Substance Name: Bis(2-ethylhexyl) phthalate (DEHP)

EC Number: 204-211-0

CAS Number: 117-81-7

SUPPORT DOCUMENT TO THE OPINION

OF THE MEMBER STATE COMMITTEE

FOR IDENTIFICATION OF

BIS(2-ETHYLHEXYL) PHTHALATE (DEHP)

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

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[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): Bis(2-ethylhexyl) phthalate (DEHP)

EC Number(s): 204-211-0

CAS number(s): 117-81-7

- Bis(2-ethylhexyl) phthalate (DEHP) 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

Bis(2-ethylhexyl) phthalate (DEHP) 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.

DEHP 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, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia.

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 DEHP have also been identified in non-mammalian wildlife where the sex ratio and reproductive output was affected in fish. Furthermore, several studies in fish indicate that DEHP has an estrogenic MoA which may cause the sex reversal of male fish to female fish and / or affect the reproductive output. Hence the current data indicates also in fish that DEHP has endocrine disruptive properties leading to adverse effects related to sexual development and reproduction.

In conclusion, when available information from toxicological and ecotoxicological studies are combined, DEHP 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.

DEHP 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

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:	204-211-0
EC name:	Bis(2-ethylhexyl) phthalate
CAS number (in the EC inventory):	117-81-7
CAS number:	117-81-7
CAS name:	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
IUPAC name:	Bis(2-ethylhexyl) phthalate
Index number in Annex VI of the CLP Regulation	607-317-00-9
Molecular formula:	$C_{24}H_{38}O_4$
Molecular weight range:	390.6 g/mol
Synonyms:	*DEHP*

Structural formula:
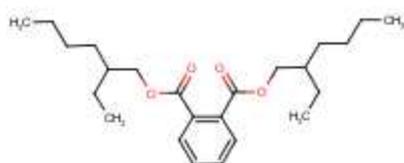
1.2 Composition of the substance

Name: DEHP

Description: DEHP is a well-defined substance containing all possible stereoisomers as main constituents.

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 oily liquid	3.1	VII, 7.1
Melting/freezing point	-55C or -50C	3.2	VII, 7.2
Boiling point	385C at 1013 hPa	3.3	VII, 7.3
Vapour pressure	0.000034 Pa at 20C	3.6	VII, 7.5
Water solubility	3ug/l at 20C	3.8	VII, 7.7
Partition coefficient n-octanol/water (log value)	7.5	3.7	VII, 7.8
Dissociation constant	-	3.21	XI, 7.16
Henry's constant	4.43 Pa m3/mol		

2 Harmonised classification and labelling

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

Classification and labelling of DEHP 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)	
607-317-00-9	bis(2-ethylhexyl) phthalate; di-(2-ethylhexyl) phthalate; DEHP	204-211-0	117-81-7	Repr. 1B	H360FD	GHS08 Dgr	H360FD	

Classification and labelling of DEHP 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
607-317-00-9	bis(2-ethylhexyl) phthalate; di-(2-ethylhexyl)	204-211-0	117-81-7	Repr. Cat. 2; R60-61	T R: 60-61	

phthalate; DEHP				S: 53-45	

3 Environmental fate properties

Information 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 Environmental fate

The environmental fate of DEHP as concluded in the Summary risk assessment report for DEHP for degradation, distribution and bioaccumulation is cited in the sections below (JRC 2008).

"Release of DEHP to the environment occurs during production, transport, storage, formulation and processing of PVC and non-polymers. Furthermore, plasticisers are not chemically bound to the matrix polymer in flexible PVC (or other materials). Therefore the plasticiser will to some extent be lost from the finished article during its use and after its final disposal. DEHP enters the environment mainly via direct releases to air and waste water, from sewage sludge and from solid waste. In air, DEHP may occur both in vapour phase and as solid particles. The nature of these particles can be either aggregated pure DEHP or polymer particles containing DEHP. Particles formed by weathering of polymer products probably represent an important route of DEHP distribution. It is estimated that around 800 industrial sites in EU use DEHP or preparations containing DEHP. Releases from these sources are expected to cause higher local exposure" Cited from JRC 2008.

3.2 Degradation

"Photodegradation of DEHP (reaction with OH radicals) is important in the atmosphere (T½ = 1 day) but is assumed to be of little importance in water and soil. DEHP does not hydrolyse in water. The biodegradation of DEHP is varying in available studies. Based on the results of standard biodegradation test DEHP is readily biodegradable. Experimental data indicates a biodegradation half-life for DEHP in surface water of 50 days, and 300 days in aerobic sediment. Anaerobic conditions and low temperature further reduce the degradation rate. Results from degradation studies of DEHP in agricultural soil are variable, but indicate moderate to low biodegradation rates. MEHP is the primary biodegradation product of DEHP." Cited from JRC 2008.

3.3 Distribution

"With a log Kow of 7.5, DEHP is expected to be strongly adsorbed to organic matter. DEHP is therefore expected to be found in the solid organic phase in the environment. The log Koc for DEHP is 5.2 L/kg. Hence, DEHP will be strongly adsorbed to the sludge in sewage treatment plants. DEHP has a vapour pressure of 3.4 . 10-5 Pa (at 20 to 25°C), which indicate a low evaporation rate from its pure state, and a Henry's law constant of 4.4 Pa m3/mol, indicating a moderate evaporation from a pure water solution ('semi-volatile')." Cited from JRC 2008.

3.4   Bioaccumulation

"DEHP is found to bioaccumulate in aquatic organisms, and the highest BCF values are observed for invertebrates e.g. 2,700 for Gammarus (BCFfish 840). This indicates that uptake via the food chain might be an important exposure route (secondary poisoning). BCF, as well as monitoring data for different trophic levels, indicate that DEHP does not bio-magnify. This may in part be due to a more effective metabolisation rate in higher organisms. Due to its high affinity to organic matter only a limited bioaccumulation of DEHP in plants is expected. The environmental studies confirm this with BCF ranging between 0.01 and 5.9.
For earthworms a BCF of 1, based on experimental results and modelled (EUSES) data, has been used in the risk assessment. "Cited from JRC 2008.

4   Human health hazard assessment

4.1   Toxicokinetics   (absorption,   metabolism,   distribution   and   elimination)

The toxicokinetics as described in the EU RAR of DEHP has been summarized and discussed by the ECHA Member State Committee in 2008 (ECHA 2008):

"Generally, DEHP is rapidly absorbed from the gastrointestinal tract following oral administration. The extent of absorption in rats is around 50% for doses up to about 200 mg/kg bw. At higher doses, it appears that absorption in non-human primates is dose-limited in contrast to rodents. For humans, information is not, however, available concerning the dependency of oral uptake on dose. Also, the extent of oral absorption at doses which humans are expected to be exposed is not known. Absorption may be 100% at daily exposure levels. Limited data on toxicokinetics, following inhalation or dermal exposure, indicate that DEHP can be absorbed through the lungs whereas absorption through the skin appears to be limited. Following intra peritoneal injection most of the administered dose remains in the peritoneal cavity.

Distribution studies in rat indicate that DEHP is widely distributed in the body without evidence of accumulation in the tissues in rats. A comparative study of rats and marmosets showed similar distribution patterns in the two species (oral administration) whereas rats had higher tissue levels than marmosets. Thus, the difference in distribution between species is quantitative rather than qualitative.

The metabolism of DEHP involves several pathways and yields a variety of metabolites. The major step in the metabolism of DEHP is hydrolysis by lipases to MEHP (mono(2-ethylhexyl)phthalate) and 2-ethylhexanol, which is common to all investigated species.
MEHP is a relatively major component in urine of monkeys, guinea pigs and mice but was in most cases not detected in rat urine. However, MEHP is present in plasma in all species tested. The substance is excreted via the urine, mainly as MEHP-metabolites, but some excretion via bile also occurs in rodents. The elimination of DEHP largely depends on its metabolism and it might take 5-7 days to eliminate 80% of the DEHP administrated. The half-life for DEHP and its metabolites was 3-5 days in the adipose tissue and 1-2 days in the liver. The elimination is most rapid in rats. In the DEHP data base, it has been observed that the oral absorption of DEHP to some extent is agedependent, and the EU RAR is concluding on oral absorption percentages of 100 % in young animals and 50 % in adult animals.

DEHP can cross the placenta barrier and distribute into foetal tissues. In addition, DEHP can be transferred through the milk from lactating rats to their pups. Since the immature liver may have a lower metabolising capacity than that of older children and adults, infants and foetuses might be especially vulnerable to exposure to DEHP and MEHP."

The oral absorption fraction of adults was adjusted to 70% by the ECHA Risk Assessment Committee in 2011 and RAC confirmed the EU RAR absorption percentages of 5% for dermal absorption and inhalatory absorption percentages of 75% for adults and 100% for children (ECHA 2012).

The toxicokinetics as described in the EU RAR for human health is cited below (EU RAR 2008): "DEHP is readily absorbed and distributed in the body, but there is no evidence of accumulation. The metabolism of DEHP involves several pathways and yields a variety of metabolites. The major step in the metabolism of DEHP is hydrolysis by lipases to MEHP (mono(2-ethylhexyl)phthalate) and 2-ethylhexanol. The substance is excreted via the urine, mainly as MEHP-metabolites, but some excretion via bile also occurs in rodents.
Additionally, there are animal and human data showing that DEHP is transferred to mothers' milk. The relative extent to which different metabolites are produced and excreted is very complex and may depend upon the species, the age of the animal, sex, inter-individual differences, nutrition state, prior exposure to DEHP, the amount of DEHP administered, and the route of administration."

4.2   Other effects: Endocrine disruption

4.2.1 General approach

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 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 DEHP have been described for the male reproductive system and most work performed to elucidate the mode of action of DEHP 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. DEHP 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 DEHP.

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

a) Background

DEHP is classified as a substance toxic to reproduction (Repr. 1B, H360FD) based on evidence of adverse effects on the reproductive organs in adult and developing rodents. The spectrum of 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 indicative of an anti-androgenic endocrine disrupting mode of action was described as follows in the EU risk assessment report from 2008 (EU RAR, 2008):

"Available data demonstrate that exposure to DEHP affects both fertility and reproduction in rodents of both sexes and also produces developmental effects in offspring. In males, DEHP induces severe testicular effects, including testicular atrophy. (…) Irreversible effects occur in rats exposed prenatally and during suckling (Arcadi et al., 1998). (…) Both *in vivo* and *in vitro* study results indicate that DEHP can interfere with the endocrine function and also influence the sexual differentiation (e.g. Gray et al., 1999 and Jones et al., 1993). Due to the effects on the Leydig cells as measured by a decreased testosterone output, it cannot be excluded that DEHP may exert an antiandrogen effect. The results of recently performed *in vivo* studies in rats exposed to DEHP or DBP support the hypothesis that exposure to phthalates may be provoked by an antiandrogen mechanism (Gray et al., 1999, Mylchrest and Foster, 1998). The present data in experimental animals are of concern for humans." (EU RAR, 2008, pp. 478-481)."

Here, "antiandrogenic mechanism" is used to describe a reduced activation of the androgen receptor. For some chemicals, this is achieved by antagonism of the androgen receptor, but for DEHP and other phthalates the reduced activation of the androgen receptor is caused by interference with steroid hormone synthesis, as will be discussed below. In addition to the studies by Gray et al., 1999 and Jones et al., 1993, other studies included in the EU risk assessment report (EU RAR, 2008) showed effects related to endocrine disruption, e.g. increased nipple retention in male pups (Schilling et al. 1999). Nipple retention in male pups is generally known to be associated with an anti-androgenic mode of action (Imperato-McGinley et al. 1985 and Imperato-McGinley et al. 1986, Wolf et al., 1999), and the findings by Schilling et al. (1999) thus strengthens the hypothesis of DEHP as an endocrine disruptor.

The reproductive toxicity of DEHP 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 EU risk assessment report for DEHP (see below).

Furthermore, the EU risk assessment report from 2008 (EU RAR, 2008) highlighted that monoester metabolites of DEHP, such as MEHP, may be important in relation to the reported adverse effects: "MEHP is believed to be the active metabolite of DEHP affecting testes and reproductive functions both *in vivo* and *in vitro*. The possible role of other metabolites is, however, not fully elucidated."

An overview of the key studies on effects of DEHP on reproduction and development were given in the EU risk assessment report for DEHP (2008) and are presented in the table in Annex 1 to this report. These studies are considered reliable (i.e. in most cases with a Klimisch score 1 or 2). Detailed study summaries can be found in the EU risk assessment report.

b)   Adverse effects indicative of endocrine disruption

Several studies on reproductive and endocrine effects of DEHP *in vivo* have been published since data was collected for the EU risk assessment report. Key studies showing adverse effects and/or showing an *in vivo* endocrine mode of action of DEHP are summarized in table 3 below or can be found in Annex I (studies described in the EU risk assessment report).

The studies included in table 3 are generally evaluated as reliable (Klimisch score 1 or 2). The reliability of a few of these studies are evaluated as somewhat limited, because they use a rather low number of animals and only one dose level (Gazouli et al., 2002; Parks et al., 2000; Wilson et al., 2004), but these studies have anyway been included in the overview table because the findings of these studies in general are accordance with the more comprehensive studies shown in the table and hence can be used as supportive evidence. 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.

Species, strain and number of animals	Protocol	Results	Reference
		Studies showing adverse effects *in vivo*	
Rat, Wistar, n=11 to 16 litters per dose	Pregnant rat dams gavaged from GD 6 to PND 21 (in utero and lactational exposure) with 0, 0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135 and 405 mg DEHP/kg bw/day.	Effects on daily sperm production from 15 mg/kg bw/day and a low, but increased incidence of cryptorchidism at 5 mg/kg bw/day. Effects on hormone levels were seen at low doses, but did not exhibit monotonic dose-response relationships. In males exposed to 1.215 mg/kg bw/day and at doses from 15 mg/kg bw/day and above (i.e. not at 5 mg/kg bw/day), daily sperm production was reduced compared to controls from the same study and compared to historical controls. The authors concluded a LOAEL of 15 mg/kg bw/day for this effect. Three animals exposed to 5, 135, and 405 mg/kg bw/day of DEHP, respectively, had undescended testes (chryptorchidism). The authors concluded a NOAEL of 1.215 mg/kg bw/day based on cryptorchidism despite the low number of affected animals, as chryptorchidism is less common in Wistar rats compared to other rat strains.	Andrade et al., 2006
Rat, Long-Evans, n=12 dams/group	Pregnant rat dams exposed via drinking water from GD 1 to PND 21 to DEHP at doses corresponding to 3-3.5 and 30-35 mg/kg bw/day. Organ weights of pups were determined at 21, 28, 35, 42 and 56 days after birth.	Significant decreases in absolute weights of testes and kidneys and an increased relative liver weight were seen at all examined ages and at both doses. Histological changes in testes, kidney and liver were observed. In testes, disorganization of seminiferous epithelium and testicular atrophy was seen already at 3 weeks of age at both doses of DEHP, whereas the normal testicular maturation occurring in controls from 5 weeks of age was absent or delayed in exposed animals at both doses of DEHP.	Arcadi et al., 1998
Rat, Wistar, n = 8-16 dams/ group	Pregnant rat dams, gavaged from GD 7 to PND 16. Two studies: Study 1 included 16 mated dams in the control group and 8	The critical effect of the combined evaluation of the two studies were effects on anogenital distance and nipple retention in males, as the anogenital distance was significantly decreased and the number of nipples significantly increased at 10 mg/kg bw/day of DEHP with a NOAEL of 3 mg/kg bw/day. At the same dose (10 mg/kg) and	Christiansen et al., 2010

Species, strain and number of animals	Protocol	Results	Reference
	mated dams per group in six exposure groups receiving 10, 30, 100, 300, 600 or 900 mg/kg bw/day of DEHP. Study 2 included 16 mated dams in the control group, 16 mated dams receiving 3 mg/kg bw/day of DEHP, and 8 mated dams per group receiving either 10, 30, or 100 mg/kg bw/day of DEHP. A number of reproductive endpoints were investigated postnatally and at PND 16.	above, decreased weights of ventral prostate and levator ani/bulbocavernosus muscle were observed, though these effects did not show a clear dose-response relationship. The findings in the study by Christiansen et al. (2010), is considered acceptable and supports the findings by Wolfe & Layton (2003).	
Rats, Sprague-Dawley, n = 5-10 dams/group in each block	Pregnant rats gavaged GD 14 to PND 3 with 0 or 750 mg/kg bw/day of DEHP, BBP, DINP, DEP, DMP or DOTP.	Reproductive effects including nipple retention and genital malformations were seen in male rats exposed perinatally to 750 mg/kg bw/day of DEHP, BBP or DINP, but not in those exposed to 750 mg/kg bw/day of DEP, DMP or DOTP. DEHP and BBP exposed males also had reduced anogenital distance.	Gray et al., 2000
Studies showing an endocrine *in vivo* mode of action			
Rat, Long-Evans. Dams n=7, prepubertal rats n= 10, adult rats n = 10	Pregnant or nursing rats dams gavaged with 100 mg/kg/ day of DEHP from GD 12 to GD 21or PND1 to PND 21. Or peripubertal rats were gavaged PND 21 to 34 (14 days) or PND 35 to 48 (14 days) or PND 21-48 (28 days) with 0, 1, 10, 100, or 200 mg/kg bw/day of DEHP. Or adult rats were gavaged PND 62 to 89 with 0, 1, 10, 100, or 200 mg/kg bw/day of DEHP.	Serum T and LH levels were significantly reduced in male offspring, compared to control at the dose tested (100 mg/kg), at 21 and 35 days of age, but not at 90 days of age. In peripubertalrats gavaged with DEHP for 14 days from PND 21 or 35, steroidogenesis was reduced at 10 (from PND 34 only), 100 and 200 mg/kg DEHP as seen by decreased testosterone production *ex vivo* and decreased activity of steroidogenic enzymes. In peripubertal rats exposed for 28 days from PND 21, increased testosterone production was seen at 10, 100 and 200 mg/kg DEHP, No effects on serum testosterone or testosterone production *ex vivo* were seen with exposure from PND 62 to 89. It was concluded that DEHP effects on Leydig cell steroidogenesis are influenced by the stage of development at exposure and may occur through modulation of T-biosynthetic enzyme activity and serum LH levels	Akingbemi et al., 2001
Rat, Wistar, n = 8 dams/ group	Pregnant rats gavaged from GD 7 to 21 with 300 or 750 mg/kg bw/day of DEHP, or 750 mg/kg bw/day of DINP, or a combination of DINP and diethylhexyl adipate (DEHA) (750	DEHP and DINP reduced testicular testosterone production ex vivo and testicular testosterone content in fetal males and DEHP also reduced plasma testosterone levels, increased plasma LH levels. DEHP reduced anogenital distance of male pups at GD 3 and increased the number of nipples in males at pup day 13.	Borch et al., 2004

Species, strain and number of animals	Protocol	Results	Reference
	+ 400 mg/kg bw/day) or a combination of DEHP and DINP (300 and 750 mg/kg bw/day). One group of offspring were examined at GD 21, and another group of offspring was kept for analysis of anogenital distance at PND 3 and determination of nipple retention at PND 13.		
Rat, Wistar, n = 8 dams/group	Pregnant rats dams gavaged from GD 7 to 21 with 10, 30, 100 or 300 mg/kg bw/day of DEHP. Male fetuses were examined at GD 21.	In DEHP exposed males, reduced testicular testosterone production ex vivo and testicular testosterone content was reduced at the highest dose. At the two highest doses, histological changes were seen in testes (multinucleated gonocytes, increased diameter of chords with a larger number of gonocytes) and clustering of Leydig cells was seen at the highest dose. The intensity of immunostaining for StAR, PBR and P450scc was reduced in Leydig cells of DEHP exposed rats compared to controls and corresponded with reduced mRNA levels of StAR, PBR, P450scc and SR-B1 indicating downregulation of steroidogenic pathways	Borch et al., 2006.
Mouse, SV129 wild-type and PPARα(-/-)-null, n = 4	Male mice, 7 days gavage with 1 g/kg bw/day of DEHP.	DEHP reduced circulating testosterone and mRNA levels of PBR (peripheral-type benzodiazepine receptor), which is involved in cholesterol uptake. As the same effects were not seen in PPARalpha-null mice, it was suggested that the influence on PBR gene expression in Leydig cells is PPARalpha dependent.	Gazouli et al., 2002
Rat, Sprague-Dawley, n = 3-4 dams/group	Pregnant rats, gavage GD 14 to 18 with 0, 100, 300, 500, 625, 750 or 875 mg DEHP /kg/day.	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. Despite differences in testosterone production values in the two strains, the same response was seen, i.e. a decrease in testosterone production at 300 mg/kg bw/day and above with a NOAEL of 100 mg/kg bw/day.	Hannas et al., 2011
Rat, Sprague-Dawley, n = 5 to 8 dams/group	Pregnant rats gavaged GD 8 to 18 with 0, 100, 300, 600, or 900 mg/kg bw/day of DEHP, BBP, DEP or DIBP or 33, 50, 100, 300 or 600 mg/kg/day DBP or 25, 50, 100, 200, 300 or 600 mg/kg/day DPP.	DEHP decreased fetal testosterone production in rats at doses from 300 mg/kg bw/day (NOAEL 100 mg/kg bw/day). 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 DEHP and the other tested phthalates (BBP, DBP, and DIBP) from 300 mg/kg bw/day and above, and for DPP (dipentyl phthalate) from 100 mg/kg bw/day	Howdeshell et al., 2008
Rat, Sprague-Dawley, n = 4-5	Neonatal male rats gavaged with a single dose of DEHP (20, 100, 200 or 500	24 hours after exposure, multinucleated gonocytes were seen in testes of DEHP and MEHP exposed animals, but not in testes of 2-EH-exposed or unexposed animals. Reduced Sertoli cell	Li et al., 2000

Species, strain and number of animals	Protocol	Results	Reference
	mg/kg), MEHP (monoethylhexyl phthalate, 393 mg/kg) or 2-EH (2-ethylhexanol, 167 mg/kg) on PND 3. Diethyl phthalate (DEP, 500 mg/kg) was used as a negative control.	proliferation was seen in DEHP exposed animals compared to controls, while no reduction in Sertoli cell proliferation was seen for DEP. These findings indicate that effects of DEHP can be attributed to the metabolite MEHP.	
Rat, Sprague-Dawley, n = 10 (control) and 5 (exposed) dams/ group	Pregnant rat dams gavaged from GD 12 to 19 with DEP, DMP, DOTP, DBP, DEHP, DPP or BBP at 500 mg/kg bw/day. Gene expression analysis in testes was performed at GD 19.	The effects of DBP, BBP, DPP and DEHP on global gene expression were similar, whereas no change in gene expression was detected for DMP, DEP and DOTP. The affected gene pathways involved cholesterol transport, steroidogenesis, lipid and cholesterol homoeostasis, insulin signaling, transcription regulation, oxidative stress and cell-cell communication. These findings clarify that DBP, BBP, DPP and DEHP share the same modes of action associated with changes in steroid synthesis and cell-cell communication	Liu et al., 2005
Rat, Sprague-Dawley, n = 4 dams/group for necropsies on GD 17, GD 18 and GD 20; and n = 5 dams/group for necropsies on PND2	Pregnant rats gavaged from GD 14 to PND 3 with 750 mg/kg bw/day of DEHP. Offspring were examined at GD 17, GD 18, GD 20 and PND 2.	Reduced anogenital distance was seen in male offspring at PND 2. Testicular testosterone production and testicular testosterone concentration were reduced at all examined ages. Carcass testosterone was reduced at GD 17 and 18. Reduced testis weight was seen at GD 20 and PND 2. Increased intensity of staining for 3bHSD was seen in Leydig cells of DEHP exposed animals, and DEHP exposed testes had increased focal Leydig cell hyperplasia and increased numbers of animals with multinucleated gonocytes	Parks et al., 2000
Rat, Sprague-Dawley, n = 5 dams/group	Pregnant rat dams gavaged GD 14 to 18 with 0 or 750 mg/kg/day of DEHP.	Effects of DEHP, DBP and BBP on steroid hormone production and insl3 gene expression were examined in fetal rats exposed orally from gestation day 14 to 18. Reduced testosterone production and insl3 gene expression was seen in males at GD 18	Wilson et al., 2004

Overall, several rodent studies have demonstrated adverse reproductive effects of DEHP (Agarwal et al., 1986a,b; Arcadi et al., 1998; Andrade et al., 2006; Christiansen et al., 2010; Dostal et al., 1988; Gray et al., 1999; Gray et al., 2000; Jones et al., 1993; Lamb et al., 1987; Moore, 1996; NTP 1982; Parmar et al., 1995; Poon et al., 1997; Schilling et al., 2001; Wolfe & Layton, 2003). Most well-described are the effects on the male reproductive system, e.g. increased nipple retention, decreased anogenital distance, reduced number of spermatocytes and testicular changes, including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia (Table 3 and Annex 1). It is well-known that these types of effects can be induced via endocrine disrupting modes of action (Wolf et al., 1999). Chemicals acting as androgen receptor antagonists can induce comparable effects (Wolf et al., 1999), but in the case of phthalates it is highly plausible that interference with steroid hormone synthesis in fetal testis is responsible for the anti-androgenic effects.

The female reproductive system and the thyroid hormone system may also be affected by DEHP. *In vivo*, DEHP has been found to alter steroidogenesis in perinatally exposed female rats leading to increased ovary weight, poor oocyte development and altered expression of steroidogenesis related genes in ovaries (Pocar et al., 2012). In adult rats, prolonged treatment with DEHP increases follicle atresia and decreases populations of ovarian follicles, as reviewed by Martinez-Arguelles et al., 2013. Moreover, effects of DEHP on thyroid histology indicating hyperactivity of the gland have been described in rat studies (Poon et al., 1997; Hinton et al., 1986; Howarth et al., 2001).

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

4.2.3 Endocrine mode of action

The studies in table 3 show adverse effects of DEHP 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 show effects on steroidogenesis, e.g. effects on testosterone production, further substantiated by mechanistic *in vivo* data showing changes in activity of steroidogenic enzymes and effects on gene pathways of steroidogenesis. Mechanistic data / data on mechanism of action are defined as effects at the cellular/sub-cellular/organelle/biochemical level (genes, receptors, enzymes etc). Several studies on testosterone production and steroidogenesis in fetal male rats indicate an endocrine disrupting mode of action of DEHP and its monoester metabolite methylhexyl phthalate (MEHP) *in vivo* (Borch et al., 2004, Borch et al., 2006; Hannas et al., 2011, Howdeshell et al., 2008; Parks et al., 2000; Wilson et al., 2004). This is further substantiated by an *in vitro* study showing decreased testosterone secretion from Leydig cells incubated with MEHP (Jones et al., 1993, Annex 1). 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. Several target genes involved in the development and function of fetal Leydig and Sertoli cells have been identified and several studies have shown reduced expression of genes in the steroid biosynthesis pathway (Hannas et al., 2011, Howdeshell et al., 2008; Parks et al., 2000; Wilson et al., 2004).

It should be noted that phthalates are absorbed as monoesters and/or rapidly metabolized to monoesters. Monoesters are transported across the placenta and reach the fetus (David 2006). Thus, it is the metabolites of phthalate diesters that are endocrine disrupting and mainly effects of metabolites such as MEHP are relevant.

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 tract malformations and semen quality (Fig. 1).
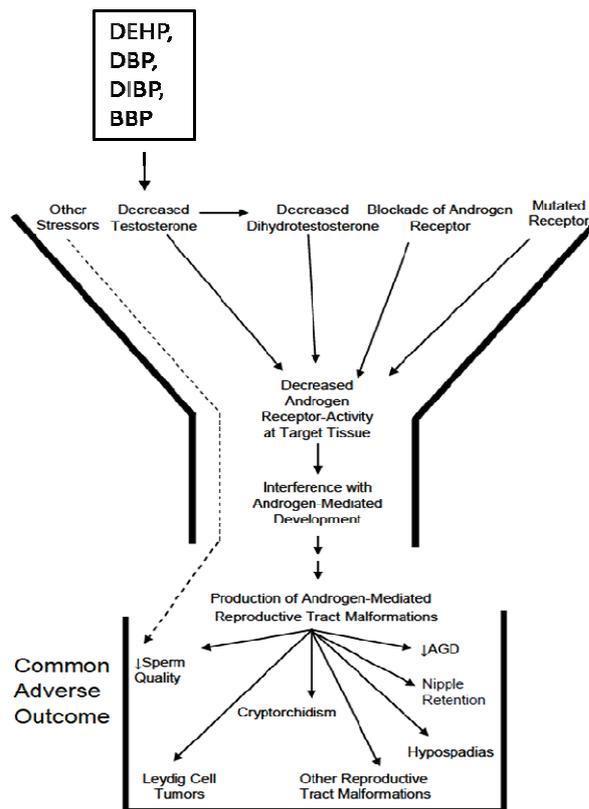
Fig. 1. Modified from NRC, 2008.

A possible estrogenic mechanism of action of DEHP has also been discussed as well as effects on the thyroid system. Studies on interaction of DEHP and MEHP with the estrogen or the androgen receptors have been carried out *in vitro*. DEHP antagonized the androgen receptor in some assays (Takeuchi et al., 2005), but not in others (Krüger et al., 2008; Kim et al., 2010; Parks et al., 2000). Although diesters may in some assays interact with the androgen receptor as well as the estrogen receptors, no interactions with monoesters have been found, as reviewed by David 2006. The DEHP metabolite monoethylhexyl phthalate (MEHP) altered steroidogenesis in cultured ovary follicles *in vitro* (Lovekamp and Davis 2001, Lenie and Smitz 2009) and DEHP interferes with binding of thyroxine to the thyroid receptor *in vitro*, as reviewed by Jugan et al., 2010. Potential interactions of DEHP or its metabolites with steroid receptors are not considered to be relevant mechanisms of action for inducing the observed adverse effects on male reproduction.

Overall, the adverse effects of DEHP on the male reproductive system are considered to be primarily related to effects on 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, indicating an anti-androgenic mode of action of DEHP and the metabolite MEHP due to effects on steroidogenesis. It is biologically highly plausible that the suggested anti-androgenic mode of action gives rise to the adverse reproductive effects of DEHP 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. The adverse effects of DEHP on male reproductive system can be attributed to decreased testosterone

levels, i.e. an anti-androgenic mode of action (EU RAR 2008). Investigation of toxicological effects of DEHP in rat studies have provided convincing evidence that exposure can cause changes in 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 DEHP and the metabolite MEHP.

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 DEHP, a review paper by David, 2006, describes alternative cascades of events that could lead to the adverse health effects observed for DEHP.

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.

Recent studies have further elaborated on the possible mechanisms/ modes of action behind the observed adverse health effects. Rat studies have shown changes in fetal testis proteome indicating a central role of estradiol; findings that corresponded with increased plasma estradiol levels in male rat fetuses (Klinefelter et al., 2012). Supplementing the proposed role of reduced testosterone production, the authors thus suggest a role for estradiol in the induction of testicular effects of phthalates and as a cause of the testicular dysgenesis syndrome in humans (Veeramachaneni and Klinefelter, 2014).

Effects of DEHP on thyroid histology have also been described in rat studies (Poon et al., 1997; Hinton et al., 1986; Howarth et al., 2001), and this may be related to interference of DEHP

with binding of thyroxine to the thyroid receptor, as reviewed by Jugan et al., 2010, and it is thus possible that DEHP may act as an endocrine disrupter via a thyroid hormone disrupting mode of action.

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 DEHP.

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 DEHP 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 vivo* endocrine modes of action and (3) a plausible link between the adverse effects and the endocrine 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 monobutyl phthalate (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 *in 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 shown 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).

For female reproductive effects related to steroidogenesis interference and for thyroid disrupting effects of DEHP, the issue of human relevance has not been addressed. It is, however plausible that the endocrine disrupting effects of DEHP may be of relevance to humans whether related to steroidogenesis interference (in males or females) or to thyroid disruption. It is therefore assumed that these effects may also be 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 European Commission's 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 EU risk assessment report from 2008 (EU RAR, 2008) acknowledges that "it cannot be excluded that DEHP may exert an antiandrogen effect" and that based on the studies available at the time "The results of recently performed *in vivo* studies on rats exposed to DEHP or DBP support the hypothesis that exposure to phthalates may be provoked by an antiandrogen mechanism". More recent studies published after data was collected for the EU risk assessment report confirm this hypothesis.

Rodent studies have demonstrated adverse reproductive effects, especially in male reproductive organs, such as testicular changes, decreased number of spermatocytes and decreased anogenital distance and nipple retention, and it is considered as highly plausible that these effects are induced by an endocrine mode of action of DEHP. Further, studies on DEHP and the metabolite MEHP also showed decreased levels of testosterone and other effects on steroidogenesis such as e.g. reduced expression of genes in the steroid biosynthesis pathway, confirming an endocrine disrupting mode of action of DEHP. There is convincing evidence of a biologically plausible link between the adverse effects observed in males and the anti-androgenic mode of action of DEHP and its metabolite MEHP.

Effects on female reproduction have also been reported as well as effects on the thyroid system. An estrogenic and a thyroid mode of action of DEHP cannot be excluded. The anti-androgenic related effects of DEHP that are evaluated to be relevant in humans include 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, DEHP 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 DEHP. Thus, DEHP 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

To clarify how DEHP fulfills the definition of being an endocrine disrupter, the topics described in chapter 4 will be covered in relation to the environment. It is important to emphasize that the results from chapter 4 are also relevant for mammalian wildlife, especially to wildlife species with low reproductive output (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.

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 analyzed. 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 DEHP on mammals are of regulatory relevance for the environment.

5.1.2 Effects in the aquatic compartment (including sediment)

Overviews of the key studies on effects of DEHP on wildlife were given in the EU risk assessment report for DEHP (2008). Studies from the report which include endpoints relevant for the assessment of endocrine disrupting effects are presented in table 4 below. Detailed study summaries can be found in the EU risk assessment report.

Table 4. Key studies on effects of DEHP on wildlife including endpoints relevant for the assessment of endocrine disrupting effects from the EU risk assessment report for DEHP (2008).

Species	Vehicle	Exp. period	Endpoint	Effect conc. (mg/l)		Comment	Reference and estimated reliability score (Klimisch)
Japanese medaka, (Oryzias latipes (larval 1- 3 d))	Acetone 0.25 ml/l	168 days	Growth survival	NOEC	<0.554	Significant (p= 0,05) weight reduction, 13,4%, when compared to control. Tested concentrations were above water solubility. No effects on survival	DeFoe et al. (1990)* (Score 2)[3]
Japanese medaka, (Oryzias latipes) (Embryo-larval)	Ethanol <100µg/l	newly fertilised eggs exposed until hatch	Hatching time, hatching success, sex ratio, GSI, mortality, body weight			Test concentrations 0, 0.01, 0.1, 1.0 and 10.0 µg/l. No dose dependent effects were seen except for decreased body weights in male fish.	Chikae et al. (2004a) (Score 2)
Japanese medaka, (Oryzias latipes) (Embryo-larval)	Ethanol	3 weeks after hatching	mortality, body weight, sex ratio, GSI,			Test concentrations 0, 0.01, 0.1, 1.0 and 10.0 µg/l. Effects on body weight, GSI and mortality were observed, however, not dose dependent.	Chikae et al. (2004b) (Score 2)
Japanese medaka, (Oryzias latipes) (Embryo-larval)	acetone	5 days	Blood samples analysed for vitellogenin	NOEC	<0.01	Test concentrations 0, 10, 50 and 100 µg/l. Decline in vitellogenin in females. Semi-quantitative method. The reporting of the study is poor, affecting the reliability of the study.	Kim et al. (2002)* (Score 2)[4]
Japanese medaka, (Oryzias latipes) (Embryo-larval)	Acetone	1 or 2 dph to 3 months	Vitellogenin, GSI and histological analysis of reproductive organs	NOEC	0.001	Test concentrations 1, 10 and 50 µg/l. Declined female GSI at 10 µg/l and inhibition of maturation of gonads . The reporting of the study is poor, affecting the reliability of the study.	Kim et al. (2002) (Score 2)[5]

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* The nominal aquatic exposure concentrations applied in the study were above the non-colloidal solubility. For further details see section 5.1.2

[3] The study is reliable with restrictions (not GLP) according to Klimisch score 2 but only one DEHP concentration was tested and although the concentration was measured it was above water solubility and the study was not used to derive a NOEC.

[4] The study is reliable with restrictions (not GLP) according to Klimisch score 2 but the endpoint vitellogenin is not fully reliable because of an un-validated semi-quantitative method performed on too few animals and lack of proper statistics.

[5] The study is reliable with restrictions (not GLP) according to Klimisch score 2 but the endpoint vitellogenin is not fully reliable because of an un-validated semi-quantitative method performed on too few animals and lack of proper statistics.

Species	Vehicle	Exp. period	Endpoint	Effect conc. (mg/l)		Comment	Reference and estimated reliability score (Klimisch)
Japanese medaka, (Oryzias latipes) (Embryo-larval)	Acetone	1-90 dph	Sex ratio, intersex morphometry	NOEC	>5	Test concentrations 0, 500, 1000 and 5000 µg/l (nominal).	Metcalfe et al, (2001)* (Score 2)
Japanese medaka, (Oryzias latipes) (Embryo-larval)	Acetone <100 µg/l	Adult males exposed 2 weeks	No. of eggs, hatching rate	NOEC	>0.39	Test concentrations 39, 120 and 390µg/l. No effect on number of eggs or hatching rate	Shioda and Wakabashi (2000)* (Score 2)
Zebrafish, (Danio rerio)	In food	90 days	Reproduction rate Fry survival	NOEC LOEC	<50 50 (in food)	Test conc,.50 and 100 mg/kg in food Considered invalid due to 49% mortality in control	Mayer and Sanders (1973) (Score 3)
Fathead minnow (Pimephales promelas)	Water and food triethylene glycol and acetone	F0, F1 and, F2 (472 days)	Hatchability, survival, growth, vitellogenin, sex ratio			5 µg/l in water + 125 or 500 mg/kg in food. Significant increase in female F2 vitellogenin at high dose.	Caunter et al., 2004 (Score 2)
Guppy, (Poecilia reticulata)	In food	90 days	Reproduction rate	NOEC	100 (in food)	Test conc:100 mg/kg in food, Non-significant effects were observed	Mayer and Sanders (1973) (Score 2)
Cod, (Gadhus morhua)	In food	121 days	Steroid metabolism	NOEC LOEC	10 100 (in food)	No significant differences in steroid metabolic profiles in male fish at highest dose (1000 µg DEHP/g food) compared to control. In female fish there was a significant alteration of steroid biosynthetic pathways in the head kidneys and ovaries of the DEHP-fed fish. The ratios of 11-deoxycortisol from 100 and 1000 µg DEHP/g groups were greater than twice the observed ratios obtained from the control and 10 µg DEHP/g.	Freeman et al. (1981) (Score 2)
Atlantic salmon, (Salmo salar)	In food	4 weeks	Sex ratio and liver somatic index	NOEC LOEC	300 1,500 (in	Test conc.: 300 and 1500 mg/kg food (nominal concentrations)	Norrgren et. al (1999) (Score 2) [6]

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* The nominal aquatic exposure concentrations applied in the study were above the non-colloidal solubility. For further details see section 5.1.2

Species	Vehicle	Exp. period	Endpoint	Effect conc. (mg/l)		Comment	Reference and estimated reliability score (Klimisch)
					food)	2-4 Injections of 160 mg/kg DEHP during 17 days caused no vitellogenin induction in juvenile salmon (7.5 g). The result is not reliable because of an un-validated method.	
Atlantic salmon, (Salmo salar)	In food	4 weeks	Intersex (ovotestis)	NOEC LOEC	800 1,500 (in food)	Test conc.: 400, 800 and 1500 mg/kg food. The EU RAR (2008) used a NOEC of 160 mg/kg for ovotestis by dividing 800 with 5 to normalize the dry food used, to food with normal water content	Norman et al (2007) (Score 2)
Xenopus laevis (newly spawned eggs)	methyl alcohol (10ml/6L),?	3 months	larval development, growth, survival	See comment		Nominal concentrations: control, solvent control, 0.5, 1, 5, 20 mg/l. The test organisms were exposed from newly spawned eggs until fully developed frogs The results indicate an embryotoxic effect of DEHP, but there was no dose-response relationship The observed effects were also seen for the solvent control (methyl alcohol)[*] No conclusion regarding effects of DEHP can be drawn from this study	Dumpert and Zietz (1983), (Score 2)* Dumpert. (1981) (Score 2)
Xenopus laevis (newly spawned eggs)	DEHP in 1/5 Holfreter solution	200 days	retarded development time, reduced pigmentation of tadpoles	NOEC LOEC	< 2 2	Measured, the only concentration tested (single application and repeated weekly application), tap water control, Holfreter control. Effect at repeated application, no effect at single application of DEHP The test organisms were exposed from newly spawned eggs until fully developed frogs.* The time from egg to fully developed frog was 78	Dumpert and Zietz (1983), (Score 2)* Dumpert. (1981) (Score 2)*

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[6] Vitellogenin was investigated with an un-validated semi-quantitative method. The result is not reliable because the data are not presented in the publication.

[*]The nominal aquatic exposure concentrations applied in the study were above the non-colloidal solubility. For further details see section 5.1.2

Species	Vehicle	Exp. period	Endpoint	Effect conc. (mg/l)	Comment	Reference and estimated reliability score (Klimisch)
					days in the control, 96 days in the 1/5 Holtfreter solution and 184 days in the 2 mg/l DEHP group. A high frequency of unhatched eggs (> 50%) in all treatments as well as in the controls.	

It is not possible to definitively conclude whether DEHP is an endocrine disrupter in fish based on the studies reported in the report (EU RAR 2008) and a conclusion is not drawn in the report. Vitellogenin was investigated in three fish studies with contradicting results and the use of un-validated methods (Kim et al., 2002, Norrgren et al., 1999) or a vitellogenin method not reported (Caunters et al 2004) make it impossible to conclude if DEHP impact vitellogenin in fish and hence may exert estrogenic/anti-estrogenic properties.

As regards endocrine disruptive adverse effects, the only endocrine specific adverse effect recorded is a slightly, but yet significant, skewing of sex ratio (from 49% females to 64% females) in Atlantic salmon after feeding 1500 mg/kg dwt DEHP for 4 weeks after yolk sac resorption followed by a 4 month depuration period (Norrgren et al., 1999). The result on sex ratio could however not be repeated in a study with increased power and measured concentrations of DEHP (Norman et al., 2007), where ovotestis was observed in males but no effect on sex ratio was seen. The authors hypothesize that the changed sex ratio observed by Norrgren et al (1999) may have been caused by a higher exposure concentration than that used (and measured) in the study by Norman et al (2007). This may be a plausible hypothesis, because ovo-testis can be characterized as a mild form of phenotypic sex reversal. Summaries of the two studies can be found in the RAR (2008).

According to the EU RAR (2008) the studies available at that time on amphibians (Dumpert and Zietz, 1983, Dumpert. 1981) where significant effect on development was observed indicate that amphibians might be sensitive to DEHP at high concentrations. The possibility that these effects could indicate adverse anti-thyroidal potential of DEHP was not discussed in the report.

The ED relevant aquatic studies published on DEHP after the EU RAR (2008) are evaluated below.

The studies were identified by searching the Web of Science™ including the following databases: Web of Science™ Core Collection (1900-present), MEDLINE® (1950-present) and SciELO Citation Index (1997-present) using the search phrases: DEHP and endocrine, DEHP and fish, DEHP and environment, Bis(2-ethylhexyl) phthalate and endocrine, Bis(2-ethylhexyl) phthalate and fish, Bis(2-ethylhexyl) phthalate and environment.

According to the EU 2008 RAR, the water solubility of DEHP found in the literature are highly variable with values ranging from 0.0006 to 1.3 mg/L at 20-25°C. The probable explanation is that DEHP readily forms more or less colloidal dispersions in water. A non-colloidal solubility of 3 µg/l was chosen for the Risk assessment in 2008. This should be taken into account when the studies below (where concentrations of DEHP often exceed the non-colloidal concentration of 3 µg/l) are evaluated. In these cases the exposure might be to a combination of colloidal and non-colloidal DEHP which may affect the uptake and bioavailability of DEHP. This solubility of DEHP is also affected by use of different carrier solvents as DMSO and ethanol. DEHP may however also under natural conditions be taken up by oral ingestion of DEHP contaminated organic material. Focus here is on hazard identification relative to serious population relevant

effects related to ED and not risk assessment where quantification of the DEHP expsoure according to exposure routes might be relevant. Hence ED related serious population relevant effects only found above the water solubility limit of DEHP is therefore regarded as of some relevance. Such studies have been marked with an* in study reviews.

Several studies have been performed with endocrine relevant endpoints included since the EU 2008 RAR.

5.1.2.1 Studies conducted after the EU RAR (2008) - Fish

5.1.2.1.1 Short-term toxicity to fish

No endocrine relevant endpoints are included in the short-term toxicity tests to fish and therefore these studies are not discussed in this part of the dossier.

5.1.2.1.2 Long-term toxicity to fish

An estimated reliability score (Klimisch 1997) is given at the end of each study summary. In this respect is should be noted that significant effects from studies with only nominal exposure concentrations are also seen as reliable. One reason is that the water solubility of DEHP as mentioned above is somewhat uncertain and that exposure above a water solubility level of 3 ug/L still may expose the organisms to the substance, but then as well as to truly dissolved DEHP also to DEHP micelles. Even in case much higher concentrations than also the critical micelle concentration was used and effects observed reliably, it can be concluded that DEHP had these effects even though an effect concentration cannot be established (besides referring to the nominal concentrations employed, meaning that the likely true exposure concentration was probably to both fully dissolved DEHP (around 3 ug/L) and DEHP micelles). Finally use of also test data with only nominal concentrations are regarded as acceptable for hazard identification and hence because the conclusions regarding whether DEHP fulfils Article 57(f) of REACH may be reached without necessarily also being able referring to a LOEC, ECx or NOEC value in each of the references used as the scientific background information.

Zanotelli et al. (2010): Different concentrations of DEHP (0.1–10 µg/l nominal concentrations) applied continuously for 91 days were tested in guppy (*Poecilia reticulate*) less than one week old at the beginning of the treatment (5 test concentrations, one replicate of 31 fish per concentration). DMSO was used as solvent. From 14 days after the start of exposure, guppies treated with 10 µg/L showed significantly reduced body length as compared with control fish. The inhibitory effect of DEHP was concentration-dependent and increased with time, leading to a maximal reduction in body length of 15 and 40% at 1 and 10 µg/l DEHP, respectively. The effect was even more pronounced for body weight, which was diminished by up to 40 and 70% at 1 and 10 µg/l DEHP, respectively. The reduction in growth was still significant at 91 days of DEHP treatment, whereas the Fulton's condition factor was unaffected. While DEHP significantly blocked growth in both male and female guppies, no shift in the sexual development was observed. These data show that DEHP can profoundly affect development in fish but it is not clear whether the effect is endocrine mediated or not. (Klimisch Score 2 (non-guideline study)).

Ye et al. (2014)*: Newly hatched Marine medaka larvae (*Oryzias melastigma*) were exposed to either DEHP (0.1 and 0.5 mg/L) or MEHP (0.1 and 0.5 mg/L) nominal values for 6 months in a semi-static system with three water exchanges per week. *O. melastigma* is not a widely used

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* The nominal aquatic exposure concentrations applied in the study were above the non-colloidal solubility. For further details see section 5.1.2

OECD model fish but has been used in some ecotox studies as a marine model – probably because it is closely related to Japanese medaka (*Oryzias latipes*) which is a well described test model. *O. melastigma* exposure was conducted in 3 L aquaria for the first month, 6 L aquaria for the next two months and 10 L aquaria for the last three months of exposure. DMSO was used as carrier solvent (0.1%). Three replicates of 50 larvae were used for each exposure concentration. The effects on reproduction, sex steroid hormones, liver vitellogenin (VTG), gonad histology and the expression of genes involved in the hypothalamic-pituitary-gonad (HPG) axis were investigated. Exposure to DEHP from hatching to adulthood accelerated the start of spawning and decreased the egg production of exposed females at both 0.1 and 0.5 mg DEHP. Moreover, exposure to both DEHP and MEHP resulted in a reduction in the fertilization rate of oocytes spawned by untreated females paired with treated males. A significant increase in plasma 17β-estradiol (E2) along with a significant decrease in testosterone (T)/E2 ratios was observed in males, which was accompanied by the upregulation of ldlr, star, cyp17a1, 17βhsd, and cyp19a transcription in the testis. Increased concentrations of T and E2 were observed in females, which was consistent with the upregulation of ldlr. The expression of brain gnrhr2, fshβ, cyp19b and steroid hormone receptor genes also corresponded well with hormonal and reproductive changes. The liver VTG level was significantly increased after DEHP and MEHP exposure in males. DEHP induced histological changes in the testes and ovaries: the testes displayed a reduced number of spermatozoa, and the ovaries displayed an increased number of atretic follicles. In addition, the tissue concentrations of MEHP, MEHHP (mono-(2-ethyl-5-hydroxyhexyl)-phthalate) and MEOHP (mono-(2-ethyl-5-oxohexyl)-phthalate) in DEHP-exposed groups were much higher than those in MEHP-exposed groups, and there were no dose- or sex-specific effects. Thus, DEHP exerts more obvious toxic effects compared with MEHP. There seemed to be some commonalities in the toxic effects and molecular mechanisms of DEHP and MEHP, suggesting that at least some of the toxic effects of DEHP may be induced by both DEHP itself and DEHP metabolites (including MEHP). Taken together, these results indicate that exposure to DEHP and MEHP from hatching to adulthood causes endocrine disruption with sex-specific effects in marine medaka, with males being more sensitive than females. (Klimisch Score 2 (non-guideline study)).

Wang et al. (2013)*: Adult Chinese rare minnow (*Gobiocypris rarus*) were exposed to DEHP (0 µg/L, 3.6 µg/L, 12.8 µg/L, 39.4 µg/L, and 117.6 µg/L (measured concentrations, DMSO as solvent)) for a 21-d period. Three replicates of each 8 females and 8 males were used per concentration. After 21 days, concentrations of sex hormones in the plasma and relative transcription of various associated genes were measured in the hypothalamic–pituitary–gonadal (HPG) axis and liver of the fish. Exposure to DEHP resulted in a significantly higher circulating concentrations of testosterone (T) and lower concentrations of estradiol (E2), which were accompanied by upregulation of Cyp17 mRNA and downregulation of Cyp19a mRNA in the gonads of females. In males, increases of T and E2 levels were consistent with upregulation of Cyp17 and Cyp19a in the gonads. Furthermore, the T/E2 ratio was significantly increased in females but reduced in males. A significant increase in the levels of hepatic vitellogenin (VTG) gene transcription was observed in both females and males. This study showed that waterborne exposure to DEHP altered plasma sex hormone levels and modulated gene transcription profiles of associated genes in the HPG axis and liver. No endocrine related or systemic adverse effects were investigated nor observed. (Klimisch Score 2 (non-guideline study).

Uhren-Webster et al. (2010): Investigated the effects of di(2-ethylhexyl) phthalate (DEHP) on the reproductive health of male zebrafish (*Danio rerio*). The study may be useful from a mechanistic perspective but with a not natural exposure route. As long as severe systemic toxicity is not occuring at the tested doses, the effects related to ED at these doses have been regarded as of some relevance. Males were treated with 0.5, 50 and 5000 mg DEHP kg^{-1} (body weight) for a period of 10 days via intraperitoneal injection. Four replicates of two males and two females were used per concentration. The effects of the exposure were assessed by analysing fertilisation success, testis histology, sperm DNA integrity and transcript profiles of the liver and testis. A significant increase in the hepatosomatic index and levels of hepatic vitellogenin transcript were observed following exposure to 5000 mg DEHP kg^{-1}. Exposure to

5000 mg DEHP kg^{-1} also resulted in a reduction in fertilisation success of oocytes spawned by untreated females. However, survival and development of the resulting embryos were unaffected by all treatments, and no evidence of DEHP-induced sperm DNA damage was observed. Exposure to 50 and 5000 mg DEHP kg^{-1} caused alterations in the proportion of germ cells at specific stages of spermatogenesis in the testis, including a reduction in the proportion of spermatozoa and an increase in the proportion of spermatocytes, suggesting that DEHP may inhibit the progression of meiosis. In parallel, exposure to 5000 mg DEHP kg^{-1} increased the levels of two peroxisome proliferatoractivated receptor (PPAR) responsive genes (acyl-coenzyme A oxidase 1 (acox1) and enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase (ehhadh). These data demonstrated that exposure to high concentrations of DEHP – in a test with a not natural exposure route - disrupts spermatogenesis in adult zebrafish with a consequent decrease in their ability to fertilise oocytes spawned by untreated females. Furthermore, the data suggest that the adverse effects caused by exposure to DEHP are likely to occur preferentially via PPAR signalling pathways in the testis and oestrogen signalling pathways in the liver. (Klimisch Score 2 (non-guideline study).

Mankidy et al. (2013)*: The study investigated cytotoxicity, endocrine disrupting effects mediated via AhR, lipid peroxidation and effects on expression of enzymes of xenobiotic metabolism caused by di-(2-ethy hexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) in developing embryos of fathead minnow (*Pimephales promelas*). DMSO was used as solvent. Oxidative stress was identified as the critical mechanism of toxicity (CMTA) in the case of DEHP and DEP, while the efficient removal of DBP and BBP by phase 1 enzymes resulted in lesser toxicity. DEHP caused cytotoxicity at 10 mg/L (P < 0.01) and in developing fathead minnow embryos Exposure to 1 mg DEHP/L resulted in 30% mortality. DEHP and DEP did not mimic estradiol (E2) in transactivation studies, but at concentrations of 1 mg/L in H295R cells, synthesis of E2 was affected and T synthesis was affected (decline) at 0.01 mg/L. Exposure to 10 mg BBP/L resulted in weak transactivation of the estrogen receptor (ER). All phthalates exhibited weak potency as agonists of the aryl hydrocarbon receptor (AhR). The order of potency of the 4 phthalates studied was; DEHP > DEP > BBP » DBP. It should be noted that some of the exposure concentrations causing effect exceeded the water solubility of DEHP by several orders of magnitude and exposure via other routes than water may have occurred.– see also discussion of this above (Klimisch Score 2 (non-guideline study)).

Crago & Klaper (2012)*: In this study male fathead minnows (FHM) (*Pimephales promelas*) were exposed to 12 µg/l, nominal concentration (methanol as solvent) of two anti-androgens, the herbicide linuron, and the plasticizer di(2-ethylhexyl) phthalate (DEHP) individually and as part of a mixture of the two for a 28-day period. A total of 40 male FHM were divided evenly among the four exposures. At the end of this period there was a reduction in plasma testosterone (T) concentrations in male FHM exposed to the mixture, but not in FHM exposed individually to linuron or DEHP or the control FHM. There was also a significant reduction in 17β-estradiol (E2) in the DEHP-only and mixture exposed groups as compared to the control. Contrary to what has been previously published for these two chemicals in mammals, the lower plasma T concentrations in male FHM exposed to the mixture was not a result of the inhibition of genes involved in steroidogenesis; nor due to an increase in the expression of genes associated with peroxisome proliferation. Rather, an increase in relative transcript abundance for CYP3A4 in the liver and androgen and estrogen-specific SULT2A1 and SULT1st2 in the testes provides evidence that the decrease in plasma T and E2 may be linked to increased steroid catabolism. Feedback from the pituitary was not repressed as the relative expression of follicle stimulating hormone β-subunit mRNA transcript levels in the brain was significantly higher in both DEHP and mixture exposed FHM. In addition, luteinizing hormone β-subunit mRNA transcript levels increased but were not significant in the mixture as

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*The nominal aquatic exposure concentrations applied in the study were above the non-colloidal solubility. For further details see section 5.1.2

compared to the control. Hormone receptor mRNA transcript levels in the liver and testes were not significantly different across all four exposure groups. Overall this study suggests that DEHP around its water solubility level may decrease serum E2 concentration in fathead minnow probably as a result of induction of the mixed function oxygenase iso-enzymes involved with the catabolism of E2. (Klimisch Score 2 (non-guideline study)).

Carnevali et al. (2010): Female zebrafish (*Danio rerio*) were exposed for three weeks, in semi-static conditions, to nominal 0.02, 0.2, 2, 20 and 40 µg/l concentrations of DEHP. Ethanol was used as solvent. Each exposure concentration had three replicates of 30 fish. After three weeks, a significant decrease in ovulation and embryo production was observed. The effects of DEHP on several key regulators of oocyte maturation and ovulation including bone morphogenetic protein-15 (BMP15), luteinizing hormone receptor (LHR), membrane progesterone receptors (mPRs) and cyclooxygenase (COX)-2 (ptgs2) were determined by real time PCR (3-4 repetitions). The expressions of BMP15 and mPR proteins were further determined by Western analyses to strengthen molecular findings. Moreover, plasma vitellogenin (vtg) titers were assayed by an ELISA procedure to determine the estrogenic effects of DEHP and its effects on oocyte growth. A significant reduction of fecundity in fish exposed to all DEHP concentrations was observed with 50% control group fecundity at 0.02 µg/l down to 1% control group fecundity at 40 µg/l. The reduced reproductive capacity was associated with an increase in ovarian BMP15 levels. This rise, in turn, was concomitant with a significant reduction in LHR and mPRb levels. Finally, ptgs2 expression, the final trigger of ovulation, was also decreased by DEHP. By an *in vitro* maturation assay, the inhibitory effect of DEHP on germinal vesicle breakdown was further confirmed. In conclusion, DEHP affecting signals involved in oocyte growth (vtg), maturation (BMP15, LHR, mPRs,) and ovulation (ptgs2), impairs ovarian functions with serious consequences on embryo production. (Klimisch Score: Due to the lack of information on water renewal period the reliability of the two studies are evaluated to Klimisch cat 2/(4) – generally acceptable but certain documentation of the test procedure is missing. The results of the studies are therefor considered of some relevance and can contribute to the overall environmental ED evaluation of DEHP (non-guideline study)).

Corradetti et al (2013) evaluated the effect of DEHP (nominally 0.2 and 20 µg/L) on the reproductive biology of adult male zebrafish (*Danio rerio*). For each concentration, three replicates of 30 fish each were exposed for one or three weeks. Ethanol was used as solvent. The effects of DEHP and 17b-ethynylestradiol were determined after one or three weeks of exposure by terminal deoxyribonucleotidyltransferase-mediated dUTP Nick-End Labeling assay (TUNEL assay), histomorphometric analysis and evaluation of reproductive performance. DEHP impaired reproduction in zebrafish by inducing a mitotic arrest during spermatogenesis, increasing DNA fragmentation in sperm cells and markedly reducing embryo production (up to 90%) at 0.2 µg/l DEHP. In conclusion, relatively short term exposure below the water solubility level of DEHP is able to severely inhibit spermatogenesis and to affect reproduction in zebrafish. The EE2-treated fish in this study acted as positive controls. The same spermatogonia and spermatid accumulation was observed on histomorphometric analysis of testis collected from fish exposed to EE2 (25 ng/ml), as in the testes from fish exposed to both concentrations of DEHP (0.2 and 20 µg/l). This indicates that even the lowest exposure concentration of DEHP (0.2 µg/l), which was at least one order of magnitude less that the water solubility level, had negative effects on reproduction, probably acting via anti-androgenic or pro-estrogenic effects. The authors hypothesize that the histological abnormalities caused by DEHP may result from DNA damage in addition to estrogenic effects and that the >90% decreased embryo production (P<0.01) may have been a result of impaired male behavior and testes male hormone production. Following the exposure to DEHP at 0.2 and 20 µg/l, male reproductive capacity recovered in untreated water after 9 and 13 days suggesting reversibility of effects on male reproduction after one to three weeks DEHP exposure. (Klimisch Score: Due to the lack of information on water renewal period the reliability of the two studies are evaluated to Klimisch cat 2/(4) – generally acceptable but certain documentation of the test procedure is missing. The results of the studies are therefor considered of some relevance and can contribute to the overall environmental ED evaluation of DEHP (non-guideline study)).

Medaka studies from the Ministry of Environment Japan:

The Ministry of Environment in Japan published two reports in relation to their investigation about EDCs. The first report (http://www.env.go.jp/en/chemi/ed/extend2005_full.pdf) is a brief summary of 61 chemicals undergoing investigation in different assays. It is correct that a medaka vitellogenin assay was performed as well as partial and full lifecycle studies with medaka. The conclusions in the report were as follows: frequency is low, but the appearance of testis-ova was confirmed. There did not appear to be a negative effect on fertilization rates. Clear endocrine disrupting effects were not recognized. As no details on test concentration, number of animals, mortality etc. is provided it is not possible to evaluate these studies in the current dossier.

The second report (http://www.env.go.jp/en/chemi/ed/extend2010_full.pdf) named "Further Actions to Endocrine Disrupting Effects of Chemical Substances" does not include new experimental data and is a more generic approach to ED testing. This report also concludes for DEHP that testis-ova were observed in low appearance frequency to the extent that would not have caused adverse effects on fecundity. Thus, clear endocrine disrupting effects were not recognized. Because no new data on the medaka experiments are provided, these studies are not evaluated in the current dossier. The studies are therefore assigned Klimish cat. 4 (unassignable).

5.1.2.2 Studies conducted after the EU RAR (2008) - Aquatic invertebrates

5.1.2.2.1 Short-term toxicity to aquatic invertebrates

Planelló et al. (2011)[*] investigated the effects of DEHP and BBP in the larvae of *Chironomus riparius* under acute short-term treatments. Fourth instar larvae, were experimentally exposed to five concentrations (0.01, 0.1, 1, 10, and 100 mg/l) of DEHP and BBP for 24 h. Ethanol was used as solvent. Three independent experiments were carried out in each concentration for each phthalate, using 10 larvae arising from three different egg masses and each sample consisted of at least three replicates (n = 9). Solvent control was included. The potential effect of DEHP and BBP on the ecdysone endocrine system (molting hormone system) was studied by analysing the two genes, EcR and usp, of the heterodimeric ecdysone receptor complex. It was found that BBP provoked the overexpression of the EcR gene, with significant increases from exposures of 0.1 mg/l and above, while DEHP significantly ($P<0.05$) decreased the activity of this gene at the highest concentration. (Klimisch Score 2 (non-guideline study).

Otherwise, no endocrine relevant endpoints are included in the short-term toxicity tests to aquatic invertebrates and therefore these studies are not discussed in this part of the dossier.

5.1.2.2.2 Long-term toxicity to aquatic invertebrates

No endocrine specific endpoints are included in the long-term toxicity tests to aquatic invertebrates. Endpoints as reproduction rate could though inform about adverse effects without describing the causality of an endocrine derived mode of action. In the EU RAR (2008) 12 long term toxicity tests to invertebrates exposed via water (predominantly using *Daphnia magna*) were presented and it can be concluded that overall no reproductive effects were seen below the level of systemic toxicity and that toxicity occurred at levels above DEHP water solubility. Therefore it is decided not to include these data in the present dossier.

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[*]The nominal aquatic exposure concentrations applied in the study were above the non-colloidal solubility. For further details see section 5.1.2

5.1.3 Adverse effects related to endocrine disruption

Below, the endocrine disruptive effects in non-mammalian aquatic (vertebrate) species are summarized from the above mentioned studies.

In fish, two studies fulfill the requirement of adversity and a related endocrine MoA: Norrgren et al. (1999) see a skewed phenotypic sex ratio in Atlantic salmon and this endpoint is considered to be both endocrine specific and adverse (OECD, 2012) and Carnevali et al. (2010) observed increased female vitellogenin in combination with a decline in embryo production to about 1% of control production. In the last study more investigations on proteins and genes related to oocyte maturation confirmed the adverse effect of DEHP. Corradetti et al 2013 observed a >90% decreased embryo production after exposure of male zebrafish to 0.2 µg/l and 20 µg/l DEHP.

Conclusion: Overall DEHP acted as a weak estrogen and/or anti-androgen changing the sex ratio of fish in one study, inducing ovo-testis in another, decreasing reproductive output in combination with Vtg induction in a third study as well as decreasing male reproductive output in a fourth study. Based on these studies and supported by rodent studies also describing DEHP as anti-androgenic it can be concluded that DEHP causes adverse effects that most likely occur through an endocrine mediated action.

Reduced growth in fish: Zanotelli et al. (2010) exposed guppy (Poecilia reticulate) from less than one week old at the beginning and 91 days ahead to 0.1 – 10 µg/L DEHP. From 14 days after the start of exposure, guppies treated with 10µg/L showed significantly reduced body length as compared with control fish. The inhibitory effect of DEHP was concentration-dependent and increased with time, leading to a maximal reduction in body length of 15 and 40% at 1 and 10 µg/l DEHP, respectively. The effect was even more pronounced for body weight, which was diminished by up to 40 and 70% at 1 and 10 µg/l DEHP, respectively. The reduction in growth was still significant at 91 days of DEHP treatment. The MoA for the reduced growth cannot be definitively concluded but the findings are not in contradiction to an anti-thyroid effect s hypothesis supported by the *in vitro* anti-thyroid effects of DEHP observed by Sun et al. (2012).

Conclusion: One study reports a significant and concentration dependent growth reduction. This might be induced by DEHP through a thyroid disrupting mode of action, but a final conclusion cannot currently be drawn because no specific thyroidal endpoints have been developed for fish.

Changed developmental time in amphibians: Dumpert and Zietz (1983) observed the development time (egg to fully developed frog) was doubled compared to the control in a long term study on the clawed toad *Xenopus laevis* at 2 mg/L DEHP (measured concentration).

Conclusion: A changed developmental time in an amphibian species was caused by DEHP exposure and could be anti-thyroidal but a final conclusion of MoA cannot be drawn as the effects is not specific to only substances causing thyroidal effects.

Reduced reproductive output in fish: Ye et al. (2014) observed decreased egg production of female Marine medaka (*O. melastigma*) after 6 month exposure from the larval stage to either DEHP (0.1 and 0.5 mg/L) or MEHP (0.1 and 0.5 mg/L). Moreover, exposure to both DEHP and MEHP resulted in a reduction in the fertilization rate of oocytes spawned by untreated females paired with treated males. Besides, DEHP induced histological changes in the testes and ovaries: the testes displayed a reduced number of spermatozoa, and the ovaries displayed an increased number of atretic follicles. Uren-Webster et al. (2010) investigated the effects of DEHP on the reproductive health of male zebrafish (*Danio rerio*). Males treated with 5000mg DEHP kg^{-1} (body weight) for a period of 10 days via intraperitoneal injection resulted in a reduction in fertilization success of oocytes spawned by untreated females. Carnevali et al. (2010) exposed female *Danio rerio* to environmentally relevant doses of DEHP (20 ng – 40 µg/L) and a significant decrease in ovulation and embryo production was observed for all doses. The embryo production in the 40 µg/l dose was about 1% of control

production. Corradetti et al 2013 observed a >90% decreased embryo production (P<0.01) after exposure of male zebrafish (Danio rerio) to 0.2 µg/l DEHP for three weeks. The authors hypothesize that the effect is a result of impaired male reproductive behaviour and testes hormone production. Norman et al. (2007) observed a statistical significant induction of ovo/testis in the highest exposure group of 1500 mg/kg in male *S. salar*.

Conclusion: DEHP reduced the reproductive output in fish significantly, especially by affecting males and a likely MoA is estrogenic and/or anti-androgenic effects on the male reproductive system but no definitive conclusion can be made.

Overall conclusion all available references taken together:

Overall DEHP acts as a weak estrogen and/or anti-androgen changing the sex ratio of fish in one study, inducing ovo-testis in another, decreasing reproductive output in combination with Vtg induction in a third study as well as decreasing male reproductive output in a fourth study. Further available studies support this conclusion and other studies suggest that DEHP may possibly also have thyroid effects in fish and/or amphibian species.

5.1.4 Endocrine mode of action

Several scientific papers describe the influence of DEHP on the endocrine system in fish. Most well-described are the effects on vitellogenin concentrations, steroidogenesis and the reproductive system of both male and female fish.

Effects on vitellogenin (Vtg) *in vivo*: Vitellogenin induction or reduction as seen in several studies (Kim et al., 2002; Caunter et al., 2004; Carnevali et al., 2010; Wang et al., 2013; Ye et al., 2014) is regarded as an endocrine specific effect at concentrations below systemic toxicity, even though it is not regarded as adverse. Effects on vitellogenin concentration in fish after waterborne and/or food exposure to DEHP has been investigated in several studies: Caunter et al. (2004) observed increased Vtg concentrations in F2 female fathead minnow exposed to 5 µg/l DEHP in water and 500 mg/kg in food for three generations. Also male fish from the same study had increased Vtg concentrations although not significant using conservative statistics. Vtg was analyzed semi-quantitatively in Japanese medaka (*Oryziaz latipes*) by Kim et al. (2002) after a chronic exposure from 1 or 2 days post-hatch (dph) until the age of 3 months to the nominal concentrations 1, 10 or 50 µg DEHP/l. A decline in female Vtg concentration was observed but n=1 per treatment concentration hampers the result and the study results are not included in the conclusion. Juvenile salmon were injected intraperitoneally (total dose of 160 mg DEHP kg^{-1} bw during 17 days). No vitellogenin was detected in the blood of the DEHP injected fish (Norrgren et al., 1999). Ye et al. (2014) observed significant increase in liver Vtg concentration in males after exposure of marine medaka (*Oryzias melastigma*) from hatch to 6 month post hatch to 0.1 and 0.5 mg/l DEHP. A significant increase in the levels of hepatic vitellogenin (Vtg) gene transcription was observed in both female (LOEC 12.8 µg/l) and male (LOEC 39.4 µg/l) adult Chinese rare minnow (*Gobiocypris rarus*) after exposure to DEHP for a 21-d period (Wang et al., 2013). Carnevali et al. (2010) reported increase of plasma vitellogenin levels in female zebrafish (*Danio rerio*) exposed for three weeks, in semi-static conditions, to nominal 0.02, 0.2, 2, 20 and 40 mg/l concentrations of DEHP.

Conclusion: Summarizing the weight of evidence DEHP induces vitellogenin protein or genes in most of the tested fish species, indicating an estrogenic mode of action and/or interference with steroid hormone synthesis as also observed in rodent studies described in section 4.

Effects on steroidogenesis *in vivo*: Steroidogenic effects were also reported in several fish studies (Crago & Klaper, 2012; Wang et al., 2013; Ye et al., 2014). These effects are regarded as endocrine specific, but are on their own not regarded as adverse. Wang et al. (2013) demonstrated several effects on steroidogenesis in Chinese rare minnow (*G. rarus*): ERα was significantly up-regulated in the liver of males and females and the authors argue that DEHP might act directly on ER genes, especially ERα, to stimulate Vtg synthesis. Exposure to DEHP

caused a significant decrease of E2 and an increased T/E2 ratio in females but a significant increase of E2 and decreased T/E2 ratio in males. These results could be explained by significant changes in both CYP17 and CYP19a gene transcriptions. Crago & Klaper (2012) observed decreased plasma E2 concentrations in male fathead minnow (*P. promelas*) after 28 days exposure to 12 µg/l DEHP. Ye et al. (2014) observed a significant increase in plasma 17β-estradiol (E2) along with a significant decrease in testosterone (T)/E2 ratio (to less than 1/3 of control ratio) in male *O. melastigma* (LOEC 0.1 mg/L). A significant decrease in testosterone (T)/E2 ratios was also observed in males. Increased concentrations of T and E2 were observed in females. Han et al. (2009) studied the effect of DEHP on sex hormones in common carp (*Cyprinus carpio*) but because the tested concentrations from 5.5 to 20.5 mg/L DEHP are several orders of magnitude above DEHP water solubility and no solvent information is presented, the results of the paper are not discussed.

Conclusion: Overall DEHP up-regulate ER-genes, changing T/E2 ratios and affecting aromatase transcriptions, indicating an estrogenic mode of action.

Mechanistic information from in vitro studies: Beside the above presented *in vivo* studies a few *in vitro* studies using fish hepatocytes have shown significant increase in Vtg mRNA transcripts levels or Vtg protein levels, which could give mechanistic support to an estrogenic mode of action of DEHP exposure (Uhren-Webster et al., 2010; Maradonna et al., 2013). Interaction with the estrogen receptor (ERα reporter gene assay – LOEC 80 µg/l) as well as the thyroid receptor (TR-mediated reporter gene assay – LOEC 800 µg/l) of DEHP has been demonstrated *in vitro* (Sun et al., 2012). In a MCF-7 breast cancer cell line, DEHP significantly affected concentrations of E2 in media where exposure to 10 mg DEHP/L resulted in 4-fold greater concentration of E2 (Mankidy et al., 2013). In the latter case, it should be noted that the exposure concentration causing effect exceed the water solubility of DEHP by orders of magnitude.

Conclusion: Overall the mechanistic studies show that DEHP acts as an estrogen agonist increasing Vtg mRNA and/or Vtg proteins as well as E2 levels *in vitro*. Interaction with the estrogen receptor and the thyroid receptors has also been reported, supporting the hypothesis about estrogenic and thyroid disrupting modes of action.

5.1.5 Plausible link between adverse effects and endocrine mode of action

As seen from the ecotoxicological studies described above, several endocrine pathways could be affected by DEHP. Estrogenic MoA is evident from several studies (Norrgren et al., 1999; Norman et al., 2007; Carnevali., et al 2010; Corradetti et al 2013) but in addition as for the rodent studies, an anti-androgenic MoA may also be involved based on the observed decline of the male serum T/E2 ratio by Wang et al., 2013 and Ye et al., 2014.

Thyroid disrupting effects were not confirmed in any of the *in vivo* studies but could be the MoA causing effects on growth and development in both amphibians and fish (Dumpert & Zietz, 1983; Zanotelli et al., 2009). Mechanistic studies *in vitro* support a possible thyroid disrupting mode of action of DEHP.

Looking at the studies overall, it is biologically highly plausible that the adverse effects on the phenotypic sex and reproductive output in both male and female fish are induced by an estrogenic MoA (Norrgren et al., 1999; Norman et al., 2007; Carnevali., et al 2010; Corradetti et al 2013). The estrogenic MoA of DEHP is further supported by observations of ovotestis (Norman et al., 2007), affected Vtg and steroidogenesis *in vivo*, and mechanistic studies *in vitro*.

5.1.6   Summary - Environment

Fish: DEHP acts as a weak estrogen agonist *in vivo* by 1) inducing vitellogenin in several species, including fathead minnow (*P. promelas*), marine medaka (*O. melastigma*), Chinese rare minnow (*G. rarus*) and zebrafish (*D. rerio*) and 2) affecting stereoidogenesis by up-regulating ER-genes, changing T/E2 ratios and affecting aromatase transcriptions also in several species, including fathead minnow (*P. promelas*), marine medaka (*O. melastigma*) and Chinese rare minnow (*G. rarus*). This alteration of the endocrine system can be coupled to a study observing skewing of the phenotypic sex of *S. salar* and another study on *S. salar* observing ovotestis and observed effects on a decrease in reproductive output for some species, including both sexes of the zebrafish (*D. rerio*) and both sexes of the marine medaka (*O. melastigma*). It should be noted that no effects on sex ratio or gonads were seen in Japanese medaka (*O. latipes*) after exposure from 0-90 days post hatch (Metcalfe et al., 2001). A few *in vitro* studies are available using fish hepatocytes have confirmed the hypothesis of DEHP being an estrogen agonist by increasing vitellogenin mRNA and/or vitellogenin proteins and E2 levels. Anti-estrogenic and anti-thyroid effects were reported in one study.

Looking at the studies overall, it is biologically highly plausible that the adverse effects on the phenotypic sex and reproductive output in both male and female fish are induced by an estrogenic MoA (Norrgren et al., 1999; Norman et al., 2007; Carnevali., et al 2010; Corradetti et al 2013). The estrogenic MoA of DEHP is further supported by observations of ovotestis (Norman et al., 2007), affected Vtg and steroidogenesis *in vivo*, and mechanistic studies *in vitro*.

Thyroid disrupting effects were not confirmed in any of the *in vivo* studies but could be the MoA causing effects on growth and development in both amphibians and fish (Dumpert & Zietz, 1983; Zanotelli et al., 2009). Mechanistic studies *in vitro* support a possible thyroid disrupting mode of action of DEHP.

Mammals: The severity of effects of DEHP on rodents (impact on development and reproduction) as presented in chapter 4 are generally accepted as endpoints of concern and as such accepted for reaching conclusions in regulatory hazard (and risk) assessment. Furthermore developmental and reproductive effects such as those of DEHP are 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.

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 working 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, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia. DEHP causes adverse –and serious – reproductive toxicity effects in rodents and a harmonized classification Rep. 1 B has been concluded.

Adverse effects caused by DEHP have also been identified in non-mammalian wildlife where change of the sex ratio or induction of ovo-testes in male fish have been observed in some studies and in addition decrease of the reproductive output was observed in other fish studies.

Re 2) DEHP 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. In fish DEHP also shows clear estrogenic activity. Several studies in fish indicate that DEHP has an estrogenic MoA which is likely to cause the observed sex reversal of male fish and / or to affect the reproductive output. Hence the current data indicates also in fish that DEHP has endocrine disruptive properties leading to serious effects related to sexual development and reproduction.

Re 3) The link between the endocrine mode of action of DEHP has been concluded in numerous investigations in rodents (mode of action on the steroidogenic biosynthesis pathway) and has also been shown in fish (estrogenic mode of action). It is considered biologically highly plausible that the observed adverse effects in rats and fish are linked to the endocrine disrupting mode of action of DEHP and it metabolite MEHP.

Re 4) DEHP 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 lack of human relevance has not been concluded for the adverse effects. In respect to the environment sex reversal and reduced reproductive output are both regarded as serious population relevant adverse effect endpoints in fish. The ED MOA and reproductive toxicity data from laboratory rodents are highly relevant for at least some mammalian wildlife species due to the similarities between mammalian species and their hormone systems. The laboratory ED MOA and reproductive toxicity data on fish are relevant for at least some wildlife fish species. Hence both human and environmental relevance can be concluded.

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, DEHP 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."

Human health

With regard to assessing whether DEHP, 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

DEHP 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 DEHP 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 DEHP

Re. probable serious effects

DEHP 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: DEHP 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 DEHP.

Environment

When assessing whether DEHP, fulfilling 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

In fish, DEHP acts as a weak estrogen agonist in vivo by 1) inducing vitellogenin in several species, including fathead minnow (P. promelas), marine medaka (O. melastigma), Chinese rare minnow (G. rarus) and zebrafish (D. rerio) and 2) affecting stereoidogenesis by up-regulating ER-genes, changing T/E2 ratios and affecting aromatase transcriptions also in several species, including fathead minnow (P. promelas), marine medaka (O. melastigma) and Chinese rare minnow (G. rarus).

Re. scientific evidence

Change in sex ratio of fish is regarded both as a population relevant adverse effect and also as a highly likely marker of endocrine disruption (in particular estrogenic MOA). This is further substantiated by *in vitro* studies where changes in vitellogenin level confirmed the hypothesis of DEHP as an estrogen agonist The estrogenic mode of action of DEHP is further supported by observations of ovotestis and affected vitellogenin and steroidogenesis *in vivo*. It is biologically highly plausible that the adverse effects on the phenotypic sex and reproductive output observed in some studies in fish are caused by an estrogenic MoA of DEHP.

Re. probable serious effects.

DEHP has adverse effects on the phenotypic sex and reproductive output in both male and female fish. Skewing of the phenotypic sex was seen in *S. salar* and another study on *S. salar* observed ovotestis.Further, a decrease in reproductive output for some species, including both sexes of the zebrafish (*D. rerio*) and both sexes of the marine medaka (*O. melastigma*) was also observed. It is noted that no effects on sex ratio or gonads were observed in Japanese medaka (*O. latipes*) after exposure in 0-90 days post hatch so significant differences in the sensitivity between fish species or not fully known exposure or bioavailability related factors in the conduced tests may have been involved. A few *in vitro* studies available using fish hepatocytes have confirmed the hypothesis of DEHP being an estrogen agonist by increasing vitellogenin mRNA and/or vitellogenin proteins and E2 levels.

Re. equivalent level of concern

- *Potential severity of ecotoxicological effects* –DEHP may adversely affect the reproductive ability of fish populations by changing male fish into female fish and may also according to some investigations directly reduce fish fecundity. Such reproductive effects are considered an adverse and serious effect with population level relevance. DEHP 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 fish and mammalian species. Change in sex ratio of fish populations is an irreversible effect with long term implications on both the population itself and populations of other species dependent on this population. If for example the sex ratio of a fish population becomes is significantly skewed and male fish becomes too scarce 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.

- *Broad environmental relevance* : Effects on reproductive ability via an estrogenic mode of action has a broad environmental relevance. Due to the conservatism of estrogen receptors it is very likely that a wide range of wildlife species with different function in the ecosystems could be affected. Further, the severity of effects of DEHP 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 DEHP.

6.3 Conclusion

Bis(2-ethylhexyl) phthalate (DEHP) 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.

DEHP 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, reduced number of spermatocytes and testicular changes including multinucleated gonocytes, tubular atrophy and Leydig cell hyperplasia.

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 DEHP have also been identified in non-mammalian wildlife where the sex ratio and reproductive output was affected in fish. Furthermore, several studies in fish indicate that DEHP has an estrogenic MoA which may cause sex the reversal of male fish to female fish and / or affect the reproductive output. Hence the current data indicates also in fish that DEHP has endocrine disruptive properties leading to adverse effects related to sexual development and reproduction.

In conclusion, when available information from toxicological and ecotoxicological studies are combined, DEHP 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.

DEHP 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 - DEHP. Studies considered most important in EU RAR 2008

The following table presents the studies considered most important (EU RAR 2008):

Species, strain, number of animals	Protocol	Results	References
Rat, Sprague-Dawley 17/males/group	3 generations via diet; 1.5, 100, 300, 1,000, 7,500 and 10,000 ppm (0.1, 0.5, 1.4, 4.8, 14, 46, 359 and 775 mg/kg/day	dose-dependent effets on numerous testis-related parameters. NOAEL for test.tox and dev. tox. 4.8 mg/kg/day, and 46 mg/kg/day for fertility	Wolfe et al, 2003
Rat, Wistar, 25 animals/group	0, 1,000, 3,000 or 9,000 ppm DEHP via the diet (corresponding to approximately 0, 113, 340, or 1,088 mg/kg/day)	3,000 ppm; a reduced testis weight in F2, focal tubular atrophy and a feminisation of 49% of the male offspring. Minimal focal tubular atrophy also occurred at 1,000 ppm (113 mg/kg and day)	Schilling et al., 2001
Rat, Wistar 10 males/group	4 weeks, *inhalation*, 0, 10, 50 or 1,000 mg/m3	no effects on male fertiliy, no testicular toxicity NOAEL 1,000 mg/m3	Klimisch et al. (1992)
Rat, F344 24 males/group	60 days, *diet* 0, 320, 1,250, 5,000 or 20,000 ppm (0, 18, 69, 284 or 1,156 mg/kg bw/day)	Dose-dependent ↓in total body, testis, epididymis, and prostate weights from 5,000 ppm ↓mean litter size at 20,000 ppm correlated with degenerative testicular changes, ↓testicular zinc content, epididymal sperm density and motiliy, ↑number abnormal sperm cells NOAEL 320 ppm (69 mg/kg bw/day)	Agarwal et al. (1986a,b)
rat, F344 48 males/group	*gavage*, 13 days 0, 330, 1,000 or 3,000 mg/kg bw/day and a diet containing 2, 20 or 20 ppm zinc	Testis: dose-dependent tubular degeneration and atrophy from 1,000 mg/kg bw DEHP combined with low-zinc diet (2 ppm) NOAEL 330 mg/kg bw/day	Agarwal et al. (1986a)
rats, Sprague-Dawley 7-10 males/group	gavage, corn oil 5 days 0, 10, 100, 1,000 or 2,000 mg/kg bw/day at 1, 2, 3, 6 and 12 weeks of age neonatal exposure on days 6-10 0, 100, 200, 500 or 1,000 mg/kg bw/day	↓absolute and relative testis weights at 1,000 mg/kg bw/day in 1, 2, 3,and 6-week old rats; ↓Sertoli cell nuclei in 1-weekold rats and loss of spermatocytes in 2- and 3-week old rats; ↓testis weight also in 6- and 12-week old rats at 2,000 mg/kg bw/day; fatalities in suckling rats at 2,000 mg/kg; NOAEL 100 mg/kg bw/day Testis: ↓ number of Sertoli cells in adult rats at 500 and 1,000 mg/kg bw, no effect on fertility after mating to untreated females	Dostal et al. (1988)
rat, F344 10 rats/sex/group	13 weeks, diet 0, 1,600, 3,100, 6,300, 12,500 or 25,000 ppm (0, 80, 160, 320, 630, or 1,250 mg/kg/day)	↓bw at 25,000 ppm testis atrophy from 12,500 ppm NOAEL 6,300 ppm (320 mg/kg/day)	NTP (1982)

rat, F344 50 rats/sex/group	103 weeks, diet 0, 6,000, or 12,000 ppm (0, 322, or 674 mg/kg/day [males])	↓bw at 12,000 ppm Anterior pituitary: hypertrophy at 12,000 ppm (22/49 males, 45%) Testis: seminiferous tubular degeneration at 6,000 ppm (2/44, 5%) and 12,000 ppm (43/48 males, 90%), histologically devoid of germinal epithelium and spermatocytes	NTP (1982)
rat, Wistar 6 males (25-day-old) per dose group	0, 50, 100, 250, or 500 mg/kg bw for 30 days	dose-dependent and significant ↑LDH and GGT and ↓SDH from 50 mg/kg bw; ↑ β-glucuronidase and ↓acid phosphatase testis: marked destructive changes in the advanced germ cell layers and vacuolar degeneration at 250 and 500 mg/kg	Parmar et al. (1995)
rat, F344 70-85/sex/group recovery group: 55/sex	104 weeks, diet 0, 100, 500, 2500, or 12500 ppm (0, 5.8, 28.9, 146.6, or 789.0 mg/kg bw/day [males]; 0, 7.3, 36.1, 181.7, or 938.5 mg/kg bw/day [females] or 12500 ppm for 78 weeks, followed by a recovery period of 26 weeks	Pituitary: ↑castration cells (30/60 males) at 12500 ppm; Testis: ↓weight, ↑incidence and severity of bilateral hypospermia at 12500 ppm; Epididymis: ↑immature or abnormal sperm forms and hypospermia from 12500 ppm; Changes in the testis and pituitary were not reversible upon cessation of exposure NOAEL for testicular effects 500 ppm (28.9 mg/kg bw/day)	Moore (1996)
rat, Sprague-Dawley 10 rats/sex/group	13 weeks, diet 0, 5, 50, 500, or 5,000 ppm (0, 0.4, 3.7, 37.6, or 375.2 mg/kg bw/day [males])	testis: mild Sertoli cell vacuolation at 500 ppm (7/10); decreased absolute and relative testicular weight, mild to moderate Sertoli cell vacuolation, testicular atrophy and complete loss of spermatogenesis at 5,000 ppm (9/10), in-creased liver and kidney weights (all rats of both sexes), and mild histological changes of the thyroid at 5,000 ppm NOAEL 50 ppm (3.7 mg/kg bw/day)	Poon et al. (1997)
mouse, B6C3F1 70-85/sex/group; recovery group: 55/sex	104 weeks, diet 0, 100, 500, 1,500 or 6,000 ppm (0, 19.2, 98.5, 292.2 or 1,266.1 mg/kg bw/day [males] or 6,000 ppm followed by a recovery period of 26 weeks	Testis: from 1,500 ppm ↓weight, ↑incidence and severity of bilateral hypospermia; Epididymis: from 1,500 ppm ↑ immature or abnormal sperm forms and hypospermia; Changes in testes partially reversible; NOAEL 500 ppm (98.5 mg/kg bw/day)	Moore (1997)
Rat, Sprague-Dawley 17/males/group	3 generations via diet; 1.5, 100, 300, 1,000, 7,500 and	dose-dependent effets on numerous testis-related parameters. NOAEL for test.tox	Wolfe et al, 2003

	10,000 ppm (0.1, 0.5, 1.4, 4.8, 14, 46, 359, and 775 mg/kg/day	and dev. tox. 4.8 mg/kg/day, and 46 mg/kg/day for fertility	
rat, Wistar 25 females/ group	*inhalation*, head-nose, gestation day 6-15 0, 0.01, 0.05, or 0.3 mg/litre (0, 10, 50, or 300 mg/m3)	↓number of live foetuses/dam and ↑percentage of resorptions/dam at 50 mg/m3; the effects showed, however, no dose-response relationship. NOAEL for maternal and developmental toxicity 300 mg/m3	Merkle et al. (1988)
rat, F344/CrlBr 34-25 females/group	*Diet* 0, 0.5, 1.0, 1.5, or 2% gestation days 0-20	↓maternal food intake and mean foetal bw from 0.5%; ↓maternal bw gain, ↑absolute and relative liver weights, ↓foetal bw/litter from 1.0% ↑number and percentage of resorptions, nonlive and affected implants/litter at 2%; NOAEL for maternal and developmental toxicity 0.5% (~357 mg/kg bw/day)	NTIS, 1984; Tyl et al. (1988)
rat, Wistar 9-10 females/group	*gavage*, oil 0, 40, 200 or 1,000 mg/kg bw/day on gestation days 6-15	↓maternal bw and ↑maternal relative kidney and liver weights at 1,000 mg/kg bw ↓number of live foetuses/dam, ↓ foetal body weights, ↑number of malformed foetuses/dam (tail, brain, urinary tract, gonads, vertebral column, and sternum) at 1,000 mg/kg bw; NOAEL for maternal and developmental toxicity 200 mg/kg/day	BASF (1995); Hellwig et al. (1997)
mouse, 1-CR 30-31 females/group	*diet*; 0, 0.025, 0.05, 0.10 or 0.15% (0, 44, 91, 190.6 or 292.5 mg/kg bw/day); gestation days 0-17	↓maternal body weight gain from 0.10% (mainly due to ↓ uterine weight, ↓foetal body weight and number of live foetuses per litter); ↑ number and percent of resorptions, late foetal deaths, dead and malformed foetuses, and percent malformed foetuses/litter from 0.05% (open eyes, exophtalmia, exencephaly, short, constricted or no tail); visceral malformations and skeletal defects (fused and branched ribs, mis-alignement, and fused thoracic vertebral centra); NOAEL for maternal toxicity 0.05% (91 mg/kg bw/day) and for develop-mental toxicity 0.025% (44 mg/kg bw/day)	NTIS, 1984; Tyl et al. (1988)
mouse, CD-1 15 females/dose group30 controls	*oral*, gavage 0, 40, 200 or 1,000 mg/kg bw/day gestation days 6-15	foetotoxic effects at 200 mg/kg bw/day ↓number of viable foetuses ↑number of resorptions and post-implantation losses at 1,000 mg/kg bw/day and also	Huntingdon (1997)

		cardiovascular abnormalities, tri-lobed left lungs, fused ribs, fused thoracic vertebral centres and arches, immature livers, and kidney abnormalities NOAEL 200 mg/kg bw for maternal toxicity and NOAEL 40 mg/kg bw/day for developmental toxicity	
breeding studies mouse, ICR 20 animals/sex/dose group, 40 control animals of each sex	*diet*, 98 days 0, 0.01, 0.1, or 0.3% (0, 20, 200 or 600 mg/kg bw/day)	dose-dependent ↓in the number of litters and proportion of pups born alive from 0.1% (0.1%: 14/19 fertile, 0.3%: 0/18); ↑ absolute and relative liver weight (both sexes) and ↓reproductive organ weights and atrophy of seminiferous tubules at 0.3%; no effect on bw NOAEL for maternal and developmental toxicity 20 and 600 mg/kg bw/day, respectively. Crossover mating trial: treated males and control females: 4/20 fertile; control males and treated females: 0/16 fertile	Lamb et al. (1987)
Rat, Sprague-Dawley 17/males/group	3 generations via diet; 1.5, 100, 300, 1,000, 7,500 and 10,000 ppm (0.1, 0.5, 1.4, 4.8, 14, 46, 359, and 775 mg/kg/day	dose-dependent effets on numerous testis-related parameters. NOAEL for test.tox and dev. tox. 4.8 mg/kg/day, and 46 mg/kg/day for fertility	Wolfe et al, 2003
Rat, Wistar, 25 animals/group	0, 1,000, 3,000 or 9,000 ppm DEHP via the diet (corresponding to approximately 0, 113, 340 or 1,088 mg/kg/day)	3,000 ppm; a reduced testis weight in F2, focal tubular atrophy and a feminisation of 49% of the male offspring. Minimal focal tubular atrophy also occurred at 1,000 ppm (113 mg/kg and day), which thus constitutes a conservatively chosen LOAEL	Schilling et al. (2001)
Rat, Wistar 10 rats/sex/group	*diet, (range finding study)* 0, 1,000, 3,000 or 9,000 ppm (0, 110, 339 or 1,060 mg/kg bw/day)	↑relative liver weight in F0 females from 1,000 ppm and in F0 males from 3,000 ppm (negative histopathology); ↓food consumption, body weight, and body weight gain and ↑postimplantation loss in females at 9,000 ppm; F1 pups : ↓number of delivered and live born pups and ↓viability index neonatally at 9,000 ppm; loss of spermatocytes at 3,000 ppm (2/10) and 9,000 ppm (7/9); ↑presence of areolas/nipple anlagen; retarded preputial separation and vaginal opening at 9,000 ppm; F1 parental animals : ↓ food consumption, body weight, and mortality in both sexes initially at 9,000 ppm and ↓ body weight	Schilling et al. (1999)

		gain in females; ↓fertility, ↓testicular and epididymal weight and size, atrophy of the testes, Leydig cell hyperplasia, interstitial oedema, and altered spermatogenesis and aspermia at 9,000 ppm; doserelated decrease of prostate weight from 1,000 ppm; F2 pups: ↑number of still born pups from 3,000 ppm, ↓number of delivered pups and mean umber of pups/dam at 9,000 ppm	
mouse, CD-1 (number not specified)	*diet*, 0.01, 0.025, or 0.05% (0, 19, 48 or 95 mg/kg bw/day)	↑prenatal mortality for F1-litters at 0.05% ↓number of viable pups neonatally at 0.05% NOAEL for parental toxicity and F2-offspring: 0.05% (95 mg/kg bw/day) NOAEL for F1-offspring: 0.025% (48 mg/kg bw/d)	NTIS (1988)
rat, Sprague-Dawley 10 males/group	*Gavage*, corn oil 5 days from the age of 1 week, 2 weeks, 3 weeks, 6 weeks, or 12 weeks 0, 10, 100, 1,000 or 2,000 mg/kg bw/day	two doses of 2,000 mg/kg bw were fatal for most pups in the three youngest age groups, ↓bw for 6- and 12-week-old rats but no mortalities; 5 doses of 1,000 mg/kg bw: ↓bw gain in 1-, 2-, and 3-week-old rats; ↑absolute and relative liver weights at 100 mg/kg bw/day in all age groups (exept for 1-weekold rats) and in all age groups at higher dose levels; ↓plasma cholesterol levels in weanling and adult rats from 1,000 mg/kg/day	Dostal et al. (1987b)
Rat, Sprague-Dawley, 8 pregnant dams/group	Pregnant rats, *gavage*, corn oil, GD 14 to PND 3 750 mg/kg bw/day Male offspring were killed at about 5 months of age.	DEHP was considerably more toxic than was DBP to the reproductive system of the male offspring. ↑ incidence of both reproductive and non-reproductive malformations including decreased anogenital distance, areolas (88%), hypospadias (67%), vaginal pouch (45%), ventral prostate agenesis (14%), testicular and epididymal atrophy or agenesis (90%), and retained nipples. Several 8-day old pups displayed haemorrhagic testes by gross examination. ↓weight of the gonads, the accessory sex organs, and the muscle Levator ani-bulbocavernosus (5 months old offspring). The chemicals investigated could be clustered into three or four separate	Gray et al., 1999

		groups, based on the resulting profiles of reproductive effects. DBP and DEHP induced a higher incidence of testicular and epididymal abnormalities, including atrophy and agenesis, which is not generally found with flutamide or Vinclozolin even at high dose levels.	
Rat, Wistar, 3 males/group	*In vivo* study: *gavage* 2 consecutive days, 6-8 weeks old 2,000 mg/kg bw Exposure to phthalate diesters: DEHP, DPP (di-n-pentyl phthalate), DOP (di-n-octyl phthalate), and DEP (diethyl phthalate) Sacrifice 24 hours after the final dose. *In vitro* study: Primary cultures of Leydig cells incubated with 1,000 µM monoester for 2 hours. The corresponding monoesters were investigated *in vitro*: MEHP, MPP, MOP, and MEP.	*In vivo* study: The changes observed were present in all animals in each group. Leydig cells stained more densely than other cell types, generally displaying an elongate profile often with thin lamellar processes. In Leydig cell cytoplasmatic ultrastructure, several subtle but highly significant alterations were produced. DEHP administration also resulted in slight rarefaction or vacuolation of a few Sertoli cells in seminiferous tubules, while treatment with DOP or DEP produced no change in seminiferous tubular structure or Leydig cell morphology. Exposure to DPP produced the most severe changes in Sertoli cells but no changes in Leydig cells. *In vitro* study: Phthalate esters exerted a direct effect on Leydig cell structure and function as determined by testosterone output with correlation of the *in vitro* and *in vivo* effects of MEHP and DEHP, respectively. MEHP and MPP produced marked effects on structure and function including decreased LH-stimulated secretion of testosterone from Leydig cells incubated with MEHP while MOP caused decreased secretion and MEP was without effect.	Jones et al., 1993

GD: gestation day, PND: post natal day.