European Plasticisers – Comments on the CLH Report Proposal for Harmonised Classification and Labelling – Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2; Substance Name: 1,2-benzenedicarboxylic acid, di-C8-C10-branched alkylesters, C9-rich; [1] di-"isononyl"phthalate; [2] [DINP]; EC number: 271-090-9 and 249-079-5; CAS number: 68515-48-0 and 28553-12-0; Dossier Submitter – Danish EPA

European Plasticisers[1] represents the major producers of plasticisers in Europe (BASF, Evonik, ExxonMobil, Deza, Grupa Azoty, Lanxess, Perstorp, and Proviron). The opinion of European Plasticisers on the classification proposal is as follows:

1. Based on the scientific evidence, European Plasticisers does not agree with the proposal for classification of DINP as a Reproductive toxicant Category 1B (Development).

2. Based on the scientific evidence, European Plasticisers does not agree with the proposal for classification of DINP as a Reproductive toxicant Category 2 (Fertility).

3. European Plasticisers would note that the endpoints brought forward by the dossier submitter have not been interpreted and/or documented in the dossier versus the detailed criteria established under the CLP for effects warranting classification (see Section 3.7.2.2. – Basis for classification). In the attached comments scientists from European Plasticisers member companies have applied these detailed criteria to the data with the conclusions shown under point 5.

4. European Plasticisers would note that a key study central to the CLH dossier is by Boberg et al (2011). This study is used in four of the nine key points in the "Short scientific justification" which the dossier submitter includes to support the classification proposal. A re-analysis of the raw data from this study using the methods in the published paper (Boberg et al. 2011) has shown that the results of statistical significance for the effects of DINP in animals cannot be reproduced for several parameters (Morfeld et al., 2017[2]). A Corrigendum[3] from Boberg et al. in 2016 and letters to the editor (Morfeld et al., 2017; Boberg et al. 2017[4]) have confirmed that the original published methods were not in fact followed and that non-standard methods are now proposed, thereby maintaining the original results. When the statistics are performed according to the original methods, the statistical significance of AGD, histopathology outcomes, and sperm parameters is lost in almost all instances, calling into question the statistical and toxicological significance of these observations. A Data in Brief article (Chen et al., submitted) and a letter to the editor (Morfeld et al. 2017) have been written by scientists from

[1] www.europeanplasticisers.eu
[2] https://doi.org/10.1016/j.reprotox.2017.03.013
[3] http://doi.org/10.1016/j.reprotox.2016.07.001
[4] https://doi.org/10.1016/j.reprotox.2017.03.014

European Plasticisers member companies, which clarify the reproducibility discrepancies and their significance to interpretation of this particular study. The editor and an independent reviewer engaged by the editor confirmed in writing their agreement with nearly all of the points in the European Plasticisers letter (Morfeld et al, 2017). European Plasticisers would also note that according to the metadata in the dossier, Dr. Boberg is also the author of the classification dossier.

5. The evidence and rationale brought forward by the dossier submitter does not justify classification of DINP as a reproductive toxicant according to the criteria of the CLP. **Based on the extensive scientific evidence demonstrating a lack of adverse reproductive effects following exposure to DINP, per the CLP criteria as detailed in section 3.7.2. of Annex I of the CLP and the detailed criteria in section 3.7.2.2. (Basis for classification), European Plasticisers proposes that classification for development and fertility effects is not required**. This proposal for no classification is consistent with the statement in the current DINP IUCLID REACH registration dossiers (updated December 2015) which uses IUCLID standardized language – that is, the reason for no classification is that the reproductive data on DINP is "conclusive but not sufficient for classification".

The above opinion is supported by the attached detailed comments which are provided in five parts:

Part 1 – Short Summary of the Scientific Justification for No Classification

Part 2 – Structured Summary on Danish EPA Reproductive Classification Proposal – DINP does not fulfill the criteria of Annex I of the CLP and therefore does not warrant classification

Part 3 – Detailed comments on the dossier

Part 4 – Scientific Appendices providing detailed information relevant to Parts 1 and 2

Part 5 – Additional Background Information

The above comments (Parts 1 to 5) are provided as 5 individual pdf documents in the zip file attached to this submission to the ECHA web page.

1. Short Summary of the Scientific Justification for No Classification

European Plasticisers (formerly ECPI) respectfully submits that classification for reproductive toxicity according to CLP is not justified based on evaluation of the scientific evidence in the context of the regulatory criteria (REGULATION (EC) No 1272/2008), and requests that the RAC reconsider the recommendation made by the dossier submitter.

For an effect to warrant classification, CLP criteria require, first and foremost, that the evidence supporting an effect is causally related to exposure to the chemical (CLP Annex I, section 3.7.2.1.1); and secondarily requires a determination that the effect is adverse (CLP Annex I, section 3.7.2.1.1), which is additionally characterized by a number of criteria including assessment of the biological significance (CLP Annex I, section 3.7.2.3.1), toxicological significance (CLP Annex I, section 3.7.2.3.3), nature, severity, and incidence (CLP Annex I, section 3.7.2.3.1) of the effect. Furthermore, conclusions on the inherent ability of a chemical to induce a specific adverse effect (CLP Annex I, section 3) should be based upon the available data and an assessment of total weight of evidence (CLP Annex I, section 3.7.2.3.1) which includes assembling together both positive and negative results. As described below, the extensive scientific evidence from animal studies involving oral exposure to DINP demonstrates a lack of adverse reproductive effects per the CLP criteria (as detailed in section 3.7.2. of Annex I of the CLP) and therefore classification for development and fertility effects is not required. This conclusion of no classification is consistent with the statement in the current DINP IUCLID REACH registration dossiers (updated December 2015) which uses IUCLID standardized language – that is, the reason for no classification is that the reproductive data on DINP is "conclusive but not sufficient for classification". The conclusion of no classification for reproduction is also consistent with the outcome of a quantitative weight of evidence evaluation of all relevant reproductive studies on DINP in the context of CLP criteria (Dekant and Bridges, 2016b).

A. Based on sufficient data, adverse effects on development are not observed following exposure to DINP and classification per CLP is not warranted

The results of developmental toxicity studies involving in utero exposure to DINP throughout gestation, during organogenesis, and during the androgen-sensitive male programming window, demonstrate that DINP does not induce adverse effects[1]. Of particular relevance, oral exposures of DINP up to (~1100mg/kg bw/d) during the male programming window fail to induce hypospadias, cryptorchidism, under developed Wolffian duct, or decreased accessory sex organ weight changes which are observed following exposure to LMW phthalates such as DEHP and DBP. Likewise, exposures of DINP during organogenesis do not induce the severe malformations (e.g. cleft palate, neural tube defects) observed following exposure to DEHP and DBP. There are studies reporting that exposure of pregnant dams to DINP during the male programming window leads to slight and reversible changes in biological markers of testosterone

[1] ADVERSITY (WHO, 2004). A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences.

perturbation (i.e. delayed regression of the nipple anlagen postnatally) and non-androgenic endpoints (i.e. multinucleated gonocytes). However, any observed change (statistically significant or not) does not automatically warrant classification. Only those changes concluded as relevant per the criteria outlined in section 3.7.2. of Annex I of CLP justify a classification decision; these criteria include consideration of the nature (3.7.2.3.1), severity (3.7.2.3.1), incidence (3.7.2.3.1) and toxicological significance (3.7.2.3.3) of an observation, as well as its occurrence below a limit dose (3.7.2.5.7-3.7.2.5.9). In the case of DINP, the observed activity does not result in adverse reproductive effects (i.e. effects with functional consequences) as demonstrated in the extensive reproductive toxicity studies on DINP. While these effects are indicators of biological activity, they themselves are of minimal toxicological consequence and as such do not warrant classification per CLP.

A.1. Adverse effects on development are not observed following treatment with DINP during the period of organogenesis.

In the studies characterizing developmental effects observed following DINP exposures (up to 1000 mg/kg bw/d) during the defined period of organogenesis (GD6-15), effects on implantation, number of live births or gross malformations were not observed. Increases in skeletal variations were reported in these studies, however the nature of these (e.g. rudimentary ribs) are characterized as common fetal variants and do not support classification (CLP 3.7.2.3.3). This is consistent with the interpretation of these observations reflected in the EU RAR for DINP (ECB, 2003), i.e. "*the effects observed in the available studies, do not justify classification.*" These data are described further in Part 4, Appendix II of this submission and in the EU RAR for DINP (ECB, 2003).

A.2. Adverse effects on development are not observed in studies assessing in utero exposures to DINP during the androgen sensitive male-programming window.

In the multitude of studies that have examined the post-natal consequence of in utero (and perinatal) exposure to DINP during the androgen responsive male programming window (GD15.5-18.5 in rats) (Adamsson et al., 2009; Boberg et al., 2011; Clewell et al., 2013a,b; Gray et al., 2000; Lee et al. 2006; Li et al., 2015; Masutomi et al., 2003; Waterman et al., 2000 a,b) effects observed included reductions in fetal testosterone, delayed regression of the nipple anlagen postnatally and, in a few instances, a statistically significant reduction in anogenital distance. These effects are all transient effects that do not persist into adulthood and have no functional consequences. Importantly, severe developmental/reproductive effects of hypospadias, cryptorchidism, under developed Wolffian duct, or decreased accessory sex organ weight have not been reported at doses up to ~1100 mg/kg bw/d of DINP. Furthermore, reproducible evidence of testicular atrophy, malformations of the testis or accessory organs (Boberg et al., 2011; Gray et al. 2000; Clewell et al., 2013b; Masutomi et al., 2003; McKee, R., 2002; Waterman et al., 2000 a b), or reliable impacts on sperm following in utero exposure (Boberg et al. 2011, 2016; Morfeld et al. 2017) are not observed.

As discussed in more detail in Part 4, Appendix VII of this submission, exposure to DINP during the male programming window frequently results in an increased frequency of multinucleated gonoctyes (MNGs) in pups (Boberg et al., 2011; Clewell et al., 2013 a, b; Li et al., 2015). However, the toxicological significance of these observations in the context of classification is questionable as they are a normal occurrence in development, a process which culminates in their normal elimination from the seminiferous epithelium within 1–2 weeks postnatally. While studies with DINP observe an increased frequency compared to controls at a given time point in development, they are no longer present in older animals exposed to DINP in utero (Boberg et al. 2011, Clewell et al. 2013); and therefore are eliminated just as they are in normal development. The disappearance of MNGs from rat testes, early in the perinatal period provides evidence that they are unlikely to be associated with any structural or functional deficits. The lack of functional deficits resulting from an increased frequency of MNGs is corroborated by the results from a two-generation reproductive toxicity study showing no functional effect on reproduction following exposure of the F2 generation throughout the entirety of in utero development and postnatal development (Waterman et al. 2000b). Thus, in the context of CLP criteria (3.7.2.3.1, 3.7.2.3.3), the appearance of MNGs do not warrant classification.

It has been inferred by the dossier submitter that doses higher than those used in in utero studies on DINP (>1100 mg/kg bw/d) could cause more severe effects (e.g. hypospadias, cryptorchidism testicular dysgenesis). The relevance of this inference to a classification decision for DINP is questionable in the base case, as observations above the limit dose[2] are outside the criteria which lead to classification (per CLP 3.7.2.5.7-3.7.2.5.9). Importantly, however, simple extrapolation to adverse outcomes observed following exposure to the LMW phthalates such as DBP and DEHP based on testosterone potency is not supported by the extensive empirical data for DINP. For example, Hannas et al. (2011) describes DEHP to be 2.3 fold more potent than DINP for inhibiting testosterone production, which is not linearly correlated to the predicted 10-20 fold difference in potency between DINP and DEHP by Gray et al. (2000) for observations downstream of testosterone modulation. Additionally studies by Gray et al. (2016) and Zirken et al. (1989) indicate large reductions in fetal testosterone (>80%) are required to induce postnatal reproductive alterations. DINP has never been observed to reduce testosterone to this magnitude up to doses of 1500 mg/kg/d (Boberg et al. 2011; Clewell et al. 2013a; Hannas et al. 2011,2012; Furr et al. 2014;). Finally, the extensive data on DINP, including evidence of absence of adverse outcomes above the limit dose (Adamsson et al., 2009; Boberg et al., 2011; Clewell et al., 2013a,b; Gray et al., 2000; Lee et al. 2006; Li et al., 2015; Masutomi et al., 2003; Waterman et al., 2000 a,b) support DINP is not capable of inducing adverse effects (e.g. hypospadias, cryptorchidism testicular dysgenesis). These observations are consistent with pharmacokinetic data which demonstrate that absorption of DINP would be saturated above 750 mg/kg/day, such that the use of higher treatment levels would not lead to higher systemic absorption or increased effects (Clewell et al. 2013 a; Mckee et al. 2002). The observation of reversible

[2] 1000 mg/kg, i.e. a defined limit dose in OECD guidelines for repeat-dose studies is an acceptable limit dose for DINP, because expected human exposure does not indicate a need for higher dose levels (CLP 3.7.2.5.7 to 9). Additionally data demonstrate that absorption of DINP is saturated above 750 mg/kg bw/day, such that the use of higher treatment levels would not lead to higher systemic absorption (Clewell et al. 2013a; Mckee 2002).

endocrine activity without adverse reproductive effects following exposure to DINP is also consistent with what the structure activity relationship associated with low molecular weight and high molecular weight phthalates would predict. Low molecular weight phthalates with 3 – 6 carbons in the straight chain backbones in the alkyl side chains cause adverse reproductive effects in animal studies (Fabjan et al 2006). High molecular weight phthalates with the longest straight chain backbone being 7 – 13 carbons in the alkyl side chains do not cause adverse reproductive effects in animal studies (OECD, 2004).

In short, **the consistently observed effects that have been associated with DINP exposure (i.e. retained nipples and MNGs in early development) are not adverse as they are mild, not permanently retained into adulthood and not associated with any long term consequences; and therefore do not warrant classification according to CLP criteria outlined in section 3.7.2.2.** Detailed discussion of the endpoints observed following exposure to DINP during the male programming window can be found in Part 4, Appendix II of this submission.

A.3. DINP does not cause adverse reproductive effects comparable to LMW phthalates.

The effects observed following exposure to DINP in reproductive toxicity studies are not comparable to those seen with the classified low molecular weight phthalates such as DEHP, DBP, DIBP and BBP. As shown in Table Ia below, DINP does not induce the developmental malformations or loss of fertility observed with DBP and DEHP when tested in comparable studies. The following effects are seen with DEHP and are the basis for the classification decision for this substance (ECBI/37/99-Add.55): cleft palate, neural tube defects, testicular tubular atrophy, complete ablation of spermatogenesis, fetal death. These effects are not seen with DINP. This is convincingly demonstrated in both regulatory guideline and research studies on DINP.

Extensive structure activity data support that adverse reproductive effects are observed in animal studies with alkyl phthalates where the longest straight chain is composed of 3 to 6 carbons with or without methyl or ethyl branching (Fabjan et al. 2006; Saillenfait et al. 2011, 2014). Extensive data also show that adverse reproductive effects are not observed with alkyl phthalates where the longest straight chain backbone is composed of 7 to 13 carbons with or without methyl, ethyl or propyl branching as confirmed by specific studies on relevant substances and as agreed and documented by OECD SIAM (OECD, 2004) and also Clewell et al. (2013b). In this context it is also noted that several other past CLH proposals for phthalates by different EU Member States have included clear reference to the structure activity relationship associated with alkyl phthalates where the longest straight chain is composed of 3 to 6 carbons (see CLH dossiers for DnHP, Di-isohexyl phthalate, DIPP). As noted above, the observation of reversible endocrine activity following exposure to DINP without manifestation of adverse reproductive effects, is consistent with what the structure activity relationship associated with low molecular weight and high molecular weight phthalates predicts.

A.4. Human data provide further scientific justification for no classification.

The current body of epidemiologic literature on DINP and reproductive endpoints (summarized in Part 4, Appendix XII of this submission) provides further scientific justification for no classification. Although relatively small and evolving, the epidemiologic literature on DINP covers many reproductive developmental and fertility endpoints, including those referenced in the CLP proposal: cryptorchidism and hypospadias (Jensen et al. 2015; Main et al. 2006), anogenital distance (AGD) (Bornehag et al. 2015; Jensen et al., 2016), pubertal development (Mieritz et al., 2012), time to pregnancy (Specht et al. 2015), as well as reproductive hormones and testicular function (Joensen et al. 2012; Specht et al. 2014; Axelsson et al. 2015). The largely null findings observed across the many endpoints examined in these existing studies is informative, despite methodological limitations, and when viewed in total suggests that DINP does not alter reproductive development or impair fertility.

Support for this conclusion comes from a recent and comprehensive systematic review and meta-analysis conducted by Bonde et al. (2017) in which researchers rigorously evaluated published data on the risk of cryptorchidism, hypospadias, low sperm counts and testicular cancer following in utero or infant exposure to chemicals that were previously included in the European Commission prioritization exercise “towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption”. Based on the (null) results of their analyses, researchers concluded that their study challenged “the widely stated view that ubiquitous endocrine disrupting chemicals in our environment play a substantial role in the development of male reproductive disorders through prenatal and perinatal mechanisms”. Notably, DINP was not associated with any of the outcomes considered in their systematic review. Furthermore, these conclusions from Bonde et al. (2017) comport with a quantitative weight of evidence evaluation (Dekant, Bridges, Swaen, 2016), in which CLP criteria were used to evaluate the epidemiologic research specific to DINP and reproductive developmental and fertility endpoints. Researchers concluded that the epidemiologic literature was “consistent with the assessment based on animal toxicity data” and “do not support the hypothesis that DINP causes either developmental or fertility problems in humans”.

Therefore, the human data do not provide support for classification.

Reproductive Outcomes	DBP	DEHP	DINP
Increased fetal death	Yes	**Yes**	No
Increased resorptions	Yes	Yes	No
Reduced fertility index	Yes	**Yes**	No
Reduced spermatogenesis in adults	Yes	**Yes**	No
Severe malformations (cleft palate, neural tube defects)	Yes	**Yes**	No
Skeletal malformations (missing ribs, fused sternebrae)	Yes	**Yes**	No
Severe testicular histopathology adults (e.g. tubular atrophy/degeneration)	Yes	**Yes**	No
Hypospadia	Yes	Yes	No
Cryptorchidism	Yes	Yes	No
Wolffian duct underdeveloped	Yes	Yes	No
Accessory sex organ changes for intact rats (Epididymis, LABC, prostate)	Yes	Yes	No
Delayed puberty	Yes	Yes	No
Testis tubular atrophy (juvenile)	Yes	Yes	No
Reduction in testis weights (juvenile)	Yes	**Yes**	No
Permanent reduction in AGD	Yes	Yes	No
Permanent nipple retention	Yes	Yes	No
Presence of MNGs in testis (juvenile)	Yes	Yes	Yes
AGD decrease in males (juvenile)	Yes	Yes	Yes
Nipple retention (juvenile)	Yes	Yes	Yes
Skeletal variations	Yes	Yes	Yes
Testosterone reduction	Yes	Yes	Yes

fertility index=%matings that result in pregnancy; anogenital distance (AGD); multinucleated gonocytes (MNGs). Reported indicators of effects on sperm following exposure to DINP (Boberg et al. 2011; Kwack et al. 2009) are not included in this table as the study designs are not comparable to those observing effects on spermatogenesis following exposure to DBP and DEHP, see Part 4 Appendices VIII and XI for discussion on these studies.

Table Ia. Qualitative comparison of reproductive test outcomes for DINP with DBP and DEHP shows DINP does not cause adverse reproductive effects comparable to LMW phthalates

DINP has been studied extensively in guideline and non-guideline studies for reproductive toxicity. DINP consistently fails to induce the adverse effects identified in similar studies conducted with LMW phthalates such as DEHP and DBP. LMW phthalates produce significant and consistent adverse effects relevant to classification. DINP causes small and inconsistent changes that fail to meet the WHO definition of adversity and are not relevant for classification when following the CLP criteria (section 3.7.2.2 of Annex I of the CLP). DINP also fails to induce the adverse effects that provided the basis for classification of DEHP according to ECBI/37/99-Add.55 (as shown in **Bold**).

B. Based on sufficient data, adverse effects on fertility are not observed following exposure to DINP and classification per CLP is not warranted.

The results of one- and two-generation guideline GLP reproductive toxicity studies show that DINP does not impair male fertility, female fertility, mating behavior, onset of puberty, mating behavior, live births or survival indices. Additionally, DINP does not induce effects of relevance (CLP 3.7.2.5.3) in reproductive tissues following repeated exposure of adult animals in subchronic and chronic studies. Based on this extensive scientific evidence demonstrating a lack of adverse effects on sexual function and fertility following exposure to DINP (as summarized in Figure Ia), per the CLP criteria detailed in section 3.7.2. of Annex I of the CLP, classification for fertility effects is not warranted.

B.1. The extensive evidence in adult animals following repeat exposure to DINP (sub-chronic, chronic, and sub-acute) do not provide some or clear evidence of an adverse effect on sexual function or fertility following exposure to DINP at exposures below the limit dose.

As shown in Figure Ia below, the extensive evidence in adult animals following exposure to DINP (which include assessment of reproductive tissue weights and histopathology) clearly indicates that DINP does not induce effects on reproductive tissues warranting classification. As pointed out by the dossier submitter, some studies have reported an effect on reproductive organ weights. However these effects are infrequently observed across studies, and when reported, primarily occur at doses well above the limit dose and therefore outside the criteria for classification (CLP 3.7.2.5.7). Furthermore, no effects on histopathology in the testes, epididymis, ovary or uterus were observed at doses below the limit dose (while studies with DEHP clearly indicate testicular atrophy in adult animals (ECB 2008), and those with DBP indicate severe testicular atrophy and degeneration (ECB 2004)). This conclusion is consistent with the interpretation of these data reflected in the EU RAR for DINP (ECB, 2003) that no overt toxicity was observed in reproductive organs and no adverse effects on fertility may be anticipated from these studies. Based on CLP criteria, observations in repeated dose toxicity studies above the limit dose (CLP 3.7.2.5.7) and judged unlikely to impair reproductive function (CLP 3.7.2.5.3) do not warrant classification.

B.2. DINP does not impair fertility in one-and two-generation studies.

The potential for DINP to affect sexual function and fertility has been assessed in one- and two-generation reproductive toxicity studies (Waterman et al. 2000 a,b; these studies have also been summarized in the EU RAR for DINP (ECB. 2003). These studies demonstrate that male and female rat reproductive function and structure of reproductive organs are unaffected by exposure to DINP at maternal doses of 555-1,129 mg/kg bw/day during gestation and lactation, respectively, and adult doses as high as 1,676 mg/kg bw/d in males and 1,694 mg/kg bw/d in females. There were no effects on male fertility parameters or reproductive performance in either the parental (P) or first filial (F1) generation. No significant differences in male mating, male fertility, female fertility, female fecundity, or female gestational indices were noted. Number of live births, survival indices, mean days of gestation were unaffected by treatment as well as the mean sex ratio of the treated offspring when compared with controls. These studies demonstrate that adult males (P) exposed to DINP prior to mating are successfully able to reproduce. More importantly,

the reproductive capacity of the F1 generation males, which were exposed to DINP throughout their lifetime, is unaltered. This is consistent with the interpretation reflected in the EU RAR for DINP (ECB, 2003) that the effects observed in Waterman et al. (2000a, b) do not justify classification.

B.3. The existing data on sperm are unreliable and do not support a DINP-mediated effect on fertility.

It is acknowledged that rodents have an excess of sperm in their ejaculates while human males have highly variable sperm counts (OECD, 2008). Thus, in a case of human subfertility it is possible that even a small change in sperm count or sperm motility may lead to infertility. For this reason, a statistically significant change in sperm count in a rodent study may be indicative of a potential effect on fertility in humans (OECD, 2008). However, an observed statistical change in sperm count does not automatically support a conclusion of biological/toxicological relevance; and likewise does not automatically warrant classification under CLP. CLP criteria requires first and foremost that evidence exists to support the effect is causally related to exposure to the chemical (CLP Annex I, section 3.7.2.1.1); and secondarily requires that the effect occurs to a severity and magnitude of toxicological significance (CLP Annex I, section 3.7.2.3.3) indicative of an adverse effect. Furthermore, conclusions on the inherent ability of a chemical to induce a specific adverse effect (CLP Annex I, Section 3.7) should be based upon the available data and an assessment of the total weight of evidence (CLP Annex I, 3.7.2.3.1) . In the base case, the two studies assessing sperm (Kwack et al. 2009; Boberg et al. 2011) have limitations in study design questioning their usefulness for informing the intrinsic properties of DINP and relevance to classification for fertility. As specified in CLP Annex I, section 3.7.1.4, studies involving in utero exposure are of more relevance to classification decisions under development than fertility. Therefore, the developmental toxicity study of Boberg et al. (2011) is not relevant to classification for fertility. Nonetheless, and described in more detail in Part 2 of this submission, the reliability of the sperm measurements in Boberg et al. (2011) are questionable as they fail to meet the minimum quality criteria in controls as required by OECD (2008), and the changes observed are within historical control ranges of the lab. Kwack et al. (2009) only reports on one-dose (500 mg/kg bw/day, no detailed data on test substance supplier and purity) and does not include histopathological examination of testes (see Part 4, Appendix VIII of this submission for more details). The results reported by Kwack et al. (2009) are ambiguous as the data presented are inconsistent with already existing data for some phthalates tested. Consequently, Kwack et al (2009) on its own, is of insufficient reliability to inform a classification decision (See Part 4, Appendix VIII for more discussion on this study). In the presence of data demonstrating an absence of effects on functional fertility and reproduction in the one and two generation reproduction studies on DINP (Waterman et al., 2000 a, b), these low reliability findings are inadequate to justify classification.

B.4. DINP does not induce adverse effects on fertility observed with DEHP and DBP.

In 1999, Sweden developed a classification proposal for DEHP (ECBI/37/99 – Add. 25) under Europe's previous classification system of a Cat 2 (equivalent to Cat1B under the CLP); R60 (May impair fertility). The effects providing the basis for this conclusion included a dose dependent decrease in fertility index in

a continuous breeding study in mice, with a decrease in fertility at ~ 141 mg/kg bw/day (0.1 % in the diet) and no fertile pairs at the highest dose (~600 mg/kg, 0.3% in the diet). In addition all but one of the males had some degree of bilateral atrophy of the seminiferous tubules with decreased sperm motility, decreased sperm concentration and increased incidence of abnormal sperm forms. Significantly reduced weights of the reproductive organs in parental animals of both sexes were also observed at 0.3% in the diet (testes, epididymis, prostate, and seminal vesicles in males and ovaries, oviducts, and uterus in females). Effects from a 13-week sub-chronic study in rats also provided the basis for this conclusion and included a dose dependent increase in incidence of Sertoli cell vacuolization (375.2 mg/kg bw/d) with atrophy of the seminiferous tubules and complete loss of spermatogenesis at the highest dose (375.2 mg/kg bw/d) was observed. Furthermore, DBP was found to alter testicular structure and function (Zhou et al., 2010). As shown in a variety of regulatory and scientific studies, DINP does not lead to such adverse effects.

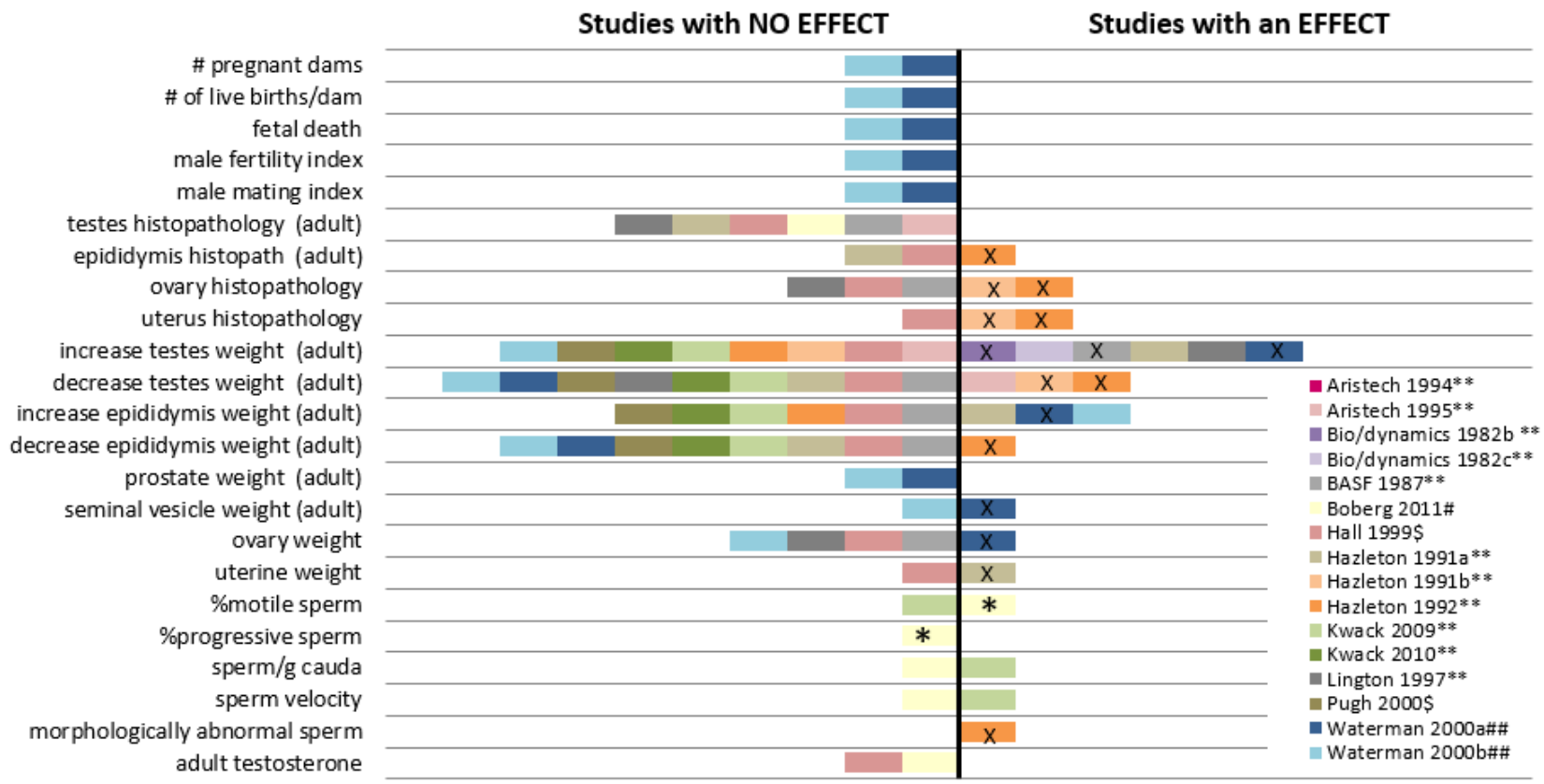

Figure Ia: The evidence for DINP does not support some or clear evidence of an adverse effect on sexual function or fertility following exposure to DINP according to CLP criteria. Studies of relevance to fertility hazard classification were reviewed and observations recorded. Endpoints were captured on the right-hand side of the chart as reporting "an EFFECT" if they were concluded in the study to be either statistically significant or qualitatively significant (for endpoints where statistics were not performed) from controls. Boxes marked with an "X" indicate effects outside the criteria for classification per CLP 3.7.2.5.7-3.7.2.5.9 (occur above a limit dose). Boxes marked with an "∗" indicate endpoints of questionable reliability (as described in Part 4, Appendix XI of this submission); for % progressive sperm the reported statistical significance could not be reproduced following reanalysis and for %motile sperm controls were well below the minimum quality criteria (Morfeld et al. 2016; Chen et al. submitted). In the figure legend, **indicates subacute/subchronic/chronic studies in rodents. $ indicates studies in nonhuman primates; #indicates developmental toxicity studies; ## indicates fertility studies.

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2. STRUCTURED SUMMARY ON DANISH EPA REPRODUCTIVE CLASSIFICATION PROPOSAL - DINP does not fulfill the C&L criteria of Annex I section 3.7.2 of the CLP and therefore does not warrant classification

A. The provided basis for developmental classification category 1B by the dossier submitter does not justify classification as described below.

The "Short summary of the scientific justification for the CLH proposal" from pages 8 –10 of the Danish EPA dossier (points a to i) are repeated below with a short summary as to why these points are not sufficient to warrant classification. Reference is made to more detailed justifications supporting these summary points.

a) ***"Structural abnormalities: skeletal effects (rudimentary ribs) were seen [in] two developmental toxicity studies (Hellwig et al., 1997;Waterman et al., 1999) (1000 mg/kg bw/day),"***

Rudimentary ribs are a common fetal variant, these effects are known to be reversible. Variations should not be mixed up with structural malformations and the wording "*structural abnormalities*" used in the CLH dossier is ambiguous. Variations are of minimal toxicological significance and therefore do not lead to classification. This is explicit in the CLP criteria, (i.e. CLP 3.7.2.3.3 "*[i]n some reproductive toxicity studies in experimental animals the only effects recorded are considered of low or minimal toxicological significance…These effects include...common foetal variants such as are observed in skeletal examinations*"). More discussion on this point can be found in Part 3 of this submission. These effects were already considered in a prior regulatory evaluation and a decision taken that they do not warrant classification (EU Risk Assessment Report (RAR) on DINP (ECB, 2003).

b) ***"Effect on altered growth: decreased body weight in offspring in a two-generation study (Waterman et al, 2000) (from 159 mg/kg bw/day),"***

As indicated by Waterman et al. (2000), the changes to offspring body weights observed in this study during lactation were very likely a consequence of palatability rather than a result of inherent toxicity of DINP and therefore do not warrant classification. Furthermore, the weights were within the historical control range (with the exception of the F2 high-dose males and females on PND 0 and F2 high dose males on PND 1) and the effects were reversible even with continued treatment. The only treatment related toxicological impacts on body weight in this study occurred at a dose of 1.5% at PND 0, the estimated exposure from 1.5% DINP in the diet is approximately 1100 mg/kg/day, i.e. above the limit dose. As effects occurring above the limit dose are outside the criteria for classification (CLP 3.7.2.5.7) and the body weight changes below the limit dose do not reflect inherent toxicity of DINP, these observations are not relevant to a classification decision. This is consistent with the interpretation reflected in the EU RAR on DINP (ECB, 2003) that the effects observed in the Waterman et al. (2000) do not justify classification. More details on this point can be found in Part 4, Appendix III of this submission.

c) ***"Functional deficiency: dose-dependent long-lasting decrease in sperm motility in rat offspring exposed perinatally (Boberg et al., 2011) (from 600 mg/kg bw/day),"***

This statement is not supported by the scientific data as the reported result for DINP (≥ 600 mg/kg bw/day) is 48 % - 52 % motile sperms which is fully within the historical control data range of the lab (61 % ± 14 %), questioning the biological significance of the statistical significance. Further, the low percentage of motile sperm in control animals indicates a problem within the lab with executing the method. A percentage of less than 70 % of motile sperm in the control animals does not fulfill minimum experimental quality criteria of the OECD (OECD, 2008), which is consistent with recommendations in the peer reviewed literature for optimization of experimental protocols for these parameters (Seed et al. 1995). To be eligible for the US NTP program, even 75 % motile sperms (NTP, 2011) in the controls are mandatory. Note the authors did not observe any effects of significance on any other measured sperm parameters, nor histopathology effects in adults (Boberg et al. 2011, 2016, 2017; Morfeld et al. 2017). The authors in fact even reported increased sperm counts for DINP treated animals at the highest dose level tested (900 mg/kg bw/d). The study by Boberg et al. (2011) has also been subject to a Corrigendum (Boberg et al. 2016) and letters to the editor (Morfeld et al. 2017; Boberg et al 2017), with questionable statistical practices being highlighted (see Part 4, Appendix XI for more detail on this point). Therefore, the reliability of this outcome as supporting a chemically mediated effect due to DINP is questionable.

d) ***"Structural abnormalities: increased nipple retention and decreased anogenital distance in infant or prepubertal male rats exposed perinatally (Boberg et al., 2011; Gray et al., 2000, Lee et al., 2006; Clewell et al., 2013b) (mostly from 750 mg/kg bw/day),"***

Nipple retention in juvenile males commonly occurs at a low level in control animals. The toxicological significance of a small increase in the frequency of this effect in juvenile animals is questionable. As discussed in more detail in Part 4, Appendix IV of this submission, the magnitude increase (i.e. severity) in the number of nipples per male (when observed) after DINP exposure was minimal, i.e. 1.98 nipples/male in control animals vs. 3.17 nipples/male in the highest dose group (Boberg et al. 2011). According to OECD (OECD, 2008) *"[p]ermanent nipples in males constitute a permanent structural change, i.e., a malformation*", with measurements in pups allowing for a correlation with parameters recorded in adulthood. Small differences in postnatal developmental assessments that are not correlated to impacts in adulthood are considered of minimal toxicological significance and do not warrant classification (CLP 3.7.2.3.3).

The lack of consistent studies reporting a change on anogenital distance (AGD) following exposure to DINP during the male programming window (i.e. observed in only 1/6 studies in perinatal animals) questions the intrinsic capacity of DINP to modulate this endpoint. An evaluation of the single positive study (Boberg et al. 2011), indicates the magnitude of the observed change in AGD was minimal (i.e. within the range of control variation, see Part 4 Appendix V of this submission) and transient (i.e. does not support a permanent structural change). Furthermore, the effect was only statistically significant when adding the day of measure as a block factor (Boberg et al. 2016, 2017; Morfeld et al. 2017) due to AGD being measured by different technicians on different days. According to OECD (2013) "*it is important that all pups are measured on the same postnatal day because the rapid growth of pups will also affect AGD*". Furthermore, according

to OECD (OECD, 2008), a change in AGD observed at birth and into adulthood constitutes a permanent change in AGD. The available evidence indicates that DINP does not cause a change in AGD in adults (Clewell et al. 2013b, Boberg et al. 2011). Therefore, the single observation of a statistically significant change in AGD in perinatal animals is not itself a determinant of adversity (see Part 4, Appendix V of this submission for further details) and does not warrant classification. One study (Clewell et al. 2013b) observed a statistically significant change at the highest dose (~750mg/kg bw/d) in juvenile males, but did not observe an effect directly after birth in perinatal males nor in adults. A seventh study (Lee et al. 2006) reported statistically significant changes in AGD at all dose levels, however this study is of insufficient reliability to inform a classification decision for a number of reasons as discussed by ECHA (ECHA, 2013) in the evaluation of new scientific evidence on DINP and further detailed in Part 4, Appendix V of this submission (e.g. critical limitations in reporting of the methodology and statistical analysis; as well as stark inconsistencies with the broader literature). Therefore a transient, statistically significant change in AGD reported in only one of six studies does not support that DINP is intrinsically capable of inducing an adverse effect warranting classification (CLP 3.7.2.3.1).

e) ***"Structural abnormalities: increased incidence of permanent changes (permanent nipples, malformations of testes and epididymis, histological changes in testes and epididymides) in rats exposed perinatally (Gray et al., 2000; Masutomi et al., 2003) (at 750 and 1165 mg/kg bw/day, respectively),"***

Two studies (Gray et al. 2000; Boberg et al. 2011) reporting a single adult animal with a larger number of retained nipples is not appropriate for informing a chemicals intrinsic ability to induce the effect, as a low incidence of males with up to 7 retained nipples are known to occur in control animals in the literature.

The scientific data do not support that DINP is intrinsically capable of causing permanent nipple retention for the following reasons:

- the incidence and severity of nipple retention in adults noted in Gray et al. (2000) and Boberg et al. (2011) is very low and comparable to the incidence/severity of nipples that has been reported in controls in the literature;
- the occurrence of retained nipples in adults was not dose responsive in Boberg et al. (2011), where they were not reported at the highest dose tested (Gray et al. (2000) only tested at one dose);
- retained nipples in adults were not observed in a study with much higher statistical power (Clewell et al. 2013b);
- the incidence and severity of nipples reported in Gray et al. and Boberg et al. was substantially lower from what is seen in studies with DBP and DEHP.

Likewise, the collective evidence supports the absence of androgen-mediated adverse morphological and histological effects on reproductive tissue warranting classification according to CLP. The testicular findings in two animals in Gray et al. (2000) (i.e. small testes/epididymis in one animal and epididymal agenesis in another animal) are difficult to interpret within the context of this one exploratory study as they are inconsistent in their nature, and there is a lack of dose response data as DINP was only tested at one dose. The observations reported in Masutomi et al. (2003) were of a different nature than those observed in Gray

et al. (as noted in the discussion section of Masutomi et al.) and consisted of minimal to slight degeneration of meiotic spermatocytes and Sertoli cells, scattered cell debris in ducts in epididymis in male offspring on PND71 at the highest administered dose of DINP of 1165/2657 mg/kg (GD15-20 / PND2-10). These effects were of minimal severity (CLP 3.7.2.3.1), and were only observed above the limit dose (CLP 3.7.2.5.7), and as such do not warrant classification. Importantly, in a robust dietary study using 100 pregnant rats (Clewell et al. 2013b) designed to provide strong statistical power for analyzing malformations of the male reproductive tract including detailed observations into adulthood, no evidence of effects on these tissues were reported on PND49. The minimal severity of the observations reported by Gray et al. (2000) and Masutomi et al. (2003) is corroborative of results from a two-generation reproductive toxicity study showing no functional effect on reproduction following exposure of the F2 generation throughout development (Waterman et al. 2000). On the basis of the available information, the evidence is insufficient to support DINP is intrinsically capable of causing a structural abnormality of a consistency (CLP 3.7.2.3.1) and severity (CLP 3.7.2.3.1) warranting classification.

As presented by the dossier submitter, observations of varying nature, that are insufficiently supported on their own as chemically mediated or of a severity warranting classification are being compiled with the aim of concluding sufficient evidence in support of an adverse effect. However, weight of evidence (CLP 3.7.2.3.1) in the context of CLP classification refers to sufficient evidence in support of a chemically mediated adverse effect from independent sources, or may also refer to a collection of changes observed within one study to support the occurrence of an adverse effect. In the case of DINP, it is clear, that the collective evidence supports the absence of permanent adverse morphological and histological effects on reproductive tissue and classification is not warranted.

f) ***"A comparable pattern of adverse effects and of mode of action as seen for other phthalates classified as reproductive toxicants in category 1B, e.g. DEHP, DBP, DIBP and BBP (Boberg et al., 2011; Borch et al., 2004; Hannas et al., 2011; Clewell et al., 2013a, Li et al., 2015)."***

This statement is not supported by well accepted structure activity relationships or the empirical evidence on DINP. Toxicity to reproduction is highly dependent on the longest linear carbon backbone chain of the esterified alcohol. It is well accepted (e.g. Saillenfait et al., 2011) that for ortho-phthalates, toxicity to reproduction is limited to linear C3-C6 carbon backbones. DINP does not show the developmental malformations or loss of fertility observed with these four phthalates when tested in comparable studies (as summarized in Part 1, Table Ia of this submission). Importantly, exposures of DINP above 1000 mg/kg bw-d during the male programming window fail to induce hypospadias, cryptorchidism, underdeveloped Wolffian ducts, or decreased accessory sex organ weight changes which are observed following exposure to LMW phthalates such as DEHP and DBP. Further discussion on this point can be found in Part 4, Appendix I of this submission.

B. The provided Basis for fertility classification category 2 by the dossier submitter does not justify classification as described below.

g) ***"reduced absolute and relative testes weights at high doses in a 2-year study in mice (Aristech Chemical Corporation, 1995) (742 and 1560 mg/kg bw/day), and at higher doses in studies with shorter durations of exposure, i.e. a 4-week study in mice (Hazleton 1991) (1377 mg/kg bw/day), and a 13-week study in mice (Hazleton 1992) (2600 and 5770 mg/kg bw/day), "***

According to the original study report (Aristech Chemical Corporation, 1995), "the significant decreases in mean absolute and relative to brain weights for the testis/epididymis were an indirect effect resulting from the treatment [explanation: *which caused significant depression of body weight gain]*," The testis/epididymis weights were not decreased on a body weight basis. No histopathological changes were identified for the testes. Further, the high dose of the chronic study with mice as well as all the other cited studies are well above the limit dose. Therefore, these effects do not warrant classification (CLP 3.7.2.5.7-3.7.2.5.9) It should also be noted that these observations are selectively highlighted out of an extensive database within which the majority of studies do not observe a significant reduction of testes weights (as depicted in Part 1, Figure Ia of this submission). These effects were already considered in a prior regulatory evaluation and a decision taken that they do not warrant classification (EU Risk Assessment Report (RAR) on DINP (ECB, 2003).

h) ***"reduced sperm count and effects on sperm motion parameters after 28 days of exposure of juvenile rats (Kwack et al., 2009) (one dose only, 500 mg/kg bw/day), "***

Kwack et al. is of limited reliability to inform a classification decision based on inconsistency of outcomes with broader literature on phthalates tested, limited information on study design, limited endpoints assessed, and only one dose tested. The results reported by Kwack are ambiguous as the data presented are inconsistent with already existing data for some phthalates tested. In the case of DEP, in a full two-generation study no effects on fertility, especially, no effects on the sperm counts and % motile sperm (approx. 85 %) were reported up to the highest dose levels of DEP tested (721 to 1901 mg/kg bw-d for the F0 and F1 generations). In contrast, Kwack et al. (2009) reported a reduction in sperm count and % motility for DEP and MEP (with only MEP being statistically significant). Considering DEP is quantitatively metabolized to MEP (Kao et al., 2012), the results reported by Kwack, i.e. significant reduction in sperm count and % motility for MEP, are inconsistent with the lack of functional fertility deficits for DEP reported in the literature. Furthermore, the observed effects for MEP in Kwack et al. (2009) are comparable to those observed for MEHP, the metabolite of DEHP, which has been shown to functionally impair fertility and is thus classified. Thus the results reported by Kwack et al. (2009) are ambiguous because they do not reproduce well-known scientific results (See Part 4, Appendix VIII for more discussion on this study). In the presence of data demonstrating an absence of effects on fertility in the one and two generation reproduction studies on DINP (Waterman et al., 2000), the low reliability findings reported in Kwack et al. (2009) are insufficient to support a chemically mediated effect that leads to toxicity to fertility and sexual function to justify classification.

i) ***"dose-dependent long-lasting reduced sperm motility in rats exposed perinatally (Boberg et al., 2011) (from 600 mg/kg bw/day),"***

It should be noted that as specified in CLP section 3.7.1.4, for pragmatic reasons, effects induced during in utero exposure are not considered for classification for sexual function and fertility. Such effects are rather evaluated under the developmental toxicity endpoint.

Nonetheless, this statement is not supported by the scientific data as the reported result for DINP (≥ 600 mg/kg bw/day) is 48 % - 52 % motile sperms which is fully within the historical control data range of the lab (61 % ± 14 %), questioning the biological significance of the statistical significance. Further, the low percentage of motile sperm in control animals indicates a problem within the lab with executing the method. A percentage of less than 70 % of motile sperm in the control animals does not fulfill minimum quality criteria of the OECD (OECD, 2008), which is consistent with recommendations in the peer reviewed literature for optimization of experimental protocols for these parameters (Seed et al. 1995). To be eligible for the US NTP program, even 75 % motile sperms (NTP, 2011) in the controls are mandatory. Note the authors did not observe any effects of significance on any other measured sperm parameters, nor histopathology effects in adults (Boberg et al. 2011, 2016; Morfeld et al. 2017). The authors in fact even reported increased sperm counts for DINP treated animals at the highest dose tested (900 mg/kg/day). The study by Boberg et al. (2011) has also been subject to a Corrigendum (2016) and a letter to the editor (Morfeld et al. 2017), with questionable statistical practices being highlighted (see Part 4, Appendix XI for more detail on this point).

C. Conclusion

The results of developmental toxicity studies involving in utero exposure to DINP throughout gestation, during organogenesis, and during the androgen-sensitive male programming window, demonstrate that DINP does not induce adverse effects. Of particular relevance, exposures of DINP up to 1000 mg/kg bw-d during the male programming window fail to induce hypospadias, cryptorchidism, underdeveloped Wolffian ducts, or decreased accessory sex organ weight changes which are observed following exposure to LMW phthalates such as DEHP and DBP. Likewise, exposures of DINP during organogenesis do not induce the severe malformations (cleft palate, neural tube defects) observed following exposure to DEHP and DBP. There are studies reporting that exposure of pregnant dams to DINP during the male programming window leads to slight and reversible changes in biological markers of testosterone perturbation (i.e. delayed regression of the nipple anlagen postnatally) and non-androgenic endpoints (i.e. multi-nucleated gonocytes). However, any observed change (statistically significant or not) does not automatically warrant classification. Only those considered relevant per the criteria outlined in section 3.7.2. of Annex I of CLP justify a classification decision, these criteria include consideration of the nature (CLP 3.7.2.3.1), severity, incidence and toxicological significance (CLP 3.7.2.3.3) of an observation. In the case of DINP, the observed activity does not result in adverse reproductive effects (i.e. effects with functional consequences) as demonstrated in the extensive reproductive toxicity studies on DINP. While these effects are indicators of biological activity, the empirical evidence for DINP supports they are of minimal toxicological consequence and as such do not warrant classification for development.

The dossier submitter emphasizes reduced absolute and relative testes weights and effects on sperm parameters as the key findings supporting a classification conclusion of some evidence of an adverse effect on fertility. These

endpoints have been isolated from the larger knowledge base on DINP and have not been compared to the detailed criteria established under CLP for effects warranting classification. Classification decisions according to CLP should be based upon the available data and an assessment of the total weight of evidence (CLP, section 3.7.2.3.1). Additionally, the consistency, nature and severity of effects, level of statistical significance, toxicological significance and occurrence below a limit dose must be considered (CLP 3.7.2). Therefore, classification decisions are to be based only on changes that have been consistently observed and of a nature and severity that affect the ongoing functioning of the organism. Extensive data in adult animals together with the results from one-and two-generation reproductive toxicity studies, show a lack of adverse effects to fertility and sexual function and therefore classification for fertility is not warranted.

Table IIa: Interpretation of key findings proposed by the dossier submitter in the context of CLP criteria for developmental toxicity

Endpoint	Assessment under CLP criteria	Brief Justification
a) Rudimentary ribs	**Classification not warranted** Effect outside of CLP criteria (3.7.2.3.3) for classification based on established categorization as a variation.	The insufficiency of these observations to justify classification is consistent with the decision reflected in the EU RAR on DINP (ECB, 2003), i.e. do not warrant classification.
b) Decreased body weight	**Classification not warranted** Body weight effects are due to decreased food consumption and therefore outside CLP criteria (3.7.2.2.1) as they do not reflect an intrinsic property of DINP. Effects considered treatment related only occur above the limit dose (CLP 3.7.2.5.7-9).	The insufficiency of these observations to justify classification is consistent with the decision reflected in the EU RAR on DINP (ECB, 2003), i.e. do not warrant classification.
c) Dose dependent decrease in sperm motility	**Classification not warranted** Effects outside CLP criteria for classification based on study quality (CLP 3.7.2.3.1) and effects occurring within historical control ranges (CLP 3.7.2.3.3).	The control value for sperm motility in the study reporting this effect (Boberg et al. 2011) does not meet the OECD minimum requirements, questioning optimization of the experimental protocol and the reliability of outcomes for informing classification. Further, observed changes reported for DINP are within historical control ranges of the lab.
d) Nipple retention	**Classification not warranted** Small differences (i.e. low magnitude/incidence of effect) in postnatal developmental assessments are of minimal toxicological significance and are outside of CLP criteria (3.7.2.3.3)	The magnitude of the induced effect in juveniles (Boberg et al. 2011) is minimal (1.98 nipples/male in control animals vs. 3.17 nipples/male in the highest dose group). OECD guidance (OECD, 2008) states permanent nipples in adult males constitutes a permanent structural change. Permanent nipple retention has not been observed (see lower box in this table).
d) Decreased AGD	**Classification not warranted** Effect outside CLP criteria (CLP 3.7.2.3.1) as it is not a replicable finding (observed in only in 1/6 studies in perinatal animals)	In the presence of 5 studies reporting no effect on AGD in perinatal animals, a single statistically significant finding (Boberg et al. 2011) in perinatal animals is insufficient to conclude the effect is treatment related. OECD guidance (2008) states permanent change in AGD into adulthood as indicative of a structural change. Permanent changes in AGD are not observed (Clewell *et al.* 2013b, Boberg *et al.* 2011).
e) Permanent nipples	**Classification not warranted** Effects outside CLP criteria based on lack of dose response and severity/incidence within control ranges (CLP 3.7.2.3.3).	Gray et al. 2000 tested one dose, and reported a very low incidence; Boberg et al. 2011 reported a very low incidence in the low- and mid- dose animals but not at the high dose. The incidence and severity in these studies is within that reported in literature controls. Permanent nipples were not observed in a large study by Clewell et al. 2013b.
e) Permanent malformations/ histological changes in testes	**Classification not warranted** Effects outside CLP criteria based on the infrequent and inconsistent nature of observations between studies, minimal severity (CLP 3.7.2.3.1), and occurrence above the limit dose (CLP 3.7.2.5.7-3.7.2.5.9).	Gray et al. reported effects in 2 animals that differed in nature (small, malformed testes w/ atrophic tubules, small epididymis in one male; epididymal agenesis, fluid filled testes in one male). Masutomi et al. reported different effects from Gray (i.e. minimal to slight vacuolar degeneration of Sertoli cells) only above the limit dose (>1000mg/kg bw/d). No permanent malformations or histology were observed in 2 studies (Clewell et al. 2013b; Boberg et al. 2011).
f) Comparable pattern of adverse effects and mode of action w/ other phthalates	This statement is not supported by the empirical evidence on DINP. Anticipation of effects from higher exposures does not justify classification as effects above the limit dose are outside CLP criteria for classification (CLP 3.7.2.5.7-9) and classification is to be based on the available data.	Harmonised classification decisions are to be taken on a case-by-case basis (per Recital 52 of the CLP regulation). The database on DINP is extensive: the substance specific data need to be considered and read-across is not appropriate (i.e ECHA RAFF (2017): read-across is intended to fill data gaps). DINP has been shown to impact a testosterone to an extent insufficient for leading to adverse effects. Adverse effects are not observed for DINP.

Table IIb: Interpretation of key findings proposed by the dossier submitter in the context of CLP criteria for fertility

Endpoint	Assessment under CLP criteria	Brief Justification
g) Reduced absolute and relative testes weights	**Classification not warranted** Effect is outside of CLP criteria for classification based on occurrence above the limit dose (CLP 3.7.2.5.7-9), lack of effect when corrected for body weight (CLP 3.7.2.3.3), low severity, and marked inconsistency with collective DINP database (CLP 3.7.2.3.1)	The insufficiency of these observations to justify classification is consistent with the decision reflected in the EU RAR on DINP (ECB, 2003), i.e. do not warrant classification
h) Reduced sperm count and effects on sperm motion parameters	**Classification not warranted** Study quality and reliability too limited to inform a classification decision (CLP 3.7.2.3.1).	In the presence of data demonstrating an absence of effects on fertility in the one and two generation reproduction studies on DINP (Waterman et al., 2000), these low reliability findings are inadequate to justify classification. Kwack is considered of low reliability due to lack of expected outcomes for other tested substances in the study, and limitations in scope and reporting of methodology.
i) Dose dependent long lasting reduced sperm motility	**Classification not warranted** As specified in CLP section 3.7.1.4, for pragmatic reasons effects induced during in utero exposure are not relevant to classification decisions on fertility but should be considered under development.	Refer to Table IIa, above for lack of relevance to developmental classification.

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3. DETAILED COMMENTS ON PROPOSAL

See Part 4, Appendix XIII for the full list of references supporting the comments made in this section of the submission

Test Substance Identity and Characterisation Is Critical for Interpretation

EU RAR on DINP (ECB, 2003) starts with an explanatory note (page VII) explaining the importance of the isomer composition of the DINPs and explaining why one risk assessment was undertaken. The isomer composition which is based on the starting materials can have an important influence on the toxicological properties. Page 9 and 10 of the EU RAR (ECB, 2003) described the production process and resulting DINPs in detail. The DINP 3 has not been included in the EU RAR (2003) but is mentioned, e.g. on page 233:

"*DINP CAS 28553-12-0 (Palatinol DN, DINP3) results: results for DINP3 are reported here to show the difference among the others, but it is not included in the risk characterisation report, because not any more manufactured since 1995 (cf. Explanatory note*)."

Having in mind that isomer composition of DINP is crucial, it is hard to understand why the DTU lab used since 2004 (Borch et al., 2004) a DINP from Sigma Aldrich, product number 376663 (and referred to in either Borch et al., 2004 nor Boberg et al., 2011 publications) without further detailed characterization. The source of the DINP from Sigma-Aldrich is unclear as the supplier claimed confidential business information upon a request by ECPI (now European Plasticisers) in 2016.
All of the DTU publications (Borch et al., 2004; /Boberg et al., 2011) give a purity of greater than 99% and refer to the supplier (Sigma-Aldrich).

Imbedded Safety Data Sheet of Sigma-Aldrich MSDS:

However, if we check the Safety Data Sheet MSDS of Sigma-Aldrich for DINP, product number 376663, Section 3 of the MSDS indicates **DEHP, CAS Nr. 117-81-7, as an impurity:.**

SECTION 3: Composition/information on ingredients

3.2 Mixtures

Formula : $C_{26}H_{42}O_4$

Molecular weight : 418,61 g/mol

Hazardous ingredients according to Regulation (EC) No 1272/2008

Component		Classification	Concentration
bis(2-Ethylhexyl) phthalate Included in the Candidate List of Substances of Very High Concern (SVHC) according to Regulation (EC) No. 1907/2006 (REACH)			
CAS-No.	117-81-7	Repr. 1B; H360FD	< 0,3 %
EC-No.	204-211-0		
Index-No.	607-317-00-9		
Registration number	01-2119484611-38-XXXX		
bis(2-Ethylhexyl) phthalate Included in the Candidate List of Substances of Very High Concern (SVHC) according to Regulation (EC) No. 1907/2006 (REACH)			
CAS-No.	117-81-7	Repr. 1B; H360FD	>= 0,1 - < 0,3 %
EC-No.	204-211-0		
Index-No.	607-317-00-9		
Registration number	01-2119484611-38-XXXX		

For the full text of the H-Statements mentioned in this Section, see Section 16.

The product streams coming from the high production volume processes leading to DINP1, CAS No 68515-48-0 or DINP2, CAS No 28553-12-0, do not contain DEHP as an impurity. Some other studies (e.g. Kwack et al., 2009) used material from laboratory chemical distributors without chemical characterization that is mandatory for regulatory GLP guideline studies.

European Plasticisers recommends that any of these academic studies with insufficient substance characterisation be interpreted with extreme care as based on the EU RAR on DINP (ECB, 2003) the isomer composition of the product streams is of utmost importance.

Generic statement regarding the use of the right fixative for testes.

The CLH proposal states several times that formaldehyde fixation of the testes would not be sensitive enough, e.g. on page 20 regarding the Hellwig et al.(1997) study: “Histological examination of testes and ovaries showed no adverse changes (but method is not considered sensitive due to fixation in formaldehyde and not Bouin’s fixative”.

Such a generic statement must be rejected as unjustified. End of the 1990’s, Bouin’s fixative was promoted to have some advantages. However, there are several basic publications on the adverse effects of low molecular weight phthalates where formaldehyde fixation was used and clear adverse effects on testes morphology and histopathology were reported (e.g. Foster et al. ,1980 and 2001; Gray et al. 1999). Further, it should be kept in mind that the highest dose level tested was 1000 mg/kg bw/d. Today’s preferred fixative for testes is modified Davidson’s fixative (also referred to as Hartmann’s (Harleman et al., 1997) fixative) and no more Bouin’s, despite the fact that it is still listed in current testing guidelines.

CLH dossier lacks in-depth discussion on species differences (CLP 3.7.2.3.2)

CLH dossier lacks an in-depth discussion on species differences regarding metabolism of DINP and species differences. While metabolites in rats are in the free form in blood and urine, in humans, the metabolites are predominantly existing in the glucuronidated form (Albro, 1986). As the glucuronidated monoester may not be active this could have a major impact on the risk assessment.

PAGE-BY-PAGE DETAILED COMMENTS

Page 8 and page 56

"Structural abnormalities: skeletal effects (rudimentary ribs) were seen in two developmental toxicity studies (Hellwig, 1997; Waterman; 1999)"

Remark: Rudimentary ribs are a common fetal variant, these effects are known to be reversible. Variations should not be mixed up with structural malformations and the wording "*structural abnormalities*" used in the CLH dossier is ambiguous. Variations are of minimal toxicological significance and therefore do not lead to classification. This is explicit in the CLP criteria (.e. CLP 3.7.2.3.3 "*[i]n some reproductive toxicity studies in experimental animals the only effects recorded are considered of low or minimal toxicological significance…These effects include...common foetal variants such as are observed in skeletal examinations*").

Distinguishing between malformations and variations is discussed in OECD guidance 43 (OECD, 2008), with a clear recommendation for reliance on study author interpretation, as follows:
Malformations versus Variations (*page 27* ***of OECD*** *guidance 43****, 2008)***
"Classification of foetal and neonatal observations into malformations and variations is a common practice. *A commonly used definition of a* ***malformation is a permanent structural change****, which may* ***adversely affect survival, development, or function****, while a common definition of a* ***variation is a divergence beyond the usual range of structural constitution****, which may not adversely affect survival or health (US EPA, 1991; Chahoud et al., 1999; Solecki et al., 2001, 2003)".*

Regarding Hellwig et al. (1997), the only substance-related foetal effects for DINP1 and DINP2 were increased incidences of a skeletal variation [accessory 14th rib(s)]. This is a variation and not a malformation, i.e. it is not a structural abnormality in the sense of Annex I of the CLP and therefore does not support classification and labelling as a developmental effect. It is unclear how the dossier submitter can stray from the interpretation of the study authors, who have clearly identified this change as a variation.
Another DINP tested, i.e**. DINP3** which was based on n-/iso butenes and consequently had a high degree of branching in the alcohols, is not on the market and has therefore consequently already been excluded from the EU Risk assessment of the DINPs in 2003 (cf explanatory note in the EU RAR (ECB, 2003). **Results from DINP3 cannot be taken into account for the evaluation of DINP1 or DINP2** as the alcohols in the DINP3 consisted of at least 60 % alkyl-substituted Hexanols, while in the other two DINPs the alcohols consist of substituted heptanols and octanols (please refer to EU RAR, 2003). This is consistent with the structure activity relationships described elsewhere in these comments, where adverse reproductive effects are seen with alkyl phthalates with straight carbon backbone chains of C3-C6 carbons in the alkyl side chains, and where adverse reproductive effects are not seen with alkyl phthalates with straight carbon backbone chains of C7-C13 carbons in the alkyl side chains

In the discussion, Hellwig et al. concluded:
"The failure of [….] DINP to induce significant developmental toxicity may be due to several reasons such as the intrinsic' impact of the alcohol moiety, namely type and degree of branching , a lower resorption rate and lower peak level s of the relevant agent, and/or to lower interference with an essential homoeostatic mechanism"……

Page 8

"Effect on altered growth: decreased body weight in offspring in a two-generation study (Waterman et al, 2000) (from 159 mg/kg bw/day)"

Remark: As described briefly in Part 2, point b and discussed in more detail in Part 4, Appendix III of this submission, and noted by the dossier submitters on page 30 in the CLH proposal, the changes to offspring bodyweight observed in Waterman et al. (2000) during lactation were likely a consequence of palatability rather than a result of inherent toxicity of DINP and all weights of all F1 and F2 treated offspring were in the historical control range of the laboratory with the exception of some of the high dose animals (~1100 mg/kg bw/d) at two time points. Therefore it is unclear how these changes are concluded as indicative of inherent toxicity of DINP and relevant to a classification decision. This is consistent with the interpretation reflected in the EU RAR on DINP (ECB, 2003) that the effects observed in the Waterman et al. (2000) do not justify classification.

Page 8, page 9 and page 56 -

Page 8-*"A comparable pattern of adverse effects and of mode of action as seen for other phthalates classified as reproductive toxicants in category 1B, e.g. DEHP, DBP, DIBP and BBP (Boberg et al., 2011; Borch et al., 2004; Hannas et al., 2011; Clewell et al., 2013a, Li et al., 2015)."*

Page 9-*"The structural and functional adverse effects and the mode of action of DINP are comparable to those of other phthalates classified as reproductive toxicants. DEHP, DBP, DIBP and BBP are classified as reproductive toxicants based on the same developmental effects listed above for DINP (ECHA 2008a, b, c, ECHA 2014)."*

Page 56-*"A comparable pattern of adverse effects and of mode of action as seen for the reproductive toxicants (category 1B) DEHP, DBP, DIBP and BBP. In foetal testes, several studies describe presence of multinucleated gonocytes and reduced testosterone production, as also described for DEHP, DBP, DIBP and BBP (Boberg et al., 2011; Borch et al., 2004; Hannas et al., 2011; Clewell et al., 2013, Li et al., 2015)."*

Remark: The effects of DINP are not comparable to those seen with the classified low molecular weight phthalates such as DEHP, DBP, DIBP and BBP as is inaccurately stated in the dossier on numerous occasions. DINP does not show the developmental malformations or loss of fertility observed with these four phthalates when tested in comparable studies. Importantly, exposures of DINP (including doses above 1000mg/kg bw/d) during the male programming window fail to induce hypospadias, cryptorchidism, under developed Wolffian duct, or decreased accessory sex organ weight changes which are observed following exposure to LMW phthalates such as DEHP and DBP. Furthermore, the effects that provided the basis for classification of DEHP (and presumably DBP, DIBP and BBP) are not being accurately or transparently portrayed. This is important as there are considerable differences in the nature, incidence, magnitude, severity, variability and reproducibility between the observations in studies following exposure to DINP and DEHP for example. The following effects are seen with DEHP and are the basis for the classification decision for this substance (ECBI/37/99-Add.55): cleft palate, neural tube defects, testicular tubular atrophy, complete ablation of spermatogenesis, fetal death. These effects are not seen with DINP, therefore the referenced studies provided in the quotes from the CLH proposal referenced above are not appropriate. Please refer to Part 4, Appendix I of this submission for additional details on this point.

Moreover, the mode(s) of action (MOA) leading to the anti-androgenic mediated effects included in the hypothesized "rat phthalate syndrome" are not fully elucidated (Foster, 2005;, Makris *et al.,* 2015;, Euling *et al.,* 2015) as discussed by ECHA (2013) in the evaluation of new scientific evidence on DINP. At best, there is evidence that DINP impacts a common measurable event (i.e. testosterone). As events can be common to more than one MOA; multiple MOAs can be linked via common events in a complex network; and key events are necessary but not necessarily sufficient for defining a MOA it is not evident that DINP shares a common MOA. Furthermore, a simple potency extrapolation from reductions in testosterone to adverse outcomes is not supported by the extensive empirical data for DINP. More discussion on this can be found in Part 4, Appendices I and IX of this submission.

The manner in which the statement on **page 56** of the CLH proposal (quoted above) is written and referenced is misleading., Hannas et al. (2011) reports only fetal testis testosterone production, StAR mRNA levels, and Cyp11a mRNA levels after exposure to DINP, and does not constitute support for reproductive effects of DINP (they are listed elsewhere in the proposal as evidence for developmental toxicity) as it did not measure MNGs or testes histopathology. Similarly, Borch et al. (2004) did not report on adverse effects but only on testosterone; and Li et al. (2015) reports on RNA modifications. See Part 4, Appendix I of this submission for more discussion on comparability of effects between DINP and DEHP and DBP.

Additionally, the statement on **page 56** of the CLH proposal (quoted above) mischaracterizes MNGs and testosterone as adverse effects. With respect to MNGs (and described in Part 4 Appendix VII of this submission) they are not considered adverse as they are eliminated from the seminiferous epithelium within 1–2 weeks postnatally (Johnson *et al.,* 2012), and do not manifest into any observable functional consequence. **The dossier submitters should provide the definition of adversity that serves as their basis for conclusions**. With respect to testosterone, while a reduction in testosterone may be interpreted as a marker for an anti-androgenic effect, as stated by ECHA in the evaluation of new scientific evidence on DINP (ECHA, 2013) "*the adversity of this effect depends on the impact it has on the sexual development, sexual behaviour, hormonal control, sperm parameters, functional fertility and structural abnormalities in later life*". Extrapolating reversible effects on testosterone to adverse outcomes for DINP is counter to what the collective empirical data on LMW phthalates and DINP support in longer term studies that assess functional outcomes as discussed in more detail in Part 4, Appendix I of this submission. Therefore, while effects on both testosterone and MNGs are observed following exposure to DINP, these effects are not themselves determinants of adversity particularly in the context of the CLP criteria and the WHO (2004) definition for adversity (i.e. A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences).

The observation of reversible endocrine activity without adverse reproductive effects observed following in utero exposure to DINP, is consistent with what the structure activity relationship associated with low molecular weight and high molecular weight phthalates would predict. But these observations are by no means comparable to the adverse effects that provide the basis for classification of the LMW phthalates. Again this is discussed in more detail in Part 4 Appendix I of this submission. Extrapolating from indicators of endocrine activity to support a classification determination is not warranted as it is not consistent with the empirical data on DINP demonstrating a lack of adverse effects; nor is it consistent with what we know about

the potency relationship between decreases in testosterone and adverse outcomes (Hannas et al., 2011; Furr et al., 2014; Boberg et al., 2011; Clewell, 2013a; Gray et al., 2016; and Zirken et al., 1989)

Page 8

"*Structural abnormalities: increased nipple retention and decreased anogenital distance in infant or prepubertal male rats exposed perinatally (Boberg et al., 2011; Gray et al., 2000, Lee et al., 2006; Clewell et al., 2013b) (mostly from 750 mg/kg bw/day),*"

Remark: Lee et al. (2006) did not report food consumption, thus so mg/kg/day cannot be calculated and "(mostly from 750 mg/kg/day)" is an inaccurate representation. The proposal submitter repeats this statement on **page 56.**

ECHA discussed the deficiencies in the Lee et al., (2006) study in the 2013 evaluation of new scientific evidence concerning DINP and DIDP (ECHA, 2013) in relation to entry 52 of Annex XVII to REACH regulation (EC) No 1907/2006 and as stated in Table 12 of CLH proposal concluded "*This study was not considered sufficient by ECHA 2013 to change the developmental NOAEL. No details on corrections for litter effects."* Referencing a study that contains such deficiencies as reported by ECHA as a key study for a key finding in support of a classification decision is not appropriate (see Recital 21 of the CLP regulation referring to the importance of quality and comparability of results in the basis of classification decisions). The CLH proposal also cites the following concluding comment from ECHA on **page 41** of the CLH proposal: "In the ECHA 2013 review, the limitations of this study are discussed with the conclusion that the drastically reduced female sexual behavior observed in this study need to be followed up before any firm conclusions can be drawn." ECHA's statement is in reference to two separate Lee et al., (2006) studies only one of which was reviewed and included in the CLH proposal (referenced in these comments as Lee et al. 2006). The second Lee et al., publication (not included in the reference list of these comments and therefore provided here i.e. Lee et al. (2006) Effects of phthalate/adipate esters exposure during perinatal period on reproductive function after maturation in rats. J Anim Sci & Technol 48: 651-662) was not included in the CLH proposal and contains information on the study design and additional findings. For transparency ECHA's commentary to both Lee et al., (2006) publications and final concluding sentence for these studies are as follows: "In conclusion, there is limited evidence that perinatal exposure to DINP (as well as to DBP and DEHA) change the expression of grn and/or p130 genes in the hypothalamus in neonatal animals might lead to decreased sexual behavior after maturation, without affecting the endocrine system of the HPG axis. DINP seems to have anti-androgenic activity causing reduced AGD in males but also weak androgenic activity increasing the AGD in females. For several reasons, however, the results of this study are not considered the primary driving force for the NOAEL/LOAEL setting: 1) there are critical limitations in reporting of the methodology and statistical analysis relating to the findings, such as reduced AGD in males and lordosis quotient; 2) the low LOAEL for AGD is not supported by other studies (Boberg et al. 2011; Clewell et al. 2011b) and the change in AGD is minor; 3) the gene expression findings show no clear dose response and would need to be confirmed; 4) the reduced lordosis reflex warrants replication due to limitations in the methodology and statistical analysis; 5) The rather low lordosis quotient of control females (75%) may indicate that the timing of the measurement may not have been optimal for females (proestrous); 6) measurement of lordosis reflex is not included in internationally accepted standard test methods; and 7) the results from one- and two-generation reproductive toxicity studies do not indicate affected fertility it is however acknowledged that fertility parameters measured in the available one- and two-generation reproductive toxicity studies may not adequately reflect lordosis quotient. Mating and pregnancy

may be successful in spite of reduced lordosis reflex. Overall, the drastically reduced female sexual behavior observed in this study need to be followed up before any firm conclusions can be drawn."

Page 8

"Structural abnormalities: increased incidence of permanent changes (permanent nipples, malformations of testes and epididymis, histological changes in testes and epididymides) in rats exposed perinatally (Gray et al., 2000; Masutomi et al., 2003) (at 750 and 1165 mg/kg bw/day, respectively),"

Remark: The manner in which this point is referenced is misleading as Masutomi et al. (2003) did not report observations of nipple retention.

Furthermore, the nature of this statement and subsequent brief narrative is misleading as to the nature and consistency of the observations supporting a conclusion of DINP-mediated 'structural abnormalities'. It would improve understanding of the relevance and weight of the reported histology and gross malformation findings to a classification decision if information on the severity of these effects was reported consistently and transparently throughout the CLH proposal. For example, additional detail similar to that reported in the narrative on **page 39** of the CLH proposal should be included here in the summaries i.e. the observations reported in Masutomi et al. (2003) consisted of minimal to slight changes that reached statistical significance only at the highest administered dose of DINP of 1165/2657 mg/kg. Furthermore and as noted in the ECHA (2013) evaluation of new scientific evidence on DINP, these effects were only observed at dose levels causing systemic toxicity in the dams. Additionally a statement regarding how the effects are consistent with one another between the exploratory Gray et al. (2000) study and Masutomi et al. (2003) should be provided, i.e. the effects reported by Masutomi et al. are of a different nature than those observed in Gray et al. (as noted by the authors in the discussion section of Masutomi et al., 2003). This detail is not captured in the summary sections of the CLH proposal and therefore a through, descriptive and sound scientific rationale regarding conclusions of adversity on the study level, nor integration across studies has not been provided to justify a classification decision based on these observations. Furthermore, the nature of these observations in the context of those expected to result from the postulated anti-androgenic mode of action should be provided (i.e. how do these compare to the observations observed for 'other phthalates'). As discussed in more detail in Part 4, Appendix VI of this submission, the evidence is insufficient to support DINP is intrinsically capable of causing a structural abnormality of a consistency (CLP 3.7.2.3.1), severity (CLP 3.7.2.3.1) and toxicological significance (CLP 3.7.2.3.3) warranting classification.

Page 9 and 58

g) " reduced absolute and relative testes weights at high doses in a 2-year study in mice (Aristech Chemical Corporation, 1995) (742 and 1560 mg/kg bw/day)……"

Remark: Summary table from CERHR Monograph DINP (2003) regarding the Carcinogenicity study with DINP on B6C3F1 mice (referred to as Moore et al., 2001, or Aristech study, 1995).

DINP was administered *daily* in the diet to B6C3Fl mice for at least 104 weeks at concentrations of 0, 500, 1,500, 4,000, and 8,000 ppm (Groups 1, 2, 3, 4, and 5, respectively). Mice in Group 6 were administered DINP

at a dietary concentration of 8,000 ppm for 78 weeks followed by a 26-week recovery period, during which they were administered the basal diet alone.

Summary Table from the US CERHR Monograph on DINP (2003).

Table 7-4: DINP, General Toxicity, Mice

Species, Strain, and Source	*Experimental Regimen*	*Animal Number/ Sex*	*Dose**	*Body Weight*	*Organ Weight****	*Liver Effects*	*Hematology*	*Other*
$B6C3F_1$/ CrlBR mice Moore 1998 (4)	Chronic study: 2 years. 6-week-old mice were fed diets with 0, 500, 1,500, 4,000, and 8,000 ppm DINP. 15 mice/dose/ sex were evaluated and sacrificed at 79 weeks and 55 mice sex/group at 105–106 weeks. Clinical evaluations (hematology, serum chemistry, and urinalysis) were conducted every 26 weeks. Peroxisome proliferation was examined in 5 mice/sex in the highest dose group and controls during the midpoint and end of study.	70	0					
		70	90.3(M) 112(F)	NE	NE	NE	NE	NOAEL (F)
		70	276(M) 336(F)	NE	NE	↑Neoplasia (F)	NE	NOAEL (M)
		70	742(M) 910(F)	↓ (wk 1–104)	↓Ki(M), ↑Li(M) (wk 79-104)	↑Neoplasia (M)	NE	NE
		70	1,560(M) 1,888(F)	↓ (wk 1–104)	↓Ki (M), ↑Li (wk 79-104)	↑Neoplasia and non-neoplastic changes ↑Serum ASAT, ALAT (M) ↑PCoA (wk 79–104)	↓WBC (wk 26–98)	↓Survival (M) ↑Nephropathy(F) ↑Serum protein (M, week 104) ↑Urinary vol with ↓Na, Cl, K (week 52–104) No effects on testicular histology
	A group of 55 mice/sex was exposed to the high dose for 78 weeks and sacrificed at 105–106 weeks to study recovery effects.**	55	1,377(M) 1,581(F)	↓(M)	↓Ki(M)	↑Neoplasia	NE	NE

*Dose measured in mg/kg bw/day.
**Only effects observed by week 104 listed
***Organ to body weight ratio
↑–Statistically Significant Increase
↓–Statistically Significant Decrease

NE–No Effects
F–Female
M–Male
wk–Week
vol–Volume

Ki–Kidney
Li–Liver
WBC–White Blood Cell

ALAT–Alanine aminotransferase
ASAT–Aspartate aminotransferase
PCoA–Palmitoyl-CoA Oxidase

Cl–Chloride
K–Potassium
Na–Sodium

II-41

Appendix II

According to the original study report by Covance, ***"the significant decreases in mean absolute and relative to brain weights for the testis/epididymis were an indirect effect resulting from the treatment [which caused] significant depression of body weight gain,"*** **[not decreased on a body weight basis].** No histopathological changes were identified for the testes. Further, the high dose of the chronic study with mice is well above the limit dose and are therefore not relevant for classification (CLP 3.7.2.5.7.)

The study has already been evaluated within the EU RAR, there is no need to re-discuss the results again as a formal C&L check has been completed by the Technical Progress Committee at that time. Citations from the original Aristech study report (COVANCE STUDY NUMBER: 2598-105), reports are now owned by BASF and report as follows:

> **Page 56 of Aristech report:** "*In male mice at the Terminal Sacrifice,* ***decreased*** *mean kidney (Groups 3-6) and* ***testes (Groups 4-6) weights*** *were noted* ***with no histomorphologic correlate****. Increased mean liver weights (absolute and relative) were noted in male mice of Groups 4 and 5 and correlated with test article-related findings*"

Page 57 of Aristech report: *"Sections of testes with epididymides* ***revealed no evidence of test article effect on spermatogenic activity."***

While with DINP there were no effects on testicular histology, with **DEHP** you can see clear effects (Reel et al., 1984; Lamb et al., 1987) on testicular histopathology and function (unfortunately, these were continuous breeding protocols i.e. functional fertility tests. However, if there was an effect, the highest dose group was subject to histopathology. In the Lamb study (1987), dietary concentration of 0.3 % (ca. 425 mg/kg bw/d.) resulted in histopathological changes (bilateral atrophy of the seminiferous tubules).

Please note that based on a significant body weight reduction at the highest dose level, the testicular weights were also found to be decreased, however, not on an organ weight to body weight ratio, only with reference to brain weight.:

Other statistically significant mean organ weight changes **were decreased testis weights** (absolute and relative to brain weight) in Groups 4, 5, and 6, **with no histomorphologic correlate**; increased lung-to body-weight ratio for Group 5 and 6 males; and increased brain-to-bodyweight ratio for Group 4, 5, and 6 males.

Other than liver and kidney, there was no histopathologic evidence of a treatment-related effect in any other tissues. In Group 4, 5, and 6 males killed at study termination, mean absolute and relative to brain weights **for testis/epididymis**, as well as the mean terminal body weight were significantly decreased compared to the control male values, **but organ-to-body-weight ratios were similar.**

From Pages 304/305 of the study report:

TABLE 10B
ONCOGENICITY STUDY IN MICE WITH DI(ISONONYL)PHTHALATE INCLUDING ANCILLARY HEPATOCELLULAR PROLIFERATION AND BIOCHEMICAL ANALYSES
ORGAN WEIGHT DATA

TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=T;SUBSET=ALL

TESTIS/EPIDIDYMIDES

SEX	DOSE GROUP		TERMINAL BODY WT (g)	ORGAN WEIGHT (g)	ORGAN-TO-BODY WT (%)	ORGAN-TO-BRAIN WT RATIO
M	1					-RT-
		NUMBER IN GROUP:	46	42	42	42
		MEAN:	32.2	0.39	1.222	0.827
		STANDARD DEV:	3.2	0.06	0.154	0.123
M	2					
		NUMBER IN GROUP:	46	41	41	41
		MEAN:	31.4	0.39	1.263	0.821
		STANDARD DEV:	2.8	0.05	0.114	0.105
M	3					
		NUMBER IN GROUP:	41	36	36	36
		MEAN:	31.2	0.38	1.250	0.803
		STANDARD DEV:	3.5	0.06	0.136	0.120
M	4					
		NUMBER IN GROUP:	40	35	35	35
		MEAN:	29.0*	0.35*	1.262	0.735*
		STANDARD DEV:	3.6	0.05	0.150	0.103

* Significantly different from control value, $p \leq 0.05$.

ORGAN WEIGHT DATA

TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=T;SUBSET=ALL

TESTIS/EPIDIDYMIDES

SEX	DOSE GROUP	TERMINAL BODY WT (g)	ORGAN WEIGHT (g)	ORGAN-TO-BODY WT (%)	ORGAN-TO-BRAIN WT RATIO
M	5				RT
	NUMBER IN GROUP:	32	27	27	27
	MEAN:	26.6*	0.31*	1.222	0.660*
	STANDARD DEV:	2.7	0.03	0.100	0.063
M	6				
	NUMBER IN GROUP:	43	38	38	38
	MEAN:	29.2*	0.35*	1.214	0.730*
	STANDARD DEV:	3.1	0.06	0.139	0.120

* Significantly different from control value, $p \leq 0.05$.

Organ data from the interim sacrifice (study week 79):

Covance 2598-105

TABLE 10A
ONCOGENICITY STUDY IN MICE WITH DI(ISONONYL)PHTHALATE INCLUDING ANCILLARY HEPATOCELLULAR PROLIFERATION AND BIOCHEMICAL ANALYSES
ORGAN WEIGHT DATA

TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=1;SUBSET=ALL

TESTIS/EPIDIDYMIDES

SEX	DOSE GROUP	TERMINAL BODY WT (g)	ORGAN WEIGHT (g)	ORGAN-TO-BODY WT (%)	ORGAN-TO-BRAIN WT RATIO
M	1				
	NUMBER IN GROUP:	15	10	10	10
	MEAN:	33.8	0.39	1.202	0.792
	STANDARD DEV:	3.7	0.06	0.238	0.133
M	2				
	NUMBER IN GROUP:	15	10	10	10
	MEAN:	33.1	0.41	1.277	0.858
	STANDARD DEV:	2.4	0.05	0.144	0.100
M	3				
	NUMBER IN GROUP:	15	10	10	10
	MEAN:	32.7	0.37	1.153	0.762
	STANDARD DEV:	2.5	0.02	0.071	0.056
M	4				
	NUMBER IN GROUP:	15	10	10	10
	MEAN:	30.3*	0.37	1.284	0.779
	STANDARD DEV:	2.8	0.04	0.104	0.083
M	5				
	NUMBER IN GROUP:	15	10	10	10
	MEAN:	29.0*	0.35	1.294	0.736
	STANDARD DEV:	3.4	0.03	0.082	0.059

* Significantly different from control value, $p \leq 0.05$.

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After 79 weeks of dietary DINP, body weights were found to be decreased, however, there was no statistical significant testicular weight decrease (neither on organ weight nor on organ weight scaled to body weight or brain)

Organ weight data at terminal sacrifice (105/106 weeks):

Covance 2598-105

TABLE 10B
ONCOGENICITY STUDY IN MICE WITH DI(ISONONYL)PHTHALATE INCLUDING ANCILLARY HEPATOCELLULAR PROLIFERATION AND BIOCHEMICAL ANALYSES
ORGAN WEIGHT DATA

TABLE INCLUDES:
SEX=ALL;GROUP=ALL;WEEKS=ALL
DEATH=T;SUBSET=ALL

TESTIS/EPIDIDYMIDES

SEX DOSE GROUP		TERMINAL BODY WT (g)	ORGAN WEIGHT (g)	ORGAN-TO-BODY WT (%)	ORGAN-TO-BRAIN WT RATIO
					-RT-
M	1				
NUMBER IN GROUP:		46	42	42	42
MEAN:		32.2	0.39	1.222	0.827
STANDARD DEV:		3.2	0.06	0.154	0.123
M	2				
NUMBER IN GROUP:		46	41	41	41
MEAN:		31.4	0.39	1.263	0.821
STANDARD DEV:		2.8	0.05	0.114	0.105
M	3				
NUMBER IN GROUP:		41	36	36	36
MEAN:		31.2	0.38	1.250	0.803
STANDARD DEV:		3.5	0.06	0.136	0.120
M	4				
NUMBER IN GROUP:		40	35	35	35
MEAN:		29.0*	0.35*	1.262	0.735*
STANDARD DEV:		3.6	0.05	0.150	0.103

* Significantly different from control value, $p \leq 0.05$.

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Page 9

"DEHP, DBP, DIBP and BBP are classified as reproductive toxicants based on the same developmental effects listed above for DINP (ECHA 2008a, b, c,…ECHA 2014)"

Remark: The references listed here could not be located. However based on the titles of the references provided it does not appear that they are the original references for the classification basis for DEHP, DBP, DIBP and BBP as they are not RAC nor ECB documents but are references to MSC SVHC reports. The original references for the classification decision are needed to confirm the factual basis for this statement. As was discussed in Part 1 of this submission, and described in detail in Part 4, Appendix I of this submission, the 'same developmental effects' that provided the basis for classification for DEHP (as described in ECBI/37/99 Add 25), for example, are not observed following exposure to DINP.

Page 9 and page 62

Page 9 *"A harmonised classification of the phthalate DCHP (Dicyclohexyl phthalate) as Repr. 1B, H360D (RAC 2015) has recently been adopted (cf. the 9th ATP to the CLP Regulation)….the above mentioned arguments further support classification of DINP in category 1B."*

Page 62 *"Furthermore, a harmonized classification of Dicyclohexylphthalate (DCHP) as toxic to reproduction in category 1B (H360D) has recently been adopted (cf. the 9th ATP to CLP). The classification proposal for DCHP was based on effects that are parallel to those of DINP (RAC 2015). The opinion adopted by RAC for DCHP thus suggests a category 1B classification for developmental toxicity"*

Remark: Harmonised classification decisions are to be taken on a case-by-case basis as stated in Recital 52 of the CLP regulation. The existence of a harmonised classification for DCHP is not a scientific justification for classifying DINP. The database for DINP is much more extensive than that for DCHP and a classification decision on DINP should therefore be based on the empirical data for DINP. If read-across to DCHP is being proposed, the CLH submitter should provide an read-across justification in accordance with the ECHA guidance (ECHA, 2017: https://echa.europa.eu/documents/10162/13628/raaf_en.pdf) including justification for why substance specific data should be overridden by read-across). However, according to ECHA (2017), read-across is intended to fill data gaps and therefore in light of the extensive data on DINP it is unclear why information on DCHP is relevant in this case.

Page15-16

Remark: The TK section should include discussion of the study by Clewell et al (2013a), i.e. Disposition of diisononyl phthalate and its effects on sexual development of the male fetus following repeated dosing in pregnant rats. Reproductive Toxicology 35:56-69. Particularly because these data demonstrate a saturation of oral absorption between 250 and 750 mg/kg bw/d such that the use of higher treatment levels >750mg/kg bw/d not lead to higher systemic absorption. A consideration very relevant to a classification decision.

Furthermore, the CLH dossier lacks an in-depth discussion on species differences regarding metabolism of DINP and species differences. While metabolites in rat are in the free form in blood and urine, in humans, the metabolites are predominantly existing in the glucuronidated form. As the glucuronidated monoester may not be active this could have a major impact on the risk assessment.

Page 17-26

Remark: Chronic toxicity and carcinogenicity studies have no need to be cited in the CLH proposal. Repeated dose toxicity studies up to 13 weeks are relevant for the evaluation of maternal toxicity in the discussion of toxicity to reproduction.

Page 18-22 Table 9

Remark: All Tables in Section 4 of the report and the accompanying narrative lack transparent reporting/discussion of study quality. It is unclear how limitations of low quality/low reliability studies (e.g. Lee et al., 2006) have been considered when determining the weight the outcomes bring to bear on the classification decision. Additionally the tables in the CLH proposal serving to summarise key findings are imbalanced in the reporting of positive and negative outcomes (selective focus on positive outcomes) whereas the most helpful information to inform this decision would be balanced reporting of endpoints of most relevance to the classification determination which include clear indications of incidence, severity and statistical significance, as well as comparison to historical controls; with particular focus of the outcomes for endpoints of relevance to the mode of action for other phthalates

For example:

- **page 22** Hall et al. (1999), reported outcomes are small effects on BW and liver, whereas the lack of any observed effects on testes is not emphasized in this table. The lack of effects on testes are more relevant to this classification decision than the positive effects on liver.

- **Page 22**, Kwack et al. (2009): The derivation of a LOAEL is not feasible because a dose-response curve is not applicable with one dose group.

Page 22, Table 9 and **page 60**

Page 22- Kwack et al. (2009): "*Decreased body weights (to 88% of control)*"

Page 60- "DINP exposed males weighed 88% of controls in study by Kwack et al., 2009…"

Remark: The specification of decreased body weights to 88% of control (p. 22, 60) is estimated, because the value is derived from a graph (Fig. 2, Kwack et al., 2009).

Page 25

"Sperm counts were lowered to 75.2% by DINP, whereas e.g. DEHP decreased sperm counts to 34.3% of controls."
"Overall, DINP appeared to affect sperm motion in a similar manner as e.g. DEHP, DBP and BBP…"

Remark: For DEHP, 30.3 % (and not 34.3 %) would be the correctly calculated value of decreased sperm counts of controls.

This comparison in sperm count changes to DEHP is not reasonable. In DEHP the testis weight is significantly reduced, which confirms the decreased sperm counts. There were no effects on the testes weight with DINP and there was no testes histology conducted to confirm whether or not histopathological effects were seen or not.

While for DINP the sperm count was 75.2 %, s.(significant), i. e. reduced as compared to control rats; the counts for phthalates known and accepted to be void of fertility effects, e.g. DEP (84%, n.s. (not significant)), MEP (59 %, s (significant)), DMP (91 %, n.s.), MMP (98 %), n.s., indicate that the values need to be critically evaluated, as outcomes for these substances are not what would be expected based on the larger database.

The same is true for the sperm motility parameter:
For DINP, 63 % (84 % of the control value) of motile sperms are reported vs 75 % for controls (please note: not statistically different from control) and therefore the wording "effect" is misleading.
Further, Kwack et al. (2009) measured the %motile sperm for phthalates known to be devoid of fertility effects, i.e. DEP (55,7 %, n.s.), MEP (33 %, s.), DMP (69.3 %, n.s.) and MMP (55,8 %, n.s.)

Regarding the sperm velocity the same significant reduction to controls is only seen in DBP. The monoester of Di-ethyl phthalate, MEP, known not to cause adverse reproductive effects was reported as showing a statistically significant reduction in sperm counts to 59% of controls; this confirms that these data should be very critically evaluated.

In conclusion, the results presented by Kwack et al. (2009) are evaluated to be ambiguous. For very short chain phthalates and their monoesters, which all do not impair fertility, are presented to show much higher "effects"

as those which are seen for DINP. See Part 4, Appendix VIII of this submission for more discussion on Kwack et al. (2009).

Typo: DEHR reduced the sperm counts to 30,3 % of the controls and not 34,3 %.

Page 30

"These two studies together indicate effects of DINP on development (decreased offspring body weight), but no clear conclusions regarding toxicity to fertility can be drawn as sperm parameters and reproductive organ histology were not examined. Furthermore, findings in repeated dose studies (see section 4.7) showed reductions of testis weights at high doses of DINP, whereas the one-generation study showed increases of testis and epididymis weights. Discussion of effects of DINP on fertility is presented in section 4.11.4".

Remark: As discussed in detail in Part 4 Appendix III of this submission and elsewhere in these comments, the offspring body weight effect observed in Waterman et al. (2000) was likely a consequence of palatability rather than inherent toxicity. The broader literature on this topic (see Part 4, Appendix III of this submission) supports this conclusion. Furthermore the differences were reversible once exposures were terminated as shown by comparative data from studies in which exposure is continued versus those in which rats were exposed for limited periods of time and then held without treatment until terminal sacrifice. This is consistent with the interpretation reflected in the EU RAR on DINP (ECB, 2003) that the effects observed in the Waterman et al. (2000) do not justify classification.

Additionally, a multigenerational reproductive study conducted according to guidelines is designed to provide information on the male and female reproductive systems, including gonadal function, the oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and the growth and development of the offspring. The study also provides information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on prenatal and postnatal developmental toxicity. In addition to studying growth and development of the F1 generation, this study also assesses the integrity and performance to assess reproductive performance and fertility. Therefore it is unclear why the power and significance of such a study to inform toxicity to fertility is dismissed due to a lack of assessment on sperm parameters. This study clearly shows no effects on any of the multitude of endpoints assessing fertility/sexual function, as reflected in the EU RAR (ECB, 2003) that effects observed in this study do not justify classification.

Page 31

"*For the DINP metabolite MINP, statistically significant reductions in free androgen index (serum levels of total testosterone relative to levels of sex hormone binding globulin) was seen in each of the three highest quartiles compared to the lowest quartile (15% lower free androgen index in highest compared to lowest MINP quartile; p<0,001). In the highest quartile of DINP exposure, FSH levels were 13% lower (p<0.05) and the total testosterone to LH ratio was 9% lower (p<0.05) than in the lowest quartile. The free androgen index relative to LH levels were 19% lower in the highest versus the lowest MINP quartile, whereas there were no significant differences in LH or testosterone levels as such*."

Remark: This statement of results from Joensen et al. (2012) is both inaccurate and incomplete. First, MINP (or any of the other three DINP metabolites or molar sum examined: MHiNP, MOiNP, MCiOP, ∑DINP) were **NOT associated** with any of the reproductive hormones or sperm quality parameters examined. The few associations (reported in the excerpt above) were only observed for %MINP (percentage of total DINP expressed as MINP), a computed, invalidated measure of "susceptibility to DINP exposure", the significance of which is not established. Second, **%MINP was also associated with relatively higher values of the sperm quality indicators** among men in the highest quartile versus the lowest quartile. This result is missing from the description in the CLH proposal.

Page 32-36 Table 12 Page 32-34

"*Table 12: Summary table of relevant experimental studies on developmental toxicity (chronol. order). Supporting studies on effects on foetal testosterone production are included here.*"

Remark: An assessment of study quality should be included in all summary tables throughout the CLH proposal and consideration given to decreasing weight from low quality studies if the study limitations warrant doing so.

Summary table 12 contains selected information from the supporting studies identified by the CLH proposal submitter and represent a bias in positive outcome reporting. An objective representation of the available data should be incorporated in the study summaries and data that may be contrary to the proposal submitters' hypothesis should be addressed. This is exemplified below.

- For Lee et al. (2006) ECHA comments in the evaluation of new scientific evidence concerning DINP (ECHA, 2013), the decrease in AGD following exposure to DINP at the lowest dose tested is not supported by studies included in the CLH proposal including Boberg et al. (2011) and Clewell et al. (2013). The dose dependency of AGD in male neonates seen in all dietary concentrations of DINP is of minimal significance compared to the control and is not worth mentioning. The proposal submitter fails to address or mention these discrepancies. Furthermore, a litter size of n=4 cannot be found in the publication of Lee et al. (2006). Lee et al. (2006) written on page 344 that "the litter size was adjusted to eight on PND 5". Importantly, and as stated many times in these comments the limited reliability of this study and its impact on how it impacts the weight of the outcomes for informing a classification decision are not addressed in the CLH proposal.
- For Borch et al. (2004) it should be noted in the remarks section for the Borch et al., 2004 study that no change in plasma testosterone or LH was observed and no significant difference in DEHP plus DINP compared to either compound alone for any endpoint was observed.
- For results from Boberg et al. (2011) reduced percentage of motile sperm are not statistically significant when properly corrected for multiple comparisons (Morfeld et al., 2017). And the nature of histology changes should be described in this table beyond simply stating 'histological changes in fetal testis"
- Similarly, the incidence of malformations reported in Gray et al. (2000) should be quantitatively reported and qualitatively described, particularly as these are noted as key effects in support of the classification decision; same comment with respect to the key histological changes noted in this table in the Masutomi et al. (2003) study, with reference to the dose at which they were observed.
- In the table detailing non-human developmental toxicity studies, the results as written for Adamsson, et al. (2009) reflect only a lack of effect observed on testosterone production on GD19 (exposure to 250 or 750mg/kg/d DINP GD13 to GD17). Adamsson, et al. (2009) also reported the following additional negative results, which are not reflected in the proposal: no change in plasma

corticosterone; no change in testis mRNA for StAR, 3β-HSD, or SF-1; no corresponding change in protein levels in testis or adrenal for StAR, 3β-HSD, AR, or P450scc. Thus, the proposal's premise that exposure to DINP causes shifts in steroidogenic enzymes is not supported by Adamsson, et al. (2009), as no corresponding changes in protein levels were observed in testis or adrenal for the steroidogenic enzymes assayed in this study (StAR, 3β-HSD, AR, or P450scc) even at 750mg/kg/d.

The following errors in the reporting in Table 12 need correcting:

- Masutomi et al. 2003: The NOAEL in this study was 307 mg/kg (GD15-20) / 657 (PND2-10); on page 33 it says mistakenly 230 mg/kg. For the LOAEL it is important to mention both values. Since food consumption increased after parturition, the dose also increased postnatally. LOAEL was 1165 mg/kg (GD15-20) / 2657 mg/kg mg/kg (PND2-10).
- Masutomi et al., 2003: Source for DINP was WAKO Pure Chemical Industries Ltd. (Osaka, Japan; CAS #28553-12-0, lot #ELR2418, purity: >98%).
- Li et al., 2015: The table gives the information that DINP (CAS 28553-12-0) was used for the study. This is not mentioned in the publication, instead the authors say "DINP (>99% mixture of C9 isomers with <0.15% dioctyl phthalate) was purchased from Sigma (St. Louis, MO, USA)".

Page 33, and Table 12 and page 33

Remark: The malformations observed in Gray et al. (2000) are provided as key observations supporting classification yet they are not described in any detail in the CLH proposal. Therefore there relevance to classification per the CLP criteria is not at all clear or transparently justified by the dossier submitter.

Page 38, Table 13

Remark: For Masutomi et al. (2003) the table header of Table 13 reads: "*Data from birth to prepubertal (PND21) necropsy.*" However, necropsy of DINP treated animals was conducted on PND 27.

Page 41

Remark: The summary of Borch et al. (2004) study should include the authors conclusions as stated in the discussion of the manuscript "no modulating effects of DINP on the effects of DEHP can be inferred on the basis of these data as fetal testosterone levels were not significantly lower with exposure to DINP in combination with DEHP than with DEHP or DINP alone (Borch et al. 2004)." Testicular testosterone content and product was significantly decreased however, no change in plasma testosterone or LH was observed. The materials and methods section states that the dose of DEHP was chosen for submaximal effects on hormone levels. There was no significant difference in DEHP plus DINP compared to either compound alone for any measured endpoint.

Lee et al., 2006: The dose dependency of AGD in male neonates seen in all dietary concentrations of DINP is of minimal significance compared to the control and is not worth mentioning.

Page 42:

Remark: The CLH proposal states "*Adamsson et al. (2009) found increased mRNA levels of P450scc and Insl3, genes that are known to be reduced by other phthalates and that are likely involved in the anti-androgenic*

effects of these compounds." It fails to include the mention of a lack of change in mRNA levels in StAR, 3β-HSD, or SF-1, and fails to include the mention of an increase in mRNA levels of GATA-4 as well as the other two genes they referenced. Additionally, there were no increases in the protein levels of P450scc in testis or adrenal, so the relative increase in P450scc mRNA in testis may have questionable biological relevance. The CLH proposal submitter emphasizes one explanation for the lack of consistency of the findings of Adamsson et al. (2009) with those reported for LMW phthalates, i.e. a 'rebound effect' due to low testosterone production at the time of dosing a few days earlier. However, another explanation is that DINP does not have the same impact on these response pathways as 'other phthalates', which is consistent with the conclusion of Adamsson et al. (2009) that exposure to DINP from ED 13.5 to 17.5 'has only little effects on testicular and adrenal steroidogenesis' and DINP 'does not down-regulate the activity of steroidogenesis'; and is also consistent with the lack of observed adverse effects in the larger database on DINP.

Page 42-45

"*A corrigendum from Boberg et al., 2016a provided further description of the statistical methods than in the 2011 paper. Furthermore, minor errors in descriptive statistics (but not in statistical results) were corrected in a letter from Boberg et al."*

Remark: As published in detail in a letter to the editor (Morfeld et al., 2017), the Corrigendum by Boberg et al. (2016) does not provide a further description of the statistical methods, but reflects a deviation of the statistical methods from those originally reported to a non-standard statistical approach. These changes to the statistical methods reflected in the Corrigendum do not reflect the best-practice in statistics and are much more impactful to the statistical significance of the results and overall interpretation of data than this statement reflects. Briefly, Boberg et al. selectively eliminated a post-hoc correction for multiple comparisons [(a necessary correction supported by EFSA scientific opinion on Statistical Significance and Biological Relevance (EFSA, 2011)] from the statistical approach for sperm parameter endpoints (yet included post hoc correction for multiple comparison in the analysis of AGD and testosterone). Inclusion of this post-hoc test impacts the statistical significance of the outcomes.

Part 4 Appendix XI of this submission describes in more detail the results from a re-analysis of the raw data from Boberg et al. (2011) and outlines where statistical significance could not be reproduced.

Remark: The statement "*In foetal testes histopathological effects known for certain other phthalates were observed (statistically significant from 600 mg/kg bw/day)"* (page 42) should be changed to "'In foetal testes androgen independent histopathological effects ~~for certain other phthalates~~ were observed (statistically significant from 600 mg/kg bw/day)".

Remark: The last sentence of the table legend for Table 15 (page 42) should read "Results in bold are significantly different from controls in a one-sided Fishers exact test without correction for multiple comparisons".

Remark: The paragraph starting *"'slight effects on testicular testosterone" on page 43* should include mention of "no statistically significant effects were observed on ex vivo testicular testosterone production, plasma testosterone, or plasma LH".

Remark: The paragraph starting "*in adulthood (PND 90) a dose-dependent reduction.." (page 43) should be corrected in several points:*

- It should be noted that the biological relevance of the reduction in sperm motility are questionable as the minimum requirement of 70% motility established by the OECD (OECD, 2008) and in the peer reviewed literature (Seed et al., 1996) has not been met since sperm parameters are sensitive to sampling techniques, analysis techniques (Schlel et al., 2013), and environmental conditions (Seed et al., 1996), interpretation of results is contingent upon experimental optimization. Importantly, reliance in support of regulatory decisions should comply with OECD standards. The lack of optimization of this experimental set-up in this lab is demonstrated in other publications from this lab, which similarly fail to meet this minimum requirement. Furthermore, the changes in sperm motility reported for DINP in Boberg et al. (2011) are within the unexplained variance of the control animals of the two other studies (Taxvig et al., 2007; Jarfelt et al., 2005) undertaken in the same laboratory. Therefore, we conclude they do not differ substantially from historical controls within this laboratory. This is discussed in further detail in Part 4, Appendix XI of this submission.

- A statistically significant outcome for sperm count per g cauda epididymis was only observed when the outcomes were not corrected for multiple comparisons.

- "'*borderline significant"* is not an accepted statistical characterization. This terminology should be removed. A cut-off for statistical significance is determined a priori, and results are either above or below that cut-off.

- The paragraph should conclude with a statement "'due to the minimal quality standards in controls these outcomes would need to be followed up before conclusions can be drawn".

Remark: Regarding Table 17 (page 44): It should be noted that the statistical significance for sperm/g cauda at 900 mg/kg bw /day DINP could not be reproduced upon independent analysis (Morfeld et al., 2017) following correction for multiple comparisons. Morfeld et al. (2017) calculate a p-value of 0.072 (i.e.>0.05).

Remark: The statement "*however four adult DINP exposed animals had permanent malformations such as **epididymal and testicular dysgenesis***" is not accurately reflecting what was observed in the study, i.e. small epididymis in one animal and small testis in another. The term 'testicular dysgenesis' carries a specific connotation in this context, yet it is being used here to merely reflect a change in size (or weight, as it is unclear in the publication what parameter was used to conclude small testes and epididymis). Dysgenetic testes in a broad context refers to testes of variable histopathological presentations. Therefore the use of this term in general, leads to confusion. In the context of LMW phthalate toxicity (particularly DBP), testicular dysgenesis is characterized by specific histopathology hallmarks (e.g. intratubular leydig cells, malformed seminiferous chords)(as described in Hutchison et al., 2007; Van den Driesche S. et al., 2017 and elsewhere). As noted in Boberg et al. (2011) "'histopathology of male reproductive organs at PND90 was not altered by DINP treatment", therefore the basis for concluding testicular and epididymal dysgenesis was observed in adult animals is not supported. Furthermore, the lack of dose response suggests these are not DINP mediated. This is consistent with the conclusion reflected in the ECHA report of new scientific evidence of DINP (ECHA, 2003), that the males having small testes and epididymides are considered as 'suggestions' of an effect only because there is no dose-response.

Remark: The meaning and toxicological relevance of the behavioral outcomes from Boberg et al. (2011) are highly questionable. First, there is only statistical significance at the top dose for females and there was no consistency in this finding, as the effects were not seen on "memory day 2" or when the platform was moved. Second, there was no impact on male behaviour which is inconsistent with the authors' hypotheses of anti-

androgenic effects of DINP. Third, the conclusion that better performance than controls in the Morris Water Maze test indicates "masculinization of behavior in DINP exposed females" is particularly without merit as no explanation for what pathways link learning and memory with potentially disrupted hormone pathways is provided, nor is there any reconciliation between the contradiction between masculinization of females and the anti-androgenic MOA proposed for DINP. The data and underlying rationale are weakly supported. This is consistent with ECHAs evaluation (ECHA, 2013) i.e. "'*the suggested masculinization of female behavior needs further clarification*".

Page 49-50:

Remark: Li et al. (2015) reports no significant effect of DINP on testis volume, Leydig cell number per testis, and nuclear size in fetal Leydig cells; however, the proposal fails to mention these negative results in context with the reported induction of increased clustering of Leydig cells after in utero DINP exposure at all dose levels (10, 100, 500, 1000mg/kg/d). Additionally, the proposal reports a dose-dependent decrease in testicular mRNA levels in Cyp11a1, HSD3b1, and Cyp17a1; the physiological relevance of the decrease in expression for Cyp11a1 and HSD3b1 is questionable, since there was not an observed dose-dependent decrease in their protein levels in testis at this time point (GD21.5) following in utero exposure to DINP (Cyp17a1 protein levels were not measured).

Page 54:

"Several studies described in section 4.11.2 on developmental toxicity have examined the influence of DINP on fetal testosterone production, testicular histopathology and/or anogenital distance", with reference to Adamsson, et al. (2009) and Hannas et al. (2011) in support of the statement.

Remark: Adamsson, et al. (2009) had not measured testicular histopathology, nor AGD; further, DINP exposure in this study had no effect on fetal testicular testosterone content. Similarly, Hannas, et al. (2011) was cited in support of this, when, Hannas et al. (2011) did not measure AGD nor investigate testicular histopathology. As has been mentioned numerous times in these comments, the cited referencing for critical statements is misleading in many instances throughout the CLH proposal.

Page 54

"*The reduction in testosterone synthesis is associated with structural changes in testes, i.e. Leydig cell aggregation and presence of multinucleated gonocytes, as described above (Borch et al., 2004; Boberg et al., 2011; Clewell et al., 2013a; Li et al., 2015).*"

Remark: As outlined in greater detail in Part 4, Appendix VII of this submission, (and supported by a publications referenced by within the CLH proposal (Gaido et al., 2007; Lehraiki et al., 2009) multinucleated gonocytes (MNGs) are not androgen dependent. Therefore this statement is not reflecting the current state of the science on the postulated mode of action for MNGs. nor the empirical evidence for DINP.

Additionally this statement is not reflecting the empirical evidence on DINP and the references as listed are misleading with respect to how they provide support for this statement. The Borch et al., (2004) study measured fetal testosterone production and plasma testosterone; the Boberg et al., (2011) study include various measurements of testosterone with varying outcomes; the Clewell et al., (2013a) study measured testosterone content, presence of multinuclear gonocytes and Leydig cells clustering; and Li et al., (2015) measured Leydig cell clustering and multinucleated gonocytes but did not observe an effect on testosterone.

Furthermore, the referenced studies themselves do not support an association between testosterone changes and changes in testes as they either report a decrease in testosterone as a single observation without further assessment of testis changes (Borch et al., 2004) or do not observe a change in testosterone but do observe leydig cell aggregation and/or MNGs" (Li et al., 2015); or have conflicting outcomes on the multitude of testosterone measurements in the study (Boberg et al., 2011). Associations are not themselves evidence of causation, and to support that testosterone leads to the purported changes through integration of observations across the above referenced studies requires a weight of evidence analysis of these data according to considerations as those outlined in the MOA/HR framework (Meek et al., 2014a). Testosterone is necessary but not sufficient to impact development during the male programming window. As discussed in Part 4, Appendix I of this submission, the empirical evidence for DINP is inconsistent with the hypothesis that DINP is capable of eliciting a change in testosterone great enough to lead to/or be responsible for adverse effects on the testes.

Page 54, section 4.11.3.1:

"The reduction in testosterone synthesis is associated with structural changes in testes, i.e. Leydig cell aggregation and presence of multinucleated gonocytes, as described above."

Remark: This statement is not supported by the information currently available which demonstrates that multinucleated gonocytes, are not influenced by a reduction in testosterone synthesis. This conclusion is supported by a few publications referenced within the CLH proposal itself (i.e. Gaido et al., 2007; Lehraiki et al., 2009) and summarized in greater detail in Part 4, Appendix VII of this submission.

Page 55:

Remark: In reference to Lee & Koo (2007) (which reports on a Hershberger assay) the CLH proposal submitter highlights "Additionally, serum testosterone levels were decreased and LH levels were increased at 500 mg/kg bw/day (approximately 85 and 130% of testosterone exposed control values, respectively, as estimated from graphical presentation)". The reason why the OECD recommends measuring testosterone levels is to differentiate between a true endocrine mediated anti-androgenic effect and an effect secondary to liver metabolism of steroids. Paragraph 53 TG 441 states "Serum T levels are useful to determine if the test substance induces liver metabolism of testosterone, lowering serum levels. Without the T data, such an effect might appear to be via an anti-androgenic mechanism." The animals used in the Hershberger assay are castrated and thus do not produce any endogenous testosterone. The circulating testosterone is from administered testosterone propionate. The finding highlighted by the CLH proposal submitters indicate that any potentially observed "anti-androgenic effects" are secondary to liver metabolism.

In reference to OECD TG 441 the CLH proposal states "*According to the test guideline, a substance should be considered as positive in the test if at least two of the five organs show an effect*." This is an incomplete depiction of how the outcomes from a TG 441 study should be interpreted. From paragraph 61 of TG 441 "A statistically significant reduction ($p \leq 0.05$) in any two or more of the five target androgen dependent tissue weights (VP, LABC, GP, CG and SVCG) relative to TP treatment alone should be considered a positive androgen antagonist result and **all the target tissues should display some degree of reduced growth**." DINP has increase statistical significance in two target tissues at 500 mg/kg/d but does not display "some degree of reduced growth" in the three other target tissues.

The conclusion by the CLH proposal submitter "[*t]hus, the dose level of 500 mg/kg bw/day can be considered as anti-androgenic in this test"* is highly flawed. When interpreting this study the CLH proposal submitter ignores two critical aspects of the OECD 441 protocol. First, that evidence of decreases in testosterone levels indicate a secondary mechanism likely due to liver toxicity and second, that in addition to two2 statistically significant weight decreases the remaining tissue needs to display some degree of reduced growth. The data from Lee and Koo (2007) should not be interpreted as a positive indicator of anti-androgenicity. Which is consistent with the rest of the literature that indicate phthalates do not directly interact with the androgen receptor and is not 5α-reductase inhibitor, which are the anti-androgenic MoA's evaluated in the Hershberger assay.

Page 57

"The effects on male pup anogenital distance (Boberg et al., 2011; Gray et al., 2000, Clewell et al., 2013b) are considered a clear adverse effect on development not considered to be secondary to toxic effects on the dam or reductions of offspring body weight, as they are analysed by taking body weight into account, i.e. inclusion of either offspring weight as a covariate in the analysis or using the anogenital index for the analysis. This means that the significant effects described for male anogenital distance cannot be explained by effects on pup body weight, if such an effect is present"

Remark: The basis for this statement is scientifically unfounded as discussed in detail in Part 4, Appendix V of this submission as the data on DINP largely fail to demonstrate an effect on this endpoint (i.e. 5/6 studies do not report an effect in perinatal animals).

- Gray *et al.* (2000) report no effect on AGD at the dose tested in the study (750 mg/kg-d) and also were not analyzed by taking body weight into account. . They include commentary on an unpublished study at 1,000 mg/kg/d and 1,500 mg/kg/d and indicate effects on AGD were observed at 1,500 mg/kg/d (but these data are not published as part of the Gray et al. (2000) report; and it is not specified if these are absolute or corrected for body weight; and doses above the limit dose are not relevant for CLP classification decisions (CLP 3.7.2.5.7-9)).

- Clewell *et al*. (2013b) did not see an effect at PND2 and only saw an effect at PND14 in conjunction with a significant decrease in weight. The authors concluded the effect at PND14 was likely due to the weight loss. This is supported by the fact that effects on AGD due to decreased testosterone in utero would already be apparent at PND2 and a change in AGD on PND2 was assessed, but no observed in the Clewell et al. (2013b) study. In OECD guideline studies, the key timing for measurement of AGD is PND 0-4

Furthermore, it is unclear on what basis a change in AGD is being concluded as a determinant of adversity, as the magnitude of the change in AGD that is needed to be a determinant of adversity has not been established (see Part 4, Appendix V of this submission for more discussion on this point). Importantly, according to OECD guidance document 43, a permanent change in AGD (i.e., observed at birth and into adulthood) constitutes a permanent structural change (OECD 2008) and is therefore of utmost relevance in the context of classification. Permanent changes in AGD have not been reported for DINP (Boberg et al. 2011; Clewell et al. 2013b)

It is important to recognize that adversity has different determinants in different contexts. For classification purposes under CLP, the considerations outlined in section 3.7.2.2 clarify the context of adversity under the regulation. The reliance on an endpoint for NOAEL setting is not comparable to a determination of adversity in the context of CLP as NOAEL setting is often established based on statistical significance alone without consideration of biological significance or toxicological significance (3.7.2.3.1, 3.7.2.3.3). Furthermore, NOAEL

and NOEL are often used interchangeably (ECETOC, 2002) for a number of reasons because the nature of effects that can provide the basis of a NO(A)EL are limited by the study design (e.g. studies assessing only molecular endpoints versus a multi-generation study with functional reproductive endpoints) and as just mentioned, the definition of adversity is context dependent. Classification decisions according to CLP should be based on the available data demonstrating the inherent ability of a chemical to induce a specific adverse effect (CLP, Section 3), according to the additionally delineated CLP criteria (3.7.2.2).

Page 57

"The observed permanent structural effects in offspring observed after perinatal DINP exposure (degeneration of meiotic spermatocytes and Sertoli cells, scattered cell debris in ducts in epididymis, decrease in number of corpora lutea (Masutomi et al., 2003); reduced percentile of motile sperm, permanent nipples/areolae in male rats, small testes and epididymides (Boberg et al., 2011; Gray et al., 2000)) are considered to be specific and not secondary non-specific consequences of maternal toxicity or other toxic effects."

Remark: The manner in which the evidence is compiled cited in the last paragraph on page 57 of the CLH proposal is particularly misleading.

- For Masutomi et al. (2003)
    - Reporting effects on epididymis and decrease number of corpora lutea are mentioned here in a continuous sentence ending with *"these are considered to be specific and not secondary consequences of maternal toxicity or other toxic effects"*. Yet this is not consistent with the reporting by Masutomi et al.(2003) or by ECHA in the evaluation of new scientific evidence concerning DINP (ECHA, 2013), i.e. The observations reported in Masutomi et al. (2003) consisted of minimal to slight changes that reached statistical significance only at the highest administered dose of DINP of 1165/2657 mg/kg. Furthermore and as noted in the ECHA restriction from 2013, these effects were only observed at dose levels causing systemic toxicity in the dams.
    - As noted previously the effects observed by Masutomi et al. (2003) on the epididymis were minimal to slight and only observed at the highest dose tested (>1000mg/kg bw/d).
    - Histopathological examination reported no statistically significant effects on corpora lutea. Effects were only statistically significant when reported per unit area of ovarian tissue and the authors concluded these to be marginal. Note these marginal effects occurred at the highest dose (>1000mg/kg bw/d).
- Gray et al. (2000) did not report changes in sperm. Importantly, Gray et al. (2000) reported having examined sperm in the methodology section of the manuscript (i.e. spermatid head count and total cauda epididymal sperm reserves for some rats >4 months of age) but did not report the outcome of this analysis for DINP in this study [(whereas the outcomes for these parameters were reported in the Gray et al. (2000) text for DEHP and BBP]).
- Boberg et al. (2011) reported small testes and epididymis in one1 animal in each of the low and mid dose (so not dose responsive). Without dose response data, it is unclear on what basis the CLH submitters can conclude the effect is 'specific' to DINP. Furthermore, and as discussed in Part 4, Appendix VI of this submission it is unclear what the determinant was for characterizing the tissues as 'small' (gross morphology or weight, as the observation is listed under the heading 'organ weights PND90 males and females').

Page 57

"*Decreases in anogenital distance and increases in nipple retention in male offspring are clearly related to adverse reproductive effects in offspring such as altered development of reproductive organs, impaired semen quality, smaller penis size and increased incidence of hypospadias and cryptorchidism*"

Remark: This statement is misleading. Permanent decreases in AGD and increases in nipple retention (i.e. effects present in adult animals after in utero exposure) have been associated with adverse reproductive effects. No permanence of these effects are observed after treatment with DINP. The toxicological significant of small variations in perinatal and juvenile animals is less clear and have not been established to be related to adverse effects. (Bowman et al., 2003; van den Driesche et al., 2017).

"*the OECD Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment that statistically significant changes in neonatal AGD that cannot be explained by the size of the animal can be considered adverse (OECD 2008)*"

Remark: This statement is incorrect. This and misconstrues what is stated in the OECD guidance document. The actual statement in the document is "statistically significant change in AGD that cannot be explained by the size of the animal indicates effects of the exposure and should be used for setting the NOAEL." The reliance on an endpoint for NOAEL setting is not comparable to a determination of adversity in the context of CLP as NOAEL setting is often established based on statistical significance alone without consideration of biological significance or toxicological significance (3.7.2.3.1, 3.7.2.3.3). Furthermore, NOAEL and NOEL are often used interchangeably (ECETOC, 2002) for a number of reasons (as discussed earlier in these comments) one being that adversity has different determinants in different contexts. Classification decisions according to CLP should be based on the available data demonstrating the inherent ability of a chemical to induce a specific adverse effect (CLP, Section 3), according to the additionally delineated CLP criteria (3.7.2.2).

Page 57

Remark: The second2nd paragraph includes an incorrect reference. The reference to Hellwig et al. (1997) is wrong. In See EU RAR, page 238, the LOAEL of 159 mg/kg bw/day is based on Waterman et al. (1999 and 2000).

Page 57 - 58

"Furthermore, the finding of reduced testosterone levels in foetal life may be considered adverse: " *the justification for considering foetal reduced testicular T concentration as adverse is that during the critical developmental window it has shown to induce male reproductive developmental effect" (OECD 2008). This is also in line with the conclusions of the ECHA review from 2013, stating that the anti-androgenic effects observed for DINP (reduced testicular testosterone and foetal testicular histopathology) support the findings of permanent changes and lower semen quality in DINP exposed males observed with higher doses of DINP exposure."*

Remark: The referenced quote could not be located in the OECD, (2008) reference provided so it is difficult to know the context within which the quote justifying testosterone as an adverse effect was made. If defining adversity was removed from a discussion regarding NOAEL setting this might be a reasonable statement, as NOAEL setting is often established based on statistical significance alone without consideration of biological significance (i.e. a magnitude change of biological importance), and also is constrained to the endpoints measured in the study (i.e. only molecular endpoints may have been measured). Classification decisions

according to CLP should be based on the available data demonstrating the inherent ability of a chemical to induce a specific adverse effect (CLP, Section 3), according to the additionally delineated CLP criteria (3.7.2.2).

In general, to broadly declare changes in testosterone are themselves "adverse" in the context of a classification decision is counter to the fundamental underpinnings of the pathway-based toxicology concepts of mode of action (WHO, 2007) and adverse outcome pathways (AOP) (OECD 2013, 2014), as modulation of events on a pathway are not necessarily sufficient for triggering the cascade of events needed to manifest in the adverse outcome.. See Part 4, Appendix IX of this submission for more details on MOA. The data on DEHP, DBP and other substances indicate a reduction of >80% in testosterone would be needed to elicit adverse outcomes associated with androgen mediated effects (Gray et al., 2016; Zirkin et al., 1989). This magnitude of testosterone reduction is larger than the database collectively supports DINP is capable of mediating at exposures up to 1500mg/kg/d (Hannas et al., 2011; Furr et al., 2014; Boberg et al., 2011; Clewell et al., 2013a).

The dossier submitter infers that it is appropriate to conclude a change in testosterone during the male programming window is adverse because we do not have enough knowledge of the human situation (pages 59, 63, 67 in the CLH proposal). However, based on the currently available information on DINP (and in the context of classification per CLP), such a leap is not supportable without speculation that is counter to the available experimental data across species (see Part 4, Appendix X of this submission for further discussion on this point). Classification decisions according to CLP should be based on the available data demonstrating the inherent ability of a chemical to induce a specific adverse effect (CLP, Section 3). In the case of DINP 1) the data on phthalates extensively suggests that a >80% reduction (or near ablation) is required to lead to adverse effects; 2) this magnitude of testosterone reduction is larger than the database collectively supports DINP is capable of mediating at exposures up to 1500mg/kg/d (Hannas et al., 2011; Furr et al., 2014; Boberg et al., 2011; Clewell et al., 2013a); 3) the extensive developmental toxicity data on DINP is consistent with point 2 as a lack of androgen-mediated adverse effects are observed following exposure during the male programming window; and 4) DINP has not been shown to impact androgen responsive pathways in any other stage of development based on outcomes in the 2-generation study (Waterman et al., 2000).

Page 58:

Remark: In reference to ECHA review in which a NOAEL of 50mg/kg/d for reproductive toxicity is derived, the proposal references three different Hannas, et al. (2011a, b, 2012) manuscripts. Only one of these manuscripts from 2011 is actually referenced in the bibliography; thus, this part of the proposal is not properly delineating the specific endpoints that are supposed to support this claim.

Page 60

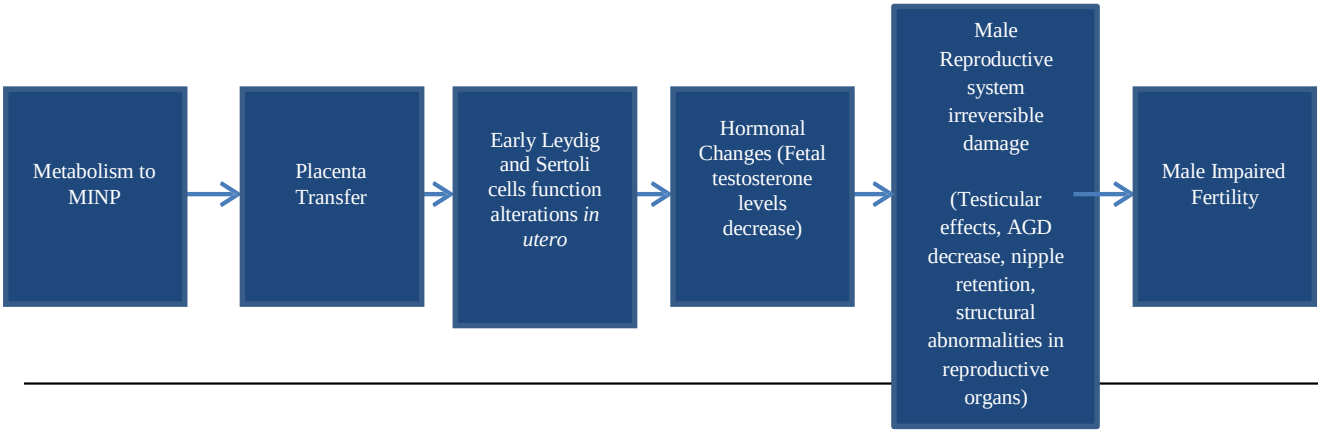
"*ECHA (2013) concluded that "the in vivo findings suggest that DINP has anti-androgenic potency but may also exhibit its effects through other modes of action". It was further concluded that the permanent changes seen after exposure to high doses of DINP are "likely to be linked to the reduced perinatal testicular T levels*".

Remark: The CLH proposal submitter does not provide the full context of the ECHA conclusions. The full conclusion from ECHA (2013) is "The decrease in testicular T levels seems to be transient and permanent changes were not generally seen in all studies with DINP. However, low incidences of permanent changes after exposure to high doses at and above 500 mg/kg bw/day have been described. Most of these changes are likely to be linked to the reduced perinatal testicular T levels." They note permanent changes are not often seen and offer a speculative statement on rare incident findings. They do not do an assessment of study quality, or a

weight of the evidence evaluation of DINP's intrinsic capacity to induce adverse effects as required by CLP.

Page 60

"*The following mode of action is hypothesised based on the WHO/IPCS Mode of Action/Human Relevance Framework description which is presented in the SVHC identification proposal for DCHP (Annex XV report DCHP, 2016)*":



Remark: The mode of action (MOA) framework developed by IPCS is often used to provide a stronger technical justification for the use of a particular effect in animals as the basis of a human health risk assessment. The credibility of a postulated MOA is judged by an assessment of the key events as a whole, asking how well the data support involvement of each key event in an MOA by considering dose response and temporal concordance, consistency, specificity, biological plausibility, and coherence of the entire pathway (Sonich-Mullin et al., 2001; Cohen et al., 2003; Seed et al., 2005; Boobis et al., 2006; Dellarco et al., 2012; Meek et al., 2014a, 2014b). In order to conduct this credibility assessment, individual key events must be defined as empirically observable steps that are necessary (but not necessarily sufficient) for the progression of an MOA (IPCS, 2007). That is, key events must be single, measurable events; if not defined with specificity, it becomes difficult to assess the necessity of a specific key event within the progression of the MOA. As defined in the above figure, key events 3, 4, and 5 are not described with sufficient specificity to assess their necessity within the biological pathway progressing to impaired male fertility. Specifically, key event 3 should identify a single cell type, key event 4 should identify the key hormonal change, and key event 5 should clearly identify which of the individual observations listed are essential for progression to the outcome (impaired male fertility). The lack of specification of true 'key events' as depicted in the Figure on page 60 of the CLH proposal obscures the nature of data that supports the hypothesis that specific chemicals act via this (largely unspecified) mode of action. Without specificity of key events, it is difficult to assess their causal linkages (i.e. define key event relationships) and incredibly difficult to judge if a chemical operates via the non-specified MOA.

As noted in the publications describing how to apply the MOA framework (e.g. Meek et al., 2014a, b), MOA analysis is often done in an iterative fashion, where a postulated MOA is judged according to the causality criteria listed above. What is depicted in the MOA figure proposed by in the CLH proposal is actually a merging of multiple MOAs into one. As such, the MOA listed in the Figure is not a MOA, and requires refinement to be informative. The next step in the assessment of this MOA should consist of defining key events in terms of measurable empirical events that can then be assessed for their essentiality within the overall network of MOAs. The network of MOAs depicted by France in the harmonised classification proposal for DIOP is an example of improved refinement of the Figure on page 60 of the CLH proposal for DINP (https://echa.europa.eu/harmonised-classification-and-labelling-previous-consultations/-/substance-

rev/16109/term). However, further refinement of this network of MOAs, is depicted in Part 4, Appendix IX of this submission.

Page 61

"There is strong evidence that these male reproductive system irreversible effects (e.g. sperm quality effects, structural abnormalities in reproductive organs, and decrease in anogenital distance) are linked to fertility adverse effects in mammalian species, including humans. Overall, fetal disturbance of the developing male reproductive system can have multiple effects in mammalian species as described by Skakkebæk et al. (2001) and summarized as the testicular dysgenesis syndrome (TDS)."

Remark: This section indicates the CLH proposal submitter is confusing Adverse Outcome Pathways (AoP), and mode of action. There is strong evidence for an AoP in humans where adverse outcomes in the male reproductive tract are related to alterations in androgen signaling in utero. However, there is no evidence that the mode of action for phthalates can activate this pathway and evidence to the contrary (i.e. that phthalates are not capable of causing the suppression of androgens in the human fetal testis). AOPs are species specific, but not chemical specific, whereas MOA is used to assess a chemicals ability to mediate a biological pathway.

Page 61

"The same effects as reported in male pups following exposure to DINP were also reported following in utero exposure to other phthalates with a harmonized classification for development as Repr. 1B, and an antiandrogenic mode of action was also suggested for these phthalates"

"However, DINP has been found to induce the same adverse effects and to share the same mode of action as the phthalates classified as reproductive toxicants (Boberg et al., 2011; Borch et al., 2004; Hannas et al., 2011; Clewell et al., 2013)."

Remark: These statements are not supported by the evidence. See Part 4, Appendix I of this submission for discussion on this point.

Page 61

"For DINP, one study has similarly indicated combination effects between DINP and DEHP (Borch et al., 2004)"

Remark: The above statement needs to be modified. The authors conclusion in the Borch et al paper are "no modulating effects of DINP on the effects of DEHP can be inferred on the basis of these data as fetal testosterone levels were not significantly lower with exposure to DINP in combination with DEHP than with DEHP or DINP alone". In addition this paper assessed testosterone measures and LH levels and no other effects. The broad use of the term "effects" in the CLH proposal suggests a broader range of endpoints having been evaluated. No significant difference in DEHP plus DINP compared to either alone for any group. The only significant difference is in comparison to control.

Page 62-Table 26

Remark: Table 26 lacks relevant information for DINP and needs to be completed to give a transparent overview on the weight of evidence as requested by 3.7.2.3.1 of the CLP:

Table 26: Similarity between effects of DINP and other phthalates classified as toxic to reproduction

Substance	Areola/nipple retention	Decreased fetal or neonatal male AGD	Hypospadias	Harmonized Repr 1B (H360D) classification	Effects on fetal testis testosterone production or -content	References
DIBP	Yes	Yes	Yes	Yes	Yes	Saillenfait et al., 2008, Borch et al., 2006
DBP	Yes	Yes	Yes	Yes	Yes	Fabjan et al., 2006 (review)
DINP			**No**	**No**	**Yes**	Gray et al., 2000, Boberg et al., 2011, Clewell et al., 2013a

The "Yes" for decreased fetal or neonatal male AGD does not differentiate between observations in perinatal animals versus adults. Assuming it is referring to effects in perinatal animals, it does not reflect the weight of the evidence (See Part 4, Appendix V of this submission). Of the studies that measure this effect, only 1/6 observed a statistically significant effect in the perinatal period (Boberg et al. 2011). This effect was of minimal magnitude and only observed at one dose. In another of the six total studies (Clewell et al. 2013b) the effect was only observed in PND14 animals but not those same animals measured at PND2, nor in adults. If the effect were due to anti-androgenicity it would have been observed at PND2; and been observed consistently across studies. Four other studies do not observe an effect up to doses ~1100 mg/kg bw/day (Masutomi et al. 2003; Gray et al. 2000; Li et al. 2015; Clewell et al. 2013a). A seventh study (Lee et al. 2006) reported a change in AGD however this study is severely flawed as discussed many times in these comments and therefore not considered this assessment.

The "yes" for areola/nipple retention does not differentiate between observation of the effect in juveniles and adults. Permanent nipples are not observed in DINP treated animals (see Part 4, Appendix IV of this submission).

Further, other adverse effects which are most important for low molecular phthalates, and provide the basis for their classification i.e. severe testicular atrophy, severe malformations during organogenesis, undescended testes, hypospadias, cleft palates, agenesis of prostate, etc. are missing in the table. By omitting these LMW specific effects, a comparison of the different effects by phthalates is incomplete. This is of specific importance for DINP which does not lead to these effects. See Part 4, Appendix I for more details on this point and Table 1 in Part 1 of this submission which clearly shows these differences

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Remark: In the comparison to DEHP, DBP and BBP it is concluded that "*the overall evidence for effects of DINP on fertility is limited, as one study in young adult rats showed reduced sperm count and sperm motility*" This statement is inconsistent with the 'Short Justification for classification' provided in the CLH proposal given that Kwack et al. (2009) is proposed as the one key finding of DINP for effects on sperm count and sperm motion parameters relevant to fertility (page 9 and 58 of the CLH proposal). Also Kwack et al (2009) does not report a statistically significant effect on sperm motility and as has been explained elsewhere in these comments it is

not possible to conclude on the basis of this study that the effects on sperm counts are directly causally related to DINP dosing.

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*"**Species similarities** are seen between mice, rats and marmosets in the foetal germ cell effects seen with prenatal exposure in vivo (McKinnell et al., 2009; Gaido et al., 2007)".*

Remark: The above statement needs to be modified. The publication by McKinnell et al. (2009) reports species differences and not similarities. Please refer to **Results** section of the abstract in the publication by McKinnell et al. (2009) which reads:

"**Fetal exposure of marmosets to MBP did not affect gross testicular morphology, reproductive tract development or testosterone levels at birth, nor were germ cell number and proliferation, Sertoli cell number or germ: Sertoli cell ratio affected**. In two of six MBP exposed animals, unusual clusters of undifferentiated germ cells were found, but their significance is unclear. Neonatal MBP treatment did not affect germ cell numbers or differentiation. Fetal exposure to MBP did not affect testis size/morphology, germ cell numbers or fertility in adulthood. There was no evidence for CIS or TGCT"

Gaido et al. (2007) report "species differences", and note that every odd page in the journal has the title "SPECIES DIFFERENCES IN TESTICULAR RESPONSE TO PHTHALATES".

This statement also needs to be modified: *"**Species similarities** are seen regarding germ cell changes in in vitro using testes from rats, mice or humans (Chauvigne et al., 2009; Habert et al., 2009; Lambrot et al., 2009; Lehraiki et al., 2009)."*

Habert et al. (2014b) in his review concludes species **differences**:
"Conversely, MEHP induced the appearance of multinucleated gonocytes only in mouse and rat but not in human fetal testis explants. Moreover, MEHP did not affect testosterone production in human fetal testis explants (Lambrot et al., 2009)"

Chemical	Concentration (M)	Effect on germ cell development			Effect on Leydig cell function			References
		Species			Species			
		Human	Rat	Mouse	Human	Rat	Mouse	
MEHP	10^{-6}	No	No	No	No	No	No	Lambrot *et al.* (2009), Lehraiki *et al.* (2009), Muczynski *et al.* (2012), Data in this paper.
	10^{-5}	Negative	Negative	Negative	No	Negative	Positive	
	10^{-4}	Negative	Negative	Negative	No	Negative	Positive	

This is discussed in more detail in Part 4, Appendices VII and X of this submission.

Remark: Furthermore, the manner in which section 4.11.4.4 is structured is questionable.

For example, the first paragraph of this section reads "[a]*s the mode of action of DINP is similar to that of DEHP, DBP, DIBP and BBP, this discussion is equally relevant for DINP*". As has been mentioned many times in these comments, it has to be demonstrated through evaluation of the data upon consideration of a number of "causal criteria' whether or not DINP operates via 'the same mode of action' (i.e. a biologically plausible series of chemical-specific KEs starting with exposure and proceeding through the interaction of an agent within a cell, subsequent physiological and tissue or organ changes, resulting in an adverse effect). An impact on one

event in a postulated mode of action is not evidence that a chemical operates via a mode of action, as events are necessary but no necessarily sufficient for causing an adverse effect. As discussed further in Part 4 Appendix IX of this submission, the extensive data are inconsistent with DINP operating via the same androgenic mode of action as DEHP, for example. While studies show DINP reduces testosterone, the evidence also supports that DINP is not capable of perturbing testosterone to a level of sufficient severity to cause the further cascade of events in the mode of action sequence i.e. it does not lead to subsequent tissue changes that result in an adverse effect.

This section combines information from many modes of action to support relevance of all modes of action to humans. For example, the evidence is strong that effects on multinucleated gonocytes are not androgen dependent. Therefore, species similarities in the occurrence of multinucleated gonocytes cannot be used to support species similarities in androgen mediated modes of action.

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"species similarities and differences have been described in the ability to reduce testosterone production…This may related to different windows of sensitivity."

Remark: The difference in the windows of sensitivity within which testosterone is affected in rats versus in marmosets (and humans) is of critical importance in this context. Unfortunately, the dossier submitter has not given this careful enough consideration. The developmental effects that are described in rat pups following exposure to DBP (for example) occur because testosterone is impacted during a particular period of development in gestation, i.e. the male programming window. If a chemical is not capable of impacting testosterone during the male programming window, than the anti-androgenic mode(s) of action relevant during the male programming window will not be impacted and likewise subsequent adverse effects will not manifest. Chemical effects on testosterone outside the male programming window are of questionable importance for informing the relevance of a mode of action occurring in the male programming window. Instead it presents a new question (i.e. what is the mode(s) of action in neonates that this key event resides within and what effects does it lead to), one that has not been adequately addressed in this proposal and is possibly already informed from studies in mice, rats, rabbits involving neonatal exposures.

The data in multiple species strongly support that DBP can impact testosterone to an extent manifesting to adverse effects during the male programming window in rats but not other species. It is unclear why the dossier submitter considers the impact of DBP on testosterone in neonatal animals as support for DBPs ability to impact testosterone in marmosets during gestation, or why it is viewed as support for relevance of the mode of action during gestation in general.

For classification decisions, the key question is if the chemically-mediated mode of action leading to adverse outcomes in rodents is of relevance in humans (either qualitatively or quantitatively). It is scientifically not supportable to infer or conclude that a change in testosterone in neonatal marmosets provides support for the human relevance of a mode of action important during gestational development. Furthermore, it is speculative to infer 1) that an observed impact on testosterone in neonatal marmosets following exposure to DBP will lead to adverse developmental effects in humans and 2) that this testosterone change for DBP reflects the intrinsic properties of DINP particularly in the presence of data demonstrating an absence of effects on development following exposure to DINP in neonatal animals (Waterman et al., 2000).

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Remark: Regarding Lee et al. (2006): It is written that the key findings for developmental toxicity are observed in absence of maternal toxicity. For Lee et al. (2006) in point d) only the pups were examined and not the dams, thus the maternal toxicity was not considered.

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"A case-control study found an association between levels of a DINP metabolite in amniotic fluid from second trimester and cryptorchidism or hypospadias in the sons (Jensen et al., 2015)"

Remark: This statement mischaracterizes the study's findings and gives it greater weight than is justifiable. Levels of a DINP metabolite (7cx-MMeHP) were reported as having an elevated odds of hypospadias and cryptorchidism, however the confidence intervals were very wide and the finding was non-significant. In addition no association with steroid hormones or insulin-like factor 3, was found. See Part 4, Appendix XII of this submission. "

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Remark: The "comparison with "criteria" in the CLH proposal are lacking any discussion of the criteria outlined in section 3.7.2.2 of the CLP regulation. It is unclear how a decision can be reached for Table 3.7.1(a) of the CLP legal text, if the considerations under the 'basis for classification' have not been considered.

References

See Part 4, Appendix XIII for the full list of references supporting the comments made in this section of the submission

4. Appendices

Contents

A.I.: DINP does not show a comparable pattern of adverse effects and mode of action as seen for classified phthalates

The effects of DINP are not comparable to those seen with the classified low molecular weight phthalates such as DEHP, DBP, DIBP and BBP. DINP does not show the developmental malformations or loss of fertility observed with these four phthalates when tested in comparable studies. Importantly, exposures of DINP up to 1000 mg/kg bw/d during the male programming window fail to induce hypospadias, cryptorchidism, under developed Wolffian duct, or decreased accessory sex organ weight changes which are observed following exposure to LMW phthalates such as DEHP and DBP. The following effects are seen with DEHP, DBP, DIBP and BBP and are the basis for the classification: cleft palate, neural tube defects, cryptorchidism, hypospadias, testicular tubular atrophy, complete ablation of spermatogenesis, fetal death. These effects are not seen with DINP.

Moreover, the mode(s) of action (MOA) leading to the observed effects included in the hypothesized "rat phthalate syndrome" is/are not fully elucidated (as discussed by ECHA (2013) in the evaluation of new scientific evidence on DINP). As molecular targets or initiating events of the phthalates have not been identified and likely differ based on the phthalate (*i.e.*, toxicodynamic differences). At best, there is evidence that these substances impact a common measurable event (i.e. testosterone). As events can be common to more than one MOA and multiple MOAs can be linked via common events in a complex network, it is not evident that DINP shares a common MOA (see Part 4, Appendix IX of this submission for further discussion on MOA).

The concepts outlined by the OECD and WHO for developing AOP/MOA (OECD 2013, 2014; Meek et al. 2014a) clearly support that the observation of a common precursor event across chemicals is not adequate to support a chemical operates via a MOA, nor is it adequate to predict that a chemicals alteration of the event will lead to an adverse effect. As key events are necessary but not necessarily sufficient for defining a MOA nor for concluding the culmination of an MOA, indications of an effect on a key event is not itself evidence of adversity. Without a quantitative understanding of the key event relationships that define a mode of action, decisions on adversity, particularly in the context of CLP, call for pathological effects that are observed, not hypotheticals.

In this context existing empirical evidence on phthalates supports a complex network of MOAs and an inability to predict outcomes based on impacts to testosterone (Gray et al. 2016, Makris *et al.* 2013, Johnson *et al*. 2012, Howdeshell *et al.* 2016). Importantly, a simple potency extrapolation from reductions in testosterone to adverse outcomes is not supported by the extensive empirical data for DINP. For example, Hannas et al. (2011) describes DEHP to be 2.3 fold more potent than DINP on inhibiting testosterone production, which is not linearly correlated to the predicted 10-20 fold difference in potency between DINP and DEHP by Gray et al. (2000) for effects downstream of testosterone modulation. DINP is also incapable of reducing testosterone to an extent (Hannas et al. 2011; Furr et al. 2014; Boberg et al. 2011; Clewell 2013a) deemed necessary to culminate in adversity, i.e. > 80% (Gray et al. 2016, Zirkin et al. 1989). Additionally, the compiled mechanistic data presented to the US Consumer Product Safety Commission by the Chronic Hazard Advisory Panel on Phthalates and Phthalate Alternatives (CPSC, 2014, see Part 4, Appendix IX of this submission) shows effects on a variety of mechanistic markers following DINP exposure are directionally reversed from the effect observed following exposure to LMW phthalates. All of these data together support that DINP either does not share a common MOA(s), or does not impact the common MOA(s) similarly as a number of events are impacted in a directional manner that is inconsistent with the impact of other phthalates.

The term "rat phthalate syndrome" was coined to encompass a group of effects observed in male rats from exposures by certain phthalates during the critical window of male reproductive tract development (Gray and Foster, 2003; Foster, 2005; Foster, 2006). These effects include reproductive abnormalities characterized by malformations of the epididymis, vas deferens, seminal vesicles, prostate, external genitalia (hypospadias), cryptorchidism and testicular injury together with permanent changes (feminization) in the retention of nipples/areolae (sexually dimorphic structures in rodents) and demasculinization of the growth of the perineum resulting in a reduced anogenital distance (AGD) in adulthood (Gray and Foster, 2003). All of the effects may not co-occur in a single animal and each have their own prevalence. This indicates complimentary and potentially overlapping pathways but the likelihood of more than one mode of action (see Part 4, Appendix IX of this submission for more discussion on MOA). Of note, in the determination of phthalate syndrome permanent nipple retention (i.e. present in adult males) is the key factor (not a delay in agenesis of the areola which may be observed in adolescent animals (PND17)). In addition nipple retention and alterations of AGD should be noted "together" with the other factors. In isolation AGD is not a marker of rat phthalate syndrome. This is highlighted in analyses by Gray et al. (2009) and Blystone et al. 2010 which assessed the dose response for male reproductive tract malformations (RTM) as a combined endpoint rather than assessing individual endpoints. In the Gray assessment AGD was not included as an indicator of RTS and only permanent nipple retention was included[1]. In the Blystone assessment neither AGD nor Nipple retention were include instead focusing on malformations of the reproductive tract.

The basis for classifying this group of effects as a "syndrome" specifically attributable to phthalates as a class is imprecise. There are significant differences in toxicity between the low molecular weight phthalates (LMW) such as DBP and DEHP and the high molecular weight phthalates (HMW), such as DINP. When these differences in toxicity are qualitatively and quantitatively compared, it is clear that the inclusion of DINP in a hazard classification category based on potential to induce adversities related to the "rat phthalate syndrome" is not warranted. DINP has been tested at doses above 1000 mg/kg/day (Waterman et al. 2000, Masutomi et al. 2003) with no induction of the adverse outcomes on development of the male reproductive tract that are observed with certain other phthalates most notably DEHP and DBP. This clearly differentiates the DINP dataset. It has been established that effects above the limit dose are not considered relevant for Classification and Labeling unless expected human response indicates the need for a higher dose level (CLP 3.7.2.5.7). This is not the case for endpoints of interest in LMW or HMW phthalates, where data suggests that humans are refractory to the in-utero anti-androgenic effects of phthalates, which further supports that lack of relevance for the use of these endpoints for classification of DINP (see Part 4, Appendix X for more discussion on species differences).

The observation of reversible endocrine activity without adverse reproductive effects observed following in utero exposure to DINP is consistent with what the structure activity relationship associated with low

[1] Endpoints included in analyses by Gray et al: (1) morphology and histology of the testes (fluid filled and flaccid, atrophic, undescended or displaying seminiferous tubular atrophy), (2) epididymidal agenesis or hypoplasia (similar to, but less severe than aplasia) of the caput, corpus and/or the cauda (confirmed histologically, not including tissues only reduced in size) and epididymal granuloma, (3) malformed seminal vesicles or coagulating glands (agenesis of both horns or a single horn or coagulating glands detached completely from the seminal vesicles, not including tissues only reduced in size), (5) permanent nipples at adult necropsy (not including areolae without nipples), (6) gubernacular agenesis or elongation (greater than 15 mm), (6) agenesis of the ventral prostate (not including tissues only reduced in size), (7) cleft phallus with hypospadias, (8) vaginal pouch, and (9) retained cranial suspensory ligaments attaching the undescended testis to the kidney.

molecular weight and high molecular weight phthalates would predict. But these observations are by no means comparable to severe adverse effects that provide the basis for classification of the LMW phthalates. LMW weight phthalates with 3 – 6 carbons in the straight chain backbones in the alkyl side chains cause adverse reproductive effects in animal studies (Fabjan et al 2006). High molecular weight phthalates with the longest straight chain being 7 – 13 carbons in the alkyl side chains do not cause adverse reproductive effects in animal studies (OECD 2004, Clewell et al. 2013b). Extrapolating from indicators of endocrine activity outcomes to support a classification determination is not warranted as it is not consistent with the empirical data on DINP demonstrating a lack of adverse effects; nor is it consistent with what we know about the potency relationship between decreases in testosterone and adverse outcomes (Hannas et al. 2011; Furr et al. 2014; Boberg et al. 2011; Clewell 2013a; Gray et al. 2016 and Zirken et al. 1989)

A.II: DINP does not induce effects on development warranting classification.

The results of developmental toxicity studies involving in utero exposure to DINP throughout gestation, during organogenesis, and during the androgen-sensitive male programming window, demonstrate that DINP does not induce adverse effects. Of particular relevance, exposures of DINP above 1000 mg/kg bw/d during the male programming window fail to induce hypospadias, cryptorchidism, under developed Wolffian duct, or decreased accessory sex organ weight changes which are observed following exposure to LMW phthalates such as DEHP and DBP. Likewise, exposures of DINP during organogenesis do not induce the severe malformations (cleft palate, neural tube defects) observed following exposure to DEHP and DBP. There are studies reporting that exposure of pregnant dams to DINP during the male programming window leads to slight and reversible changes in biological markers of testosterone perturbation (i.e. delayed regression of the nipple anlagen postnatally) and non-androgenic endpoints (i.e. multi-nucleated gonocytes). However, any observed change (statistically significant or not) does not automatically warrant classification. Only those that are determinants of adverse effects and considered relevant per the criteria outlined in section 3.7.2. of Annex I of CLP justify a classification decision, these criteria include consideration of the nature (3.7.2.3.1), severity, incidence and toxicological significance (3.7.2.3.3) of an observation. In the case of DINP, the observed activity does not result in adverse reproductive effects (i.e. effects with functional consequences) as demonstrated in the extensive reproductive toxicity studies on DINP. While these effects are indicators of biological activity, they themselves are of minimal toxicological consequence and as such do not warrant classification.

DINP Does Not Induce Adverse Effects Following Exposures During the Male Programming Window.

Gross male reproductive tract malformations, such as cryptorchidism or hypospadias, have not been reported in any studies for DINP; including, the definitive two-generation reproductive toxicity study (Waterman *et al.*, 2000), and a number of other *in vivo* studies previously mentioned (Adamsson *et al.*, 2009; Boberg *et al.*, 2011; Borch *et al.*, 2004; Gray *et al.*, 2000; Hellwig *et al.*, 1997; Kwack *et al.*, 2009; Lee and Koo, 2007; Lee *et al.*, 2006a; Lee *et al.*, 2006; Masutomi *et al.*, 2004; Masutomi *et al.*, 2003; Waterman *et al.*, 1999).

Likewise, DINP does not induce general reproductive tract malformations manifested as decreased weights in androgen sensitive tissues: levator ani/bulbocavernosus muscles (LABC), seminal vesicles, ventral prostrate, glans penis, bulbouretral gland, and epididymis (Adamsson *et al.*, 2009; Boberg *et al.*, 2011; Gray *et al.*, 2000, Clewell *et al.* 2013b).

DINP exposure during gestation had no effect on the age of preputial separation in male rats (Gray *et al.*, 2000; Masutomi *et al.*, 2003). Furthermore, as reported by Lee *et al.* (2006) in an unreliable study, the frequency of copulatory behaviors in post natal week 20 animals was unaffected by DINP at doses of 400 or 4000 ppm (number of mountings, number of intromissions, number of ejaculations, and post ejaculation interval).

The Clewell et al (2013b) study included evaluation of preputial separation (PPS) at PND 49. The PPS score was not altered with any of the DINP treatments.

The data evaluating the effect of DINP on reduced AGD, and nipple retention, and reproductive histopathology are discussed in detail in other sections in this submission (i.e. Part 4, Appendices IV, V, and VI)

The outcomes reported in two robust developmental studies of DINP, consisting of a gavage study using 144 pregnant rats and a dietary study using 100 pregnant rats (Clewell *et al.*, 2013a, b) are fairly representative of the collective data on DINP in this window of development. These studies were designed to provide strong statistical power for analyzing, collectively, the kinetics and fetal testes effects of DINP and post-natal effects including nipple retention and AGD as well as any malformations of the male reproductive tract including hypospadias, cryptorchidism, and epididymal malformations, both gross and histological and the endpoints attributed to the hypothesized "rat phthalate syndrome." Investigation of effects at GD 19 gave a no observed effect level (NOEL) of 50 mg/kg/day based on increased MNGs and reduced testes testosterone concentration in the fetal rat. As discussed in Part 4, Appendix VII of this submission, multinucleated gonocytes (MNGs) are not a consequence of reduced testosterone synthesis and are not determinants of adversity and therefore not considered relevant to classification under CLP. Additionally, in these studies global endpoint analysis showed no evidence of a rat "phthalate syndrome" on PND 49 with DINP administration. (Clewell et al. 2013a, b).

Boberg et al. 2011 is cited extensively as key study supporting the CLH proposal. As discussed in Part 4, Appendix XI of this submission, a re-analysis of the raw data from this study using the methods in the published paper has shown that the results of statistical significance for the effects of DINP in animals cannot be reproduced for several parameters (Morfeld et al., 2017), including AGD, histopathology outcomes, and sperm parameters, calling into question the statistical and toxicological significance of these observations. Despite the errors in statistical significance the outcomes in Boberg et al. (2011) do not differ greatly from those reported in the Clewell et al. (2013a, b) studies. The only distinction is that Boberg et al. (2011) also reported outcomes on sperm. As discussed previously in the short summary sections of these comments (Parts 1 and 2 of this submission), and described in more detail in Morfeld et al. (2017), the statistically significant change in sperm motility detected only at the highest dose in Boberg et al. (2011) is not relevant to classification due to the unreliability of the experimental procedures which makes it difficult to interpret the toxicological significance, i.e. according to OECD guidance (OECD 2008) a percentage of at least 70% motile sperm for untreated control rats is a standard requirement, which is consistent with recommendations in the peer reviewed literature for optimization of experimental protocols for these parameters (Seed et al. 1995). Additionally, statistically significant changes are not alone determinants of adversity warranting classification.

DINP Does Not Induce Effects Following Exposures during the Developmental Period of Organogenesis

DINP has been repeatedly assessed in rats for developmental toxicity potential in studies involving in utero exposures during the period of organogenesis (GD6-15). These studies indicate no adverse effects on development even at doses above 1000 mg/kg bw/d. There is some indication of increased frequency of developmental variations, but these effects are mild in nature and widely held to be non-adverse as discussed in Parts 1 and 2 of this submission and; OECD, 2008; and, ECB, 2003) and not relevant to classification (CLP 3.7.2.3.3). The most generic indicator of fetal toxicity, average fetal body weight, is unaffected by DINP treatment even at exposures of 1000 mg/kg bw/d in these studies. Consistent with the conclusions reflected in the EU RAR (ECB, 2003), the effects observed in these studies do not justify classification.

(1) Waterman 1999- DINP was administered to Sprague-Dawley female rats (n ≥ 23) once daily by oral gavage at 0 (corn oil), 100, 500, or 1000 mg/kg bw/d from GD6-15. Fetal exams were conducted on GD21. Maternal weight gain and food consumption was significantly decreased in rats exposed to 1000 mg DINP/kg bw/d. There were no effects of treatment on mean number of corpora lutea, total implantation sites, post-implantation loss, and viable fetuses. There were no effects of treatment on fetal body weights or sex ratios. Under the test conditions DINP exposure increased visceral variations per litter (primarily dilated renal pelvis)

significantly increased at 1000 mg/kg bw/d. Overall occurrence of skeletal variations per litter was significantly increased at 500 (but not 1000) mg/kg bw/d for DINP. Malformations were not reported. When considering specific skeletal variations the frequency of rudimentary lumbar ribs per litter was significantly increased at 1000 mg/kg bw/d for DINP. The observation of significant increases in visceral and skeletal variations in fetuses from 1000 mg DINP/kg bw/d exposed occurred concomitantly with significant effects on maternal weight gain and food consumption. The absence of malformations or evidence of fetotoxicity (no effects on fetal body weight or survival) in the presence of some evidence for maternal toxicity suggests under the test conditions DINP did not cause adverse effects on developmental during organogenesis.

(2) Hellwig 1997 –Two different DINP CAS #s of relevance to this assessment were tested in this study[2]. For both, DINP was administered to female Wistar rats (n = 10) once daily by oral gavage at 0 (olive oil), 40, 200, 1000 mg DEHP/kg/d during GD6-15. Fetal exams were conducted on GD20. At 1000 mg/kg bw/d of DINP 1 food consumption was reduced. One animal had vaginal haemorrhage and urine-smeared fur, but all rats delivered litters and there was no effects on pre- or post-implantation loss, resorptions, number of liver fetuses/dam, or foetal weight. An increased frequency of foetal skeletal variations (primarily rudimentary cervical and/or accessory 14th rib[s]) was reported. When reviewed on both foetal and litter basis, frequency of foetal malformations in DINP 1 exposed dams was similar to controls. At 1000 mg DINP 2/kg bw/d one dam showed vaginal haemorrhage on GD14 and 15, but all rats delivered litters and there was no effects on pre- or post-implantation loss, resorptions, number of liver fetuses/dam, or foetal weight. An increased incidence of a skeletal variation (accessory 14th rib[s]) was observed. A single foetus presented with multiple malformations (globular-shaped heart, unilobular lung, hydrocephaly, dilatation of the aortic arch and anasarca) regarded as spontaneous in nature. When reviewed on both foetal and litter basis, frequency of foetal malformations in DINP 2 exposed dams was lower than controls.

(3) Hazleton 1981-DINP was administered to female Sprague Dawley rats once daily by oral gavage at 0 (corn oil), 10, 500, or 1000 mg DINP/kg/d on GD6-15. Fetal exams were conducted on GD20. No exposure related effects occurred in dams when considering maternal body weights, clinical signs, pregnancy rates, mean number of corpora lutea, implantation efficiencies, gross pathology or uterine weight changes. At 1000 mg/kg/d resorption incidence was marginally increased (10.2% compared to 4.8% in controls) and fetal viability was slightly lower (values not specified) compared to control, although not statistically significant. Fetal body weights were unaffected by exposure. Incidence of visceral variations (dilated ureters and/or kidneys) was generally higher in the treated groups but not statistically significant and did not appear to be exposure-related.

DINP Does Not Induce Developmental Effects in Multi-Generational Studies

Effects on offspring were not observed following exposure in multigenerational toxicity studies (Waterman et al. 2000). Briefly, offspring survival was reduced at the 1.5% level (~1100 mg/kg bw/day but unaffected at the 1% level (~760 mg/kg/day). No gross malformations were observed nor were any macroscopic effects seen in a number of selected organs and tissues, there were no testicular effects in either P1 males exposed as juveniles and young adults or the P2 (F1) offspring exposed in utero, through lactation and continuously to

[2] "DINP 3" (CAS 28553-12-0), which was based on n-/iso butenes and consequently had a high degree of branching in the alcohols, is not on the market and is excluded from relevance to this assessment . This is consistent with the conclusion in the EU Risk assessment of DINP (ECB, 2003) to exclude data on DINP3 (cf explanatory note in the EU RAR (2003). **Results from DINP3 cannot be taken into account for the evaluation of DINP1 or DINP2** as the alcohols in the DINP3 consisted of at least 60 % alkyl-substituted Hexanols, while in the other two DINPs the alcohols consist of substituted heptanols and octanols).

terminal sacrifice. As described in Part 4, Appendix III of this submission, there were deceases in offspring bodyweight, however the effects were only concluded to be treatment related at the 1.5% level. Consistent with conclusion expressed in the EU RAR on DINP (ECB, 2003), classification based on the observations in this study is not warranted (in addition to the publication, a detailed summary of these data can be found in the EU RAR on DINP (ECB, 2003); and the REACH dossier for this substance).

A.III: Changes in bodyweight do not reflect inherent toxicity of DINP and are not relevant for classification

The CLH proposal identifies a decreased body weight in offspring in a two-generation study (Waterman et al. 2000) as a key finding supporting a classification conclusion of clear evidence of an adverse effect on development. Closer consideration of these body weight changes in the context of the study as well as the larger database on DINP, supports that changes induced by DINP below the limit dose are very likely a consequence of palatability rather than inherent toxicity of DINP and as such do not justify classification. Furthermore, the weights in the Waterman et al. (2000) study were within the historical control range (with the exception of the F2 high-dose males and females on PND 0 and F2 high dose males on PND 1) and the effects were reversible even with continued treatment. The only treatment related toxicological impacts on body weight in this study occurred at the high dose of 1.5% at PND 0, the estimated exposure from 1.5% DINP in the diet is ~ 1100mg/kg bw/day, above the limit dose. **As effects occurring above the limit dose are outside the criteria for classification (CLP 3.7.2.5.7) and the body weight changes below the limit dose do not reflect an intrinsic toxicity of DINP (CLP3.7.2.2.1), these observations are not relevant to a classification decision per CLP. This is consistent with the interpretation reflected in the EU RAR for DINP (ECB, 2003) that the effects observed in the Waterman et al. (2000) do not justify classification.**

Effects on Offspring Bodyweight are Not Observed in All Studies and do Not Reflect an Intrinsic Property of DINP

Effects following in utero exposure to DINP on offspring bodyweight are inconsistently observed across studies. Among the relevant studies, 5 of 11 studies report some effect on fetal body weight. Of the positive studies, 4 of 5 studies involve dietary exposure, whereas all negative studies involve exposure via gavage. Of the 4 positive gavage studies, one study (Lee et al. 2006) is of considerably low quality for a number of reasons (as described in ECHA, 2013 pp120-121) including the lack of clarity as to whether statistical outcomes are reported on a per animal or per litter basis. Therefore that study was not evaluated further. The details of the other 3 positive dietary studies and the positive gavage study are outlined below.

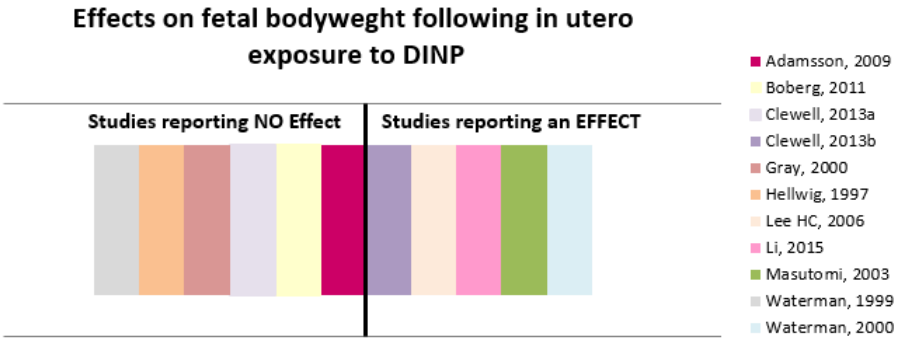


Figure IIIa Weight of the evidence for effects on body weight is equivocal. Reproductive toxicity studies involving in utero exposure to DINP and that reported outcomes on fetal bodyweight are represented above. Studies reporting a statistically significant difference in fetal body weight at any time point appear on the right-hand side of the chart as reporting "an EFFECT"(n=5 studies). Studies that did not have a statistically significant impact on fetal bodyweight appear on the left-hand side of the figure as reporting 'NO effect" (n=6 studies). Therefore positive outcomes in this figure indicate effects of statistical significance only and do not include consideration of dose response, variation, percent change, etc.

(1) Waterman et al. (2000) – Although the Waterman paper describes both a one-generation study (conducted for dose-selection purposes) and a two-generation study, it is the two generation study data that is most useful for purposes of this discussion. In this study rats were given DINP by dietary administration at levels of 0.2, 0.4, or 0.8% in the diet. Dosing was initiated 10 weeks prior to mating and continued to post-natal day 21 of the second generation. By reference to Waterman Tables 8, it is apparent that in the first generation there was little effect of DINP treatment on birth weights (which were related to pre-natal exposures) or body weights in the perinatal period (when exposure would be via lactation), but differences begin to appear at PND 7 (when the pups start eating solid food) and by PND 21 (weaning) statistically significant differences in body weights were apparent in all treatment groups. The results of the second generation (Waterman Table 11) were similar but the differences in the lowest dose group were not statistically different from control values. Waterman et al. considered that the effect on body weight gain was due to palatability and addressed this issue in the discussion section of the publication. As pointed out by Waterman, the offspring body weights throughout lactation were within the historical control range at the 0.2 and 0.4% levels in the two-generation study and the 0.5% level in the one-generation study. At the 0.8% level, the weights were within the historical control range except for PND0 and PND1 in the second generation. Additionally, recovery occurred in all groups even though dietary exposure continued, with no long-term consequences noted. Therefore the only treatment related toxicological impacts on body weight in this study occurred at a dose of 1.5% at PND 0 in the 1-generation study, the estimated exposure from 1.5% DINP in the diet is ~ 1100mg/kg/day, above the limit dose.

(2) Masutomi et al. (2003) - In this study rats were given DINP by dietary administration starting at GD15 and continuing to PND10. The offspring was subjected to prepubertal necropsy on All survivors were sacrificed on PND27 and to adult examination on PND71. Dietary levels were 0.04%, 0.4% and 2% (corresponding to approximately 31, 307 or 1165 mg/kg/day (GD15-20) and 66, 657, or 2657 mg/kg/day (PND2-10)30, 300, or 1500 mg/kg/day). As shown in Masutomi Table 4, food consumption and maternal weight gain in the highest dose group (above the limit dose) was significantly reduced on GD15-GD20 and during the lactational period (PND2-PND10). Body weights of offspring were not significantly different from control values on PND 2 at any dose. Weight gain of offspring was significantly reduced in the high dose group (above the limit dose) during the period PND2-10 (dosing was discontinued on PND10), but there were no significant differences in offspring body weights during the period PND10-PND21, Body weights of offspring were not significantly different at termination (PND771). These data provide evidence that the reduction in body weight gain is a reversible effect, and show no significant effect on offspring bodyweight below limit doses.

(3) Clewell et al. (2013a) – In this study rats were given DINP by dietary administration between GD12-PND14 at dietary levels of 760, 3800, or 11400 ppm (corresponding to ~50, 250, or 750 mg/kg/day). There was a significant reduction in maternal weight gain in the high dose group and offspring body weights in the high dose group were significantly lower than control values at PND2. At PND 14 (the last day of dosing), the body weights of offspring in the mid and dose groups were significantly below control values, but at terminal sacrifice (approximately PND50), the body weights were not significantly different. Clewell et al. (2013) considered this to have been related to palatability and not due to the intrinsic hazardous properties of DINP as discussed in paragraph 4.2 of their discussion, and captured here:

- A concurrent reduction in food consumption indicates that food palatability is likely responsible for reduced weight gain in 11,400 ppm DINP dams.

- When DINP was administered via oral gavage at doses up to 900 mg/kg-day during gestation, no alteration in maternal or fetal bodyweight was observed [Boberg et al. (2011), Clewell et al. (2013b)].
- Palatability of milk and feed is also likely responsible for the temporary reduction in bodyweight of the PND 14 pups, as body weight was within control values in either PND 2 or PND 49 pups (Clewell et al. 2013a).
- Masutomi et al. (2003) had similar findings: Offspring sacrificed on PND 27 rats had decreased body weights after administration of 4000 ppm and 20,000 ppm DINP (males) and after 20,000 ppm (females) in the diet from GD 15 to PND 10, while PND 2 and PND 71 bodyweights were similar to controls.
- Cross fostering studies with diisodecyl phthalate (DIDP) supported the hypothesis that the reduced palatability of milk contributes to low postnatal bodyweight (Nikiforov et al. 1997).

(4) A statistically significant change was reported in one (Li et al. 2015) of seven studies involving in utero exposure to DINP via gavage. As shown in Table 1 in the publication, DINP did not affect the duration of pregnancy, birth rates, number of pups per dam. There was no effects on the body weight of the dams. However, the body weight of the male pups was significantly (but not dose dependently) reduced by all doses of DINP (~10%, ~16%, ~8%, ~9% at doses of 10, 100, 500 and 1000mg/kg bw/d). The lack of dose responsiveness indicates that this statistically significant difference is unlikely to be an effect of treatment.

Changes in Fetal Bodyweight Observed Following in utero Exposure to DINP are Not Relevant to Classification According to CLP

In summary, DINP is not consistently observed as impacting fetal body weight following in utero exposure. In general, when DINP was given by dietary administration, the offspring in the treated groups gained weight less rapidly than the corresponding controls, particularly at dietary levels corresponding to daily doses > 200 mg/kg/day. These effects were most notable when the animals were starting to transition from lactation to solid food (~PND7). Furthermore the differences are reversible both under continued exposure as seen in Waterman et al. (2000) and once exposures were terminated as shown by comparative data from studies in which exposure is continued versus those in which rats were exposed for limited periods of time and then held without treatment until terminal sacrifice. As detailed above, the body of evidence supports that the offspring body weight effects occurring below the limit dose are likely a consequence of palatability rather than inherent toxicity of DINP. Therefore this effect is not relevant to the classification of DINP as a reproductive agent per CLP.

A.IV: Observations of nipple retention in juveniles that are not correlated with impacts in adulthood are of minimal toxicological significance and do not warrant classification

According to CLP 3.7.2.3.3 small differences in postnatal developmental assessments are considered of minimal toxicological significance and do not warrant classification. The magnitude of the induced effect in juveniles by DINP (when observed) is small (1.98 nipples/male in control animals vs. 3.17 nipples/male in the highest dose group). A characterization of minimal significance is consistent with observations that 1-3 retained nipples/areolae on PND13 is associated with a minimal response in the testes (Barlow 2004). In addition, observations of nipple retention in juveniles occur at low incidence in controls, are known to be reversible effects (Barlow, 2004) and OECD guidance (OECD, 2008) emphasizes permanent nipples in males (i.e. presence in adults) to be the hallmark of a permanent structural change (i.e. malformation).

Observations of one adult animal with a larger number of retained nipples in a dose group is not appropriate for informing a chemicals intrinsic ability to induce the effect, as males with up to 7 retained nipples are known to occur in control animals. Based on control data one would expect a male with 4-7 nipples to occur 8.1% of the time (i.e. 1.6 animals in a dose group with 20 animals) and 1-7 nipples 23% of the time (i.e. 4.7 animals in a dose group with 20 animals). The low incidence of permanent nipples observed in Boberg *et al*. (2011) and Gray *et al.* (2000) (i.e. # of animals with nipples) is consistent with what one would expect by chance (i.e. 1/35 and 1/18 with 4-7 nipples and 1/52 with 4-7 nipples respectively). The likelihood of these observations occurring by chance is supported by the lack of males with >2 nipples in the highest dose group (900 mg/kg/d) tested by Boberg *et al.* (2011), i.e. the occurrence is not dose responsive in this study as the number of nipples retained/male and the number of males retaining them is not higher than controls. The data demonstrate DINP does not increase the number of permanently retained nipples in adult males (0/3).

Therefore small differences in postnatal developmental assessments that are not correlated to impacts in adulthood are considered of minimal toxicological significance and do not warrant classification (CLP 3.7.2.3.3).

Nipple retention as a toxicological indicator of effect & its relevance to classification

Nipple retention in males is thought to be a sensitive endpoint downstream of a reduction in fetal testosterone and has been assessed in several studies. The development of the rodent nipple is sexually dimorphic (Kratochwil, 1971; Kratochwil and Schwartz, 1976). Although mammary gland development begins similarly in both male and female rodent fetuses, offspring female rats and mice have nipples but males typically do not. In the developing rodent fetus, di-hydroxy testosterone produced locally from fetal testosterone causes regression of the nipple anlagen (Imperato-McGinley *et al.*, 1986; Kratochwil, 1977, 1986). This process can be disrupted, and the affected male offspring subsequently display nipples. Nipple retention data are reported as litter incidence (# of litters/total litters per dose group) or pup incidence (#males/total #males per dose group) and/or average number of nipples/areolas per male. Not all studies report both measures and it is not always clear if the number of nipples/male is litter averages or individual averages per dose group. Incidence is not considered a sensitive measure as it is highly dependent on control values. Average # of nipples /male is considered a more sensitive indicator (Beekhuijzen *et al*. 2016) which also gives an indication of the severity of the effect. Female rats normally have 12 nipples and theoretically males should have 0 nipples, however

retained nipples in males does vary in control populations. When evaluating this endpoint in toxicological studies it is important be evaluated on the same PND as differences in the maturation process could artificially identify or obscure treatment related effects (Beekhuijzen *et al*. 2016). Observations of nipples in juvenile animals may be indicative of a delayed regression of the nipple anlagen which is transient and in the lexicon of developmental toxicity should be characterised as a variation which would be considered of minimal toxicological significance. The OECD guidance document on mammalian reproductive toxicity testing and assessment (OECD 2008) highlights "permanent nipples in males (i.e. found in adults) constitute a permanent structural change". Assessment in juveniles is of use to assess correlation with parameters recorded in the same animals in adulthood (OECD 2008).

Permanence of the effect (nipple retention observed in adults) is a marker of "rat phthalate" syndrome (Foster 2005) and may be associated with adverse effects on the male reproductive tract. Though the severity, and not just the mere occurrence, of the nipple retention (i.e. # of nipples retained) is important (McIntyre *et al.* 2001). One study found that male rats with malformations of DHT-dependent tissue had 6 or more nipples as adults, and an almost completely feminized phenotype (10-12 nipples) was observed in male rats with malformations in T-dependent tissues (McIntyre *et al.* 2001) Variations in the presence/absence (incidence) and degree of observed nipples (#/12) occurs in the control population. Reporting of the number of retained nipples (12 nipples being fully feminized) is important for understanding the severity of the effect and it is recommended to use the number of areolas in each male for the assessment (OECD 2008) to inform conclusions on treatment-related effects.

Nipple retention associated with DINP treatment

Out of three studies that evaluated nipple retention in juvenile animals (PND12 or 14) one study reported an increase incidence of nipples retained (Gray et al. 2000) with no statistically significant increase in severity (# of nipples per animal); one study reported a small statistically significant increase in severity (# of nipples per male) but did not report incidence (Boberg et al. 2011); and one study observed no statistically significant increase in severity (# of nipples per male) and did not report incidence (Clewell et al. 2013b). Each of the studies evaluated the animals for permanence of the effect by scoring nipple retention in adults and none of the studies (0/3) found a statistically significant effect on either parameter (Boberg et al. 2011; Clewell et al. 2013b; Gray et al. 2000). Table IVa below summarized the nipple retention data for DINP.

Table IVa: Summary of Nipple Retention data present in the literature

DINP Treated				Age at Nipple Retention Measurement			
	Route/ Strain	Exposure Duration	Dose (mg/kg/d)	PND13-PND14		Adult	
				% Incidence	Avg. # per male	% Incidence	Avg. # per male
(Gray *et al.*, 2000)*	G/SD	GD 14 – PND 3	0	0	n.d.	0	0
			750	**22.4**		4	0.11
(Boberg *et al.*, 2011)*[1]	G/W	GD 7 – PND 17	0	n.d.	1.98	10.5**	0.09**
			300		2.00	0	0.00
			600		2.91	8.6	0.17

			750		**3.14**	16.7	0.44
			900		**3.17**	13.4	0.14
(Clewell *et al.*, 2012b)	D/SD	GD 12 – PND 14	0	n.d	1.8	n.d.	0.6
			56		1.7		0.8
			288		1.9		0.4
			720		2.1		0.4

G: Gavage D: Diet SD: Sprague Dawley W: Wistar, **Bolded** values were reported as statically significant by authors.
*In these studies data is provided as pup incidence or pup mean. Otherwise data is reported as either litter incidence or litter mean
1. There were 2 animals with 1 nipple per animal in the control group, comparable to 3 males per group in the three highest dose groups with 1-6 nipple per animal, w/ one with 4 in the 600 and one with 6 in the 750 mg/kg group. It was assumed animals not given a specific nipple value had 1 nipple i.e. (2 with 1 at 600, 2 with 1 at 700, and 3 with 1 at 900). Range of males per group given as 18-35. Assumed pup number examined for nipple retention was equivalent to pup number assessed for testicular histopathology reported in Table 5 based on the methodology for controls and 300 mg/kg/d dose groups. The number of animals reported in Table 5 are consistent with the reported animals evaluated for nipples in the 600 (n=35) and 750 (n=18) mg/kg/d groups. The reported number of animals examined for nipples in the 900 mg/kg/d group is slightly higher (n=22) compared to the number reported in the table for testis histopath (n=18). Incidence not reported in publication. Looking at the raw data from Boberg *et al.* 2011 (available on EPA's HERO database) it appears litter incidence for nipple retention was 100% across all groups at PND14. For pup incidence the values appear to be 94.6, 97.1, 98, 100, 100 for 0, 300, 600, 750, and 900 mg/kg/d respectively. Adult incidence for Boberg *et al* 2011 was estimated from litter numbers and effected animals reported in the text.

As reported in Gray *et al.* (2000), data for DINP indicated that at 13 days of age, infant males with areolas were observed at an incidence of 22% compared with controls (0%) (Table IVa). Incidence is an insensitive measure of nipple retention and nipples per male is the recommended measure to use for assessment (OECD, 2008) as it gives a more precise indicator of effect and information on the severity of effect. At approximately 5 months of age, 2/52 male pups displayed permanent nipples where the number of nipples equaled 1 and 6 for each of the two males. The range of control values in the literature important for understanding the low incidence observed in adults. In the public literature McIntyre *et al.* (2001) list 2/76 control animals with permanent nipples (1 and 4), and Boberg *et al.* (2011) reports 2 out of 23 animals (1 each) with permanent nipples in adults. In the study report from Clewell *et al.* (2013b) it can be determine that 26/111 adults males had retained nipples ranging from 1-7 and one would expect a male with 4-7 nipples to occur 8.1% of the time (i.e. 1.6 animals in a dose group with 20 animals) and 1-7 nipples 23% of the time (i.e. 4.7 animals in a dose group with 20 animals (Table IVb). The number of animals and nipples per animal reported in Gray *et al.* (2000) is not outside what might be expected by chance. Additional dose-response information would be helpful to further inform interpretation.

Boberg *et al.* (2011) reported a significant increase in the average number of nipples in males exposed to DINP at 750 and 900 mg/kg bw/day (average of 3 nipples in each dose group) as compared to controls (average of 2 nipples) on post natal day 13. On post natal day 90, study authors reported 2 animals with 1 nipple per animal in control group compared to 3 males per group in the three highest dose groups with 1-6 nipples per animal (one male with 4 in the 600 and one with 6 in the 750 mg/kg group; and presumably two males with 1 nipple in 600, two males with 1 nipple in the 750, and three males with 1 nipple in the 900mg/kg group). These values, however, were not statistically significant, do not follow a dose response, and give an average number of nipples per male in each dose group of 0.0, 0.17, 0.44, and 0.14 nipples per male for 300, 600, 750, and 900

mg/kg/d respectively and approximately 0.09[3] for the controls. Given the comparable number of nipples per male across dose groups and the lack of a dose response there is no clear evidence that DINP is intrinsically capable of causing permanent nipple retention. The single animals with retained nipples of 4 and 6 are likely chance findings. A tabulation of control data from the study report for Clewell *et al.* (2013b) indicates the number of nipples retained in the adult control animals ranges from 1-7 and one would expect a male with 4-7 nipples to occur 8.1% of the time (i.e. 1.6 animals in a dose group with 20 animals) and 1-7 nipples 23% of the time (i.e. 4.7 animals in a dose group with 20 animals (Table IVb, below). The low incidence and magnitude of effect reported by Boberg *et al.* (2011) is within these bounds and cannot be interpreted as toxicologically meaningful. Particularly in light of the data from Clewell et al. (2013b) where no significant difference of nipples in males exposed to DINP at approximately 50, 250, and 750 mg/kg/day in either juvenile or adult animals. This study included 100 pregnant females and was designed to provide strong statistical power for analyzing post-natal effects including nipple retention.

Table IVb: Incidence in the control population from Clewell et al. *2013b for the number of nipples per male retained as an adult*

# of retained nipples	# of control males
0	85
1	12
2	4
3	1
4	2
5	3
6	1
7	3

DINP Does Not Induce Permanent Nipple Retention in contrast to DBP and DEHP

DINP exhibits a different pattern of effect on measures of nipple retention than DBP and DEHP. Nipple retention in juveniles are not consistently observed in studies with DINP (2/3) compared to studies with DBP (8/8) and DEHP (8/8) where doses greater than 150 mg/kg/d were tested (Table IVc below). No study reports a statistically significant effect on incidence or an increase in severity of effect (avg # per animal) in adults (0/3) for DINP. This contrasts with DBP (3/3 studies report effects) and DEHP (4/4 studies report effects). Additionally all studies with DBP and DEHP which report information on both incidence and severity of effect (avg. # per male) found statistically significant effects in both parameters (see Table IVd at end of this Appendix). Which contrasts with DINP where the statistically significant effect was found for one measure but not the other.

[3] The number of animals in the control group was estimated from numbers reported in Table 5 of Boberg et al. (2011) for Testis histopath. These values were consistent with values reported in the text for group numbers of animals evaluated for nipple retention at 600 and 750 mg/kg/d.

Table IVc. Nipple retention following in utero exposure to DINP

		Nipple retention (# positive studies/total # of studies
DINP	Adolescent	2/3[1]
	Adult	0/3
DBP[2]	Adolescent	8/8
	Adult	3/3
DEHP	Adolescent	8/10[3]
	Adult	4/4

Reproductive toxicity studies involving in utero exposure to either DINP, DBP or DEHP and that assessed and reported nipple retention outcomes are represented in the table above. A study was considered positive if either a statistically significant increase in nipple retention (either in juveniles or adults) was reported at any dose; or a greater than 10% increase in incidence (statistically significant or not) over control was observed. Incidence refers to either the number of males with retain nipples/total number of males per dose group or the number of litters that have a male with retained nipples/total number of litters per dose group.

1. 1/3 studies reported a statistically significant increase in severity (Boberg *et al.* 2011). Raw data for Boberg *et al.* 2011 obtained from the EPA's HERO data base indicate there is no statistically significant difference in incidence. Gray *et al.* (2000) reported a statistically significant increase in incidence and no effect on severity, however this study had an unusually low (0%) incidence in the control animals which questions the relevance of this finding.
2. Statistical analyses of the endpoint was not conducted in Saillenfait 2008 and Mylchreest 1999. The endpoint was assumed as a significant effect for the chart given the magnitude of the reported incidence in comparison to control (0 vs 75% and 0 vs 88.9% respectively).
3. The two studies where no effects were observed for DEHP the highest doses tested were 100 mg/kg/d and 150 mg/kg/d. These doses are below that at which one would reliably expect to see an effect. It can be considered that these studies would have identified an effect if tested at a higher dosage.

The incidence of nipple retention and severity of effect (avg. #/male) noted in Gray *et al.* (2000) and Boberg *et al.* (2011) for nipple retention in juveniles are notably different from what is seen in studies with DBP and DEHP (Figure IVa).

Figure IVa: The ability of DINP to induce nipple retention (incidence) and the severity of the induced effect (avg. #/male) in juvenile males differs from DBP and DEHP.

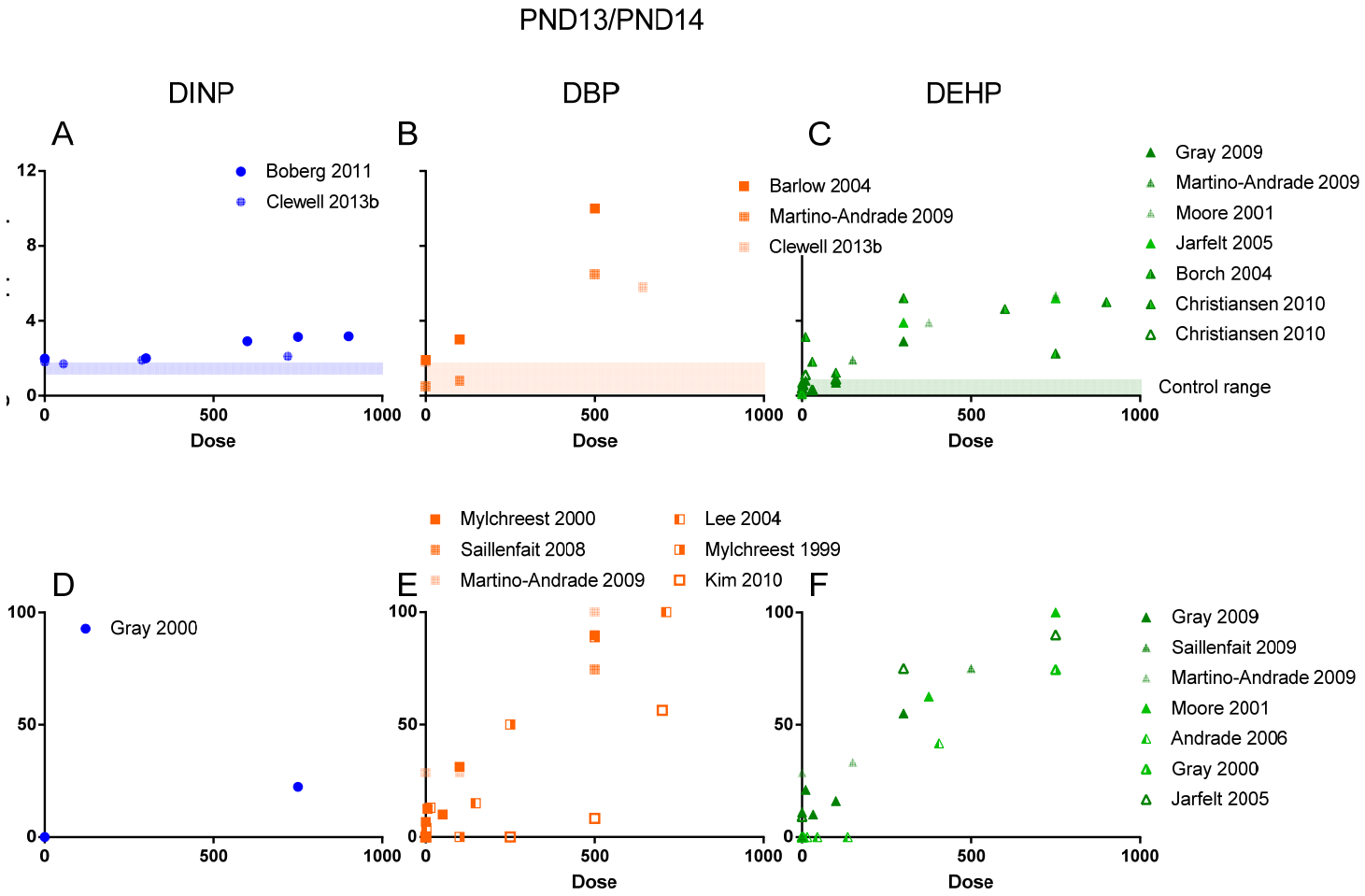


Figure IVa. Average number of nipples per male (A, B, C) or incidence of retained nipples (D, E, F)as measured on either PND 13 or PND 14 after exposure during the male programming window (MPW) with either (A, D) DINP (B, E) DBP or (C, F) DEHP. Some studies in this figure use pup incidence rather than litter incidence. Table IVd indicates which studies report incidence is by pup rather than litter. Both the magnitude of the induced retention and severity are lower for DINP than DBP and DEHP

Though transient nipple retention (present on PND13/14) has been observed after treatment with DINP it is important to note that the literature considers permanent nipple retention in adulthood (e.g. presence in sexually mature animals ~5 wks or >PND35) the biologically relevant endpoint (Foster 2005, Foster 2006, OECD2008). A statistically significant increase in the avg. number of retained nipples has not been reported in

any of the DINP studies (0/3) in comparison to all of the DBP (3/3) and DEHP (3/3[4]) studies where statistical significance of permanent nipples has been reported. The average retained nipples per male in adults for DBP and DEHP is 2 or more, compared to the maximum reported value at any dose of DINP of 0.44 (Clewell *et al.* 2013b), and 0.11 (Boberg *et al.* 2011), values that are comparable to control ranges of 0-0.6 in the DINP studies, Table IVd. The low incidence of permanent nipples observed in Boberg et al. (2011) and Gray et al. (2000) is consistent with the~8% incidence of nipples in this range one would expect by chance based on the control data from Clewell *et al.* 2013b (i.e. 1/35 (~3%) and 1/18 (~5.6%) with 4-7 nipples in Boberg and 1/52 (~2%) with 4-7 nipples in Gray). The likelihood of these observations occurring by chance is supported by the lack of males with >2 nipples in the highest dose group (900 mg/kg/d) tested by Boberg et al. (2011). Though less studies evaluated the endpoint in adults the differences in nipple retention induced by DBP and DEHP is strikingly different from DINP (Figure IVb below).

[4] One additional DEHP study assessed permanence but did not report on significance and is therefore not reflected in this tally but the dose group was elevated over control 2.13 vs 0.07

Figure IVb: The DINP does not have a biologically or toxicologically meaningfully effect on nipple retention in adults (incidence and severity of effect). This contrasts with DBP and DEHP which clearly increase permanent nipple retention in adults following exposures during the male programming window.

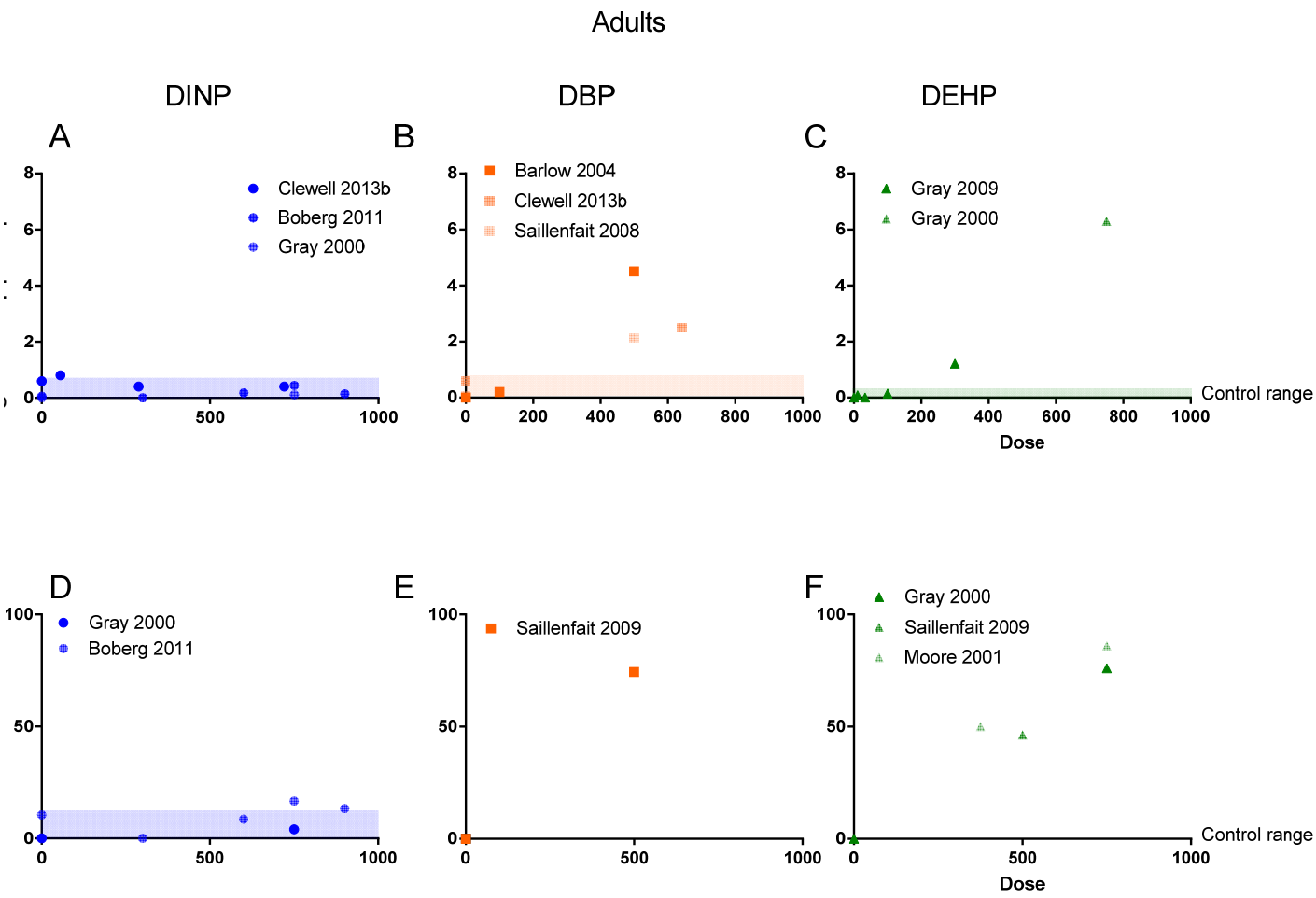


Figure 2. Average number of nipples per male (A, B, C) or incidence of retained nipples (D, E, F) as measured in adults after exposure during the male programming window (MPW) with either (A, D) DINP (B, E) DBP or (C, F) DEHP. Some studies in the figure use pup incidence rather than litter incidence. Table IVd indicates which studies report incidence is by pup rather than litter. Both the magnitude of the induced retention and severity are lower for DINP than DBP and DEHP

Nipple retention data for DINP, and DBP was compiled from relevant references identified by the US EPA in the preliminary IRIS evidence tables for DINP[5] and DBP[6].For DEHP, no recent IRIS documents were available,

[5] http://ofmpub.epa.gov/eims/eimscomm.getfile?p_download_id=525505

[6] http://ofmpub.epa.gov/eims/eimscomm.getfile?p_download_id=524735

therefore the 2006 NTP-CERHR monograph (Shelby 2006) on the potential human reproductive and developmental effects of di (2-ethylhexyl) phthalate (DEHP) was mined for references. Once all references from those sources were reviewed and the data tabulated a Pubmed search using the search terms DINP, DBP, or DEHP, and phthalate, and nipple retention was conducted. Any references that had not already been collected were reviewed for potential data. The information on nipple retention was tabulated in Table IVd.

Table IVd Compilation of dose response data from reviewed literature.

					Age at Nipple Retention Measurement			
	Phthalate	Route/ Strain	Exposure Duration	Dose (mg/kg/d)	PND13-PND14		Adult	
					% Incidence	Avg. # per male	% Incidence	Avg. # per male
(Gray *et al.*, 2000)*	DINP	G/SD	GD 14 – PND 3	0	0	n.d.	0	0
				750	**22.4**		4	0.11
(Boberg *et al.*, 2011)*[1,3]	DINP	G/W	GD 7 – PND 17	0	n.r.	1.98	10.5**	0.09**
				300		2.00	0	0.00
				600		2.91	8.6	0.17
				750		**3.14**	16.7	0.44
				900		**3.17**	13.4	0.14
(Clewell *et al.*, 2012b)	DINP	D/SD	GD 12 – PND 14	0	n.r.	1.8	n.r.	0.6
				56		1.7		0.8
				288		1.9		0.4
				720		2.1		0.4
(Lee *et al.* 2004)*	DBP	D/SD	GD15 - 20	0	0	n.d.	n.d.	
				2-3	4			
				14-29	13			
				148-291	15			
				712-1372	**100**			
(Mylchreest *et al.*, 2000)	DBP	G/SD	GD12- 21	0	26	n.d.	n.d.	
				0.5	25			
				5	42			
				50	50			
				100	**80**			
				500	**100**			
(Clewell *et al.*, 2013)	DBP	D/SD	GD 12-PND14	0	n.r.	1.8	n.r.	0.6
				642		**5.8**		**2.5**
(Martino-Andrade *et al.*, 2009)[2]	DBP	G/W	GD 13 – 21	0	28.6	0.5	n.d.	
				100	28.6	0.8		
				500	100	6.5		
(Mylchreest *et al.*, 1999a)	DBP	G/SD	GD 12 – 21	0	0	n.d.	n.d.	
				100	0			
				250	50			
				500	88.9			
(Barlow *et al.*, 2004)*	DBP	G/SD	GD 12-21	0	n.d.	1.9	n.d.	0
				100		**3**		0.2
				500		**10**		**4.5**
(Saillenfait *et al.,* 2008)	DBP	G/SD	GD 12 – 21	0	0	n.d.	0	0.07
				500	74.6		74.4	2.13

(Kim *et al,*. 2010)*	DBP	G/SD	GD 10 – 19	0	0	n.d.	n.d.	
				250	0			
				500	8.3			
				700	56.4			
(Gray *et al.*, 2000)	DEHP	G/SD	GD 14 – PND 3	0	0	n.d.	0	0
				750	86.9		**76**	**6.3**
(Borch *et al.*, 2004)	DEHP	G/W	GD 7 – PND 17	0	n.r.	0.25	n.d.	
				750		**2.25**		
(Moore *et al.*, 2001)*	DEHP	G/W	GD 6 – PND21	0	0	0	0	n.d.
				375	62.5	3.9	50	
				750	**100**	**5.375**	86	
				1500	**100**	**8.7**	100	
(Saillenfait *et al.*, 2009)	DEHP	G/SD	GD 12 - 21	0	0	n.d.	0	n.d.
				500	75		46.3	
(Martino-Andrade *et al.*, 2009)[2]	DEHP	G/W	GD 13 – 21	0	28.6	0.5	n.d.	
				150	33	1.9		
(Gray *et al.*, 2009)*	DEHP	G/SD	GD8 – PND17	0	11	0.7	n.r.	0.0
				11	21	0.3		0.08
				33	10	0.8		0.0
				100	16	0.7		0.15
				300	**55**	**2.9**		**1.22**
(Jarfelt *et al.*, 2005)[4]*	DEHP	G/W	GD - PND 17	0	9	0.1	n.d.	
				300	**75**	**3.9**		
				750	**90**	**5.2**		
(Christiansen *et al.*, 2010)[5]	DEHP	G/W	GD 7 – PND 16	0	n.r.	0.22	n.d.	
				10		**3.14**		
				30		**1.81**		
				100		**1.23**		
				300		**5.21**		
				600		**4.63**		
				900		**5.01**		
(Christiansen *et al.*, 2010)[5]	DEHP	G/W	GD 7 – PND 16	0	n.r.	0.38	n.d.	
				3		0.59		
				10		1.13		
				30		0.31		
				100		0.86		
(Andrade *et al.*, 2006b)*	DEHP	G/W	GD 6 – PND21	0	0	n.d.	n.d.	
				0.015	0			
				0.045	0			
				0.135	0			
				0.405	0			
				1.215	0			
				5	0			
				15	0			

				45	0		
				135	0		
				405	41.67		

G: Gavage, D: Dietary, SD: Sprague Dawley, W: Wistar, n.r.: not reported, n.d. not determined. **Bolded** values were reported as statistically significant by study authors.

*Incidence (# of animals w nipples) is per pup not per litter,

1: There were 2 animals with 1 nipple per animal in the control group, comparable to 3 males per group in the three highest dose groups with 1-6 nipple per animal, w/ one with 4 in the 600 and one with 6 in the 750 mg/kg group. It was assumed animals not given a specific nipple value had 1 nipple i.e. (2 with 1 at 600, 2 with 1 at 700, and 3 with 1 at 900). Range of males per group given as 18-35. Assumed pup number examined for nipple retention was equivalent to pup number assessed for testicular histopathology reported in table 5 based on the methodology. The number of animals reported in Table 5 are consistent with the reported animals evaluated for nipples in the 600 (35) and 750 (18) mg/kg/d groups. The reported number of animals examined for nipples in the 900 mg/kg/d group is slightly higher (22) compared to the number reported in the table for testis histopath (18).

2: In the table 75th percentile values are depicted. The study also reported median nipples/animal which were 0, 0, and 4 for 0, 100, and 500 mg/kg/d respectively for DBP. Median values for DEHP were 0 for both 0 and 100 mg/kg/d

3: Incidence not reported in publication. Looking at the raw data it appears litter incidence for nipple retention was 100% across all groups at PND14. For pup incidence the values appear to be 94.6, 97.1, 98, 100, 100 for 0, 300, 600, 750, and 900 mg/kg/d respectively. Adult incidence was estimated from litter numbers and effected animals reported in the text.

4: Authors grouped 0-1 nipples into the same category for the dose groupings. Only reporting incidence for more than 2 nipples so incidence may be higher. Authors separately indicated 9.1 as non-zero value for control.

5: Authors reported two separate studies in single publication.

A.V: Changes in AGD do not meet the criteria for classification as they are inconsistently observed across studies, transient when observed, and do not meet a criteria of biological significance

DINP Does Not Induce Changes in Anogenital Distance Relevant for Classification

According to OECD guidance document 43, a permanent change in AGD (i.e., observed at birth and into adulthood) constitutes a permanent structural change (OECD 2008). While it has been assessed, there is no evidence to support that DINP causes a permanent change in AGD (Clewell *et al.* 2013b, Boberg *et al.* 2011).

A change in AGD at birth following *in utero* exposure to DINP during the male programming window (MPW) is inconsistently and infrequently reported.

- Anogenital distance was reported to be unaltered in 4 studies in which: a single dose of 750 mg/kg/day DINP was administered by gavage GD 14-PND 3 (Gray *et al.*, 2000); doses up to ~2500 mg/kg/day (~1166 mg/kg/day in the MPW) were administered via the diet GD15-PND 10 (Masutomi *et al.*, 2003); doses up to 1000mg/kg bw/day were administered via gavage from GD 12 to 21 (Li et al. 2015); and doses up to 750 mg/kg bw/day were administered via gavage from GD12 to 19 (Clewell et al. 2013a)

- Boberg *et al.* (2011) reported a small (6%) but statistically significant decrease in anogenital distance[7] in males exposed to DINP at 900 mg/kg/day on post-natal day 1, following exposure starting on GD 7. However, the authors reported there was no difference between treated animals and controls on post-natal day 90 supporting the change in AGD was transitory.

- Clewell *et al.* (2012b) reported no statistically significant decreases in anogenital distance on post-natal days 2 and 49 in males exposed up to 750 mg/kg/day from GD12-PND14. There was a slight statistically significant difference at post-natal day 14 in the highest dose group. Anogenital difference is highly dependent on animal size. At post-natal day 14 the pup weights for males in this group were also statistically different than controls, however these animals were no longer different for either weight or anogenital distance at post natal day 49. Given that anogenital differences induced by anti-androgenic influences *in utero* would already be apparent at birth, the difference observed at post-natal day 14 (and not PND 2) was likely due to a difference in pup size, and not evidence of an anti-androgenic effect. This conclusion is supported by the lack of difference at post-natal day 2 and further supported by the return to control values by post-natal day 49.

- Lee *et al.* (2006b) reported a significant decrease in anogenital distance at all doses tested (0, 40, 400, 4000, or 20000 pm in the diet on GD 15 through PND 21) on post natal day 1. However, the results of this study are of questionable reliability for a number of reasons (as detailed in ECHA, 2013 pp120-121) including, the very small difference between the control (2.5) and the treated (< 0.1 below 2.5) normalized AGD values for all dose groups; the lack of clarity regarding if the litter was the statistical unit for this calculation; and because no effect was reported for the potent anti-androgen, DBP, that was also studied in this report.

[7] Of note, a recently published Corrigendum (Boberg et al. 2016) to this paper states that AGD 'was measured by different technicians on different days' in this study. According to OECD (OECD 2013b) "*it is important that all pups are measured on the same postnatal day because the rapid growth of pups will also affect AGD*".

The lack of consistency of the data for anogenital distance questions if the reported statistical significance is indeed a treatment related effect. Importantly, neither of the studies that looked at AGD in adults (Boberg *et al.* 2011; Clewell *et al.* 2013b) found a statistically significant effect or any trend in the dose response that might indicate a biological effect that did not reach significance.

Anogenital distance (AGD) is a sexually dimorphic trait in laboratory rodents and humans; rodent males exhibit a distance 2 – 2.5 fold greater than females. Androgens are responsible for normal AGD elongation in neonatal males (Clemens *et al.*, 1978; Hotchkiss *et al.*, 2007; Imperato-McGinley *et al.*, 1985). In laboratory animals, agents that are androgen receptor antagonists will induce a decrease in AGD in males. Historically AGD has been used as a means of sexing pups at birth, generally with histological confirmation at sacrifice (Hood 2012). In the classic two generation reproductive toxicology test (i.e. OECD 416) AGD was a triggered endpoint which was investigated in the F2 generation when alterations in sex ratios or timing of sexual maturation were observed in the F1 generation. Therefore if a change in AGD was detected in the second generation, it would have been accompanied by an effect on sex ratio or sexual maturation in the first generation. In more recent protocols (OECD 443) AGD is automatically measured between PND0 and PND4. Given its responsive nature AGD is an indicator of an endocrine mode of action and, as a part of a pattern of effects, can be indicative of mediation of effects via the endocrine system. In males feminization of AGD (i.e. reduction to the length of control females) has been considered adverse. However, outside of a change of a large enough magnitude resulting in lengths comparable to those in females, the magnitude of the change in AGD that is needed to be a determinant of adversity has not been established. However, according to OECD guidance document 43, a permanent change in AGD (i.e., observed at birth and into adulthood) constitutes a permanent structural change (OECD 2008) and is therefore of utmost relevance in the context of classification.

A weight of the evidence assessment of the data on AGD following *in utero* exposure to DINP indicates it is highly questionable that DINP is chemically mediating changes on AGD. The majority of studies exposing during the male programming window do not report an effect (4/6 reliable studies are negative for an effect). In the 2 studies that show an effect, the magnitude of the reported statistically significant changes are minimal (i.e. within the range of control variation, and considerably lower than the magnitude change reported following exposure to DBP and DEHP) and transient (i.e. do not support a permanent structural change); and therefore the reported statistically significant change in AGD is not itself a determinant of adversity .Therefore changes in AGD do not meet the criteria for classification as the rare incidence of statistically significant observations of minimal magnitude change (CLP 3.7.2.3.1) do not support DINP is intrinsically capable of inducing an adverse effect warranting classification.

In contrast to DINP, the Effects on Anogenital Distance of DBP and DEHP are Both Consistently Observed and Biologically Significant

Effects following *in utero* exposure during the male programming window to DBP and DEHP on AGD are consistently observed across studies and are permanent. Studies that dose animals at levels higher than 375 mg/kg bw/d reliably report statistically significant effects on AGD in perinatal animals (Table Va). In contrast to DINP, permanent reductions in AGD after *in utero* exposure to DBP and DEHP (statistical significance in adult animals) are observed. The pattern of results after treatment with DBP and DEHP differs from that seen after treatment with DINP as highlighted in Table Va below.

Table Va. The pattern of results after treatment with DBP and DEHP differs from that seen after treatment with DINP as can be seen from the tabulation of statistically significant increases in effect reported by study authors.

		Effects Observed
DINP	Perinatal	1/6[1]
	Adolescent	1/1
	Adult	0/2
DBP	Perinatal	9/9[1]
	Adolescent	3/3
	Adult	2/3
DEHP	Perinatal	8/9[2]
	Adolescent	4/4
	Adult	1/1

1: Lee *et al.* 2006 is excluded from the table as it is has been deemed a low quality study and the AGD results are unreliable (as detailed in ECHA, 2013 pp120-121)

2: In the one study where no effect was observed for DEHP, the highest dose tested was 150 mg/kg bw/d. This dose is below that at which one would reliably expect to see an effect. It can be considered that 8/8 studies which tested at a dose high enough to observe effects did see effects.

It is important to recognize that there is a large degree of biological variation in the length of AGD in males, with a 28% difference in control values between the smallest and largest AGD measurements reported in the same strain of rat on the same day (PND2) from two separate studies on DINP (Masutomi *et al.* 2003 and Clewell *et al.* 2013b). Some of the between lab variation could be due to differences in landmarks chosen for measuring the effect (Hood 2012). However, a large degree of intra study variability is also present where landmarks (i.e. measurement protocols) should be standardized. The standard deviation of control values within a given study, evaluating effects of DINP, ranges from 6.5% - 16.7% of the mean. It is important to evaluate small statistically significant changes in the context of biological significance, as a statically significant finding does not itself inform the size, importance or biological relevance of the effect. In addition to being able to consistently induce statically significant effects on AGD, DBP and DEHP induce a magnitude change that is much greater than DINP. This is depicted in Figure Va below which shows that the magnitude of effect for changes in AGD reported in studies for DBP and DEHP always exceeds variation of the control measurements (control standard deviation) in dose groups ≥ 300 mg/kg/d. This is in contrast with DINP where the variation of control measurements always exceeds any potential treatment effect (control mean vs. treatment mean) even at dose levels up to 1000 mg/k/d. This type of comparison to variance in controls, provides some perspective on the meaningfulness of the reported statistical significance in the 2 studies on DINP in the absence of historical control data in these studies. This data suggests that DBP and DEHP can induce a statistically as well as biologically meaningful decrease in AGD (Figure Va below).

Figure Va: DEHP and DBP perturbed anogenital distance outside the bounds of normal biological variability in contrast to DINP.

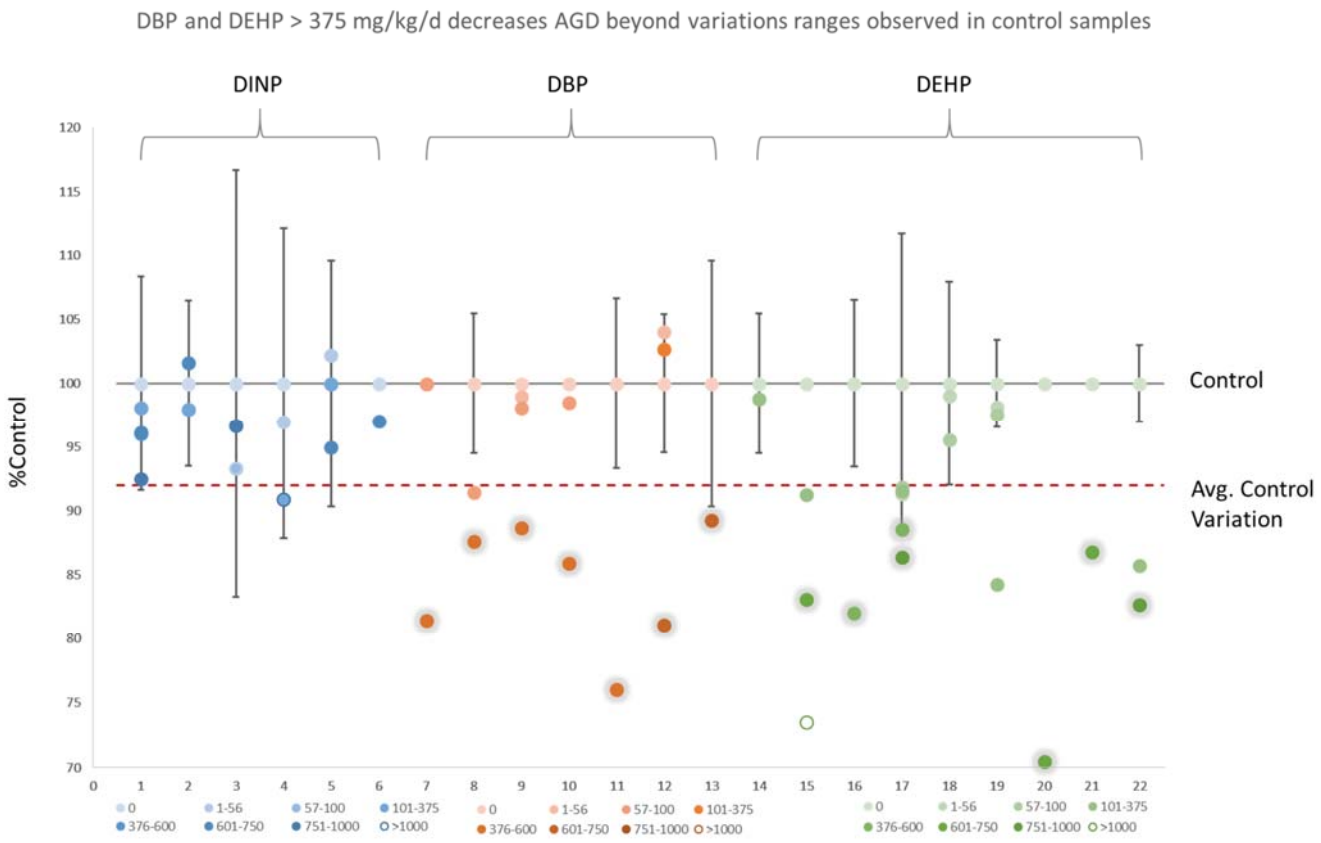


Figure Va: Absolute measures of anogenital distance were transformed to percent control values for each study. Increasing dose is reflected by darkening color and doses for DBP and DEHP >375 mg/kg/d are highlighted with a grey outline. Doses above the limit dose are shown as an open circle as these are outside the bounds for classification and labeling. The red dashed line indicates the average standard deviation of the control values across all studies and treatments. This could be considered an approximate boundary of normal biological variation for the endpoint, where effects sizes larger than this may be biologically significant. Effect sizes near or below this level could be considered of minimal, if any, toxicological significance. Treatment with DBP and DEHP exceeding 375 mg/kg/d induces effects sizes greater than the average standard deviation across all control groups (~8%). One study for DINP exceeds this boundary and did not have a dose response (9% decrease at ~307 and 1166 mg/kg/d). In all other studies, DINP at doses up to 1000 mg/kg bw/d, any potential treatment effect does not exceed natural variation in the endpoint. (1: Boberg *et al*. 2011, 2: Clewell *et al*. 2013a, 3: Li *et al*. 2015, 4: Masutomi *et al.* 2003, 5, 13: Clewell *et al*. 2013b, 6, 20: Gray *et al*. 2000, 7: Johnson *et al.* 2011, 8, 14: Martino-Andrade *et al.* 2009, 9: Mylchreest *et al*. 1999, 10: Barlow *et al*. 2004, 11: Saillenfait *et al*. 2008, 12: Lee *et al*. 2004, 15: Moore *et al* 2001, 16: Saillenfait *et al*. 2009, 17, 18: Christiansen *et al.* 2010 19: Gray *et al*. 2009, 21: Borch *et al.* 2004, 22: Jarfelt *et al.* 2005)

In contrast to DBP and DEHP, DINP exposure does not perturb anogenital distance outside an estimated bounds of normal biological variability in all but one study (5/6). In the one study (Masutomi *et al.* 2003) where DINP exceeds this boundary a small non statistically significant trend (decrease from 3.3 mm in controls to 3.2, 3.0, 3.0 mm in low, mid, high dose groups) is seen with no dose response between the mid (~306 mg/kg/d) and high dose groups (~1166 mg/kg/d). A similar non statistically significant trend in body weight at

PND2 (day of AGD measure) is also present in this study. The body weight-corrected anogenital distance was not determined in Masutomi, and it is likely that the trend in decrease of AGD in the Masutomi study is due to the differences in body weight between the treatment groups. An important confounder in AGD measure is animal size and weight (Kawashima 1975, Gallavan *et al.* 1999, Hood 2012) and the appropriate between study group comparison is weight corrected (mm/bw$^{1/3}$). Only the most recent studies report data in this manner and therefore is a smaller sample size. When the confounders of weight and size are accounted for by the appropriate bodyweight$^{1/3}$ correction the average of the control value standard deviations is 6.2%, compared to 8% for uncorrected data, as some of the variation in the absolute measurements is due to differences in size/weight. For the bodyweight corrected data the magnitude of effect for DINP are all below this measure of natural variation (Figure Vb below). In contrast treatment with DBP and DEHP exceeding 375 mg/kg/d induces a magnitude of effect greater than this measure of natural variation.

Figure Vb. Bodyweight corrected data decrease the effect size of DINP further supporting a lack of biological significance. For studies with dose >375 mg/kg/d both DBP and DEHP exceed the estimated measure of biological significance.

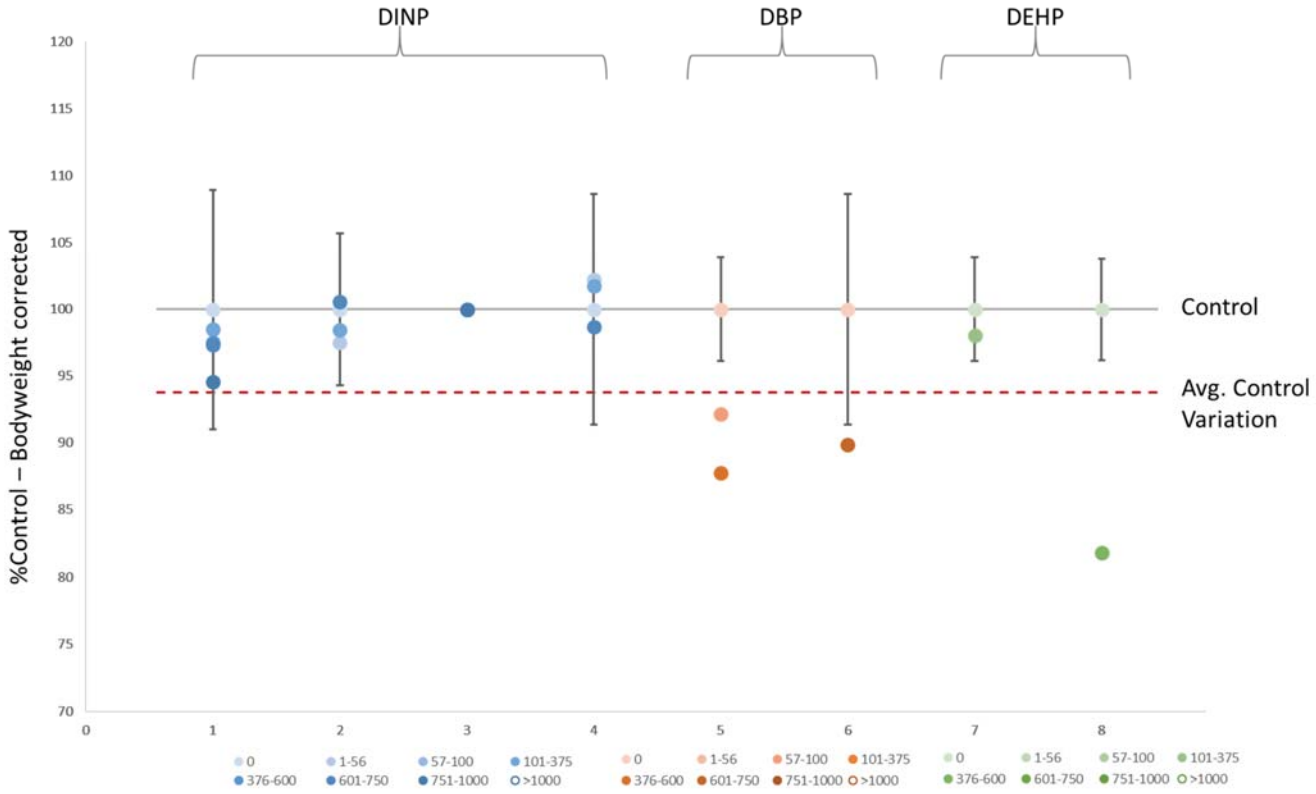


Figure Vb: Bodyweight corrected measures of anogenital distance were transformed to percent control values for each study where the data were available to facilitate such a correction. Increasing dose is reflected by darkening color. The red dashed lined indicates the average standard deviation of the control values. For the bodyweight corrected data all effect sizes for DINP are within this measure of control variation. In contrast treatment with DBP and DEHP exceeding 375 mg/kg/d induces effects sizes greater than this measure of control variation. (1: Boberg *et al*. 2011, 2: Clewell *et al*. 2013a, 3: Li *et al*. 2015, 4, 6: Clewell *et al*. 2013b, 5, 7: Martino-Andrade *et al.* 2009, 8: Saillenfait *et al*. 2009)

All together the infrequent reporting of statistically significant reductions in AGD in males of a minimal magnitude change do not support that DINP is intrinsically capable of inducing an adverse effect warranting classification. Both DBP and DEHP, in contrast, consistently perturb this endpoint to a larger magnitude in males, and this effect persists into adult. The difference in pattern of effect highlights that effects observed after treatment with DINP are not consistent with DBP and/or DEHP treatment related effects from a consideration of consistency and magnitude of effect.

Anogenital Distance is a Marker of in utero Androgen Environment, Not an Adverse Effect in and of itself

AGD is a sensitive indicator of in utero androgen levels and is a useful measure for making decisions in live animals (e.g. sexing at birth, triggering additional study considerations). In rodent studies an evaluation of potential adversity that may be caused by altered in-utero androgen levels will have been extensively evaluated. In and of itself anogenital distance (e.g. in the absence of histopathology, structural malformations, and significant decreases in organ weight size) is not an adverse effect[8], as outlined in detail below.

External genital development is a complex process with many potential areas where disruption can occur. There are two essential phases, with Phase I being hormone independent and Phase II being hormone dependent. Disruption during Phase I can lead to severe malformations such as imperforate anus[9], persistent cloaca[10] and severe hypospadias with the urethral opening located on the edge of the perineum (region between the anus and genitals). Disruptions during Phase I are due to non-hormonally regulated processes. Disruptions during Phase II lead to malformation/disruption of the male or female patterning of the external genitalia. In XY males this can be phenotypically female (complete feminization of the external genitalia generally due to complete loss of androgen signaling, or the ability to respond to androgen), ambiguous genitalia, or hypospadias with a varying degree of severity. The progression of genital formation is temporal and the observed adversity is related to both the severity and timing of the developmental insult (e.g. disruptions at an earlier stage of Phase II would lead to a more severe phenotype and disruptions at a later stage would lead to a less severe phenotype). Disruption of hormones has to occur in a specific critical time window[11] to have an effect on external genital development. Disruptions of androgens before this window, or after, have no effect on normal progression of the male external genital development (Welsh et al 2008, Foster and Harris 2005, van den Driesche 2012).

Once the genital tubercle has entered the sexually dimorphic phase, there are two distinct processes which are operated by different signaling pathways, which are important for appropriate male genital development. These are growth of the genital tubercle including ventro-lateral growth of the preputial swellings to form the prepuce, and remodeling of the bilaminar urethral plate into an epithelial tube. The set of signaling responsible for growth of the GT are the same that are responsible for the increased anogenital distance. The

[8] ADVERSITY (WHO, 2002). A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences.
[9] An imperforate anus is the lack of an opening for the anorectal tract. This is thought to be due to improper outgrowth of the perineum (Guo et al 2014).
[10] A persistent cloaca is where the separation of the anorectal sinus and urogenital sinus fails to form. The contents of the intestines and bladder empty through the same opening (Seifert 2009)
[11] This time period has been termed the" male programing window" (MPW) and has been identified as GD15.5 to GD18.5 in rats (Welsh et al 2014, Mcleod et al 2010, Welsh et al 2008). This time period corresponds to 8 to 14 weeks in humans (Welsh et al 2014), and is roughly similar in mice though the boundaries of the window have not been as clearly defined as they have been for rats (Seifret et al 2008, Cohn 2011, Miyagawa 2009).

anogenital distance relates to the final size of the perineum. The perineum is fully functional[12] by the start of the sexual differentiation phase (phase II), however it is responsive to androgens and does grow further during the androgen responsive phase. The growth of the perineum is one of the first indicators of sexual differentiation and appears to be one of the first tissues to respond to androgens. However, this growth does not appear to be essential to normal male reproductive tract development. AGD is a sensitive indicator of in utero androgen levels and is a useful measure for making decisions in live animals (e.g. sexing at birth, triggering additional study considerations). In humans the measure is useful as a non-invasive indicator of potential adversity, but in rodent studies an evaluation of potential adversity that may be caused by altered in-utero androgen levels will have been extensively evaluated. In and of itself anogenital distance does not alter functionality or structure of the reproductive tract and is therefore not an adverse measure.

[12] The perineum provides physical separation between the urogenital and the urorectal tracts. The perineum serves the same function in males and females, though the structural integrity plays a larger role in females as it can be disrupted during birthing.

A.VI: DINP does not induce permanent changes in morphology or histology of reproductive tissues.

Structural abnormalities: increased incidence of permanent changes (permanent nipples, malformations of testes and epididymis, histological changes in testes and epididymides) in rats exposed perinatally (Gray et al., 2000; Masutomi et al., 2003) (at 750 and 1165 mg/kg bw/day, respectively),

Morphology and histology observations need to be interpreted within the context of the study they were observed in, across studies, and then considered with respect to their relationship to one another as indicators of the same adverse effect. This is supported under the CLP criteria (3.7.2.3) that states classification as a reproductive toxicant should be made on the basis of an assessment of total weight of evidence. Weight of evidence (CLP 3.7.2.3.1) in the context of CLP classification does not refer to piecing together insufficient information of observations to come to a classification conclusion; but rather observations from independent sources, or several observations within one source, which all support the occurrence of a particular, chemically mediated adverse effect. In the case of DINP, it is clear, that the collective evidence supports the absence of androgen-mediated adverse morphological and histological effects on reproductive tissue.

The testicular findings in two animals in Gray et al. (2000) (i.e. small testes/epididymis in one animal and epididymal agenesis another animal) are difficult to interpret within the context of just this one study as they are inconsistent in their nature, and there is a lack of dose response data as DINP was only tested at one dose. The observations reported in Masutomi et al (2003) consisted of minimal to slight changes (degeneration of meiotic spermatocytes and Sertoli cells, scattered cell debris in ducts in epididymis) that reached statistical significance only at the highest administered dose of DINP of 1165/2657 mg/kg (GD15-20/PND2-10). These effects are of a different nature than those observed in Gray et al. (as noted in the discussion section of Masutomi et al. 2003), of minimal severity (CLP3.7.2.3.1) and occur above the limit dose (CLP 3.7.2.5.7), therein not relevant for classification by themselves. Importantly, in a robust dietary study using 100 pregnant rats (Clewell et al. 2013b) designed to provide strong statistical power for analyzing, malformations of the male reproductive tract including retention into adulthood, reported no evidence of effects on these tissues on PND 49. The minimal severity of the observations reported by Gray and Masutomi is corroborative of results from a two generation reproductive toxicity study showing no functional effect on reproduction following exposure of the F2 generation throughout development (Waterman et al. 2000).

Gross male reproductive tract malformations, such as cryptorchidism or hypospadias, have not been reported in any studies for DINP; including, the definitive two-generation reproductive toxicity study (Waterman *et al.*, 2000), and a number of other *in vivo* studies previously mentioned (Adamsson *et al.*, 2009; Boberg *et al.*, 2011; Borch *et al.*, 2004; Gray *et al.*, 2000; Hellwig *et al.*, 1997; Lee *et al.*, 2015; Masutomi *et al.*, 2004; Masutomi *et al.*, 2003; Waterman *et al.*, 1999). Likewise, DINP does not induce general reproductive tract malformations manifested as decreased weights in androgen sensitive tissues: levator ani/bulbocavernosus muscles (LABC), seminal vesicles, ventral prostate, glans penis, bulbouretral gland, and epididymis (Adamsson *et al.*, 2009; Boberg *et al.*, 2011; Gray *et al.*, 2000, Clewell et al. 2013b).

In general, interpretation of adversity of most pathologic changes is not clearly adverse or non-adverse but depends on specific characteristics of the pathology defined by severity, incidence, degree of change, related lesions, etc (Palazzi et al. 2016). To conclude on classification there is the added burden of determining the

strength of the evidence to support the identified adverse effect was indeed a result of an intrinsic property of the chemical. **On the basis of the information available, the evidence is insufficient to support DINP is intrinsically capable of causing a structural abnormality of a consistency (CLP 3.7.2.3.1), severity (CLP 3.7.2.3.1) and toxicological significance (CLP 3.7.2.3.3) warranting classification.**

(1) Gray et al. – In this study, pregnant rats were exposed to DINP, DEHP, BBP, DEP, DMP and DOTP at single dose of 750 mg/kg/d in corn oil as vehicle from GD14-3. With respect to malformations, four of 52 adult males (from three litters) exposed perinatally to DINP were reported as exhibiting a malformation at >5 months of life: 1 male displayed bilateral testicular atrophy (not fluid filled or flaccid), small atrophic testes, paired testicular epididymal atrophy, malformed testes, atrophic tubules, lack of spermatids; 1 male displayed epididymis agenesis and fluid filled testes; the other 2 males both exhibited retained nipples, with one male having 1 nipple; and the other male having 6. The low incidence of reported effects was without any dose response, using a small number of rats, and effects are of unclear significance. The collective incidence of effects in DINP treated animals was 7.7% on an individual animal basis (4/52 pups $p<0.05$) and $p<0.06$ on litter basis for 3/14 litters) (compared to 82% with DEHP treated animals). No endpoint on its own was significantly different from control values (0/19 litters; 0/80 animals); rather, different effects were pooled to produce the 7.7% incidence and the pooled effects were only found statistically significant on an individual animal basis and not when corrected for litter effects. If we consider the observed effects to testes and epididymis observed in the 2 animals, they are not of a consistent nature, questioning their causality. In one animal small (not fluid filled) testes with tubular atrophy and small epididymis were observe; versus in the other animal which had fluid filled testes and epididymal agenesis. This is compared to the effects observed following DEHP and BBP where complete agenesis of the seminal vesicles were observed; and 67% of males were observed to have fluid filled testes and epididymal agenesis versus 2% in the DINP group (Figure 6 in the publication). Based on the very low incidence of inconsistent observations, lack of dose response, lack of historical control data, the relevance of these observations to DINP mediated toxicity are hard to interpret within the context of this one study alone.

(2) Masutomi et al. 2003-Masutomi et al (2003) tested 3 doses of DINP (400, 4000, 20000 ppm) in pregnant Sprague-Dawley rats from gestational day (GD) 15 to postnatal day (PND) 10; administration was via diet and 5 to 6 dams were allocated to each group. Measured doses were 31, 307, 1165 mg/kg (GD15-20) and 66, 657, 2657 mg/kg (PND2-10). At prepubertal necropsy on PND 27 testes weight was reduced at high dose (reduction to 46 and 81% of controls in absolute and relative weight) (n = 5 males per group, 1 per litter). No histopathological investigations were conducted at that time point. The authors concluded DINP as having exerted severe toxic effects on the dams at the highest dose based on reductions in body weight gain, that were considered particularly severe during lactation. On PND 71 at adult necropsy (n = 5 males and females per group, 1 per litter), minimal to slight degeneration of stage XIV meiotic spermatocytes and vacuolar degeneration of Sertoli cells (and scattered cell debris in epididymal ducts) were observed in the high dose group in 4 of 5 males ; organ weights of male reproductive organs were unchanged.

In conclusion, minimal to slight changes (degeneration of meiotic spermatocytes and Sertoli cells, scattered cell debris in ducts in epididymis) that reached statistical significance were observed in male offspring at adult examination at the highest administered dose of DINP of 1165/2657 mg/kg. Based on their toxicological significance (CLP section 3.7.2.3.3) together with their occurrence above the limit dose (CLP3.7.23.5.7), they do not justify classification.

Generally, the statistical power of the Masutomi study is low, which is also stated in the CLH proposal when discussing changes in AGD. In this study, only 5 to 6 dams were allocated to each group, and 5 litters were examined. For comparison, OECD guidelines for the investigation of developmental changes (OECD414) recommend the investigation of 20 liters per dose group in order to achieve statistically powerful results. Especially, for adult examination of offspring, only 1 male and female animal each per litter were investigated for histopathology, so that the conclusion on developmental changes in the Masutomi study is based on the investigation of 5 animals in total. This is in comparison to the large statistical power of the Clewell et al. (2013a, b) studies described below.

(3) Clewell et al. (2013 a, b). Robust developmental studies of DINP, consisting of a gavage study using 144 pregnant rats (Clewell et al. 2013a) and a dietary study using 100 pregnant rats (Clewell et al. 2013b), were designed to provide strong statistical power for analyzing, collectively, the kinetics and fetal testes effects of DINP and post-natal effects including nipple retention and AGD as well as any malformations of the male reproductive tract including hypospadias, cryptorchidism, and epididymal malformations, both gross and histological and the endpoints attributed to the hypothesized "rat phthalate syndrome." Investigation of effects at GD 19 gave a no observed effect level (NOEL) of 50 mg/kg/day based on increased multinucleated gonocytes (MNGs) and reduced testes testosterone concentration in the fetal rat. There is evidence that the MNGs are not a consequence of reduced testosterone synthesis and as such there relevance to classification is discussed in more detail in Part 4, Appendix VII of this submission. The dietary study (Clewell *et al.*, 2013b) included evaluation of phallus malformation, preputial separation, a full suite of reproductive organ weights at PND 49 and a comprehensive review of testes and epididymal histopathology at PND 2 and PND 49. Global endpoint analysis showed no evidence of effects on these tissues on PND 49 with DINP administration (Clewell *et al.*, 2012b).

(4) Boberg et al. (2011) reported small testes and epididymis in 1 animal in each of the low (600) and mid (750) dose groups, however it is unclear what provided the basis for this determination (gross morphology or organ weight), as the observation is listed under the heading 'organ weights PND90 males and females'. The severity of this observation is also not clearly reported (slight, moderate, etc). These same observations are later referred to in the discussion section of the manuscript as epididymal and testicular dysgenesis. However, it is unclear what parameters provide the basis for characterization as 'dysgenesis'. Dysgenetic testes in a broad context refers to testes of variable histopathological presentations. In the context of LMW phthalate toxicity, testicular dysgenesis is characterized by specific histopathology hallmarks (e.g. intratubular leydig cells, malformed seminiferous chord) as described in Hutchison et al. (2007); Van den Driesche S. et al (2017) and elsewhere. As noted in Boberg et al. (2011) 'histopathology of male reproductive organs at PND90 was not altered by DINP treatment", therefore the basis for concluding testicular and epididymal dysgenesis in adult animals is not supported by the details in the publication. Furthermore, the lack of dose response suggests these are not DINP mediated. Based on the very low incidence of poorly characterized observations, the lack of dose response, and lack of historical control data, these observations do not provide a basis for classification. This is consistent with the conclusion reflected in the ECHA report of new scientific evidence of DINP (ECHA, 2003), that the males having small testes and epididymides are considered as 'suggestions' of an effect only because there is no dose-response.

Effects not explicitly mentioned by the CLH proposal submitter include the appearance of MNGs. As discussed in Part 4, Appendix VII of this submission, exposure to DINP during the male programming window consistently induces an increased frequency of MNGs, however the toxicological significance of these observations in the context of classification is questionable as they occur as a normal part of development with their elimination

occurring from the seminiferous epithelium within 1–2 weeks postnatally. This non-permanence is similarly observed in studies with DINP (Boberg et al. 2011, Clewell et al. 2013b). Therefore whether an increased frequency of MNGs affect reproductive health in adulthood (e.g. reduced germ cells) is currently not clear from available data.

A.VII: MNGs do not warrant classification due to their nature, severity, and toxicological significance.

An increased frequency of multinucleated gonocytes (MNGs) has been reported in fetal/neonatal animals following in utero exposure to DINP in four studies (Boberg et al. 2011; Clewell et al 2013a,b; Li et al, 2015), with significant increases observed at ~100 mg/kg bw/day and above. Importantly, these effects were transient as statistically significant increases in MNGs were not maintained into adulthood in the two studies that assessed their permanence (Boberg et al. 2011; Clewell et al. 2013b); and sexual function and fertility in a multigenerational study involving in utero exposure that covered the same time window were not impaired (Waterman et al. 2000). While MNGs are likely chemically related, any chemically mediated change should not automatically be considered relevant for the outcome of an assessment, but an independent evaluation of the effect in the context of CLP criteria (CLP section 3.7) is required[13]. **Consideration of what is known about the toxicological significance of MNGs in general together with the DINP specific data support the view: In the context of CLP criteria (e.g. nature and severity of effect (3.7.2.3.1), toxicological significance (3.7.2.3.3) supporting a conclusion of clear evidence of an adverse effect on reproductive function, fertility or development), the appearance of MNGs in the DINP studies do not warrant classification.**

- **MNGs are part of normal development.** The relevance of MNGs to classification is questionable in the base case, based on what is known in general about these cells. As described in more detail below, evidence supports that formation of MNGs is a normal part of development, meaning they occur at a certain frequency and are eliminated fairly rapidly after birth with no apparent consequence to reproductive function or development in the absence of chemical exposure. Therefore, the transient occurrence of an increased frequency of MNGs is of questionable toxicological significance.
- **Presence of MNGs following exposure to DINP does not impact function.** Classification of DINP on the basis of MNGs is not warranted in the context of CLP based on their questionable toxicological significance (3.7.2.3.3). In addition to what is known in general about the toxicological relevance of MNGs, DINP-mediated MNGs do not manifest into any apparent effects on development (Boberg et al. 2011; Clewell et al 2013a,b; Li et al, 2015); do not persist into adulthood (Boberg et al. 2011; Clewell et al. 2013b); and lack any functional impact to sexual function and fertility (Waterman et al. 2000).
- **MNG formation is not androgen dependent.** Increased MNG frequency and alterations in seminiferous cord diameter do not appear to be androgen dependent as discussed in more detail below. Studies of both human and mouse fetal testis indicate that the seminiferous cord effects (including MNGs) following DBP and MEHP (the principal metabolite of DEHP) exposures, are androgen independent, as seminiferous cord alterations are observed even in the absence of a measureable anti-androgenic effects (Gaido et al. 2007; Lehraiki et al. 2009; Heger et al. 2012; Spade et al. 2014; Mitchell et al. 2012 Habert et al. 2014a, b; Albert et al. 2014; Johnson et al. 2012). Therefore the nature,

[13] The CLP legal text lists a number of criteria relevant to classification decisions for reproductive toxicity. These include the nature, severity, and toxicological significance of the effect (CLP Part 3 section 3.7.2.3). The nature of the effect, can be either adaptive, or beneficial and may occur at different levels, e.g. molecular cell, organ, individual, population or ecosystem; the severity can range from inconsequential to severe; and the toxicological significance can range from low to high. According to the WHO (2004), an effect is considered "adverse" when leading to a *change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity to compensate for additional stress or an increase in susceptibility to other influences"*.

severity and toxicological significance of MNGs and their relevance as a determinant of an adverse effect on reproductive function, fertility or development must be considered separately from effects that are considered androgen responsive.

The Underlying Biology of Multinucleated Germ Cells (MNGs) Reflects their Transient Occurrence during Development

MNGs are, simply, gonocytes with more than one nucleus which occur spontaneously at low frequency in perinatal rat testis. They arise from differentiated gonocytes by a non-proliferative process (Spade et al., 2015) and occur normally during development. MNG frequency is increased by treatment with DBP and DEHP (Barlow and Foster, 2003; Boekelheide et al., 2009; Fisher et al., 2003; Kleymenova et al., 2005; Mylchreest et al., 2002; Parks et al., 2000) and the toxicological significance of this observations can be informed from these studies. As reported by Parks et al. (2000), MNGs most often contained 2—3 nuclei but some contained 4 and occasionally 5-6. The process leading to MNG formation is unknown (Kleymenova et al., 2005), but, according to Johnson et al. (2012), there are two possible mechanisms, either nuclear division without cytoplasmic division or the collapse of intercellular bridges. Based on the report by Kleymenova et al. (2005) and later confirmation by van den Driesche et al. (2015) interference with intercellular contact is the current working hypothesis (Spade et al., 2015). The MNGs are removed early in the postnatal period (Barlow and Foster, 2003; Boekelheide et al., 2009) by a process at least partially dependent on P53 signaling (Saffarini et al., 2012).

MNGs are Not Androgen Dependent

Gaido et al (2007) noted that although reproductive tract development has a similar pattern and time course in rat and mouse, previous investigations had shown that the adult mouse is less sensitive to the testicular effects of DBP than is the adult rat, suggesting that some of the reproductive tract changes could be species-specific. Accordingly, they examined development of fetal rat and mouse following DBP treatment and found that the early pattern of gene expression was similar in the two species. However, reductions in the expression of genes involved in cholesterol and lipid homeostasis and steroidogenesis which were characteristic responses in rats to active phthalates, were not observed in mice. These findings provided supporting evidence that androgen-dependent processes in mice are not altered by *in utero* exposure to phthalate esters, and are, most likely, rat-specific. However, there were also alterations in seminiferous cord development with an increase in MNGs which occurred in the absence of down-regulation of testosterone synthesis, providing initial evidence that some changes including increased MNG frequency were androgen-independent and not species-specific.

Investigations of the potential for androgen-dependent and androgen-independent changes in human fetal testes associated with DBP and DEHP exposure.

To assess the potential effects of DBP exposure on the development of human fetal testis, experiments were conducted in 3 independent laboratories in which human fetal tissue was exposed under either *in vivo* (Heger et al., 2012; Mitchell et al., 2012; Spade et al., 2014; van den Driesche, 2015) or *in vitro* (Habert et al., 2014a; 2014b; Lambrot et al., 2009) conditions. More specifically, Heger et al. (2012) implanted fetal rat, mouse and/or human testes into the renal capsules of nude mice. Twenty-four hours after surgery, the host mice were given DBP by oral administration in doses of 100, 250, or 500 mg/kg. Most commonly, test material was administered as a single dose, but multiple dosing was used in some experiments. All animals were sacrificed 6 hours after the final dose, and the fetal testes were recovered and examined. Responses observed in the explanted fetal rat testes mirrored those in previous, whole animal studies, i.e., genes involved in steroid synthesis were down-regulated and there was a significant decrease in fetal testosterone synthesis.

Additionally, there was a reduction in a Leydig cell-specific gene for testicular descent (Insl-3) and an increase in MNGs. In the fetal mouse testes there was also an increase in MNG frequency, but no changes in steroidogenic gene expression, and only a slight (but not statistically significant) increase in fetal testosterone. In human fetal testes there was also an increase in MNGs, but, as in the mice, there was no reduction in steroidogenic gene expression and no decrease in fetal testosterone. The authors concluded that DBP treatment produced non-androgen-dependent effects in fetal testes from all three species but that the steroidogenic gene expression changes were specific to the fetal rat testis.

The following work by Mitchell et al. (2012), Spade et al. (2014) Van den Driesche et al. (2015), and Habert and colleagues (2014a, 2014b; Lambrot et al., 2009) further support the conclusion that MNGs are androgen independent and that androgen-dependent changes are specific to the fetal rat testis. In the studies by Mitchell et al. (2012), human fetal testes were implanted under the dorsal skin of male nude mice. Implants of rat fetal testis were also examined for comparative purposes. The host mice were given either vehicle or DBP (500 mg/kg) on a daily basis for 16 days (days 4-21 following human fetal testis explant). In the studies of explanted fetal rat testis, the animals were given DBP (500 mg/kg) over a 4 day period, and the testes were harvested 4 hours after the last treatment. The authors reported that DBP treatment reduced expression of steroidogenic genes and reduced fetal testosterone levels in rat testes, but that none of the androgen-dependent parameters that were measured were significantly affected in the explanted fetal human testis. MNG frequencies were not reported.

Spade et al. (2014) compared the effect of DBP (500 mg/kg) to those of abiraterone, a CYP17A1 inhibitor (a key enzyme in testosterone synthesis, the inhibition of which results in decreased circulating testosterone), on human fetal testis xenografts. Following treatment with abiraterone, there was a significant decrease in serum testosterone in the hosts along with reductions in weights of seminal vesicles and anterior prostate, although the difference in prostate weight was not statistically significant ($p = 0.054$). In contrast, DBP treatment had no effect on levels of serum testosterone in the host or weights of seminal vesicles or prostate. The frequency of MNGs was increased but was not significantly different ($p = 0.051$) after DBP treatment. No change in, MNG frequencies occurred following abiraterone treatment. This confirms that the increase in MNGs is not associated with a decrease in testosterone levels and this is an androgen-independent observation. These studies were a useful control for the previous work of Heger (2012) and Mitchell (2012), confirming that the absence of effects of DBP on human fetal testosterone synthesis was due to inherent differences between rats and humans and not to a problem with the experimental design. The experiment also supports that the increase in MNG frequency was due to DBP treatment in both species.

Van den Driesche et al. (2015) extended the comparison of the effects of DBP on fetal testes from rat and human, primarily through an examination of Sertoli- and germ-cell markers. Rat testes were harvested either prior to (gestational days 17.5 and 21.5) or shortly after (postnatal day 4) birth. The fetal human testes were treated using the explant model as in the previous experiments by Mitchell et al. (2012). In the rat, DBP exposure resulted in a significant increase in germ cell aggregates which were present at GD21.5 but had resolved by PND 4. In contrast, germ cell aggregates were found in only 4 (of 54) human fetal testis explants; all of which were from a single donor. Immunohistochemical staining revealed that in the fetal rat testis there was a reduction in Sertoli cell-germ cell interactions but this had been restored by PND4. In the human fetal testis explants, there also appeared to be a reduction in Sertoli cell – germ cell interactions, but only in the 4/54 explants in which germ cell aggregates were observed. There was a significant increase in MNG frequency (control = 0%, treated = 3.9%) in rat fetal testis; a significant increase in MNG frequency was also observed in the human fetal explants but at a lower frequency (control = 0.8%, treated = 1.76%). The authors

also reported small but statistically significant reductions in MNGs in rat testis and in number of germ cells per explant. An overall conclusion was that DBP treatment increased MNG frequencies by a "non-androgen-dependent" process in fetal rat and fetal human testis. Although there were substantial differences in the way in which the dose was delivered, DBP appeared to be quantitatively more effective in increasing MNG frequency in fetal rat testis than in fetal human testis.

Habert and colleagues (2014a, 2014b; Lambrot et al., 2009) reported a reduction in fetal testosterone in fetal rat testes exposed to mono (2-ethyl hexyl) phthalate (MEHP, the principal metabolite of DEHP) under *in vitro* conditions. In contrast, there was no reduction in testosterone production in human fetal testes treated under the same conditions.

There was agreement between the explant studies with DBP and the *in vitro* studies of MEHP on the principal point, phthalate exposure did not reduce testosterone levels in fetal testes. In other aspects, however, differences in outcome between the experimental systems were reported. Both Mitchell et al. (2012) and van den Driesche (2015) reported that DBP treatment increased the frequency of MNGs in fetal human testes in their explant experiments, but Lambrot et al. (2009) did not observe an increase in MNGs in fetal human testes exposed under *in vitro* conditions. There may also have been inconsistencies in regard to apoptosis. Data from Boekelheide et al. (2009) and later Saffarini et al. (2012) suggest that the increase in MNG frequency could be due to an inhibition of apoptosis early in the perinatal period but that in a few more days, apoptosis is restored and the MNGs are removed. Lambrot et al. (2009) reported that apoptosis was slightly but not significantly elevated when human fetal testes were exposed to 10^{-4} M MEHP under *in vitro* conditions. However, in a later paper Muczynski et al. (2012) reported that apoptosis was increased in human fetal testes exposed to 10-5 M MEHP under *in vitro* conditions. Whether these differences are due to differences in experimental conditions or other factors is unknown.

In summary, these experiments provided consistent and reproducible evidence that DBP -induced down-regulation of testosterone is a species-specific response which occurs in fetal rat testes but not in fetal testes from mouse or human and that increased MNG frequency and alterations in seminiferous cord diameter do not appear to be androgen-dependent.

MNGS are of unknown Toxicological Significance

Fisher et al. (2003) discussed the similarity of phthalate-induced effects in rats to a hypothesized human condition referred to as "testicular dysgenesis syndrome" (TDS). Of direct importance is that one of the endpoints of this hypothesized syndrome is testicular germ cell cancer and its precursor, carcinogenesis in situ (CIS). It is believed that in humans, CIS originates from gonocytes that have failed to differentiate normally. In contrast, testicular germ cell cancer is very rare in rats. **Fisher et al. (2003**) had noted the parallels between increased MNG frequencies in rats and CIS induction, and suggested that these might be related. However, the recent data from **Spade et al. (2015**) do not provide support for that view. More specifically, MNGs arise from differentiated germ cells by a non-proliferative mechanism, best described as degenerative. Further, as MNGs are rapidly removed, there is no plausible means by which they could be involved in a carcinogenic process. Rather, the disappearance of MNGs from rat testes, early in the perinatal period provides evidence that they are unlikely to be associated with any structural or functional deficits. Other plausible toxicological consequences of transient increases in MNG frequency are unclear.

Occurrence of Non-Androgen-Dependent Effects Associated with DINP Treatment

As summarized by Spade et al. (2015), the principal non-androgen-dependent effects associated with DBP exposure are increases in multinucleated germ cells (MNG) and increases in the diameter of seminiferous tubules.

Following exposure to DINP during the male programming window, Boberg et al. (2011) reported an increase in MNGs in male rats from dams treated with DINP at 300 mg/kg. An increase in seminiferous cord diameter was reported in animals treated with 600 mg/kg. Clewell et al. (2013a) reported a dose-dependent increase in MNGs at doses ≥250 mg/kg with 50 mg/kg as a no effect level. There was also an increase in the number of animals with large Leydig cell (LC) aggregates in animals exposed at 750 mg/kg, but significant differences in seminiferous tubule diameter were only found in animals treated with doses ≥ 750 mg/kg. Li et al (2015) reported an increase in LC aggregates at all doses tested, and an increased frequency of MNGs at 100mg/kg and higher. Both Boberg and Clewell reported that MNGs were not observed in pathological investigations of rats examined after puberty, confirming the reversibility of the MNGs (permanence was not assessed in Li et al. (2015)). This observation is consistent with earlier investigations (Barlow and Foster, 2003; Saffarini et al., 2012) that MNGs are removed early in the post-natal period. Pathological investigations of sexually mature rats exposed to DINP during the gestational phase did not provide any consistent evidence of persistent testicular effects (Boberg et al., 2011; Clewell et al., 2013b; McKee et al., 2002; Waterman et al., 2000), and no reductions in fertility (Waterman et al., 2000).

MNGs are Not Relevant to Classification of DINP According to CLP Based on Nature, Severity and Toxicological Significance

MNGs arise from differentiated germ cells by a non-proliferative mechanism, best described as degenerative (Spade et al. 2015). Further, the disappearance of MNGs from rat testes, early in the perinatal period provides evidence that they are unlikely to be associated with any structural or functional deficits. Of relevance here, the increased frequency of MNGs following treatment with DINP, do not persist into adulthood and have no apparent toxicological consequences. More specifically, the increase in MNG frequency was not associated with adverse pathological findings such as changes in seminiferous cord diameter or reductions in sperm count, and there were no reductions in fertility in adults. Further, the MNGs did not persist and were not associated with any functional changes. In the context of CLP criteria (e.g. nature and severity of effect, toxicological significance supporting a conclusion of clear evidence of an adverse effect on reproductive function, fertility or development), the appearance of MNGs do not warrant classification.

A.VIII: A Key study, Kwack et al 2009 is ambiguous and not sufficiently reliable to support a fertility classification.

The objective of the study by Kwack et al. (2009) was to evaluate comparatively nine phthalate diesters including DINP and five metabolites for toxicological risk assessment. Doses of 250 (metabolites) or 500 (phthalate diesters) mg/kg body weight per day were administered orally to juvenile Sprague-Dawley male rats for 28 days.

The study design of this repeated dose toxicity study is not consistent with guidelines. The study was not conducted under GLP and documentation (e.g. supplier and purity of DINP not described) is not sufficient to judge the methods and reliability of outcomes. Moreover, Kwack et al. (2009) used only one dose group with 6 male rats, whereas in a classical repeated dose toxicity study (e.g. OECD TG No. 407) at least 10 animals per dose levels, female and males and at least three test groups are used. As a result, they reported a reduction of 25 % to the control in sperm counts but no significant decrease in motility for DINP and do not include histopathological examination of testes. These sperm parameters were regarded relevant for the fertility hazard classification in CLH report as key findings of adverse effects on male reproductive organs. However, the results reported by Kwack et al. (2009) are ambiguous as the data presented are inconsistent with already existing data for some phthalates tested. In the case of DEP, in a full two-generation study with DEP (source: Wako Pure Chemicals, purity 99,8 %), Fujii et al. (2005) report no effects on fertility, especially, no effects on the sperm counts and % motile sperm (approx. 85 %) up to the highest dose levels tested (15000 ppm which corresponds to 721 to 1901mg/kg bw/d for the F0 and F1 generations). In contrast, % sperm motility is reported to be 56 % (55.67 % ± 0.58 %, but not statistically significant from controls 74.67 % ± 4.51 %) in Kwack et al. (2009). DEP is quantitatively metabolized to MEP (Kao et al., 2012). Thus, the results reported by Kwack, i.e. significant reduction in sperm count and % motility for MEP, the primary metabolite of DEP, but not for DEP itself, is strange. The statistical significant reductions in sperm counts and % motile sperms with MEP are reported to be in the same range as with MEHP, the monoester of DEHP, which is classified for reproductive toxicity. Kwack et al (2009) is therefore methodologically limited and the internal inconsistency make it of questionable reliability for supporting a classification decision on DINP.

A.IX: Mode of Action assessment for phthalate induced effects indicates only markers of cellular perturbations are observed after *in utero* exposure to DINP. No adverse effects on organs or the organism are observed.

There are at least three known modes of action (MoA's) operating to produce the constellation of effects associated with in utero exposure to specific phthalates (i.e. DBP and DEHP) (Foster 2005, Makris *et al.* 2015, Euling *et al.* 2015).

(1) Lowered fetal testicular testosterone production (related to malformations of the male reproductive tract – such as the epididymis, vas deferens, prostate, etc.);
(2) Lowered production of the Leydig cell product insl3 (related to the development of the gubernaculum and development of cryptorchidism) and
(3) Effects on fetal Sertoli and germ cell function leading to the generation of multinucleated gonocytes in the fetal testis.

The precise initiating mechanisms for these events is unclear. Testosterone synthesis occurs following the steroidogenic enzymes converting cholesterol to testosterone. For this process to occur cholesterol needs to be transported (SB-1) into the Leydig cells and the appropriate steroidogenic enzymes (StAR, Cyp11a1 "CYP450scc", and Cyp17a1) need to be present for conversion to testosterone. Gene expression studies have indicated DBP and DEHP can decrease expression of genes involved in steroidogenesis and cholesterol transport including those indicated above (Euling *et al.* 2013). INLS3 protein has been shown to be critical for testicular descent, with the predominant phenotype of *Insl3* knock-out mice being primary cryptorchidism (Ivell and Anand-Ivell 2009). Both DEHP and DBP decrease gene expression of *Insl3* in a dose range that makes it plausible that *Insl3* is on the pathway responsible for mediating downstream effects (Wilson *et al.* 2004, Howdeshell *et al.* 2007, and Borch *et al.* 2006). This is in contrast to DINP (Lambright *et al.* 2011, Hannas *et al.* 2012) where the ED50 is approximated at 1488 mg/kg/d (Hannas *et al.* 2012), a dose well above the limit dose. Based on the literature a putative mode of action can be established for the *in utero* effects of certain phthalate esters (Figure IXa).

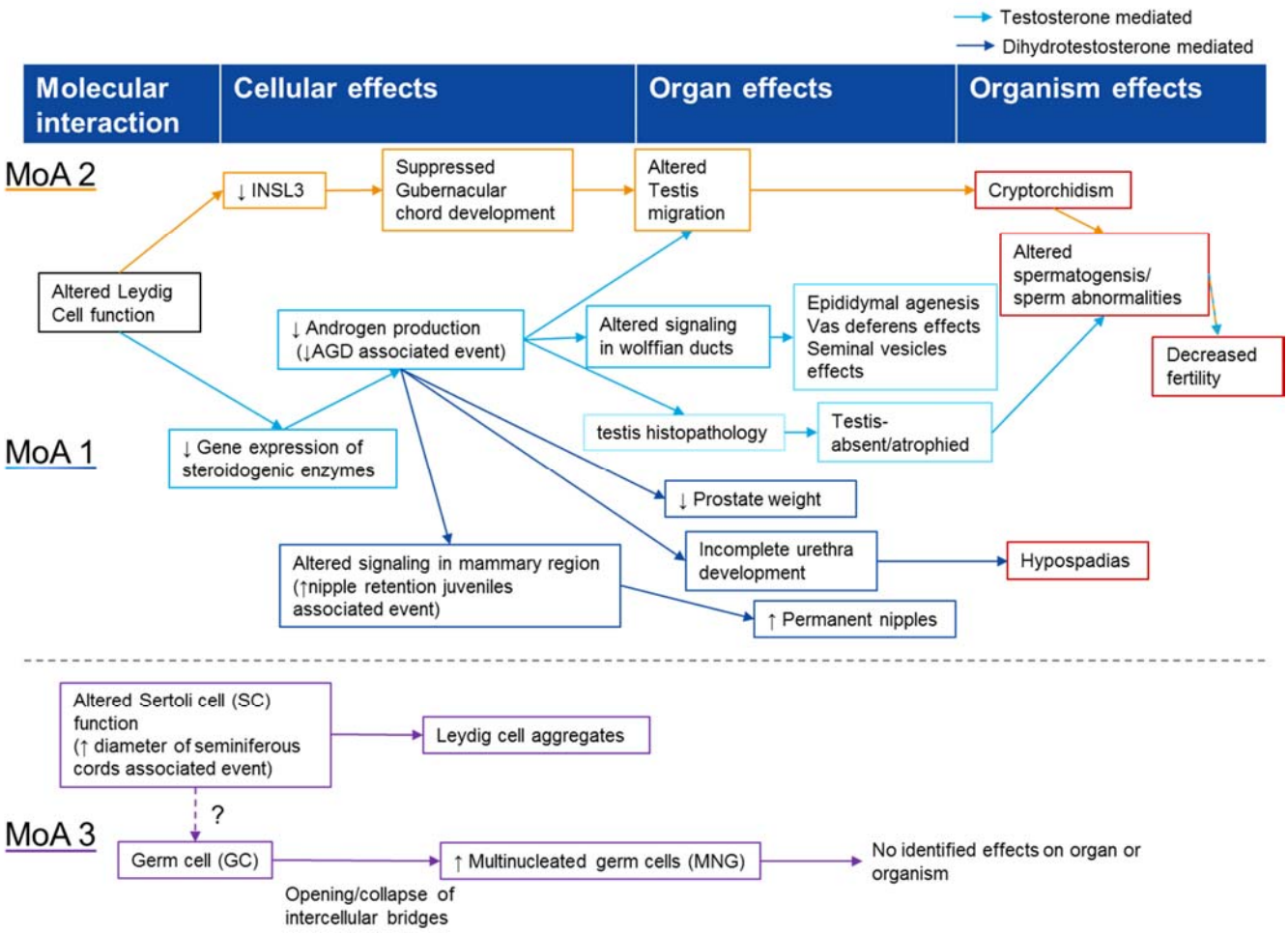


Figure IXa Proposed Mode of Action for observed developmental effects after in utero exposure to DBP and DEHP

If you map the data for DBP and DEHP to the proposed mode of action each key event and adverse outcome have been consistently observed after *in utero* exposure (Figure IXb)

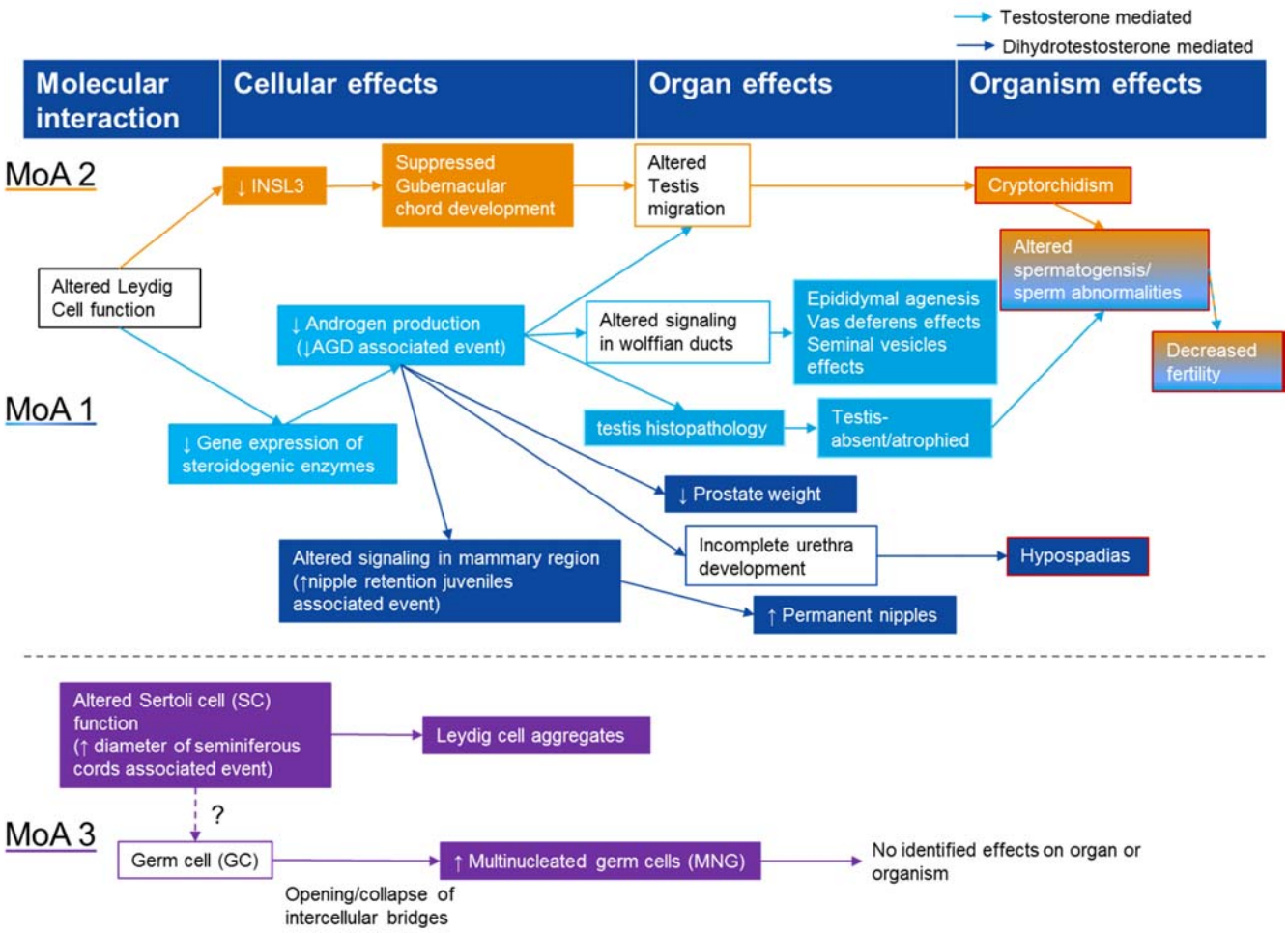


Figure IXb: DBP and DEHP consistently initiate each key event and adverse outcome following in utero exposure

In contrast to DBP and DEHP, the same pattern of effects are not observed after *in utero* exposure to DINP.

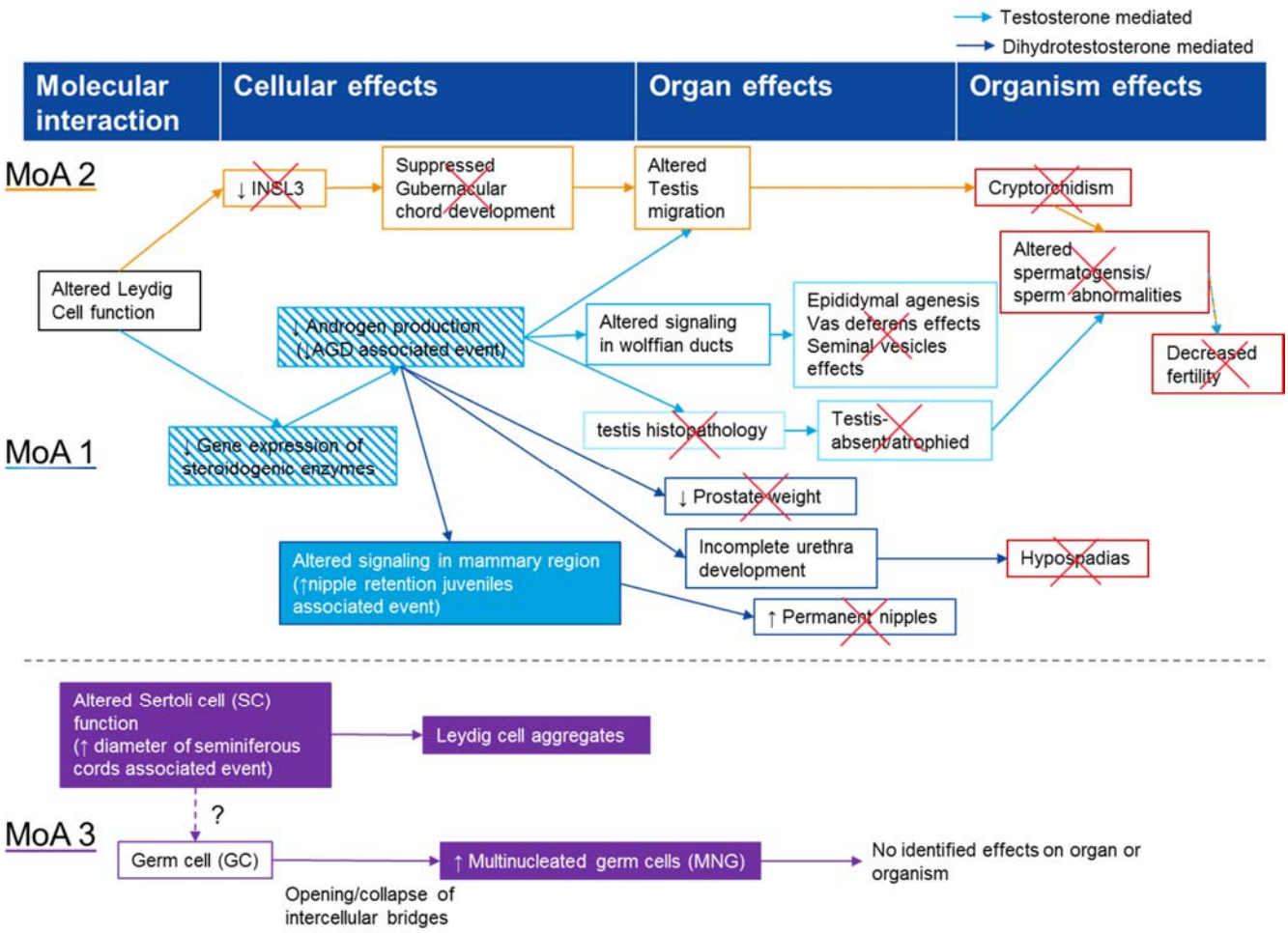
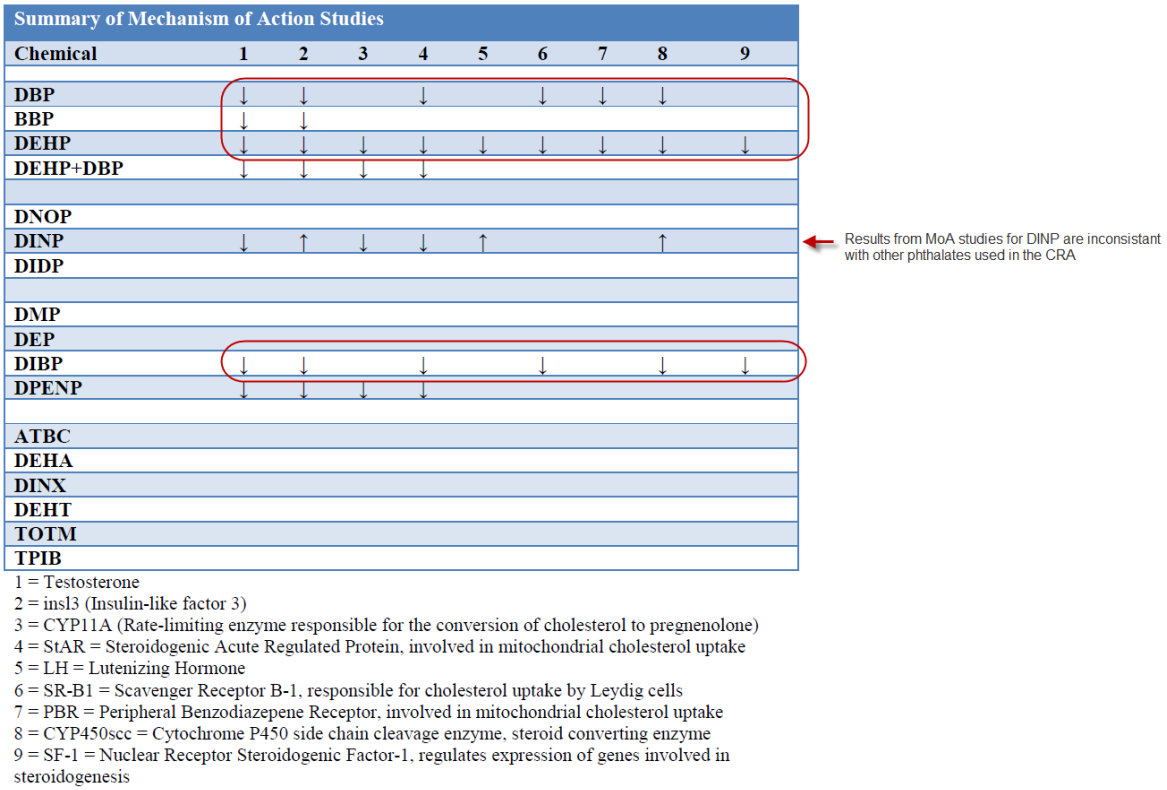


Figure IXc. Only markers of cellular effects are observed after in utero exposure to DINP. No adverse effects on organs or the organism are observed.

Figure IXc depicts the hypothesized modes of action for the *in utero* effects of DBP and DEHP. The portions of the modes of action for which observations have been reported for DINP have been shaded in. The portions of the modes of action that have been specifically measured and not observed have been marked with a red X. Decreases in gene expression of steroidogenic enzymes is hashed, and not shaded in, because some, but not all, of the genes affected by DBP and DEHP are down regulated by DINP and reports are conflicting. Decreased androgen production is hashed because though decreases in testosterone levels have been observed after DINP treatment the critical window for anti-androgenic effects is GD15.5-GD18.5 (Male Programming Window [MPW], Welsch *et al.* 2008), and testosterone has never been measured during this window; also AGD has been shown to be a sensitive measure of testosterone levels during the MPW and studies evaluating AGD changes in perinatal animals after DINP exposure have only rarely reported an statistically significant change in AGD(2/7 studies). This could mean that DINP does not decrease testosterone during the critical window or the decrease is minimal.

As can be observed in Figures IXb and IXc there are key differences in DINP's ability to initiate the various modes of action necessary to elicit the effects observed after *in utero* exposure to DBP and DEHP. When the Chronic Hazard Advisory Panel (CHAP) convened by the U.S. Consumer Product Safety Commission to study the effects on children's health of all phthalates and phthalate alternatives as used in children's toys and child care articles published their final report they noted similar inconsistencies between DINP and the other phthalates (Figure IXd below).



Summary of Mechanism of Action Studies									
Chemical	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
DBP	↓	↓		↓		↓	↓	↓	
BBP	↓	↓							
DEHP	↓	↓	↓	↓	↓	↓	↓	↓	↓
DEHP+DBP	↓	↓	↓	↓					
DNOP									
DINP	↓	↑	↓	↓	↑			↑	
DIDP									
DMP									
DEP									
DIBP	↓	↓		↓		↓		↓	↓
DPENP	↓	↓	↓	↓					
ATBC									
DEHA									
DINX									
DEHT									
TOTM									
TPIB									

1 = Testosterone
2 = insl3 (Insulin-like factor 3)
3 = CYP11A (Rate-limiting enzyme responsible for the conversion of cholesterol to pregnenolone)
4 = StAR = Steroidogenic Acute Regulated Protein, involved in mitochondrial cholesterol uptake
5 = LH = Lutenizing Hormone
6 = SR-B1 = Scavenger Receptor B-1, responsible for cholesterol uptake by Leydig cells
7 = PBR = Peripheral Benzodiazepene Receptor, involved in mitochondrial cholesterol uptake
8 = CYP450scc = Cytochrome P450 side chain cleavage enzyme, steroid converting enzyme
9 = SF-1 = Nuclear Receptor Steroidogenic Factor-1, regulates expression of genes involved in steroidogenesis

Figure IXd. Summary of Mechanism of Action studies for phthalates published by the U.S. Consumer Product Safety Commission's Chronic Hazard Advisory Panel (CHAP) in Appendix A of their final report.

Figures IXa-c were developed based on the following references

Borch, J., Metzdorff, S.B., Vinggaard, A.M., Brokken, L., Dalgaard, M., (2006). Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223, 144–155.

Carmichael, S. L., Witte, J. S., Ma, C., Lammer, E. J., & Shaw, G. M. (2014). Hypospadias and variants in genes related to sex hormone biosynthesis and metabolism. Andrology, 2(1), 130-137.

Euling, S. Y., White, L. D., Kim, A. S., Sen, B., Wilson, V. S., Keshava, C., ... & Androulakis, I. P. (2013). Use of genomic data in risk assessment case study: II. Evaluation of the dibutyl phthalate toxicogenomic data set. Toxicology and applied pharmacology, 271(3), 349-362.

Foster, P. M. (2005). Mode of action: impaired fetal Leydig cell function—effects on male reproductive development produced by certain phthalate esters. Critical reviews in toxicology, 35(8-9), 713-719.

Hannas, B. R., Lambright, C. S., Furr, J., Evans, N., Foster, P. M., Gray, L. E., & Wilson, V. S. (2011). Genomic biomarkers of phthalate-induced male reproductive developmental toxicity: A targeted rtPCR array approach for defining relative potency. Toxicological Sciences, kfr315.

Howdeshell, K. L., Hotchkiss, A. K., & Gray, L. E. (2016). Cumulative effects of antiandrogenic chemical mixtures and their relevance to human health risk assessment. International Journal of Hygiene and Environmental Health.

Howdeshell, K.L., Furr, J., Lambright, C.R., Rider, C.V., Wilson, V.S., Gray, L.E. Jr., (2007). Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: Altered fetal steroid hormones and genes. Toxicol Sci 99, 190–202.

Ivell, R., & Anand-Ivell, R. (2009). Biology of insulin-like factor 3 in human reproduction. *Human reproduction update*, *15*(4), 463-476.

Johnson, K. J., Heger, N. E., & Boekelheide, K. (2012). Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent. Toxicological Sciences, 129(2), 235-248.

Makris, S. L., Euling, S. Y., Gray, L. E., Benson, R., & Foster, P. M. (2013). Use of genomic data in risk assessment case study: I. Evaluation of the dibutyl phthalate male reproductive development toxicity data set. *Toxicology and applied pharmacology*, *271*(3), 336-348.

Sinisi, A. A., Pasquali, D., Notaro, A., & Bellastella, A. (2002). Sexual differentiation. Journal of endocrinological investigation, 26(3 Suppl), 23-28.

Spade, D. J., Hall, S. J., Wilson, S., & Boekelheide, K. (2015). Di-n-butyl phthalate induces multinucleated germ cells in the rat fetal testis through a nonproliferative mechanism. Biology of reproduction, 93(5), 110.

Wilson, V.S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., Gray, L.E. Jr., (2004). Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. Toxicol Lett 146, 207–215.

A.X: Existing data do not support phthalates can reduce fetal testosterone in humans, which is a necessary step for the proposed mode of action

Species differences in response to phthalates have become more apparent in the recent literature. The presence or absence of anti-androgenic effects has not been consistent across species. Toxicological studies have identified sensitive species (rats) and insensitive species (mice, marmosets). In utero exposure of marmosets to MBP had no effects on testosterone levels, or gross abnormalities of the male reproductive tract (McKinnell *et al.* 2010). In utero exposure of mice and rats to DBP results in multinucleated germ cell formation and an increase in seminiferous tubule diameter, yet rats only exhibit suppression of fetal Leydig cell steroidogenesis (Gaido et al., 2007). This difference could be a species specific effect of DBP exposure on fetal Leydig cell SREBP2 activity; however the underlying mechanism is unknown (Johnson *et al.* 2011)

The fact that in-utero exposure to phthalates has not shown an anti-androgenic effect in mice and marmosets (effects which are commonly found in rats) raises the question whether humans are more like rats, or more like these insensitive species (Gaido et al., 2007, McKinnell *et al.* 2010, Tomonari *et al.* 2006). Mechanistic studies evaluating this question have shown that human fetal tissue does not respond to phthalates that may have an anti-androgenic effect[14] in the same manner as rat fetal tissue, and these findings of insensitivity of human fetal tissue have been repeated in multiple labs using several different methodologies.

Lambrot *et al*. (2008) investigated the effect of MEHP on human fetal testes recovered during the first trimester[15] (male programming window in humans) of gestation in an organ culture system. MEHP had no effect on basal or LH-stimulated testosterone, no down regulation of genes in the androgen biosynthetic process, and did not affect proliferation and apoptosis of Sertoli cells. Reduced mRNA expression of anti-Müllerian hormone was reported and a reduced number of germ cells (via increased apoptosis) were also seen. Similarly, Hallmark *et al.* (2007) reported no effect on human fetal testis explants cultured with 10-3M MBP for up to 48hrs. This included measurement of intra-testicular testosterone levels and cytochrome P450 side chain cleavage enzyme expression as well as Leydig cell aggregation. However, the authors of the paper questioned the utility and validity of the in vitro system. Drs. Boekelheide and Sharpe independently developed model systems, in which embryonic human testes and embryonic rat fetal testes are implanted (xenograft) into adult immunosuppressed rodents to test for anti-androgenicity. These investigators showed that when the host animals were treated with DBP, testosterone production was reduced in the implanted fetal rat testis, but testosterone levels were unaffected in the implanted human testis (Heger *et al.* 2012; Johnson *et al.* 2012; Mitchell *et al.* 2012). An additional study by Spade *et al.* (2014) used this model system to demonstrate that reduction in testosterone is observed after exposure to a compound known to reduce testosterone in humans. These findings address concerns that too much variability existed in the models to identify a positive effect and suggest the model is sensitive enough to identify a positive effect if one exists. Spade *et al.* (2014) also evaluated the effect of DBP and again found no effect of treatment on testosterone levels.

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[14] Anti-androgenicity has been defined as the ability to antagonize male hormones (androgens). In a broad sense, the ability of a phthalate to affect testosterone levels after *in-utero* fetal exposures has been referenced as a mode of action in the classification proposal. This reference to mode of action is overly simplified to the point of inaccuracy. First, the ability to impair testosterone synthesis must cross a threshold in order to have a biological relevance (Gray *et al.* 2016). Second, the male developmental effects associated with phthalate exposure are likely due to multiple modes of action, which include anti-androgenic mechanisms (both decreased testosterone and decreased Insl-3), as well as non-anti-androgenic mechanisms (Foster 2006).

[15] 7-12 weeks

The ex-vivo xenograft results from Heger *et al.* (2012), Johnson *et al.* (2012), Mitchell *et al.* (2012), and Spade *et al.* (2014) confirm the results from the in vitro model systems, highlighted in Habert et al. (2014), that demonstrated after treatment with MEHP, the active metabolite of DEHP, there were no effects on testosterone production in human testis maintained under in vitro conditions. The tissue used in Lambrot et al. 2009 was taken from 1st trimester tissue and gave similar results to those seen in the ex-vivo xenograft models. The lack of effects in human testes in different experimental models is supported by the in vivo studies in non-human primates (McKinnell et al. 2010). These data demonstrate the relative insensitivity of human fetal testes to phthalate induced anti-androgenicity. The authors and results are quite clear; the potent phthalates DBP and DEHP do not affect testosterone production in the developing human testis as is seen in the rat testis.[16]

This may be explained by the observation that fundamental control of steroidogenesis in the fetal rat differs from that in the human fetus.[17] This point is important since it is frequently claimed that the pathway (sexual differentiation) that certain phthalates disrupts in the fetal male rat is highly conserved in all mammals, and is known to be critical for human reproductive development. Indeed, commonalities exist between humans and rodents during the period of sexual differentiation (i.e., the time when a fetus can be morphologically distinguished as being male) and to some extent masculinization. However, a clear difference is noted in the stimulatory mechanisms for testicular steroidogenesis during the critical period when masculinization of the reproductive tract is being programmed. Specifically, the 3 day time period (GD 15.5-18.5) during which testosterone is produced and masculinization occurs for the rat is largely luteinizing hormone (LH)-independent (Scott *et al.* 2009). Conversely, human fetal testosterone production begins around gestational week 8, and is mainly controlled by human chorionic gonadotropin (hCG) secreted by the placenta, a hormone not produced by rats. By gestation week 12, hCG begins to decline and LH levels are seen to rise. (Dufau *et al.* 1972, Lee and Ryan, 1973). Unlike rats, paracrine factors likely have a secondary or supporting role in human testosterone secretion and do not initiate production. In order to effect in utero testosterone levels in humans, phthalates would have to effect hCG levels directly, as suppression of testosterone by phthalates does not affect a pathway downstream of hCG as demonstrated by the xenograft studies. In a study by Culty *et al*. 2008, testosterone production in response to hCG stimulation was similar between control samples and fetal rat tissue exposed to DEHP, whereas in tissue exposed to DEHP at similar doses without hCG stimulation, testosterone production was significantly reduced. In addition a recent epidemiological study (Adibi *et al.* 2015) found a significant effect between 1st trimester hCG levels and fetal AGD measurements.

The data on species differences in the ability of phthalates to effects testosterone levels has been so compelling that multiple reviews have been authored on the subject questioning the utility of this model system for human health risk assessment: Man is not a big rat: concerns with traditional human risk

[16] An increase in multinucleated gonocytes (MNGs) per total number of germ cells was reported although the significance of this effect is discussed in Part 4 Appendix VII of this submission.

[17] Basic differences in the steroidogenic cascade are also noted. The principle form of circulating cholesterol differs between rats and humans: high-density lipoprotein (HDL) is the primary source taken up by the SRB-1/HDL receptor on the Leydig cell in rats and low-density lipoprotein (LDL) is the primary source taken up by the LDL receptor on the Leydig cell in humans. In addition, the preferred steroid biosynthetic pathway converting cholesterol to testosterone differs; the Δ4 pathway (*i.e.,* progesterone and its intermediate 17α-hydroxyprogesterone) predominates in rats, while the Δ5 pathway (*i.e.,* pregnenolone and its intermediates, 17α-hydroxypregnenolone and DHEA) is the predominant mechanism of testosterone synthesis in humans. These differences must be considered when characterizing the relevance of reported rodent effects and their extrapolation to human hazard characterization and risk assessment.

assessment of phthalates based on their anti-androgenic effects observed in the rat foetus. (Habert *et al.* 2014a); Concerns about the widespread use of rodent models for human risk assessments of endocrine disruptors (Habert *et al*. 2014b) and; Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent. (Johnson *et al.* 2012). The evidence developed to date is strong enough to show that the decrease in testosterone observed in rodents following exposure to anti-androgenic phthalates is not relevant to in utero human male reproductive tract development and using effects caused in rats subsequent to testosterone decrease as an endpoint for human health assessments, including classification, is not appropriate.

A.XI: Boberg et al. (2011) cannot be reproduced using raw data and the statistical methods in the original publication.

The CLH proposal cites Boberg et al (2011) in three of the six points justifying the proposed developmental classification - points c), d) and f) on page 8 of the DINP CLH report - and in one of the three points – point i) on page 9 of the DINP CLH report - justifying the proposed fertility classification.

A re-analysis of the raw data from this study using the methods in the published paper (Boberg et al 2011) has shown that the results of statistical significance for the effects of DINP in animals cannot be reproduced for several parameters (Morfeld et al, 2017). This section briefly reports the outcome of that reanalysis.

A Corrigendum (Boberg et al. 2016) to the Boberg et al. publication (Boberg et al. 2011) was recently published to 'avoid misunderstanding' regarding the statistical and experimental approaches as described in Boberg et al. (2011). The changes in the Corrigendum selectively modified the originally published statistical approach for sperm parameters and changed the experimental protocol for AGD, leaving all of the originally published results intact. The primary change to the statistical protocol involved selective omission of a post-hoc test with correction for multiple comparisons. The accepted statistical practice is to apply correction for multiple comparisons in all instances when multiple comparisons have been performed. The importance of this correction is recognized, for example, by EFSA in their scientific opinion on distinguishing statistical significance from biological relevance (EFSA, 2011).

While we recognize that statistical outcomes do not constitute the only input into inferences about treatment-related effects or biological significance, we consider the scientifically debatable changes in the Corrigendum [2] more impactful to the results and interpretation than the Corrigendum implies. A Data in Brief (Chen et al. submitted) and a letter to the editor (Morfeld et al 2017) have been written by European Plasticisers scientists (formerly ECPI) which clarify the reproducibility discrepancies and their significance to interpretation of this particular study.

Following the description of the statistical methods in Boberg et al. 2011, we reanalyzed the raw data (made publically available in the US EPA HERO database (USEPA 2016)), for the following endpoints: testosterone, nipple retention, sperm motility, sperm/g cauda, percent progressive sperm, and anogenital distance (index) (AGD/AGDi) measurements. The results of this reanalysis are detailed in a publication (Chen et al. 2017 submitted). We were unable to confirm the reported statistical significance as published by Boberg et al. (2011) for four of these six parameters:

1. Percent progressive sperm: Boberg et al. reported a significant difference in percent progressive sperm between the control and the 750 mg/kg/day exposure group. Our reanalysis concluded that there is no significant difference between control animals and those treated with DINP.

2. Sperm/g cauda: Boberg et al. reported a significant increase in sperm/g cauda epididymis at the highest dose group (900 mg/kg/day). Our reanalysis concluded that there is no significant difference between control animals and those treated with DINP at all doses.

3. Sperm motility: Boberg et al. reported a significant decrease in sperm motility between control and the 600, 750 and 900 mg/kg/day exposure groups. Our reanalysis confirmed statistical significance at the two highest

doses (750 and 900 mg/kg/day); however, the reanalysis concluded that there is no significant difference between control animals and those treated with 600 mg/kg/day DINP.

4. AGD and AGDi; Boberg et al. reported a statistically significant difference in both ADG and AGDi on PND 13 between the control and 900 mg/kg/day exposure group. Our reanalysis concluded that there is no significant difference in AGD and AGDi between control animals and those treated with DINP.

Also of critical importance, the low percentage of motile sperm in the control animals (60% or less) does not comply with literature standards (OECD, 2008; Seed et al. 1996). Since sperm parameters are sensitive to sampling techniques, analysis techniques (Scleh et al. 2013), and environmental conditions (Seed et al. 1996) interpretation of results is contingent upon experimental optimization. OECD published in 2008 a respective guidance document (OECD, 2008) indicating a percentage of at least 70% motile sperm for untreated control rats as a standard requirement, consistent with recommendations in the peer reviewed literature (Seed et al. 2010). To better understand the potential issues related to the sperm parameter evaluation in this specific laboratory, we extracted control data from two other published studies from this laboratory. The motile sperm control animal data of Boberg et al. (mean = 59.3%), Jarfelt et al. (2005) (mean = 66.9%) and Taxvig et al. (2007) (mean = 53.8%) do not meet the minimum quality requirements of the OECD. In addition, we found that the percentage of motile sperm varied statistically significantly between the controls in these three studies (F test: p-value = 1.3%). Thus, the variance in motile sperm percentage for control animal readings cannot be explained by physiological (random) fluctuations. As no further explanation from the authors is available, the variance points to the conclusion of a lack of standardization of this parameter in this lab. It is important to note that the changes in sperm motility reported for DINP in Boberg et al (2011) are within the unexplained variance of the control animals of the two other studies undertaken in the same laboratory. Therefore, we conclude they do not differ substantially from historical controls within this laboratory. The authors failed to account for the non-adherence to scientific standards and unexplained systematic variation in control sets from their laboratory, hindering meaningful interpretation of biological relevance of reported sperm measurements.

Additionally, the Corrigendum (Boberg et al. 2016) reports for the ‘Sperm per gram cauda epididymis’ endpoint at the highest dose a p-value of 0.047, whereas we calculate a p-value of 0.072 and conclude that this is not statistically significant.

A.XII: The Epidemiologic Data Do Not Support Classification

Summary of the literature on DINP and reproductive (fertility and development) endpoints

The current body of epidemiologic literature on DINP and reproductive/fertility endpoints **does not** provide scientific justification for classification. Although relatively small and evolving, the epidemiologic literature on DINP covers many reproductive developmental and fertility endpoints, including those referenced in the CLP proposal: cryptorchidism and hypospadias (Jensen et al. 2015; Main et al. 2006), anogenital distance (AGD) (Bornehag et al. 2015; Jensen et al., 2016), pubertal development (Mieritz et al., 2012), time to pregnancy (Specht et al. 2015), as well as reproductive hormones and testicular function (Joensen et al. 2012; Specht et al. 2014; Axelsson et al. 2015). As a whole, the epidemiologic database is characterized by uncertainty due to methodological limitations and substantial variation in study design, population, sample size & selection, exposure assessment & timing, outcome ascertainment, study conduct and overall quality. Most of the studies that comprise the literature report null (no) effects; studies that did observe associations report very small effect sizes, are the result of hundreds of models and are unsupported by/inconsistent with other epidemiologic studies. Moreover, the clinical significance (i.e. adversity) of the outcomes under investigation is unclear. Nonetheless, the largely (and repeatedly) null findings observed across the many endpoints examined in these existing studies is informative, despite the noted methodological limitations, and viewed in total suggest that DINP does not alter reproductive development or impair fertility.

Unlike the relatively large number of reproductive/developmental epidemiologic studies that have been published on low molecular weight phthalates, including DEHP, only sixteen studies published in the peer reviewed literature have examined DINP. Among these studies a wide range of reproductive endpoints have been examined, with less than a handful addressing any one endpoint. The Danish EPA considered a subset (9 of 16) studies, with the basis for selection unknown. Comments on the studies listed in the proposal are provided below.

Comments on fertility studies cited in the proposal

With few exceptions, studies of fertility (e.g. sperm quality, hormonal levels, pubertal development, time to pregnancy) did not observe associations between DINP metabolite concentrations and any of the endpoints examined. This conclusion has been stated by the study investigators and the Danish EPA concurs in their proposal. The few associations reported (e.g. testosterone decrements) are very weak, inconsistent with results of similar studies and are generally not observed in concert with decrements in more proximate measures of fertility (e.g. sperm quality). In addition, the clinical significance of single specimen semen samples and hormonal levels is uncertain, especially in light of the very small effect sizes being observed. As such, the data do not support a conclusion of adversity nor classification. Detailed comments are below for the fertility studies reference in the proposal.

1. Joensen et al. 2012

The paper by Joensen et al. (2012) describes the methods and results of a study designed to examine testicular and endocrine function in relation to phthalate exposure among a cross section of 881 healthy, young Danish

men recruited from 2007 to 2009, during compulsory examination to evaluate their fitness for military service. Exposure was estimated based on urinary metabolite concentrations of fourteen phthalate metabolites: MEP, MnBP, MiBP, MBzP, MEHP, MEHHP, MEOHP, MECPP, MOP, MCPP, MiNP, MHiNP, MOiNP and MCiOP. In addition to examining individual metabolite concentration, several constructed exposures were also examined: (1) Molar sums of low-molecular-weight phthalate metabolites (MEP, MiBP, MnBP, DEHP), high-molecular-weight phthalate metabolites (MBzP, MCPP, , DiNP) and total metabolites, (2) molar sums of DEHP and DiNP metabolites, (3) percentage of total DEHP metabolites expressed as MEHP and (4) percentage of total DiNP expressed as MiNP.

Results indicated few associations between any measure of phthalate exposure (either single metabolites or composite/constructed variables) and alterations in reproductive hormone levels, hormone ratios or sperm quality indicators. Exceptions were observed **only** when phthalate exposure was specified as %MEHP or %DiNP in the regression models. For hormone levels, %MINP was associated with a 15% lower FAI [95% confidence limit (CL) -23% to -8%], 9% lower total testosterone/LH ratio [CL: -18% to -0.4%] and 19% lower FAI/LH ratio [CL: -30% to -8%] among men in the highest v. the lowest quartiles. %MEHP was associated with lower FAI [-9%; CL: -16% to -1%], lower total testosterone [-7%; CL: -13% to -1%], lower free testosterone, [-7%; CL: -12% to -0.3%]. Both %MiNP and MEHP were negatively associated with FSH, corresponding to a 13% lower level [CL: -25% to -1%] and 14% lower [CL: -25% to -3%] FSH in the highest v. lowest quartiles, respectively. For sperm quality, however, **both %MiNP and %MEHP were associated with relatively higher values of the sperm quality indicators** among men in the highest quartile v the lowest quartile; other sperm quality results were not statistically significant.

The interpretation of this study is complicated by limitations of these invalidated susceptibility measures. First and most importantly, primary metabolites, MEHP and MiNP are non-specific biomarkers of exposure to their respective phthalate diester (i.e. DEPH and DiNP) because they share the same metabolic pathway as other chemicals that require cytochrome p450 Phase I enzymes for detoxification. As such**,** MEHP, MiNP and any measure on which they are computationally based may be confounded by other chemical exposures. Secondly, MiNP is an “insensitive” biomarker of DiNP exposure because it further metabolizes to the oxidative metabolites for which no environmental sources are known (Calafat et al., 2011). Finally, %MEHP and %DiNP are largely un-validated measures reflecting a metabolic pathway that is still not completely understood.

Another significant limitation of Joensen et al. relates to the appropriateness of examining hormonal levels (or sperm quality), using a cross-sectional study design, in light of the exceedingly short biological half-lives of the exposure markers. It seems biologically questionable that the exposures represented from a spot urine (largely reflecting the prior 18 to 24 hours) would be etiologically relevant to endpoints such as serum hormone levels and indicators of sperm quality.

Beyond questions of validity, the clinical significance of the results seems, as suggested by the authors and noted in the proposal, negligible. Reported effects were modest and alterations were not observed to be in conjunction with any of the sperm quality parameters. To the contrary, Joensen et al. reported a positive association between %MiNP and semen volume and progressively motile sperm. In addition, the authors struggled to explain the pattern of their results, relying largely on conjecture given the extraordinarily complex nature of the male endocrine and reproductive systems and the many unknowns about the potential mechanisms of toxicity by which these metabolites may act. Finally, the results of Joensen et al. were not consistent with the only other report to have utilized %MEHP as an exposure variable, with Meeker et al.

(2009) reporting a negative association between %MEHP and E_2 and a positive association between %MEHP and total testosterone/E_2 ratio.

2. Specht et al. (2014)

In this cross-sectional study of 589 fertile adult men attending an prenatal clinic with their currently pregnant partners, investigators examined serum phthalate (DEHP and DiNP) concentrations in relation to a series of 17 reproductive endpoints (semen quality and reproductive hormones), including semen volume, sperm count, sperm concentration, normal morphology, motility, fructose, PSA, NAG, zinc, testosterone, estradiol, inhibin B, FSH, LH, FAI and SHBG and the T/LH ratio. Using generalize linear model (GLM) regression analysis, an estimated 136 equations were run for DINP alone. A "trend analysis" was used to compute p-values and served as the basis for determining statistically significant results, but the authors do not identify the test for trend or describe how it was applied, so it is not possible to evaluate their statistical methods. Results for DINP revealed few statistically significant associations (less than would be expected by chance) between metabolite concentrations of DiNP and the hormones or sperm quality parameters examined. The two associations observed suggested a positive association with "proxy-MINP"(composite oxidative DINP metabolites) and sperm volume and a weakly inverse association with testosterone and SHBG. Such statistical associations were observed less frequently than would be expected, suggesting that these findings could be an artifact of random error, particularly when (1) no decrements were observed in any testicular function measures (i.e. sperm motility, morphology, count, concentration and volume) and (2) the established fertility (i.e. currently pregnant partners) of the men in the study. The authors support this conclusion, as they interpreted their results as weak and without clinical implications (for DEHP) and even more so for DiNP.

3. Specht et al. (2015)

Specht et al (2015) examined time to pregnancy (TTP) in 938 women and 401 men of pregnant couples from Poland, Hungary and Greenland in relation to serum metabolite levels of DEHP and DiNP, modeled as "proxy-DEHP" (composite DEHP oxidative metabolites) and proxy-DINP (composite oxidative DINP metabolites) similar to Specht et al. 2014. Fecundity ratios were computed, using modified Cox regression models and represent the probability of conceiving in a month or cycle. For women, results for DEHP suggest increased fecundity and reduced TTP with increasing serum concentrations (both measured continuously and categorized into tertiles) overall (pooled) and trending across the three countries. With the exception of a subgroup analysis of primiparips (women with first pregnancies) from Greenland, suggesting a longer TTP for women (interpreted as a 28% reduction in probability of conception for any given cycle should the concentration of DINP increase by 2.7 times baseline), the investigators concluded that DiNP was unrelated to TTP for both women and men.

The findings of Specht et al. (2015) were confirmed in a more recent (2016) and robust (albeit smaller) and detailed prospective cohort study of phthalate concentrations and follicular phase length, luteal phase length, time to pregnancy and early pregnancy loss in US women residing in North Carolina. Of the 11 phthalate metabolites examined (including metabolites of DEHP and DiNP), no detrimental alterations were observed for either compound, with DEHP associated with a reduction in pregnancy loss (Jukic et al. 2016). Investigators for both the Specht et al. 2015 and Jukic et al. 2016 studies concluded no adverse effects of either DEHP or DiNP on fecundity/TTP, with the favorable trends reported for DEHP on TTP and decrease pregnancy loss requiring confirmation.

4. Mieritz et al. (2012)

This cross-sectional study recruited 555 healthy boys (24.7%) ages 6 to 19 years from a larger cohort of 3,101 boys that were participating in the COPENHAGEN Puberty Study from 2006 to 2008 to examine the relationship between current urinary phthalate metabolite concentrations (including ∑ DEHP and ∑DINP) and pubertal timing and presence of gynaecomastia. Serum samples were obtained to measure testosterone levels as well. Urinary phthalate concentrations were not associated with age of puberty, serum testosterone levels or presence of gynecomastia. Phthalate concentration levels were similar to those reported in other studies. The study investigators interpreted their results as indicating "no evidence for anti-androgenic effect of phthalates on healthy boys."

Comments on developmental reproduction studies cited in the proposal

Similar to the fertility database, the empirical evidence for DINP-related adversity on reproductive development is lacking. Detailed comments on the studies used by the Danish EPA in their classification proposal are found below.

1. Main et al. (2006)

As part of a Danish-Finish prospective cohort study designed to established contemporary rates of and identify factors associated with the prevalence of cryptorchidism, Main et al. conducted a case-control study, using bio-banked breast milk samples from the mothers of 130 one to three month old infant boys (62 with cryptorchidism and 68 without) and measured metabolite concentrations of six phthalates, including DINP (measured as the primary metabolite MINP) to examine the association between phthalates, hormonal levels and cryptorchidism. Researchers did not observe differences in phthalate concentrations between boys with and without cryptorchidism, but did observe associations with several phthalate metabolites, including MiNP and a mix of different hormones. Specifically, MINP was positively associated with luteinizing hormone (r=.24; p-value = 0.19).

Despite the matched case-control design, researchers used multiple regression and non-parametric statistics (Spearman correlations), breaking the case-control design. The implications of their analytical choice is unknown, but it is not conventional and calls into question the study results. Moreover, researchers state that their data should not be used to argue against breast-feeding, "because effects on reproductive hormones were subtle" and there was no increased risk for cryptorchidism.

2. Bornehag et al. (2015)

In Bornehag et al., researchers examined the relationship between concentrations of 1st trimester maternal urinary phthalate metabolites of DEP, DBP, DEHP, and DiNP and two measures of anogenital distance among 196 sons born between September, 2009 and November, 2010 to women participating in the Swedish Environmental Longitudinal, Mother and child, Asthma and Allergy (SELMA). Average age of the boys was 21 months. The participation rate was 39%. Significant associations were reported between the shorter of two AGD measures (anoscrotal distance; AGD as) and DiNP metabolites, particularly oh-MMeOP and oxo-MMeOP. Weak to no associations were observed for DEHP.

Studies from Bornehag et al. and Swan et al. both published in 2015, illustrate the uncertainty and challenges in characterizing the potential reproductive hazard associated with DINP in general and the use of AGD as an

endpoint in particular. Swan's paper was the 3rd from this group to report results from a prospective cohort study of prenatal phthalate exposure and genital morphologic development in US boys. According to the authors, this study of prenatal phthalate exposure and AGD is the largest to date with a sample size of 753 for all phthalates examined (DEHP, DEP, DBP, BBzP, DiBP, DnOP), expect for DINP and DINP for which the sample size was 464. This cohort was assembled from four areas (San Francisco, CA; Minneapolis, MN; Rochester, NY; Seattle WA). Eligible women were at least 18 years of age with healthy pregnancies, < 13 weeks of gestation, who provided urine (for urinary phthalate measurements) in the first trimester of pregnancy and completed questionnaires in each term. No specific details of recruitment, including rates of and factors associated with participation, were provided. Two AGD measurements were taken, AGD-AP and AGD-AS (as in Bornehag). Significant negative associations were observed for metabolites of DEHP (MEHP, MEOHP, MEHHP) and both measures of AGD in boys, but not girls. **No associations were observed for the DINP metabolites**.

These findings from Swan et al. are in contrast to those from Bornehag et al. who, collaborating with Dr. Swan, conducted the two studies described above. However, unlike the null findings for DINP reported by Swan, Bornehag observed a small, but statistically significant inverse correlation between DINP and one of the AGD measurements, revealing a 4% reduction in AGD associated with more than an interquartile range increase in DINP urinary metabolite concentrations. Bornehag, however, did not observe associations in relative AGD reductions with metabolites of low molecular weight phthalates or DEHP, which have been shown in previous studies, including Swan et al. 2015 and 2008 – a difference the researchers acknowledge as "largely unexplained". In addition, the 4% reduction observed is within the range of measurement or random error.

AGD as an endpoint in epidemiologic studies has several limitations and our understanding and interpretation is still evolving. Currently, there is no standard measurement for AGD; different studies often measure the distance between different anatomical structures, using different measurement tools. In addition, normative data is lacking and as such so too are clear referents by which to compare, validate and interpret study findings (Liu, 2014). In addition, there is no established method for appropriate statistical adjustment for age and weight, which are known determinants of AGD. Moreover, AGD is a very difficult measurement to (reliably if at all) obtain with young children, especially toddlers, which was the study population in Bornehag et al. 2015. Finally, the clinical relevance and adversity of AGD has not been established (Mendiola J, et al. 2015; Para MD et al., 2015; Mendiola J et al.). Given these important considerations, it is premature to use AGD as measure of reproductive developmental impacts and therefore cannot contribute information for classification.

3. Jensen et al. (2015)

This study used a nest case-control design (412 controls, 270 cryptorchidism cases and 75 hypospadias cases), using specimens selected from a Danish repository of second trimester amniotic fluid samples, to examine the relationship between exposure to DEHP and DINP and hypospadias and cryptorchidism. This study also examined the association between these phthalates and several amniotic fluid steroid hormones and insulin-like factors 3 levels. Only samples from live singleton males were included in the study. Results revealed no consistent association between amniotic fluid levels of 5cx-MEPP and cryptorchidism or hypospadias. However, 5cx-MEPP levels were positively associated with amniotic fluid steroid hormone levels and inversely associated with the insulin-like factor 3 level. Associations were dose-dependent, ranging from approximately 20% to 40% difference between first and third 5cx-MEPP tertiles. 7cx-MMeHP levels did not show consistent associations with steroid hormones or insulin-like factor 3, but elevated odds of hypospadias and, less strongly, cryptorchidism, were reported, although with very wide (imprecise), non-significant confidence intervals.

This largely null study reveals several important considerations that put the results into context. First, this study did not observe associations with DEHP, which is unexpected and may be an artifact of this dataset. Secondly, despite (very weak) alterations reported in hormones measuring Leydig cell function, **DINP was not associated with either of the reproductive malformations under investigation – hypospadias or cryptorchidism**. In addition, they did not adjust for birth weight, which was lower for "case" babies than control babies. This tracked with congenital malformations, which they adjusted for by restriction in their analyses. To the extent that birth weight is an indicator of gestational metabolism, this could be important. Nevertheless, most of the observed results were reassuring, especially when we consider similar studies collectively.

4. Jensen TK et al. (2016)

To examine the association between prenatal phthalate exposure and anogenital distance in male infants, investigators recruited Danish women 8 to 16 weeks pregnant (~43%) who were enrolled in a prospective cohort study (Odense Child Cohort). Women provided spot urine samples at ~28 weeks gestation and phthalate metabolite concentrations of DEP, DiBP, DnBP, BBzP, DEHP and DiNP were measured in 555 women of whom 293 delivered live, singleton boys; after eligibility exclusions the final sample was 293 infant boy/mother pairs. Replicating measures of Bornehag and Swan (2015), two measures of AGD were obtained (AGD-as and AGD-ap), in addition to penile length and width at three months of age, regardless of length of gestation. Associations between prenatal phthalate metabolite concentrations and AGD, penile length and width were estimated using multivariable linear regression models adjusting for age and weight for age standard deviation scores. None of the metabolites for the six parent compounds, including DINP, was associated with any of the measured endpoints considered in this study. Investigators speculated that their results may be due to the relatively low metabolite concentrations, but it is important to bear in mind that other studies with higher concentrations also did not report reduced AGD (e.g. Swan et al. 2015) and those that did (e.g. Bornehag et al. 2015) were inconclusive.

5. Axelsson J. et al. (2015)

The aim of this study was to examine the association between prenatal phthalate exposure to DEHP and DINP and male reproductive parameters. Swedish men 17 to 20 years of age presenting for military recruitment were invited to participate, with 241 or 14% assenting. An additional 73 men were recruited through school announcements, but due to loss to follow up and the availability of maternal serum samples from which to measure prenatal metabolite concentrations, the final sample was reduced to 112. Data on genital measurements, sperm parameters and reproductive hormone levels were obtained on all men. Results indicated that one of the metabolites of DiNP (MCiOP) was associated with lower testicular volume and higher FSH (highest v. lowest tertile). DEHP metabolite, MEHHP, was associated with lower semen volume, again comparing the highest to the lowest tertile of exposure.

Overall, the results of this paper suggest that prenatal exposure to DEHP and **DiNP is not associated with subsequent (long-term) adverse effects in the reproductive functioning of adolescent males**, as evidence by testicular and reproductive hormone measurements. Of the 84 associations examined [14 endpoints x 6 metabolites (3 DEHP and 3 DiNP)], only 4 (total testicular size, semen volume, and FSH for MCiOP and semen volume for MEHHP) showed significant differences, on average, between the highest (tertile) exposure group and the lowest. **However**, these few and minor decrements were (1) likely due to random error (4/84 = 0.047, which is exactly the proportion of associations you would expect to see simply by chance when the alpha for statistical significance is set to 0.05) AND (2) not clinically significant, **as the mean values in the group with the**

highest phthalate exposure were all well within published reference values (e.g. normal testicular range 15-25 mL; abnormal is considered < 12 mL; the study suggests that the average in the highest exposed group is 20.5).

In addition, given a very low response proportion ~ 12%, the potential for selection bias is heightened. In summary, this study does not raise the index of suspicion, given the limited findings across a range of metabolites and endpoints, the role of random error (chance), the small, non-clinically relevant observations, the potential for selection bias and measurement error and the lack of agreement with toxicologic data. Therefore, we do not agree with the study authors' conclusion that "prenatal levels of some metabolites of DiNP and DEHP seemed negatively associated with reproductive function of adolescent men". We agree with ECHA's re-evaluation, which considered 384 publications and scientific reports and concluded for DINP "no further risks identified" and "no further risk management measures needed" for adults and children. **The present study does not change ECHA's conclusion.**

Conclusions

On the basis of currently available data, DINP does not appear to impair normal reproductive development or fertility. As such, the epidemiologic data do not provide the evidence necessary to justify classification.

1. Epidemiologic studies of the relationship between exposure to DINP and fertility, reproductive development and testicular toxicity are largely of no finding (null); interpretation of studies that report associations are compromised by methodological limitations, weak and/or inconsistent associations, lack of biological plausibility and/or inconsistency with the toxicologic literature, and lack of (or unknown) clinical significance of the measured outcomes.

    a. High degree of variability and limitations with respect to study conduct and methods in the published literature including (1) study design and subjects, (2) exposure assessment, including choice of metabolites, timing of specimen sampling, sample matrix, (3) outcomes measured and (4) statistical/analytical methods compromise conclusions

    b. Results, despite the often inaccurate and/or over-reaching interpretation of the authors are negative (null) or weak. Reports from the adult male literature suggest that current DINP exposure does not perturb normal male reproductive/testicular function. This is consistent between human and animal studies and within the epidemiologic literature.

2. Cross-sectional reproductive studies of adult males, using measures of current phthalate exposure, are not instructive to understanding the potential fetal origins of testicular dysgenesis syndrome (TDS). Male cohort studies with valid biomarkers of phthalate exposure measured at etiologically relevant periods of fetal development are required, but currently missing, from the literature. Mendiola (2011) acknowledged gaps in the interpretation of existing literature stating "whether shorter AGD in men reflects dysgenesis and whether this is a consequence of fetal antiandrogen exposure are speculative".

    a. Prenatal phthalate exposure in humans has been examined in relation to several reproductive outcomes, including AGD, hypospadias, cryptorchidism, penis and testicular size and hormonal levels, however, their interpretation is not straight forward, due to the reasons listed above. As such, conclusions from these studies are compromised by (1) a small pool of data with (2) inconsistent findings that (3) evaluated and/or reported only one endpoint and (4) have extensive methodological limitations

b. In addition, these findings have been largely un-replicated because the subsequent studies did not observe similar associations.

c. AGD, as a specific endpoint that has received significant attention, has yet to be fully established as a marker of reproductive health and function. Moreover, the limitations described above and found in the current literature must be addressed before definitive statement can be made regarding the clinical significance of AGD.

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5. Additional Background Information

This information is provided for those who may not be familiar with the prior evaluations conducted on DINP as well as other relevant points:

1. DINP has been subject since the 1980s to extensive toxicological testing including reproductive testing and research studies. There is a large amount of relevant data on DINP. DINP has also been the subject of extensive regulatory evaluations with the conclusion that classification (Dangerous Substances Directive) is not required (EU Risk Assessment Report 2003), and that no further risk management measures are needed for children or adults (EU Risk Assessment Report - Completed 2003 (published in the Official Journal in 2006), ECHA Evaluation Report on New Data, 2013). These assessments included full hazard characterizations including the available reproductive studies. The latter includes Boberg et al. 2011, which is a key study in the dossier as already highlighted and discussed above. The ECHA Evaluation concluded also that existing restrictions for toys and childcare articles which can be placed in the mouth should be maintained, based on liver effects observed in rats.

2. A Risk Assessment Committee Opinion was developed on the ECHA Evaluation Report. RAC agreed with the ECHA conclusions.

3. DINP was REACH registered in March 2010 and a major update of the dossier was completed in December 2015, with incorporation of all relevant references from the ECHA Evaluation Report on New Data, 2013. As required by REACH over 400 pre-registrants/SIEF members were contacted to review and agree the proposed classification based on the scientific data. All agreed with the proposal which has been included in the REACH registration dossier of “conclusive but not sufficient for classification” for all CLP endpoints including reproduction.

4. There is no significant new animal data relevant to reproduction in the evidence brought forward by the dossier submitter in the DINP CLH Report that was not included in one or more of the prior evaluations referenced above. For example, Boberg et al. (2011) – one of the key studies in the Danish EPA dossier – was already assessed as part of the ECHA Restriction Evaluation and did not change the conclusion of no further risk management measures needed. Principles of Better Regulation and regulatory coherence should mean that new regulatory measures (which generate significant market uncertainty for manufacturers) should not be put forward in the absence of significant new scientific evidence.

5. DINP has been identified correctly as a major substitute for DEHP, DBP, DIBP and BBP in the Danish EPA and ECHA Restrictions Dossier. DEHP remains a major plasticiser product globally and if DEHP is to be substituted further then DINP as a major plasticiser will be needed. Therefore this proposal has the potential to have significant economic and employment impacts on producers and downstream users and to have important consequences for consumer health; particularly given that the 2013 ECHA re-evaluation

concluded that no further risk management measures are needed for DINP for adults and children. This conclusion was endorsed by the RAC Committee in 2013 as noted above. The following information was provided by European Plasticisers (formerly ECPI) into the Restrictions proposal consultation on DEHP, DBP, DIBP and BBP with regard to the investments which have been necessary over the last 20 years plus to enable the availability of DINP and other high molecular weight plasticisers as alternatives to DEHP (DBP, DIBP and BBP). The major alternatives are the high molecular phthalates and plasticisers namely: DINP, DIDP, DPHP and DINCH. The estimated approximate costs (in 2016 Euros) invested over the last 20 years by the European plasticiser industry to enable these products to be manufactured and available in sufficient quantities for downstream users is 6 – 8 Billion Euros (300 – 400 Million Euros per year on average). Because of these investments, including in health and environmental testing, alternatives have been available over the last 20 years enabling major substitution in the EU since approximately 1995. These investments then allow substitution going forward as well – it is though misleading to state that substitution is "low cost" (as has been stated in the SEAC opinion on the Restrictions dossier) in view of the significant investments which have been made to achieve this situation. The extensive scientific data on DINP and the other high molecular weight phthalates referenced above showing the clear lack of reproductive effects has guided this investment over this period. It is of course clearly recognized that the RAC process is a scientific process and the economic impact information is provided as background only; it does though underline the importance of a full and robust scientific assessment of all of the relevant scientific data versus the CLP criteria.

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