized classification Rep. 1 B has been concluded.

For the environment, no experimental data for DIBP is available in the open literature for non-mammalian wildlife species.

Re 2) DIBP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway. For the environment, no experimental data for DIBP is available in the open literature for non-mammalian wildlife species.

Re 3) The link between adverse effects and the endocrine mode of action of DIBP has been concluded in numerous investigations in rodents (mode of action on the steroidogenic biosynthesis pathway) It is considered biologically highly plausible that the observed adverse effects in rats are linked to the endocrine disrupting mode of action of DIBP. For the environment, no experimental data for DIBP is available in the open literature for non-mammalian wildlife species.

Re 4) DIBP causes serious adverse reproductive toxicity effects in rodents and based on an assessment of human relevance using also other available information, a harmonized classification Repr. 1B has been concluded. Hence it can be concluded that human relevance has been agreed for the adverse effects. For the environment, no experimental data for DIBP

is available in the open literature for non-mammalian wildlife species. Cross-species extrapolation of chemical effects seems relevant, even if apical responses vary across phyla, since the key molecular initiating events (in this case inhibition of enzymes involved in steroid synthesis) are conserved between species. In addition, read across between structural analogues for hazard identification of the endocrine disruptive properties of BBP from other phthalates with similar main metabolites, such as DEHP and DBP, and for which sufficient experimental data in fish and rodents is available, supports that DIBP has endocrine properties for which there is evidence of probable serious effects to wildlife species.

In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction has an even higher potential for causing long term negative effect at the population level for such taxa.

In conclusion, when available information from toxicological and ecotoxicological studies are combined, DIBP can be considered an endocrine disruptor for both the environment and for human health as it fulfils the WHO/IPCS definition of an endocrine disruptor, the recommendations from the European Commission's Endocrine Disrupters Expert Advisory Group for a substance to be identified as an endocrine disruptor

6.2 Conclusion on fulfilment of Article 57(f)

Article 57(f) states that: "substances – such as those having endocrine disrupting properties or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, which do not fulfil the criteria of points (d) or (e) – for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) and which are identified on a case-by-case basis in accordance with the procedure set out in Article 59."

In order to conclude on whether DIBP, in addition to fulfilling the definition of an endocrine disrupter as given by WHO/IPCS and further elaborated by the European Commission's Endocrine Disrupters Expert Advisory Group (JRC 2013), also fulfils Article 57(f), the following should be considered:

For substances with endocrine disrupting properties, the scientific interpretation of Article 57(f) for regulatory implementation can be linked to the WHO/IPCS definition. Thus,

(i) "endocrine disrupting properties" (REACH art. 57.f) is another way of formulating "altering the functions of the endocrine system" (WHO definition),

(ii) "scientific evidence of probable serious effects" (art. 57 f.) is another way to explain the cause-effect relationship (WHO definition)_between the endocrine activity and the adverse effects,

(iii) "equivalent level of concern" is another way to describe that the adverse effects (of endocrine disruptors, WHO definition) are serious at the same level as CMRs or PBT/vPvBs. (ELoC according to REACH art. 57 f)

The differences between REACH Art. 57f as regards substances with ED properties of ELoC and the WHO definition of endocrine disrupters are:

a) The requirement for proving a causal relationship between the ED MOA and adverse/ serious effects is seemingly a bit higher according to the WHO definition, because this definition (rather unreflectively) uses the term "causes" whereas REACH art. 57 f expresses that the linkage between ED properties and serious effects should be "probable" (realizing that making a definitive causal proof may in reality not be possible

even though the evidence is strong and also realizing that "waiting for the ultimate proof" may not be appropriate as basis for regulation).

b) The seriousness of the adverse effects is expressed slightly differently in the WHO definition and in the art. 57 f (ELoC part). This is probably due to the fact that the scope for the WHO definition is more narrow focussing on only endocrine disrupters ("causing adverse health effects in an intact organism, or its progeny, or (sub)populations.") whereas the scope of art. 57 f concerns both endocrine disrupters and in addition other types of serious chemical properties raising ELoC as those of CMRs or vPvB/PBTs.

Human health

With regard to assessing whether DIBP, which fulfils the WHO/IPCS definition of an endocrine disruptor for human health, also fulfils Article 57(f) the following elements are considered:

Re. endocrine disrupting properties

DIBP has systematically been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway.

Re. scientific evidence

Altered steroidogenesis is related to adverse effects in males as well as in females, and the adverse effects of DIBP may be attributed to decreased testosterone levels, i.e. an anti-androgenic mode of action. Consistent findings in rats provide convincing evidence that exposure can cause irreversible adverse reproductive effects. It is biologically highly plausible that the observed adverse effects are linked to/caused by the endocrine disrupting mode of action of DIBP

Re. probable serious effects

DIBP systematically has shown a wide spectrum of adverse effects observed in rats which include increased nipple retention, decreased anogenital distance, genital malformations, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. These development/reproductive toxicity effects have led to the harmonized classification Repr. 1B

Re. equivalent level of concern

The observed serious developmental/reproductive toxic effects are of an equivalent level of concern to substances classified with CMR Cat 1 because they have led to the harmonized classification Repr. 1B (i.e a CMR classification to which Art. 57f directly refers). In addition, the seriousness of the reproductive effects concerned can be characterized in the following way:

- Potential severity of health effects: DIBP adversely affects the normal development and the reproductive ability Irreversibility of health effects: the adverse effects concerned such as reduced ability to produce semen (Leydig cell hyperplasis) or a malformed reproductive system are irreversible / long lasting reproductive changes.
- Delay of effects: There is a long latency period between early impacts and occurrence of the adverse effects. Impacts during early development which adversely affects reproductive ability such as reduced number of spermatocytes, testicular changes, tubular atrophy and organ malformations or mis-function, will not manifest themselves fully until reproductive age.

- Quality of life: A reduced ability to reproduce considerably affects the quality of life negatively for the individuals affected as well as for their partners and families. Reduced fertility is of general concern in the EU countries.
- Negative impact on society: A reduced ability to reproduce negatively affects the society as it contributes to a significant increased financial burden on the health care sector, both providing assisted fertilisation treatments and clinical treatment for individuals with adverse reproductive effects post-natally. In addition, the fertility rate in many EU countries (including in Denmark) is decreasing.
- No toxicological threshold for the endocrine disruption caused reproductive toxic effects has yet been scientifically proposed, discussed and concluded and/or agreed for DIBP.

Environment

When assessing whether DIBP, which fulfils the WHO/IPCS definition of an endocrine disruptor for the environment, also fulfils Article 57(f), the following elements are considered:

Re. endocrine disrupting properties

For the environment, no experimental data for DIBP is available in the open literature for non-mammalian wildlife species.

Ad (ii) *scientific evidence*

For the environment, no experimental data for DIBP is available in the open literature for non-mammalian wildlife species.

Re. probable serious effects

Cross-species extrapolation for hazard identification of endocrine disruptive properties seems relevant until further information is available for non-mammalian wildlife, e.g. between rodents and fish, since the key molecular initiating events (in this case inhibition of enzymes involved in steroid synthesis) are similar between species (even though apical responses vary across phyla and some differences in sensitivity to adverse effects have been observed. In addition, read across between structural analogues for hazard identification of the endocrine disruptive properties of DIBP from other phthalates with similar main metabolites, such as DEHP and DBP, and for which sufficient experimental data in fish and rodents is available, supports that DIBP has endocrine properties for which there is evidence of probable serious effects to wildlife species.

Re. equivalent level of concern

- *Potential severity of ecotoxicological effects* – the adverse effects of DIBP on mammalian wildlife adversely affect the reproductive ability which is considered an adverse effect on the population level. DIBP also as mentioned above causes developmental and reproductive toxicity effects in laboratory rat, which due to the general conservation of hormone systems between different mammalian species is also an appropriate animal model for mammalian wildlife species.
- *Irreversibility of effects:* Endocrine modulation is a very complex feedback process that is set up during critical early life stages in non-mammalian and mammalian species. Significantt effects on the population will not be able to maintain its size or may go through "a genetic bottle neck" reducing its natural genetic variability and thereby potentially diminishing the adaptation of the population to environmental changes. In mammalian wildlife adverse effects such as reduced ability to produce semen (Leydig cell hyperplasis) or malformed reproductive systems are irreversible reproductive changes. Further, exposure during development which adversely affects reproductive ability such as reduced number of spermatocytes, testicular changes, tubular atrophy

and organ malformations or mis-function will not manifest themselves until reproductive age.

- *Broad environmental relevance*: Effects on reproductive ability has a broad environmental relevance. Due to the conservatism of receptors in the endocrine system it is very likely that a wide range of wildlife species with different functions in the ecosystems could be affected. Read across between structural analogues for hazard identification of the endocrine disruptive properties of DIBP from other phthalates with similar main metabolites, such as DEHP and DBP, and for which sufficient experimental data in fish and rodents is available, seems appropriate until further investigations are available on the effects of DIBP on non-mammalian wildlife. Further, the severity of effects of DIBP on rodents are of particular concern in relation to mammalian wildlife including top predator species and other mammals (inclusive endangered species), where the described reproductive effects are expected to cause serious effects at population level because of a natural low reproductive output of such taxa.

- Finally. no toxicological threshold for the endocrine disruption caused reproductive toxic effects has yet been scientifically proposed, discussed and concluded and/or agreed for DIBP.

6.3 Conclusion

Diisobutyl phthalate (DIBP) should be identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to human health and the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

DIBP has been shown to adversely affect the endocrine system of mammals primarily through *in vivo* findings on reduced fetal testosterone. These findings are further substantiated by mechanistic findings, also *in vivo*, of down-regulation of genes in the steroidogenic biosynthesis pathway. The spectrum of adverse effects observed in rats include increased nipple retention, decreased anogenital distance, genital malformations, effects on spermatogenesis and testicular changes including weight changes and changes in Leydig an Sertoli cells.

In relation to the environment, adverse effects concerning development and reproduction are generally regarded as endpoints of particular relevance because such effects are likely to manifest themselves at the population level. The effects observed in rats are of particular concern for wildlife species with a natural low reproductive output, including top predators and other mammals (including endangered species) as negative effects on reproduction has an even higher potential for causing long term negative effect at the population level for such taxa.

Adverse effects caused by exposure to DIBP has not been observed in non-mammalian wildlife as no fish, amphibian or invertebrate studies including endocrine relevant endpoints have been found for DIBP. However, cross-species extrapolation for hazard identification of endocrine disruptive properties seems relevant, e.g. between rodents and fish, since the key molecular initiating events (in this case inhibition of enzymes involved in steroid synthesis) are similar between species (even though apical responses vary across phyla and some differences in sensitivity to adverse effects have been observed). In addition, read across between structural analogues for hazard identification of the endocrine disruptive properties of DIBP from other phthalates with similar main metabolites, such as DEHP and DBP where experimental data in fish and rodents provide evidence of probable serious effects to wildlife species, seems appropriate until further investigations are available on the effects of DIBP on non-mammalian wildlife.

In conclusion, when available information is evaluated, DIBP can be considered an endocrine disruptor for both the environment and for human health as it fulfils the WHO/IPCS definition

of an endocrine disruptor and the recommendations from the European Commission's Endocrine Disrupters Expert Advisory Group for a substance to be identified as an endocrine disruptor.

DIBP should be considered as a substance giving rise to an equivalent level of concern because scientific evidence shows that exposure during sensitive time windows of development may cause irreversible developmental programming effects leading to severe effects on development and reproduction, regarded as particularly serious in relation to human health and wildlife species, also because these adverse effects may first manifest themselves in later life stages as a consequence of exposure during early life stages. Adverse effects on development and reproduction are in addition generally regarded as endpoints of concern, and as such frequently used for regulatory hazard and risk assessment both for human health and for environmental species.

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Annex 1 - Studies included in the SVHC support document for DIBP (ECHA 2009)

Study design	Effects	Reference
Male and female rats, 4 months feeding	*In albino rats (strain unknown), a feeding study over a period of four months is reported by Hodge (1954). Body weights and haematological parameters were measured. Organ weights were determined at autopsy. Livers and kidneys were examined histologically. Groups of rats (5/sex/group) were fed 0, 0.1, 1.0 and 5.0% DIBP in the diet. These dose levels were equivalent to 0 or to about 70, 700 and 3500 mg/kg bw/d in both sexes (calculated on an assumed daily food intake of 7% of the body weight). Retarded growth was observed at dosages of 1.0% and above DIBP in feed. Significantly decreased body weights were observed in both sexes at 5.0% (decrease up to 43% for males and 13% for females). The intake of 5.0% DIBP caused slight reduction in red blood cell counts in males and in haemoglobin values in both sexes. Both absolute and relative testes weights were considerably reduced in the 5.0% group. No statistical analyses were conducted but reductions were noted to approximately 30% and 50% of control values respectively. Absolute and relative liver weights were raised in the 5.0% groups of both sexes. For males, absolute weights were increased by 5%; relative weights by 80%. For females, absolute weights were increased by 40%; relative weights by 60%. Pathological examinations of liver and kidney were unremarkable.*	Hodge 1954
One male and one female dog, two months feeding	*Hodge (1954) also reported on a feeding study in dogs. One male and one female dog (species unknown) were fed with DIBP via diet at a daily rate of 0.1 ml/kg feed and 2.0 ml/kg feed respectively (equivalent to about 2.6 mg/kg bw/d and 51.9 mg/kg bw/d, calculated on an assumed daily food intake of 25 g/kg bw) for a period of two months. Weight loss was noted in the female dog at the last three treatment weeks. No abnormality was detected in the haematological and urine analyses as well as in gross pathology in both sexes. Organ weight assessment revealed an increase in relative liver weight compared to historical controls in female dog, no histological abnormalities in the liver were observed. In the male dog given 2.6 mg/kg bw/d DIBP, histological examination revealed abnormally few matured sperm in the testes.*	Hodge 1954
Male rats, one week feeding	*Feeding a diet containing 2.0% (approximately 1500 mg/kg bw/d, calculated on an assumed daily food intake of 7% of the mean body weight of 108 g) of DIBP to 10 male rats (JCL: Wistar, 5 weeks old) resulted in significantly decreased zinc concentrations in the testes and liver. Testosterone concentrations in the testes were increased but appeared normal in the serum. The testes of DIBP-treated rats were reduced in size when compared to controls, and organ weight assessment revealed significantly ($p<0.05$) decreased absolute and relative testicular weights in these rats. Microscopy indicated marked inhibition of spermatogenesis and desquamation of spermatocytes (Oishi and Hiraga, 1980c).*	Oishi and Hiraga 1980c
Male mice, one week feeding	*In a comparable study in male mice zinc and testosterone concentrations in tissues were determined, and body and organ weights of testes, liver and kidneys were evaluated, however microscopy was not performed. Administration of 2.0% (approximately 2000 mg/kg bw/d, calculated on an assumed daily food intake of 10% of the body weight) of DIBP in the diet to 10 young male mice (JCL: ICR) revealed significantly decreased zinc concentrations in the testes. The concentration of testosterone in the testes of DIBP-treated mice was not different from control values. The relative weights of the testes and liver of DIBP-treated mice were significantly higher, but the absolute testis weight was not different from control values (Oishi and Hiraga, 1980b). The purpose of studies with MIBP was to discover whether phthalic acid monoesters including their metabolites have similar effects to their diesters regarding effects on the testes and alterations in zinc and testosterone concentrations.*	Oishi and Hiraga, 1980b

Study design	Effects	Reference
Male rats, one week feeding	*Administration of 2.0% MIBP (corresponding to total intake of 2300 mg/kg bw/d) in the diet to 10 male rats (JCL: Wistar, 5 weeks old) for 7 days resulted in significantly suppressed food consumption throughout the experimental period, depressed body weight gains (69% of controls), and significantly decreased absolute and relative testes weights (60% of controls). Examination on the concentration of zinc in the testes, liver, kidneys and serum showed significantly decreased values in the testes and liver (60% and 90% of control values). Testosterone concentrations in the testes and serum were significantly increased by 260% and 160% of control values. Microscopy was not performed in this study.*	Oishi & Hiraga, 1980d
Male rats, six days gavage	*In a further rat study 800 mg/kg bw/d MIBP was administered by gavage to young male Sprague- Dawley rats (80-100 g) daily for six days. MIBP was given in aqueous solutions as the ammonium salt (pH 6.0). Control animals received an equivalent amount of ammonium chloride (pH 6.0). Liver, kidneys, testes and accessory sex organs were weighed, and testes and accessory sex organs were examined by light microscopy. Additionally zinc metabolism was examined in 9 rats received [65Zn]Cl2 (50 µCi/kg body wt.) i.p. 48h prior to treatment with MIBP for 4 days. The 65Zn content was determined in liver, kidney and testes. Urinary 65Zn excretion was examined over a 24-h period following 4 days of treatment. Treated rats developed markedly reduced absolute and relative testes weights (73%, P<0.001), and lowered seminal vesicle weights (not significant) compared to control values. No differences were evident from prostate weights. Microscopy revealed in all six examined animals marked testicular atrophy of the majority of the seminiferous tubules with a diminution of both spermatocytes and spermatogonia. In all instances the lesions were bilateral in origin. No abnormalities were detected in sections of prostate or seminal vesicles. The zinc metabolism was adversely altered by significantly increasing urinary zinc excretion concomitant with decreased 65Zn testicular content and elevated renal 65Zn content*	Foster et al., 1981
Male mice, one week feeding	*In a mice study body weights and organ weights of testes, liver and kidneys were evaluated; and zinc concentration in testes, liver and kidneys was determined, and testosterone concentration in the testes. Feeding of 2.0% (approximately 2000 mg/kg bw/d, calculated on an assumed daily food intake of 10% of the body weight) MIBP in the diet to 10 male mice (JCL: ICR, 5 weeks old) for 7 days resulted in significantly increased relative liver and testes weights associated with decreased body weight, whereas the absolute weights did not differ from control values. The average zinc level in the testes of MIBP-treated mice was significantly lower than the control value and did not differ in liver and kidneys. Testosterone concentration in the testes was significantly decreased*	Oishi & Hiraga, 1980a
Pregnant rats, 3 days exposure in gestation	*In a comparative study on eight different phthalate esters Singh et al. (1972) treated pregnant Sprague Dawley rats (n = 5/group) with DIBP at single doses of 0.375, 0.75 and 1.25 ml/kg bw (approximately 390, 780 and 1300 mg/kg bw) by intraperitoneal injection on three different days during gestation. Animals of the control groups were either untreated or received a similar volume of distilled water, normal saline or cottonseed oil. The pregnant females were treated on 3 days during gestation (GD 5, 10 and 15) and were sacrificed on GD 20, one day prior to parturition. Ovaries were taken for recording of the numbers of corpora lutea; uterine horns were taken for recording the numbers of resorption sites, and of dead and viable fetuses. Fetuses were weighed and examined for gross malformations. A randomly selected number of fetuses (30-50% of the total) was taken for evaluation of skeletal malformations. Investigation on any maternal parameters is not reported from the study. As a result, there was no difference observed in the number of corpora lutea at any dose level in comparison to the controls. An increase in resorptions (25.8%) was revealed at the high dose level, indicating an embryotoxic potential and leading to a decrease of the number of live fetuses. At the*	Singh et al., 1972

Study design	Effects	Reference
	dose level of 0.75 mL/kg bw 2 out of a total of 52 fetuses were found dead, however, at the low and high dose level only life fetuses were recorded. The average weight of fetuses was reduced in comparison to controls at all dose levels. Gross abnormalities (not further specified) were observed in two fetuses, however at the dose level of 0.75 ml/kg bw only and an increased incidence in skeletal abnormalities was reported for the high dose level (not further specified)	
Pregnant rats, gavage GD 7 to 19 or 21	*Mated female Wistar rats (n=8/group) were gavaged from GD 7 until GD 19 or until GD 20/21 with either vehicle (corn oil) or 600 mg/kg bw/d of DIBP (purity 99%), when they were sacrificed and their male offspring evaluated. At sacrifice on GD 19 five dams from the control and six dams from the treated group provided litters and at sacrifice on GD 20/21 six dams from the treated group provided litters. Anogenital distance (AGD) was measured in all fetuses, fetuses were decapitated and their trunk blood collected, and from males testes removed for histopathology and for immunohistochemistry, for measurement of testosterone production ex vivo, respectively measurement of testosterone content. Administration of DIBP resulted in statistically significant reduction in AGD in male pups (and increased AGD in female pups) at GD 20/21 together with 10 % reduction in bodyweights of male and female fetuses and in a significant reduction in testicular testosterone production and testicular testosterone content in the male offspring at GD 20/21. Histopathological investigations revealed testes pathology as seen with other phthalates, in particular clustering of small Leydig cells on GD19 or GD20/21 and vacuolisation of Sertoli cells on GD 20/21. Immunohistochemistry revealed reduced staining for StAR and P450scc, indicative for reduced expression of these two proteins and thereby reduced capacity of the testicular steroid synthesis. Further results from the study by Borch et al., 2006 were reported by Boberg et al. (2008), who quantified levels of insulin, leptin, MCP1, IL-1B, PAI-active, IL6, and TNFα in pooled samples of plasma. In addition, livers, adrenals and testes tissue from the male fetuses and ovaries from the females had been used for gene expression (mRNA expression) analysis and for steroid hormone measurements (estradiol, testosterone). Treatment with DIBP had resulted at GD 21 in statistically significant reduction of protein levels of insulin and of leptin, whereas no alterations were seen in plasma levels of MCP1, IL-1B, PAI-1 active, IL6 or TNFα. Gene expression analysis on genes involved in steroid synthesis revealed reduced testicular mRNA levels of SR-B1, StAR, P450c17, P450scc and Insl-3 at GD 19 and GD21. In addition testicular SF-1 mRNA levels were reduced on GD 19, whereas no alterations were seen for testicular mRNA levels of aromatase or PBR. In the ovaries of DIBP treated animals an increase in mRNA levels of aromatase was revealed at GD 21. Gene expression analysis on PPARα and on PPARγ revealed significantly reduced mRNA levels of PPARα in livers and testes of DIBP exposed males at GD 19 but not at GD 21. PPARγ mRNA levels were very low in both testes as well as livers and appeared unaltered by DIBP treatment. In the ovaries of DIBP treated animals no alterations were seen in the expression of ERα, ERβ, PPARα, or PPARγ. Besides reductions in mRNA levels there were also indications for reduced protein levels of P450c17 and of PPARγ in the Leydig cells of DIBP treated animals at GD 19 and GD21 (evidenced from reduced immunostaining intensity).*	Borch et al., 2006 and Boberg et al., 2008
Pregnant rats, gavage GD 6 to 20	*In a dose-range finding study on Sprague-Dawley rats, DIBP was administered to pregnant animals (10-14 animals per treatment group) by gavage at doses of 0 (olive oil), 250, 500, 750 and 1000 mg/kg bw/d (Saillenfait et al., 2005) on GD 6-20. Maternal body weights and clinical signs were recorded. Dams were euthanised on GD 21, and the uterine contents were evaluated for number of implantations, resorptions, fetal deaths, and live fetuses. All live fetuses were submitted to external examination and to internal gross examination of the reproductive tract. Maternal body weight gain was transiently depressed on GD 6-9 at the*	Saillenfait et al., 2005

Study design	Effects	Reference
	two higher dose levels. However, the weight gains during GD 6-21 corrected for uterine weight were comparable across groups. A marked increase in the number of resorptions of 38% and of 61% was observed at the 750 and 1000 mg/kg bw/d dose level. A dose-related reduction in fetal body weight was observed amounting to 21% at 1000 mg/kg. Gross internal examination of the reproductive tract revealed undescended testes in 56% and 70% of the male fetuses at 750 and 1000 mg/kg. No further visceral or skeletal examinations were conducted.	
Pregnant rats, gavage GD 6 to 20	*In a further guideline according prenatal toxicity study on Sprague-Dawley rats, DIBP was administered to pregnant animals (23-24 animals per treatment group) by gavage at doses of 0 (olive oil), 250, 500, 750 and 1000 mg/kg bw/d on GD 6-20 (Saillenfait et al., 2006). Endpoints included in addition were determination of the degree of transabdominal testicular migration (TTM). There were no maternal deaths. Signs of transient maternal toxicity were observed, as evidenced by reduction in body weight gain, at the beginning of treatment (GD 6-9) at 500 mg/kg bw/d and higher doses, however, overall weight gain corrected for gravid uterus was not different from controls at the end of gestation. No changes could be observed for maternal food consumption, pregnancy rate or number of implantations. The incidences of resorptions were statistically significantly increased to 28% at 750 mg/kg bw/d and to 59% at 1000 mg/kg bw/d. Mean fetal body weight was statistically significantly reduced at 500 mg/kg/d and higher doses amounting to a decrease of 24% -26% at 1000 mg/kg/d in comparison to controls. The incidence of total external malformations (neural tube closure defects, anophthalmia) and of total visceral malformations (urinary tract and vascular defects) was statistically increased at 750 and 1000 mg/kg bw/d. Skeletal evaluations revealed malformations primarily of the axial column with the incidences of fused sternebrae statistically significantly increased at 750 and 1000 mg/kg bw/d and variations (delayed ossification and supernumerary ribs) at 750 and 1000 mg/kg bw/d with supernumerary ribs in 95% of the fetuses of the 1000 mg/kg group. Visceral variations involved mainly the urinary tract with statistically significantly increased incidences of ureter variations in the 1000 mg/kg group and the male reproductive system. Unilateral or bilateral undescended testes occurred at 500 mg/kg/d and was significantly increased at 750 mg/kg/d (in 30/55 male fetuses and in 16/20 litters) and at 1000 mg/kg bw/d (in 30/34 male fetuses and in 16/17 litters). In addition the degree of transabdominal descent was significantly impaired at 500 mg/kg/d with about two third of the testes located in the upper half of the abdominal cavity at the 1000 mg/kg dose group. Thus, it appeared that alterations of the male reproductive system occurred at lower doses than those producing structural malformations/variations and embryotoxicity. No evidence of embryo or fetal effects was found at the 250 mg/kg dose level. Therefore, a NOAEL/developmental toxicity of 250 mg/kg/d can be derived from the study.*	Saillenfait et al., 2006
Pregnant rats, gavage GD 8 to 18	*In a further study on Sprague-Dawley rats, which was designed to provide dose-response information on the effects of a series of individual phthalates on fetal testosterone production and on the use of the data obtained for the prediction of effects of phthalate mixtures on fetal testosterone production, DIBP was administered to pregnant animals (5-8 animals per treatment group) by gavage at doses of 0 (corn oil), 100, 300, 600, and 900 mg/kg bw/d on GD 8-18 (Howdeshell et al., 2008). Maternal body weights were taken on GD 8 and on GD 18 at sacrifice, when the uterus was removed and the number of fetuses (live and dead) and resorptions were counted and recorded. The total number of implantations was calculated by adding together the number of live and dead fetuses with the total number of resorptions. Fetal mortality was calculated by adding together the number of resorptions and dead fetuses then divided by the total number of implantations. Testes from three males/dose group (2 replicate determinations in individual testes)*	Howdeshell et al., 2008

Study design	Effects	Reference
	in were used for investigation of ex vivo testis testosterone production. Maternal body weight gain was reduced from 73 g in controls to 48 and 43 g in the 600 and 900 mg/kg/d dose group. DIBP-induced complete litter loss in 1/5 dams at 900 mg/kg/d, and induced greater than 50% resorptions in 2/5 dams at 900 mg/kg/d and in 1/5 dams at 600 mg/kg/d resulting in increased percentages of fetal mortality of 17% at 600 mg/kg/d and of 59% at 900 mg/kg/d as compared to 1.3% in the controls. It is reported, that many of the testes collected from DiBP fetuses at dosages of 600 and 900 mg/kg/d were smaller, mucinilagous, and/or located higher in the abdominal cavity. The functional assay on testes ability for hormone production revealed that fetal testicular testosterone production was statistically significantly ($p<0.001$) reduced at dosages of 300 mg/kg/d or higher. The overall results indicated that DIBP (as well as DBP and BBP) was of equivalent potency to DEHP at reducing fetal testosterone production. Dosage levels reducing fetal testosterone production were about one-half to one-third of that required to increase fetal mortality, indicating changes in fetal testicular testosterone production to be a sensitive parameter. Based on statistically significantly lower fetal testosterone production at 300 mg/kg/d a NOAEL of 100 mg/kg bw/d can be derived from this study. BASF, 2003: *A further guideline according prenatal toxicity study on Wistar rats (BASF, 2003; cited in Saillenfait and Laudet-Hesbert, 2005) with dietary administration is indicated in the data base, for which the study report is not available to the rapporteur. It is reported that a decrease in fetal weights and an increase in skeletal variations was observed in rats that had ingested 942 mg/kg DIBP with their diet during pregnancy.*	
Pregnant rats, gavage GD 12 to 21	*In a study on Sprague-Dawley rats, which was performed to determine whether in utero exposure to DIBP would induce permanent and dose-responsive alterations of male reproductive development, DIBP was administered to pregnant animals (11-13 animals per treatment group) by gavage at doses of 0 (olive oil), 125, 250, 500, and 650 mg/kg bw/d on GD 12-21 (Saillenfait et al., 2008). Doses were based on an unpublished preliminary study in which 625 mg DIBP/(kg day) on GD 12-21 caused reproductive tract malformations in male offspring and had no effects on litter size or pup survival. Litters of the definite study were examined as soon as possible after birth to determine the number of viable and stillborn pups. Pup body weights were recorded on PND 1, 4, 7, 14 and 21. AGD was measured on PND1 and litters culled to 10 pups on PND 4. All pups were examined for the presence of areola and/or nipples on the ventral surface of the thorax on PND 12-14. At weaning on PND 21 three to four male pups from each litter were randomly selected and retained and unselected pups sacrificed and submitted to internal examination. After weaning the dams were sacrificed and the number of implantations recorded from their uteri. All retained males were examined for preputial separation (PPS) and individual body weights recorded at acquisition. Adult males were necropsied on PND 76-86 (two males in each litter) or on PND 111-122 (the remaining males in each litter). They were 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. Testes, epididymides, seminal vesicles (with the coagulating glands and seminal fluid), and prostate were weighed. Histopathology was conducted on testes and epididymides of all DIBP animals necropsied on PND 76-88. No differences in maternal body weight gain were observed between the controls and the treatment groups. All dams delivered live pups. Post-DIBP implantation loss, litter size, sex ratio, and pup survival to PND 4 and PND 21 were unaffected by treatment. AGD measured on PND 1 was dose-dependently significantly reduced in male pups from 250 mg DIBP/(kg day) to the higher doses with or without adjustment for body weight. The decrease amounted to 11% at 250 mg DIBP/(kg day) and*	Saillenfait et al., 2008

Study design	Effects	Reference
	22% at 625 mg DIBP/(kg day), compared to controls. AGD of females was not affected at any dose. Pup body weight at PND 1 of both sexes was statistically significantly decreased at 625 mg DIBP/(kg day), and remained lower in comparison to controls in the male pups at weaning. During the post weaning period mean body weights of the offspring were lower than controls at 500 and 625 mg DIBP/(kg day) (6-8% and 10-12%, respectively). On PND 12-14 or at adult necropsy retained areolas and/or nipples were apparent in males at 250 mg DIBP/(kg day) and their incidence increased with dose. No such effects were observed in animals from vehicle controls or the 125 mg DIBP/(kg day) treated group. Acquisition of PPS was delayed by approximately 4 days at 500 mg DIBP/(kg day). Evaluation of PPS was precluded in half of the males at the high dose by presence of hypospadias. Mature males displayed severe malformations (hypospadias with exposed os penis in the more severely affected animals, and non-scrotal testis) at the two high doses. Non-descended testes were always located in the inguinal or supra-inguinal area; none were in the intra-abdominal position. Markedly underdeveloped (less than 10% of control weight) or absent testes and/or epididymes were seen in 2%, 16% (7 males from 5 litters), and 13% (5 males from 4 litters) of the animals in the 250, 500 and 625 mg /(kg day) dose groups. At sacrifice (PND 76-86, resp. PND 111-122) organ weights of the testes, epididymes, seminal vesicles and prostate were significantly reduced (with or without body weight as covariate) at 500 and 625 mg DIBP/(kg day). These reductions amounted to 39-59% for the testes and the epididymes, and 28-33% for the seminal vesicles and the prostate. Histological examinations revealed testicular damage in all DIBP treated groups with moderate or severe degeneration of seminiferous tubules (including Sertoli cell only tubules). The lesions were uni- or bilateral and associated with oligospermia or total azoospermia in the corresponding epididymides. Based on these observations a NOAEL/developmental toxicity could not be determined. Therefore, a LOAEL/developmental toxicity of 125 mg DIBP/kg bw/day can be derived from this study.	
Pregnant mice, gavage GD 6 to 13	*DIBP was further evaluated in a Chernoff-Kavlock screening assay in which CD-1 mice (50 dams/group) were gavaged on GD 6-13 with a single dose level of 4000 mg/kg bw/d or corn oil (Hardin et al., 1987). Dams were allowed to litter and a postnatal evaluation was conducted. At that dose, no pregnant dams gave birth to a live litter and 27/50 exposed dams died.*	Hardin et al., 1987