# Proposition 65 Maximum Allowable Dose Level (MADL) for Reproductive Toxicity for Di(*n*-butyl)phthalate (DBP)

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## Office of Environmental Health Hazard Assessment (OEHHA) Reproductive and Cancer Hazard Assessment Section

## SUMMARY

The maximum allowable dose level (MADL) for di(*n*-butyl)phthalate (DBP) is **8.7 micrograms/day (µg/day).** This value is based on the reproductive effects of DBP as observed in the study in rats by Lee et al. (2004). In that study, dietary maternal exposure during pregnancy and the lactational period adversely affected development of the reproductive system of male and female rat offspring. Since the developmental and reproductive effects observed in pups were mediated by maternal exposure to DBP, with fetuses receiving gestational exposure, and pups receiving lactational exposures, the MADL is calculated based on maternal exposure and a human female body weight of 58 kg (Title 22, California Code of Regulations, section 12803(b))[1].

## BACKGROUND

This report describes the derivation of a maximum allowable dose level (MADL) for DBP (CAS No. 84-74-2).

DBP is widely used in consumer products (NTP-CERHR, 2003a). It is mainly used as a coalescing aid in latex adhesives, as a solvent or plasticizer or for other purposes in personal care products (e.g., cosmetics), and in other consumer products (e.g., printing inks, nitrocellulose paints, film coatings or glass fibers). DBP has also been used as an inert ingredient in medications approved by the U.S. Food and Drug Administration (Hauser et al., 2004).

DBP was listed under the Safe Drinking Water and Toxic Enforcement Act of 1986 (commonly known as Proposition 65, codified at Health and Safety Code section 25249.5 et seq.) as known to the State to cause reproductive toxicity (developmental, male, and female reproductive toxicity), effective December 2, 2005. This listing was based on formal identification of DBP as causing developmental, female, and male reproductive toxicity by the National Toxicology Program (NTP) in its final report titled "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-*n*-

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[1] All further references to regulations are to Title 22 of the California Code of Regulations, unless otherwise noted

butyl Phthalate (DBP)" (NTP-CERHR, 2003a).  The NTP, solely as to final reports of the NTP's Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR), is a body recognized as authoritative for the listing of chemicals as known to cause reproductive toxicity under Proposition 65 (Section 12306(1)).

Procedures for the development of Proposition 65 MADLs are provided in Sections 12801 and 12803.  Exposure at a level 1,000 times greater than the MADL is expected to have no observable effect.  As defined in regulation, a MADL is derived from a No Observable Effect Level (NOEL) based on the most sensitive study deemed to be of sufficient quality.  The NOEL shall be the highest dose level which results in no observable reproductive effect expressed in milligrams of chemical per kilogram of bodyweight per day.  When data do not allow the determination of a NOEL, the lowest observable effect level (LOEL) shall be divided by 10 to establish a NOEL for purposes of Proposition 65 (Section 12803).

## STUDY SELECTION

Relevant studies or reports that provide information on the developmental, female, or male reproductive toxicity of DBP have been identified through literature searches.

## Scope of Literature Review

There are numerous studies or review reports providing relevant information on the developmental, female, or male reproductive toxicity of DBP.  The current document focuses on selection of studies for MADL calculation.  Under the regulations for MADL development, the MADL is to be based on "the most sensitive study deemed to be of sufficient quality" (Section 12803).  Therefore the focus of the literature review is to identify sensitive studies of "sufficient quality."  Detailed information and characterization of the developmental and reproductive toxicity of DBP have been presented in the "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-*n*-butyl Phthalate (DBP)" (NTP-CERHR, 2003a) and in the comprehensive review, the European Union Risk Assessment Report on Dibutyl Phthalate, by the European Chemicals Bureau (ECU, 2003).  The reader is referred to the NTP-CERHR documents for detailed discussion on characteristics of the developmental and reproductive toxicity (DART) of DBP.  OEHHA relied upon information provided in these two documents to identify potential sensitive studies that are possibly of "sufficient quality."  In addition, through a comprehensive literature search, OEHHA identified and reviewed a number of epidemiological studies in humans and experimental studies in laboratory animals that were published after releases of the NTP-CERHR (2003a) or ECU (2003) documents.

All the studies that are potentially sensitive studies of "sufficient quality" were identified and carefully considered in selection of "the most sensitive study deemed to be of sufficient quality."

## Human Studies

Several recent epidemiological studies investigated the potential effects of exposure to phthalates, including DBP, on development and function of the male reproductive system in humans. Major findings from these studies are briefly summarized in Table 1.

Noted in Table 1 are the measures of exposure employed in these epidemiological studies. The studies by Reddy et al. (2006a and 2006b) analyzed concentrations of phthalate diesters (parent compounds) in blood samples. All of the other studies measured levels of phthalate monoesters – metabolites of these diesters - in biological samples. Di-esters of butyl-benzyl phthalate (BBP), di-ethyl phthalate (DEP), DBP, or di-2-ethylhexyl phthalate (DEHP), are metabolized by hydrolysis to their corresponding monoesters (e.g., NTP-CERHR, 2003a; 2003b; 2006). Concentrations of mono-ethyl phthalate (MEP), mono-benzyl phthalate (MBzP), or mono-ethylhexyl phthalate (MEHP) in human biological samples (blood, urine, breast milk, or amniotic fluids) indicate levels of human exposure to DEP, BBP, or DEHP, respectively (e.g., Blount et al., 2000; Kohn et al., 2000; Silva et al., 2004b; Hoppin et al., 2002).

Mono-butyl phthalate (MBP) is a metabolite of DBP or BBP (NTP-CERHR, 2003a; 2003b). MBP in urine samples thus reflects exposure to DBP and BBP, although concentrations of MBP in urine samples collected from the general population have been used to estimate exposure levels to DBP only (David, 2000; Kohn et al., 2000). Since both DBP and BBP contribute to MBP in biological samples, actual level of exposure to DBP could be over-estimated (i.e., higher than true exposure levels) based on urinary levels of MBP in the general population. There is no analysis on the relative contribution of DBP to the urinary level of MBP in the general population, as compared to that by exposure to BBP.

Data reported in several studies in Table 1 clearly show an association between increased levels of MBP in biological samples (urine, blood or breast milk) and developmental, male, or female reproductive effects of DBP. In addition to DBP, the exposure data reported in the studies demonstrate that the subjects in all of these studies were exposed to multiple phthalates (e.g, BBP, DBP, DEP, DEHP) and polychlorinated biphenyls (PCBs). Several phthalates exhibit similar adverse male and developmental effect. Interactions between different phthalates or between phthalates and PCBs in the male reproductive toxicity of these compounds have been observed in rats (Gray et al., 2006a) and in men (Hauser et al., 2005). These issues – concomitant exposure to multiple phthalates and interaction among DBP and other phthalates - raise questions regarding the extent to which the observed associations between DBP and reproductive effects may be confounded. Therefore, although these human studies provide strong evidence that exposure to phthalates at certain levels is associated with developmental, male, or female reproductive effects in humans, they do not provide sufficient data to determining quantitatively the relationship between DBP exposure and these effects. For this reason human data were not used as the basis for MADL calculation.

**Table 1.  Epidemiological studies on the developmental and reproductive toxicity of DBP**

| Study Citation | Subjects | Exposure Assessment | Endpoints for Developmental or Reproductive Effects | Significant Findings on DBP or MBP |
|---|---|---|---|---|
| Duty et al., 2003a | 168 male partners of sub-fertile couples | Urinary levels of phthalate monoesters including MBP | DNA integrity in sperm using the neutral comet assay | No association with comet assay parameters indicative of DNA damage in sperm. |
| Duty et al., 2003b | 168 male partners of sub-fertile couples | Urinary levels of phthalate monoesters including MBP | Sperm concentration, motility, and morphology | Increased odds ratios for all three sperm parameters. |
| Duty et al., 2004 | 220 male partners of sub-fertile couples | Urinary levels of phthalate monoesters including MBP | Computer-aided sperm motion analysis | Suggestive evidence on reduced straight-line velocity. |
| Duty et al., 2005 | 295 male volunteers (18-54 years of age) from the general population. | Urinary levels of phthalate monoesters including MBP | Levels of reproductive hormones in blood samples | No physiologically relevant association with blood hormone levels. |
| Hauser et al., 2006 | 463 male partners of sub-fertile couples (168 of them were subjects reported by Duty et al., 2003b). | Urinary levels of phthalate monoesters including MBP and oxidative metabolites of DEHP | Semen quality (sperm concentration, motility, and morphology) | MBP level is inversely correlated with decreased sperm concentration and motility. |
| Pan et al., 2006 | 74 male workers occupationally exposed to DBP and DEHP (exposed group) and 63 construction workers (control group) in China | Urinary levels of MBP and MEHP | Blood levels of reproductive hormones including FSH, LH, testosterone, and estradiol. | Decreased level of free testosterone in the exposed group and significant negative correlation between blood testosterone level and urinary concentration of MBP. |
| Jonsson et al., 2005 | 234 men 18-21 years of age from military recruits in Sweden | Urinary levels of phthalate monoesters including MBP | Testicular volume, semen quality, and levels of reproductive hormones in blood samples | No clear pattern of association with male reproductive toxicity endpoints evaluated. |

**Table 1. Continued**

| Main et al., 2006 | Nursing mothers of 62 boys (29 from Denmark and 33 from Finland) with cryptorchidism as cases and those of 68 healthy boys (36 from Denmark and 32 from Finland) as controls. | Levels of MMP, MEP, DBP, MBzP, MEHP, and MiNP in breast milk samples collected from 1 month to 3 month after birth. Daily exposure level to each phthalate was estimated. | Levels of reproductive hormones (testosterone, FSH, LH, SHBG, inhibin B) in serum samples from all boys collected at about 3 months of age. | MBP level in breast milk was significantly correlated with decreased levels of free testosterone, increased levels of SHBG, and increased ratio of LH:free testosterone. |
|---|---|---|---|---|
| Reddy et al., 2006a | 85 infertile female patients with endometriosis and 135 controls with proven fertility | Levels of DBP, BBP, DEHP, Di-noctyl phthalate and polychlorinated biphenyls (PCBs) in blood samples | Case-control study to compare levels of phthalates and PCBs between the case and the control group. | Significantly increased levels of all phthalates and PCBs in female patients with endometriosis |
| Reddy et al., 2006b | 49 infertile female patients with endometriosis as cases; 38 infertile female patients without endometriosis as control group I and 21 with proven fertility as control group II | Levels of DBP, BBP, DEHP, DiOP in blood samples | Case-control study to compare levels of phthalates between the case and the two control groups. | Significantly increased levels of all phthalates measured in the case group as compared to the control groups. |
| Swan et al., 2005 | 134 boys 2-36 months of age | Maternal urinary levels of phthalate monoesters including MBP during pregnancy | Anogenital distance measured at 15.9 months of age (mean) | Decreased anogenital distance is associated with increased MBP level. |

MMP: mono-methyl phthalate; MiNP: mono-iso-nonyl phthalate; DiOP: Di-iso-octyl phthalate; FSH: follicle-stimulating hormone; SHBG: Sex hormone binding globulin; LH: Luteinizing hormone.

A number of studies listed in Table 1 investigated the male reproductive toxicity of DBP in boys or adult men (Duty et al., 2003a; 2003b; 2004; 2005; Jonsson et al., 2005; Hauser et al., 2006; Pan et al., 2006; Main et al., 2006; Swan et al., 2005), and a clear association between increased urinary levels of MBP and abnormal changes in endpoints indicative of development or function of the male reproductive system has been observed in several studies (see discussions below). Three studies observed an association between exposure to DBP and female reproductive effects (Main et al., 2006; Reddy et al., 2006a; 2006b). One study found a strong association between prenatal exposure to DBP and developmental effects on the male reproductive system in boys after birth (Swan et al., 2005). In addition, relatively high levels of MBP in amniotic fluids from pregnant women have been reported by Silvia et al. (2004a).

*Epidemiological Studies of Male Reproductive Endpoints*

A series of studies reported by Duty et al. (2003a; 2003b; 2004; 2005) and Hauser et al. (2006), respectively, investigated the relationship between urinary levels of phthalate monoesters and semen quality or blood sex hormone levels among male partners of subfertile couples presented to an andrology laboratory the Massachusetts General Hospital in Boston. The authors found no association between urinary levels of MBP or other phthalate monoesters and sperm DNA damage (Duty et al., 2003a) or blood sex hormone levels (Duty et al., 2005). However, the urinary level of MBP was inversely associated with sperm concentration and motility (Duty et al., 2003b; 2004; Hauser et al., 2006). In the study reported by Duty et al. (2003b), the incidence of men with sperm concentration below 20 million/ml among men with MBP levels above the median (16.2 ng/ml urine, adjusted for specific gravity) was compared to that in men with MBP levels below the median. The odds ratio of increased incidence in men with MBP levels above the median was 2.4 (95% confidence interval or CI = 0.80-7.2). Similarly, men with high urinary MBP levels had higher chances of low sperm motility (OR 2.4, CI = 1.1-5.0) or sperm with poor morphology(OR =1.7, CI = 0.8-3.9). In addition, the authors found increased odds ratio for low sperm concentration in men with increased urinary levels of MBzP, indicating association of decreased sperm number with exposure to BBP in the subjects included in the study.

These findings were confirmed in another recent study by the same research group (Hauser et al., 2006) that included more subjects [a total of 463, including the 168 subjects that were reported by Duty et al. (2003b)]. In this new report, the subjects were divided into quartiles according to their urinary levels of MBP or other phthalate metabolites, including MBzP, MMP, MEP, MEHP, etc., and compared the incidence of men with low sperm concentration (< 20 million/mL), low sperm motility (<50% mobile sperm), or poor morphology (<4% sperm with normal morphology). The odds ratio for men with low sperm concentration at four quartiles (0-<25th, $25^{th}$ - 50th, 51th-75th, and >$75^{th}$ percentiles, respectively, were 1.0, 3.1, 2.5, and 3.3, respectively (p value for trend = 0.04). Significantly increased odds ratio for low sperm motility was also observed in men with urinary MBP levels above the $25^{th}$ percentile. Urinary MBP concentrations (adjusted for specific gravity) at the $25^{th}$ and $50^{th}$ percentile were 10.6 and 17.7 ng/ml, respectively (geometric mean 17.3 ng/ml for the whole study population). Based on the normal 24-hr urine volume of 800-2000 ml/day for a healthy man (Medical Encyclopedia, 2006), urinary concentration of 17.3 ng/ml MBP suggests a daily excretion of 13,840 – 34,600 ng/day MBP or 63 – 156 nmol MBP per day (molecular weight of MBP = 222). Assuming that 69% of ingested DBP is excreted in urine as MBP (Anderson et al., 2001; Koch et al., 2003), and that all MBP molecules in the urine result from exposure to DBP, this level of urinary MBP represents an exposure to DBP at a dose level of approximately 91 -226 nmol/day, or approximately 25- 63 µg/day (molecular weight of DBP = 278). Therefore, the results of these studies by Duty et al. (2003b) and Hauser et al. (2006), respectively, indicate that exposure to DBP at approximately 25-63 µg/day or higher dose levels is associated with decreased sperm concentration and motility in adult men. There was no statistically significant association between sperm parameters included in this study to the

urinary levels of any other phthalate metabolites, though there was suggestive evidence of an association between the highest MBzP quartile and low sperm concentration.

A significantly decreased blood level of free testosterone has been observed in a cross-sectional study among workers occupationally exposed to high levels of DBP and DEHP in China (Pan et al., 2006).  In this study, urinary concentrations of MBP and MEHP and levels of reproductive hormones were measured among 74 male workers at a factory producing polyvinyl chloride (PVC) flooring products (exposed group) and 63 workers working in a construction company as controls.  The two groups were matched to age (mean age 33.9 years), history of cigarette-smoking (60-62% smokers in both groups), and alcohol consumption (54-64% consumers in both groups).  The exposed group had one year (mean, SD = 0.8) of employment time at the factory surveyed, suggesting that the exposed workers had a limited time of exposure.  Urinary concentrations of MBP and MEHP in exposed workers were significantly higher than those in the control group (644.3 and 565.7 µg/g creatinine in the exposed group vs. 129.6 and 5.7 µg/g creatinine in the control group for MBP and MEHP, respectively, geometric means).  The median concentrations of MBP and MEHP in the control group were about two to seven fold higher than those in the general population in the U.S. (Silva et al., 2004b), but were similar to those observed in German males (Koch et al., 2003).  The mean concentration of free testosterone in exposed workers was significantly lower than that of the controls ($8.4\pm1.5$ vs. $9.7\pm1.4$, $\log_{10}$-transformed blood levels of free testosterone; actual concentrations of reproductive hormones were not reported).  The authors reported no significant difference in blood levels of other reproductive hormones (FSH, LH, and estradiol) between the control and exposed groups.  Statistical correlation and regression analysis conducted by the authors found that both MBP and MEHP were responsible for the decreased level of free testosterone in blood.  Thus, data from this study clearly show that occupational exposure to DBP and DEHP is associated with decreased levels of free testosterone, indicative of male reproductive toxicity of DBP in men.  However, the data presented by the authors are not sufficient to quantitatively distinguish among the effects attributable to DBP and those attributable to concurrent DEHP exposure or interaction between the two phthalates.

While the findings from the studies discussed above indicate an apparent association between exposure to DBP and the male reproductive effects in men, Jonsson et al. (2005) found no association between urinary levels of MBP, MBzP, or MEHP and parameters for semen quality or reproductive hormone levels among 234 Swedish men 18-21 years of age.  All the subjects in this study were participants in the conscript examination before military service in Sweden.  Each subject received a comprehensive evaluation of parameters for semen quality (e.g., semen volume, sperm count, motility, morphology, etc.).  Serum levels of reproductive hormones (FSH, LH, testosterone, SHBG, estradiol) and urinary levels of phthalate metabolites (MEP, MBP, MBzP, MEHP and phthalic acid) were analyzed.  Urinary levels of MBP in this study were in the same order of magnitude as those previously reported in the U.S. populations (e.g., Blount et al., 2000).  The reason(s) for the inconsistent findings between this study and those reported by Duty et al. (2003b) and Hauser et al. (2006) is unclear.  Jonsson et al. (2005) noted that the subjects in their study were from the general population in Sweden and were healthy young (18-21

years old), whereas the studies by Duty et al. (2003a; 2003b) or Hauser et al (2006) were based on male partners of subfertile couples with a wide range of age (20-54 years). Thus, the U.S. studies may have been more sensitive in design. Because of increased age and possible poor status of spermatogenesis, subjects in the U.S. studies may have been more susceptible to the adverse effects of phthalates than those who participated in the Swedish study.

Two epidemiological studies investigated potential male reproductive effects of phthalates in boys who were exposed to phthalates through their pregnant or nursing mothers (Swan et al., 2005 and Main et al., 2006, respectively). Major findings from the study by Swan et al. (2005) are discussed below in the subsections on developmental effects of DBP. In the study by Main et al. (2006), the authors evaluated the association between phthalate levels in breast milk of nursing mothers and blood levels of reproductive hormones among 62 boys (29 from Denmark and 33 from Finland) with cryptorchidism and 68 healthy boys without cryptorchidism (36 from Denmark and 32 from Finland) as controls. Aliquots of breast milk samples were collected and pooled by each nursing mother between one and three months after birth. Blood samples from all the boys were collected at 3 months of age. Concentrations of six monoesters of phthalates, including MMP, MEP, MBP, MBzP, MEHP, and MiNP were measured in breast milk samples. Milk samples from Finland showed significantly higher values for MBP, MBzP, and MEHP than in samples from Denmark. With regard to MBP, samples from Denmark and Finland had median levels of 4.3 and 12 µg/L, respectively. Based on the figure presented in the report, samples from both countries had the same concentration of MBP (approximately 30 µg/L) at the 90th percentile. Assuming an average milk consumption of 0.12 L/kg-day, the authors estimated that the boys included in the study were exposed to 0.517 (in Denmark) or 1.45 (in Finland) µg/kg-day MBP at the median level. Using the same milk consumption and a body weight of 6.6 kg for 3-month-old boys as reported by the authors, OEHHA estimated that the boys in both countries were exposed to approximately 3.6 µg/kg-day or 24 µg/day MBP at the 90th percentile via their mother's milk. This exposure level of MBP is the molar equivalent of 30 µg/day of DBP.

The authors found no significant difference in any phthalate monoester concentration in the mothers' breast milk between children with or without cryptorchidism. However, positive correlations between the levels of MBP and MEP in breast milk and the concentration of SHBG or the LH:free testosterone ratio were statistically significant. Free testosterone levels were also inversely associated with the MBP levels, with a 15% decrease in free testosterone levels over a 10-fold increase of MBP levels (p<0.01). These results suggest that exposure of boys to DBP at approximately 30 µg/day via their mother's breast milk is associated with perturbation in their free (and thus functional) testosterone levels. Significant negative effects on milk quality, whether measured directly or reflected in impaired development of young, is considered adverse female reproductive effects, according to the U. S. EPA Guidelines for Reproductive Toxicity Risk Assessment (U.S. EPA, 1996). Therefore, this study by Main et al. (2006) provided evidence on both the male and female reproductive effects of DBP in humans.

As discussed above, in the studies by Duty et al. (2003b), Hauser et al. (2006), Pan et al. (2006) and Main et al. (2006), respectively, exposure to DBP either directly in men or indirectly via breast-feeding mothers is clearly associated with abnormal changes in the male reproductive system. However, the subjects in these studies were exposed to multiple phthalates. Data provided in these studies are not sufficient to determine quantitatively relative contribution of DBP alone to the observed reproductive effects. Future research, analyzing the confounding variables or on the relative effectiveness of various phthalates and other exposures to produce these effects may provide for a more definitive dose response evaluation. Thus, at this time these studies are limited in their usefulness for quantitatively understanding the effects of DBP

*Epidemiological Studies of Female Reproductive Endpoints*

In addition to the study by Main et al. (2006), two study reports by Reddy et al. (2006a and 2006b) also reported possible associations between exposure to DBP and female reproductive effects. In these two studies, Reddy et al. (2006a; 2006b) measured blood levels of PCBs and/or phthalate di-esters in female patients with (cases) or without endometriosis (controls) from a south Indian region. It is unclear if there is an overlap between the subjects in two studies, which were reported separately. The authors of both studies found that blood levels of all the compounds measured in endometriosis patients, including four PCB congeners, DBP, BBP, DEHP, and DOP, were significantly higher than those in controls ($p<0.05$). The authors concluded that phthalates may have an etiological association with endometriosis in women. However, it has been shown that exposure to PCBs is associated with endometriosis in laboratory animals and in women (e.g., Arnold et al., 1996; Rier, 2002; Louis et al., 2005); high concentrations of DEHP have also been found in women with endometriosis (Cobellis et al., 2003). Because the authors did not control for this confounding factor, the data from this study are not sufficient to determine if or how much DBP per se contributes to development of endometriosis in women.

*Epidemiological Studies of Developmental Endpoints*

With regard to the developmental effects of DBP in humans, two studies (Silva et al., 2004a and Swan et al., 2005, respectively) provide relevant evidence. The first study, by Swan et al., indicates feminization of males by in utero exposure to DBP. The second study, by Silva et al. (2004a), provides biological plausibility to the finding by indicating the degree of possible DBP exposure in utero in the general population.

Swan et al. (2005) analyzed levels of MBP and eight other phthalate monoesters in urine samples from 85 pregnant women at a mean gestational time of 28.3 weeks. The authors also performed genital examinations and measured anogenital distance (AGD) among a total of 134 boys at 2-30 months of age. Data from boys whose mother's urine samples had been analyzed for phthalate levels were included in the statistical regression analysis. The authors found that increased levels of MEP, MBP, MBzP, and mono-isobutyl phthalate in prenatal urine samples in mothers was associated with decreased AGD in boys after birth. The subjects were divided into three groups based on the phthalate levels in

their mothers urine ($<25^{th}$, $\geq 25^{th}$ to $< 75^{th}$, and $\geq 75^{th}$ percentile for the low, medium, and high exposure groups, respectively).  The data clearly show a dose-dependent association between increased levels of MBP in maternal urine samples during gestation and reduced AGD in boys after birth.  The odds ratios for shorter than expected age- and body weight-adjusted AGD in the three groups according to MBP levels in the maternal urine samples were 1.0, 3.8 (95% CI = 1.2 – 12.3), and 10.2 (955 CI = 2.5-42.2).  Consistent with similar findings on the reduced AGD in rats following gestational treatment with DBP (e.g., Mylchreest et al., 2000), the findings in this epidemiological study suggests that prenatal exposure to DBP may cause alterations in the development of the male reproductive system in boys.  DBP exposure of mothers in this study was estimated to be approximately 0.56, 0.84, and 1.31 μg/kg-day at the $25^{th}$, $50^{th}$, and $75^{th}$ percentiles, respectively (Marsee et al., 2006).  Using bodyweight of 58 kg for a pregnant woman (Section 12803), these estimated exposure levels are equivalent to approximately 32, 49, 73 μg/day, respectively.  Thus, in this study, maternal exposure to DBP at doses 32-49 μg/day or higher during pregnancy is associated with observable effects in the male reproductive system in boys after birth.  However, reduced AGD in the boys as observed in this study was associated with multiple phthalates and available data and analyses are not sufficient to determine quantitatively the relative contribution of DBP, thus limiting this study's usefulness in providing an understanding of the dose response for DBP alone.

The study by Silva et al. (2004a) provides biological plausibility to the findings of Swan et al. (2005).  Silva et al. (2004a) measured concentrations of phthalate metabolites MEP, MEHP, and MBP in amniotic fluid samples from 54 pregnant women.  MBP was detected in 50 samples (92.6%), with 5.8 and 14.2 ng/ml at the $50^{th}$ and $90^{th}$ percentiles and a maximal concentration of 263.9 ng/ml.  The authors did not investigate any potential association of MBP in amniotic fluids with pregnancy outcome or developmental effects in the offspring.  However, the concentrations of MBP at the $90^{th}$ percentile and the maximal level observed (14.2 and 263.9 ng/ml, respectively) are only about 100- or 5-fold lower than the average concentration of MBP (1,400 ng/ml) in the amniotic fluid from rats treated with 100 mg/kg-day of DBP during gestational day (GD) 12 and 18 (Calafat et al., 2006).  Prenatal treatment with DBP at 100 mg/kg-day has been shown to cause permanent damage to the male reproductive system in male rat pups (e.g., Mychreest et al., 2000).  Moreover, MBP in the amniotic fluid of rats is predominantly present as the free form, as compared to the conjugated form of MBP-glucuronide (Calafat et al., 2006), most likely due to inadequate perinatal glucuronidation (e.g., Coughtrie et al., 1988).  Similar to that in rats, most uridinediphosphate-glucuronosyltransferase isoenzymes that are involved in the glucuronidation reactions in humans are not expressed or functioning until after birth (e.g., Rane and Tomson, 1980; Ring et al., 1999).  MBP in the amniotic fluid in women is thus most likely present in the free form, as suggested by Silva et al. (2004a).  Free MBP is believed to be the active metabolite of DBP responsible for its reproductive toxicity (e.g., NTP-CERHR, 2003a), and its clearance rate from the fetus may be slower than that of the conjugated form.  Thus, the findings from this study indicate the presence of relatively high levels of free MBP in amniotic fluids from a relatively high proportion of pregnant women in the general population raise concerns regarding the potential reproductive hazards in the general population.

Taking all the human data together, especially those reported by Duty et al. (2003b), Swan et al. (2005), Main et al. (2006), and Hauser et al. (2006), exposure to DBP at approximately 30-50 µg/day or higher levels either during development through pregnant mothers or in adult men is associated with observable developmental, male, or female reproductive effects in humans. However, since the subjects in these studies were concurrently exposed to multiple phthalates and possibly other chemicals (e.g., PCB, Hauser et al., 2005), and in general the confounding was not sufficiently addressed in the analyses in these studies, these data are not sufficient to quantitatively determine the contribution of DBP exposure to the reported effects. Therefore, while future epidemiological studies with appropriate statistical analysis may make it possible to establish a dose-response relationship between exposure to DBP alone and developmental or reproductive effects in humans, none of the epidemiological studies discussed above can be used for this purpose at the present time.

## Studies in Laboratory Animals

This section focuses on the studies available from which to select "the most sensitive study deemed to be sufficient quality" for MADL calculation. For studies in laboratory animals, OEHHA has identified a number of studies that appear to be "of sufficient quality," used multiple doses of DBP, and observed relatively low values of LOELs for developmental and/or reproductive toxicity (DART). All such animal studies and their major findings are summarized in Table 2. Following Table 2 are detailed discussions on several key developmental or reproductive toxicity endpoints and the rationale for selection of "the most sensitive study."

All the studies listed in Table 2 are oral studies. There is no inhalation study on the developmental or reproductive toxicity of DBP in laboratory animals.

**Table 2.  Major findings from key studies that may be used as the basis for MADL calculation**

| Study Reference | Animals | Treatment | General Toxicity | Major DART and LOEL | NOEL |
|---|---|---|---|---|---|
| Lee et al., 2004 | Sprague-Dawley rats, 6-8 dams per group. Soy-free diet for dams and regular rodent diet for offspring after weaning. | Feed, 0, 20, 200, 2000, and 10,000 ppm of DBP in diet from gestational day (GD) 15 until postnatal day (PND) 21. | Slight decrease in body weight gain in dams from GD15 to GD 20 at 20, and 10,000 ppm only.  No effect on feed consumption. Increased liver and kidney weights at 10,000ppm. | <u>Developmental effects excluding postnatal exposure:</u> reduced male-to-female ratio and reduced AGD in male offspring on PND 2 at 10,000ppm. <u>Female Reproductive Effects:</u> hypoplasia of alveolar buds in the mammary glands in female offspring on PND 21in all treated groups, but not in adulthood (postnatal week 11 or 20). <u>Male Reproductive Effects:</u> Histopathological changes in the testis and mammary glands at >= 20ppm. **LOEL: 20 ppm (1.5-3.0 mg/kg-day)** | Not observed, based on endpoints for male and female reproductive effects. |
| Wine et al., 1997 | Sprague-Dawley rats, NIH-07 diet NTP-RACB protocol | Feed, 0, 0.1, 0.5, and 1.0% (w/w) in diet. | Reduced body weights, increased liver and kidney weights at 1.0%. | Male and female reproductive effects: 1.0%: decreased live pups per litter and live pup weights.  Abnormalities in development of the male reproductive system. 0.5%: Similar to 1.0% group. 0.1%: Reduction in live pups per litter in F1 pups and in live pup weight in F2 pups. LOEL: 0.1% (80 mg/kg-day in females) | Not observed based on endpoints for develop-mental effects. |

**Table 2 Continued.**

| Study Reference | Animals | Treatment | General Toxicity | Major DART and LOEL | NOEL |
|---|---|---|---|---|---|
| Lehmann et al., 2004 | Sprague-Dawley rats, 7 rats for the control group and 5 for each treated group. | Gavage, 0. 0.1, 1.0, 10, 30, 50, 100, and 500 mg/kg-day from GD 12-19. For measurement of fetal testicular testosterone levels, 3-4 male fetuses from 1-4 litters per dose group were used. | Not reported. | Developmental and male reproductive effects: Dose-dependent decline in expression of genes and corresponding proteins involved in cholesterol transport, steriodogenesis, or cell survival. Significant reduction was observed at 0.1 mg/kg-day in genes for 3beta-HSD and c-Kit. Dose-dependent reduction in fetal testicular testosterone concentrations; obvious decline at 30 mg/kg-day, but statistically significant at >= 50 mg/kg-day. LOEL: 30 mg/kg-day | 10 mg/kg-day based on functional changes in the testis. |
| Mylchreest et al., 2000 | Sprague-Dawley rats, 11-20 pregnant dams per group | Gavage, 0, 0.5, 5, 50, 100, or 500 mg/kg-day, GD 12-21. Offspring examined on PND 1, 14, 21, or 38. | No apparent maternal or general toxicity at all dose levels. | Developmental and male reproductive effects: 500 mg/kg-day: retained nipples, decreased AGD, reproductive tract malformations, and histopathological changes in the testis. 100 mg/kg-day: significant increase in the incidence of retained areolas or nipples. 50 and 5 mg/kg-day: obvious, but not statistically significant increase in the incidence of retained areolas or nipples. LOEL: 100 mg/kg-day | 50 mg/kg-day, based on lack of statistically significant increase in retained areolas or nipples. More discussions on this NOEL in the text. |
| Zhang et al., 2004 | Sprague-Dawley rats, 20 pregnant dams per group | Gavage, 0, 50, 250, or 500 mg/kg-day, GD 1 – PND 21. Male offspring examined on PND 14, 21, and 70. | No apparent maternal or general toxicity at all dose levels. | Developmental, male, and female reproductive effects: 250 and 500 mg/kg-day: apparent adverse effect on the development of the male reproductive system. LOEL: 250 mg/kg-day | 50 mg/kg-day |

Findings from numerous studies in laboratory animals (mainly rats) have shown that perinatal exposure to DBP at doses ≥ 100 mg/kg-day causes a spectrum of adverse effects on the reproductive system of both sexes. In the male, these effects include increased incidences of retained nipples, decreased AGD, reproductive tract malformations (e.g.,

hypospadias or undescended testis), interstitial cell hyperplasia or adenoma, and impaired spermatogenesis (e.g., Mylchreest et al., 1998; 1999; 2000; Zhang et al., 2004; NTP-CERHR, 2003a; ECU, 2003). Primary perturbation of steriodogenesis in Leydig cells and direct damage to Sertoli cells have been proposed as primary modes of actions for the adverse effect of DBP on the male reproductive system (Mylchreest et al., 2002; Fisher et al., 2003; NTP-CERHR, 2003a; Foster, 2005). In support of this mode of action, obvious changes in endpoints indicative of perturbation of androgen production and/or metabolism - such as increases in the incidence of retained nipples, abnormal mammary gland development, or deceased AGD (Mylchreest et al., 2000; Lee et al., 2004) - or significant changes in the expression of genes critical for testosterone production (e.g., Lehmann et al., 2004), have been observed at doses lower than 50 mg/kg-day. Selection of "the most sensitive study deemed to be sufficient quality" is thus made among three studies: Mylchreest et al. (2000), Lehmann et al. (2004), and Lee et al. (2004).

### *Mychreest et al. (2000)*

The study by Mychreest et al. (2000) characterized the dose-dependent adverse effects of DBP on the male reproductive system following gestational and lactational exposure. Based on statistically significant increase on PND 14 in the incidence of retained areolas or nipples in the male offspring from dams treated with 100 or 500 mg/kg-day DBP, the authors concluded that 50 mg/kg-day was the No Observed Adverse Effect Level (NOAEL) for DBP in their study. Data from this study on nipple retention in pups are presented in Table 3.

**Table 3. Incidence of retained nipples in male pups in the study by Mylchreest et al. (2000)**

| Doses (mg/kg-day) | Incidence with pups as unit | | Incidence with litters as unit | |
|---|---|---|---|---|
| | Pups affected/total pups | Percentage | Litters affected/total litters | Percentage |
| 0 | 9/134 | 6.7 | 5/19 | 26.3 |
| 0.5 | 8/119 | 6.7 | 5/20 | 25.0 |
| 5 | 13/103[1] | 12.6 | 8/19 | 42.1 |
| 50 | 12/120 | 10.0 | 10/20 | 50.0 |
| 100 | 44/141[4] | 31.2 | 16/20[2] | 80.0* |
| 500 | 52/58[4] | 90.0 | 11/11[3] | 100.0* |

Source: U.S. EPA (2006). *: Statistically significant difference from the control group (p<0.05), based on author's analysis using F test on the least-square estimates of mean values. Numeric superscripts: pairwise comparisons between control and treated animals using Fisher's Exact Test (conducted by OEHHA): [1] $p \leq 0.1$, [2] $p \leq 0.001$, [3] $p \leq 0.0001$, [4] $p \leq 0.000001$

Abnormal retention of areolas or nipples in males indicates alteration in the mammary gland. It has been clearly shown by numerous studies in laboratory animals and in clinical observations in humans that the environment of sex hormones, mainly including androgens and estrogens during pregnancy and early postnatal life determines the development and growth of the mammary gland (Topper and Freeman, 1980; Forest, 1983; Imperato-McGinley et al., 1986; Elger and Neumann, 1966; Liao and Dickson, 2002; Neville et al., 2002; Hovey et al., 2002). Inhibition of testosterone production and/or action by treatment with anti-androgenic compounds (e.g., cyproterone acetate or cyanoketone) during the late

gestational period in rats not only causes development of breast tissues and nipples in male fetuses similar to that in females, but also results in abnormal expression of estrogen receptors and C-19 steroid aromatase (e.g., Rajendran et al., 1977). Therefore, the developmental status of the nipple and mammary gland is an indicator for the function of androgens during the critical developmental period (Mylchreest et al., 2000). Moreover, nipples, once developed, remain morphologically permanent. Ductal and lobular-alveolar development of the mammary gland during the postnatal period is characterized by hormonally controlled morphological changes involving apoptosis (Balinsky, 1950; Elger and Neumann, 1966; Imagawa et al., 1990; Humphreys et al., 1996; Hovey et al., 2002). Therefore, histological evaluation of mammary gland tissues may be more sensitive and accurate in assessing the developmental status of mammary glands, as compared to gross examination for retention of nipples during the postnatal period.

In the study by Mylchreest et al. (2000), the authors counted retained nipples in male pups at PND 14. The increase in the incidence of retained nipples is statistically significant at doses ≥ 100 mg/kg-day, but the increase at doses of 5 and 50 mg/kg-day (42.1% and 50% based on litters, respectively, compared to 26.3% in the control) is apparent. Based on the statistical significance, the authors identified 50 mg/kg-day as a NOEL for the male reproductive toxicity of DBP resulting from prenatal exposure. However, considering the proposed mode of actions for DBP, the increased incidence of retained nipples at 5 and 50 mg/kg-day may be biologically significant. Since no histopathological evaluation of the mammary gland was included in this study, it is unknown if there were morphological changes in the mammary gland of male pups treated with DBP at these low doses.

***Lehmann et al. (2004)***

In the study by Lehmann et al. (2004), testicular testosterone concentrations were significantly lower in male fetuses from dams exposed to DBP at 50 mg/kg-day and higher doses during gestation, compared to controls. Testicular testosterone concentration is an endpoint indicative of a decrease in testosterone production (a functional change) in the testis. Original data on this endpoint from the study were obtained (U.S. EPA, 2006) and presented in Table 4 below.

**Table 4. Testicular testosterone concentrations in fetuses in the study by Lehmann et al. (2004).**

| Doses (mg/kg-day) | Number of dams | Number of fetuses | Testosterone concentrations (ng/ml) | Testosterone concentrations (Log$_{10}$ ng/ml) |
|---|---|---|---|---|
| 0 | 4 | 4 | 15.8±1.55 | 1.20±0.19 |
| 0.1 | 1 | 4 | 17.4±1.41 | 1.24±0.15 |
| 1.0 | 2 | 3 | 15.8±1.38 | 1.20±0.14 |
| 10 | 2 | 4 | 15.5±1.55 | 1.19±0.19 |
| 30 | 2 | 4 | 11.7±1.62 | 1.07±0.21 |
| 50 | 2 | 4 | 6.2±2.00 | 0.79±0.30* |
| 100 | 3 | 4 | 4.9±1.51 | 0.69±0.18* |
| 500 | 3 | 3 | 1.1±2.19 | 0.06±0.34* |

Note: Source: U.S. EPA (2006). Testosterone concentration was expressed as mean±SEM. *: Statistically significant difference from control (p<0.05), based on the author's analysis using Dunntt's test.

As shown in Table 4, testicular concentration of testosterone in the 30-mg/kg-day group was apparently lower than that in the control group, but the difference was not statistically significant. There was no apparent difference in the testicular concentration of testosterone between the control group and those treated with 0.1, 1, or 10 mg/kg-day DBP. Testosterone concentrations in the testes of 4 male fetuses from dams exposed to 30 mg/kg-day were 26% lower than those of the controls (11.7 ng testosterone /ml testicular tissue homogenates in the treated group vs. 15.8 ng/ml in the control group of 4 male fetuses from 4 dams). Since only 3-4 fetuses from 1-4 dams from each dosing group were included in the experiments to measure testicular testosterone levels, the statistical power for this endpoint in this study is limited. Even though the decrease in testicular testosterone concentration in the 30-mg/kg-day group is not statistically significant, the decrease (26%) is likely to be treatment-related and appears to be biologically significant. In the same study, dose-dependent declines in expression of a number of genes and their corresponding proteins that play critical roles in testosterone production were observed at doses as low as 0.1 mg/kg-day. This indicates that DBP at doses much lower than 50 mg/kg-day can disrupt testosterone production in the testis of male fetuses. Based on the intratesticular testosterone concentration, an endpoint for testicular function, 10 mg/kg-day is an apparent NOEL for the developmental and male reproductive toxicity.

### Lee et al. (2004)

In the study by Lee et al. (2004), the lowest dose used in the study was 20 ppm of DBP in soy-free diet, estimated by the study authors to be equivalent to 1.5-3.0 mg/kg-day. At this dose, there was no difference in reproductive organ weights or AGD in male pups between the treated and control group. However, 4% of pups retained areolas or nipples on PND 14, as compared to 0% in the control, although the increase was not statistically significant. When the offspring were examined on PND 21 (the last day of treatment), a lower number of germ cells in the seminiferous epithelium and histopathological changes in the mammary gland were observed in 4 of 8 male pups examined (as compared to none of 8 pups in the control group, p<0.05). In female pups, 4 of 8 examined at this time point had hypoplasia of the alveolar bud in the mammary gland (p<0.05). These findings suggest

that DBP at 20 ppm in diet altered testicular and mammary gland development in male pups, and affected mammary gland development in female pups. The authors identified this dose as a LOEL. The effect on the mammary gland in male pups from dams exposed to 20 ppm DBP as observed in this study is consistent with the findings from the study by Mychreest et al. (2000). It is also supported by the findings from the study by Lehmann et al. (2004), which observed a coordinated decrease in expression of a number of genes critical for testosterone production or germ cell survival at doses as low as 0.1 mg/kg-day. Thus, 20 ppm, estimated by the study authors to range from 1.5 mg/kg-day during gestation to 3.0 mg/kg-day at the maximal intake during PND 10-21, should be considered as a LOEL for the male and female reproductive toxicity. The design of this study and major findings are presented below.

Five groups of pregnant IGS (Sprague-Dawley) rats, 6-8 dams per group, were treated with 0, 20, 200, 2,000, or 10,000 ppm DBP in soy-free diet from GD 15 to postnatal (PND) 21. DBP treatment was terminated on PND 21. The dams were fed soy-free diets from GD 3 to PND 21. Soy-free diets used in the study contained very low levels of phytoestrogens. Use of soy-free diets may prevent potential effects on the development of the reproductive organs in the offspring resulting from exposure to phytoestrogens in regular rodent diets or from interactions between DBP and phytoestrogens (Masutomi et al., 2003; 2004; Thigpen et al., 2004; Mead, 2006). The offspring were weaned on PND 21 and were divided into three groups: eight-ten males and eight-ten females (at least one male and one female per litter) for necropsy and histopathological examination of endocrine-linked organs at prepubertal (PND 21), postnatal week (PNW) 11, and 20, respectively. However, no male offspring in the 10,000-ppm group was examined at PNW 20, because there was not sufficient number of male pups available in this group for examination at this time point. During the treatment period, the body weight and food intake of all dams were recorded at GD 15, 20, PND 2, 10, and 21. The numbers, weights, and AGDs of all the neonates were recorded on PND 2. Each litter was culled randomly to four males and four females on PND 3.

Based on food consumption and average body weights of dams during the treatment period, the authors estimated that the dams in the five groups were exposed to 0, 1.5-3.0, 14.4-28.5, 148.2-290.9, and 712.3-1371.8 mg/kg-day of DBP for the control, 20, 200, 2,000, and 10,000 ppm groups, respectively. The average (mean) intake of DBP between PND 10 and 21 in each group was the highest and was approximately two-fold higher than that between GD 15-20, due to increased food consumption in the presence of reduced body weight gains for the period of PND 10-21 in each group.

For the developmental effects possibly associated with prenatal exposure and assessed before significant postnatal exposure, the authors evaluated the number of live offspring, the male pup ratio, body weights, and AGDs on PND 2. There was no difference in the number of live offspring between the control and any DBP-treated group. The ratio of male to female pups was decreased in a dose-dependent manner. Compared to the control group (65.6±14.2%), the decrease in the 2,000- and 10,000-ppm groups was statistically significant (43.9±15.7% and 24.7±4.5%, respectively. When measured on PND 2, the AGD in male pups in the 10,000-ppm group was significantly reduced (3.0±0.1 mm vs

3.7±0.2 mm in the control group, p<0.01).  No other effect was observed.  Thus, for the purpose of Proposition 65, the NOEL for the developmental effects excluding postnatal exposure as observed in this study is 14.4 mg/kg-day (estimated dose for the period of GD 15-20).

The major findings on the male reproductive effects are summarized in Table 5 below.

**Table 5.  Male reproductive effects in Rats Treated with DBP - Lee et al. (2004)**

| Doses (ppm) | 0 | 20 | 200 | 2,000 | 10,000 |
|---|---|---|---|---|---|
| Estimated intake during gestation | 0 | 1.5±0.1 | 14.4±0.8 | 148.2±15.3 | 712.3±128.9 |
| Estimated maximal intake (mg/kg-day) | 0 | 3.0±0.4 | 28.5±4.9 | 290.9±36.4 | 1371.8±376.9 |
| No. of live offspring | 13.3±2.2 | 11.0±2.6 | 13.7±1.9 | 12.4±1.9 | 12.8±1.9 |
| Male ratio (%) | 65.6±14.2 | 51.0±17.8 | 47.4±13.5 | 43.9±15.7* | 24.7±4.5** |
| AGD on PND 2 (mm) | 3.7±0.2 | 3.9±0.2 | 3.8±0.3 | 3.8±0.2 | 3.0±0.1** |
| Percentage of males pups with retained nipples on PND 14 | 0 | 4 | 13 | 15 | 100** |
| Onset of preputial separation (days) | 42.1±1.3 | 41.2±1.5 | 40.8±1.2 | 41.7±1.5 | 42.5±1.9 |
| Testicular weights on PND 21 (g/100 g body weights) | 0.43±0.03 | 0.41±0.04 | 0.40±0.03 | 0.40±0.04 | 0.35±0.03** |
| Testicular weights on PNW 11 (g/100 g body weights) | 0.79±0.03 | 0.80±0.06 | 0.77±0.09 | 0.84±0.12 | 0.73±0.14 |
| Testicular weights on PNW 20 (g/100 g body weights) | 0.67±0.08 | 0.62±0.09 | 0.58±0.07 | 0.67±0.09 | Not examined |
| No. of males with reduced germ cells in the testis/total no. of animals examined on PND 21 | 0/8 | 4/8* | 4/8* | 8/8** | 8/8** |
| No. of males with reduced germ cells in the testis/total no. of animals examined on PNW 11 | 0/8 | 0/8 | 1/8 | 4/8* | 9/10** |
| No. of males with reduced germ cells in the testis/total no. of animals examined on PNW 20 | 1/10 | 2/10 | 2/8 | 5/10 | Not examined |
| No. of males with dilatation of alveolar bud in the mammary gland/total No. of animals examined on PND 21 | 0/8 | 2/8 | 2/8 | 2/8 | 1/8 |
| No. of males with dilatation of duct in the mammary gland/total no. of animals examined on PND 21 | 0/8 | 2/8 | 3/8 | 1/8 | 3/8 |
| No. of males with delayed germ cell development in the testis/total no. of animals examined on PNW 11 | 0/8 | 0/8 | 1/8 | 4/8 | 9/10 |
| No. of males with degeneration of alveolar cells  in the mammary gland/total no. of animals examined on PNW 11 | 1/8 | 8/8** | 6/8** | 8/8** | 9/10** |
| No. of males with alveolar atrophy in the mammary gland/total no. of animals examined on PNW 11 | 0/8 | 6/8** | 2/8 | 6/8** | 5/10* |
| No. of males with degeneration of alveolar cells  in the mammary gland/total no. of animals examined | 2/10 | 5/10 | 6/8** | 8/8** | 9/10** |

| on PNW 20 | | | | | |
|---|---|---|---|---|---|
| No. of males with alveolar atrophy in the mammary gland/total no. of animals examined on PNW 20 | 0/8 | 6/8** | 2/8 | 6/8** | 5/10* |

**Note:** continuous data were presented as mean±SD. * and **: Statistically significant difference from control (p<0.05 and 0.01, respectively), based on the author's statistical analysis.

The data summarized in Table 5 clearly show that treatment with DBP at 10,000 ppm in diet from GD 15 to PND 21 causes: reduced number of male pups per litter, decreased AGD, reduced testicular weights, reduced germ cells and delayed germ cell development in the testis, increased number of male pups with retained nipples, and abnormal development of the mammary gland. Some of these effects were also observed in animals in the 20-, 200- or 2,000-ppm groups.

At 20 ppm, a significantly increased number of animals had a reduced number of spermatocytes, clearly indicating the effect of DBP on the testicular development at this dose level. The incidence (number of male pups with reduced germ cell development over the total number of male pups examined) and severity of reduction in germ cell development were increased in a dose-dependent manner on PND 21. By PNW 11, a statistically significant increase in the incidence of reduced germ cell development was only observed in the 2,000- and 10,000 ppm groups. By PNW 20, although reduced germ cell development was observed in 2 of 10, 2 of 8, and 5 of 10 animals examined in the 20, 200, and 2,000-ppm groups, respectively, the incidences of reduced germ cell development were not statistically significant. The data suggest that DBP-induced reduction in germ cell development at doses up to 2,000 ppm in diet as observed this study may be to some extent reversible, after exposure was discontinued for more than seven weeks. Reduction in germ cell development may result from DBP-caused damage in germ cells or Sertoli cells. It has been clearly shown that exposure to DBP during late gestation or the perinatal period causes formation of abnormally giant, multinucleated gonocytes (pre-spermatogonia), reduction in the number of gonocytes, and germ cell degeneration in developing male rats (e.g., Mylchreest et al., 1998; 1999; 2000; ECU, 2003; Ferrara et al., 2006). While the long-term effects of DBP-caused reduction in germ cell development as observed by Lee et al. (2004) remain to be determined, this abnormal histopathological change is consistent with similar observations from other studies, and is supported by the proposed modes of actions for the male reproductive effects of DBP. As noted in the U.S. EPA "Guidelines for Reproductive Toxicity Risk Assessment" (U.S. EPA, 1996), "significant and biologically meaningful histopathological damage in excess of the levels seen in control tissue of any of the male reproductive organs should be considered an adverse reproductive effect." Therefore, reduction in germ cell development as observed in the Lee et al. (2004) study is an adverse effect, although it may be reversible after the exposure was discontinued for more than seven weeks (in PNW 11 and 20). Available data do not permit a determination of whether this effect would still be reversible if the exposure continued after PND 21.

In the study by Lee et al. (2004), the number of male pups with retained nipples was increased (4%), as compared to zero incidence in the control group. However, the difference was not statistically significant. On the other hand, the authors observed a significantly increased number of male pups with abnormal development of the mammary

gland, as indicated by the histopathological examination. Since the effect of DBP on the development of the nipple and mammary gland is one of the major characteristics of male reproductive effects of this chemical and is one of the major findings from this critical study, detailed discussions on these two endpoints, nipple retention and histopathological changes in the mammary gland, are presented below.

On nipple retention, the authors found that perinatal treatment with DBP caused increased incidence of male offspring with retained nipples or areola on PND 14. The data on this endpoint reported by the authors has two limitations. First, the authors used individual pups, rather than the litter, as the unit for statistical analysis. Secondly, only 8 animals from 6-8 dams (or litters) per group were used for this study, and thus the statistical power for detecting a significant difference between groups is limited. However, the increasing incidence of male pups with retained nipples with increased doses is consistent with that reported by Mylchreest et al. (2000). For example, using 19-20 dams delivering up to 141 male pups per group (see Table 3 above), Mylchreest et al. (2000) found 12.6% and 10.0% of pups with retained nipples in groups treated prenatally with 5 or 50 mg/kg-day of DBP, respectively. In the study by Lee et al. (2004), there were 4% and 13% of pups in the group treated with 1.5 or 14.4 mg/kg-day of DBP during the gestational period (20 and 200 ppm group, respectively). At high doses [500 mg/kg-day in the study by Mylchreest et al. (2000) and 712.3 mg/kg-day in the study by Lee et al. (2004)], nearly all the male pups (90% and 100%, respectively) had retained nipples on PND 14, as shown in both studies. Thus, the data reported by Lee et al. (2004) on nipple retention are supportive of previous findings by Mylchreest et al. (2000) that DBP causes alterations in nipple development in males at very low doses.

In addition to nipple retention, Lee et al. (2004) also reported dramatic histopathological changes in the mammary gland in male offspring in all DBP-treated groups at PND 21 and PNW 11. At PND 21, the day of treatment termination, the authors observed histopathological changes indicative of growth or development of mammary gland in males. As discussed above, it is known that inhibition of testosterone production or action by treatment with anti-androgenic compounds during the late gestational period in rats causes development of breast tissues (Topper and Freeman, 1980; Forest, 1983; Imperato-McGinley et al., 1986; Elger and Neumann, 1966; Liao and Dickson, 2002; Hovey et al., 2002). Thus, the histopathological changes in mammary gland in male pups at PND 21, as observed by Lee et al. (2004) are not only consistent with the data on nipple retention, they are supported by proposed modes of action for the male reproductive effects of DBP.

In contrast to the abnormal development of mammary gland on PND 21 in males pups treated with ≥ 20 ppm, mammary glands of almost all the male pups in all DBP-treated groups underwent degenerative changes at PNW 11 (7-8 weeks post-treatment). By PNW 20 (16-17 weeks post-treatment), the degeneration in mammary gland tissues was still obvious in most male pups in all treated groups. The DBP treatment in this study was terminated on PND 21. Up to 90% of oral doses of DBP administered to rats is excreted in urine within 24-48 hours (ECU, 2003). Once the treatment was stopped on PND 21, DBP and its metabolites were eliminated from the target tissues (e.g., testis) within a few days, thus allowing the target tissues (e.g., testis, mammary gland) to recover from the damage.

The degenerative changes in the mammary gland at PNW 11 and 20 in male pups treated perinatally with DBP are clearly treatment-related. Although the underlying mechanism remains unknown, it most likely reflects the recovery of mammary gland from the abnormal development of this organ during the perinatal period. The long-term biological significance of the abnormal changes in the mammary gland in male pups remains to be determined. However, they clearly indicate abnormal effects of DBP on this organ, most likely resulting from altered production or function of testosterone in the testis. Therefore, the biological significance of abnormal effects of DBP on the mammary gland in male pups is clear.

The male reproductive effects of DBP observed in rat pups by Lee et al. (2004) result from exposure of pregnant dams during gestation and nursing dams during lactation to DBP. The relative contributions of prenatal and postnatal exposures in the pups to the observed reproductive effects cannot be reliably estimated. Therefore, it is not possible to estimate the dose that, administered directly to young pups, would result in male reproductive effects. All the male reproductive effects in the pups are mediated through the dam (either in utero or via lactation), and it is therefore possible to estimate the dose to females that would result in male reproductive effects in offspring exposed in utero and through nursing.

In addition to the male reproductive effects, Lee et al. (2004) found increased incidence of hypoplasia of alveolar bud in the developing mammary gland in female pups on PND 21. Compared to the zero incidence in control group, 4, 3, 4, and 4 out of 8 female pups examined for each group (20-, 200-, 2,000-, and 10,000-ppm, respectively) had this abnormal histopathological change in the mammary gland. Even with the small group sizes, the increases were statistically significant at 20-, 2,000-, and 10,000-ppm group, but not in the 200-ppm group. The authors reported no histopathological changes in the mammary gland in female offspring on PNW 11 or 20, suggesting that DBP-caused alteration in developing mammary glands during postnatal development may have recovered by PNW 11. The mode of action for the abnormal effect of DBP on the development of mammary gland in female animals is unknown. However, it is known that exposure to DBP causes alterations in ovarian function (Gray et al., 2006b) or expression of estrogen-metabolizing enzymes in rats (e.g., Corton et al., 1997) and development of mammary gland in females is regulated by hormones including estrogens (e.g., Topper and Freeman, 1980; Imagawa et al., 1990; Neville et al., 2002). Thus, histopathological changes in the mammary gland in female offspring exposed perinatally to DBP at concentrations of $\geq 20$ ppm in diets indicate female reproductive effects of DBP at these doses.

To summarize, both male and female reproductive effects were observed in animals treated with 20 ppm of DBP in diet, the lowest dose used in the study by Lee et al. (2004). The male reproductive effects at this dose are manifested as increased number of male pups with reduced germ cell in the testis on PND 21, increased percentage of male pups with retained nipples on PND 14, increased number of male pups with dilatation of alveolar bud in the mammary gland on PND 21, increased numbers of male pups with degeneration of alveolar cells and alveolar atrophy in the mammary gland on PNW 11 and 20. All these

effects are consistent with proposed modes of actions for the male reproductive toxicity of DBP and thus are biologically significant.  For the female reproductive effects, perinatal exposure to DBP at 20 ppm in diet caused apparent histopathological changes in the mammary gland of the female pups that are consistent with other reported female reproductive toxicity of DBP in female rats.  Therefore, 20 ppm in diet, the lowest dose used in the study by Lee et al. (2004), is considered to be a LOEL.

The reproductive effects of DBP as observed in the study by Lee et al. (2004) were the consequence of maternal exposures.  This dose, equivalent to 1.5, 2.4, or 3.0 mg/kg-day of DBP to the dam for the periods of GD 15-20, PND 2-10, or PND 10-21, respectively, is considered as the LOEL for the reproductive effects observed in this study.  The estimated dose for the gestational period, 1.5 mg/kg-day, is the lowest LOEL for the developmental, male, and female reproductive toxicity of DBP among all the relevant studies that OEHHA has reviewed for establishing a MADL for DBP.  The study by Lee et al. (2004) is thus identified as "the most sensitive study deemed to be of sufficient quality" and will be used as the basis for MADL calculation.  The LOEL (20 ppm) for the reproductive effects observed in this study are the basis for establishing a Tolerable Daily Intake (TDI) for DBP by the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) of the European Food Safety Authority (EFSA, 2005).


## MADL CALCULATION

The NOEL is the highest dose level that results in no observable reproductive effect, expressed in milligrams of chemical per kilogram of bodyweight per day.  When a NOEL is not provided from the relevant studies, the LOEL is converted to a NOEL for purposes of assessment by dividing by 10 (Section 12803(a)(7)).  The NOEL is converted to a milligram per day dose level by multiplying the assumed human body weight by the NOEL (Section 12803(b)).  When the applicable reproductive effect is upon the male, the MADL is calculated based on a human body weight of 70 kg.  When the applicable reproductive effect is upon the female, a body weight of 58 kg is used. (Section 12803(b)).  In this case, the male reproductive effects manifested in the male pups, effects which are themselves specifically relevant to Proposition 65, resulted from exposure via pregnant and nursing dams.  Thus, the MADL is calculated based on a bodyweight of 58kg.

The following calculations were performed to derive the MADLs for DBP via the oral route of exposure, based on a LOEL of 1.5 mg/kg-day for the reproductive effects as observed in rats by Lee et al. (2004).

Conversion from a LOEL to NOEL:
  1.5 mg/kg-day $\div 10 = 0.15$ mg/kg-day

Calculation of the NOEL for a 58 kg woman:
  0.15 mg/kg-day $\times 58$ kg $= 8.7$ mg/day

The MADL is derived by dividing the NOEL by one thousand (Section 12801(b)(1)). Thus, the adjusted NOEL was divided by 1,000 to obtain the MADL.

$$MADL_{adult\ oral} = 8.7\ mg/day \div 1000 = \mathbf{8.7\ \mu g/day}$$

Studies that are currently available in the literature (NTP-CERHR, 2003a; ECU, 2003) have shown that DBP causes male reproductive effects in adult male rats at doses much higher than the LOEL (1.5 mg/kg-day) observed in the study of Lee et al. (2004). This MADL therefore applies to exposure of adults, including adult men, pregnant and nursing women, to DBP by the oral route. There is no inhalation or dermal study on the developmental and reproductive toxicity of DBP. However, subcutaneous injection of DBP causes the same male reproductive effects in rats as those caused by oral administration (Kim et al., 2004). The pattern and degree of DBP-induced changes in cytochrome P-450 mediated metabolism in rat liver and lung are similar following different routes of exposure, including oral administration, inhalation, and intraperitoneal injection (Walseth et al., 1982; Walseth and Nilsen, 1984; 1986). A recent study by Kremer et al. (2005) also found that vehicle (oral versus aqueous), dose levels, and routes of exposure (oral versus I.V) did not affect the pharmacokinetic characteristics of MBP, the active metabolite of DBP, in pregnant rats. These findings indicate that DBP or its hydrolyzed metabolites exert the same effects on target organs following different routes of exposure. Therefore, it is appropriate to apply MADLs based on an oral study to exposure via other routes including inhalation and dermal contact.

DBP is nearly completely absorbed following oral administration in rats (NTP-CERHR, 2003a). Thus, the MADL proposed above for the oral route of exposure can also be considered as an absorbed dose. For the purpose of Proposition 65, exposure by dermal contact or inhalation or via multiple routes that leads to absorbed doses equivalent to the MADL proposed above should be the maximum allowable dose level.

It has been shown that developing animals are sensitive to the reproductive effects of DBP (e.g., Higuchi et al., 2003; NTP-CERHR, 2003a). Similarly, postnatal only versus adult only studies on a related phthalate, DEHP, showed the young animal to be substantially more sensitive than the adult to the male reproductive effects of the chemical (OEHHA, 2006). However, there is no study that is of sufficient quality for OEHHA to establish a dose-response relationship between direct exposure to DBP and the reproductive effects in the young. Thus, at the present time, OEHHA is unable to establish age-specific MADLs for people under 18 years of age who are directly exposed postnatally to DBP from sources other than mother's milk (Section 12703(a)(8)). OEHHA will develop MADLs specific for humans under 18 years of age when there are sufficient data for OEHHA to do so.

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