National Toxicology Program
U.S. Department of Health and Human Services

Center For The Evaluation Of Risks To Human Reproduction

NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di(2-Ethylhexyl) Phthalate (DEHP)

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PREFACE

The National Toxicology Program (NTP) established the Center for the Evaluation of Risks to Human Reproduction (CERHR) in 1998. CERHR is a publicly accessible resource for information about adverse reproductive and/or developmental health effects associated with exposure to environmental and/or occupational chemicals. CERHR is located at the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health and Dr. Michael Shelby is the director.[1]

CERHR broadly solicits nominations of chemicals for evaluation from the public and private sectors. Chemicals are selected for evaluation based upon several factors including the following:

- potential for human exposure from use and occurrence in the environment
- extent of public concern
- production volume
- extent of database on reproductive and developmental toxicity studies

CERHR follows a formal process for review and evaluation of nominated chemicals that includes multiple opportunities for public comment. Briefly, CERHR convenes a scientific expert panel that meets in a public forum to review, discuss, and evaluate the scientific literature on the selected chemical. CERHR expert panels use explicit guidelines to evaluate the scientific literature and prepare the expert panel reports. Public comment is invited prior to and during the meeting. The expert panel produces a report on the chemical's reproductive and developmental toxicities and provides its opinion of the degree to which exposure to the chemical is hazardous to humans. The panel also identifies areas of uncertainty and where additional data are needed. Expert panel reports are made public and comments are solicited.

Next, CERHR prepares the NTP brief. The goal of the NTP brief is to provide the public, as well as government health, regulatory, and research agencies, with the NTP's interpretation of the potential for the chemical to adversely affect human reproductive health or children's development. CERHR then prepares the NTP-CERHR monograph, which includes the NTP brief on the chemical evaluated, the expert panel report, and public comments on the expert panel report. The NTP-CERHR monograph is made publicly available on the CERHR web site and in hard copy or CD-ROM from CERHR.

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[1] Information about the CERHR is available on its web site <*http://cerhr.niehs.nih.gov*> or by contacting:

Dr. Michael D. Shelby
Director, CERHR
NIEHS, P.O. Box 12233, MD EC-32,
Research Triangle Park, NC 27709
919-541-3455
919-316-4511 [fax]
shelby@niehs.nih.gov

Information about the NTP is available on the web at <*http://ntp.niehs.nih.gov*> or by contacting the NTP Liaison and Scientific Review Office at the NIEHS:

liaison@starbase.niehs.nih.gov
919-541-0530

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ABSTRACT

NTP-CERHR MONOGRAPH ON THE POTENTIAL HUMAN REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF DI(2-ETHYLHEXYL) PHTHALATE (DEHP)

The National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) conducted an updated evaluation of the potential for DEHP to cause adverse effects on reproduction and development in humans. The first CERHR expert panel evaluation of DEHP was completed in 2000 by the Phthalates Expert Panel. CERHR selected DEHP for an updated evaluation because of:

(1) widespread human exposure,
(2) public and government interest in adverse health effects,
(3) recently available human exposure studies, and
(4) the large number of relevant toxicity papers published since the earlier evaluation.

DEHP (CAS RN: 117-81-7) is a high production volume chemical used as a plasticizer of polyvinyl chloride in the manufacture of a wide variety of consumer goods, such as building products, car products, clothing, food packaging, children's products (but not in toys intended for mouthing), and in medical devices made of polyvinyl chloride. The public can be exposed to DEHP by ingesting food, drink or dust that has been in contact with DEHP-containing materials, by inhaling contaminated air or dust, or by undergoing a medical procedure that uses polyvinyl chloride medical tubing or storage bags. It is estimated that the general population of the United States is exposed to DEHP levels ranging from 1 to 30 µg/kg bw/day (micrograms per kilogram body weight per day).

The results of this DEHP update evaluation are published in an NTP-CERHR monograph that includes:

(1) the NTP Brief,
(2) the Expert Panel Update on the Reproductive and Developmental Toxicity of DEHP, and
(3) public comments on the expert panel report.

The NTP reached the following conclusions on the possible effects of exposure to DEHP on human development and reproduction. Note that the possible levels of concern, from lowest to highest, are negligible concern, minimal concern, some concern, concern, and serious concern.

There is serious concern that certain intensive medical treatments of male infants may result in DEHP exposure levels that adversely affect development of the male reproductive tract. DEHP exposure from medical procedures in infants was estimated to be as high as 6000 µg/kg bw/day.

There is concern for adverse effects on development of the reproductive tract in male offspring of pregnant and breastfeeding women undergoing certain medical procedures that may result in exposure to high levels of DEHP.

There is concern for effects of DEHP exposure on development of the male reproductive tract for infants less than one year old. Diet, mouthing of DEHP-containing objects, and certain medical

treatments may lead to DEHP exposures that are higher than those experienced by the general population.

There is some concern for effects of DEHP exposure on development of the reproductive tract of male children older than one year. As in infants, exposures of children to DEHP may be higher than in the general population.

There is some concern for adverse effects of DEHP exposure on development of the male reproductive tract in male offspring of pregnant women not medically exposed to DEHP. Although DEHP exposures are assumed to be the same as for the general population, the developing male reproductive tract is sensitive to the adverse effects of DEHP.

There is minimal concern for reproductive toxicity in adults exposed to DEHP at 1–30 µg/kg bw/day. This level of concern is not altered for adults medically exposed to DEHP.

NTP will transmit the NTP-CERHR Monograph on DEHP to federal and state agencies, interested parties, and the public and it will be available in electronic PDF format on the CERHR web site <*http://cerhr.niehs.nih.gov*> and in printed text or CD-ROM from the CERHR:

Dr. Michael D. Shelby
Director, CERHR
NIEHS, P.O. Box 12233, MD EC-32
Research Triangle Park, NC 27709
919-541-3455
919-316-4511 [fax]
shelby@niehs.nih.gov

INTRODUCTION

DEHP (di(2-ethylhexyl) phthalate, CAS RN 117-81-7) is a high production volume chemical used as a plasticizer of polyvinyl chloride in the manufacturer of a wide variety of consumer products such as building products, car products, clothing, food packaging, and children's products (but not in toys intended for mouthing), and in medical devices made of polyvinyl chloride.

In 1999–2000, the CERHR Phthalates Expert Panel evaluated DEHP and six other phthalates for reproductive and developmental toxicities. Between the release of the first CERHR Expert Panel Report on DEHP in 2000 and the convening of the present panel in 2005, approximately 70 papers relevant to human exposure and reproductive and/or developmental toxicity of DEHP were published. Because most people in the United States are exposed to DEHP and it is known to cause adverse effects on reproduction and development in laboratory animals, there is considerable interest in its possible health effects on people. For these reasons, the CERHR convened an expert panel to conduct an updated evaluation of the potential reproductive and developmental toxicities of DEHP.

This monograph includes the NTP Brief on DEHP, a list of the expert panel members (Appendix I), the Expert Panel Report on DEHP (Appendix II), and all public comments received on the expert panel report (Appendix III). The NTP-CERHR monograph is intended to serve as a single, collective source of information on the potential for DEHP to adversely affect human reproduction or development.

The NTP Brief on DEHP presents the NTP's opinion on the potential for exposure to DEHP to cause adverse reproductive or developmental effects in people. The NTP brief is intended to provide clear, balanced, scientifically sound information. It is based on information about DEHP provided in the expert panel report, public comments, comments from peer reviewers [2] and additional scientific information available since the expert panel meeting.

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[2] Peer review of this brief was conducted by letter review. Reviewers were:

- Prof. Jürgen Angerer
  Institut für Arbeits-, Sozial- und Umweltmedizin
- Dr. Michael Brabec
  Eastern Michigan University
- Dr. Lynn Goldman
  Johns Hopkins University
- Dr. Mary Vore
  University of Kentucky.

DEVELOPMENTAL TOXICITY AND REPRODUCTIVE TOXICITY

The CERHR evaluation process addresses effects on both development and reproduction. While there are biological and practical reasons for considering developmental toxicity and reproductive toxicity as two separate issues, a clear separation of the two is not always possible. It is important to keep in mind that life in mammals, including humans, is a cycle. In brief, the cycle includes the production of sperm and eggs, fertilization, prenatal development of the offspring, birth, postnatal development, sexual maturity, and, again, production of sperm and eggs.

Toxic effects are often studied in a "life stage specific" manner. Thus, developmental toxicity is typically studied by exposing pregnant laboratory animals to the substance of interest and looking for adverse effects on development of the resulting offspring. Developmental toxicity can be detected as death, structural malformations, or reduced weights of the fetuses just prior to birth or abnormal structural or functional development after birth. Reproductive toxicity is often studied by exposing sexually mature animals to the substance of interest and effects are detected as impaired capacity to reproduce.

Over the years, toxicologists realized that exposure during one part of the life cycle may lead to adverse effects that are apparent only at a different stage of the life cycle. For example, exposure of a sexually mature animal to a substance capable of inducing genetic damage in eggs or sperm might have no apparent effect on the exposed individual. However, if a genetically damaged egg or sperm from that individual is involved in fertilization, the genetic damage might lead to death of offspring before they are born or a genetic disorder in the surviving offspring. In this example, chemical-induced damage in the germ cells is observed as a developmental disorder in the next generation.

In contrast, development of both the male and female reproductive systems begins well before birth and continues until sexual maturity is attained. Thus, the exposure of sexually immature animals, either before or following birth, to agents that adversely affect development of the reproductive system can result in structural or functional reproductive disorders. These effects may become apparent only when reproductive studies are conducted after the exposed individual reaches sexual maturity.

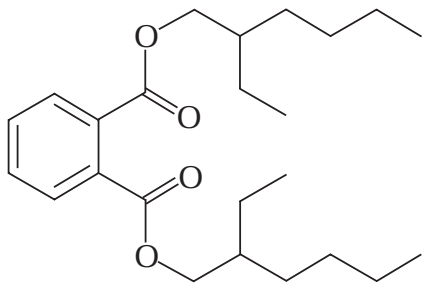
Thus, in the case of genetic damage induced in eggs or sperm, damage to reproductive cells gives rise to developmental disorders. Conversely, in the case of adverse effects on development of the reproductive tract, developmental toxicity results in reproductive disorders. In both of these examples it is difficult to make a clear distinction between developmental and reproductive toxicity. This issue is important in the present evaluation because laboratory animal studies provide evidence that DEHP exposure before or soon after birth can cause developmental toxicity affecting the reproductive system in later stages of the life cycle.

NTP BRIEF ON DEHP

What is DEHP?

DEHP is an oily liquid with the chemical formula $C_{24}H_{38}O_4$ and the structure is shown in **Figure 1**.

Figure 1.
Chemical structure of DEHP



It is one of a group of industrially important chemicals known as phthalates. Phthalates are used primarily as plasticizers to add flexibility to plastics. DEHP is used in a wide variety of products including flooring, wallpaper, auto upholstery, raincoats, toys, and food packaging. It is not used in toys intended for mouthing such as nipples or teething rings. DEHP is currently the only phthalate plasticizer used in polyvinyl chloride (PVC) medical devices such as blood bags and tubing.

DEHP is produced by reacting 2-ethylhexanol with phthalic anhydride. In 2002 the Agency for Toxic Substances and Disease Registry (ATSDR) estimated that 241 million pounds of dioctyl phthalates (including DEHP) were produced in the United States in 1999.

Are People Exposed to DEHP?[3]

Yes. There are several ways that people may be exposed to DEHP at home, at work, or through medical procedures. Human exposure to DEHP can occur during the manufacture of DEHP, the manufacture of DEHP-containing products, the use of such products, and through the presence of DEHP in the environment.

Environmental exposures can occur through air, water, or food. The primary source of DEHP exposure for most people is through food. DEHP migrates into foods, particularly fatty foods, from DEHP-containing materials that are used to process and package food. Indoor air and dust are other common sources of exposure. The 1999–2000 National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention reported that 78% of the 2541 urine samples tested contained the DEHP metabolite mono (2-ethylhexyl) phthalate (MEHP). This figure may be an underestimate of the portion of people exposed to DEHP because two other urinary metabolites of DEHP not screened for in the 1999–2000 study were subsequently reported to occur in higher concentrations in human urine than MEHP (Silva et al., 2004).

The expert panel estimated that exposure to DEHP in the United States general population is approximately 1–30 μg/kg bw/day (micrograms per kilogram body weight per day). This estimate reflects a total daily exposure of approximately 70–2100 μg DEHP for a 70-kilogram (155 pound) person.

DEHP is used in the manufacture of a number of medical devices including blood bags and bags and tubing used for intravenous administration of fluids, drugs, and nutrients. DEHP can leach from the plastic bags or tubing into the fluids being administered. Opportunities for high DEHP exposures occur during medical procedures such as hemodialysis, transfusion

[3] Answers to this and subsequent questions may be: *Yes, Probably, Possibly, Probably Not, No or Unknown*

of blood or blood products, extracorporeal membrane oxygenation, heart bypass surgery, and administration of intravenous fluids. The highest human exposures to DEHP can occur in newborns and infants undergoing extensive medical procedures such as transfusions, extracorporeal membrane oxygenation, and total parenteral nutrition. The expert panel estimated that newborns undergoing such medical procedures might be exposed to DEHP levels ranging from 130 to 6000 µg/kg bw/day. There is sufficient evidence to conclude that all age groups undergoing certain medical procedures are exposed to levels of DEHP that are far higher than those encountered by the general population.

Can DEHP Affect Human Development or Reproduction?

*Probably***.** Although there is no direct evidence that exposure of people to DEHP adversely affects reproduction or development, studies with laboratory rodents clearly show that exposure to DEHP can cause adverse effects on development and reproduction (**See Figures 2a & 2b**). Based on recent data on the extent to which humans absorb, metabolize and excrete DEHP, the NTP believes it is reasonable and prudent to

Figure 2a. *The weight of evidence that DEHP causes adverse developmental or reproductive effects in humans*

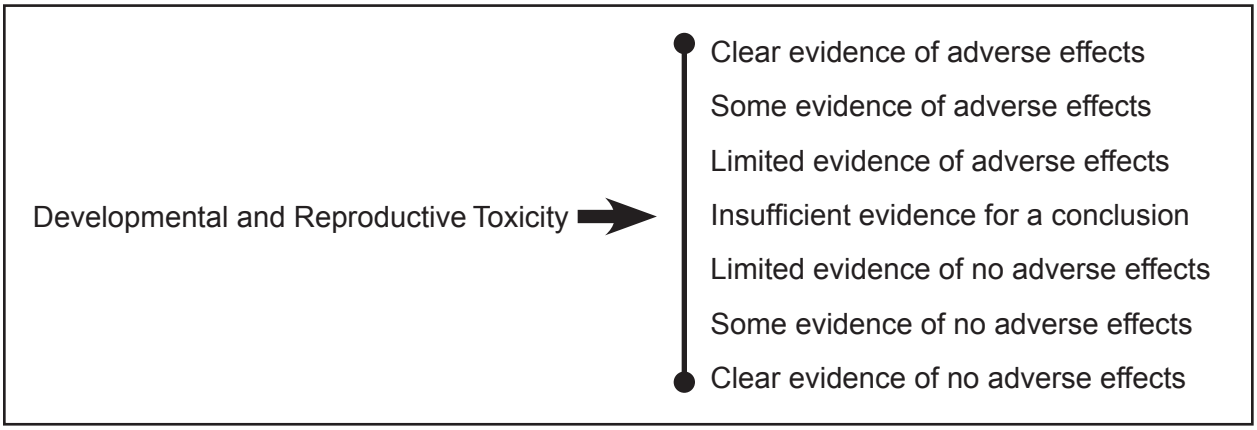
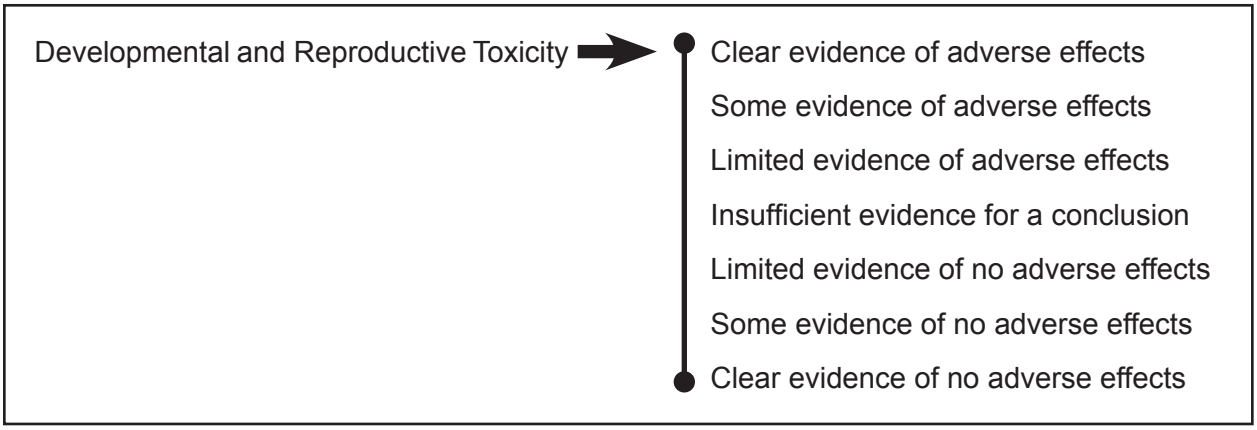


Figure 2b. *The weight of evidence that DEHP causes adverse developmental or reproductive effects in laboratory animals*



conclude that the results reported in laboratory animals indicate a potential for similar adverse effects in human populations.

Scientific decisions concerning health risks are generally based on what is known as the "weight-of-evidence." In this case, recognizing the lack of sufficient data on the effects of DEHP in humans and the clear evidence of effects in laboratory animals, the NTP judges the scientific evidence sufficient to conclude that DEHP may adversely affect human reproduction or development if exposures are sufficiently high (**See Figure 3**).

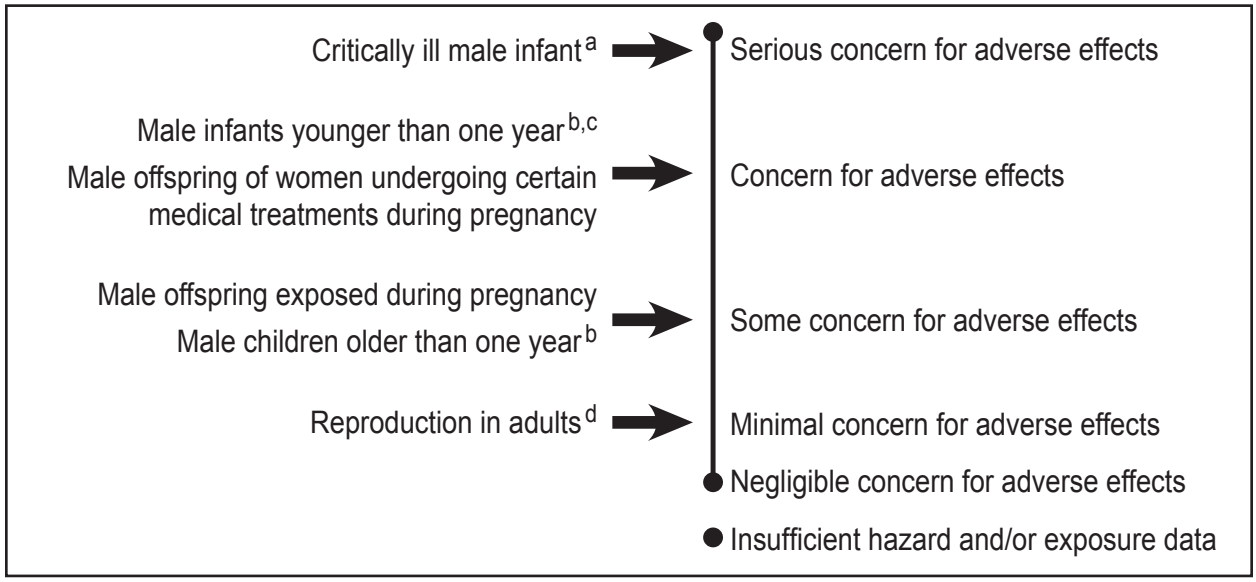
Supporting Evidence

The CERHR Expert Panel Update Report on DEHP (Appendix II) provides details and citations regarding studies on the possible reproductive and developmental toxicity of DEHP.

The expert panel evaluated several human studies but there was insufficient evidence to conclude that DEHP causes or does not cause developmental toxicity when exposure occurs prenatally or during childhood. There was also insufficient evidence to conclude that DEHP causes or does not cause reproductive toxicity in studies of DEHP-exposed adults. Some of the methodological factors that limited the usefulness of these human studies were small sample size and, in most cases, uncertainties in the exposure measurements.

As presented in both the earlier and present DEHP expert panel reports, a large body of data addresses the adverse developmental and reproductive effects of DEHP in laboratory animals. Results from developmental toxicity studies in mice and rats provide a consistent pattern of adverse effects following DEHP exposure. Oral exposure to approximately 100–200 mg/kg bw/day of DEHP during gestation typically results in skeletal and cardiovascular malformations, neural tube defects, developmental delays,

Figure 3. *NTP conclusions regarding the possibilities that human development or reproduction might be adversely affected by exposure to DEHP*



[a] Based on estimated DEHP exposures as high as 6000 µg/kg bw/day

[b] Based on exposures at the high end of an estimated exposure range of 1–30 µg/kg bw/day

[c] Includes exposure through breast-feeding in infants younger than 1 year

[d] Based on estimated exposures of 1–30 µg/kg bw/day

and intrauterine death of the offspring. Studies such as these, in which pups are examined in the prenatal or immediate postnatal period, provide only limited information on the effects of DEHP. This is because adverse effects on the reproductive tract may become apparent only at later stages of development. DEHP exposure has been shown to adversely affect reproduction in several species including mice, rats, guinea pigs, and ferrets. While effects have been reported in both males and females, the *in utero* and early postnatal development of the reproductive system of males appears to be more sensitive to the adverse effects of DEHP.

Exposure of rats to DEHP-containing feed during gestation and/or early postnatal life at 14–23 mg/kg bw/day or greater results in adverse effects on the developing male reproductive tract such as abnormally small or absent reproductive organs. Other studies at higher doses show similar adverse effects on the developing male reproductive tract. Adverse effects on the developing female reproductive tract occur in rats exposed to 1088 mg/kg bw/day DEHP in the feed.

One new reproductive toxicity study in nonhuman primates was available to the DEHP Update Panel. Marmosets were exposed orally to DEHP at doses of 100, 500, or 2,500 mg/kg bw/day from the juvenile stage through young adulthood. Following 65 weeks of exposure to DEHP, no adverse changes in the male reproductive tract were observed. Marmosets and humans differ in intestinal lipase activity, absorption and excretion of DEHP, and testosterone levels during development of the male reproductive tract. These differences lead to uncertainty as to the utility of the marmoset as a model for studying the possible effects of DEHP on development of the human male reproductive tract. In addition, this marmoset study encountered problems with the health and growth of the study animals and did not investigate the most sensitive stage in the development of the male reproductive tract, i.e., the perinatal period.

Are Current Exposures to DEHP High Enough to Cause Concern?

Yes. Potentially high exposures of fetuses and infants to DEHP may lead to adverse effects on the developing male reproductive tract. High DEHP exposures of fetuses and infants can occur when pregnant and breast-feeding women undergo certain medical procedures involving DEHP-containing polyvinyl chloride medical devices. Infants may also be exposed to high levels of DEHP through medical procedures, diet, and/or mouthing of DEHP-containing objects. In a recently published paper, Wormuth et al. (2006) predicted that infants and toddlers are exposed to higher levels of DEHP than other subgroups in the general population. The authors concluded that a major portion of this exposure, perhaps as much as 35%, results from the ingestion of DEHP-contaminated dust.

Based on the estimated high levels of exposure that can occur during intensive medical treatments of ill infants and on the apparent sensitivity of the developing male reproductive tract to DEHP, there is particular concern for this subpopulation. The adverse effects on development of the rodent male reproductive tract are attributed to abnormally low levels of testosterone induced by DEHP. Concerns for such effects do not extend to female fetuses and infants. The general adult population presently appears to be exposed to DEHP at levels that are not expected to cause adverse effects to the reproductive system. However, more data are needed to better understand human DEHP exposure levels and how these exposures vary across the population. The NTP offers the following conclusions regarding the potential for DEHP to adversely affect human reproduction and development of children.

The NTP concurs with the CERHR DEHP Update Expert Panel that there is serious concern that certain intensive medical treatments of male infants may result in DEHP levels that adversely affect development of the reproductive tract.

This conclusion is based on the apparent sensitivity of the developing male reproductive tract and the estimated high levels of DEHP exposure that can occur during intensive medical treatments of ill infants. Such exposures were estimated to be as much as 100 to 1000 times higher than exposures in the general population. The NTP also acknowledges, as did the expert panel, that the health benefits of these medical procedures may outweigh any risks. It is noteworthy that both the U.S. Food and Drug Administration (2001) and Health Canada (2002) used the CERHR Phthalates Expert Panel Report on DEHP in conducting their own assessments of the safety of DEHP-containing medical devices. Both agencies point out that infants and children undergoing certain medical procedures may be at increased risk for adverse effects of exposure to DEHP.

The NTP concurs with the CERHR DEHP Update Expert Panel that there is concern for adverse effects on development of the reproductive tract in male offspring of pregnant and breast-feeding women undergoing certain medical procedures that may result in exposure to high levels of DEHP.

DEHP exposure levels in adults undergoing certain medical procedures can be as much as 1000-fold greater than exposure of the general population. Because DEHP metabolites can cross the placenta and enter breast milk, fetuses and nursing infants may experience elevated DEHP exposures if their mothers undergo such medical procedures.

The NTP concurs with the CERHR DEHP Update Expert Panel that there is concern for effects of DEHP exposure on development of the reproductive tract for infants less than one year old.

This level of concern is based on the uncertainty regarding DEHP exposure levels in this population, the greater activity of enzymes (lipases) that convert DEHP to its toxic form, and the possibility that in infants the developing male reproductive tract will be more sensitive to the adverse effects of DEHP than in children older than one year. Although there is uncertainty regarding levels of DEHP exposure in this age group, there is the potential for DEHP exposures to exceed those of the general population. Such elevated exposures may occur through medical procedures, diet (including breast-feeding), and/or mouthing of DEHP-containing objects.

The NTP concurs with the CERHR DEHP Update Expert Panel that there is some concern for the effects of DEHP exposure on development of the reproductive tract in male children older than one year.

This level of concern is based on the apparent sensitivity of the developing male reproductive tract to the adverse effects of DEHP and the potential for DEHP exposures in children to exceed those of the general population. Recent studies provide greater confidence in estimates of DEHP exposure levels in children, exposures that may occur through medical procedures, diet, and/or mouthing of DEHP-containing objects.

The NTP concurs with the CERHR DEHP Update Expert Panel that there is some concern for adverse effects of DEHP exposure on development of the reproductive tract in male offspring of pregnant women not medically exposed to DEHP.

"Some concern" is a lower level of concern than that expressed for pregnant and breast-feeding

women by the previous CERHR Phthalates Expert Panel[4]. This lower level of concern is based on a greater confidence in the estimated DEHP exposure levels in women of childbearing age, a greater confidence in the DEHP exposure levels at which adverse effects are observed in laboratory rodents, and evidence that humans have lower levels of enzymes (lipases) that activate DEHP than rodents. Further, exposure estimates for women of childbearing age, i.e., the age group that would be pregnant or breastfeeding, not medically exposed to DEHP are the same as for the general population (1–30 µg/kg bw/day). While studies of DEHP effects in humans and non-human primates are not sufficient to draw conclusions, data from recent studies in rodents provide evidence that no adverse effects are observed in development of the male reproductive tract following DEHP exposure of the pregnant dams to less than 10 mg/kg bw/day.

The NTP concurs with the CERHR DEHP Update Expert Panel that there is minimal concern for reproductive toxicity in adults exposed at 1–30 µg/kg bw/day. This level of concern is not altered for adults medically exposed to DEHP.

This conclusion for the general population is based on an estimated range of DEHP exposures of 1–30 µg/kg bw/day. Based on data from rodent studies, the adult reproductive tract is expected to be much less sensitive to the adverse effects of DEHP exposure than the developing reproductive tract. Finally, adult rodents have higher intestinal lipase activity than adult humans and are expected to produce higher levels of MEHP, a biologically active metabolite. Thus, adult humans are expected to be less sensitive than adult rodents to the reproductive toxicity effects of a given dose of DEHP. This expert panel did not specifically address occupational exposures because no significant new information on this issue had become available since the earlier expert panel report was released.

These conclusions are based on the information available at the time this brief was prepared. As new information on toxicity and exposure accumulate, it may form the basis for either lowering or raising the levels of concern expressed in the conclusions.

[4] Following the October 2005 meeting of the DEHP update expert panel, 3 members of the panel reconsidered this conclusion and expressed the opinion that the level of concern should not have been lowered.

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Wormuth M, Scheringer M, Vollenweider M, and Hungerbühler K. (2006) What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? *Risk Analysis* 26:803 – 824.

APPENDIX I.
NTP-CERHR DEHP UPDATE EXPERT PANEL

An 11-member panel of scientists covering disciplines such as toxicology, epidemiology, and medicine was recommended by the CERHR Core Committee and approved by the Associate Director of the National Toxicology Program. Prior to the expert panel meeting, the panelists critically reviewed articles from the scientific literature, as well as a variety of other relevant documents. Based on this material, they identified key studies and issues for discussion. At a public meeting held October 10–12, 2005, the expert panel discussed these studies, the sufficiency of available data, and identified data needed to improve future assessments. The expert panel reached conclusions on whether estimated exposures may result in adverse effects on human reproduction or development. Panel assessments were based on the scientific evidence available at the time of the final meeting. The expert panel reports were made available for public comment on November 21, 2005 and the deadline for public comments was February 3, 2006 (*Federal Register* 70:220 [16 Nov. 2005] p69567; and *Federal Register* 70:239 [14 Dec. 2005] p74026). The Expert Panel Update Report on DEHP is provided in Appendix II and the public comments received on that report are in Appendix III. Input from the public and interested groups throughout the panel's deliberations was invaluable in helping to assure the completeness and accuracy of the reports. The Expert Panel Update Report on DEHP is available on the CERHR website <*http://cerhr.niehs.nih.gov*>.

APPENDIX I. NTP-CERHR DEHP UPDATE EXPERT PANEL

Robert Kavlock, Ph.D. (Chair)
U.S. Environmental Protection Agency
Research Triangle Park, NC

Dana Boyd Barr, Ph.D.
Centers for Disease Control & Prevention
Atlanta, GA

Kim Boekelheide, M.D., Ph.D.
Brown University
Providence, RI

William Breslin, Ph.D.
Eli Lilly and Company
Greenfield, IN

Patrick N. Breysse, Ph.D.
The Johns Hopkins University
Baltimore, MD

Robert Chapin, Ph.D.
Pfizer Global Research & Development
Groton, CT

Kevin Gaido, Ph.D.
CIIT Centers for Health Research
Research Triangle Park, NC

Ernest Hodgson, Ph.D.
North Carolina State University
Raleigh, NC

Michele Marcus, Ph.D.
Emory University
Atlanta, GA

Katherine M. Shea, M.D., M.P.H.
Consultant
Chapel Hill, NC

Paige L. Williams, Ph.D.
Harvard School of Public Health
Boston, MA

National Toxicology Program
U.S. Department of Health and Human Services

Center For The Evaluation Of Risks To Human Reproduction

Appendix II

NTP-CERHR EXPERT PANEL UPDATE ON THE REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF DI(2-ETHYLHEXYL) PHTHALATE

October 2005 *NTP-CERHR-DEHP-05*

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ABBREVIATIONS

ADP.....adenosine diphosphate
AGI.....anogenital index
Ah.....aryl hydrocarbon
ANCOVA .....analysis of co-variance
ANOVA.....analysis of variance
AUC .....area under the concentration–time curve
BMD_{10} .....benchmark dose, 10% effect level
$BMD_{1\ SD}$ .....benchmark dose, 1 control standard deviation
BMDL.....benchmark dose 95th percentile lower confidence limit
BrdU.....bromodeoxyuridine
bw.....body weight
CAS RN .....Chemical Abstracts Service Registry Number
cDNA .....complementary deoxyribonucleic acid
CERHR .....Center for the Evaluation of Risks to Human Reproduction
CI.....confidence interval
C_{max}.....maximum concentration
CYP.....cytochrome P450
DEHA .....diethylhexyl adipate
DEHP .....di(2-ethylhexyl) phthalate
DMSO.....dimethylsulfoxide
DNA.....deoxyribonucleic acid
ECMO.....extracorporeal membrane oxygenation
EPA .....Environmental Protection Agency
F_0.....parental generation
F_1.....first filial generation
F_2.....second filial generation
F_3.....third filial generation
fasL .....fas ligand
FDA.....Food and Drug Administration
g.....gram(s)
FSH .....follicle stimulating hormone
GC.....gas chromatography
GD.....gestation day(s)
GLP.....Good Laboratory Practice
hCG.....human chorionic gonadotropin
HPLC .....high performance liquid chromatography
H-FABP.....heart-fatty acid binding protein
IARC.....International Agency for Research on Cancer
im .....intramuscular(ly)
ip .....intraperitoneal(ly)
IU .....international unit
iv.....intravenous(ly)
kDa.....kilodalton

kg.....kilogram(s)
L .....liter(s)
LH .....luteinizing hormone
LOAEL .....low observed adverse effect level
LOD .....limit of detection
LOQ .....limit of quantification
m .....meter(s)
m^3 .....cubic meter(s)
M.....molar
MEHP .....mono(2-ethylhexyl) phthalate
mM.....millimolar
mmol .....millimole(s)
mol .....mole(s)
MRL.....minimal risk level
mRNA .....messenger ribonucleic acid
MS.....mass spectrometry
MT.....metallothionein
MTT .....3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH .....nicotine-adenine dinucleotide, reduced
ng.....nanogram(s)
NHANES .....National Health and Nutrition Examination Survey
NICU.....neonatal intensive care unit
NIEHS.....National Institute of Environmental Health Sciences
NIH .....National Institutes of Health
nmol .....nanomole(s)
NOAEL .....no observed adverse effect level
NTP.....National Toxicology Program
OR.....odds ratio
PARP.....poly(ADP-ribose) polymerase
PCB.....polychlorinated biphenyl
PCNA.....proliferating cell nuclear antigen
PMSG.....pregnant mare serum gonadotropin
PND.....postnatal day(s)
PPAR.....peroxisome proliferator-activated receptor
ppb.....parts per billion
ppm .....parts per million
PVC.....polyvinyl chloride
RAR .....retinoic acid receptor
RIA.....radioimmunoassay
RNA .....ribonucleic acid
RT-PCR .....reverse transcriptase-polymerase chain reaction
RXR .....retinoic acid X receptor
sc .....subcutaneous(ly)
SD .....standard deviation
SDS-PAGE.....sodium dodecyl sulfate-polyacrylamide gel electrophoresis

$t_{1/2}$.....half-life of elimination
T_{max}.....time to maximum concentration
TI.....tolerable intake
TNF.....tumor necrosis factor
TPN.....total parenteral nutrition
TUNEL .....terminal deoxynucleotidal transferase-mediated dUTP nick-end labeling
UDP.....uridine diphosphate
US .....United States
ZnT-1 .....zinc transporter-1
µg.....microgram(s)
µL .....microliter(s)
µm .....micrometer(s)
µM.....micromolar
µmol .....micromole(s)
2-EH.....2-ethylhexanol
2-EHA .....2-ethylhexanoic acid
2-cx-MMHP.....mono(2-carboxymethyl)hexyl phthalate
5-cx-MEPP.....mono(2-ethyl)-5-carboxypentyl phthalate
5-OH-MEHP.....mono(2-ethyl-5-hydroxyhexyl) phthalate
5-oxo-MEHP.....mono(2-ethyl-5-oxy-hexyl) phthalate
8-Br-cAMP.....8-bromo-cyclic adenosine monophosphate

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PREFACE

The National Toxicology Program (NTP) and the National Institute of Environmental Health Sciences (NIEHS) established the NTP Center for the Evaluation of Risks to Human Reproduction (CERHR) in June 1998. The purpose of the Center is to provide timely, unbiased, scientifically sound evaluations of human and experimental evidence for adverse effects on reproduction, to include development, caused by agents to which humans may be exposed.

Di-(2-ethylhexyl) phthalate (DEHP) was originally evaluated by the CERHR Phthalates Expert Panel in 1999–2000 and an expert panel report was published in 2001. DEHP was selected for re-evaluation by CERHR because of widespread human exposure and public and government interest in potential adverse health effects. Further, over 150 relevant papers on DEHP had been published since the first evaluation. This is the first time a CERHR expert panel was convened to update an evaluation conducted by a previous CERHR expert panel.

DEHP (CAS RN: 117-81-7) is a high production volume chemical used as a plasticizer in polyvinyl chloride plastics. It is found in a wide variety of consumer products, such as building products, car products, clothing, food packaging, children's products (but not in toys intended for mouthing), and in some medical devices made of polyvinyl chloride.

To obtain information about DEHP for this CERHR evaluation, the PubMed (Medline) and Toxnet databases were searched from January 1, 2000 through September 30, 2005, with CAS RNs for DEHP (117-81-7), mono-(2-ethylhexyl) phthalate (MEHP) (4376-20-9), and relevant keywords. References were also identified from databases such as REPROTOX®, HSDB, IRIS, and DART and from the bibliographies of literature being reviewed.

This evaluation results from the effort of an eleven-member panel of government and non-government scientists that culminated in a public expert panel meeting held October 10–12, 2005. This report is a product of the Expert Panel and is intended to (1) interpret the strength of scientific evidence that DEHP is a reproductive or developmental toxicant based on data from in vitro, animal, or human studies, (2) assess the extent of human exposures to include the general public, occupational groups, and other sub-populations, (3) provide objective and scientifically thorough assessments of the scientific evidence that adverse reproductive/developmental health effects may be associated with such exposures, and (4) identify knowledge gaps to help establish research and testing priorities to reduce uncertainties and increase confidence in future assessments of risk. This report has been reviewed by CERHR staff scientists, and by members of the DEHP Expert Panel. Copies have been provided to the CERHR Core Committee, which is made up of representatives of NTP-participating agencies. **The findings and conclusions of this report are those of the expert panel and should not be construed to represent the views of the National Toxicology Program.**

This Expert Panel Report will be a central part of the subsequent NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Di-(2-ethylhexyl) Phthalate. This monograph will include the NTP-CERHR Brief, the Expert Panel Report, and all public comments on the Expert Panel Report. The NTP-CERHR Monograph will be made publicly available and transmitted to appropriate health and regulatory agencies.

The NTP-CERHR is headquartered at NIEHS, Research Triangle Park, NC and is staffed and administered by scientists and support personnel at NIEHS and at Sciences International, Inc., Alexandria, Virginia.

Reports can be obtained from the website <*http://cerhr.niehs.nih.gov/*> or from:

Michael D. Shelby, Ph.D.
NIEHS EC-32
PO Box 12233
Research Triangle Park, NC 27709
919-541-3455
shelby@niehs.nih.gov

A REPORT OF THE CERHR DEHP UPDATE EXPERT PANEL:

Robert Kavlock, Ph.D., Chair	US Environmental Protection Agency Research Triangle Park NC
Dana Barr, Ph.D.	Centers for Disease Control and Prevention Atlanta GA
Kim Boekelheide, M.D., Ph.D.	Brown University, Providence RI
William Breslin, Ph.D.	Eli Lilly & Company, Greenfield IN
*Patrick Breysse, Ph.D.	The Johns Hopkins University, Baltimore MD
Robert Chapin, Ph.D.	Pfizer, Inc., Groton CT
Kevin Gaido, Ph.D.	CIIT Centers for Health Research Research Triangle Park NC
Ernest Hodgson, Ph.D.	North Carolina State University, Raleigh NC
*Michele Marcus, Ph.D.	Emory University, Atlanta GA
*Katherine Shea, M.D., M.P.H.	University of North Carolina School of Public Health, Chapel Hill NC
Paige Williams, Ph.D.	Harvard School of Public Health, Boston MA

With the Support of CERHR Staff:

NTP/NIEHS

Michael Shelby, Ph.D.	Director, CERHR
Paul M.D. Foster, Ph.D	Deputy Director, CERHR
Christopher Portier, Ph.D.	Associate Director, National Toxicology Program
Kristina Thayer, Ph.D.	NTP Liaison and Scientific Review Office

Sciences International, Inc.

Anthony Scialli, M.D.	Principal Scientist
Annette Iannucci, M.S.	Toxicologist
Gloria Jahnke, D.V.M.	Toxicologist
Vera Jurgenson, M.S.	Research Assistant

Note to Reader:

This report is prepared according to the Guidelines for CERHR Panel Members established by NTP/NIEHS. The guidelines are available from the CERHR web site <*http://cerhr.niehs.nih.gov/*>. The format for Expert Panel Reports includes synopses of studies reviewed, followed by an evaluation of the Strengths/Weaknesses and Utility (Adequacy) of the study for a CERHR evaluation. Statements and conclusions made under Strengths/Weaknesses and Utility evaluations are those of the Expert Panel and are prepared according to the NTP/NIEHS guidelines. In addition, the Panel often makes comments or notes limitations in the synopses of the study. **Bold, square brackets** are used to enclose such statements. As discussed in the guidelines, **[square brackets]** are used to enclose key items of information not provided in a publication, limitations noted in the study, conclusions that differ from authors, and conversions or analyses of data conducted by the panel.

The findings and conclusions of this report are those of the expert panel and should not be construed to represent the views of the National Toxicology Program.

1.0 USE AND HUMAN EXPOSURE

The first section of CERHR Expert Panel Reports is devoted to chemistry, use, and human exposure. The following conclusions regarding di(2-ethylhexyl) phthalate (DEHP) exposure were expressed by the Expert Panel in the CERHR Expert Panel Report released in 2000:

> While the Panel recognizes the variability and uncertainties in exposure estimates, it appears that for the general adult human population, ambient exposures may be on the order of 3−30 μg/kg bw/day. Non-dietary mouthing behaviors in infants and toddlers may result in exposures that are several-fold higher. The 3−30 μg/kg [bw]/d range may be increased by 2−3 orders of magnitude for infants undergoing intensive therapeutic interventions.

Since the initial CERHR Expert Panel Report on DEHP, no additional information on chemistry has been added.

Phthalates are used in a variety of products, including lubricants, perfumes, hairsprays and cosmetics, construction materials, wood finishers, adhesives, floorings, and paints. DEHP is typically added to building materials and medical devices made from polyvinyl chloride (PVC) to increase flexibility. When DEHP is used as a plasticizer in medical devices such as storage containers, bags, and tubing, it can leach from the device into infusate (e.g., pharmaceuticals, blood, blood products, parenteral nutrition solutions, air in ventilation tubing). A review by the European Commission *(1)* noted the use of DEHP in orthodontic retainers that are typically used by 7 – 14-year-old children. It is not known if DEHP is used in orthodontic devices in the US. The Food and Drug Administration (FDA) referenced a study stating that DEHP has been detected as a leachate from dental composites, but that plasticizers other than DEHP are most often used for such applications *(2)*.

DEHP production volume was referenced in the initial CERHR Expert Panel Report on DEHP as approaching 260 million pounds. No recent information on production volume was located.

This section reviews the literature relating to human exposure studies published after the previous CERHR Expert Panel Report on DEHP (2000) was completed. The studies reviewed in this section included estimated or calculated exposures to DEHP and its metabolites from medical devices, residential exposures, dietary exposures, and environmental exposures. Several studies reviewed the effects of temperature, contact time, and solution type in medical devices such as container bags or tubing on exposure to DEHP and various metabolites. The specific chemicals that have been measured for the estimation of DEHP exposures are listed in Table 1 and shown in Figure 1.

Table 1. Markers of DEHP Exposure Measured in a Variety of Matrices to Assess Exposure to DEHP.

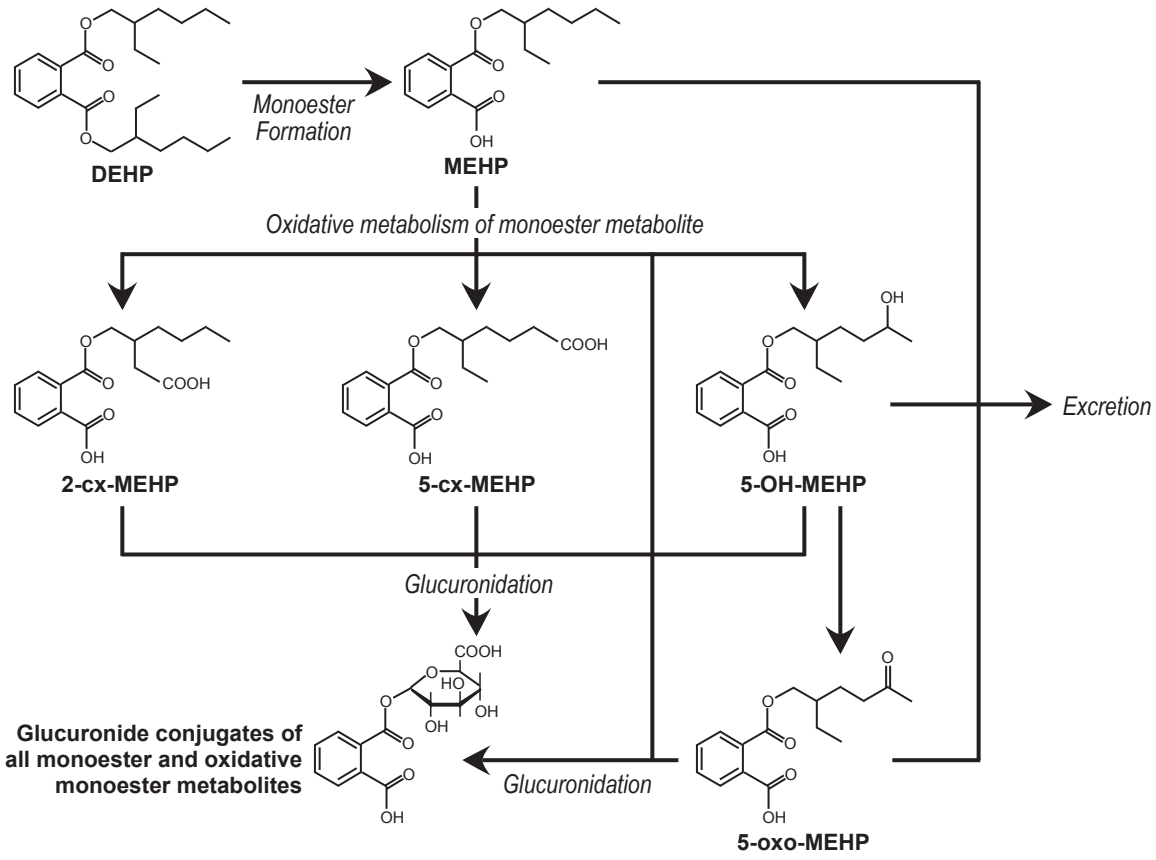
Marker	*Marker type*	*Matrices*	*Citations*
DEHP [a]	Parent diester	Environmental samples, serum	*(3)*
MEHP [b]	Monoester metabolite	Serum, urine, amniotic fluid, saliva, breast milk	*(4-7)*
5-OH-MEHP	Oxidized monoester metabolite	Serum, urine, amniotic fluid, saliva, breast milk	*(6-8)*
5-oxo-MEHP	Oxidized monoester metabolite	Serum, urine, amniotic fluid, saliva, breast milk	*(6-8)*
2-cx-MMHP	Oxidized monoester metabolite	Serum, urine, amniotic fluid, saliva, breast milk	*(9, 10)*
5-cx-MEPP	Oxidized monoester metabolite	Serum, urine, amniotic fluid, saliva, breast milk	*(9, 10)*

DEHP di(2-ethylhexyl) phthalate
MEHP mono(2-ethylhexyl) phthalate
5-OH-MEHP mono(2-ethyl-5-hydroxyhexyl) phthalate
5-oxo-MEHP mono(2-ethyl-5-oxy-hexyl) phthalate
2-cx-MMHP mono(2-carboxymethyl)hexyl phthalate
5-cx-MEPP mono(2-ethyl)-5-carboxypentyl phthalate.

[a] The ubiquitous presence of DEHP in both the environment and laboratory require extensive blank testing and preventative measures to reduce or eliminate overestimation of values from contamination. Treatment of serum samples with a preservative such as phosphoric acid to eliminate residual esterase/lipase activity is necessary to avoid preanalytic contamination of the sample leading to falsely elevated levels. In general, serum DEHP measurements are not reliable markers of exposure.

[b] Treatment of serum, milk, and saliva samples with a preservative such as phosphoric acid to eliminate residual esterase/lipase activity is necessary to avoid preanalytic contamination of the sample leading to falsely elevated concentrations.

Figure 1. DEHP and metabolites used to estimate DEHP exposure.



Abbreviations are listed in the footnote to Table 1.

1.1 General Population Exposure

1.1.1 Exposure Estimates Based on DEHP Levels in Environmental Samples and Foods

Clark et al. *(11)* compiled measurements of phthalate diesters in several environmental media from databases in Canada, the US, Europe, and Japan/Asia. **[US data for DEHP are presented here.]** Many of the measurements, including those for DEHP, were compiled by Exxon Mobil Biomedical Sciences, Inc. Medians and ranges are given in Table 2 for environmental samples and in Table 3 for food samples.

In a separate paper *(12)* the same authors presented exposure estimates using probabilistic analysis based on concentrations from an unpublished report prepared for industry. Log-normal distributions were used for most exposure sources. Estimated DEHP intakes by age group are shown in Table 4 and Table 5. Except for intake in infants, more than 90% of estimated DEHP intake was from food. Formula-fed infants were estimated to derive 43.7% of DEHP intake from food, and breast-fed infants were estimated to derive 59.6% of DEHP intake from food. Nearly all of the remainder of DEHP intake in infants was estimated to arise from ingestion of dust.

The authors indicated that exposure estimates of other authors, back-calculated based on measurements of urinary metabolites **[discussed below]**, gave lower estimates of daily intake. They suggested that the current study may have overestimated food exposure to DEHP due to use of outdated food measurements or due to failure to account for cooking-associated loss of DEHP in food. **[The Expert Panel noted that the authors summarized a number of recent estimates and all but 1 from Health Canada (1996) were within the 3 – 30 µg/kg bw/day range assumed in the original CERHR DEHP report. The difference in the Health Canada value is related to dust ingestion by children.]**

Table 2. Environmental DEHP Concentrations Measured in the US

Medium	*Mean Concentration*	*Median Concentration (Range)*	
Surface water, µg/L	0.21	0.05	*(< 0.002 – 137)*
Ground water, µg/L	15.7	15.7	*(not detected – 470)*
Drinking water, µg/L	0.55	0.55	*(0.16 – 170)*
Sediments, µg/kg	1.4	0.16	*(0.00027 – 218)*
Soil, µg/kg	0.03	median not available	*(0.03 – 1280)*
Outdoor air, ng/m3	5.0	2.3	*(< 0.4 – 65)*
Indoor air, ng/m3	109	55	*(20 – 240)*
Dust, g/kg	3.24	median not available	*(2.38 – 4.10)*
Wastewater, µg/L	27	8.3	*(0.01 – 4400)*
Sludge, g/kg	0.301	median not available	*(0.000420 – 58.3)*
Rainwater, µg/L	0.17	0.17	*(0.004 – 0.68)*

From Clark et al. *(12)*.

Table 3. Food Concentrations of DEHP

Food	*Median concentration, µg/g (Range)*
Beverages	0.043 *(0.006–1.7)*
Cereal	0.05 *(0.02–1.7)*
Dairy (excluding milk)	0.96 *(0.059–16.*8)
Eggs	0.12 (< 0.01–0.6)
Fats and oils	2.4 (0.7–11.9)
Fish	0.001 (0.00005–not given [90th percentile 0.02])
Fruits	0.02 (< 0.02–0.11)
Grains	0.14 (< 0.1–1.5)
Meat, not processed	0.05 (< 0.01–0.8)
Milk	0.035 (< 0.005–1.4)
Nuts and beans	0.045 (< 0.08–0.8)
Poultry	0.9 (0.05–2.6)
Processed meat	0.45 (< 0.1–4.32)
Vegetables	0.048 (0.0098–2.2)
Infant formula, powdered	0.12 (< 0.012–0.98)
Infant formula, liquid	0.006 (< 0.005–0.15)
Breast milk	0.062 (0.01–0.6)
Baby food	0.12 (0.01–0.6)
Other food	0.05 (< 0.01–25)

From Clark et al. *(12)*.

Table 4. Estimated DEHP Intake by Age Group

Age group	*Median DEHP intake (µg/kg bw/day)*
Adult (20–70 years)	8.2
Teen (12–19 years)	10
Child (5–11 years)	18.9
Toddler (7 months–4 years)	25.8
Infant (0–6 months) Formula-fed Breast-fed	 5.0 7.3

From Clark et al. *(11)*.

Table 5. DEHP Intake from Environmental and Food Sources

Source	Adult (20–70 yrs)	Teen (12–19 yrs)	Child (5–11 yrs)	Toddler (7 months–4 yrs)	Infant (0–6 months)	
					Formula-fed	Breast-fed
Outdoor air	0.0	0.0	0.0	0.0	0.1	0.0
Indoor air	1.0	0.9	1.0	0.9	1.5	1.1
Drinking water	0.1	0.1	0.1	0.1	0.7	0.0
Ingested soil	0.0	0.0	0.0	0.0	0.0	0.0
Ingested dust	4.3	4.2	5.0	6.6	54.1	39.3
Beverages[a]	11.2	5.2	3.3	2.2	0.0	0.0
Cereals	2.4	2.0	3.5	5.5	0.0	0.0
Dairy products[b]	13.2	11.7	12.2	12.9	0.0	0.0
Eggs	1.1	0.7	0.8	1.3	0.0	0.0
Fats and oils	16.9	19.1	16.5	11.1	0.0	0.0
Fish	1.6	0.8	0.7	0.4	0.0	0.0
Fruit products	0.9	0.8	1.1	1.4	0.0	0.0
Grains	13.4	16.6	18.1	11.1	0.0	0.0
Meats	5.5	5.2	3.7	3.3	0.0	0.0
Milk	3.1	6.7	8.6	12.6	0.0	0.0
Nuts and beans	1.0	1.0	0.9	0.8	0.0	0.0
Other foods	10.3	11.2	11.3	18.9	0.0	0.0
Poultry	3.9	3.5	3.5	3.6	0.0	0.0
Processed meats	3.4	3.4	3.4	2.5	0.0	0.0
Vegetable products	6.6	6.1	6.1	4.9	0.0	0.0
Formula/breast milk	–	–	–	–	43.7	59.6

Data expressed as µg/kg bw/day.
[a]Excluding water
[b]Excluding milk.
From Clark et al. (11)

Tsumura et al. *(13)* evaluated DEHP in prepackaged meals sold in convenience stores in Japan. In 16 meals purchased between August, 1999, and February, 2000, DEHP levels ranges from 346 to 11,800 ng/g food. Five of these meals contained enough DEHP that a 50-kg person would be estimated to receive more than the European Union tolerable daily intake value of 37 µg/kg bw/day. The authors evaluated 10 restaurant-prepared lunches, which are generally served in ceramic containers, and found DEHP levels of 12–304 ng/g food, with only 1 lunch having a DEHP level higher than 95 ng/g food. After an evaluation of preparation techniques, the authors concluded that higher DEHP content of the prepackaged meals was due to the use of PVC gloves in meal preparation. Further, spraying the gloves with an ethanol solution as a decontamination measure was believed to be associated with additional mobilization of DEHP from the gloves.

A Danish study *(14)* measured DEHP in total diet samples, baby food, and infant formulas. The total diet sample included foods consumed by 29 adults during a 24-hour period (excluding beverages and sweets). Baby food and infant formula samples were purchased in retail stores. Mean DEHP concentrations in the adult diets were 0.11 – 0.18 mg/kg diet. **[The lower value was calculated using 0 for samples below the limit of detection and using the limit of detection for samples that were above the limit of detection but below the limit of quantification. The higher value used the limit of detection for samples that were below the limit of detection and used the limit of quantification for samples that were above the limit of detection but below the limit of quantification.]** Mean DEHP levels in baby food were 0.36 – 0.63 mg/kg food, and mean DEHP levels in infant formula were 0.04 – 0.06 mg/kg reconstituted formula.

In a review article, Latini et al. *(15)* estimated from European Union reports that infants consuming formula would be exposed to 8 – 13 μg/kg bw/day from this source. Ingestion of DEHP in human milk was estimated to result in intakes of 8 – 21 μg/kg bw/day. This review also referred to an abstract *(16)* in which DEHP or mono(2-ethylhexyl) phthalate (MEHP) were measurable in 100% of milk or colostrum samples from 17 healthy mothers. Mean DEHP was 1.01 μg/mL (range 0.57 – 1.15 μg/mL). Mean MEHP was 0.68 μg/mL (range 0.28 – 1.08 μg/mL). **[Abstracts are noted for completeness but are not used in the evaluation process.]**

Main et al. *(17)* reported phthalate concentrations in milk collected from 65 Finnish and 65 Danish women as part of a study of cryporchidism and hormone levels in male children. **[The relationship between milk MEHP and infant endpoints is discussed in Section 3.1]**. Women collected aliquots of milk at the end of a feeding starting when their infants were 1 month old. Samples were collected at unspecified intervals until a total sample volume of 200 mL was reached. As each sample was collected, it was placed in a glass bottle in the subject's home freezer, with subsequent samples added to the same bottle. Mothers were instructed to collect the samples in glass or porcelain containers and to avoid breast pumping. **[Almost half the Danish mothers used a breast pump at least once; information on pumping was not available for Finnish mothers. The authors tested milk samples in 1 common Danish pump system and found no effect on phthalate monoester levels.]** High-performance liquid chromatography (HPLC)-mass spectrometry (MS) was used to quantify milk levels of MEHP as well as monomethyl, monoethyl, monobutyl, monobenzyl, and mono-isononyl phthalate. MEHP was detected in milk from all 130 women. The median (range) concentration in Danish samples was 9.5 (1.5 – 191) μg/L, and the median (range) concentration in Finnish samples was 13 (4.0 – 1410) μg/L. The difference between MEHP concentrations in Denmark and Finland was statistically significant ($P < 0.001$, Mann-Whitney U test). Estimated MEHP intake was calculated using infant weight at 3 months of age and assuming milk consumption of 0.120 L/day. For Danish children, the median (range) estimated MEHP intake was 1.14 (0.18 – 23) μg/kg bw/day and for Finnish children, the median (range) estimated MEHP intake was 1.56 (0.47 – 169) μg/kg bw/day. The authors indicated that they could not exclude contamination of samples with dust or other household sources of phthalates, and they suggested caution in interpreting the numerical values reported for milk phthalate concentrations.

Mortensen et al. *(18)* measured phthalates in milk collected from 36 Danish women from 1 to 3 months after delivery. Milk aliquots were collected in the same glass bottle at the end of a feeding and stored in a freezer. **[The methods and collection times appear to be similar to those of Main et al. *(17)*, from the same group of investigators. The Main et al. study references the Mortensen et al. study**

as involving different Danish women.] After thawing, phosphoric acid was added to half of each sample to inactivate milk esterases that might convert contaminant DEHP to MEHP. After clean-up, milk MEHP was quantified using HPLC-tandem MS. Phosphoric acid treatment was not shown to influence MEHP measurement. Median (range) milk MEHP was 9.5 (2.7 – 13) μg/L. Seven samples of commercially sold cow milk were analyzed. MEHP concentrations (range) were 7.1 – 9.9 μg/L. Ten cow milk-based baby formulas were analyzed. MEHP concentrations (range) were 5.6 – 9.1 μg/L.

Yano et al. *(19)* measured DEHP in 27 powdered formula products obtained in 11 countries. The formulas had been produced in 12 countries (Japan, Taiwan, Vietnam, Turkey, the United Kingdom, Germany, Spain, Netherlands, New Zealand, Denmark, Ireland, and the US). Phthalate levels ranged from about 32 to 533 ng/g powder **[estimated from a graph]**. A single sample produced in Turkey contained the highest level of DEHP. Excluding this sample, the highest DEHP concentrations (averaged by country of production) were around 200 ng/g **[estimated from a graph]**. The authors estimated that a 3 kg child consuming 700 mL/d formula would receive a daily DEHP dose of 2.5 – 16.1 μg/kg bw, below the European Commission tolerable daily intake of 37 μg/kg bw.

In Japan, the estimated dietary daily intake of several plasticizers, including DEHP, resulting from the preparation, packaging, and storage of food in 3 hospitals was calculated by Tsumura et al. *(20)*. This study was an update of a similar study conducted in 1999 *(21)* that found a high level of DEHP contamination from disposable gloves used by the food preparers, resulting in the regulation of these gloves by the Japanese Ministry of Health, Labor, and Welfare. DEHP concentrations from the duplicate diet samples containing predetermined amounts of protein, lipids, and carbohydrates varied by hospital and food type, but almost all (62 of 63 samples) contained measurable amounts of DEHP. The average daily DEHP intake was 160 μg/day **[3.2 μg/kg bw/day based on a 50 kg bw]**, which was lower than the 1999 average daily intake of 519 μg/day and lower than the tolerable daily intake range (40 – 140 μg/kg bw/day) set by the Japanese Ministry of Health, Labor, and Welfare.

Fromme et al. *(22)* measured concentrations of DEHP, dibutyl phthalate, butyl benzyl phthalate, diethyl phthalate, dimethyl phthalate, dimethylpropyl phthalate, di-n-octyl phthalate, dipropyl phthalate, and dicyclohexyl phthalate in indoor air and vacuum cleaner dust in 59 apartments and in indoor air in 74 kindergartens in Berlin. The median indoor air DEHP concentration was 156 ng/m3 (95th percentile 390, maximum 615 ng/m^3) in apartments and 458 ng/m^3 (95th percentile 1510, maximum 2253 ng/m^3) in kindergartens. Median dust DEHP content in apartments was 703.4 mg/kg (95th percentile 1542, maximum 1763 mg/kg). DEHP accounted for more than 80% of the phthalate content of household dust. The authors estimated DEHP intakes for children assuming a body weight of 13 kg, inhalation of 5 m^3 air/day and ingestion of dust at 100 mg/day to be 24 μg/kg bw/day, of which the largest contribution was an estimated food intake of 18 μg/kg bw/day (taken from the Canadian Environmental Protection Act). Estimated DEHP intake for adults was 5.06 μg/kg bw/day, assuming a 70 kg body weight, 23 m^3/day inhaled air, and ingestion of 10 mg/day dust. The food contribution to this estimate was 4.9 μg/kg bw/day. **[No source was given for the estimates of dust ingestion.]**

Another evaluation of DEHP and 5 other phthalates in household dust was conducted in Sweden as part of a case-control study of children with allergic disease and asthma *(23)*. Dust samples were obtained from children's rooms in 346 homes. In the 343 samples with DEHP levels above the limit of detection, the geometric mean DEHP dust concentration was 0.789 mg/g. In samples from the homes of 173

case children, the geometric mean DEHP dust concentration (95% CI) was 0.836 (0.724 – 0.964), and in samples from the homes of 176 control children, the geometric mean dust concentration (95% CI) was 0.741 (0.643 – 0.855). There was no significant difference between the DEHP dust concentration in the homes of cases and controls ($P = 0.232$, *t*-test on log-transformed data).

Koo and Lee *(24)* measured DEHP in 42 perfumes, 8 deodorants, 21 nail polishes, and 31 hair care products marketed in Korea. DEHP was detected in 2 (4.8%) of the perfumes, 2 (9.5%) of the nail polishes, and none of the deodorants or hair products. The maximum DEHP detected in perfume was 18.315 mg/L, and the maximum detected in nail polish was 25.077 mg/L. Based on questionnaires probing cosmetic use in the community, models were constructed for the estimation of DEHP exposure from these products. The 3 different models gave median exposure values of 0.6 – 26 ng/kg bw/day and 90th percentile values of 1.3 – 69 ng/kg bw/day.

1.1.2 Exposure estimates based on biomarkers

Estimates of DEHP exposure are often based on urinary concentrations of DEHP metabolites, particularly MEHP or its oxidation products. Urinary measures of metabolites provide an integrative measure across routes of exposure. By contrast, blood serum DEHP and MEHP have been found below or at limit of detection (5.7 ng/mL) in healthy adults when environmental contamination is minimized through the use of HPLC-tandem MS *(25)*. MEHP has also been measured in saliva *(7)* at up to 4.9 ng/mL, comparable to serum. The median saliva value was lower than the limit of detection.

A study of the reproducibility of urinary MEHP concentrations was conducted by Hoppin et al. *(26)*. The study sample consisted of 46 African American women between the ages of 35 and 49 years. The women collected first-morning urine samples on each of 2 consecutive days, timed to the onset of menses. Urine samples were frozen until analyzed. MEHP was determined using HPLC- tandem MS with both urine samples from each woman evaluated in the same laboratory run. The median (range) urinary MEHP concentration was 7.3 (1.0 – 143.9) ng/mL. Adjusted for urinary creatinine, the median (range) MEHP concentration was 6.4 (0.4 – 77.3) µg/g creatinine. The intraclass correlation coefficient (95% CI) for urinary MEHP was 0.52 (0.32 – 0.68). The intraclass correlation coefficient (95% CI) for creatinine-adjusted urinary MEHP was 0. 67 (0.49 – 0.79). Interperson variability was greater than intraperson variability. The authors indicated that the spot urine samples were a reliable biomarker of individual exposure, but because the urine collections were first-morning voids from consecutive days, the reproducibility represented in this study was a best-case example. Most women's patterns of exposure may be sufficiently stable to assign an exposure level based on a single first-morning urine biomarker measurement. However, the authors also noted that no data exist to correlate these monoester urinary markers to total exposure over time since the biological half-life of MEHP is around 12 hours.

The National Health and Nutrition Examination Survey (NHANES) 1999 – 2000 measured monoester metabolites of 7 phthalate esters in 2540 urine samples from adults and children older than 6 years *(4)*. NHANES was updated in 2005 with data for the period 2001 – 2002 (n = 2782), and phthalate levels in the 2 periods were similar *(27)*. In 1999 – 2000, MEHP was found in more than 75% of the samples: 87% from 6 – 11 year olds (n = 328), 84% from 12 – 19 year olds (n = 753), and 76% from adults ≥ 20 years old (n = 1461). **[The Expert Panel noted that no children under age 6 were tested in either time period, and it is most likely that MEHP would be detected in younger children.]** Data from the 2001 – 2002 samples are summarized in Table 6.

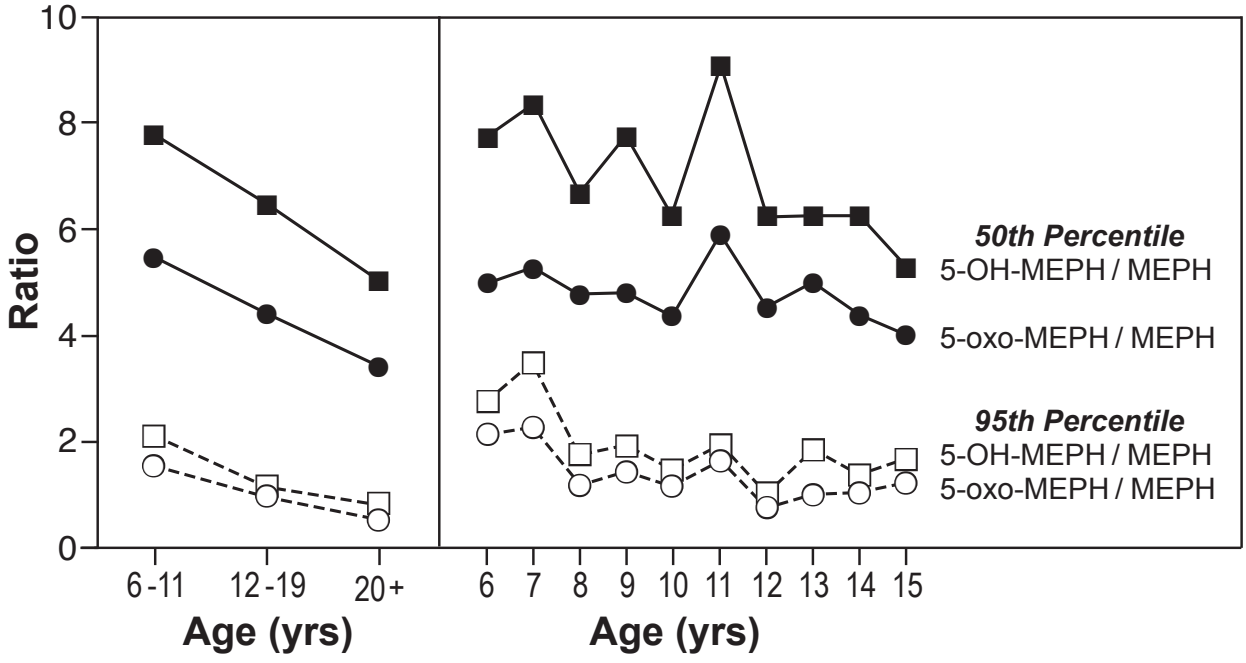
Table 6. DEHP Metabolites in Urine in the NHANES 2001–2002 Sample

Group		n		Geometric mean (95% CI)					
		Total	Creatinine Corrected	MEHP		5-OH MEHP		5-oxo-MEHP	
				µg/L	µg/g creatinine	µg/L	µg/g creatinine	µg/L	µg/g creatinine
Total Sample	—	2782	2772	4.27 (3.80–4.79)	3.99 (3.57–4.46)	20.0 (17.8–22.5)	18.8 (17.0–20.8)	13.5 (12.0–15.0)	12.6 (11.5–13.9)
Age Group (years)	6–11	393	392	4.41 (3.90–5.00)	5.02 (4.47–55.64)	33.6 (29.7–37.9)	38.3 (34.3–42.6)	23.3 (20.9–26.1)	26.6 (24.0–29.4)
	12–19	742	742	4.57 (3.96–5.27)	3.53 (3.09–4.03)	24.9 (21.3–29.1)	19.2 (17.0–21.8)	17.5 (15.1–20.3)	13.5 (12.0–15.2)
	20+	1647	1638	4.20 (3.63–4.86)	3.96 (3.48–4.50)	18.1 (15.7–20.9)	17.2 (15.2–19.4)	12.0 10.5–13.9)	11.4 (10.2–12.8)
Sex	Male	1371	1367	4.31 (3.84–4.83)	3.49 (3.06–3.98)	22.0 (19.5–24.7)	17.9 (16.2–19.7)	14.5 (13.0–16.2)	11.8 (10.7–13.0)
	Female	1411	1405	4.23 (3.67–4.86)	4.53 (4.01–5.11)	18.3 (15.7–21.4)	19.7 (17.3–22.5)	12.5 (10.8–14.6)	13.5 (11.9–15.3)
Race/ Ethnicity	Mexican American	677	674	4.32 (3.75–4.98)	4.05 (3.57–4.61)	18.5 (16.2–21.1)	17.5 (15.9–19.2)	13.1 (11.6–14.9)	12.4 (11.4–13.5)
	Non-Hispanic Black	703	702	6.60 (5.57–7.82)	4.63 (3.95–5.42)	29.8 (26.1–34.1)	21.0 (18.8–23.3)	19.6 (17.1–22.5)	13.8 (12.3–15.4)
	Non-Hispanic White	1216	1211	3.85 (3.37–4.40)	3.80 (3.33–4.33)	19.1 (16.7–21.9)	19.0 (17.1–21.1)	12.8 (11.2–14.6)	12.7 (11.4–14.1)

From Centers for Disease Control and Prevention (27).

NHANES noted that urinary MEHP levels were roughly comparable to those in previous reports **[discussed below]** for US residents *(28)*, pregnant women in New York *(29)*, and in men from an infertility clinic *(30)*. The 2001–2002 report indicated that levels of MEHP, 5-oxo-MEHP, and 5-OH-MEHP, the last 2 of which were evaluated for the first time in this report, were similar to or up to 2-fold higher than samples obtained in German adults and children *(31-33)*. **[The Expert Panel noted that in the NHANES reports, levels below the detection limit were imputed by dividing the limit of detection by the square root of 2. The procedure was unlikely to skew conclusions for children, because most levels were above the limit of detection, but the Panel noted a possibility for error. The Expert Panel considers the NHANES data representative and thus generalizable to the entire US population. From the publicly available NHANES 2001–2002 data (www.cdc.gov/nchs/nhanes), the Expert Panel calculated the ratio of the oxidative monoester metabolites 5-OH-MEHP and 5oxo-MEHP to the monoester metabolite MEHP using standard procedures for analyzing NHANES data (e.g., SAS and SUDAAN PROC DESCRIPT procedure). The ratio of oxidative metabolites to monoester metabolites changed almost linearly with age group but not with sex and race/ethnicity (Figure 2). Children aged 6–11 years produced a larger fraction of oxidative metabolites than adolescents or adults. A further analysis of children and adolescents aged 6–15 years stratified by year of age showed a similar though less pronounced relation with the metabolite ratios. Although the differences were most notable at the high end of the ratio distribution (95th percentile) the trend was clearly still evident even at the median of the distribution.]**

Figure 2. Age-dependent changes in primary and secondary metabolite ratios



Obtained from the publicly available NHANES 2001–2002 data <www.cdc.gov/nchs/nhanes>.

Itoh et al. *(34)* measured MEHP in urine samples collected from 36 Japanese adults. HPLC-tandem MS was used after enzymatic deconjugation. Estimates of DEHP exposure were based on the method of David (discussed below). The median (range) MEHP urine concentration was 5.1 (0.76–25) µg/L. The creatinine-adjusted median (range) urine level was 4.5 (0.79–27) µg/g creatinine. The estimated

median DEHP intake ± geometric SD (range) was 1.8 ± 2.17 (0.37 – 7.3) μg/kg bw/day.

Brock et al. *(35)* measured urinary phthalate monoesters in 19 children aged 12 – 18 months at a clinic visit and about 4 weeks later at a home visit. Phthalate-free adhesive collection bags were used to obtain the samples. Determinations were made using HPLC-tandem MS. Eight samples from 6 children had detectable levels of MEHP ranging from 6.1 to 47.3 ng/mL **[12 – 202 μg/g creatinine, calculated from data presented in the study]**.

Koch et al. *(31, 36)* estimated exposures to DEHP based on first-morning urine samples from 85 urban Germans aged 7 – 34 years (median age 33 years). Concentrations of MEHP and of the secondary metabolites 5-OH- and 5-oxo-MEHP were used with metabolite excretion factors to estimate exposure. Levels of the DEHP metabolites measured in urine are summarized in Section 1.7. MEHP concentrations predicted a median DEHP daily intake level of 10.3 μg/kg bw/day. The range of estimated DEHP daily intake was from the limit of quantification to 165 μg/kg bw/day, with a 95th percentile estimate of 38.3 μg/kg bw/day. The authors believed that the primary metabolite, MEHP, was susceptible to contamination, and that the low urinary MEHP concentrations made it difficult to estimate accurately DEHP exposures. Concentrations of the secondary metabolites were 3 – 5 times higher than MEHP concentrations and gave a median DEHP intake estimate of 13.8 μg/kg bw/day with a 95th percentile estimate of 52.1 μg/kg bw/day. The secondary metabolites were considered by the authors to give a more accurate estimate of DEHP exposure, and any fluctuation in 1 metabolite was also seen in the other. Men had higher daily intake estimates than women (95th percentile 65.0 μg/kg bw/day for men and 27.4 μg/kg bw/day for women). No significant relationships were found between estimated DEHP daily intake and lifestyle habits obtained from a questionnaire.

David *(37)* argued in a letter-to-the-editor that Koch's daily intake estimate was too high. David's estimation of DEHP, based on a different MEHP molar excretion fraction, was approximately 5 times lower (median daily intake 1.76 μg/kg bw/day compared to the Koch et al. estimate of 10.3 μg/kg bw/day). Koch responded stating that conservative fractions were used because there were limited studies regarding molar extraction fractions *(38)*. In addition, Koch noted that if the higher molar extraction values were chosen and the secondary metabolites also considered, the metabolite dose would exceed 100% of the DEHP dose. Koch also pointed to his conclusion that the secondary metabolites were better predictors of DEHP exposure than was MEHP.

Koo and Lee *(39)* measured DEHP, MEHP, and other phthalates (diethyl, dibutyl, and benzyl butyl) in the urine of 150 Korean women 20 – 73 years old and 150 Korean children 11 – 12 years old **[method of subject selection not specified except as "hospital visitors"]**. Geometric mean urinary DEHP was 12.5 ± 17 μg/L in women and 9.5 ± 8 μg/L in children **[error assumed to be geometric SD]**. Geometric mean urinary MEHP was 41.3 ± 50 μg/L in women and 13.3 ± 24 μg/L in children. Geometric mean DEHP adjusted for creatinine (μg/g creatinine) was 16.0 in women and 7.8 in children. Geometric mean MEHP adjusted for creatinine (μg/g creatinine) was 39.6 in women and 9.6 in children. The authors estimated median daily DEHP intake to be 21.4 μg/kg bw in women and 6.0 μg/kg bw in children with a 95th percentile estimated daily DEHP intake of 158.4 μg/kg bw in women and 37.2 μg/kg bw in children. They noted that more than 40% of women had an estimated daily intake above the tolerable intake level of 37 μg/kg bw/day set in 1998 by the EU Scientific Committee for Toxicity, Ecotoxicity, and the Environment.

Koch et al. *(40)* evaluated urine and serum levels of DEHP metabolites after a single oral dose of deuterium-labeled DEHP. A 61-year-old male volunteer weighing 75 kg (the senior author) consumed 48.10 mg **[641 µg/kg bw]** labeled DEHP. The DEHP was incorporated into butter and eaten on bread. Urine samples were collected prior to dosing and for 44 hours thereafter. Blood samples were collected prior to dosing and every 2 hours thereafter, for a total of 5 blood samples, the final of which was 8 hours post-dosing. Blood was immediately centrifuged. Urine and serum samples were frozen until analyzed. MEHP, 5-oxo-MEHP, and 5-OH-MEHP were determined by reverse phase HPLC-tandem MS. The peak urine concentration of labeled MEHP was 3.63 mg/L, 2 hours after the dose. The peak urine concentration of labeled 5-OH-MEHP was 10.04 mg/L, and the peak urine concentration of 5-oxo-MEHP was 6.34 mg/L. The peak urinary concentrations of these MEHP oxidation products occurred 4 hours after the dose. Over the course of the 2-day study period, 47% of the DEHP dose was represented in urine (on a molar basis) by 1 of the 3 measured metabolites. On a molar basis, 7.34% of the administered DEHP dose appeared in the urine as MEHP, 24.7% of the administered DEHP dose appeared in the urine as 5-OH-MEHP, and 14.9% of the administered DEHP dose appeared in the urine as 5-oxo-MEHP. Serum concentrations of MEHP were higher than those of its oxidation products at all time points, consistent with the more rapid urinary elimination of the polar metabolites. Estimated serum elimination half-lives for the 3 measured DEHP metabolites were all less than 2 hours.

Koch et al. *(9)* published a further characterization of DEHP urinary metabolites that may be useful in estimating DEHP exposure. The focus of the study was 2 ω-oxidation products, mono(2-ethyl-5-carboxypentyl) phthalate and mono[2-(carboxymethyl)hexyl] phthalate. This paper presented urine and blood measurements of 5 DEHP metabolites obtained from a single 61-year-old German male (the senior author) after oral ingestion of 3 different doses of deuterium ring-labeled DEHP in butter (doses were separated by 1 week). The proportional metabolite excretion relative to the DEHP dose did not vary by dose (Table 7).

Table 7. Urinary Metabolite Excretion 24 Hours after Oral Ingestion of DEHP

Metabolite	*Estimated Elimination $t_{1/2}$ (h)*	*DEHP dose (µg/kg bw)*		
		4.7	*28.7*	*650*
MEHP	5	6.2	4.3	7.3
5-OH-MEHP	10	23.1	22.7	24.1
5-oxo-MEHP	10	17.3	13.0	14.6
mono(2-ethyl-5-carboxypentyl)phthalate	12–15	15.5	19.4	20.7
mono[2-(carboxymethyl)hexyl]phthalate	24	3.7	5.2	3.8
Total percent of DEHP dose		65.8	64.6	70.5

$t_{1/2}$ = half-life.
Data expressed as percent of administered deuterium-labeled DEHP on a molar basis.
From Koch et al. *(9)*.

Over the first 2 days, 74.3% of the administered DEHP dose was excreted as metabolites, the most abundant of which, on a molar basis, was 5-OH-MEHP (24.7% of the DEHP dose), followed in descending order by mono(2-ethyl-5-carboxypentyl) phthalate (21.9%), 5-oxo-MEHP (14.9%), MEHP (7.34%), and mono[2-(carboxymethyl)hexyl] phthalate (5.4%). The authors suggested that the use of

secondary DEHP metabolites in urine would give a more accurate estimate of DEHP exposure and dose than MEHP in blood or urine. The study authors noted that serum MEHP is not a useful biomarker of DEHP exposure due to its short half-life. However, they stated that serum levels were present at the same orders of magnitude as in animal studies, despite the fact that the human dose was 50 – 1000 times lower than in animal studies. The authors noted that if it is assumed that MEHP in blood is a surrogate for toxic potential, DEHP would be 15 – 100 times more toxic in humans than in marmosets or rats.

Barr et al. *(8)* conducted a urinary metabolite study to evaluate whether the metabolites 5-OH-MEHP and 5-oxo-MEHP were better biomarkers than MEHP of DEHP exposure. In the 50 (of 62) urine samples of adults and children that had detectable levels of all 3 metabolites, the average concentration of 5-OH-MEHP was 4.3 times higher than the average concentration of MEHP; 5-oxo-MEHP concentration was approximately 3 times higher than the MEHP concentration. The median concentration of 5-OH-MEHP was 36 ng/mL, the median concentration of 5-oxo-MEHP was 28 ng/mL, and the median concentration of MEHP was 4.5 ng/mL. Concentrations of 5-OH- and 5-oxo-MEHP were highly correlated to one another (r2=0.984), and both were correlated with MEHP (r2=0.944 for 5-oxo-MEHP and 0.892 for 5-OH-MEHP). 5-OH- and 5-oxo-MEHP appeared to be formed consistently within each individual subject (5-OH/5-oxo ratio 1.4, relative standard deviation [SD] 22%), but there appeared to be variations between individuals in the oxidization of MEHP (5-OH-MEHP/MEHP ratio 8.2, relative SD 80%; 5-oxo-MEHP/MEHP ratio 5.9, relative SD 74%). The authors concluded that 5-OH- and 5-oxo-MEHP are "more sensitive indicators" than MEHP due to higher urinary concentrations and frequency of detection, although MEHP was considered a valid biomarker for health endpoints. The authors also noted that because NHANES used only MEHP as a biomarker for DEHP, exposure levels may have been higher than previously calculated.

Kato et al. *(6)* analyzed 127 paired human serum and urine samples for MEHP and the secondary metabolites 5-OH- and 5-oxo-MEHP. The volunteers in this experiment were aged 6 years and older and had no known previous DEHP exposure (Silva, M personal communication June 29, 2005). The concentrations of the secondary metabolites were 10 times the concentrations of MEHP in urine; metabolite levels are summarized in Section 1.7. 5-OH- and 5-oxo-MEHP were excreted primarily as their glucuronide conjugates, and their concentrations were highly correlated with one another ($r = 0.928$, $P < 0.0001$). Fewer than half of the serum samples had detectable levels of 5-OH- and 5-oxo-MEHP, and unlike the urinary samples, sera contained higher concentrations of MEHP than of 5-OH- and 5-oxo-MEHP. The authors noted that because lipases that convert DEHP to MEHP are present in the serum samples, MEHP concentrations may have been artifactually increased by any DEHP introduced during blood collection and storage. The authors' conclusions were similar to those of Barr et al. *(8)* that 5-OH- and 5-oxo-MEHP appear to be more sensitive urinary biomarkers than MEHP of DEHP exposure, but that MEHP remains important in studying the health effects of DEHP exposure.

Becker et al. *(33)* measured MEHP, 5-OH-MEHP, and 5-oxo-MEHP in first-morning urine samples collected from 254 German children aged 3 – 14 years. House dust samples were collected from ordinary vacuum cleaner bags and extracted with toluene for analysis of DEHP. Questionnaires were used to collect information on age, gender, nutrition, time spent on the floor, floor coverings, furniture, urban versus rural residence, diet, and the presence of orthodontic braces. The non-creatinine-adjusted geometric mean urinary MEHP concentration was 7.91 µg/L (range 0.74 – 226 µg/L), the geometric mean urinary concentration of 5-OH-MEHP was 52.1 µg/L (range 1.86 – 2590 µg/L), and the geometric mean urinary

concentration of 5-oxo-MEHP was 39.9 μg/L (range < 0.5 – 1420 μg/L). As in the previous studies by Koch et al. *(31, 36*), urinary concentrations of 5-OH- and 5-oxo-MEHP were higher than those of MEHP and correlated with one another (r = 0.98). MEHP concentrations correlated significantly but less closely with 5-OH-MEHP (r = 0.72). Geometric mean concentrations of all 3 metabolites were 19 – 34% higher in boys than girls. When 2-year age blocks were considered, children at 13 – 14 years of age had the lowest geometric mean urinary concentration of 5-OH- and 5-oxo-MEHP. The ratios of secondary metabolites to MEHP also decreased with increasing age, suggesting age-dependent metabolism. None of the factors identified by questionnaire were significant determinants of urinary DEHP metabolites. House dust contained a geometric mean DEHP concentration of 508 mg/kg **[ppm]** (range 22 – 5530 mg/kg). There was no correlation between house dust concentration of DEHP and urinary concentrations of MEHP (r = 0.06) or 5-OH-MEHP (r = 0.00). The authors concluded that failure to show a correlation between house dust DEHP and urinary DEHP metabolites may have been due to consideration of the entire sample of children (ages ranging from 3 to 14 years). They proposed that evaluation of very young children, who are more likely to spend time on or near the floor, might show such a correlation. They indicated that their study had too few children in this age group to evaluate this possibility. **[The Expert Panel noted that an alternative explanation for the lack of correlation between dust and urinary measures is that dust is not the only exposure medium.]**

Koch et al. *(32*) measured MEHP, 5-oxo-MEHP, and 5-OH-MEHP in first-morning urine samples collected from 36 German nursery-school children aged 2.6 – 6.5 years. Four teachers and 15 parents also provided urine samples. Determinations were made using multidimensional liquid chromatography and tandem MS. Urinary concentrations in adults and children were compared using the Mann-Whitney *U*-test. The results are shown in Table 8. The authors interpreted these results as demonstrating that DEHP exposure was greater **[double]** among children than adults living in the same environment. The difference between children and adults was particularly evident when creatinine adjustment was used. The authors indicated that there was no relationship between urinary DEHP metabolite concentration in children and parental reports (by questionnaire) of mouthing activities. The authors speculated that the difference between children and adults might be attributable to dust inhalation or to differences in food phthalate exposures. The study authors concluded that exposure of children was twice as high as adults when body weight was considered, and that measures to reduce exposure of children need to be considered. The authors also suggested that using 5-oxo- and 5-OH-MEHP as biomarkers of exposure in children may be preferable to using MEHP because the oxidation products are present at higher concentrations and less likely to be affected by environmental contamination. **[The Expert Panel noted that this important study suggested double the exposure in pre-school aged children compared to adults living in the same environment. However, it was noted that highly significant differences between creatinine-adjusted child and adult concentrations were likely due to natural differences in creatinine excretion between children and adults. Because creatinine excretion is a function of lean muscle mass, smaller individuals excrete less than larger individuals, and children excrete half the levels of adults. Thus, exposures based upon creatinine-corrected results would lead to overestimation of exposure in children compared to adults. However, creatinine-corrected results may be a reasonable surrogate for body weight-adjusted dose. The study demonstrated no correlation between biomarker-measured exposure and mouthing behavior (by report, no data shown). Oxidation to secondary metabolites was observed to be much higher in children compared to adults. Study authors noted the lack of toxicity data on secondary metabolites at the time this report was written.]**

Table 8. DEHP Metabolites in the Urine of Nursery-school Children and Adults

Metabolite		Urine Concentration, Median (Range)		P (children vs adults)
		Unadjusted (µg/L)	Adjusted (µg/g creatinine)	Unadjusted/ Adjusted
MEHP	Children	6.6 (1.5–18.3)	8.7 (1.7–48.4)	0.045/0.908
	Adults	9.0 (2.6–43.1)	8.6 (3.8–26.6)	
5-OH-MEHP	Children	49.6 (2.7–129)	55.8 (15.4–258)	0.038/< 0.0001
	Adults	32.1 (10.7–103)	28.1 (10.9–63.6)	
5-oxo-MEHP	Children	33.8 (2.2–90.6)	38.3 (10.2–158)	0.015/< 0.0001
	Adults	19.6 (4.9–55.1)	17.2 (4.5–40.9)	
Sum of the 3 metabolites	Children	90.0 (6.3–221)	98.8 (28.7–464)	0.074/< 0.0001
	Adults	59.1 (21.1–201)	50.9 (20.5–124)	

From Koch et al. (32).

Ten men participated in a study by Hauser et al. *(41)* in which 8 phthalate monoesters were measured monthly in urine for 3 consecutive days over the course of 3 months. The measured monoesters were the monoethyl, -butyl, -benzyl, -methyl, -octyl, -isononyl, and -cyclohexyl phthalates and MEHP. Five of the phthalates were identified in more than 90% of the urine samples with substantial variation between day-to-day and month-to-month levels. More variation was observed for urine samples collected 1–3 months apart compared to 1–2 days apart. The authors concluded that the predictive value of a single urine measurement in characterizing exposure as high, medium, or low over the course of 3 months was highest for monoethyl phthalate and lowest for MEHP. Of men in the highest tertile for MEHP exposure based on the 3 months of urine monitoring, 56% would have been identified by a single urine sample. A single MEHP measurement would have identified 83% of men in the lower 2 tertiles. The study authors noted that the poor predictive value of MEHP levels in single urine samples could lead to misclassification of exposure over a 3-month period and bias towards the null hypothesis when assessing exposure-response relationships.

Adibi et al. *(29)* measured phthalate diesters in 48-hour air monitoring samples collected by 30 pregnant women in New York city and 30 pregnant women in Krakow, Poland. The New York women were Dominican or African-American, were of low socioeconomic status, and were in the third trimester of pregnancy. The Polish women had been residents of the city for at least a year, were middle-class, and were in the second trimester. Spot urine samples were collected at the end of the personal air sampling period from 25 of the New York women and analyzed for monoester metabolites of the phthalate diesters. Spearman rank correlation was used to evaluate the relationship between the diester concentration in air and the corresponding monoester concentration in urine. All 60 personal

air samples contained measurable concentrations of diethyl, di-*n*-butyl, di-isobutyl, and butyl benzyl phthalate and DEHP. The median DEHP air concentrations (ranges) were: New York 0.22 (0.05 – 0.41) μg/m^3, Krakow 0.37 (0.08 – 1.1) μg/m^3. Median (range) urine MEHP in the subset of 25 New York women was 4.60 (1.80 – 449) μg/g creatinine. The study authors noted that urinary MEHP levels reported for the New York group were similar to values reported for the NHANES sample. There was no significant relationship between urinary MEHP and air sample DEHP concentrations (Spearman correlation coefficient 0.37). **[The Expert Panel noted that the lack of association between air levels and MEHP urine levels may be due to the fact that air is not the only exposure medium.]** Significant correlations were reported for personal air samples and urinary concentrations of butyl benzyl, dibutyl, and diethyl phthalate.

Latini et al. *(42)* reported placental transfer of DEHP and MEHP. They found either DEHP or MEHP in 87.5% of 24 maternal plasma samples and 76% of 25 umbilical cord samples (1 set of twins). Samples were collected at delivery at 35 – 42 weeks gestation. DEHP was measurable in 17 (71%) of the 24 maternal samples and 11 of 25 cord samples ($P = 0.024$, chi-squared). MEHP was measurable in 18/24 (75%) of maternal samples and 18/25 (72%) cord samples (*P* NS). Mean ± SD DEHP concentrations were higher in cord plasma than in maternal plasma (2.05 ± 1.47 μg/mL [n = 11] compared to 1.15 ± 0.81 μg/mL [n = 11], $P = 0.042$, *t* test). MEHP concentrations in maternal and umbilical cord plasma were similar with mean ± SD values of 0.68 ± 0.85 for maternal plasma and 0.68 ± 1.03 μg/mL for cord plasma. The authors concluded that variation in plasma concentrations could have been due to different environmental exposures, and that fetal bioaccumulation may have been due to lack of maturation of excretory organs. Although no statistical correlations were found between DEHP and MEHP levels in either the mother or infant, the authors noted that exposure to phthalates begins prenatally and suggested that fetal exposure is "closely related to maternal exposure." **[The Expert Panel was concerned that there had been pre-analytic contamination in this study because the measured levels of MEHP were 3 orders of magnitude above levels obtained in other studies.]**

An abstract *(43)* reported maternal and cord blood phthalate concentrations in samples collected from 50 maternal-child pairs at cesarean section. MEHP and its oxidative metabolites were said to be present at higher concentrations in fetal than maternal blood. **[Data were not given. Abstracts are noted but are not used in the evaluation.]**

Silva et al. *(44)* measured phthalate monoesters, including MEHP and its oxidative metabolites, in amniotic fluid samples from 54 women. The samples were described as having been obtained during "routine amniocentesis." **[The Expert Panel notes that amniocentesis is performed for clinical indications and is never routine.]** No demographic or clinical information, including gestational age, was available for any of the samples. Quantification was performed using HPLC-tandem MS. MEHP was detected in 24% of the samples, but its oxidative metabolites were not present above the limit of detection in any sample. The median MEHP amniotic fluid level was below the limit of detection, with a maximum detected vale of 2.8 ng/mL. The authors noted that MEHP levels in amniotic fluid, which is fetal urine, were lower than NHANES reported for urine in adults and children. They further noted that the fetus may not be able to biotransform MEHP to its oxidative metabolites due to immaturity of the liver.

1.2 Exposure Assessed Through Toys

Bouma et al. *(45)* measured DEHP released from 47 PVC-containing toys bought in the Netherlands after mixing with a saliva stimulant **[composition not specified]**. DEHP was found in 20 (43%) of the 47 toys at 3–44% by weight. Migration of DEHP into saliva simulant increased with increasing DEHP content. Six toys exceeded the Dutch guidance release value (2.3 μg/min/10 cm^2) for children younger than 1 year. Six toys also exceeded the Scientific Committee on Toxicity, Ecotoxicity and the Environment migration guidance of 1.7 μg/min/10 cm^2; 5 of these toys were intended for children older than 3 years of age.

Niino et al. *(46)* identified migration into simulated saliva of DEHP from a sample of a PVC ball that contained DEHP 190 mg/g. The simulated saliva contained sodium chloride 4.5 g/L, potassium chloride 0.3 g/L, sodium sulfate 0.3 g/L, ammonium chloride 0.4 g/L, urea 0.2 g/L, and lactic acid 3.0 g/L dissolved in distilled water and adjusted to pH 6.5–7.0 with 5 M sodium hydroxide. The ball portion was shaken with the simulated saliva at 35ºC for 15 minutes, yielding DEHP 315 ± 25.0 μg/hour/10 cm^2 (mean ± SD, n = 5). When 4 volunteers **[age not specified]** chewed a ball segment for four 15-minute sessions separated by rest periods, the amount of mobilized DEHP measured in saliva was 44.4 ± 12.3 μg/hour/10 cm^2. Hydrolysis to MEHP occurred to a limited extent. Over the 60-minute chewing period, salivary DEHP decreased from about 42 to 32 nmol while MEHP increased from 0 to about 2 nmol **[estimated from a graph]**.

1.3 Exposure Through Building Materials

Otake et al. *(47)* measured indoor air concentrations of common phthalates and phosphate esters in Japanese homes. Twenty-seven homes in the Tokyo metropolitan area had indoor air concentrations of DEHP ranging from < 0.001 to 3.13 μg/m^3. The mean ± SD DEHP concentration was 0.32 ± 0.6 μg/m^3, the second highest concentration next to dibutyl phthalate, which was 0.75 ± 1.17 μg/m^3. DEHP levels were 100–1000 times higher than ambient outdoor air concentrations reported in articles cited by the author: 2.0 ng/m^3 in Sweden and 16 ng/m^3 in Japan.

Danish authors *(48, 49)* studied the DEHP emission and sorption characteristics of PVC flooring material in an emission cell and in an emission test chamber. Airborne DEHP concentrations increased up to 150 days, at which point emissions leveled off at approximately 1 μg/m^3. Dust on soiled PVC material increased the emission rate of DEHP; dust sorbed 3700 μg of DEHP compared to 900 μg emitted from unsoiled PVC material over 68 days. The authors concluded that resuspended dust may be an important route of DEHP exposure. A similar evaluation of PVC-coated wall coverings showed variable chamber air DEHP concentrations with a maximum air concentration of just under 1 μg/m^3 *(50)*.

1.4 Exposure Through Wastewater

Marttinen et al. *(51, 52)* found DEHP to be the most frequently encountered phthalate in sewage in Finland, with DEHP concentrations of 98–122 μg/L compared to < 5 μg/L for other phthalates. The highest concentration, 160–166 μg/g, was found in treated sewer sludge; this concentration exceeded the maximum value set by the European Commission for the use of sludge in agriculture. In 4 sewage treatment plants, 80–96% of DEHP was removed, but the remaining DEHP accumulated in treated sludge. In leachate from 11 landfills, DEHP was the most commonly identified of 14 measured waste chemicals. When landfill leachate was handled in the same treatment plants as sewage, the contribution of leachate DEHP to total sewage leachate was low, on the order of 1%.

Sewage sludge in Spain contained DEHP at levels of 180–267 ppm dry matter **[µg/g]** *(53)*. Composts made with sludge had DEHP contents of 38–99 ppm dry matter, and soil mixtures with sludge or compose contained DEHP 3–21 ppm at mixing, declining to 0.4–2.5 ppm 9 months later. A study from Scotland did not find a difference in tissue levels of DEHP in sheep grazing on pastures fertilized with sewage sludge compared to pastures treated with inorganic fertilizer *(54)*.

1.5 Medical Exposures

Loff et al. *(55)* quantified DEHP leaching from PVC infusion set tubing during infusion of parenteral nutrition, blood products, and selected pharmaceuticals at room temperature (27ºC) using neonatal intensive care (NICU) protocols employed in treating sick neonates. The highest DEHP concentration was found in lipid-containing solutions used for parenteral nutrition (424.4 µg/mL over 24 hours) resulting in an exposure of 5 mg/kg bw for a 2 kg infant (25 mL solution). Small amounts of DEHP were found in an amino acid/glucose solution (0.83 µg/mL, 24 hours). Blood products stored in 20 mL PVC bags contained 7–339 µg/mL DEHP. When the blood product in the PVC bags was administered through PVC tubing, a single 20 mL dose of a blood product for a 2 kg baby was estimated to contain 608 µg DEHP for packed red blood cells, 928 µg DEHP for platelet-rich plasma, and 552–8108 µg DEHP for fresh frozen plasma. **[MEHP levels were not measured.]** Administration of 1% propofol (10 mL) resulted in a daily DEHP dose of 6561 µg. Administration of 28.8 mL fentanyl resulted in a DEHP dose of 132.5 µg and administration of 24 mL midazolam resulted in a DEHP dose of 26.4 µg. The study authors concluded that the dose of DEHP for a typical preterm neonate requiring total parenteral nutrition (TPN) and additional therapy can range from 10 to 20 mg/day.

Data from the Loff et al. *(55)* study were used by theFDA *(2)* to estimate infant exposures to DEHP through administration of sedatives (discussed below). Because propofol is not approved for sedation in pediatric patients, the intake value from fentanyl (0.03 mg/kg bw/day) was used as the upper-bound estimate of DEHP exposure of 4 kg neonates receiving conscious sedation. **[The Expert Panel notes that the FDA report was dated 2001 on the FDA web site, although the cited publication in Government Reports, Announcements, & Index was dated 2004.]**

Loff et al. *(56)* updated their previous study *(55)* by evaluating the effects of temperature on DEHP release. Temperature and contact time greatly affected the release of DEHP from PVC-infusion lines into a lipid-containing infusion solution. An increase in temperature from 27ºC (the temperature used in the earlier study) to 33ºC increased the amount of released DEHP by approximately 30% (422 µg/mL at 27ºC and 540 µg/mL at 33ºC). The administration of 24 mL of this infusion to a 2-kg newborn resulted in a DEHP dose of 13 mg (6.5 mg/kg bw) at 33ºC compared to 10 mg (5 mg/kg bw) at 27ºC. The rate of extraction of DEHP from PVC tubing was directly related to the length of contact time between the solution and the tubing. The concentration of DEHP in the infusion solution increased from 25 µg/mL at 4 hours to 478 µg/mL between 20 and 24 hours. The authors noted that these findings were important because neonatal ICUs are typically maintained at 30º, and incubator temperatures can reach 37ºC. Loff et al. *(56)* also noted that these exposure estimates were only from 1 type of medical device, and that newborns in these units can be exposed through other devices as well.

[The Expert Panel noted limitations in the Loff et al. studies *(55, 56)*. First, the authors did not address prevention of DEHP contamination or mention if contamination was a problem. Second, because blood products contain enzymes that can metabolize DEHP to MEHP, measurement

of DEHP alone may underestimate total blood product exposure to DEHP-related chemicals. Several important observations were made by the Expert Panel. The first is that even though glass containers were used to store lipophilic substances that were slowly infused through PVC tubing, significant DEHP exposure was estimated. Second, data demonstrated that at NICU temperatures (33ºC), perfusion of TPN through PVC tubing increased DEHP extraction by 20 – 30%, compared to extraction observed at room temperature (27ºC). A study from another group also showed the influence of temperature on DEHP release from PVC *(57)*. Third, extraction rates increased for the first 12 hours and then slowed. Lastly, rinsing lines did not affect leaching.]

Loff et al. *(58)* reported the extraction by lipid emulsions of DEHP from different brands of PVC infusion tubing and different lengths of tubing. Emulsions were run through the lines at 1 mL/minute for 24 hours and were collected in glass flasks. Glassware was rinsed with solvents and heated for removal of possible DEHP contamination. After infusion through PVC tubing, DEHP was present in emulsions at concentrations of 69 – 117 mg/L. When PVC tubing with a polyurethane lining was used, the post-infusion emulsion concentration was 67 – 78 mg/L, and when PVC with a polyethylene liner was used, the emulsions concentration was 32 – 52 mg/L. The amount of DEHP in the emulsion after infusion increased with tubing length.

Another study *(59)* measured the extent of DEHP leaching into a lipid-containing TPN solution from ethyl vinyl acetate bags with PVC connectors and tubing. The bags and tubing were stored at 4ºC for 24 hours or 1 week prior to simulated use. The 1-week storage period simulated conditions that can occur in home parenteral nutrition programs. For both storage periods, DEHP concentrations were highest in solutions with the highest lipid content (3.85%) and decreased for simulated infusions as flow rate decreased. After storage for 24 hours, DEHP content per infusion set ranged from 0.2 to 0.7 mg in the ethyl vinyl acetate bags and from 0.8 to 2 mg in the outlet tubing. The authors concluded that the DEHP dose from a TPN infusion could range from 0.8 to 2 mg/day for an infant or child depending on the lipid content and flow rate. **[The Expert Panel noted that the study provides new information on DEHP leaching during home TPN use.]**

Kambia et al. *(60)* used an HPLC method to measure the amount of DEHP leaching into lipid-containing TPN solutions stored in ethyl vinyl acetate bags with PVC outlets and infused through PVC tubing. The amount of DEHP leaching into TPN solutions was estimated at 0.2 ± 0.008 to 0.7 ± 0.02 mg from bags and 0.8 ± 0.09 to 2 ± 0.07 mg from tubing. **[Variances assumed to be SD as reported in other parts of the paper.]** DEHP was measured at 0.3 – 6.9 μg/mL in blood samples from 4 children receiving TPN. **[Levels of DEHP metabolites in blood were not measured.]**

PVC tubing designed to reduce DEHP leaching by using an "inert" polyethylene inner lining did not show significant differences in the amount of DEHP released into solution compared to standard PVC tubing *(61)*. Three types of multi-layer tubing (PVC, PVC/polyethylene, and PVC/ethyl vinyl acetate/polyethylene) were tested using an etoposide solution containing a polysorbate excipient. DEHP concentration increased nonlinearly with polysorbate concentration and linearly with temperature and contact time. DEHP leaching was particularly evident during the first 2 hours of contact. The authors concluded that polysorbate was responsible for the release of DEHP into etoposide solutions, and that the polyethylene linings did not prevent the release of DEHP into solutions. They noted that DEHP was found on the inert lining even before coming in contact with either solution and suggested that DEHP

might gain access to the tubing lumen through pores in lining materials. The authors suggested the use of polyethylene-only tubing for infants in incubators (37ºC) who receive solutions with polysorbate. **[The Expert Panel noted that the study demonstrates that multilayer tubing does not prevent leaching as marketed and is not a solution to DEHP exposure problems. However, failure to address possible contamination could have been the reason why no differences were observed between lined and unlined tubing.]**

Haighton et al. *(62*) published an abstract in which DEHP exposure from a closed inhalation spray container was estimated at 0.0037 µg/kg bw/day. Details of the assumptions made in this estimation were not available in the abstract. **[The Expert Panel notes this abstract for completeness, but the abstract will not be considered in the evaluation.]**

Calafat et al. *(5*) conducted a study to measure DEHP exposures in infants receiving multiple treatments in the NICU. Six premature newborns undergoing intensive care interventions for more than 2 weeks were tested for the 3 DEHP urinary biomarker metabolites MEHP, 5-oxo-MEHP, and 5-OH-MEHP. All 3 metabolites were found in 33 of the 41 urine samples collected from these infants. 5-OH- and 5-oxo-MEHP were found in all 41 samples, and measurements of these 2 metabolites were an order of magnitude higher than those for MEHP. Urinary concentrations varied widely among the infants. **[The Expert Panel noted that the high variability in the ratio of MEHP to oxidative metabolites suggests metabolic variation. Less than 25% of the metabolites were present in "free form," which is the putative biologically active species.]** Geometric mean 5-oxo-MEHP was 1617 ng/mL, 5-OH-MEHP was 2003 ng/mL, and MEHP was 100 ng/mL. Urinary concentrations of 5-OH-MEHP and 5-oxo-MEHP were highly correlated. The author notes that the geometric means found in this study were several-fold higher than the MEHP geometric mean in the general US population 6 years and older (3.43 ng/mL). **[The Expert Panel notes the high importance of this study because it is the first to quantify real-world exposures resulting from the use of multiple DEHP-containing devices in a contemporary NICU. The study documents DEHP exposures that are orders of magnitude higher than the general population, including children ≥6 years. Three metabolites were examined, and metabolism in premature infants was partially elucidated. Results suggested that MEHP may not be the best marker of exposure. The study also enumerated persistent data gaps regarding possibly increased susceptibility of children, including the effects of high gastric lipase activity, lower capacity for glucuronidation, increased permeability of the blood-testis barrier, and possibly increased absorption in the gastrointestinal tract. Limitations of the study included small sample size, no measurement of exposure from individual sources, and no discussion of primary MEHP exposure (i.e., MEHP infused directly into the patient).]**

Green et al. *(63*) measured urinary DEHP metabolites in 54 infants in a NICU. The infants were hospitalized in 1 of 2 hospitals. One of the investigators observed the care of each infant for a total of 3 – 12 hours (1 – 4 hours/day on 1 – 3 days) and noted the products used in the care of the infants. DEHP exposure was rated low, medium, or high based on the kind of medical devices used and the length of time used. Medical records were not consulted in evaluating infant exposures. Urine was collected from diaper liners or from cotton gauze placed in the diaper. The urine was collected during the observation period. Some infants had 2 or 3 urine specimens collected; in these instances, the urinary MEHP concentrations were highly correlated within infants. Urine was assayed for MEHP, 5-oxo-MEHP, and 5-OH-MEHP using HPLC-tandem MS. **[Only MEHP results were given in the paper.]** Specimens

with MEHP levels below the limit of detection were assigned a value of half the limit of detection. Statistical analysis of urinary MEHP by sex, institution, and DEHP exposure group was performed using the Mann-Whitney-Kruskal-Wallis test, multiple linear regression, and quartile regression.

Urinary MEHP levels are shown in Table 9. DEHP exposure group was described as a substantial predictor of urinary MEHP levels ($P = 0.09$ after adjusting for infant sex and institution). Infants in the medium-exposure group had urinary MEHP concentrations twice as high (calculated from the regression model) as infants in the low-exposure group ($P = 0.3$), and infants in the high-exposure group had urinary MEHP concentrations 5.1 times as high as infants in the low-exposure group ($P = 0.03$). **[The Expert Panel noted that urinary MEHP levels were quite different between infants at the two hospitals and suggested that it may be due to different products used at the two hospitals.]**

Table 9. Urinary MEHP in Infants in Two NICUs by DEHP Exposure Group

Exposure Group (n)	*Urinary MEHP, µg/L, by percentile*		
	25th	*Median*	*75th*
Low (13)	< 0.87	4	18
Medium (24)	3	28	61
High (17)	21	86	171

Exposure status assigned by observing use of medical device during 3 – 12 hours of the child's care.
$P = 0.001$ for exposure class (i.e., low, medium, high)
From Green et al. *(63)*.

The Expert Panel is aware of recent reviews in which exposure to DEHP through medical devices was estimated. The most thorough estimates were conducted by the FDA *(2)* and are summarized in Table 10. **[The Expert Panel notes that the estimates were conducted using data that were available to the CERHR Expert Panel during their first phthalates review and does not provide new data.]**

The FDA noted a lack of data to estimate exposure through breast milk for infants of mothers who had undergone or were undergoing medical procedures like hemodialysis. The FDA believed that few infants were exposed to breast milk from women undergoing these kinds of medical procedures.

A 1 m segment of PVC tubing was used to measure DEHP release into polysorbate 80 solutions *(64)*. Physiological saline and distilled water solutions of polysorbate 80 resulted in greater DEHP release from tubing than did glucose solutions. Use of a flow rate of 90 mL/hour resulted in greater DEHP release than did 60 mL/hour. After 5 hours of infusion of 2 mg/mL polysorbate 80 at a rate of 90 mL/hour, the cumulative amount of DEHP recovered was 850 µg **[estimated from a graph]**. Recovery of DEHP was greater at 90 mL/hour than at 60 mL/hour, even when the concentration of polysorbate 80 was increased 1.5-fold at the lower flow rate, suggesting to the authors that the amount of polysorbate passing through the tube segment was less important than the speed of the polysorbate micelles interacting with the walls of the tubing. **[The Expert Panel noted that the study is pertinent considering the very slow flow rate of TPN administered to neonates in NICU settings.]**

Table 10. FDA Estimates of DEHP Exposures Resulting from Medical Treatments

Medical procedure	*Estimated DEHP dose (mg/kg bw/day)*	
	70 kg adult	*4 kg neonate*
Crystalloid intravenous (iv) solution infusion	0.005	0.03
Infusion of pharmaceuticals with solubilization vehicles		
Administered according to manufacturer instructions	0.04	0.03
Mixed and stored at room temperature for 24 hours	0.15	
TPN administration		
Without added lipid	0.03	0.03
With added lipid	0.13	2.5
Administered via ethyl vinyl acetate bag and PVC tubing	0.06	
Blood transfusion		
Trauma patient	8.5	
Transfusion/extracorporeal membrane oxygenation (ECMO) in adult patients	3.0	
Exchange transfusion in neonates		22.6
Replacement transfusions in neonates in NICU		0.3
Replacement transfusions to treat anemia in chemotherapy and sickle cells disease patients	0.09	
Replacement transfusions in patients undergoing coronary artery bypass grafting	0.28	
Treatment of cryodisorders with cryoprecipitate	0.03	
Cardiopulmonary bypass		
Coronary artery bypass grafting	1	
Orthotopic heart transplant	0.3	
Artificial heart transplant	2.4	
ECMO		14
Apheresis	0.03	
Hemodialysis	0.36	
Peritoneal dialysis	< 0.01	
Enteral nutrition	0.14	0.14
Aggregate exposures of NICU infants undergoing iv administration of sedatives, iv administration of TPN, and replacment transfusion		2.83

From FDA (2).

Polyethoxylated hydrogenated castor oil, an emulsifier used in pharmaceuticals to increase solubility, was found to increase the release of DEHP from PVC tubing when given in distilled water, glucose, or physiological saline *(65, 66*). Release appeared to increase linearly over time, reaching an approximate cumulative value of 776 µg DEHP after 4 hours. Sugar solutions (ribose, fructose, or glucose) containing

polyethoxylated hydrogenated castor oil resulted in less DEHP release from tubing. DEHP levels increased with increasing polyethoxylated hydrogenated castor oil concentrations in all solutions. A decrease in release of DEHP from tubing was shown when paclitaxel in polyethoxylated hydrogenated castor oil was replaced by paclitaxel in polymeric micelles *(67*). Cyclosporine preparations, which use polyethoxylated hydrogenated castor oil in ethanol, have been shown to contain DEHP at concentrations of 3 – 4 mg/L after storage in PVC bags for 12 hours *(68*).

Demore et al. *(69*) studied the release of DEHP from containers when the antineoplastic drug etoposide was stored. Etoposide was evaluated because it is prepared with the surfactant polysorbate 80, which is believed to release DEHP from PVC containers. After 24 hours at room temperature in PVC containers, etoposide in saline contained 18 – 25 µg/mL DEHP, and etoposide in 5% dextrose contained 17 – 25 µg/mL DEHP. Etoposide in glass or polyolefin containers did not contain detectable levels of DEHP after similar time periods. Another study using etoposide infused through PVC tubing found that flow rate, tubing length, and etoposide concentration influenced DEHP leaching, with DEHP concentrations in the solutions of 54 – 155 mg/L after 6 hours of infusion *(70*). As noted above, triple-layer tubing, with a PVC outer layer, a polyvinyl acetate middle layer, and a polyethylene inner layer, offered no advantage in preventing access of DEHP to the solution. Haishima et al. *(71*) evaluated the relationship of DEHP released from medical-grade PVC and physical chemical properties of 53 medications that are administered by injection. The most important predictor of DEHP release was lipid solubility of the medication preparation, which could be easily assessed by solubility of the lipophilic pigment methyl yellow.

DEHP and MEHP in stored whole blood or red blood cells have been simultaneously measured and showed ranges of 6.8 – 83.2 mg/L for DEHP and 0.3 – 9.7 mg/L for MEHP *(72*). Platelets and fresh frozen plasma contained lower concentrations of both phthalates. The concentration in blood products increased with storage time.

In Japan, DEHP was measured in a blood circuit system used to simulate hemodialysis and pump-oxygenation therapy using heparin-coated and uncoated PVC tubing *(73*). In the hemodialysis system, the bovine blood used as the simulant had a baseline DEHP concentration of 249 ppb **[µg/L]**. After 4 hours of circulation, the DEHP concentration was 1718 ppb **[µg/L]**, a 7-fold increase. In the pump-oxygen system, PVC tubing with covalently bonded heparin coating resulted in DEHP levels approximately 50% lower than tubes with ionic-associated heparin coating or no coating at all. The DEHP daily dose for an 11 kg child exposed to 6 hours of pump-oxygenation therapy without heparin-coated tubes was estimated (using bovine blood) at 0.7 mg/kg bw/day and using heparin-coated tubing between 0.3 and 0.6 mg/kg bw/day. An adult exposure was estimated at approximately 0.3 mg/kg bw/day for uncoated tubing and between 0.16 and 0.3 mg/kg bw/day for heparin-coated tubings. These values were noted by the authors to be above the upper limit of the tolerable daily intake established by the Japanese Ministry of Health, Labor and Welfare. In the pump-oxygenation system, the authors estimated that 3 – 4% of DEHP was converted to MEHP. MEHP was also found to decrease with the use of covalently bonded heparin-coated PVC tubing, but concentrations increased over time. In the hemodialysis system, approximately 80 ppb **[µg/L]** MEHP was measured after 4 hours. The authors concluded that the use of PVC tubing for high-risk patients and for long-term therapy should be questioned. **[The Expert Panel noted that this study does not provide much new information, with the exception that covalent and not ionic bonding of heparin to tubing is necessary to prevent significant leaching.]**

Mettang et al. *(74)* compared serum, urine, and dialysate levels of phthalate acid esters in 5 adult peritoneal dialysis patients before and 42 days after the use of plasticizer-free bags and tubing. Following the switch to plasticizer-free materials, significant changes included reductions in phthalic acid levels in serum and MEHP and phthalic acid levels in effluent dialysate. Serum concentrations of DEHP decreased non-significantly. There was no effect on levels of MEHP or 2-ethylhexanol in serum or phthalic acid in urine. The study authors concluded that peritoneal dialysis patients are likely exposed to sources of phthalates in addition to dialysis equipment.

In an effort to simulate exposure during respiratory therapy, Hill et al. *(75)* measured the concentrations of phthalates including DEHP in air after passage through PVC medical tubing. DEHP was detectable in an unspecified proportion of samples but was not above limit of quantification or was not present at concentrations demonstrably higher than background. The presence of 2-ethylhexanol was interpreted as due to DEHP breakdown. The authors concluded that for most adults, exposure from respiratory therapy is small compared to other exposures, but that sensitive populations, particularly those with allergies to plasticizers or with asthma, may be at "significant risk" from respiratory therapy exposures. **[The Expert Panel noted that findings are consistent with previous studies.]**

Platelet pheresis donors (n=36) were evaluated for DEHP exposure by measuring serum DEHP concentrations before and after pheresis sessions of 38–89 minutes *(76)*. In 4 donors, additional serum samples were evaluated for up to 48 hours after the pheresis session. Median (range) serum DEHP increased from a baseline of 92.2 ng/mL (5.9–219.6 ng/mL) to 213.8 ng/mL (7.3–716.1 ng/mL). The authors estimated a median (range) DEHP dose of 6.46 (1.8–20.3) µg/kg bw. In the subjects with further serum monitoring, DEHP serum concentrations returned to baseline within 3 hours of the procedure. Serum triglyceride concentration was correlated with the relative increase in serum DEHP ($r2=0.24$, $P=0.03$).

Koch et al. *(77)* measured urinary concentrations of the DEHP metabolites MEHP, 5-OH-MEHP, 5-oxo-MEHP, 5-cx-MEPP, and 2-cx-MMHP in 1 platelet pheresis donor before and for 24 hours after the pheresis procedure. Over the 24-hour period, the total molar excretion of DEHP metabolites was 4.508 µmol. Urinary excretion ratios from different authors yielded estimates of DEHP dose of 2.6–4.0 mg or 31.6–48.1 µg/kg. The same authors evaluated 18 pheresis donors and 5 non-donor controls using 24-hour urine samples for measurement of MEHP, 5-OH-MEHP, and 5-oxo-MEHP *(78)*. The first urine samples in the donors were collected just prior to the pheresis procedure. There were 6 donors who underwent plasma pheresis and 12 donors who underwent platelet pheresis (6 with a dual-needle continuous-flow technique and 6 with a single-needle discontinuous flow technique). Mean metabolite concentrations in urine shortly after pheresis were about twice as high for continuous flow techniques as for discontinuous flow techniques. Most metabolite excretion occurred during the first 5 hours after the pheresis procedure. Using metabolite excretion factors, DEHP doses were calculated as summarized in Table 11. The authors compared the weight-adjusted dose with the European Union tolerable daily intake values of 20–48 µg/kg bw. They suggested that the DEHP dose associated with plasma pheresis may not be elevated above background because the lipid-rich plasma removed by the procedure may contain most of the DEHP associated with exposure to the pheresis tubing.

Table 11. DEHP Dose with Pheresis Procedures

Procedure		*Median Dose (mg)*	*Mean Dose (Range) µg/kg bw*	
Platelet pheresis	Continuous technique	2.10	32.1	(28.2–38.1)
	Discontinuous technique	1.18	18.1	(14.3–23.8)
Plasma pheresis		0.37	5.7	(3.1–9.6)
Controls (no procedure)		0.41	6.2	(3.0–11.6)

From Koch et al. (78).

A study of recipients of platelet concentrate, derived from pheresis procedures, identified an increase in serum DEHP from a median (range) of 192 (10–532) ng/mL to 478 (142–1236) ng/mL 5 minutes after transfusion *(79)*. Storage time of the platelet concentrates was related to DEHP concentration in the product, increasing from a median (range) of 1.88 (0.41–3.2) mg/L shortly after collection to 6.59 (2.09–10.67) mg/L 5 days later. Washing of platelets 5 days after collection with resuspension in saline resulted in a 31–80% reduction in DEHP concentration in the preparation.

The amount of DEHP retained by dialysis patients during a 4-hour dialysis treatment was estimated by measurement of DEHP blood levels in blood coming to the patient from the dialysis machine and in blood coming from the patient to the dialysis machine *(80)*. In all patients, a higher concentration of DEHP was present in blood entering the patient than in blood leaving the patient. The mean amount of DEHP retained by the patient after 4 hours of dialysis was 16.4 mg (range 3.6–59.6 mg). The authors used their data to construct a toxicokinetic model of DEHP transfer during dialysis. **[There was no discussion of MEHP infused during dialysis or created** *in vivo* **during dialysis.]**

Ito et al. *(81)* noted that release of DEHP from medical-grade PVC could be reduced by ultraviolet irradiation without altering the material's strength or flexibility. The authors attributed the reduction in DEHP release to alterations in the surface structure of the material. **[The Expert Panel notes that ultraviolet-irradiated PVC is not currently used in medical devices.]**

1.6 Utility of Exposure Data

Estimates of DEHP exposures from medical devices have been made using simulated medical procedures with a variety of media and by measuring urinary metabolites in patients undergoing medical procedures. General population exposures have been estimated from urinary concentrations of DEHP metabolites. Other exposure estimates have been derived from measurements of DEHP or MEHP in food, blood, air, or environmental media.

Exposures to DEHP can be estimated using environmental contamination/exposure data coupled with estimates of inhalation and ingestion rates. This probabilistic method will provide accurate estimates of exposure in situations where the routes of exposure and environmental concentrations are well characterized. In the case of DEHP, for which it has been estimated that more than 90% of the intake is from food, probabilistic models are more straightforward, requiring accurate data on food contamination and intake rates. There is considerable variability in the degree of DEHP contamination of foods based upon packaging and processing practices and lipid content of foods. There are situations in which non-food exposure pathways may contribute significantly to exposure, including medical exposures,

occupational exposures, some indoor air exposures, and, potentially, exposure from mouthing of DEHP-containing objects. Probabilistic models are attractive because they provide a distribution of probable intakes; however, the uncertainties described above can lead to large variability in dose estimation.

An alternate approach is to use direct measures of DEHP metabolites in urine samples and back calculate to the DEHP dose (or dose reconstruction). There are uncertainties to this approach as well. The metabolite back calculation approach, when it relies on a single urine measure, assumes a steady state exposure and cannot differentiate between peak levels and background, which is particularly important in small studies. This method also assumes that the metabolite excretion fraction is known and is constant across and within populations with diverse demographic characteristics such as age, sex, and ethnicity. A study by Hauser et al. *(41)* indicated that there is large intra-person variability in excretion of MEHP. Kohn et al. *(82)*, cited in the initial CERHR Expert Panel Report on DEHP, concluded that fractional metabolite excretion is highly variable and that exposure estimates based on metabolite excretion calculations provide order of magnitude estimates of exposure. Another limitation of urinary measurements is that spot samples vary in the degree of dilution based upon hydration state of individuals. Several methods have been evaluated to "correct" for the variability in urine dilution across spot samples, the most popular being creatinine *(83)*. Creatinine excretion varies due to many factors, including the size of the participant, so inter-individual variation, especially among diverse populations, is large. Thus, creatinine-adjusted DEHP metabolite concentrations should never be compared among individuals of vastly different age groups (i.e., children versus adults); however, creatinine-adjusted measurements may serve as a surrogate for a weight-related dose. Changes in creatinine excretion during pregnancy should be thoroughly evaluated before comparing to other women in similar age groups. Similarly, creatinine adjustment has not been standardized for neonates or small children. The validity of creatinine adjustment may also be metabolite-dependent based upon the renal excretion of the metabolite. Thus, caution should be exercised when using creatinine-adjusted concentrations for comparisons among exposure populations or dose reconstruction using urinary metabolite data.

Both methods for estimating dose (probabilistic and dose reconstruction) suffer from uncertainty. Dose calculations using urinary measures tend to be lower than probabilistic estimates. However both methods tend to agree within an order of magnitude, suggesting that the probabilistic methods account for the major routes of exposure in the general population or in known exposure scenarios. The Expert Panel finds current dose estimates robust because both methods provide estimates within a close range.

1.7 Summary of Exposure Data

General population intake estimates for DEHP have been developed using probabilistic analysis *(11)*. More than 90% of estimated daily DEHP intake in people over the age of 6 months is from food. Median estimates are given in Table 4 and range from 8.2 μg/kg bw/day in adults to 25.8 μg/kg bw/day in toddlers. These estimates are similar to those assumed for the general population, not occupationally exposed, by the first DEHP Expert Panel.

Since the first Expert Panel Report on DEHP, 2 population-based surveys of DEHP exposure have been conducted on representative samples of the US population over age 6 years have been completed. NHANES 1999–2000 measured MEHP, and NHANES 2000–2001 measured MEHP as well as 5-OH-MEHP and 5-oxo-MEHP. Mean MEHP, 5-OH-MEHP, and 5-oxo-MEHP vary by age with younger ages groups having both higher MEHP concentrations (both corrected for creatinine and whole

volume) and higher proportions of secondary metabolites (5-OH-MEHP and 5-oxo-MEHP) than older children and adults. In addition, a number of investigators have evaluated urinary metabolites in small populations for a variety of purposes, and these are summarized in Table 12 and shown graphically in Figure 3 and Figure 4. Which metabolite(s) are optimum for estimation of exposure is an issue that is currently being discussed, but 5-oxo- and 5-OH-MEHP may be more sensitive predictors of DEHP exposure due to their relatively high concentration in urine and their lack of susceptibility to contaminants in the sample collection process *(8, 31, 36*). Calculations of population exposure based on urinary metabolites are generally within the original range assumed of 3 – 30 ug/kg/day, but estimates made from the upper 95th percentile of measured ranges exceed this range by up to a factor of 2 in some studies. For example, estimates of general population exposures (95th percentile) using these urine metabolites are 65.0 µg/kg bw/day for men and 27.4 µg/kg bw/day for women *(31, 36*). Not all investigators agree with the methods used to derive these estimates, and alternative estimates have been as much as 5-fold lower *(37*). Exposure to DEHP from medical devices is summarized in Table 13**.**

Appendix II

Table 12. Summary of DEHP Metabolite Levels Measured in Human Urine

Population		*Data Presentation*	*Urinary levels of metabolites (µg/L unless otherwise specified)*			
			MEHP	*5-OH-MEHP*	*5-oxo-MEHP*	*Reference*
46 women 35–49 years old from the US		Median (Range)	7.3 (1.0–143.9 6.4 (0.4–77.3)[a]			Hoppin et al. *(26)*
2540 urine samples Adults and children older than 6 years from the US	Children 6–11 years old	Geometric Means	5.12 (5.19 [a])			NHANES 1999–2000 Presented by Silva et al. *(4)*
	Adolescents 12–19 years old		3.75 (2.53 [a])			
	Adults ≥20 years		3.21 (3.03)[a]			
2782 urine samples Adults and children older than 6 years from the US	Children 6–11 years old	Geometric Means	4.41 (5.02)[a]	33.6 (38.3)[a]	23.3 (26.6)[a]	NHANES 2001–2002 *(27)*
	Adolescents 12–19 years old		4.57 (3.53)[a]	24.9 (19.2)[a]	6–11, 17.5 (13.5)[a]	
	Adults ≥20 years		4.20 (3.96)[a]	18.1 (17.2)[a]	12.0 (11.4)[a]	
150 Korean women 150 children	Women 20–73 years	Geometric Means	41.3±50			Koo and Lee *(39)*
	Children 11–12 years old		13.3±24			
19 children 12–18 months old in the US			6.1 to 47.3			Brock et al. *(35)*
85 volunteers Age 7–34 years in Germany		Median (Range)	10.3 (<LOQ–177)[b] 9.2 (<LOQ–123)[a]	46.8 (0.5– 818) 40.2 (6.9–449)[a]	36.5 (0.5–544) 30.4 (6.4–262)[a]	Koch et al. *(31, 36)*
62 urine samples Adults and children in the US		Median	4.5	36	28	Barr et al. *(8)*
127 adults or children ≥ 6 years in the US		Median (5th–95th percentile)	<LOD[c] (<LOD–20.4)	17.4 (<LOD–220)	15.6 (<LOD–243)	Kato et al. *(6)*

Population	*Data Presentation*	*Urinary levels of metabolites (µg/L unless otherwise specified)*			
		MEHP	*5-OH-MEHP*	*5-oxo-MEHP*	*Reference*
254 children age 3–14 years in Germany	Geometric Means	7.91 (range 0.74–226)	52.1 (1.86–2590)	39.9 (< 0.5–1420)	Becker et al. *(33)*
36 children (age 2.6–6.5 years) in Germany	Median (Range)	6.6 (1.5–18.3) 8.7 (1.7–48.4) [a]	49.6 (2.7–129) 55.8 (15.4–258)[a]	33.8 (2.2–90.6) 38.3 (10.2–158)[a]	Koch et al. *(32)*
19 adults in Germany	Median (Range)	9.0 (2.6–43.1) 8.6 (3.8–26.6) [a]	32.1 (10.7–103) 28.1 (10.9–63.6)[a]	19.6 (4.9–55.1) 17.2 (4.5–40.9)[a]	Koch et al. *(32)*
25 pregnant women in the US	Median (Range)	4.60 (1.80–449) [a]			Adibi et al. *(29)*
Urine samples randomly selected from 289 adults	Median (Range)	Total urinary 2 ethylhexyl metabolites: 2.7 (<1.2 to 66.6)			Blount et al. *(28)*
234 young Swedish males	Median (Range)	<15 (<15–150)			Jönsson et al. *(85)*
369 men presenting for a fertility examination	Median (5th–95th percentile)	5.2 (0.1–110); Specific gravity-adjusted 6.5 (0.8–120)			Hauser et al. *(41)*

[a] µg/g creatinine
[b] LOQ = limit of quantification
[c] LOD = limit of detection.

Figure 3. Urinary MEHP Concentrations

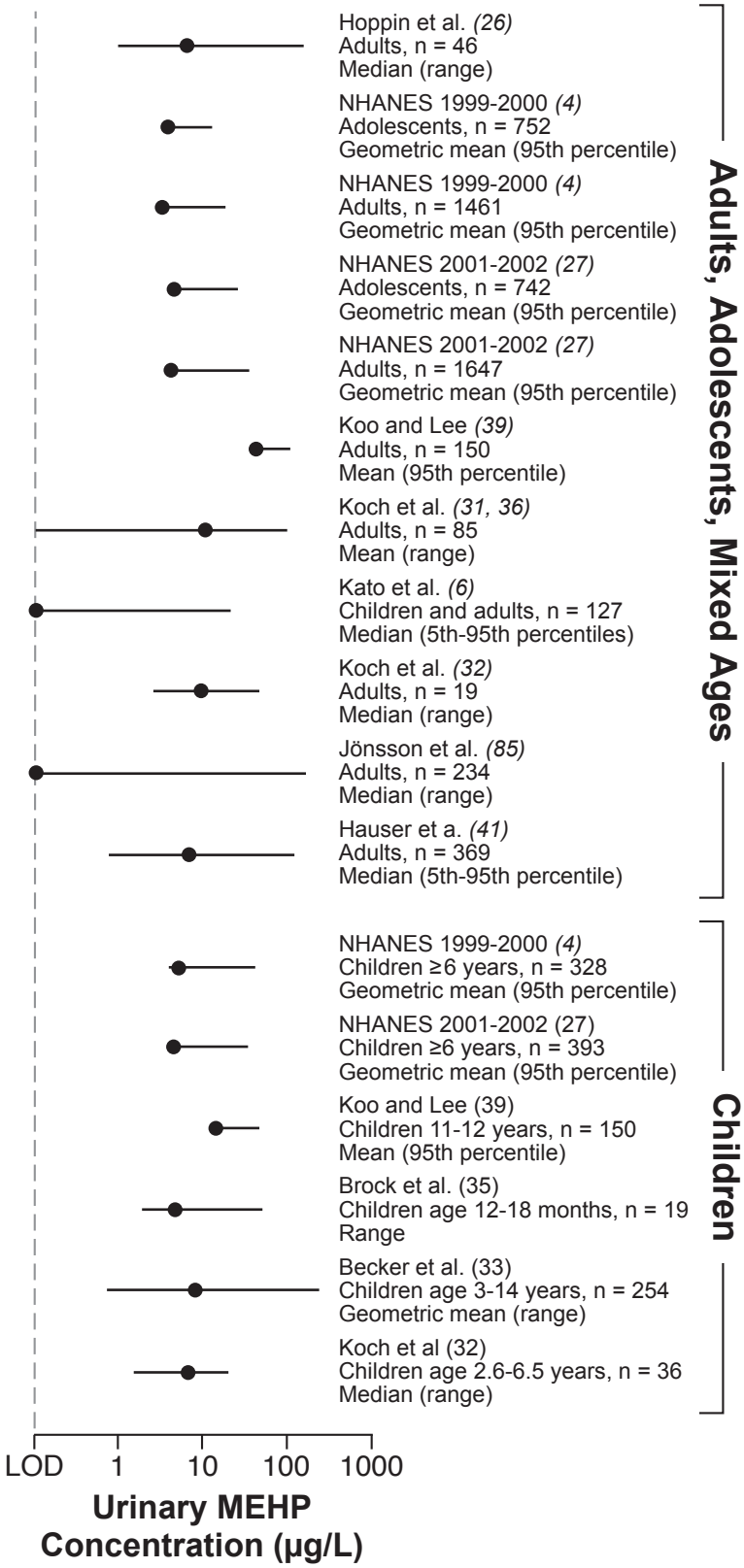
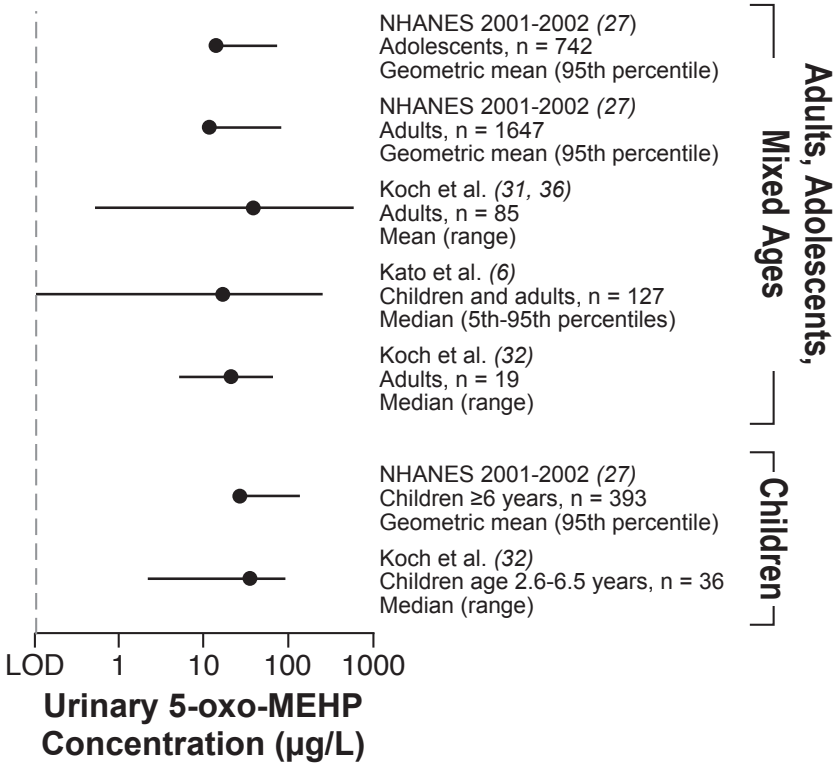
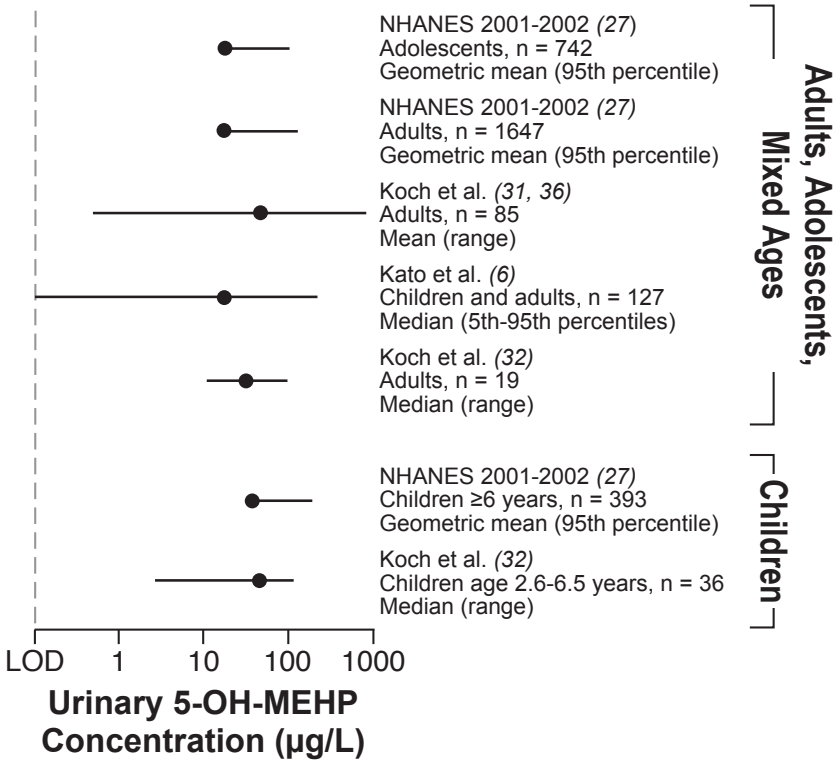


Figure 4. Urinary 5-oxo- and 5-OH-MEHP Concentrations





Appendix II

Table 13. Summary of DEHP Exposure Estimates from Medical Devices

Population	*Medical device*	*Medium*	*Estimated DEHP exposure*	*Reference*
Infants	IV tubing	Lipid-containing solutions used for parenteral nutrition	424.4 µg/mL over 24 hours; 5 mg/kg bw for a 2 kg infant receiving 25 mL solution	Loff et al. *(55)*
		Amino acid/glucose solution	0.83 µg/mL, 24 hours	
		1% propofol, continuous	6561 µg for a 2 kg infant	
		Fentanyl solution 28.8 mL	132.5 µg for a 2 kg infant	
		Midazolam 24 mL	26.4 µg for a 2 kg infant	
	Blood bag + tubing	Packed red blood cells, 20 mL	608 µg for a 2 kg infant	
		Platelet-rich plasma	928 µg for a 2 kg infant	
		Fresh frozen plasma	552 – 8108 µg for a 2 kg infant	
	IV tubing	Lipid-containing infusion solution, 27°C	422 µg/mL 10 mg for a 2 kg infant receiving 24 mL	Loff et al. *(56)*
		Lipid-containing infusion solution, 33°C	540 µg/mL 13 mg for a 2 kg infant receiving 24 mL	
Not specified	IV tubing	Hydrogenated castor oil in saline or water	775 µg after 4 hours	Hanawa et al. *(65)*
		Hydrogenated caster oil in sugar solutions	150 µg over 4 hours	
Not specified	PVC bags	Etoposide in polysorbate 80-containing solution	17 – 25 µg/mL after 24 hours	Demore et al. *(69)*
Children	Ethyl vinyl acetate bags with PVC connectors and tubing	Lipid-containing parenteral nutrition solution stored at 4ºC for 24 hours or 1 week	0.8 – 2 mg/day	Kambia et al. *(59)*
Not specified	PVC multi-layer iv tubing	Polysorbate solution	26 – 30 mg/mL after 2 hours; 62 – 70 mg/mL after 24 hours	Bourdeaux et al. *(61)*

Population	*Medical device*	*Medium*	*Estimated DEHP exposure*	*Reference*
Children and adults	Hemodialysis simulation	Bovine blood	1718 µg/L after 4 hours, compared to 249 µg/L at baseline. MEHP 80 µg/L after 4 hours. Estimated adult dose 0.067 mg/kg bw/day.	Haishima et al. *(73)*
	Pump-oxygen-ation simulation		Estimated child's dose of 0.3–0.7 mg/kg bw/day Estimated adult dose of 0.16–0.3 mg/kg bw/day. MEHP 200–400 µg/L after 4 hours.	
Adults	Hemodialysis	Blood from 11 patients on therapy	Patients retained DEHP 16.4 mg (range 3.6–59.6 mg) after a 4-hour dialysis session	Dine et al. *(80)*
Adults	Platelet pheresis	Blood from 36 healthy donors	Median dose retained after pheresis session was 6.46 µg/kg bw (range 1.8–20.3 µg/kg bw)	Buchta et al. *(76)*
Adults	Platelet pheresis	Blood from 12 healthy donors	Median dose retained after pheresis session was 18.1–32.3 µg/kg bw (range 14.3–38.1 µg/kg bw)	Koch et al. *(84)*
Infants	All ICU exposures	Aggregate exposures	2.83 mg/kg bw/day	FDA *(2)*
Adults	Medical treatment		0.005–8.5 mg/kg bw/day	
Adults	ECMO, multiple transfusions	Blood	≥ 4 mg/kg bw/day	
Not specified	Respirator PVC tubing	Air	Below limit of quantification	Hill et al. *(75)*

At the time of publication of the first DEHP Expert Panel report, there was concern that infants undergoing multiple medical procedures might have exposures 3 orders of magnitude higher than the population exposure level. The previous report based this concern upon studies that were either several decades old or in which exposures were calculated based upon single source DEHP exposure. Since then, 3 studies, 2 from the US *(5, 63*) and 1 from Germany *(84*) have confirmed that doses assessed using urinary metabolites do indeed reach levels up to about 6 mg/kg bw/day. These studies are summarized in Table 14.

Table 14. Summary of DEHP Metabolite Levels Measured in Medically-Exposed Infants

Population	***Urinary levels of metabolites (µg/L)***			
	MEHP	***5-OH-MEHP***	***5-oxo-MEHP***	***Reference***
54 neonates admitted to NICU for at least 2 days Presented as median (25th – 75th percentile)	*Total Group:* Female: 20 (3 – 64) Male: 39 (19 – 75) *DEHP Exposure Group:* Low: 4 (< Lod – 18) Medium: 28 (3 – 61) High: 86 (21 – 171)			Green et al. *(63)*
41 urine samples from 6 premature newborns who were potentially given IV infusions for >2 weeks Presented as median (5th – 95th percentile)	129 (6.22-704)	2221 (290 – 13,161)	1697 (243 – 10,413)	Calafat et al. *(5)*
45 neonates treated with various medical procedures: • *Blood transfusions* • *Intubation* • *Continuous positive airway pressure* • *Intralipids feeding* • *Orogastric tubing* • *IV nutrition* Presented as 95th percentile		557	406	Koch et al. *(84)*

Another data gap that has been filled since the last report is represented by the work by Loff *(55, 56)* showing that DEHP leaches from PVC iv tubing used to deliver TPN at levels in the 5 – 10 mg/kg bw/day dose range. DEHP extraction from iv tubing increases with increasing temperature, of note since most NICUs are kept warm and babies are often under warmers or in heated isolettes, and varies with the nature of the solution being administered. Infant ICU exposures were estimated by FDA *(2)* at 2.83 mg/kg bw/day from only 3 potential sources using only data available at the time of the original Expert Panel Report on DEHP. Blood transfusion is an important source of DEHP exposure, and FDA *(2)* estimated adult exposures at ≥4 mg/kg bw/day resulting from ECMO or multiple transfusions.

General population exposures of concern can include fetal and neonatal exposure via general exposures to pregnant and lactating women. The initial Expert Panel Report on DEHP assumed both placental

and mammary transfer of DEHP based upon experimental animal studies. Human data are now available and document both placental transfer in humans *(44*) as well as breast milk transfer *(5, 17*). **[The Expert Panel notes that another potential source of infant exposure is breast milk expressed using DEHP-containing breast pumps.]** These data are still scant, but may be of particular concern if the toxic metabolites of DEHP are present in breast milk or amniotic fluid in free (unconjugated) form. DEHP is also present in some infant formulas *(12, 14, 18, 19*).

Dietary intake has been identified as an important route of exposure *(12-14, 20-22*). Reported daily intakes are variable and generally cover the range of exposures expected for the general population (i.e., 1 – 30 μg/kg bw/day).

Since the initial Expert Panel Report on DEHP, 2 studies have estimated DEHP release from toys due to mouthing behavior. Bouma et al. *(45)* measured DEHP released from 47 toys containing PVC after mixing with a saliva simulant. DEHP was found in 20 (43%) of the 47 toys at 30 – 45% by weight. Six toys exceeded the Dutch guidance release value (2.3 μg/min/10 cm^2) for children younger than 1 year. Niino et al. *(46)* identified migration into simulated saliva of DEHP from a sample of a PVC ball that contained DEHP 190 mg/g. DEHP leaching rate was found to be 315 ± 25.0 μg/hour/10 cm^2 (mean ± SD, n = 5).

Three publications reported on inhalation as a route of exposure. Otake et al. *(47)* measured concentrations of common phthalates in 27 homes in the Tokyo metropolitan area. Indoor air concentrations of DEHP ranged from < 0.001 to 3.13 μg/m^3. The mean ± SD concentration was 0.32 ± 0.6 μg/m^3. DEHP levels were 100 – 1000 times higher than ambient outdoor concentrations Adibi et al. *(29)* measured phthalate diesters in 48-hour personal air samples collected by 30 pregnant women in New York city and 30 pregnant women in Krakow, Poland. The median DEHP air concentrations (ranges) were: New York 0.22 (0.05 – 0.41) μg/m3 and Krakow 0.37 (0.08 – 1.1) μg/m^3. The median indoor air concentration reported by Fromme et al. *(22)* in German apartments was 0.16 μg/m^3 and 0.458 μg/m^3 in kindergartens.

DEHP has also been shown to be a constituent of dust in households. Fromme et al. *(22)* found DEHP 775.5 mg/kg in dust collected from 30 apartments in Germany. Bornehag et al. *(23)* measured DEHP concentration in dust samples from children's bedrooms in 346 homes. The geometric mean was 0.789 mg/g of dust.

2.0 GENERAL TOXICOLOGY AND BIOLOGIC EFFECTS

Section 2 of this report contains summaries of toxicokinetics, general toxicity, or carcinogenicity studies that may be especially relevant to the interpretation of developmental and reproductive effects associated with DEHP exposure. Since the initial CERHR Expert Panel Report on DEHP, there have been additional studies on toxicokinetics in rats and marmosets. There have also been studies using systems designed to assess the anti-androgenicity and estrogenicity of DEHP.

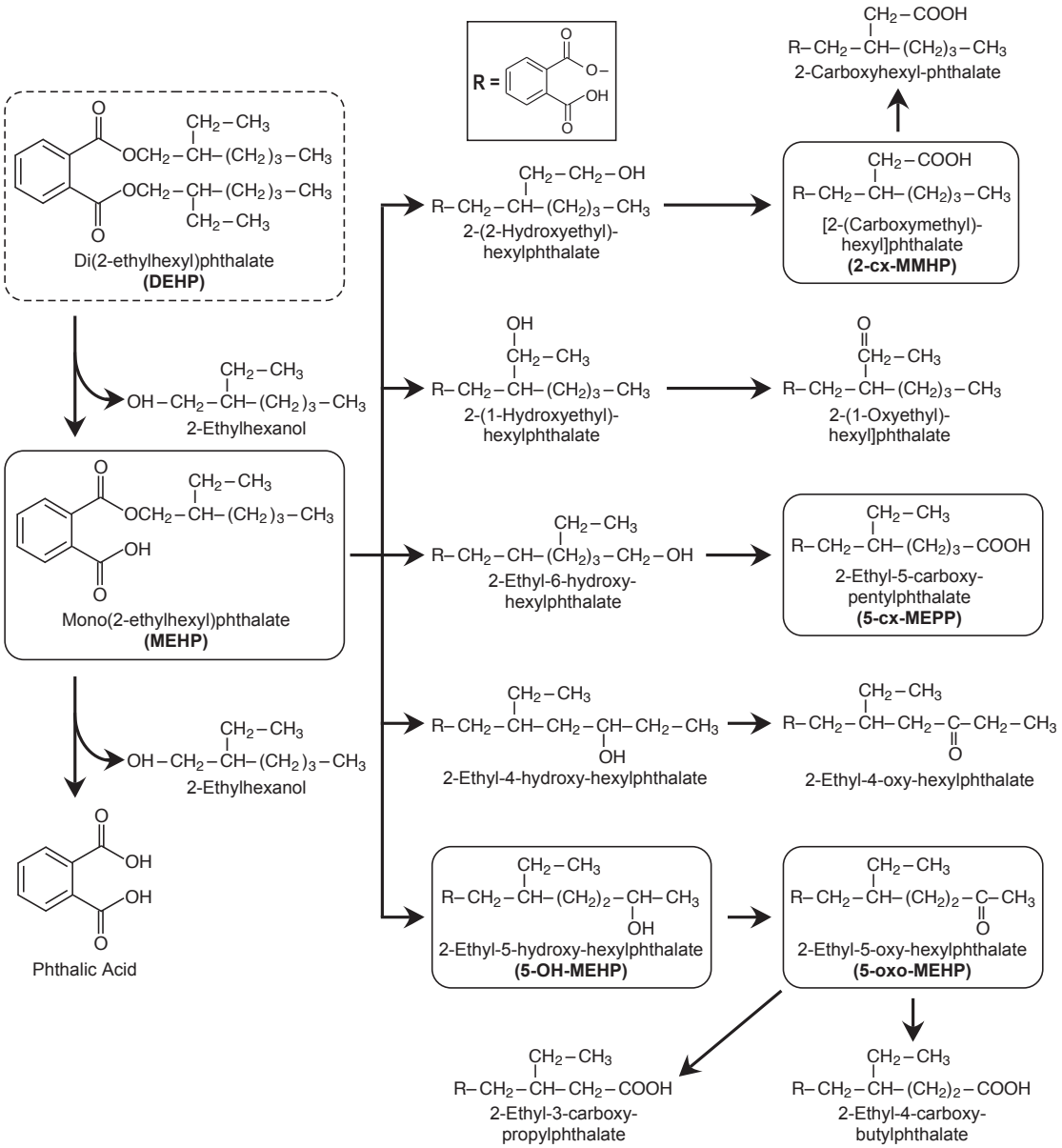
2.1 Toxicokinetics

The metabolism of DEHP in humans is discussed in Section 1 with respect to the use of urinary metabolite concentrations in the estimation of DEHP exposures and is illustrated in more detail in Figure 5. Metabolism has also been summarized by Koch et al. *(84*). By 24 hours after an oral DEHP dose, about 70% appears in the urine as 5 major metabolites (see Table 7). According to this review, metabolism is similar after IV exposure to DEHP.

It is important to distinguish between *in vivo* **and** *in vitro* **metabolism. The former is based by analogy on excreted metabolites and provides no definitive information on reaction mechanisms, on the enzymes involved, or their polymorphic forms.** *In vitro* **studies, on the other hand, provide this information and, in addition, information on the proper sequence of secondary and tertiary metabolites, as well as revealing reactive, but short-lived, metabolites. A significant data gap is the lack** *in vitro* **metabolic studies in general but particularly in the case of** *in vitro* **studies of human metabolism. Although such studies have been carried out extensively for other xenobiotics, they have not yet been carried out for DEHP. Only human studies can reveal the extent of metabolic variation within the human population and shed light on the metabolic parameters involved in the identification of populations or individuals at greater or lesser risk. These studies may also help in the selection of the most appropriate surrogate animal for** *in vivo* **studies and help in estimating uncertainty factors in risk analysis.]**

Calafat et al. *(86*) measured MEHP in maternal urine and amniotic fluid after gavage administration of DEHP **[purity not specified]** in corn oil to pregnant Sprague-Dawley rats on gestation day (GD) 8, 10, 15, 16, and 17. **[The abstract indicates administration also on GD "5/7".]** Doses were 0, 11, 33, 100, and 300 mg/kg bw (n=2/dose group). Urine was collected approximately 6 hours after dosing, and amniotic fluid was collected at necropsy on GD 18. MEHP was analyzed by HPLC-tandem MS after solid-phase extraction and enzymatic hydrolysis. There was no temporal trend in urinary MEHP levels over the collection period, and the 5 urine MEHP levels were combined for each animal. Creatinine-corrected and uncorrected urinary MEHP and uncorrected amniotic fluid MEHP were highly correlated with maternal DEHP dose (*r* values 0.964–0.998). **[Data were presented only in graphic form. At the 300 mg/kg maternal DEHP dose level, urinary MEHP was estimated from a graph at 16.4 mg/L and amniotic fluid MEHP was estimated at 2.8 mg/L.]** Maternal urinary MEHP was only 13.3% unconjugated, while amniotic fluid MEHP was 88.2% unconjugated. The authors observed that the finding that MEHP was largely conjugated in urine did not agree with reports of other studies on urinary MEHP in rats. The authors also indicated that the lack of measurement of more oxidized MEHP metabolites may lead to an underestimation of exposure to DEHP and its biotransformation products.

Figure 5. DEHP Metabolism



The metabolites discussed in this report are circled. From Koch et al. (84), used with kind permission of Springer Science and Business Media and of Prof. Dr. J. Angerer.

Toxicokinetic studies using radiolabeled DEHP by gavage in pregnant and non-pregnant female Wistar rats and CD-1 mice appeared in unpublished reports sponsored by the European Council for Plasticizers and Intermediates *(87-91)*. Determinations were made after single doses of 200 or 1000 mg/kg bw and after 5 daily doses at these levels. Results are summarized in Table 15.

Table 15. Toxicokinetic Parameters in Pregnant (GD 6) and Non-pregnant Female Rats and Mice Given Oral Radiolabeled DEHP

Model	*C_{max} (nmol-eq/mL)*	*AUC_{0-48} (nmol-eq-h/mL)*	*$t_{½}$ (hours)*
Single 200 mg/kg bw Dose			
Non-pregnant rat	64	1426	7.1
Pregnant rat	58 (32.1/36.4)[a]	983 (217/511)	7.8 (82.1/5.9)
Non-pregnant mouse	154	2069	7.1
Pregnant mouse	91 (28/84)	1078 (171/816)	7.3 (–/4.0)
Single 1000 mg/kg bw Dose			
Non-pregnant rat	353	5825	10.2
Pregnant rat	249 (90.8/146.3)	6254 (1180/2445)	5.5 (–/14.0)
Non-pregnant mouse	1339	6838	10.3
Pregnant mouse	227 (103/215)	6745 (1107/3526)	9.7 (–/14.2)
Repeated 200 mg/kg bw Dose			
Non-pregnant rat	77	1007	8.7
Pregnant rat	98	1606	10.3
Non-pregnant mouse	197	2252	7.1
Pregnant mouse	90	1083	7.3
Repeated 1000 mg/kg bw Dose			
Non-pregnant rat	405	6398	13.5
Pregnant rat	518	7410	6.6
Non-pregnant mouse	396	5672	7.9
Pregnant mouse	551	4890	11.4

[a] Figures are given for total radioactivity with (DEHP/MEHP) in parentheses, as determined by gas chromatography (GC).
C_{max} = maximum concentration
t_{max} = time to maximum concentration
$t_{1/2}$ = half-life
AUC = area under the concentration – time curve
From Laignelet and Lhuguenot *(87-91)*.

An unpublished report from Mitsubishi Chemical Safety Institute, Ltd. *(92)* described a 65-week oral-dose toxicity study of DEHP in marmosets (discussed in Section 4.2.3) and included a toxicokinetic study. **[Some data from this study were published in abstract *(93)*.]** The study was sponsored by the Japan Plasticizer Industry Association. Ring-labeled ^{14}C-DEHP (99.6% purity) in corn oil was given to 3 groups of marmosets. The first group was treated at 3 months of age. The second group was treated

at 18 months of age. The third group was treated for 65 weeks from 3 months of age with unlabeled DEHP and studied at 18 months of age. There were 3 animals of each sex in each treatment group. Treatments were by gavage at dose levels of 100 or 2500 mg/kg bw. Blood samples were collected 1, 2, 4, 8, 12, 24, 48, 72, 120, and 168 hours after dosing. Spontaneous urine and feces were collected for radioactivity determination. At least 2 weeks after the kinetic studies, animals were dosed again and tissues collected 2 hours later for determination of radioactivity. Radioactivity determination was by liquid scintillation counting. Toxicokinetic parameters are shown in Table 16.

Table 16. Toxicokinetic Parameters after Oral Dosing of Marmosets at Age 3 and 18 Months with Radiolabeled DEHP

Dose group			C_{max} (µg eq/mL)	t_{max} (hour)	$t_{1/2}$ (hour)	AUC (µg eq-hour/mL)	AUC/dose (hour-kg/L)
Age (months)	Dose (mg/kg bw)	Sex					
3	100	Male	6.86 ± 4.86	4.0 ± 3.5	8.0 ± 4.0	37.4 ± 19.3	0.374 ± 0.193
		Female	17.08 ± 10.69	1.3 ± 0.6	6.0 ± 3.5	78.7 ± 67.2	0.787 ± 0.672
	2500	Male	36.00 ± 37.47	10.0 ± 12.2	21.3 ± 23.1	270.2 ± 194.5	0.108 ± 0.078
		Female	66.00 ± 22.34	4.0 ± 0.0	8.0 ± 0.0	347.7 ± 66.5	0.139 ± 0.027
18	100	Male	13.53 ± 6.07	2.3 ± 1.5	5.3 ± 2.3	99.0 ± 57.4	0.990 ± 0.574
		Female	19.49 ± 16.71	1.0 ± 0.0	4.7 ± 3.1	150.8 ± 137.8	1.508 ± 1.378
	2500	Male	50.00 ± 39.23	1.0 ± 0.0	2.7 ± 1.2	444.7 ± 197.8	0.178 ± 0.079
		Female	62.67 ± 38.73	1.3 ± 0.6	4.7 ± 3.1	952.8 ± 1093.3	0.381 ± 0.437
18 (after 65 weeks pretreatment)	100	Male	14.77 ± 17.04	2.3 ± 1.5	5.3 ± 2.3	83.0 ± 104.9	0.830 ± 1.049
		Female	4.81 ± 3.61	2.3 ± 1.5	6.7 ± 4.6	48.2 ± 46.0	0.382 ± 0.460
	2500	Male	32.33 ± 6.43	1.0 ± 0.0	2.7 ± 1.2	153.7 ± 18.9	0.061 ± 0.008
		Female	4.33 ± 2.31	1.0 ± 0.0	2.7 ± 1.2	11.5 ± 10.3	0.004 ± 0.004

Data are mean ± SD, n = 3 animals/sex/group.
From Mitsubishi Chemical Safety Institute, Ltd. *(92)*.

Reproductive organ radioactivity contents 2 hours after dosing are shown in Table 17. The authors found the highest level of radiation in the kidneys after a single oral dose and considered that high radioactivity levels in the prostate and seminal vesicles of some animals may have been due to urine contamination. Repeated dosing for 65 weeks did not appear to alter the distribution of DEHP in 18-month-old animals. The authors called particular attention to the small amount of label distributed to the testis and postulated that differences in access of DEHP metabolites to the testis may explain a lack of testicular toxicity in marmosets compared to rodents, in which large amounts of MEHP are distributed to the testis after DEHP treatment.

Table 17. Reproductive Organ Radioactivity Content 2 Hours after Oral Dosing of Marmosets with Radiolabeled DEHP

Dose group			Concentration (µg eq/mL or µg eq /mg)			Organ/ Plasma Ratio	Distribution (% of dose)
Age (months)	Dose (mg/kg bw)	Tissue	Male	Female	Organ		
3	100	Plasma	26.54 ± 35.56	33.55 ± 29.22			
		Testis			5.47 ± 7.35	0.21	0.002 ± 0.003
		Epididymis			10.25 ± 13.28	0.39	0.002 ± 0.002
		Prostate			13.04 ± 8.79	0.49	0.001 ± 0.002
		Seminal vesicle			8.03 ± 4.57	0.30	0.001 ± 0.001
		Ovary			4.84 ± 4.33	0.14	0.000 ± 0.000
		Uterus			11.28 ± 13.16	0.24	0.003 ± 0.004
	2500	Plasma	45.51 ± 31.47	45.50 ± 45.92			
		Testis			10.52 ± 4.63	0.23	0.000 ± 0.000
		Epididymis			14.09 ± 2.54	0.31	0.000 ± 0.000
		Prostate			34.22 ± 30.49	0.75	0.000 ± 0.000
		Seminal vesicle			23.14 ± 15.18	0.51	0.000 ± 0.000
		Ovary			ND	–	–
		Uterus			8.91 ± 9.49	0.20	0.000 ± 0.000
18	100	Plasma	9.01 ± 9.97	16.88 ± 13.48			
		Testis			0.83 ± 0.81	0.09	0.003 ± 0.004
		Epididymis			1.97 ± 1.29	0.22	0.001 ± 0.001
		Prostate			4.59 ± 4.52	0.51	0.002 ± 0.002
		Seminal vesicle			16.26 ± 21.74	1.80	0.005 ± 0.006
		Ovary			5.93 ± 4.25	0.35	0.004 ± 0.004
		Uterus			3.79 ± 2.64	0.22	0.002 ± 0.002
	2500	Plasma	65.48 ± 95.58	123.74 ± 33.78			
		Testis			8.47 ± 11.95	0.13	0.001 ± 0.001
		Epididymis			15.98 ± 20.15	0.24	0.000 ± 0.001
		Prostate			10.64 ± 14.53	0.16	0.000 ± 0.000
		Seminal vesicle			13.84 ± 15.79	0.21	0.000 ± 0.001
		Ovary			36.11 ± 11.39	0.29	0.001 ± .000
		Uterus			33.52 ± 12.62	0.27	0.001 ± 0.001

Dose group			Concentration (µg eq/mL or µg eq /mg)			Organ/ Plasma Ratio	Distribution (% of dose)
Age (months)	Dose (mg/kg bw)	Tissue	Male	Female	Organ		
18 (65 weeks pretreatment)	100	Plasma	29.92 ± 6.61	47.28 [a]			
		Testis			3.03 ± 0.97	0.10	0.011 ± 0.004
		Epididymis			9.79 ± 6.91	0.33	0.008 ± 0.008
		Prostate			7.34 ± 3.34	0.25	0.002 ± 0.002
		Seminal vesicle			12.60 ± 11.95	0.42	0.004 ± 0.004
		Ovary			14.12 [a]	0.30	0.006 [a]
		Uterus			9.24 [a]	0.20	0.004 [a]
	2500	Plasma	102.78 ± 81.44	41.70 ± 29.53			
		Testis			12.40 ± 9.07	0.12	0.002 ± 0.002
		Epididymis			27.98 ± 20.66	0.27	0.001 ± 0.001
		Prostate			20.38 ± 14.74	0.20	0.000 ± 0.000
		Seminal vesicle			23.73 ± 18.80	0.23	0.000 ± 0.000
		Ovary			13.18 ± 8.51	0.32	0.000 ± 0.000
		Uterus			9.97 ± 6.12	0.24	0.000 ± 0.000

Data are mean ± SD, n = 3 animals/sex/group, ND = Not determined.
[a] There were only 2 females in this group.
From Mitsubishi Chemical Safety Institute, Ltd. *(92)*.

Kessler et al. *(94)*, sponsored in part by the American Chemistry Council, compared blood levels of DEHP and MEHP in pregnant and non-pregnant Sprague-Dawley rats and marmosets **[strain not indicated]** in a Good Laboratory Practice (GLP) study. DEHP or deuterium-labeled DEHP were dissolved in an aqueous Tween 80/Methocel/saccharose solution that was fed to marmosets through a syringe following their first meal and administered to Sprague-Dawley rats by gavage. In most cases, the deuterated-DEHP was administered in at least 1 dose group on the days that time-course experiments were conducted in order to differentiate between background DEHP and MEHP. Non-pregnant female rats (n = 3 – 4 group) were dosed with 30, 500, or 1000 mg/kg bw. Rats in the 500 mg/kg bw group were dosed for 7 days, and time-course experiments were conducted on study days 1, 4, and 7. Pregnant rats were dosed with 30 or 500 mg/kg bw/DEHP on GD 14 – 20, and concentration time courses were determined on GD 14 and 19 **[of a 21 – 22 day gestation]**. In rats, blood samples were collected over a 24 – 48 hour period following dosing. Non-pregnant marmosets (n = 8/dose) were treated with 30 or 500 mg/kg bw/day DEHP for 29 days; concentration time courses were determined on treatment days 1 and 29. Pregnant marmosets (n = 4/dose) were dosed with 30 or 500 mg DEHP/kg bw/day on GD 96 – 125; concentration time courses were determined GD 96, 103, 117, and 124 **[of a 140 – 148 day gestation]**. On days when concentration-time courses were determined, blood samples were drawn over 15 hours following exposure of non-pregnant marmosets and 8 hours following exposure of pregnant marmosets. Because blood could be drawn only once per week from the arm vein of the marmosets, each time point of the blood sampling curve was represented by 1 animal. Blood levels of DEHP and MEHP were determined by GC/MS.

Area under the concentration–time curve (AUC) values determined in rat studies are listed in Table 18. Authors concluded that concentration time courses were similar in pregnant and non-pregnant rats and that repeated dosing had no marked effects on kinetics in either group of rats. In both groups of rats, MEHP blood AUCs were about 2 orders of magnitude higher than DEHP blood AUCs. For the non-pregnant rats, maximum concentrations for DEHP were obtained at about 1 hour following dosing; maximum concentrations of MEHP following dosing were reached at 30 minutes in the 30 mg/kg bw group, 2 hours in the 500 mg/kg bw group, and 4 hours in the 1000 mg/kg bw group. Based on normalized AUCs that were not dose dependent, the authors concluded that kinetics were linear for DEHP. The authors concluded that kinetics for MEHP were saturated based on AUC values and increased time to reach maximum concentration at higher doses.

Table 18. Normalized AUCs for Blood DEHP and MEHP in Rats Treated with DEHP

Rats	*DEHP dose (mg/kg bw/day)*	*Treatment Day*	*MEHP C_{max} (μM)*	*DEHP AUC (nmol-h/mL per mmol DEHP/kg)* [a]	*MEHP AUC (nmol-h/mL per mmol DEHP/kg)* [a]
Non-Pregnant	30	1	10	ND [b]	695 ± 113
	500	1	210	5.9 ± 3.1	1058 ± 60
		4		7.1 ± 3.1	1104 ± 423
		7		4.7 ± 0.7	1237 ± 636
	1000 [c]	1	500	8.4 ± 4.4	1756 ± 838
Pregnant	30 [d]	1 (GD 14)		8.5 ± 3.6	606 ± 77
		6 (GD 19)		21.0 ± 7.9	646 ± 42
	500 [d]	1 (GD 14)		10.0 ± 5.4	1537 ± 158
		6 (GD 19)		12.7 ± 6.3	1106 ± 230

[a] Total normalized AUC presented as mean ± SD.
[b] ND = Not determined.
[c] Deuterated DEHP administered to 2 of 4 animals.
[d] Deuterated DEHP administered to all animals.
From Kessler et al. *(94)*.

AUC values for marmosets are listed in Table 19. Concentration time courses were similar in pregnant and non-pregnant marmosets with the exception that MEHP values in the 500 mg/kg bw DEHP group were lower compared to the non-pregnant animals at GD 103 and beyond. In the non-pregnant marmosets, DEHP concentrations peaked at 2 hours following dosing; MEHP concentrations returned to starting levels within 15 hours following dosing. MEHP AUCs in pregnant and non-pregnant marmosets were more than an order of magnitude higher than DEHP AUCs and were independent of dose.

In a comparison of species differences, maximum concentrations of MEHP in rats were an average of 3.2 times higher (range 1.3–7.5) than those of marmosets. MEHP AUCs were an average of 7.3 times higher (range 2.6–15.6) in rats compared to marmosets. Based on maternal blood levels, the study authors concluded that the MEHP burden in marmoset fetuses is lower than in rat fetuses. **[The Expert Panel notes that the burden to rats compared to marmosets was not determined. The Expert Panel also notes that species differences in C_{max} and AUC between marmosets and rats are less at the lower dose levels.]**

Table 19. AUCs for Blood DEHP and MEHP in Marmosets Treated with DEHP

DEHP dose (mg/kg bw/day)	*Treatment Day*	*MEHP C_{max} (μM)*	*DEHP AUC (nmol-h/mL per mmol DEHP/kg)* [a]	*MEHP AUC (nmol-h/mL per mmol DEHP/kg)* [b]	*MEHP AUC (nmol-h/mL per mmol DEHP/kg)* [a]
Non-pregnant Marmosets					
30 [c]	1	8	8.9	172	181
	29		6.5	112	118
500	1	66	1.2d	100 d	ND
	29		2.5	123	130
Pregnant Marmosets					
30 [c]	1 (GD 96)		5.6	178	ND
	8 (GD 103)		5.2	258	ND
	22 (GD 117)		3.5	154	ND
	29 (GD 124)		6.4	245	ND
500	1 (GD 96)		12.3	170	ND
	8 (GD 103)		4.1	31	ND
	22 (GD 117)		2.8	63	ND
	29 (GD 124)		3.4	71	ND

[a] Total normalized AUC (unless otherwise indicated).
[b] Normalized AUC (up to 8 hours unless otherwise indicated).
[c] Deuterated DEHP administered to all animals.
[d] Normalized AUC up to 6 hours.
ND = Not determined.
From Kessler et al. *(94)*.

Ito et al. *(95)* evaluated enzyme activities in tissues from rats, mice, and marmosets to assess possible specifies differences in the biotransformation of DEHP. CD-1 mice and Sprague-Dawley rats were 11 weeks old and Common marmosets were 18 months old when liver, kidney, lung, and small intestine were harvested. Tissues were stored at −85ºC until used. Tissue homogenates or microsomal fractions were assayed for lipase activity based on hydrolysis of DEHP to MEHP and uridine diphosphate (UDP)-glucuronyl transferase by measuring glucuronidation of MEHP, naphthol, and bisphenol A. Alcohol dehydrogenase was measured using 2-phenoxyethanol and 2-ethylhexanol as substrates, and aldehyde dehydrogenase was measured using 2-phenylpropionaldehyde and 2-ethylhexanal as substrates. Lipase activity was highest in liver, small intestine, and kidney in mice. The lowest lipase activity was found in marmosets. Marmoset hepatic lipase activity was 4–5% that of mouse activity, and small intestine lipase activity in marmosets was < 1% of mouse small intestine activity. Rat lipase activities

in these organs were intermediate between mouse and marmoset. Lipase activities were comparably low in rat and mouse lung and were undetectable in marmoset lung. UDP-glucuronyl transferase was detectable only in liver in the 3 species. Although activity was greater in mouse than marmoset, the difference between species was not as great as for lipase. Alcohol and aldehyde dehydrogenases were higher in marmoset than in rodents; however, the authors concluded that the possible increased ability of marmosets over rodents to convert MEHP to its ω-oxidation products was unlikely to be important given the small amount of MEHP that would be expected to be generated in marmosets from oral or IV exposures.

An earlier study *(96)* evaluated the hydrolysis of phthalates, including DEHP, in rat, ferret, baboon, and human liver and intestine. While the rates for intestinal hydrolysis in rat, ferret, and human were similar, with ferret > rat > human, the rate for baboon intestine was some 3-fold higher than that of the ferret.

Ono et al. *(97)* evaluated the testicular distribution of DEHP in 8-week-old Sprague-Dawley rats. The rats were given a single gavage dose of DEHP 1000 mg/kg bw, radiolabeled either in the ring or the aliphatic side chains. The animals were perfusion-fixed with paraformaldehyde and glutaraldehyde under anesthesia 6 or 24 hours after DEHP administration (n = 4 animals/time point). Testis, liver, and kidney were collected and processed for light and electron microscopic autoradiography. After ring-labeled DEHP was given, light microscopy showed preferential distribution of grains to the basal portions of stage IX – I tubules at 6 hours. Grain counts were high in the kidney at 6 hours at the epithelial brush border and the abluminal cytoplasm of the proximal tubule. At 24 hours, grain counts in testis and kidney were much reduced, and hepatic grain counts were increased in a centrilobular distribution in the liver. Electron microscopic autoradiography of Stage IX – I seminiferous tubules 6 hours after ring-labeled DEHP showed grains in Sertoli cell smooth endoplasmic reticulum and mitochondria. There were also grains at cell-junctions involving neighboring Sertoli cells and Sertoli-germ cells. Fewer grains were seen in the Sertoli cell Golgi apparatus and lysomes and in spermatocyte cytoplasm. By contrast, administration of side arm-labeled DEHP resulted in few grains in the seminiferous epithelium and 6 hours and no grains in any tissue examined at 24 hours. The authors concluded that phthalic acid is transported into tissue after DEHP administration and is responsible for the testicular toxicity of both DEHP and MEHP.

2.2 General Toxicity and Carcinogenicity

Conclusions in recent reviews by the FDA, Health Canada, and the European Commission are summarized in Table 20. **[The Expert Panel notes the conclusions in this table are based on the presumed lack of peroxisome proliferator-activated receptor (PPAR)α-mediated toxicity. It may be premature to decide that effects mediated through PPARα are not relevant in humans (FDA, 2004 #200). Although peroxisome proliferation, mediated by PPARα, occurs in rodents but not in humans, nevertheless, humans do have a functional PPARα nuclear receptor.]**

Table 20. DEHP Conclusions by US, Canadian, and European Agencies

Topics	*Agency*			
	FDA (2)	*ATSDR (101)*	*Health Canada (102)*	*European Commission (1)*
Most sensitive target organ	Testis	Testis	Testis and conceptus	Testis
Other possible targets of toxicity	One study suggested that DEHP could contribute to hyaline membrane disease in mechanically ventilated children. Factors such as poor bowel perfusion more likely contribute to necrotizing enterocolitis in newborns than DEHP.	Although confounded, there is some evidence suggesting that DEHP released from PVC tubing during respiratory ventilation can cause lung disorders in children.		Suspicions about development of polycystic kidney disease in patients undergoing hemodialysis have not been confirmed by clinical evidence. Causation cannot be determined for the role of DEHP in pathological lung effects in ventilated preterm infants. Evidence suggests that DEHP is not a causative agent of hepatoblastoma.
Genetic toxicity		The weight of evidence indicates that DEHP is not genotoxic.		
Cancer		Mechanisms of liver cancer in rats and mice are not relevant to humans.	Concurs with IARC conclusion that mechanisms of liver tumors in rodents are not relevant to humans.	There are no concerns about carcinogenicity in humans, based on animal studies.
Sensitive populations	Children receiving some medical treatments may receive a higher dose on a mg/kg bw basis than adults. Compared to adults, children may absorb greater amounts of DEHP due to greater intestinal permeability, may more effectively convert DEHP to MEHP (the toxic metabolite) due to higher levels of intestinal lipases, and may less effectively excrete MEHP due to reduced glucuronidation.	Infants have higher levels of gastric lipases and may be more able to convert DEHP to MEHP. Permeability of blood-testis barrier is higher in children. There appear to be few indications of biological polymorphisms that increase sensitivity. Younger animals appear to be more sensitive to DEHP-induced toxicity than older animals.	Populations at highest risk of DEHP toxicity include newborns, infants, toddlers, and children with critical illnesses. Populations with unknown risk of toxicity include breast-fed children, the fetus, and pre-pubescent males.	There is evidence of greater DEHP sensitivity in immature compared to mature animals.

Topics	*Agency*			
	FDA (2)	*ATSDR (101)*	*Health Canada (102)*	*European Commission (1)*
Sensitive populations *(continued)*	Children may be more pharmacodynamically sensitive to DEHP than adults (e.g., increased permeability of the blood-testis barrier). DEHP may exacerbate zinc and vitamin E deficiencies, which are not uncommon in preterm infants. There are polymorphisms in genes coding for pancreatic lipase. There are polymorphisms in several UDP-glycuronyltransferase genes.			
Species differences	Liver effects mediated through PPARα do not appear relevant to humans. **[The Expert Panel does not necessarily concur with this conclusion inasmuch as a functional PPARα receptor does occur in humans.]**	Liver effects mediated through PPARα do not appear relevant to humans. DEHP hydrolysis rates are highest in mouse > rat > guinea pig > hamster > humans and primates. Primates are more efficient at glucuronidating metabolites but less effective at oxidizing metabolites than rodents.		It is believed that DEHP toxicity in rodents is mediated through the PPARα receptor, which is less relevant in humans; however, there is also evidence that PPARα-independent toxicity also occurs. Mechanisms of adverse effects in rodents do not appear to be of great significance in non-human primates; evidence that those mechanisms apply to humans is lacking.
Acceptable limits	TI (oral) = 0.04 mg/kg bw/day. TI (parenteral) = 0.6 mg/kg bw/day.	MRL = 0.1 mg/kg bw/day for oral exposures of intermediate duration (15–364 days). MRL = 0.06 mg/kg bw/day for oral exposures of chronic duration (≥ 365 days).		No Tolerable Intake Value can be recommended regarding use of DEHP in medical devices.

Topics	*Agency*			
	FDA (2)	***ATSDR (101)***	***Health Canada (102)***	***European Commission (1)***
Situations where DEHP exposures may be of toxicological concern	". . . children undergoing certain medical procedures may represent a population at increased risk for the effects of DEHP." Medical procedures of possible concern include: TPN in infants and pregnant women, enteral nutrition, exchange transfusions in infants, ECMO in infants and adults, aggregate exposures of neonates in NICU; cardiopulmonary by-pass surgery may lead to high exposure but exposures vary widely depending on use of heparin-coated tubing.		Subpopulations at greatest risk: ECMO patients, cardiopulmonary by-pass patients, infants, and children receiving exchange transfusions, patients receiving some IV therapies such as TPN and lipophilic drug formulations. Subpopulations with possible but undetermined risk: trauma patients receiving multiple blood transfusions, hemodialysis patients, patients receiving oxygen therapy.	Premature infants are a particular risk group because they can be exposed to high DEHP concentrations through blood transfusions, ECMO, and respiratory therapy.
Situations not likely to result in toxicological concern	Infusion (IV) of crystalloid fluids and drugs. TPN in adults. Blood transfusions. Hemodialysis and peritoneal dialysis.	Ambient levels in environment.	DEHP levels do not pose a danger to the environment on which human life is dependent.	

ECMO extra corporeal membrane oxygenation
MRL minimal risk level
TI tolerable intake.

Stroheker et al. *(98*) evaluated the anti-androgenic activity of DEHP in a modified Hershberger assay using Wistar rats. Male offspring were weaned and randomized by weight at 20 days of age **[day of birth not defined]**. On the following day, the animals were castrated and allowed to recover for 1 week. DEHP (>99% purity) in corn oil was given by gavage for 10 days at 0, 200, 400, 600, 800, or 1000 mg/kg bw/day in the first experiment and 0, 4, 20, or 100 mg/kg bw/day in the second experiment (n=8/treatment group). In both experiments, testosterone propionate 0.4 mg/kg bw/day was given subcutaneously (sc) on the same days as the DEHP treatments. The animals were weighed and killed 24 hours after the last treatment and relative weights were determined for the seminal vesicles, prostate, and bulbocavernosus/levator ani muscles. As expected, testosterone propionate treatment produced a significant increase in the relative weight of all accessory sex organs compared to vehicle-treated control. A significant impairment of the testosterone propionate-induced organ weight increase occurred with DEHP treatment beginning at 100 mg/kg bw/day for the bulbocavernosus/levator ani muscles, 200 mg/kg bw/day for the prostate, and 400 mg/kg bw/day for the seminal vesicles. The authors concluded that DEHP treatment has anti-androgenic effects but does not inhibit 5α-reductase because bulbocavernosus/levator ani muscles, the most sensitive organs, are only testosterone-responsive, whereas prostate is only dihydrotestosterone-responsive, and seminal vesicles are responsive to both androgens. **[The Expert Panel noted that it is not clear if testosterone propionate data were combined or compared separately for the two blocks. The lack of dose-response was noted.]**

In the same report, Stroheker et al. *(98*) evaluated DEHP, MEHP, 5-oxo-MEHP, and 5-OH-MEHP in an androgen receptor-positive breast cancer cell line stably transfected with a luciferase reporter gene. The cell line showed an 81% decrease in dihydrotestosterone-induced luciferase activity after exposure to the positive control nilutamide, an androgen receptor antagonist, at 10^{-6} M. DEHP and MEHP were added to cultures at log unit concentrations ranging from 10^{-10} to 10^{-5} M. The secondary DEHP metabolites 5-oxo- and 5-OH-MEHP were added at 10^{-10} to 10^{-8} M, limited by solubility or cytotoxicity. **[The method for evaluating cytotoxicity was not described.]** There was no inhibition of dihydrotestosterone stimulation of luciferase activity at any tested concentration of DEHP or MEHP. Both 5-oxo- and 5-OH-MEHP inhibited luciferase activity at all tested concentrations to 40–70% of control levels **[estimated from a graph]**. The authors concluded that although *in vivo* anti-androgenic activity of DEHP could be indirect, due to increased catabolism of testosterone, it might alternatively be due to the anti-androgenic activity of the 5-oxo- and 5-OH-MEHP metabolites. **[The lack of a dose response with the oxidative metabolites, combined with a lack of clear understanding of the mechanism by which these compounds reduced luciferase activity, reduces the usefulness of these data.]**

Roy et al. *(99*) evaluated DEHP in a recombinant cell-based *in vitro* assay for anti-androgenicity. Chinese Hamster ovary cells were stably transfected with human androgen receptor and an androgen-dependent luciferase reporter. The androgen receptor agonist R1881 was used at a half maximally stimulating concentration of 0.1 nM. Cyproterone acetate and hydroxyflutamide were used to check that the assay responded to anti-androgens. A panel of 60 compounds was tested, including DEHP, which was negative in the assay. **[The report did not give the tested concentration(s) of DEHP. The Expert Panel notes that testing of MEHP would have been preferable to the testing of DEHP in this assay.]**

Hwang et al. *(100*) evaluated DEHP in a novel double-transgenic mouse assay for anti-androgenicity. The transgenic animal co-expressed the tetracycline-controlled transactivator and human CYP1B1. Expression of human CYP1B1 in this model was high during the neonatal period and decreased in

adult males. Castration resulted in an increase in CYP1B1, which could be suppressed with testosterone treatment. Flutamide, an anti-androgen, was shown to increase CYP1B1 in intact adult transgenics. DEHP [purity not given] in corn oil was administered as a single sc dose to 10-week-old transgenic mice at 0, 100, 500, or 1000 mg/kg bw (5 mice/group). Total ribonucleic acid (RNA) was extracted from livers 3 days later and amplified by reverse-transcriptase polymerase chain reaction (RT-PCR). Microsomal protein was harvested, and human CYP1B1 was detected by Western blotting. CYP1B1 activity was determined by measurement of the dealkylation of benzyloxyresorufin. Statistical analysis used 1-way analysis of variance (ANOVA) **[post hoc test not specified]**. In a separate experiment **[described in the Results section]**, transgenic mice were treated with DEHP 0 or 1000 mg/kg bw/day on days 1, 3, and 9 or for 1, 3, or 9 consecutive days. **[The text of the Results section describes the first dosing schedule, and a figure legend describes the second dosing schedule. Evaluation of RNA, protein, and activity were performed at unspecified times after dosing.]**

There was a dose-related increase in CYP1B1 transcript, CYP1B1 protein, and CYP1B1 activity, with a significant increase in transcript at 500 and 1000 mg/kg bw and an increase in protein and activity at all doses compared to the control values for each of the assays. Transcript, protein, and activity showed a duration-related increase with treatments labeled 1, 3, and 9 day. **[The Expert Panel notes that di(n-butyl) and diethyl phthalate were tested in the same model and showed responses in the graphic representation of the 1, 3, and 9-day results that were similar to the DEHP response. Linuron also showed a duration-related increase in response.]**

The authors concluded that the double-transgenic model they described was a useful test for anti-androgenic activity. **[The Expert Panel notes lack of a readily discernable androgen-dependent link between the double-construct and its response to anti-androgens; the Panel was unable to discern why this construct should react to anti-androgens. The lack of flutamide in the group of test compounds, as well as the absence of any metabolism-requiring androgen-receptor negatives, only raises the level of concern that this construct is really reporting a metabolic need and has nothing to do with androgenicity. Diethyl phthalate should have served as a negative control in the Hwang paper *(100)*, and the fact that it did not indicates that the assay is not specific for anti-androgenic activity.]**

Kim et al. *(103)* evaluated DEHP and butyl benzyl and dibutyl phthalate for the ability to inhibit tamoxifen-induced apoptosis in MCF-7 cells in culture. Tamoxifen caused a concentration-related decrease in MCF-7 cell viability. The phthalates increased MCF-7 cell proliferation with DEHP 10 μM **[3.9 mg/L]** for 24 hours, giving rise to 133% of the control number of cells **[estimated from a graph]**. 17β-Estradiol, the positive control, gave rise to 158% of the control number of cells **[estimated from a graph]** at a concentration of 1 nM. By contrast, none of the treatments affected the number of estrogen receptor-negative MDA-MB-231 cells. Coadministration of DEHP 10μM and tamoxifen for 24 hours resulted in 72% survival compared to the control culture, compared to 93% survival after coadministration of 17β-estradiol 1 nM and tamoxifen. Tamoxifen alone resulted in 59% survival. Tamoxifen was shown to decrease the anti-apoptotic Bcl-2 protein and increase the pro-apoptotic Bax protein in the MCF-7 cells. The Bcl-2:Bax ratio was increased by 17β-estradiol and by the phthalates, including DEHP.

Hong et al. *(104)* evaluated the activity of DEHP and diethyl, benzylbutyl, dibutyl, and dicyclohexyl phthalate on MCF-7 cells in culture and on uterine calbindin-D9k in preweaning Sprague-Dawley rats.

Both MCF-7 proliferation and an increase in uterine calbindin-D_{9k} were considered to be estrogenic endpoints. In the MCF-7 assay, ethinyl estradiol and 17β-estradiol were used as positive controls and induced a 9-fold increase in cell proliferation (relative to vehicle control) at concentrations of 10^{-5} M. DEHP produced a 6-fold increase in proliferation at a concentration of 10^{-4} M **[39 mg/L]** and no significant increase in proliferation at 10^{-5} M. In the calbindin-D_{9k} assay, DEHP in corn oil was given at 0 or 600 mg/kg bw/day on postnatal day (PND) 14–16 and uteri were harvested on PND 17. Calbindin-D_{9k} messenger RNA (mRNA) and protein were assayed. Ethinyl estradiol and diethylstilbestrol, the positive controls, increased calbindin-D_{9k} mRNA and protein, but DEHP and the other phthalates had no effect. The authors suggested that the phthalates may have been metabolized in the tissues of the intact rats with consequent loss of their estrogenic activity. **[The Expert Panel notes that these authors did not evaluate whether the MCF-7 cell response to phthalates was estrogen receptor-mediated.]**

Voss et al. *(105)* administered DEHP (>99% purity) in the diet to male Sprague-Dawley rats beginning at an age of 90–110 days and continuing for the entire lifetime of the animals (up to 159 weeks). DEHP was administered in feed at 0, 600, 1897, and 6000 mg/kg diet, given in 5 g feed/100 g bw/day 6 days/week. On the 7th day of the week, animals received DEHP-free feed after their DEHP-treated feed had been completely consumed. DEHP dose levels were 0 (n=390), 30 (n=180), 95 (n=100), and 300 (n=60) mg/kg bw/day **[6 days/week unless residual treated feed was consumed on the 7th day. Daily feed consumption was not reported.]** The number of animals in each group was chosen based on anticipated tumor incidence, with larger numbers of animals in groups expected to have a lower incidence of tumors. Animals were killed when moribund if they did not die spontaneously, and all animals were necropsied after death. Brain, liver, adrenals, testes, thyroid, lungs, spleen, and macroscopic lesions were fixed in 7% formalin, sectioned in paraffin, and examined by light microscopy after staining with hematoxylin and eosin. Livers were weighed, and liver slices were fixed in Carnoy fluid. In addition to hematoxylin and eosin-stained sections, liver evaluation included treatment with periodic acid-Schiff with orange G and iron hematoxylin counterstaining. Statistical evaluation was performed with Kruskal-Wallis and chi-squared tests.

The animals fed DEHP in all dose groups experienced a transient absolute weight reduction compared to control animals about 300 days after the beginning of the experiment, but weights were comparable thereafter. The authors described a dose-dependent increase in liver weight, reaching 108% of control values in the highest dose group but indicated that liver weights were not statistically different from controls. There was no effect of DEHP treatment on survival time of the animals. The proportion of animals with malignant and benign tumors, overall, was not affected by treatment; however, detailed evaluation of livers of the sacrificed animals showed a 29.0% incidence of all neoplasms in the highest DEHP dose group compared to 9.0% of control animals ($P=0.005$). Although the lower 2 DEHP dose groups did not have a statistically increased incidence of hepatic neoplasms on pair-wise comparison with the control, trend testing showed a significant trend over the dose ranges ($P=0.001$).

Leydig cell tumors occurred in 28.3% of animals in the highest dose group compared to 16.4% of control animals ($P=0.038$), and a dose-related trend was identified in Leydig cell tumors over the dose range ($P=0.019$). The association of DEHP treatment with Leydig cell tumors extended to analysis of unilateral, bilateral, and multifocal unilateral tumors. When the lifetimes of the animals were divided into 3 periods (0–750, 750–950, and 950–1250 days), the associations between total and unilateral Leydig

cell tumors and DEHP dose level were most evident during the middle period. Bilateral and multifocal unilateral tumors showed dose-related DEHP increases during the third period. The authors postulated that the DEHP-associated increase in Leydig cell tumors might be due to an increase in gonadotropin production secondary to decreased testosterone synthesis or increased testosterone aromatization.

2.3 Summary of General Toxicology and Biologic Effects

2.3.1 Toxicokinetics

As discussed in Section 1, exposure studies in humans measuring primary and secondary urinary metabolites (MEHP, 5-OH-MEHP, 5-oxo-ME HP) suggest aged-related differences in production and/or clearance. Younger children produce higher proportions of 5-OH-MEHP and 5-oxo-MEHP compared to MEHP, and this difference increases with decreasing age. Furthermore, as noted in the first Expert Panel Report on DEHP, premature and term infants have reduced renal clearance based on decreased glomerular filtration rates and immature glucuronidation, which may increase the internal doses of toxic metabolites. Data from Calafat *(106)* and Silva *(44)* (reviewed in Section 1) show that oxidative metabolites are present in free (unconjugated) form in breast milk and amniotic fluid, which may pose additional risk from these metabolites. Finally, the Expert Panel notes, as mentioned in the initial Expert Panel Report on DEHP, that neonates have lingual, gastric, and intestinal lipases that would need to be quantified in comparison to adults levels in order to assess DEHP conversion rates. Breast milk also contains lipases. The relative activities of these combined systems would determine gut absorption in the newborn and young infant and need to be elucidated.

An unpublished report from Mitsubishi Chemical Safety Institute, Ltd. *(92)* included a toxicokinetic study in marmosets at 3 and 18 months of age. The 18-month-old animals included a group that had been pretreated with radiolabeled DEHP for 65 weeks and a treatment-naïve group. Blood was drawn at intervals during the week following a single dose of 100 or 2500 mg/kg bw. Two weeks later, an additional oral dose was given and tissues were sampled 2 hours later. The report presented radioactivity contents, expressed as μg equivalents, without characterization of unchanged DEHP or DEHP metabolites. There was no apparent effect of chronic DEHP treatment on toxicokinetic parameters or organ distribution at 18 months of age. Concentrations of radiolabel in testis were 9–23% of plasma concentrations, and the authors remarked that the small amount of DEHP and metabolites distributed to the marmoset testis might explain the lack of testicular toxicity noted by them in a 65-week feeding study (discussed in Section 4.2.3). **[The Expert Panel noted that given the large variability in t_{max} (1–10 hours, Table 16), the organ to plasma ratio of radiolabeled DEHP (Table 17) collected at a fixed time (2 hours) may not accurately reflect age- and dose-related differences. Furthermore, the small sample size combined with the large inter-individual variability complicates interpretation of the data and may not accurately reflect dose- and age-related differences.]**

A GLP study compared blood levels of DEHP and MEHP in pregnant and non-pregnant rats and marmosets dosed with 30 or 500 mg/kg bw/day DEHP *(94)*. The study authors concluded that concentration time courses were similar in pregnant rats receiving both dose levels by gavage on GD 14–20 and non-pregnant rats given 500 mg/kg bw/day for 7 days; repeated dosing had no marked effects on kinetics in either group of rats. In both groups of rats, MEHP AUCs were about 2 orders of magnitude higher than DEHP AUCs. The authors concluded that kinetics for DEHP were linear, while kinetics for MEHP were saturated. In marmosets fed 30 or 500 mg/kg bw/day DEHP through

a syringe, concentration time courses (based on 1 marmoset per time point) were similar in pregnant animals dosed on GD 96–125 and non-pregnant animals dosed for 29 days; the exception was that MEHP values in the 500 mg/kg bw DEHP group were lower compared to the non-pregnant animals on GD 103 and beyond. MEHP AUCs in pregnant and non-pregnant marmosets were more than an order of magnitude higher than DEHP AUCs and were independent of dose. In a comparison of species differences, maximum blood concentrations of MEHP in rats were an average of 3.2 times higher (range 1.3–7.5) than those of marmosets. MEHP AUCs were an average of 7.3 times higher (range 2.6–15.6) in rats compared to marmosets**. [However, the small marmoset sample size prevents a lack of a good understanding of inter-individual variability and reduces confidence in the comparisons of C_{max} and AUC between marmosets and rats, which is reflected by the wide range in rat to marmoset AUC ratios. The Expert Panel also notes that species differences in C_{max} and AUC between marmosets and rats are less at the lower dose levels.]**

A study using tissues from rats, mice, and marmosets evaluated the activities of enzymes involved in the metabolism of DEHP *(95)*. Marmoset small intestine, liver, and kidney appear unable to convert DEHP to MEHP to any great extent, based on enzyme activities *in vitro*. [**The Expert Panel believes that it would have been better, from an experimental point of view, if lipase secreted into the lumen of the gut had been measured.**]

2.3.2 General Toxicity and Carcinogenicity

The anti-androgenic activities of DEHP and some of its metabolites were evaluated by Stroheker et al. *(98)*. Treatment of castrated 21-day-old Wistar rats with DEHP prevented an increase in accessory sex-organ weight after testosterone propionate. The bulbocavernosus/levator ani muscles were the most sensitive to this DEHP effect, and the prostate was the least sensitive, leading the authors to conclude that DEHP did not inhibit 5α-reductase. Evaluation of DEHP, MEHP, and the secondary DEHP metabolites, 5-oxo- and 5-OH-MEHP, in an androgen receptor-positive cell line showed no antagonism by DEHP or MEHP of the activity of the androgen receptor agonist dihydrotestosterone. Both 5-oxo- and 5-OH-MEHP showed significant dihydrotestosterone antagonism. The authors concluded that the *in vivo* anti-androgenic effects of DEHP could be mediated through the secondary metabolites. **[The lack of a dose response with the oxidative metabolites combined with a lack of clear understanding of the mechanism by which these compounds reduced luciferase activity reduces the usefulness of these data.]**

Voss et al. *(105)* administered DEHP at 0, 600, 1897, and 6000 mg/kg feed in the diet to male Sprague-Dawley rats beginning at an age of 90–110 days and continuing for the entire lifetime of the animals. DEHP dose levels were 0, 30, 95, and 300 mg/kg bw/day **[6 days/week unless residual treated feed was consumed on the 7th day]**. The animals fed DEHP experienced a transient weight reduction compared to control animals about 300 days after the beginning of the experiment, but weights were comparable thereafter. The authors described a dose-dependent increase in liver weight, reaching 108% of control values in the highest dose group, but indicated that liver weights were not statistically different from controls. There was no effect of DEHP treatment on survival time of the animals. The proportion of animals with malignant and benign tumors, overall, was not affected by treatment; however, detailed evaluation of livers of the sacrificed animals showed a 29% incidence of all neoplasms in the highest DEHP dose group compared to 9% of control animals ($P=0.005$). Although the lower 2 DEHP dose groups did not have a statistically increased incidence of hepatic

neoplasms on pair-wise comparison with the control, trend testing showed a significant trend over the dose ranges ($P = 0.001$). Leydig cell tumors occurred in 28.3% of animals in the highest dose group compared to 16.4% of control animals ($P = 0.038$), and a dose-related trend was identified in Leydig cell tumors over the dose range ($P = 0.019$). The authors postulated that the DEHP-associated increase in Leydig cell tumors might be due to an increase in gonadotropin production secondary to decreased testosterone synthesis or increased testosterone aromatization.

3.0 DEVELOPMENTAL TOXICITY DATA

3.1 Human Data

Since the initial CERHR Expert Panel Report on DEHP, there have been several studies in humans in which development of the male reproductive system has been evaluated with respect to estimates of DEHP exposure during pregnancy or early childhood. There has also been a study addressing premature breast development and DEHP exposure.

Latini et al. *(107)*, funding not indicated, conducted a study to examine the effects of prenatal exposure to DEHP and MEHP. Cord blood samples were collected from 84 consecutive newborns (including a set of twins) delivered at an Italian hospital. Ages of mothers ranged from 18 to 42 years. DEHP and MEHP levels were measured in cord blood serum by HPLC. Glass equipment was used in sample preparation and analyses to avoid phthalate contamination. Analyses were conducted to determine possible relationships between phthalate exposure and adverse neonatal outcomes. Relationships between phthalates in cord blood and outcomes in infants were assessed by Fisher exact test and unpaired *t*-tests. Significance levels for multiple *t*-tests were Bonferroni corrected. Multivariable logistic regression models were used to evaluate significant differences in univariate analyses and effects from potential confounders. DEHP and/or MEHP were detected in 74 of 84 cord blood samples. Mean (range) cord blood serum concentrations were 1.19 (0–4.71) μg/mL for DEHP and 0.52 (0–2.94) μg/mL for MEHP. Mean gestational age was significantly lower in MEHP-positive neonates (38.16 ± 2.34 **[SD]** weeks) versus MEHP-negative neonates (39.35 ± 1.35 weeks; $P = 0.033$). There were no significant associations between DEHP or MEHP concentrations and infant sex, delivery mode, maternal smoking, premature membrane rupture, cord loops, neonatal jaundice, small infant size, birth weight, 1- or 5-minute Apgar scores, or maternal age. **[With the exception of birth weight, data were not shown for these endpoints.]**

The study authors concluded that their study demonstrated the presence of DEHP/MEHP in most newborns, and that phthalate exposure is associated with shorter pregnancy duration.

Strengths/Weaknesses: Use of cord blood, which reflects infant exposure *in utero*, is a strength as is the use of consecutive births at same hospital. Outcome assessment determination blind to exposure status is a strength. However, the levels measured in blood were unusually high and led the Expert Panel to wonder whether pre-analytic contamination occurred or the wrong units were reported. In addition, the blood samples were not pretreated and in a previous publication on a subgroup of the current study *(42)*, these authors saw no significant correlations between maternal DEHP/MEHP and cord blood DEHP/MEHP.

Utility (Adequacy) for CERHR Evaluation Process: Because the Expert Panel cannot resolve whether the unusually high reported blood levels represent an error in units (μg instead of ng) or were the result of pre-analytic contamination, this study cannot be used in the evaluation process.

Swan et al. *(108)*, supported by the Environmental Protection Agency (EPA), National Institutes of Health (NIH), and the state of Iowa, evaluated anogenital distance in children and maternal urinary phthalate monoester concentrations. Pregnant women in 1 of 3 US cities were recruited as part of a larger study. Urine samples were collected from women at a mean gestational age of 28.3 weeks. Data from the 134 sons of 172 women who were eligible for this part of the study were used to assess the associa-

tion between anogenital index (AGI; anogenital distance adjusted for body weight) and other genital parameters such as testicular descent. Exclusion criteria included mother-son dyads with incomplete information, lack of consent for genital examination, and child age greater than 18 months, considered to make anogenital distance measurement unreliable due to movement. Of these 134 sons, 85 had phthalate measurements used in the analyses of association between phthalates and anogenital index. The urine analytes included metabolites of DEHP (MEHP, 5-oxo-MEHP, and 5-OH-MEHP) and monobutyl, monobenzyl, mono-3-carboxypropyl, monoethyl, mono-isobutyl, and monomethyl phthalate. Analysis was performed by HPLC-tandem MS after enzymatic deconjugation. Infant evaluation included height, weight, head circumference, skin-fold thickness, anogenital distance (measured from the midpoint of the anus to the anterior base of the penis), anoscrotal distance (measured from the midpoint of the anus to the posterior insertion of the scrotum), and detailed examination of the breasts and genitalia.

The relationship between maternal pregnancy urinary phthalate concentration (logarithmically transformed) and anogenital distance was evaluated using general linear models. Analyte concentrations below the limit of detection were considered to be the limit of detection value divided by the square root of 2. Data were also analyzed using a categorical approach based on 25th and 75th percentiles for age- and weight-adjusted anogenital distance and 25th and 75th percentiles for analyte concentrations. **[Categorical analysis was reported only for the 4 analytes associated with decreased anogenital index.]** Potential confounders considered in regression analysis included mother's ethnicity, smoking status, time and season of urine collection, gestational age at time of urine collection, and infant weight at examination. **[Confounding by clinic site, calendar time, and maternal education was not assessed.]** In addition to using individual urinary phthalate monoester concentrations in the analysis, the authors constructed a total phthalate score based on quartiles of individual phthalate concentrations **[using only the concentrations of monobutyl, monobenzyl, monoethyl, and mono-isobutyl phthalate, which had been shown to be associated with anogenital index, discussed below]**. Individual phthalate concentrations in the lowest quartile made no contribution to the total phthalate score. One point was given for each quartile above the lowest quartile. The calculation of total phthalate score led to the trichotomizing of samples into categories of low (score 0 – 1), intermediate (2 – 10), and high (11 – 12).

The final regression model, including only age and age-squared as covariates, showed an inverse relationship between logarithmically transformed analyte concentration and weight-adjusted anogenital distance for monobutyl ($P = 0.031$), monobenzyl ($P = 0.097$), monoethyl ($P = 0.017$), and mono-isobutyl phthalate ($P = 0.007$). The regression coefficient for MEHP was – 0.051 ($P = 0.833$). The regression coefficient for 5-oxo-MEHP was – 0.412 ($P = 0.114$), and the regression coefficient for 5-OH-MEHP was – 0.398 ($P = 0.145$). The authors noted that the regression coefficients for the oxidative MEHP metabolites were of similar magnitude to the coefficients for monobutyl and monobenzyl phthalate (– 0.592 and – 0.390). The authors indicated that DEHP shortens anogenital distance in rodents and that it was not possible to tell if the urinary concentrations of MEHP and its oxidative metabolites failed to be associated significantly with anogenital index in children because of sample size limitations or because humans and rodents responded differently to DEHP.

The relationship between testicular descent (normal or normal-retractable versus one or both testicles incompletely descended) and anogenital index was assessed in 134 boys. The proportions of boys with one or both testicles incompletely descended were 20.0, 9.5, and 5.9% for boys with short AGI (below the 25th percentile), intermediate (25th – 75th percentile), and long (75th percentile or higher) (*P* value

for short AGI vs. all others <0.001.) **[The Expert Panel was not able to confirm this *P* value from the data presented in the paper.]**

Strengths/Weaknesses: The prospective nature of the study and the collection of urine for exposure assessment during pregnancy, reflecting *in utero* exposure, are strengths as is the measurement of urinary metabolites rather than parent compounds, avoiding contamination issues. The masking of clinicians measuring anogenital distance to the laboratory assessment of phthalates and vice versa are additional strengths. The choice of anogenital distance as an endpoint is consistent with a sensitive endpoint in rodents. However there were no data presented on the reliability of the measurement of anogenital distance or other variables that may be associated with anogenital distance. Methods used to determine independent or combined effects of various phthalates (creation of summary score) were not appropriate for that purpose. A weakness of the study is that potential confounding by clinic, education, and calendar time was not assessed.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

Main et al. *(17)*, supported by the European Commission, the Danish Medical Research Council, the Svend Andersen and Velux Foundation, the Turku University Central Hospital, and the Academy of Finland, studied the association of breast milk levels of MEHP and other phthalates and blood levels of reproductive hormones in 3-month-old boys. **[Milk concentrations were discussed in Section 1.1.1.]** Pooled milk samples were obtained from each of 130 women (half from Denmark and half from Norway) when their children were 1–3 months old. Milk was analyzed using HPLC-MS for MEHP as well as monomethyl, monoethyl, monobutyl, monobenzyl, and mono-isononyl phthalate. Cryptorchidism was identified in 62 of the 130 children of these women; however, there was no significant association between milk phthalate concentrations and cryptorchidism. The children had venous blood sampled at 3 months of age for determination of sex hormone-binding globulin, total and free testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), and inhibin B. Individual hormone levels were used to calculate LH/testosterone, LH/free testosterone, and FSH/inhibit B ratios. Multiple regression analysis was used to explore relationships between log-transformed milk phthalate concentrations and hormone levels using gestational age at birth, weight for gestational age, parity, smoking, diabetes, and country of origin as potential confounders. Only country of origin was retained as a confounder. Associations between milk phthalate levels and hormone levels were then tested with country-adjusted partial Spearman correlations with exact *P*-values obtained using Monte Carlo permutation.

MEHP was found in all milk samples. Milk concentration of MEHP was observed to have a marginally significant correlation with free testosterone (Spearman $r=-0.169$, $P=0.107$) and inhibin B ($r=0.185$, $P=0.075$). In addition, associations were observed with three ratios: LH/testosterone ($r=0.180$, $P=0.087$), LH/free testosterone ($r=0.175$, $P=0.095$), and FSH/inhibin B ($r=-0.204$, $P=0.050$). **[The Expert Panel places more weight on individual hormone measures rather than the hormone ratios (LH/testosterone, LH/free testosterone), because these ratios are not biologically relevant in a non-clinical setting. The Expert Panel notes that 9 hormones or ratios of hormones were evaluated for each of 6 phthalate monoesters, yielding multiple comparisons without adjustment. The Expert Panel also has concerns about the adequacy of control for country differences both as a potential confounder and as an effect modifier. The Expert Panel is also concerned about possible contamination by use of breast pumps after the feeding of the infant.]**

The authors concluded that there were "subtle, but significant, dose-dependent associations between neonatal exposure to phthalate monoesters in breast milk and levels of reproductive hormones in boys at three months of age."

Strengths/Weaknesses: Strengths included collection of breast milk to assess exposure during the first 3 months of life, drawing of blood samples at the 3-month visit to assess hormone levels, the analyses of phthalates conducted blind to case status and hormone levels, and the assessment of hormone levels conducted blind to case status and phthalate levels. Weaknesses include possible contamination of breast milk samples. Women who used a breast pump in Denmark had significantly higher levels of monoethyl and monobutyl phthalate. Breast pump-associated levels of other phthalates were not significantly different, but data were not shown and breast pump use was not reported for the Finnish population. Confounding was not assessed for comparison of phthalate levels between cryptorchid cases and controls, although the authors stated that there was no significant difference stratified by country. The small sample size may have yielded limited power for stratified analyses, and confounding was not assessed by other variables. (It appears that cases and controls may have differed on prevalence of maternal diabetes and gestational age even though differences did not reach statistical significance.) Statistical analyses of associations between phthalate levels and hormone levels were not presented clearly. Confounding was assessed with multiple regression on log transformed data, but it appears that associations were assessed with a rank-based model adjusting for country. It was also not stated what criteria were used to assess confounding. The authors stated that parity, maternal smoking, gestational age, and weight for gestational age were not "significant" confounders. Confounding is different than statistical significance; therefore, it is not clear that confounding was adequately assessed. The sample size was small.

Utility (Adequacy) for CERHR Evaluation Process: This study is of some utility for suggesting an association between testosterone and MEHP, but concerns remain about assessment of confounding and contamination by breast pump use.

Rais-Bahrami et al. *(109)*, sponsored by NIH, examined onset of puberty and sexual maturity parameters in 14 – 16-year-old adolescents who had been subjected to ECMO as neonates; the procedure potentially led to high DEHP exposure. The adolescents included 13 males and 6 females. Measurements taken during physical examinations included height, weight, head circumference, testicular volume, and phallic length. Pubertal staging was conducted according to the Tanner method. Laboratory tests were conducted to assess thyroid, liver, and renal function. LH and FSH levels were measured in both sexes, estrogen levels were measured in females, and testosterone levels were measured in males. Except for 1 female with Marfan syndrome, growth percentiles were normal for age and sex. Pubertal development was stated to be normal. **[The authors did not state whether testicular volume and phallic length were normal.]** Laboratory results indicated normal thyroid, liver, and renal function. LH, FSH, testosterone, and 17β-estradiol levels were normal for stage of pubertal development. **[A control group of children who did not receive ECMO treatment as neonates was not included for comparison. In addition, the authors did not state the criteria they used for determining if parameters were within normal ranges.]** The study authors concluded that their study ". . . did not show long-term adverse outcome related to physical growth and pubertal development in adolescents previously exposed to DEHP in the neonatal period."

Strengths/Weaknesses: The extensive assessment of endocrine function to supplement Tanner stages

is a strength of this study; however, there were no measurements of phthalate exposure, and there was no comparison group to compare to children presumed to be exposed. Another weakness is the very small sample size (13 males and 6 females) and the inability to detect changes in hormone levels that were still within the normal range.

Utility (Adequacy) for CERHR Evaluation Process: Given the small sample size and lack of a comparison group, this study is of minimal utility for the evaluation process.

Colón et al *(110)*, supported in part by the EPA Minority Academic Institutions Traineeship programs, compared blood phthalate levels in premature thelarche patients and controls. Cases consisted of girls between the ages of 6 months and 8 years with premature breast development. Controls consisted of females aged 6 months to 10 years who displayed no evidence of premature sexual development or other endocrine disease. Blood samples from premature thelarche patients were taken between January 1994 and April 1998. **[It is not specified if blood samples were collected from controls during the same time period.]** Forty-one samples were obtained from premature thelarche patients and 35 samples from control patients. **[It was not stated if each sample was obtained from a different subject.]** Levels of phthalates, including DEHP, were measured in serum by GC/MS; numerous blank samples were analyzed to rule out contamination through solvents, water, or medical or laboratory equipment. Phthalates were detected in 28 of 41 samples from premature thelarche patients. DEHP was detected in 25 of the samples at concentrations ranging from 187 to 2098 μg/L (ppb); average concentration was reported at 450 μg/L. MEHP was detected in 5 of the samples at concentrations of 6.3–38 μg/L. In the control group, DEHP was detected in 5 of 35 blood samples at concentrations of 276–719 μg/L; average concentration was reported to be 70 μg/L. Di-n-octyl phthalate was the only other phthalate detected in 1 control sample. The difference in average blood DEHP level in cases versus controls was found to be statistically significant using the 95% confidence interval. **[Methods of statistical analyses were not discussed.]** Study authors concluded "This study suggests a possible association between plasticizers with known estrogenic and antiandrogenic activity and the cause of premature breast development in a human female population."

[The Expert Panel notes a letter by McKee *(3)* in response to the Colón et al. study. This letter identified the blood DEHP and MEHP concentrations as being difficult to reconcile with published studies on phthalate blood levels. The very high blood levels of DEHP and low blood levels of MEHP were described as "anomalous" and consistent only with sampling immediately after introduction of substantial amounts of DEHP into the bloodstream, as might occur after a medical procedure. This scenario would not be consistent with a picture of chronic DEHP exposure levels such as might be hypothesized to affect thelarche. The Expert Panel agrees with McKee that the DEHP concentrations reported in this study are unreliable.]

Strengths/Weaknesses: This study used a clinically relevant outcome, but phthalates detected in serum specimens may have been unreliable due to laboratory contamination or to medical procedures conducted because of the diagnosis. It was not stated whether phthalate laboratory analyses were conducted blind to case status.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful because of the lack of confidence in the reported DEHP measurements.

3.2 Experimental Animal Data

Since the initial CERHR Expert Panel Report on DEHP, several experimental animal studies have addressed the mechanisms by which fetal and neonatal DEHP exposure interferes with development of the male reproductive system in rodents. There has also been a multigeneration continuous-breeding study in rats using 8 dietary dose levels to evaluate dose-response relationships for developmental and reproductive endpoints.

3.2.1 Developmental Studies Focusing on Reproductive System and Endocrine Effects

This section examines reproductive or endocrine effects occurring in animals dosed during gestation or during the pre-weaning stage. Studies examining reproductive effects in animals dosed subsequent to the lactational stage (≥21 days of age) are summarized in Section 4.

3.2.1.1 In Vivo Exposures

Akingbemi et al. *(111)*), supported by NIEHS, evaluated the effect of DEHP on Leydig cell function in male Long-Evans rats exposed *in utero*, during nursing, or during post-weaning stages (the post-weaning results are presented in Section 4.2.2.2). Pregnant rats were exposed to DEHP (>99% purity) on GD 12–21 **[plug day not indicated]**, and lactating rats were exposed on PND 1–21 (day after birth=PND 1). DEHP was administered to dams by gavage in corn oil at 0 or 100 mg/kg bw/day. Males were obtained for evaluation on PND 21, 35, or 90 (n=7 dams/group/stage) **[no information was provided on culling or rearing]**. Male offspring were decapitated within 24 hours of the final dose, and trunk blood was collected for measurement of LH and testosterone by radioimmunoassay (RIA). Testes and seminal vesicles were weighed, and testicular interstitial fluid was collected for measurement of fluid testosterone by RIA. Testicular histology was evaluated. Cultures of Leydig cells or, in 21-day-old animals, progenitor Leydig cells, were prepared from testes by Percoll density gradient preceded in 90-day-old rats by centrifugal elutriation. The resulting preparations were 90% pure for progenitor Leydig cells and 95–97% pure for PND 35 or 90 Leydig cells, as evaluated by staining for 17-hydroxysteroid dehydrogenase. Cultures were evaluated after 3-hour incubation with and without a maximally stimulating concentration of ovine LH. Testosterone was measured in the medium. **[According to the methods section, the activity of different enzymes in the testosterone biosynthesis pathway were evaluated by incubating Leydig cells for 3 hours with saturating concentrations of substrate for the enzyme of interest; however, results of these experiments were given only for males treated during the post-weaning period (summarized in Section 4.2.2.2).]** Statistical analysis was by ANOVA and Duncan multiple range test.

There were no effects of treatment during gestation or lactation on dam weight or weight gain or on offspring weight. Offspring testis and seminal vesicle weights were also not affected by treatment during either developmental period. Serum testosterone was reduced 31–33% and serum LH was reduced 50–64% **[estimated from a graph]** in 21- and 35-day-old males exposed to DEHP during gestation. There were no prenatal DEHP effects on serum testosterone or LH in 90-day-old males. Prenatal exposure to DEHP resulted in decreased testosterone production by cultured progenitor Leydig cells obtained from 21-day-old males. Basal testosterone production was reduced 47%, and LH-stimulated testosterone production was reduced 56%. There were no treatment effects on cultured Leydig cells derived from 35- and 90-day-old offspring. Lactational exposure to DEHP was associated with a 13% decrease in serum testosterone on PND 21. There were no significant changes in serum LH on PND 21 or in testosterone or LH on PND 35 or 90. **[No results were presented for cultured**

Leydig cells derived from males exposed during lactation.] Testicular histology was described as normal in all treatment groups.

The authors concluded that exposure to DEHP during gestation or lactation resulted in suppression of pituitary LH in the presence of reduced serum testosterone, and that growing rats were more susceptible to the effects of developmental exposure than adult rats.

Strengths/Weaknesses: This report contains good descriptions of experimental design and methods with some exceptions. The studies used an appropriate route and time of exposure, and chemical source and purity were described. Multiple dose levels in the second study (discussed in Section 4.2.2.2) allowed for dose-response analyses. The comparison of responses to postnatal exposure at 3 different ages is a strength. Weaknesses include inadequate detail on the numbers of animals and numbers of litters per group used for histopathologic examination. The litter was not utilized as the unit of analysis following maternal/gestational exposure. For prepubertal and young adult rats, animals were randomly selected and assigned to treatment groups, but correction for potential litter effects was not conducted. Although the lack of control for litter effects is a weakness in this study, it is less compromising for prepubertal rats directly dosed with DEHP, because litter effects diminish somewhat with age post-weaning, and the animals were given a standardized dose based on individual body weight. The single, high dose level used in the first experiment is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: The histopathology results are of limited value because no data were presented, and it was not clear how many animals from each treatment group were evaluated. The gestational and lactational results from this study are not useful for the evaluation because the study design did not control for litter effect following maternal exposure. The lactational data were not presented. The *ex vivo* testosterone production data are not useful for the evaluation process due to the artificial *in vitro* environment in which these data were generated, which has an uncertain application to human risk. The enzyme activity and testosterone production information are useful for providing insight into potential mechanisms of action (Section 4.2.2).

Shirota et al. *(112)*, support not indicated, evaluated testicular pathology after intrauterine exposure of Sprague-Dawley rats to DEHP. In experiment 1, pregnant rats were treated by gavage with DEHP **[purity not given]** in corn oil at 0, 500, or 1000 mg/kg bw/day on GD 7–18 **[plug=GD 0]**. Ethinyl estradiol 0.25 or 0.5 mg/kg bw/day was used as a positive control. There were 28–30 dams/treatment group. Six dams/treatment/time point were killed on GD 12, 14, 16, 18, or 20 and live fetuses processed for light or electron microscopic examination. An additional 5 dams/treatment group given DEHP 500 or 1000 mg/kg bw/day were permitted to deliver and raise their young. Male offspring from these litters were killed at 7 weeks of age for histologic evaluation of testes and epididymides. In experiment 2, designed to identify a no-effect level, 11 or 12 pregnant rats/treatment group were given DEHP in corn oil at 0, 125, 250, or 500 mg/kg bw/day on GD 7–18. Fetuses were delivered by cesarean section on GD 20 in 3 dams/treatment group. The remaining dams were permitted to deliver and rear their offspring. Four male offspring per treatment group per time point were killed at 5 or 10 weeks of age for light microscopic examination of testes and epididymides, 2 male offspring/treatment group/time point were killed at 5 or 10 weeks for electron microscopic examination of the testes, and 4 male offspring/treatment group were killed at 10 weeks of age for evaluation of testicular and epididymal sperm. **[Litter of origin of the offspring at 5 and 10 weeks was not mentioned and the**

data tables suggest that each offspring was considered an independent treatment unit.] Light microscopy was performed after fixation of testes in Bouin fluid and then formalin. Tissues were embedded in paraffin and stained with hematoxylin and eosin. In experiment 2, a testicular section was also stained with periodic acid Schiff to confirm acrosomal status of sperm. Immunohistochemistry was performed with antibody to androgen receptor. Epididymal sperm were assessed in experiment 2 using computer-assisted sperm motion analysis. Epididymal sperm counts were also assisted using an automated method. Statistical analysis was performed using ANOVA or Kruskal-Wallis rank-sum test with post hoc Dunnett test.

Dam weight was decreased about 10% by DEHP 1000 mg/kg bw/day and by 17β-estradiol. There were no effects of lower DEHP dose levels on dam weight. Fetal weight and mortality were increased by DEHP treatment of the dam at 1000 mg/kg bw/day. The developmental lowest-observed adverse effect level (LOAEL) was 1000 mg/kg bw/day based on increased intrauterine mortality and decreased live fetuses/litter). **[Statistical differences were not marked in the data table in the paper, but were apparent by ANOVA with post hoc Dunnett test performed by CERHR. The BMD_{10}[1] was 734 – 755 mg/kg bw/day for the developmental endpoints. The $BMDL_{10}$ was 334 mg/kg bw/day for the decrease in live fetuses. $BMD_{1\ SD}$ was 846 – 874, and the $BMDL_{1\ SD}$ was 490 mg/kg bw/day for decrease in live fetuses. Due to the large SD for the litter percent intrauterine mortality, BMDLs computed for this endpoint were not meaningful.]**

In experiment 2, pup birth weight was increased in the groups exposed to DEHP at 250 and 500 mg/kg bw/day. On PND 4, there were no group differences in pup weight. Histologic examination of GD12 fetuses did not show identifiable testicular tissue. On GD 14, germinal ridges with germ cells were distinguishable. There were no treatment-related effects at this time point. On GD 16, testicular cords were evident and germ cell degeneration was apparent in 1 of the 12 fetuses of the DEHP 1000 mg/kg bw/day group. Germ cell degeneration was shown by electron microscopy **[whether in this fetus or in others was not stated]**. On GD 18 and 20, fetal testes in the DEHP-treated groups were small and showed hyperplasia of interstitial cells and multinucleated germ cells. Testes from 17β-estradiol exposed fetuses were also small and contained multinucleated germ cells. At 7 weeks of age, 1 offspring in the DEHP 500 mg/kg bw/day group showed multinucleated giant cells in the seminiferous tubules, but otherwise, testicular histology was normal. Testes from the DEHP 1000 mg/kg bw/day group showed branched and dilated tubules, atrophic tubules, multinucleated giant cells, and dilatation of the rete testis at 7 weeks. There was also epididymal atrophy, dilatation, and inflammation.

In experiment 2, there were multinucleated germ cells in fetal testes from all groups exposed to DEHP. Interstitial hyperplasia was also seen in the groups exposed to DEHP 250 and 500 mg/kg bw/day, with some degenerated germ cells in the 500 mg/kg bw/day group. Androgen receptor immunohistochemistry in the fetal testes was consistent with Leydig cell hyperplasia in the 500 mg/kg bw/day group. At 5 and 10 weeks of age, there were no abnormalities in the testes in any of the treatment groups by

[1] Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide 1 kind of description of the dose-response relationship in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making.

light or electron microscopy. Epididymal sperm counts and sperm motility parameters did not show treatment effects.

The authors concluded that DEHP was toxic to the fetal testis with histologic findings of germ cell degeneration and interstitial cell hyperplasia. These effects were seen at maternal dose levels of 250 mg/kg bw/day but not 125 mg/kg bw/day, which the authors identified as a no-observed effect level. **[The Expert Panel notes that multinucleated germ cells were identified in 0/15 fetuses in the control group, 6/16 fetuses in the 125 mg/kg bw/day group, 15/19 fetuses in the 250 mg/kg bw/day group, and 25/28 fetuses in the 500 mg/kg bw/day group. Benchmark dose analysis for this endpoint gives a BMD_{10} of 73 mg/kg bw/day and a $BMDL_{10}$ of 54 mg/kg bw/day.]**

Strengths/Weaknesses: The multiple exposure levels allow for a dose-response evaluation. The presentations of most methods and data are fairly good. The study shows the developmental progression for testicular injury following *in utero* exposure and recovery postnatally, through sexual maturity. This study also evaluates lower doses in order to establish a no adverse effect level. The statistical evaluation and study design are weak. Although the investigators started with sufficient animals within the treatment groups of experiment 1 (28–30 dams/group), breaking the groups into multiple sampling time points resulted in relatively small group sizes per time point (5 or 6 dams for experiment 1 and 3–9 dams for experiment 2). In experiment 2, the GD 20 groups were limited to 3 dams/group. Since the dams were directly dosed, the dam or litter should have been the unit of analysis. The investigators failed address the unit of analysis in the statistical analyses section of the Methods and appeared to use the fetus or offspring as the unit of analysis for most parameters. The endpoints that appear to be analyzed correctly, with dam as the unit of analysis, included number of implantations, intrauterine mortality, survival indices, number of live fetuses, and sex ratio; all other endpoints were either analyzed on a fetal/offspring basis or it could not be determined how the endpoints were analyzed. For pathologic observations noted at high incidences (high percentage of fetuses), the unit of analysis deficiency has little impact in drawing conclusions regarding clear effect levels, due to the lack of similar findings in the control animals. However, for extrapolating no-observed adverse effect levels (NOAELs), benchmark dose, or LOAELs, these data are not useful. The investigators failed to identify how offspring within a group, or tubules within a tissue section, were selected for evaluation (random, 1st 4/5?). For the "recovery" evaluation in experiment 2, low confidence is placed in these conclusions due to the small sample size (4 offspring/group). In addition, it could not be determined if each offspring was from a different dam or all were from the same dam, further confounding the interpretation. The number of dams in each group did not add to the number stated to be assigned to study. Under experimental design, the authors state "In experiment 1, 28–30 dams per group were given… Each 6 of these dams were killed…on G12, 14, 16, 18 and 20… In addition, each 5 dams… were allowed to deliver…" Given this assignment (5 gestation day kills of 6 dams/day = 30, plus one delivery group of 5 dams) a total of 35 dams/group appear to have been used, not 28–30. This confusion may be due to the wording of the text, as a result of translation from Japanese.

Utility (Adequacy) for CERHR Evaluation Process: The data on number of implantations, intrauterine mortality, survival indices, number of live fetuses and sex ratio are useful for the evaluation process. However, the number of dams/group is somewhat small, reducing the confidence in the NOAELs. Although the pathology data were not presented on a litter basis, the high incidence findings can be used to establish effects levels, but should not be used for benchmark dose calculations.

Moore et al. ***(113)***, supported by NIH and University of Wisconsin, examined rat sexual development in offspring of dams dosed with DEHP during gestation and lactation. In an experiment conducted in 2 blocks, at least 8 pregnant Sprague-Dawley rats/group were orally dosed **[presumed gavage]** with DEHP (99% purity) at 0 (corn oil vehicle), 375, 750, or 1500 mg/kg bw/day from GD 3 (GD 1 = day after sperm detected) to PND 21. One group of rats was dosed with 3000 mg/kg bw/day in the first block of the study, but that dose was not used in the second block due to excess toxicity consisting of nearly complete prenatal or postnatal mortality. Dams were allowed to litter, and the litters were adjusted to 10 pups 1–2 days following birth. Litters were maintained at 10 pups by replacing any pups that died with pups from litters exposed to the same or lower concentrations of DEHP; data from replacement pups were not reported. Parameters examined in all pups (time period examined) included pup weight (PND 1, PND 7, and then weekly), anogenital distance (PND 1), presence of areolas (from PND 11), vaginal opening (from PND 24), time to first estrus (starting from vaginal opening), preputial separation (from PND 38), and male sex organ weight (PND 21, 63, and 105). In PND 63 rats, 1 epididymis and testis were fixed in neutral-buffered formalin, and the other testis and epididymis were used to determine daily sperm production. Sexual behavior with a sexually receptive female rat was assessed in males that were later necropsied on PND 105. The litter was considered the experimental unit in statistical analyses that included Levene test for homogeneity of variance, ANOVA, least significant difference test, chi-squared test, and/or Fisher exact test.

Results achieving statistical significance or displaying dose-response relationships are summarized in Table 21.

Table 21. Results Achieving Statistical Significance or Dose-response Relationships Following DEHP Prenatal and Lactational DEHP Exposure

Endpoint	*Maternal DEHP dose (mg/kg bw/day)*			
	0	*375*	*750*	*1500*
Maternal prenatal weight gain, g (% of control value)	128 ± 4	123 ± 7 (96)	99 ± 10* (77)	87 ± 13* (68)
Parturition incidence (%)	100	100	89	75
Pups born/dam (% of control value)	12.5 ± 1.0	11.4 ± 0.8 (91)	9.6 ± 1.3 (77)	7.7 ± 1.4* (62)
Pups surviving/dam (% of control value)	10.9 ± 1.0	9.8 ± 0.8 (90)	7.5 ± 1.3* (69)	5.0 ± 1.3* (46)
Mean male anogenital distance, mm [a]	3.5	3.3	3*	2.5*
Mean no. areolas per male [a]	0	2	7*	9.5*
Mean % litters containing males with:				
Areolas or nipples on PND 14 [a]	0	62*	100*	100*
Areolas or nipples as adults [a]	0	50*	85*	100*
Incomplete preputial separation [a]	0	10	25	80*
Undescended testis on PND 21 [a]	0	42	75*	100*
With undescended testis as adults	0	25	58	40
Daily sperm production, 10^6/testes (% of control value)	34.2 ± 1.5	36.5 ± 1.2 (107)	25.6 ± 4.5 (75)	24.4 ± 5.4 (71)

Endpoint	*Maternal DEHP dose (mg/kg bw/day)*			
	0	*375*	*750*	*1500*
Epididymal sperm number in 10^6/cauda (% of control value)	55.5 ± 3.7	46.5 ± 5.1 (84)	29.8 ± 8.7* (54)	19.3 ± 7.5* (35)
Litters (pup) with abnormality/no. examined				
Ventral prostate agenesis	0/8 (0/42)	1/8 (1/32)	2/8 (5/29)	2/5 (3/12)
Dorsolateral prostate agenesis	0/8 (0/42)	1/8 (1/32)	0/8 (0/29)	1/5 (2/12)
Anterior prostate agenesis	0/8 (0/42)	1/8 (1/32)	5/8* (9/29)	4/5* (6/12)
Seminal vesicle agenesis	0/8 (0/42)	0/8 (0/32)	0/8 (0/29)	2/5 (2/12)
Litters with reproductive defects, %[a, b]	0	65*	88*	100*
Index of abnormalities, %[a, c]	0	18*	55*	75*
Absolute testes weight, % of control value				
PND 21		90	78*	62*
PND 63		103	73*	59*
PND 105		99	71	39
Absolute epididymides weight, % of control value				
PND 63		98	66*	69*
PND 105		91	61*	55*
Absolute glans penis weight, % of control value				
PND 21		90	82*	71*
PND 63		97	89*	83*
PND 105		97	86*	79*
Age at vaginal opening, days	31.1 ± 0.9	29.7 ± 0.9	29.7 ± 1.2	27.2 ± 1.1
Body weight at vaginal opening, g (% of control value)	94 ± 6	87 ± 6 (92)	86 ± 9 (91)	64 ± 9* (68)
Age at first estrus in days	33.5 ± 0.3	33.1 ± 0.4	35.8 ± 2.0	34.4 ± 0.7
Body weight at first estrus, g (% of control value)	108 ± 4	105 ± 2 (97)	116 ± 11 (107)	98 ± 2 (91)

From: Moore et al. *(113)*. Mean ± SEM unless otherwise stated.

*$P < 0.05$.

[a] Estimated from graph by CERHR;

[b] Reproductive defects included missing, malformed, or small sex organs; incomplete preputial separation; or undescended testis

[c] Index of abnormalities is score based on missing, pathological, or small reproductive organs; presence of nipples or undescended testis; incomplete preputial separation; and failed ejaculation.

DEHP treatment reduced prenatal maternal weight gain at the middle and high dose. There was no significant effect on implantation sites, though the number appeared to be slightly reduced by DEHP treatment. All the rats with implantation sites gave birth to litters except for 1 mid-dose and 2 high-dose rats. Number of pups born was reduced at the high dose, and postnatal survival was decreased at the middle and high dose. Adult male offspring exposed to DEHP experienced a 6% reduction in body weight at the middle dose and 12% reduction at the high dose **[data not shown]**. An 8% reduction in body weight of adult female offspring of the high-dose group was reported as not significant. **[Data were not shown. A significant reduction in female body weight was reported for day of vaginal opening, as discussed below.]**

DEHP treatment caused numerous effects on the reproductive systems of male rats, outlined in detail in Table 21. Areolas or nipples were not observed in any control male rats but were increased according to dose in all treated rats. Effects first noted in male rats of the mid-dose group were reduced anogenital distance, increased numbers of undescended testes, reduced sperm counts, and agenesis of anterior prostate. Incidence of incomplete preputial separation was significant at the high dose, but the authors considered the effect to be biologically significant at all doses due to the rarity of the effect in rats. Agenesis of prostate, seminal vesicles, and epididymis was noted in some treated rats. Agenesis of anterior prostate was significant at the middle and high dose; the study authors suggested that the effect was biologically significant at the low dose due to rarity of prostate agenesis. DEHP treatment reduced absolute weights of testes, epididymides, and glans penis at PND 21, 63, and/or 105, and in most cases, statistical significance was obtained at the middle and high dose. Absolute weight effects are outlined in Table 21. In most cases, effects on relative weights were similar in terms of direction and statistical significance of effect. Weights of accessory male organs, which are not illustrated in Table 21, were also reduced by DEHP treatment. The organs affected (day and dose that statistical significance was achieved for absolute organ weight) were ventral prostate (PND 21: middle and high dose; PND 105: mid dose), dorsolateral prostate (PND 21 and 105: middle and high dose; PND 63: high dose), anterior prostate (PND 21: all doses; PND 63 and 105: middle and high dose), and seminal vesicles (PND 21: middle and high dose). Effects on relative organ weights were similar in most cases. In sexual behavior tests, there were fewer rats from all dose groups that did not mount, intromit, and/or ejaculate. The authors stated that statistical significance was not obtained due to the small numbers of animals tested (n = 7 – 8 at control and 2 lower dose levels and n = 2 at high-dose level).

Statistically significant and dose-related effects for female offspring are also listed in Table 21. In female pups, DEHP treatment had no effect on anogenital distance. At the high dose, vaginal opening was described as having occurred slightly earlier than in control rats, but age at first estrous was described as slightly higher; neither effect was statistically significant. Body weight of high-dose females was 68% that of control body weight on the day of vaginal opening, and the effect was statistically significant. The study authors attributed the effect to DEHP-induced toxicity and not to an estrogenic effect.

[CERHR estimated benchmark doses for endpoints when there was evidence of a dose-response relationship and for which the authors reported sufficient data for benchmark dose modeling. Benchmark dose values are presented in Table 22.] The study authors identified a LOAEL of 375 mg/kg bw/day for this study based on a significant decrease in anterior prostate weight and an increase in permanent nipple retention. Other biologically significant effects observed by study authors at 375 mg/kg bw/day were non-descent of testes, incomplete preputial separation, and agenesis of accessory sex organs. The study authors noted that DEHP exposure adversely affected reproductive system development and sexual behavior in male rats, but there was no evidence of estrogenic activity in female rats.

Strengths/Weaknesses: This paper includes a good, detailed description of methodology and statistical analyses using the litter as the unit of analysis. The study was well-designed, using an appropriate route and timing of exposure and multiple dose levels. Even thought the sample size was small, there was sufficient magnitude of effects to obtain statistical significance. The study demonstrated a pattern of effects consistent with other study findings and the establishment of NOAELs/LOAELs. The dose-response data are appropriate for benchmark dose evaluation. It is a strength that animals were followed into early adulthood. The observation of effects on reproductive behaviors in the absence of gross external changes

suggested additional effects on the central nervous system. This study reinforced the increased sensitivity of the fetal male compared to the pubertal or adult male, although only by reference to existing literature.

Table 22. Benchmark Dose Values for Offspring of Rats Exposed to DEHP During Gestation and Lactation

Endpoint		Benchmark dose (mg/kg bw/day)[a]			
		BMD10	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Maternal prenatal weight gain		433	317	754	509
Pups born/dam		378	269	872	565
Pups surviving/dam		269	205	696	479
Absolute testis weight	PND 21	384	298	562	400
	PND 63	421	283	452	258
	PND 105	374	204	342	203
Epididymis weight	PND 63	394	291	395	233
	PND 105	262	196	220	135
Glans penis weight	PND 21	503	393	526	379
	PND 63	806	623	445	321
	PND 105	642	500	278	171
Daily sperm production/testes		490	289	686	389
Epididymal sperm number		213	167	612	428
Body weight at vaginal opening		780	343	1157	611

From: Moore et al. *(113)*.

[a]The BMD_{10} is the benchmark dose associated with a 10% effect, estimated from a curve fit to the experimental data. The $BMDL_{10}$ represents the dose associated with the lower 95% confidence interval around this estimate. A 10% alteration in a continuously distributed parameter is an arbitrary benchmark that may not be comparable to a similar alteration in any other endpoint. The $BMD_{1\ SD}$, which represents an alteration equivalent to 1 SD of the control distribution, may permit more appropriate comparisons of the responses of continuously-distributed parameters. Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide 1 kind of description of the dose-response relationship in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making. Values were calculated using the power model by CERHR using EPA Benchmark Dose Software version 1.3.2. The program offers models based on homogeneity of variance, and CERHR was guided by the program in this regard.

Strengths/Weaknesses: Weaknesses include the small sample size, and negative effects cannot be accepted with high confidence. For example, there appeared to be a significant biological effect of reduced implantation sites at 1500 mg/kg but no statistical significance. Professional experience and judgment would lead to the conclusion of an effect on the number of implantation sites. The use of relatively high dose levels is another weakness. The authors used a post hoc statistical design for grouping of all reproductive abnormalities/defects and for behavioral effects. Post hoc (after generation and visual assessment of the data) statistical analyses are generally considered inappropriate, however, because the signal for reproductive abnormalities/defects is overwhelming (60–80% of litters affected in the

DEHP group compared to zero in the control), this oversight has no significance for these endpoints. It is a common practice when conducting embryo/fetal toxicity studies to group malformations and variations by system or type (external, visceral, or skeletal; organ system). For the behavioral assessment, the post hoc grouping of findings across DEHP treatment groups was inappropriate and not valid for risk assessment but was valuable for hypothesis generation. In addition, no data were presented for the behavioral evaluation.

Utility (Adequacy) for CERHR Evaluation Process: This study was well conducted and used a comprehensive battery of relevant developmental endpoints, multiple dose levels, and a relevant route of exposure during critical periods of sexual development. Even though sample size and power were limited, clear treatment-related (and in many cases dose-responsive) effects on maternal toxicity, reproductive parameters, measures of sexual differentiation/development, and sexual function were identified. Given the variability in response, small sample size, post hoc grouping of results across treatments, and lack of presentation of data, the behavioral data are not appropriate for the evaluation process. The negative findings are not viewed with high confidence given the small sample size.

The National Toxicology Program (NTP) *(114)* sponsored a multigeneration continuous breeding study in rats. **[Because developmental effects were reported, particularly on the male reproductive system, the study is included in this section. This summary with additional details concerning the reproductive effects is also presented in Section 4.2.2.2.]** Sprague-Dawley rats (17/sex/group) were randomly assigned to diets containing 1.5 (control group exposed to background DEHP levels in feed), 10, 30, 100, 300, 1000, or 7500 ppm DEHP (99.8% pure) from the first day of the study until the day of necropsy. Due to a lack of reproductive effects in the first litter produced, the study was repeated with 2 additional doses, 1.5 (control) and 10,000 ppm. Ranges of DEHP intake in the F_0, F_1, and F_2 animals were estimated at 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day. At about 5 weeks of age, F_0 rats were fed the DEHP-containing diets for 6 weeks prior to mating and were then cohabitated for 9 weeks. Concentrations of dosing solutions were verified. The first 2 litters delivered during the cohabitation period (F_{1a} and F_{1b}) were counted, weighed, assessed for anogenital distance, and discarded. The third litter (F_{1c}) was raised by the dam until weaning on PND 21 **[designation for day of birth not specified]**. Following weaning of pups, vaginal cytology was monitored in F_0 females for 14 days. After completion of crossover studies described below, at least 10 F_0 rats sex/group were necropsied. Sperm analyses were conducted, and organs were collected for histopathological evaluation. Ovaries were preserved in Bouin fluid. Testes and epididymides were preserved in 2% paraformaldehyde/3% glutaraldehyde. F_1 pups were counted, weighed, and examined for anogenital distance and nipple retention during the lactation period. On PND 16, 1 female per litter was evaluated for vaginal opening, and a second was selected for F_1 mating. One male per litter was selected for mating, and 4 or 5 males per litter were evaluated for testicular descent and preputial separation; both groups of rats were necropsied. At weaning (PND 21), pups were given diets containing the same DEHP concentrations as their parents. On PND 81, the F_1 rats chosen for mating (17/sex/group) were randomly assigned to breeding pairs (preferably non-sibling) and cohabited for 9 weeks. The study conducted in F_0 parents and F_1 offspring was repeated in F_1 parents and F_2 offspring, except that the third F_3 litter born (F_{3c}) did not undergo the continuous-breeding protocol. Selected F_{3c} males were necropsied on PND 63–64 and selected females on PND 60–74. Statistical analyses included Jonckheere test to determine if data should be analyzed by Shirley or Dunn test. Shirley test was used to evaluate data that consistently increased or decreased according

to dose. Dunn test was used to evaluate data with severe departures from monotonicity. Additional statistical analyses included Wilcoxon, Cochran-Armitage, and chi-squared tests.

Some systemic effects were consistent across all generations. During numerous time periods of the study and especially at necropsy, body weight gains were decreased in rats from the 7500 and 10,000 ppm groups. Dam body weights during delivery and lactation were decreased by 8–20% in the F_0 10,000 ppm group. Increases and decreases in feed intake were observed at most dose levels. In the F_0 7500 and 10,000 ppm groups, feed intake was decreased during lactation. The liver was identified as a target of toxicity, with increases in liver weight and hepatocellular hypertrophy observed at dose levels ≥1000 ppm. Changes in organ weights and lesions were also observed in kidney at ≥7500 ppm and adrenal gland at 10,000 ppm.

The lowest dose level producing dose-related effects in F_1 offspring was 7500 ppm, and those effects included decreases in number of live pups/litter, reduced male anogenital distance, and delays in vaginal opening, preputial separation, and age of testicular descent. Additional effects noted in the F_1 offspring from the 10,000 ppm group included decreased live pup weight at birth and during the lactation period and increased ratio of female anogenital distance to body weight. In the non-mating F_1 adult males of the 300 ppm group, there was a small increase in the number of animals (3 of 45 with small testes and/or epididymides. The effects were not observed at the next higher dose (1000 ppm), but small testes were observed in 10 of 30 males of the 7500 ppm non-mating group. Small testes and epididymides were observed in 21 of 21 animals of the 10,000 ppm non-mating group. A small percentage (3–7%) of non-mating rats treated with ≥1000 ppm had small ventral prostates. In rats that were mated, the only decreases in reproductive organ size occurred in testes at 7500 and 10,000 ppm (8 of 10 and 10 of 10 affected at each dose) and epididymides at 7500 ppm (2 of 10). Histopathological findings observed in all animals of the 7500 and 10,000 ppm groups were consistent with those observed in the F_0 generation and included minimal-to-marked seminiferous tubule atrophy and occasional sperm release failure. Minimal seminiferous tubule atrophy was observed in 1 of 10 males in the 100 and 300 ppm groups. Reductions in numerous reproductive organ weights were observed in mating and non-mating F_1 males treated with ≥7500 ppm. Additional reproductive effects observed in F_1 rats were reduced sperm counts at 7500 ppm and higher and increased uterus and ovary weights at 10,000 ppm. Estrous cycle length was slightly increased at 10,000 ppm. In the F_2 pups, delays in preputial separation and testicular descent occurred at every dose level above the control. **[In no other generation did delays in preputial separation and testicular descent occur at such low doses, but the study authors did not offer any explanations for this observation. The Expert Panel believes these findings are consistent with a problem with the control group in that generation.]** All other effects occurred in F_2 pups of the 7500 ppm group and included delayed vaginal opening and reductions in live pup weight at birth and during the lactation period, male anogenital distance, and survival during the lactation period.

In non-mating F_2 male rats, small testes and epididymides were observed at ≥300 ppm (1/21), 1000 ppm (1/25), and 7500 ppm (7–11/20). However, in males that were mated, small epididymides and testes were only observed at the 7500 ppm dose level (8/10). Seminiferous tubule atrophy was observed in 10/10 males of the 7500 ppm group. In F_3 pups, a decrease in postnatal survival of females was observed only on PND 7 in the 300 ppm group but was not observed on any other day or dose level. All other effects in F_3 pups occurred at 7500 ppm and included decreases in male anogenital distance, delayed vaginal opening, preputial separation, and testicular descent, and an increase in male pups with

nipples. F_3 pups were the only generation of rats to experience an increase in males with nipples. At necropsy of adult F_3 rats, effects were only observed at the 7500 ppm dose level and included reduced sperm counts and weights of dorsolateral prostate, testis, and epididymis.

The study authors discussed the relevancy of small male reproductive organ sizes observed in both F_1 and F_2 rats of the 300 ppm groups. They noted that although incidences were low, the effects were consistent with phthalate-induced developmental toxicity. The incidence of small testes and epididymides exceeded historical control data from the laboratory. Therefore, the study authors considered the effects as potentially treatment-related. However, the study authors concluded that the overall significance of the effects could not be determined due to lack of histopathological data and lack of adverse reproductive effects at 300 and 1000 ppm.

A crossover breeding study was conducted to investigate the decrease in F_3 pup body weight in the 7500 ppm group. High-dose rats of each sex (n = 17/sex/group) were mated with naïve animals for 7 days or until a vaginal plug was detected. Pups were counted, weighed, assessed for anogenital distance, and discarded. Implantation sites were examined in naïve females. The crossover study demonstrated a decrease in pup weight and male anogenital distance in offspring born to females treated with 7500 and 10,000 ppm DEHP and mated to naïve males.

The study authors concluded, "The findings obtained in this study indicate that DEHP is clearly a reproductive and developmental toxicant at 7500 and 10,000 ppm based upon changes in fertility and pregnancy indices, litter data, sperm parameters, sexual development, and/or histopathological changes in testes." Intake at 7500 ppm was estimated at 392 – 592 mg/kg bw/day, and intake at 10,000 ppm was estimated at 543 – 775 mg/kg bw/day. **[The lowest BMD_{10} is 787 ppm based on F_3 sperm/cauda. The $BMDL_{10}$ for this endpoint is 728 ppm. The $BMD_{1\ SD}$ is 1188 ppm and the $BMDL_{1\ SD}$ is 970 ppm. Extrapolating from the authors' estimates of intakes at 1000 mg/kg bw/day, the BMD_{10} intake level is 36 – 61 mg/kg bw/day, the $BMDL_{10}$ intake level is 33 – 56 mg/kg bw/day, the $BMD_{1\ SD}$ intake level is 54 – 92 mg/kg bw/day, and the $BMDL_{1\ SD}$ is 45 – 75 mg/kg bw/day. The Expert Panel carefully considered the finding of small reproductive organ sizes by gross observations in both F_1 and F_2 rats. The combined F_1 and F_2 data were reviewed to determine the occurrence of these alterations on a per animal and per litter basis across the dose range, as shown in Table 23. Based on the incidence of small reproductive organ size at necropsy, the Expert Panel considered 300 ppm (about 14 – 23 mg/kg bw/day) to be an effect level, giving a NOAEL of 100 ppm, about 3 – 5 mg/kg bw/day.]**

Strengths/Weaknesses: Clearly, a major strength of this study is the number of doses evaluated. The relatively small group sizes were compensated by the unusually large numbers of groups and the very low doses used. An additional strength is the fact that more offspring were evaluated early for alterations in the development of the reproductive system; a weakness might be that not all animals were so evaluated. The quality of the histology is another strength.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process and show that 10,000 and 7500 ppm are clearly toxic to the developing reproductive system in rats. The Expert Panel considers 300 ppm and 1000 ppm to represent the tail of the dose-response curve in this study based on the incidence of testicular abnormalities, which would put the NOAEL for these developmental effects at 100 ppm, in the 3 – 5 mg/kg bw/day range.

Table 23. Reproductive Organ Abnormalities in Combined F_1+F_2 Non-breeding Males in NTP Multigeneration Study

Organ	DEHP dose level, ppm in feed (n)						
	1.5 (39)	10 (36)	30 (39)	100 (41)	300 (45)	1000 (43)	7500 (30)
Testis	0	0	0	0	4	3	21
Epididymis	0	0	0	0	3	3	7
Seminal vesicles	0	1	0	0	2	0	0
Prostate	0	0	0	0	0	4	1
Any reproductive organ	0	1 (1)	0	0	5 (4)	7 (5)	22 (14)

Data expressed as number of animals (litters) affected. From NTP *(114)*

Borch et al. ***(115)***, support not indicated, conducted a series of studies in Wistar rats to examine anti-androgenic effects of DEHP (99% purity) alone or in combination with diisononyl phthalate or diethylhexyl adipate. Diisononyl phthalate was examined alone in some cases, but this summary focuses on DEHP. Dams were gavage dosed with vehicle (peanut oil) or DEHP in peanut oil during gestation and/or lactation. Endpoints examined in male offspring included hormone levels in testes or blood and *ex vivo* testicular testosterone production. To measure *ex vivo* testosterone production, the left testis was incubated in media for 3 hours and the supernatant was saved for testosterone analysis. Following extraction from incubation media, testis, or blood, quantification of hormone levels was performed using fluorometric or immunofluorometic methods. Testicular testosterone production was measured in testes from 2 males per litter, and testicular testosterone content was measured in testes from 1 male per litter. Plasma samples were pooled from 1 or 2 litters in the case of fetal or immature offspring or were obtained from 9–16 males per group in the case of mature offspring. Data were evaluated by ANOVA, analysis of covariance (ANCOVA), Dunnett test, and /or Pearson correlation. Litter was included as an independent random factor in ANOVA analyses.

The first study appears to have been previously reported in an abstract *(116)*. Approximately 8 dams/group were gavage dosed during gestation with vehicle, 300 mg/kg bw/day DEHP, or 300 mg/kg bw/day DEHP+750 mg/kg bw/day diisononyl phthalate on GD 7–21. **[Criteria for determining day of gestation were not stated, but it is assumed that GD 1 was the day following mating, as in study 2 described below.]** Endpoints examined in GD 21 male fetuses included testicular testosterone content and production and plasma testosterone and LH levels. Compared to control values, testicular testosterone content and production were significantly reduced in the DEHP and DEHP+diisononyl phthalate groups. Plasma testosterone was significantly reduced, and plasma LH was significantly increased in the DEHP+diisononyl phthalate group; similar effects on plasma testosterone and LH levels were described in groups receiving DEHP or diisononyl phthalate alone, but statistical significance was not achieved. Factorial statistical analyses revealed no significant interactions between DEHP and diisononyl phthalate.

In the second study, 16 pregnant Wistar rats per group were gavage dosed with vehicle, 750 mg/kg bw/day DEHP, or 750 mg/kg bw/day DEHP+400 mg/kg bw/day diethylhexyl adipate from GD 7 to PND 17. GD 1 was the day following mating, and PND 1 was the day following birth. Eight dams per

group were randomly selected and killed on GD 21. The fetuses were also killed for collection of blood and testes. The remaining 8 dams per group were allowed to litter; anogenital distance was measured in their male offspring on PND 3, and nipple retention was assessed on PND 13. In GD-21 male fetuses exposed to DEHP or DEHP + diethylhexyl adipate, testicular testosterone content and production and plasma testosterone levels were significantly reduced compared to the control group. Plasma LH level was significantly increased in GD 21 fetuses exposed to DEHP. Plasma LH levels were not measured in the DEHP + diethylhexyl adipate group. Anogenital distance was significantly reduced in PND 3 male offspring from the DEHP and DEHP + diethylhexyl adipate groups, and the effect was not related to birth weight. Numbers of nipples per animal were significantly increased in PND offspring from the DEHP group, but the increase in the DEHP + diethylhexyl adipate group was not statistically significant.

In the third study, 80 Wistar rats were randomly assigned to 1 of 4 groups that were treated with DEHP 0, 300, or 750/kg bw/day or DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day from GD 7 to PND 17. Blood and testes were collected from male offspring killed on PND 22 and PND 190. In PND 22 offspring treated with 750 mg/kg bw/day DEHP, there was a dose-related, significant reduction in serum inhibin B level. A non-significant "tendency" for increased serum FSH levels in the DEHP-treated groups was described by the study authors. There were no significant findings for serum LH levels, but about half the litters in the 750 mg/kg bw/day DEHP-treated group had LH levels several-fold higher than those of control rats. Correlations were noted between serum LH and serum FSH ($r = 0.61$, $P < 0.001$) and serum FSH and serum inhibin B ($r = -0.51$, $P < 0.001$). There were no significant differences in plasma testosterone levels on PND 22. No statistically significant differences in serum testosterone levels, testicular testosterone content, or serum inhibin B levels were observed in PND 190 rats.

The study authors concluded that in neonatal rats, endocrine-modulated effects following gestational or lactational exposure to DEHP were similar to those previously reported in the literature, including a reduction in anogenital distance and an increase in nipples. However, hormonal effects were less evident in prepubertal and adult animals. Administration of diisononyl phthalate or diethylhexyl adipate in combination with DEHP caused no significant modulation of endocrine effects.

Strengths/Weaknesses: The use of the Wistar rat was a strength in extending the evaluation of DEHP to a strain different from that used in most other studies. The paper included a good, detailed description of methods including test material source and purity, appropriate statistical evaluation of the data using the litter as the unit of analysis, and nesting of individual animals/litter. Study 2 demonstrated clear treatment-related effects on plasma testosterone and LH in fetuses following maternal gestational exposure to DEHP 750 mg/kg bw. Although the sample size was small (n = 2–6 samples/group), the magnitude of the DEHP effect was sufficient to show statistical significance. Gestational DEHP exposure to 750 mg/kg bw also resulted in decreased anogenital distance and increased number of nipples per male. Study 3 employed multiple dose groups (300 and 750 mg/kg DEHP), allowing for a dose-response evaluation. Maternal animals were dosed from GD 7 through PND 17, the period of sensitivity for male sexual development. The only treatment-related effect noted following maternal exposure from GD 7 through PND 17 was decreased inhibin B on PND 22, demonstrating a Sertoli cell effect. The small sample size (< 8 litters/group/time point) was a weakness in these studies. *Ex vivo* testosterone production is of questionable relevance to human risk, especially when no *in vivo* plasma testosterone or LH changes were noted. Study 1 demonstrated no treatment-related effects on plasma testosterone and plasma LH in 21-day-old rat fetuses following maternal gestational DEHP exposure at 300 mg/kg bw. However, given

the small sample size (n=3–7), there is a low level of confidence in the lack of findings. In studies 1 and 2, a single dose level of 300 or 750 mg DEHP/kg bw was used, precluding a dose-response evaluation.

Utility (Adequacy) for CERHR Evaluation Process: Studies 1 and 2 utilized single doses of DEHP and have low value for a quantitative evaluation. The results from these studies support findings reported by other authors. The *ex vivo* testosterone production data are of questionable relevance to human risk assessment, having no direct human correlate. Study 3 utilized multiple doses of DEHP (300 and 750 mg/kg bw/day) during a critical period of male sexual development and is useful in the evaluation process. This assay is best used for studying potential mechanisms/modes of action and for screening for potential activity.

Jarfelt et al. *(117)*), supported by the Denmark Directorate for Food, Fisheries and Agro Business, evaluated the effects of perinatal exposure of Wistar rats to DEHP with or without diethylhexyl adipate. Timed-mated pregnant animals were treated by gavage from GD 7 to PND 17 **[plug day unspecified; date of birth = PND 0]**. Dose groups were vehicle control, DEHP 300 mg/kg bw/day, DEHP 750 mg/kg bw/day, and DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day (n=20/group). Chemical purity was 99%. Unadjusted litters were raised by their dams until weaning on PND 21, after which 1 male and 1 female per litter were retained. Anogenital distance was assessed on PND 3, and retention of nipples/areolae was assessed on PND 13. Retained offspring were observed for vaginal opening and balano-preputial separation, and males underwent evaluation of epididymal sperm parameters and testicular histopathology on PND 190. **[Later in the Methods section, histopathologic evaluation was described for 14–16 adult males representing 14–16 litters.]** Non-retained pups and dams were killed on PND 22 and evaluated for macroscopic lesions, and 3–5 males/litter underwent histopathologic evaluation of the testes. **[Later in the Methods section, histopathologic evaluation of testes was described for about 5% of PND 22 males, representing 10 litters. The results section presents histolopathology data for 18–21 PND 22 males/dose group.]** Testes were fixed in Bouin fluid (half of PND 22 testes and all adult testes) or in formalin (half of PND 22 testes). Embedding material was not specified for testes. Staining was hematoxylin and eosin. Immunohistochemistry for 3β-hydroxysteroid dehydrogenase and smooth muscle actin was performed using 1 section/testis. Brain, liver, kidney, adrenal, testis, epididymis, seminal vesicle, ventral prostate, bulbourethral gland, and levator ani/bulbocavernosus muscle weights were recorded in all males. Histologic sections of accessory sex organs were prepared for 10 males representing 10 litters. Statistical analysis was performed using ANOVA with post hoc Dunnett test or, for data not satisfying conditions for ANOVA, Kruskall-Wallis or Fisher test. Litter was included in the analysis. **[Data were shown in the results for 11–15 litters/dose group, with 12 litters in the control group, although 20 timed-mated animals/dose group had been treated. In the group receiving DEHP + diethylhexyl adipate, there were 3 dams with total litter loss. The other missing litters were not explained.]**

Results are summarized in Table 24. Although there were no significant alterations in sperm count and motility parameters, the study authors reported that "a few animals" were severely affected with regard to these parameters. The study authors indicated that males exposed to DEHP with or without diethylhexyl adipate had histologically normal testicular tissue with small foci of malformed tubules associated with interstitial cell hyperplasia. Within these malformed tubules, the seminiferous epithelium was disorganized with decreased spermatogenesis and tubular anastomoses. Immunohistochemistry for 3β-hydroxysteroid dehydrogenase and smooth muscle actin showed Leydig cells inside the malformed

tubules. The authors called attention to the higher incidence of malformed tubules among young animals, and they concluded that "dysgenic" testicular tissue was surrounded by normal tissue during pubertal testis growth. There were no abnormal histopathologic findings in accessory sex organs in PND 22 males and only 4 males with mild prostate changes among the 3 DEHP-exposed dose groups on PND 190.

The authors concluded that their study confirmed the anti-androgenic effects of DEHP identified in previous studies, and they called attention to the variability in response among their animals.

Table 24. Outcomes after Perinatal Exposure of Rats to DEHP With or Without Diethylhexyl Adipate

Endpoint		*Treatment Group (mg/kg bw/day to the dam)*			*DEHP Benchmark Dose[a] (mg/kg bw/day to the dam)*			
		DEHP 300	*DEHP 750*	*DEHP 750+ DEHA 400*	*BMD_{10}*	*$BMDL_{10}$*	*BMD_{1SD}*	*$BMDL_{1SD}$*
Maternal pregnancy weight gain		↔	↔	↓21%				
Birth weight	Males	↔	↓8%	↓9%	770	701	735	405
	Females	↔	↓8%	↓11%	758	638	750	533
Litter size		↔	↔	↓28%				
Postnatal death		"↑" 4-fold	"↑" 5-fold	↑17-fold	622	127	754	504
Postimplantation loss		↔	↑2.3-fold	↑3-fold	72	13	1127	574
Male anogenital distance		↓14%	↓17%	↓17%	432	315	338	179
Male retained nipples		↑39-fold	↑52-fold	↑39-fold	BMD computation failed			
Sperm/g cauda		↔	↔	↔				
Sperm motility parameters		↔	↔	↔				
Macroscopic malformations		↔	↔	↔				
PND 22								
Body weight		↔	↔	↔				
Paired testis weight		↔	↓8%	↔	748	538	761	652
Other organ weights		↔	↔	↔				
Abnormal testis histology		↑2.6-fold	↑2.6-fold	↔				
PND 190								
Body weight		↔	↔	↔				
Ventral prostate weight		↓20%	↓21%	↓17%	388	250	753	454
Sexual muscle weight		↓15%	↓18%	↓16%	428	290	635	406
Other organ weights		↔	↔	↔				
Abnormal testis histology[b]		44%	27%	31%				

DEHA = diethylhexyl adipate.

↑, ↓, ↔ Statistical increase, decrease, no change compared to control.

"↑" Increase identified by study authors, although not statistically different from control.

[a] Benchmark dose calculations performed using the groups treated with control and DEHP only. See footnote to Table 22 for information on the benchmark dose calculations

[b] control rate 0%.

From Jarfelt et al. *(117)*.

Strengths/Weaknesses: Multiple doses of DEHP were used (300 and 750 mg/kg), allowing for dose-response analyses. This study provided a moderately comprehensive evaluation of reproductive and developmental effects following maternal gestational and lactational exposure during the critical period of male sexual development. Assessment of offspring was carried out through sexual maturity. The paper presented good detail on general methodology and test material source and purity, with the exception of suboptimal tracking of litters and lack of consistent use of the litter as the unit of analysis. When multiple pups/litter were used, the litter was included as an independent random and nested factor; however, it was not clear in the paper when, and for what specific parameters, litter was used as an analysis factor. Clear treatment-related findings in male offspring (anogenital distance, nipple retention, reproductive organ weights) were apparent and adequately analyzed. These results also supported findings from other investigators. The use of the Wistar rat was a strength in extending the evaluation of DEHP to a strain different from that used in most other studies. Weaknesses include the relatively high DEHP dose levels that were used. Because the pathology data apparently were not analyzed on a litter basis, these data are not optimal for benchmark dose evaluation. There is significant confusion as to the sample size used for specific parameters and the lack of litter as the unit of analysis for the pathologic data. The authors stated that 1 male and 1 female/litter were kept after weaning (PND 21) to investigate sexual maturation, sperm quality, and histopathology of the testes at adulthood, but this statement contradicts a subsequent statement that "sixteen animals per group were used, one to two males per litter," in reference to sperm count evaluations and the statement that 1–4 males per litter from 10–16 litters were used to analyze sperm quality at PND 190 and terminal body weight. Table 1 of the paper lists only 11–15 litters/group, yet the methods describe males representing 14–16 litters. With the exception of addressing 3 litters with total loss of pups, there is no mention of the loss of litters in the remaining dams. A total of 27 litters are unaccounted for, assuming the 3 litters with total pup loss are included in Table 1 of the paper.

Utility (Adequacy) for CERHR Evaluation Process: The dose-response data for reproductive results (postnatal death, postimplantation loss), offspring growth, nipple development, anogenital distance, sperm quality, and organ weights are suitable for use in the evaluation process. Although NOAELs were not observed for some of these endpoints, the benchmark dose methodology can be applied. The pathology data cannot be used for the benchmark dose evaluations because the litter effect was not controlled and findings in the DEHP groups did not demonstrate a dose-response.

Borch et al. *(118)*, supported by the Denmark Directorate for Food, Fisheries and Agro Business, evaluated early testicular effects of perinatal exposure to DEHP with or without diethylhexyl adipate in Wistar rats. In the first experiment, pregnant females were treated by gavage with vehicle, DEHP 750 mg/kg bw/day or DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day beginning on GD 7 (plug = GD 0; n = 18/dose group). Chemicals were of 99% purity. On GD 21, 8 dams/group were killed and fetal testes were harvested. The remaining 8 dams/group continued to receive treatment until PND 17. These animals were permitted to litter. Male offspring were killed on PND 26 (birth = PND 0), and testes were harvested.

A second experiment used 20 pregnant animals in each of 4 dose groups: vehicle control, DEHP 300 mg/kg bw/day, DEHP 750 mg/kg bw/day, and DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day. Treatment was from GD 7 through PND 17. On PND 22, 3 males/litter were killed and testes were harvested. On PND 190, 1 or 2 males/litter were killed and testes harvested.

Of the testes collected on GD 21, 14 – 19/dose group (2 – 4/litter) were fixed in formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin for light microscopy. Ten testes/dose group (1 or 2/litter) from PND 22 and PND 26 animals were processed in the same manner. Another 10 testes from these age groups as well as 16 testes/dose group (1 or 2/litter) were fixed in Bouin fluid, and stained with hematoxylin and eosin for light microscopy **[embedding material not specified]**. Tubule diameters were measured, and a 10% increase over the control maximum was defined as enlarged. Terminal deoxynucleotidal transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using a commercial kit, and immunostaining was performed for caspase-3, proliferating cell nuclear antigen (PCNA), histone H3, anti-Müllerian hormone, 3β-hydroxysteroid dehydrogenase, vimentin, and smooth muscle actin. Caspase-3 activity was measured in 10 testes/dose group (from 5 – 10 litters/group) at GD 21, PND 22, and PND 26. **[The method was described only by reference to another paper.]** DNA laddering was assessed based on relative fluorescence of DNA ladders on gels. Statistical analysis was by ANOVA with post hoc Dunnett test or by Kruskall-Wallis test. Litter was included as a factor in the ANOVA.

In testes evaluated on GD 21, vacuolization of Sertoli cells, shedding of gonocytes, reduced interstitial cell cytoplasm, and enlarged tubules were identified in offspring of all dams exposed to DEHP 750 mg/kg bw/day, regardless of diethylhexyl adipate co-exposure, compared to 0 – 14% of dams exposed to vehicle. Leydig cell hyperplasia was identified in offspring of more dams with DEHP treatment than control dams **[statistical analysis not shown]**. The number of histone H3-positive cells per testis section was not altered by treatment. **[Other immunohistochemistry results were not quantified but were not reported as affected by treatment.]** Staining for anti-Müllerian hormone to identify Sertoli cells showed positive cells within Leydig cell clusters, outside the tubules. DNA laddering was increased by DEHP treatment, although TUNEL-positive cells and caspase-3-positive cells were not increased by maternal DEHP 750 mg/kg bw/day.

On PND 26, tubules without spermatocytes were found in all litters exposed to DEHP compared to 29% of control litters **[statistical analysis not shown]**. Malformed tubules were identified in 17 – 29% of DEHP-exposed litters compared to none of the control litters. There were no effects of DEHP treatment on any of the measures of apoptosis on PND 22, 26, or 190, although the authors indicated that "a few animals in the treated groups had very high numbers of TUNEL positive cells, presumably spermatocytes."

The authors concluded that the development of dysgenic tubules in response to DEHP exposure was related to interstitial changes occurring during gestation, including the presence of Sertoli cells in the interstitium. They believed that Sertoli cell dysfunction in the fetal period might underlie the focal testicular dysgenesis seen in older animals. The authors proposed that the lack of alteration in Sertoli cell structure in prepubertal rats in this study might reflect recovery from DEHP, which was last administered on PND 17.

Strengths/Weaknesses: This study included a detailed description of methods, source and purity of test material, and appropriate statistical analyses, when utilized, employing nesting of offspring within litter. Animals were dosed during a relevant period of male sexual development. Both litter and individual offspring incidence for histopathology were presented in Study 1. This single-dose study at 750 mg/kg bw demonstrated clear effects on the incidence of Sertoli cell vacuolization, shedding of gonocytes, multinucleated gonocytes, and reduced cytoplasm in interstitial cells in 21-day-old

rat fetuses, and malformed tubules and tubules lacking spermatocytes in 26-day-old (postnatal) rat pups. This effect was clear even though statistical evaluations were not conducted on these endpoints. Study 2 explored a dose-response relationship (DEHP at 300 and 750 mg/kg bw) with 20 time-mated females/group. The number of litters/group, however, was not reported. The use of the Wistar rat was a strength in extending the evaluation of DEHP to a strain different from that used in most other studies. Weaknesses include the small sample size (5 – 8 litter/group) in the first study, although effects in treated animals were observed at a high incidence and were clearly distinguishable from control. The lack of dose-response data in the first study was a weakness. The second study, which permitted dose-response evaluation, focused primarily on mechanism/mode of action and either did not present the data in a format useful for the evaluation process or included few endpoints useful for assessing risk. In addition, the dose levels used in the second study were relatively high.

Utility (Adequacy) for CERHR Evaluation Process: The single-dose study is useful in supporting previous study results but is of limited use in a quantitative evaluation. The multi-dose study is more useful for the evaluation process, although it is limited in the evaluated endpoints.

Li et al. *(119)*, supported by NIH, examined the effects of DEHP and 2 of its metabolites on neonatal rat gonocytes and Sertoli cells. Male Sprague-Dawley rat pups from 4 – 7 litters were pooled and randomly placed into groups of 4 – 5 pups. On PND 3 (day of birth = PND 1), the rats were gavage dosed with DEHP (> 95% purity) at 0 (corn oil vehicle), 20, 100, 200, or 500 mg/kg bw. Pups were killed 24 hours after dosing. One testis was collected and preserved in 2% glutaraldehyde for a morphological examination. The other testis was collected to examine Sertoli cell proliferation through bromodeoxyuridine (BrdU) uptake. Serum FSH was measured by RIA. In a second experiment, Sertoli cell proliferation was measured and a morphological examination of testis was conducted at 6, 9, 12, 24, or 48 hours after rats were dosed with 0 or 200 mg/kg bw DEHP. Statistical analyses included one-way ANOVA and Student-Newman-Keuls *t*-test. In rats treated with 100 – 500 mg/kg bw DEHP, there was a dose-related increase in abnormally large gonocytes containing 2 – 4 nuclei. Multinucleated gonocytes were first detected at 12 hours following exposure to 200 mg/kg bw DEHP, and their numbers increased with time. Sertoli cell proliferation was reduced in rats treated with ≥ 100 mg/kg bw DEHP, as noted by significant decreases in BrdU labeling. BrdU labeling indices were reported at 27.03% in control rats, 20.83% in the 100 mg/kg bw group, 9.95% in the 200 mg/kg bw group, and 4.13% in the 500 mg/kg bw group. There was a rebound in Sertoli cell proliferation at 48 hours following treatment with 200 mg/kg bw DEHP; at that time point, the labeling index was 30.5% in the DEHP group and 24.7% in controls. Dosing with up to 500 mg/kg bw DEHP did not affect serum FSH levels.

To determine the role of DEHP metabolites, measurement of Sertoli cell proliferation and a morphological examination of testis were conducted in 4 rats/group that were gavage dosed with vehicle, 393 mg/kg bw MEHP (> 95% purity) in corn oil, or 167 mg/kg bw 2-EH (> 95% purity) in phosphate-buffered saline. The doses of MEHP and 2-ethylhexanol were equivalent to 500 mg/kg bw DEHP on a molar basis (1.28 mmol/kg bw). Like DEHP, MEHP caused an increase in large multinucleated gonocytes and a decrease in BrdU labeling. Those effects were not observed following treatment with 2-ethylhexanol.

To determine if inhibited Sertoli cell proliferation is due to altered expression of cell cycle regulators, expression of D1, D2, D3, and p27kipl proteins and cyclin D2 mRNA was measured in 4 – 5 rats/group gavage dosed with 0, 200, or 500 mg/kg DEHP; rats were killed at 6, 8, 12, or 24 hours following

dosing. Statistically significant effects included a small but reproducible decrease in cyclin D2 protein level at 8 and 12 hours following treatment with 200 mg/kg bw DEHP. **[It was not clear if protein expression was also examined in rats treated with 500 mg/kg bw DEHP.]** The decrease in D2 protein expression was confirmed by a dose-related reduction in D2 mRNA expression in the 200 and 500 mg/kg bw groups.

The conclusions of the study authors were that DEHP-induced transient reductions in Sertoli cell proliferation and changes in gonocyte morphology are mediated through MEHP, alterations in Sertoli cell proliferation do not occur as a result of changes in FSH levels, and developing testes are especially vulnerable to phthalate-induced toxicity.

Strengths/Weaknesses: An appropriate route of exposure (oral) was used, and the multiple DEHP dose groups were a strength. There was a strong dose-response with statistical significance for decreased Sertoli cell proliferation as measured by the BrdU labeling index. This study has some serious design and reporting deficiencies. Sample sizes were very small for most endpoints, 4 or 5 pups per group, with a limited dosing duration (single dose) during the postnatal period that may have been too late for many of the developmental endpoints. It was not clear how many pups/group were evaluated for serum FSH or if serum samples were pooled. A quantitative evaluation of FSH did not demonstrate an effect of treatment; however, the small sample size, single-dose exposure, and uncertainty regarding pooling of samples resulted in low confidence for this conclusion. There was no quantification of the pathologic findings in the testes, such as numbers of animals with specific lesions. Without incidence data or quantitative evaluations, the effect and no-observed-effect levels for most pathologic observations cannot be substantiated.

Utility (Adequacy) for CERHR Evaluation Process: Although this study utilized multiple dose levels for dose-response evaluation, the study suffered significant design and reporting deficiencies and is not useful for the evaluation process, with the possible exception of the Sertoli cell proliferation data. Low sample size, lack of control for litter effect, and lack of quantification of pathology findings make these data unsuitable for a quantitative assessment. The primary value of these data is to characterize the known pathologic effects on the testes and provide information on mechanistism/mode of action (changes in Sertoli cell proliferation and cell cycle regulators) for these effects.

Cammack et al. ***(120)***, in a GLP study commissioned by the Advanced Medical Technology Association, examined reproductive development of Sprague-Dawley rats treated IV or orally with DEHP. Beginning at 3–5 days of age, rats were treated for 21 days with DEHP (99.8% purity) at 0 (vehicle), 60, 300, or 600 mg/kg bw/day by IV infusion or 0 (vehicle) or 300 mg/kg bw/day by oral gavage. Another set of rats was dosed with 600 mg/kg bw/day DEHP by oral gavage for 19 days; this group replaced a previous group that suffered high mortality rates following gavage dosing with 1000 mg/kg bw/day. Concentration and stability of dosing solutions were verified. The dosing vehicle was Intralipid®, a 10% fat emulsion solution for IV use. Each dose group in this study consisted of 16 animals. Seven rats/group were scheduled to be killed following the dosing period, and 9/group were to be held for a recovery period until 90 days of age, at which time they were also killed and necropsied. During each necropsy period, brain, liver, spleen, heart, kidneys, and testes were weighed. A testis from each rat was fixed in Bouin fluid. Histopathological examinations of testes were conducted during both necropsy periods; histopathological analyses of prostate, seminal vesicle, and epididymis were conducted only in 90-day-old animals. Sperm

count, motility, and morphology were examined in the 90-day-old rats. Sperm data were evaluated by Kruskal-Wallis nonparametric ANOVA, and if there was a significant finding, a Mann-Whitney *U* test was used for pair-wise comparisons. Body weight and organ weight data were assessed by determining group differences followed by pair-wise comparisons in the case of significant findings.

The only significant body weight effect reported was reduced body weight gain in rats given 600 mg/kg bw/day by IV infusion and oral gavage. Percent changes in testes and liver weight compared to control are outlined in Table 25.

Table 25. Testicular and Liver Weight Changes in Rats Treated with DEHP by IV Infusion or Oral Gavage

Weight Change	*Percent of Control Value in Each Dose Group (mg/kg bw/day)*				
	60 IV	*300 IV*	*600 IV*	*300 oral*	*600[b] oral*
Period immediately after dosing[a]					
Testis	91[a]	67***	52***	59***	36***
Liver	99	126*	133***	125	108
Following recovery period[a]					
Testis	101	88**	74***	69***	58***
Liver	98	96	89	94	89

From: Cammack et al. *(120)*.

*$P<0.01$; **$P<0.001$; ***$P<0.001$.

[a] Rats were examined immediately after the 21-day dosing period or following a recovery period at 90 days of age.

[b] Rats in this group were dosed for 19 days, while the other dose groups were dosed for 21 days.

[Several organ weight effects were noted, but data were presented only for absolute liver and testis weight. Relative weights are discussed only when differences were noted from absolute weights.] In animals killed immediately after the dosing period, absolute testis weights were significantly reduced in the 300 and 600 mg/kg bw/day oral and IV groups. **[For testis weights, CERHR calculated a BMD_{10}[2] of 122 mg/kg bw/day, a $BMDL_{10}$ of 106 mg/kg bw/day, a $BMD_{1\ SD}$ of 179 mg/kg bw/day, and a $BMDL_{1\ SD}$ of 125 mg/kg bw/day in animals treated by IV infusion. After oral exposure, the BMD_{10} was 90.7 mg/kg bw/day, the $BMDL_{10}$ was 77.4 mg/kg bw/day, the $BMD_{1\ SD}$ was 875 mg/kg bw/day, and the $BMDL_{1\ SD}$ was 628 mg/kg bw/day.]** Absolute liver weights were increased in rats given 300 or 600 mg/kg bw/day DEHP by IV infusion. **[For liver effects in IV-dosed rats, CERHR calculated a BMD10 of 163 mg/kg bw/day, $BMDL_{10}$ of 122 mg/kg bw/day, a $BMD_{1\ SD}$ of 101 mg/kg bw/day, and a $BMDL_{1\ SD}$ of 66 mg/kg bw/day. In orally dosed rats, the BMD_{10} was 712 mg/kg bw/day, the $BMDL_{10}$ was 196 mg/kg bw/day, the $BMD_{1\ SD}$ was 585 mg/kg bw/day, and the $BMDL_{1\ SD}$ was 193 mg/kg bw/day.]** Liver weight relative to body weight was reportedly increased in the 300 and 600 mg/kg bw/day oral DEHP groups. Other absolute and relative organ weight effects reported were increased spleen weight in the IV 600 mg/kg bw/day group and decreased kidney weight in the oral 600 mg/kg bw/day group. Depletion of germinal epithelium and/or decreased

[2] See footnote to Table 22 for definitions and a discussion of the use of benchmark doses in this report.

seminiferous tubule diameter was noted in all animals from the 300 and 600 mg/kg bw/day oral and IV dosing groups. **[It was not clear if all 7 rats from each dose group were examined, or how many rats were affected with each type of lesion.]** Germinal epithelium depletion was rated as moderate (51–75% reduction in thickness) in the 600 mg/kg bw/day oral group and mild (25–50% change) in all other dose groups given ≥300 mg/kg bw/day DEHP. Reduced tubule diameter was rated as mild (25–50% reduction in diameter) in the 600 mg/kg bw/day oral group and minimal (<25% change) in the other dose groups treated with ≥300 mg/kg bw/day DEHP.

In animals killed at 90 days of age, testicular weights in the 300 and 600 mg/kg bw/day IV and oral groups remained lower than those of controls. **[CERHR estimated a BMD_{10} of 222 mg/kg bw/day, a $BMDL_{10}$ of 190 mg/kg bw/day, a $BMD_{1\ SD}$ of 169 mg/kg bw/day, and a $BMDL_{1\ SD}$ of 105 mg/kg bw/day for testicular weights in the IV group. In the orally dosed group, the BMD_{10} was 138 mg/kg bw/day, the $BMDL_{10}$ was 125 mg/kg bw/day, the $BMD_{1\ SD}$ was 85 mg/kg bw/day, and the $BMDL_{1\ SD}$ was 65 mg/kg bw/day.]** Earlier increases in absolute liver weights did not persist in 90-day-old animals. The only persisting testicular lesion, apart from reduced testis weight, was a minimal (<25%) decrease in seminiferous tubular diameter in 2 of 5 rats in the 300 mg/kg bw/day oral group and 3 of 7 rats in the 600 mg/kg bw/day oral group. **[The number of animals examined in other dose groups was not indicated.]** No lesions were observed in prostate, epididymis, or seminal vesicles. There were no adverse effects on sperm count, motility, or morphology.

The study authors concluded that sperm and testicular histologic parameters resolved after DEHP treatment was discontinued. They stated that "lack of residual effects on sperm parameters found in this study will be important in the understanding of potential health risks from DEHP in patients undergoing critical procedures, such as ECMO in infants, and the management of these risks."

[The Expert Panel notes that an acknowledgement in this paper indicates that advice on design was received from an Expert Panel member "representing the NTP/CERHR Phthalate Expert Panel"; however, this Expert Panel member was rendering advice as an individual scientist and not as a representative of the Expert Panel.]

Strengths/Weaknesses: This GLP study was conducted using relevant routes of exposure, both IV and oral, during a relevant period of postnatal development. Multiple dose levels allowed for dose-response and benchmark dose assessment. Test material source and purity were provided. The dosing emulsification was characterized and evaluated for stability. Although the characterization and stability data for the dosing emulsification were not presented, the wording in this section suggested that DEHP concentration and stability were confirmed. This study demonstrated dose-responsive effects in a variety of parameters following both IV and oral exposure. Weaknesses included inadequate detail on statistical analyses; only sperm endpoints were addressed with specific details on analyses methods. The description of analyses of body and organ weights included reference to "standard operating procedures." The use of an alpha level of 0.01 is a weakness. The authors did not address assignment of animals to treatment or whether litter effect was controlled. The pathology incidence data were not presented, making it difficult to confirm effect levels and compare to background lesions in the control. However, even though no control data were presented, the incidence of findings was high in the treated animals and the types of lesions observed were fairly rare in untreated control animals. The variability in onset of dosing (PND 3–5) is an additional weakness.

Utility (Adequacy) for CERHR Evaluation Process: Portions of this study can be used for the evaluation. This study provides important dose-response information following both IV and oral exposure in young animals. The testes and liver weight and sperm assessment parameters provide quantitative multi-route dose-response data. Due to incomplete presentation of data, there is less confidence in the conclusion regarding the pathologic findings; however, pathological changes consistent with disruption of spermatogenesis are supported by the findings of other investigators. The relatively small sample size also reduces the confidence level of these conclusions, especially with regard to lack of treatment-related effects.

Gray et al. *(121)*, from the EPA, examined the effect of perinatal phthalate exposure in rats. It appears that data from this study were also reported in an abstract by Ostby et al. *(122)*. Sprague-Dawley rats were gavage dosed with 0 (corn oil vehicle) or 750 mg/kg bw/day DEHP (99% purity) from GD 14 (GD 1 = day sperm detected) to PND 3 (PND 1 = postcoital day 23). The experiment was repeated with a second block of animals. In each block of the experiment, there were 7–9 treated dams and 9–10 control dams. Parameters examined in pups (period examined) included body weight (PND 2), anogenital distance (PND 2), testicular histology (PND 2, 9–10, and 13, 3–5 months, and 4–7 months), areolas/nipples (PND 13), preputial separation (beginning on PND 28), mating behavior (adulthood), abnormalities of reproductive organs (3–5 months and 4–7 months), and sperm counts. Statistical analyses were based on litters, and blocks were pooled in cases of identical results. Analyses included 1-way ANOVA followed by post hoc *t*-tests when statistical significance was obtained. Anogenital distance and organ weight data were covaried with body weight. Categorical data were analyzed by Fisher exact test or chi-squared test.

DEHP treatment resulted in a small reduction in maternal body weight gain. Litter weight at birth was significantly reduced by 15% in the DEHP group, but there was no effect on number of live pups at birth. In DEHP-treated males on PND 2, anogenital distance was significantly decreased by ~30%, with or without adjustment for body weight, and paired testis weights were significantly decreased by 35%. There was no effect on anogenital distance in female pups. Histological examination of testes from DEHP-treated rats on PND 2–3 revealed focal interstitial hemorrhage and multinucleated gonocytes containing 3–5 nuclei or undergoing degenerative changes. Hemorrhagic testes were observed in 7 DEHP-treated males from 3 litters at PND 8–9. Histological examination of testes on PND 9–10 revealed evidence of focal hemorrhage in some testes and extensive coagulative necrosis in other testes of DEHP-treated rats; loss of seminiferous epithelium was observed in areas with hemorrhage or necrosis. Areolas were observed in 87% of DEHP-treated male pups versus none in control pups. DEHP treatment did not delay the age of preputial separation, but preputial separation was incomplete due to malformations in 19 of 56 treated pups.

DEHP did not appear to affect sexual behavior in adult rats, except that males with malformed penises were unable to achieve intromission. At necropsy, 45 DEHP-treated adult rats from 15 litters were assessed for malformations of reproductive organs, which were observed in 82% of DEHP-treated males. The types of malformations included permanent nipples, clefting of phallus and hypospadias, vaginal pouches, agenesis of prostate, seminal vesicles, or coagulating glands. Sperm production and numbers were said to be unaffected by DEHP treatment **[data not shown]**. Testicular defects included hemorrhage, granuloma, fibrosis, reduced size or atrophy, and non-descent associated with abnormal gubernacula or ligaments. Significant reductions in weight were observed for all male reproductive organs including testis, levator ani plus bulbocavernosus muscle, seminal vesicle, prostate, penis, and

epididymis. Liver, pituitary, kidney, and adrenal weights were not affected by DEHP treatment. Serum testosterone levels were unaffected in DEHP-treated rats. The study authors concluded that 750 mg/kg bw/day DEHP severely alters sexual differentiation in an anti-androgenic manner.

Strengths/Weaknesses: This paper reports a high quality comprehensive evaluation of the potential anti-androgenic effects of DEHP in rats when administered at a single dose during a period of critical sexual differentiation, from GD 14 to PND 3. The experiment included a relevant route of exposure, and the authors provided the source and purity of test material. Strengths include good detailed description of methodology, robust numbers of litters evaluated (as high as 16 litters) for the "active" phthalates, appropriate statistical analyses using the litter as the unit of analysis for most endpoints, randomization of animals into blocks, and near complete presentation of data with standard errors where appropriate. Most treatment-related changes were robust and clearly distinguishable from controls, resulting in high confidence for the findings. The authors described a characteristic phthalate phenotype, although they were not the first to do so. The suggestion of a similar mechanism of action for fetal and pubertal male effects based on the structure of active and inactive phthalates was an important contribution. Weaknesses include the single, high dose level, the small sample size for most of the "inactive" phthalate exposures, and the presentation of only fetal incidence for malformation data.

Utility (Adequacy) for CERHR Evaluation Process: The majority of these data are adequate for consideration. The study robustly demonstrates multiple adverse effects on sexual development in males, fetal growth, and maternal toxicity. Because only one dose level was used, a NOAEL was not obtained and a dose-response and benchmark dose evaluation cannot be conducted. The presentation of the pathology findings is troublesome in that no data or comparison to the controls were presented, diminishing the value of this information for the evaluation. However, given the severity of the findings and the support of other endpoints affected, this oversight may be of less importance. The behavioral evaluation is insufficient and cannot be used for risk assessment.

Parks et al. *(123)*, from the EPA, conducted a series of *in vivo*, *ex vivo*, and *in vitro* studies to examine mechanisms of DEHP-induced malformations in rat reproductive organs. A competitive androgen-binding study was conducted in monkey cells transfected with the human androgen receptor vector pCMVhAR. Radioactivity was measured following incubation of the cells for 2 hours with 5 nM 3H-R1881 (a synthetic androgen ligand) and DEHP or MEHP at concentrations of 0 or 0.05 – 10 μM. Neither DEHP nor MEHP competed with R1881 for androgen receptor binding.

In the *in vivo* study, Sprague-Dawley rats were randomly assigned to groups that were gavage dosed with 0 (corn oil vehicle) or 750 mg/kg bw/day DEHP from GD 14 (GD 1 = day after mating) until necropsy. Rats were killed and necropsied on GD 17, 18, or 20 or PND 2 (PND 1 = day after birth). The study was conducted in 2 blocks, and a total of 4 – 5 litters per group were examined at each necrospy period. At GD 17, 18, and 20 and PND 2, 1 testis from 2 or 3 males/litter was incubated in media for 3 hours to determine *ex vivo* testosterone production, and the other testis was used to measure testosterone content. In GD 17, 18, and 20 males, testosterone levels were also measured in the carcasses from which testes were removed (n = 18 – 20 per group). Testosterone levels were measured by RIA. One testis from each of 4 DEHP-treated and 6 control PND 2 males was fixed in 5% glutaraldehyde for histopathological examination. One testis from each of 4 control and 5 DEHP-exposed PND 20 males and an unspecified number of DEHP-exposed PND 3 males from a parallel study was stained

for 3β-hydroxysteroid dehydrogenase, which is specific for Leydig cells. Anogenital distance was measured in all male and female offspring on PND 2. Litter means were used in statistical analyses. Data were analyzed by ANOVA followed by 2-tailed *t*-tests if ANOVA resulted in significant findings. Testicular histopathological findings were analyzed by Fisher exact test.

Maternal weight gain during gestation was significantly reduced in the DEHP-treated group. Number of live pups at birth was not significantly affected by DEHP treatment. *Ex vivo* testicular testosterone production in GD 17, 18, and 20 and PND 2 offspring from DEHP-exposed groups was significantly lower compared to control groups. Testicular testosterone content in DEHP-exposed offspring and pups was reduced by 60–85% compared to controls examined at each necropsy period; the effect was statistically significant at all time points except GD 20. **[It appears a footnote regarding GD 20 is missing in Table 1 of the study.]** Whole body testosterone levels were significantly lower in DEHP-exposed fetuses on GD 17 (71% lower than controls) and 18 (47% lower than controls), but the reduction on GD 20 was not significant. Significant reductions in testis weight were noted in the DEHP group on GD 20 (18% lower than controls) and PND 2 (49% lower than controls). Body weights of DEHP-exposed pups were described as 23% lower than controls on PND 2, but statistical significance was not achieved. Testis weights adjusted for body weights were significantly decreased in PND 2 pups exposed to DEHP. Anogenital distance was significantly reduced by 36% in PND 2 males compared to controls but was not affected in female pups exposed to DEHP. Histopathological examination of PND 2 testes of DEHP-treated rats revealed an increased number of enlarged and multinucleated gonocytes and aggregates of hyperplastic Leydig cells. 3β-Hydroxysteroid dehydrogenase staining confirmed the presence of Leydig cell aggregates in DEHP-exposed males on GD 20 and PND 2. In contrast, 3β-hydroxysteroid dehydrogenase staining revealed an even dispersion of Leydig cells and less intense staining in testes of control fetuses and pups.

The study authors concluded that treatment with 750 mg/kg bw/day DEHP inhibited testosterone production in male pups during the period of sexual differentiation, and this inhibition was a likely cause of malformations observed in other studies. Malformations likely result from a mechanism that does not directly involve the androgen receptor.

Strengths/Weaknesses: This report includes a good, detailed description of the methods, appropriate route of exposure, and statistical methods using the litter as the unit of analysis. The authors provided excellent use of controls in the evaluation of pathology. An appropriate exposure period was used for male effects. This study demonstrated clear effects on fetal whole body and fetal and postnatal testicular testosterone levels in male offspring, testes weight, anogenital distance, and histopathology of the testes following maternal gestational or gestational and postnatal exposure, and provided clear evidence that DEHP and its principal metabolite do not bind to the androgen receptor. The description of the time course for fetal testosterone alterations is an additional strength. Weaknesses include the single, high dose level and the small sample size. The samples size was sufficient to demonstrate robust effects of treatment but not to provide high confidence in lack of effect. There was no purity information on the test material.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate in providing mechanistic information for use in the evaluation process. Because only one dose level was used, the study cannot establish a NOAEL and is not suitable for dose-response or benchmark dose evaluation. Given the small sample size, there is low confidence in negative findings.

Wilson et al. *(124)*, from the EPA, conducted 2 *in vivo* studies to determine if gubernacular lesions induced by DEHP and other chemicals result from inhibition of insulin-like hormone 3 (insl3), a hormone produced by fetal Leydig cells and considered to be a marker of cell maturation. In the first study, 10 pregnant Sprague-Dawley rats were gavage dosed with 0 (corn oil vehicle) or 750 mg/kg bw/day DEHP (99% purity) on GD 14–18 (GD 1 = day sperm detected). In the second study, 3 dams/group were dosed with 0 or 1000 mg/kg bw/day DEHP on GD 14–18. The DEHP concentrations were found to induce gubernacular lesions in previous studies. Dams were killed on GD 18, and fetal testes were removed. The testes were pooled by litter for examination of insl3 expression by a polymerized chain reaction method. The second study also examined *ex vivo* testosterone and progesterone production by incubating the testes in media for 3 hours, measuring hormone levels in media by RIA, and pooling the data by litter. Statistical analyses were conducted on a litter basis. Analyses included ANOVA, followed by paired *t*-test if statistical significance was obtained by ANOVA. DEHP treatment significantly reduced insl3 expression by about 80% in the first study and **[~60%]** in the second study. *Ex vivo* testosterone production was reduced **[~50%]** in testes from the 1000 mg/kg bw/day DEHP group compared to the control group. There was no effect on *ex vivo* progesterone production.

The study authors proposed that DEHP intake in rodents results in delayed fetal Leydig cell maturation, which leads to reduced testosterone production and insl3 production. It was stated that reduced insl3 expression results in gubernacular malformations and undescended testis, while reduced testosterone leads to malformations in testosterone-dependent tissues.

Strengths/Weaknesses: Use of the oral route of exposure and the appropriate exposure window are strengths of this study. Statistical methods were appropriate, using the litter as the unit of analysis. The data are consistent with previous findings with evaluation of a possible mode of action for impaired testis descent. The use of a single dose level is a weakness, and the *ex vivo* endpoints are not directly relevant to human risk assessment.

Utility (Adequacy) for CERHR Evaluation Process: This paper has limited utility in the evaluation process.

Liu et al. *(125)*, supported by NIH, evaluated gene expression profiles in GD 19 fetal testes after GD 12–19 gavage treatment of dams with 1 of 7 phthalates (n = 5/group) or with corn oil vehicle (n = 10; vaginal sperm = GD 0). The phthalates were DEHP or diethyl, dimethyl, dioctyl tere-, dibutyl, dipentyl, or benzyl butyl phthalate. **[Purity was said to have been verified but was not specified.]** The dose of each phthalate was 500 mg/kg bw/day. After removal by cesarean section, pups were evaluated for anogenital distance and testes were harvested from male offspring. Total RNA was extracted from the testes of 3 pups/treatment group, each from a different litter, and hybridized to a microarray gene chip. ANOVA and post hoc Dunnett test were used to evaluate differences in gene expression in phthalate-exposed and control samples. A Bonferroni adjustment was used for multiple comparisons. Selected genes were investigated further using RT-PCR on total testis RNA from 6 control fetuses and 3 phthalate-treated fetuses/group. Relative expression ratios were calculated with respect to glyceraldehyde-3-phosphate dehydrogenase and compared to control using ANOVA and post hoc Dunnett test. Comparisons between different phthalates were also evaluated using ANOVA. Immunohistochemistry was performed in formalin-fixed testicular sections from control and dibutyl phthalate-exposed fetuses **[not discussed here]**.

Anogenital distance was significantly reduced by pregnancy treatment with DEHP, dibutyl phthalate, benzyl butyl phthalate, and dipentyl phthalate. Dimethyl, diethyl, and dioctyl terephthalate did not affect anogenital distance. Of 391 significant gene probe sets, there were 167 characterized sequences. Genes related to lipid, sterol, and cholesterol homeostasis accounted for 31 of these 167 genes. There were also 10 genes involved in lipid, sterol, and cholesterol transport, 12 genes involved in steroidogenesis, 9 transcription factor genes, 22 signal transduction genes, 11 genes involved in oxidative stress, and 13 genes related to the cytoskeleton. Eighteen of these genes were evaluated using RT-PCR, and 16 of the 18 were affected by those phthalates that altered anogenital distance in comparison to the phthalates that did not alter anogenital distance (Table 26). There were few differences in relative expression between the individual phthalates in the group that altered anogenital distance. The authors concluded that the developmentally toxic phthalates were "indistinguishable in their effects on gene expression in the developing fetal testis." These phthalates were described as targeting pathways directly or indirectly related to Leydig cell production of testosterone and pathways important for Sertoli cell-gonocyte interaction.

[The Expert Panel notes that there is a study by Lehmann et al. *(126)* in which di(n-butyl) phthalate was administered to pregnant Sprague-Dawley rats on GD 12 – 19 at gavage doses of 0.1, 1.0, 10, 50, 100, or 500 mg/kg bw/day. Fetal testes were evaluated for testosterone and mRNA and protein concentration for key steroidogenic enzymes. Decreases in mRNA for key steroidogenic enzymes occurred at maternal exposure levels below those associated with a decrease in testicular testosterone. To the extent that di(n-butyl phthalate) and DEHP share molecular mechanisms of action, this study may offer insights relevant to DEHP testicular toxicity.]

Strengths/Weaknesses: This study was a well-conducted and reported and investigated the potential mechanisms of "testicular dysgenesis" and male reproductive tract abnormalities in rats following gestational exposure to DEHP and other phthalates. The dams were treated during a critical period of male reproductive tract development (GD 12 – 19) by an appropriate route (oral) of exposure. The statistical design was appropriate, using the litter as the experimental unit of analysis and incorporating body weight as a cofactor in the anogenital distance analysis. The use of both "active" and "inactive" phthalates was a strength, permitting a comparison of gene expression profiles. Weaknesses include the use of only a single dose level of 500 mg/kg bw, and small sample size (5/treatment); however, the sample was sufficient to detect strong effects. The study used only 1 endpoint (anogenital distance) that is classically used for risk assessment. The remaining endpoints were mechanistic in nature (gene expression). Evaluation at a single time point is a weakness inasmuch as the noted effects could be the consequences rather than the causes of the altered differentiation.

Utility (Adequacy) for CERHR Evaluation Process: The anogenital distance data from this study are useful for hazard identification at the relatively high dose level of 500 mg/kg bw and the study supports/confirms other reports of adverse effects on this endpoint following gestational exposure to DEHP. Because only one dose level was used, a dose-response evaluation and NOAELs/LOAELs cannot be established. The gene expression assays are valuable in helping to understand potential mechanisms of action. Based on the affected genes, a complex mode of action is suggested involving Leydig cell, Sertoli cell, and Sertoli-gonocyte interactions.

Table 26. Relative Expression of Fetal Testis Genes after Phthalate Treatment of Pregnant Rats

Gene	*Direction of Alteration*
Lipid, sterol, and cholesterol transport	
Epididymal secretory protein 1	↓
Low density lipoprotein receptor	↓
Steroidogenesis	
17b-Hydroxysteroid dehydrogenase 7	↓
17b-Hydroxysteroid dehydrogenase 3	↔
LH/chorionic gonadotropin receptor	↓
Transcription factors	
CCAAT/enhancer binding protein, beta	↓
Early growth response 1	↑
Nuclear receptor subfamily 4, group A, member 1	↑
Nuclear factor, interleukin 3, regulated	↑
Nuclear receptor subfamily 0, group B, member 1	↓
Transcription factor 1	
Signal transduction	
Insulin-induced gene 1	↓
Cytoskeleton	
Fasciculation and elongation protein	↑*
Unclassified	
Decay-accelerating factor	↔
DOPA decarboxylase	↓
Seminal vesicle secretion 5	↓
Testis-derived transcript (testin)	↑

↑,↓,↔ Statistically increased, decreased, or unchanged on comparison of the phthalates that decreased anogenital distance (DEHP, dibutyl phthalate, benzyl butyl phthalate) with the phthalates that did not (dimethyl, diethyl, and dioctyl terephthalate).

*DEHP did not show a response, although the other anogenital distance-altering phthalates did. From Liu et al. *(125)*.

Kobayashi et al. *(127)* presented an abstract describing treatment of pregnant Sprague Dawley rats by gavage with DEHP 0, 25, 100, or 400 mg/kg bw/day on GD 6–PND 20. There were no treatment effects on offspring body weight, body length, tail length, organ weights, or plasma levels of thyroxine or tri-iodothyronine at 1, 3, or 9 weeks of age. **[Abstracts are noted but are not used in reaching conclusions.]**

Wang et al. *(128)* presented an abstract describing treatment of pregnant Sprague Dawley rats by gavage with DEHP 0, 25, 100, or 400 mg/kg bw/day on GD 6–PND 20. Blood testosterone and progesterone in

male offspring were described as showing a dose-related increase at 9 weeks of age, although differences were not statistically significant. There were no treatment effects on relative testis or prostate weight at 9 or 36 weeks of age. Human chorionic gonadotropin was used to stimulate testosterone production **[age unspecified]**; blood testosterone increases were numerically lower in DEHP-exposed offspring than in control offspring. **[Abstracts are noted but are not used in reaching conclusions.]**

3.2.1.2 In Vitro Exposures

Iona et al. ***(129)***, supported by "EU BIO-CT96-0183," "MURST," and the Italian Public Health Ministry, conducted *in vitro* studies to examine the effect of MEHP and 2 other chemicals on primordial mouse germ cells. In the first and second studies, primordial germ cells were obtained from sexually undifferentiated gonadal ridges of CD-1 mouse embryos at 11.5 days post coitum and were seeded onto STO, an embryonic mouse fibroblast cell line, to allow the germ cells to proliferate. Cultures were treated with MEHP **[purity not indicated]** at 0 (dimethylsulfoxide [DMSO] vehicle), 100, 300, or 600 μM **[0, 27.8, 83.4, or 167 mg/L]** for 2 hours or 1 day. **[It was not specified if MEHP was rinsed from cells following the 2-hour and 1-day treatments.]** Numbers of primordial germ cells were counted after 1 and 3 days of culture. MEHP treatment for 2 hours did not affect numbers of primordial germ cells at any dose. Following treatment with MEHP for 1 day, there was a "slight but not significant" decrease in primordial germ cell number at 100 and 300 μM MEHP and cytotoxicity at 600 μM MEHP. It was determined that 600 μM MEHP reduced the viability of supporting STO cells by about 50%. In the third study, primordial germ cells were incubated in suspension with 100–300 μM MEHP for 2 hours. MEHP was washed from the cells, and the germ cells were then seeded onto STO cells. The numbers of primordial germ cells were measured after 1 and 3 days of culture. Numbers of germ cells were reduced by about 38% **[estimated from a graph]** at ≥300 μM MEHP **[statistical significance not indicated, but based on the graphed standard errors, the results appear to be significant]**. In a fourth study, apoptosis was not significantly increased following incubation of the primordial germ cells in 500 μM MEHP for 6 hours. In a fifth study, a short-term adhesion assay was used to determine that exposure to 300–600 μM MEHP for 2 hours reduced by about 40% the percentages of primordial germ cells capable of binding to STO cells. The study authors concluded that MEHP affected adhesion of primordial germ cells to STO cells without inhibiting growth or survival of the germ cells.

A sixth study was conducted to determine if *in vitro* effects of MEHP could be replicated *in vivo*. Pregnant CD-1 mice were gavage dosed with 1000 or 2000 mg/kg bw MEHP on day 8.5, 10.5, or 11.5 post coitum. **[One control group was used, but it was not specified if or when the control was gavaged.]** Three mice per dose were killed 12.5 days post coitum, and gonadal ridges were taken from each embryo. Sections from at least 3 randomly selected embryos/sex were examined to determine primordial germ cell numbers. Apparent reductions in primordial germ cell numbers were noted in 3 of 5 female embryos and 2 of 5 male embryos treated with 2000 mg/kg bw DEHP 8.5 and 11.5 days post coitum, but mean differences were not statistically significant in any dose group.

Strengths/Weaknesses: A range of dose levels was used for *in vitro* (100–600 μM; 28–167 mg/L) and *in vivo* (1000–2000 mg/kg) studies, but it is a weakness that the *in vitro* levels were not related to *in vivo* exposure levels. In addition, the endpoints are not typical examples of phthalate toxicity, which limits the importance of the experiments. Although the studies were insightful, potentially useful experiments to help understand the mechanisms of action of reproductive toxicants, the *in vitro* studies were primarily an exercise in methods development/methods utility. The *in vitro* systems were not standardized or

validated for their predictive value for *in vivo* effects (i.e., the artificial environments used may not be relevant to *in vivo* exposure). This opinion is supported by the authors' conclusion that their *in vitro* results were not always exactly predictive of the effects *in vivo*. The *in vivo* study used an insufficient number of litters, employed a single dose, and did not directly address any relevant functional outcome.

Utility (Adequacy) for CERHR Evaluation Process: This paper has no utility in a quantitative assessment, although it may provide supplemental mechanism information.

Li and Kim *(130)*, supported by NIEHS, examined the effects of MEHP on cultured fetal and neonatal rat testes during 3 periods: GD 13 (plug = GD 0), GD 18, and PND 3. GD 13 is the period when testicular cord formation and Sertoli cell differentiation begin. GD 18 represents the period when Sertoli cells are proliferating and gonocytes are mitotically quiescent. PND 3 represents the period when Sertoli cells continue proliferating and gonocytes migrate to the basal side of the seminiferous tubule and become mitotically active. Gonads were obtained from fetuses or pups of Sprague-Dawley rat dams (at least 3 dams/group and 4 offspring/dam) during each of the 3 periods of development. A testis from each animal was cultured for 3 days in medium containing MEHP (≥ 99% purity) 50, 100, or 200 μM **[13.9, 27.8, or 55.6 mg/L]** and the other in medium containing the DMSO vehicle. Assays were conducted to determine cell counts, cell differentiation and proliferation, and expression of testicular cell marker proteins. Data were analyzed by one-way ANOVA followed by pair-wise comparison. In GD 13 testes, MEHP had no effect on testicular cord formation or number of gonocytes. According to the study authors, the lack of effect on testicular cord formation meant that MEHP did not affect differentiation of indifferent gonad to testis. MEHP treatment also had no effect on expression patterns of Müllerian-inhibiting substance or GATA-4, Sertoli cell markers, or on mouse vasa homolog protein, a germ cell marker. In GD 18 testes, Sertoli cells were targets of MEHP as indicated by decreased Müllerian-inhibiting substance and GATA-4 expression and impaired Sertoli cell proliferation at ≥ 100 μM MEHP. Numbers of gonocytes and mouse vasa homolog protein expression were unaffected by MEHP treatment. Both Sertoli cells and gonocytes were targets of MEHP in PND 3 testes. There were significant dose-dependent reductions of Müllerian-inhibiting substance and GATA expression; Sertoli cell proliferation was inhibited at ≥ 100 μM MEHP. Reduced gonocyte numbers and Sertoli and germ cell disorganization were observed at ≥ 100 μM MEHP. Gonocyte proliferation was not inhibited, but apoptosis was increased by treatment with ≥ 100 μM MEHP. Seminiferous cord structure remained intact following MEHP exposure.

The study authors concluded that these results suggest that MEHP targets testicular cells during periods when they are mitotically active.

Strengths/Weaknesses: The use of multiple concentrations of MEHP allowed a dose-response evaluation. In general, the methods and results were well presented. The culture system was unique, without a history of use in other laboratories, and the absence of positive and negative controls was a weakness. The MEHP concentrations that were used were not related to *in vivo* exposure levels, and the assumption that exposure levels would be constant over a 3-day culture period was not verified. There were limited numbers of dams (3) and uncertain control for litter effect.

Utility (Adequacy) for CERHR Evaluation Process: The study provides evidence for a potential mode of action for adverse effects on the testes noted after *in vivo* embryonic or prepubertal exposure to DEHP. Because these studies were conducted *in vitro*, they are not directly useful in the evaluation process.

3.2.2 Developmental Studies Focusing on Non-reproductive Effects

Magliozzi et al. ***(131)***, supported by Italian "CNR" and "MURST" grants, examined the effects of prenatal DEHP exposure on rat neonatal lung. Wistar rats were fed diets containing 0 or 1% (w/w) DEHP from the week prior to delivery through 2 days following delivery. Five dams that ate at least 1000 mg DEHP/kg bw/day and 5 control dams were used for the study. Two days following delivery, pups were weighed and killed. One pup per litter was used to measure DEHP in blood by GC/MS. Lungs from 5 pups per litter were fixed in Bouin fluid for examination by light microscopy, and lungs from 1 pup per litter were fixed in 4% formaldehyde for examination by electron microscopy. Lungs from 2-day-old rat pups were stated to have similar histological features as lungs from premature infants at gestation weeks 26–36. A catalase-immunoreactivity method was used to measure number and sizes of pneumocytes, cells that are a major source of surfactant and contain high numbers of peroxisomes. Pup livers were removed and weighed. **[Statistical methods were not discussed.]** DEHP in blood was measured at 4.7±0.46 μg/mL **[error not specified]** in treated pups and 1.9±0.57 in control pups. Relative liver weight was significantly increased in the DEHP-treated pups, thus confirming DEHP exposure. Examination of lungs by light microscopy revealed reduced respiratory surface in DEHP-treated animals as a result of fewer airspace units that were dilated and units that were less branched than spaces of untreated animals. In treated compared to control pups, relative number of type II pneumocytes increased by 187% and mean diameter increased by 120% ($P < 0.01$). Pneumocyte peroxisomes were unaffected in DEHP-treated pups. The study authors concluded that the relevancy of these results to intubated preterm infants inhaling DEHP is not known due to differences in routes of exposure and interspecies metabolism. However, they stated that their study indicates a need to examine possible injury related to forced ventilation of infants.

Strengths/Weaknesses: For most procedures, this paper included excellent presentations of methodology and results. The quantitative representation of pathology using morphometrics provided value; however, no description of the statistical analyses was noted. The investigation of a new target tissue (lung) was a strength. Weaknesses included the use of a single very high dose level without an indication that the effect is relevant to lower exposure levels. Only animals consuming at least 1 g/kg bw/day were selected, resulting in low sample size. Plasma extraction validation was conducted with human blood rather than rat blood. Significant contamination of control animals with DEHP was apparent with DEHP blood levels in controls about one-third those of treated animals. In addition, only DEHP was measured, leading to the possibility that evaluation of other analytes would have suggested even greater control animal contamination. The relatively high levels of DEHP in control pups raises serious questions regarding dosing errors and quality of study conduct and/or plasma level evaluation. Because exposure of the dams continued until the pups were killed, the pups from the DEHP-treated dams should have had markedly higher levels of DEHP than the control pups. Methods for the statistical analyses were not presented.

Utility (Adequacy) for CERHR Evaluation Process: Although the evaluation of a new target tissue is potentially valuable, due to the deficiencies in presentation of statistical analyses and the potential contamination of the control pups, this study is of low utility.

Masuo et al. ***(132)***, from the Japanese National Institute of Advanced Industrial Science and Technology, studied the effects of DEHP and other chemicals on motor activity in rats. At 5 days following birth, male Wistar rats (~10 g) received DEHP **[purity not specified]** at 0 (olive oil vehicle) or 87 nmol **[3.4**

mg/kg bw] by intracisternal administration. Groups of 7 control pups and 5 DEHP-treated pups were nursed by lactating dams until weaning at 3 weeks of age. Spontaneous motor activity was measured in all 4-week-old pups using a Supermex activity-monitoring system. The rats were killed at 8 weeks of age. Multiple gene expression in striatum and midbrain of 3 rats/group was determined using an array, and an immunohistochemical technique was used to measure tyrosine hydroxylase levels in sectioned brains from 8-week-old rats. Statistical analyses included ANOVA, followed by post hoc Scheffé test.

DEHP caused a significant increase in spontaneous motor activity during the dark phase, light phase, and entire 24-hour period. DEHP treatment did not appear to affect stereotyped behavior. There was no effect on tyrosine hydroxylase immunoreactivity (data not shown). In midbrain, DEHP treatment up-regulated expression of genes for glutamate/aspartate transporter, superoxide dismutase 1, heat shock 90-kilodalton (kDa) protein beta, neuropeptide Y, fibroblast growth factor 10, and natriuretic peptide precursor C. DEHP treatment down-regulated midbrain expression of genes for c-fos proto-oncogene, cytochrome P450 17, heat shock 70-kDa protein 1A, dopamine receptor 1A, galanin receptor 2, arginine vasopressin receptor 2, and glutamate ionotropic receptor. In striatum, DEHP treatment down-regulated expression of genes for c-fos proto-oncogene, heat shock 70-kDa protein 1A, galanin receptor 2, glutamate ionotropic receptor, and PDGF B polypeptide. Based on analyses of gene expression changes, the study authors postulated that an inhibition of glutamatergic transmission in midbrain and striatum may be a mechanism of DEHP-induced changes in motor activity.

Strengths/Weaknesses: The hypothesis that DEHP would alter brain dopaminergic system, as has been reported for estrogenic chemicals, was reasonable. The comparison of a positive control (6-hydroxy-dopamine) and 4 chemicals considered capable of endocrine alterations (bisphenol A, *p*-nonylphenol, *p*-octylphenol, and DEHP), all of which caused a similar increase in diurnal spontaneous motor activity, is a strength. Weaknesses include the route of administration (intracisternal), the high dose level, the lack of information on offspring body weights, the apparent lack of control for litter effects, the small sample sizes (5 – 7 litters), and the use of a single dose level. It is not clear to what extent DEHP would be metabolized after intracisternal administration.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful in the evaluation process.

Tanaka *(133)*, support not indicated, examined neurobehavioral toxicity in mice exposed to DEHP (> 97% purity) during prenatal development. At 5 weeks of age, 10 CD-1 mice/sex/group were fed diets containing 0, 0.01, 0.03, or 0.09% DEHP for 4 weeks prior to mating and during a 5-day mating period that began at 9 weeks of age. Females continued to receive the control or DEHP-containing diets throughout the gestation and lactation periods. The authors converted DEHP doses to a mg/kg bw/day basis, and those values are summarized in Table 27. Each female was mated to 1 male, and the females were allowed to litter and rear their offspring. At birth (PND 0), litter size, litter weight, and sex ratio were determined. Offspring were individually weighed, and postnatal survival was monitored during the lactation period. Neurobehavioral parameters examined in all offspring during the lactation period included surface righting (PND 4 and 7), negative geotaxis (PND 4 and 7), cliff avoidance (PND 7), swimming behavior (PND 4 and 14), and olfactory orientation (PND 14). Weaning occurred at 4 weeks of age, and 1 male and 1 female per litter were selected to continue receiving treatment until 9 weeks of age. **[Though not specified, it is assumed that the offspring from each treatment group received the same doses as their parents.]** Doses on a mg/kg bw/day basis for offspring are

also included in Table 27. At 7 weeks of age, the F_1 mice were tested using a Biel type water T-maze. Exploratory behavior was assessed using an animal movement analyzing system in 3-week-old mice from the F_1 generation and 8-week-old mice from the F_0 and F_1 generations. Statistical analyses included Bonferroni multiple comparison, ANOVA, Kruskall-Wallis test, chi-squared test, Fisher exact test, Wilcoxon sign test, and/or Jonckheere test. **[It does not appear that statistical analyses were conducted on a per litter basis.]**

Table 27. Summary of DEHP Doses in Mice

Generation and Study Period		*Mean DEHP Doses, mg/kg bw/day*[a]		
		Diet group		
		0.01%	*0.03%*	*0.09%*
F_0 males premating		16	47	142
F_0 females	Premating	20	56	168
	Mating	15	40	126
	Gestation	17	47	140
	Lactation	60	172	493
F_1 males		16	48	145
F_1 females		19	56	171

From: Tanaka *(133)*.

a Values were presented as mean ± SD by study authors. The values presented here are means rounded to whole numbers because that information is sufficient for the CERHR evaluation process.

In F_0 mice, DEHP treatment had no effect on body weight gain, movement, or exploratory activity. As a result of non dose-related failures to become pregnant or abortions in 1 – 2 dams of the low- and mid-dose groups, 8 – 10 litters were available for evaluation in each treatment group. There were no significant effects on sex ratio or litter size or weight at birth. A 7% decrease in body weight in male offspring of the low-dose group compared to control males on PND 0 was the only significant body weight effect observed in offspring. Significant reductions in survival were noted in the high-dose group for female offspring from PND 4 to 14 and for total offspring from PND 4 to 21. Percentages of total surviving offspring at PND 21 were 98.4% in the control group and 92.8% in the high-dose group. Time for surface righting was significantly delayed in females of the low- and mid-dose groups on PND 4, in males of the high-dose group on PND 7, and in females of the low-dose group on PND 7. There were no other significant findings in neurobehavioral parameters examined during the lactation period **[data not shown]**. Compared to controls, there were no adverse effects in water T-maze performance in treated animals at 7 weeks of age, and movement and exploratory behavior were not affected by treatment at 3 or 8 weeks of age. The study authors concluded that "few adverse effects on several behavioral parameters were produced at the high-dose level of DEHP in the present study."

Strengths/Weaknesses: Source and purity of test material were provided and an appropriate route of exposure (diet) was used. The use of relatively low dietary exposure levels was a strength. Additional

strengths included the use of multiple dose levels and multigenerational exposure in mice from 5 weeks of age for the starting F_0 generation through 9 weeks of age for the F_1 generation, encompassing the premating, gestation, lactation, and sexual maturation periods. For the F_0 generation animals, the post-weaning evaluations were controlled for litter effect by selecting one male or female per litter. Litter means were also used in the evaluation of pup weight and litter size. It is unfortunate that animals were not evaluated for the classic phenotype of prenatal phthalate exposure, which would have extended the observations to another species. Sexually dimorphic behaviors should have been evaluated, given the presumed mode of action involving a reduction in fetal steroid hormone levels. There are weaknesses in the presentation and analysis of data. The preweaning data for surface righting, negative geotaxis, cliff avoidance, swimming behavior, and olfactory orientation were analyzed on an individual animal basis and were not controlled for litter effect. Of these endpoints, the authors only presented data for surface righting. Exploratory behavior data at 8 weeks of age were also not presented. Preweaning survival indices were also not analyzed on a litter basis. There was a limited set of parameters evaluated; endpoints of sexual development, fertility, and pathology were not evaluated. Sample size was limited for this type of experiment.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the specific endpoints for which data were presented and litter effect was controlled (litter size, average pup weight, and T-maze performance). None of these endpoints demonstrated a significant dose-responsive treatment effect; however, there is low confidence in these results due to use of relatively small group sizes (8 – 10 litters/group). Other endpoints from the F_1 offspring are not useful due to deficiencies in statistical evaluation and/or lack of data presentation.

James *(134)* wrote a letter to the editor to question Tanaka's *(133)* conclusion that sex ratios were not affected in offspring of mice fed diets containing 0.01 – 0.09% DEHP during pregnancy. James noted that there were a total of 190 male offspring and 152 female offspring in all DEHP treatment groups and that the difference in sex ratio was significantly higher than equality ($P < 0.05$). It was stated that DEHP can have opposite effects on endocrine parameters (e.g., testosterone and estradiol levels) in male versus female rats, and that it could potentially affect offspring sex ratio differently in treated males versus females. Because Tanaka mated treated female mice with treated male mice, James concluded that lack of effect of DEHP on sex ratio could not be ruled out. A later study by Tanaka *(135)* did examine sex ratios in DEHP-treated male and female mice mated with untreated control animals, and that study is addressed below. **[The James letter is noted for completeness but is not used in the evaluation process.]**

Tanaka *(136)* responded to the James letter *(134)* regarding sex ratios in the Tanaka *(133)* study. Tanaka re-analyzed the data on a litter basis using Steel multiple comparison test and demonstrated there were no significant differences in sex ratios of offspring from DEHP-treated versus control mice. It was noted that variations in sex ratio were much higher among litters than among treatment groups. Tanaka noted that other studies in rodents also failed to demonstrate an effect on sex ratio following DEHP treatment. Lastly, Tanaka stated that sex ratios in all treated groups were within ranges observed in control mice in his laboratory during the past 10 years. Tanaka concluded that there was no experimental evidence that DEHP adversely affects sex ratio in offspring of DEHP-treated mice.

Strengths/Weaknesses: This re-evaluation of data from the previous study used a more appropriate litter-based analysis. The re-analysis and discussion of additional studies referenced in the literature

and historical control data from the author's laboratory fully support the lack of an effect on sex ratio following exposure (as studied) in mice.

Utility (Adequacy) for CERHR Evaluation Process: This information supports previous conclusions *(133*).

Tanaka *(135*), support not indicated, examined the effects of prenatal DEHP exposure on sex ratio in mice. It appears that the study was conducted to address concerns about DEHP effects on sex ratio that were raised in letter by James *(134*). Starting at 5 weeks of age, 20 male and female CD-1 mice/sex/group were fed diets containing 0 or 0.03% DEHP (purity > 97.0%). At 9 weeks of age, each female was mated for 5 days with a male from the same or opposite treatment group (i.e., cross-mating). There were 4 treatment groups consisting of 10 mice/sex: control females × control males, control females × treated males, treated females × control males, and treated females × treated males. Females continued to receive the DEHP-containing or control diets during the mating period and throughout gestation. The study authors estimated that intake of DEHP was ~47–49 mg/kg bw/day in males and ~55–58 mg/kg bw/day in females during the preconception period. Intakes by females were estimated at ~45 mg/kg bw/day during the mating period and ~50 mg/kg bw/day during the gestation period. Females were allowed to litter, and endpoints examined on day of birth were litter size, litter weight, individual offspring weight, and sex ratio. Statistical analyses included ANOVA or Kruskal-Wallis test followed by Bonferroni multiple comparison to assess food intake, litter size, and litter and body weights. Chi-squared test was used to evaluate sex ratio based on offspring, and the Steel test was used to assess sex ratio based on litter. As a result of pregnancy failures or abortions, there were 8–10 litters delivered in each treatment group. Compared to the group consisting of control males and females, mean body weights of male offspring were increased in all groups containing a treated female and/or male parent. No significant effects were noted for litter size, litter weight, total or average sex ratio, or female offspring weights. The study authors concluded that the concentrations of DEHP used in this study did not produce adverse effects on sex ratios.

Strengths/Weaknesses: Source and purity of test material was provided, and an appropriate route of exposure (diet) was used. Exposures were conducted during the critical period of sexual differentiation. Mice were exposed from 5 weeks of age from the start of the F_0 generation through delivery of the F_1 offspring, encompassing the premating and gestation periods. Strengths are that a factorial design was used based on exposure of one sex bred with an unexposed or exposed mate and that litter was the unit of analysis for most reproductive and developmental endpoints evaluated. Weaknesses are that only a single dose level was used (0.03% in the diet) and that classical measures of phthalate toxicity in male offspring were not evaluated. Sample size was limited (8–10 litters/group) for this type of experiment, resulting in low confidence in negative outcomes or marginal effects. For example, statistically significant increases in male offspring weight were observed in all groups with DEHP parents. However, in two other studies by this author utilizing a similar study design exposing mice to equal or higher doses of DEHP, no effect on pup weights or an increase in female pup weight was found. (The author concluded that the male pup weights in the concurrent control were unusually low and the effect of increased male pup weight was not treatment-related. The Expert Panel agrees).

Utility (Adequacy) for CERHR Evaluation Process: This study is limited in scope to litter size, litter weight, pup weight and sex ratio, and to a single dose level of 0.03% in the diet (40–56 mg/kg bw/day).

Tanaka et al. ***(137)***, support not indicated, gave DEHP (>97% purity) to CD-1 mice in the diet from 5 weeks of age in the F_0 generation to 9 weeks of age in the F_1 generation. A single dietary dose level of 0.03% was used, with control animals receiving untreated basal feed (n=20/sex/treatment group). At 9 weeks of age, 10 DEHP-treated females were paired with DEHP-treated males, 10 DEHP-treated females were paired with control males, 10 control females were paired with DEHP-treated males, and 10 control females were paired with control males. The females' diet was available to males during the 5-day cohabitation phase. Females reared their own unadjusted litters, which were weaned at 4 weeks of age. One female and male from each litter were retained and fed their dam's diet until 9 weeks of age.

All F_1 offspring underwent neurobehavioral testing during the lactation period, including surface righting and negative geotaxis on PND 4 and 7, cliff avoidance on PND 7, swimming behavior on PND 4 and 14, and olfactory orientation on PND 14. Exploratory behavior was assessed in 1 male and 1 female from each litter at 3 weeks of age. Post-weaning tests included multiple-T water maze at 7 weeks of age and exploratory behavior at 8 weeks of age. Statistical analyses were performed using ANOVA or Kruskal-Wallis test followed by Bonferroni multiple comparison test. Proportions were evaluated using chi-squared or Fisher test. **[It is not stated whether litter was considered in the analysis of the preweaning neurobehavioral tests.]**

Based on measured feed consumption, mean DEHP intake by treated males **[rounded by CERHR]** was 46 mg/kg bw/day. Treated females received 53–57 mg/kg bw/day during the preconception period, ~43 mg/kg bw/day during mating, 46–49 mg/kg bw/day during gestation, and 154–171 mg/kg bw/day during lactation. DEHP had no effect on feed consumption or dam body weight. As repeated in Section 4.2.3, there were no significant treatment effects on the number of pregnant females, number of litters, number of offspring, average litter size or weight, or offspring sex ratio. Offspring body weight during the lactation period was similar between groups except for an 8% decrease in body weight on PND 14 in female offspring when the parents both had received DEHP. The author did not consider this isolated alteration to be treatment related. Swimming ability was accelerated in PND 4 female offspring when the dam received DEHP. The number of movements in the test of exploratory behavior was decreased in male offspring the parents of which both received DEHP. There were isolated differences in T-maze performance by sex, trial, and treatment group that were not considered to represent treatment-related alterations in maze-learning. None of the other behavioral tests revealed effects of DEHP treatment.

The author concluded that "few adverse effects on several behavioral parameters were produced at the dose level of DEHP in the present study."

Strengths/Weaknesses: Source and purity of test material were provided, and an appropriate route of exposure (diet) was used. The use of multigenerational exposure in mice from 5 weeks of age for the F_0 generation through 9 weeks of age for the F_1 generation encompassed the premating, gestation, lactation, and sexual maturation periods. The use of factorial design based on exposure of one sex bred with an unexposed or exposed mate was a strength. For the F_0 generation animals, the post-weaning evaluations were controlled for litter effect by selecting one male or female per litter. Litter means were also used in the evaluation of pup weight and litter size. It is a weakness that only a single dose level was used, although it is a strength that this dose level was relatively low. Sample size was limited (8–10 litters/group), resulting in low confidence in negative outcomes. For example, the author states

that no effect was observed on pup weight, with the exception of decreased female pup weights on PND 14 in group 4. However, the data suggest that decreased weight in both sexes at all preweaning time points would have been evident had a sufficient sample size been used. Sexually dimorphic behaviors were not evaluated. Other weaknesses in the presentation of data and conduct of behavioral testing are as discussed above for Tanaka *(133*).

Utility (Adequacy) for CERHR Evaluation Process: This study has limited use only for specific endpoints in which data were presented and litter effect controlled (litter size, mean pup weight, post-weaning exploratory behavior, and T-maze performance). None of these endpoints demonstrated a significant dose-responsive treatment effect; however, there is low confidence in these results due to use of a relatively small group sizes (8 – 10 litters/group). All other endpoints on the F_1 offspring are not useful for the evaluation due to deficiencies in statistical evaluation and/or lack of data presentation.

Lee et al. *(138*), supported by the South Korean Ministry of Environment, examined the expression of zinc-metabolizing enzymes in mouse dams and embryos exposed to DEHP. One hypothesis is that altered zinc homeostasis is a cause of teratogenicity following DEHP exposure. On GD 9 (9 days post-coitus), CD-1 mice were given corn oil (vehicle) or DEHP 800 mg/kg bw by gavage. Dams were killed at 3, 4.5, or 6 hours following exposure, and maternal liver, visceral yolk sac, and embryonic forebrain were collected. Polymerase chain reaction and Western blotting techniques were used to study expression of zinc-metabolizing enzymes in the collected tissues. Results were analyzed by Student *t*-test. Maternal liver expression of metallothionein (MT)-I and MT-II, enzymes that sequester zinc in liver and thus lower blood levels, were increased at 3.0 – 4.5 hours following DEHP exposure and then began returning to baseline levels at 6 hours following exposure. Maternal liver expression of zinc transporter-1 (ZnT-1), a transmembrane protein involved in zinc efflux, was not affected by DEHP exposure. Exposure to DEHP resulted in a down-regulation of MT-I, MT-II, and ZnT-1 expression in embryonic brain from 3 to 6 hours following exposure. There was no effect on visceral yolk sac.

A dose-response study was conducted in which pregnant mice were gavaged on GD 9 with 0, 50, 200, or 800 mg DEHP/kg bw. Dams were killed, and maternal liver and embryonic brains were collected at 3 hours following exposure. According to the text of the study, up-regulation of MT-I and MT-II in maternal liver reached statistical significance at 200 mg/kg bw/day DEHP. **[Table 1 of the study indicates that the increase in MT-II expression reached statistical significance at 800 mg/kg bw.]** The study authors calculated a BMD_5 of 6.7 mg/kg bw for MT-1 and 5.6 mg/kg bw for MT-II. $BMDL_5$s (lower 95% confidence limit) were calculated at 3.7 mg/kg bw for MT-I and 3.2 mg/kg bw for MT-II. In embryonic brain, reductions in MT-I and ZnT-1 were significant at 200 mg/kg bw, and reductions in MT-II were significant at 50 mg/kg bw. Study authors calculated BMD_5 responses of 11.6 mg/kg bw for MT-I, 8.9 mg/kg bw for MT-II, and 6.6 mg/kg bw for ZnT-1. $BMDL_5$s (lower 95% confidence limit) were calculated at 7.1 mg/kg bw for MT-I, 5.2 mg/kg bw for MT-II, and 3.9 mg/kg bw for ZnT-1. The study authors concluded that exposure of dams to DEHP during periods of organogenesis can alter the expression of key fetal enzymes involved in zinc homeostasis.

Strengths/Weaknesses: This paper is based on a strong hypothesis and includes good presentation of methods and results. Multiple dose levels were evaluated, and a dose-response was demonstrated. The paper lacked direct measurement of zinc levels in sensitive tissue to correlate with the gene expression/protein data. MT-I/MT-II and ZnT-1 are not validated biomarkers for the endpoints of concern

(affected by DEHP). Changes in gene expression alone are not adverse, may be adaptive, and must be linked directly to an adverse outcome, which was not done. The authors conclude "How this mechanism contributes to the overall developmental toxicity of phthalates in general and DEHP in particular remains to be further examined." This statement clearly indicates the authors believe their information is useful for hypotheses generation, preliminary in nature, and in need of further development before it can be used directly in risk assessment. An additional limitation is the time of dosing, which is not the most critical period for phthalate adverse effects on male reproductive organ development.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not directly useful in the evaluation process, although it provides good hypothesis-generating information for explaining potential mechanisms.

Rhee et al. *(139)*, supported by the Korean Food and Drug Administration, evaluated the developmental effects of DEHP using *in vitro* tests. Whole embryo culture was performed by explanting GD 9.5 Wistar rat embryos into serum-based media treated with DEHP **[purity not specified]** in 0.5% DMSO. DEHP concentrations were 1, 10, or 100 μg/mL **[mg/L],** n = 15 – 28 embryos/concentration. Comparisons were made to untreated (n = 35 embryos) and DMSO vehicle-treated (n = 30 embryos) controls. Cultures were maintained for 48 hours, following which embryos were evaluated for yolk sac diameter, crown-rump length, head length, somite number, and Maele-Fabry morphology score. Data were analyzed using ANOVA with post hoc Bonferroni or Dunnett test. Yolk sac diameter, somite number, and Maele-Fabry score were significantly decreased at all concentrations of DEHP. Crown-rump and head length were decreased by the 2 highest concentrations of DEHP. The authors also performed micromass cultures using dissociated limb bud and midbrain cells from GD 12.5 embryos. Cell suspensions were allowed to attach for 2 hours, following which they were exposed to DEHP at concentrations ranging from 7.81 to 1000 μg/mL for 96 hours. Cytotoxicity was determined using neutral red. Differentiation was determined in limb bud cells using Alcian blue uptake and in midbrain cells using hematoxylin staining followed by quantification of differentiated foci with an image analyzer. The planned endpoint was a comparison of the concentrations at which differentiation and cell survival were 50% inhibited compared to a control value. The authors reported that the planned inhibitory concentration comparison could not be carried out. **[The authors do not give a reason for not being able to report a result, but inspection of a graph in the paper suggests that a 50% effect was not achieved for both cytotoxicity and differentiation in either the limb or midbrain culture system.]** The authors concluded that the absence of evaluable effect in the micromass assay in the face of prominent effects in the whole embryo culture system was consistent with lack of metabolism of DEHP to toxic intermediate(s) in the cell culture system.

Strengths/Weaknesses: The use of multiple *in vitro* screening methods and multiple exposure levels are strengths. Comparison to other phthalates was a strength, but failure to include "inactive" phthalates was a weakness. The assays are used for potential screening and mechanistic studies, however, rather than for risk assessment. No mention was made of how the embryos were assigned to groups and whether litter effect was controlled. There was no analytical support for metabolite formation in culture media to support the hypothesis on toxic metabolite formation in the whole embryo culture.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in the evaluation process.

3.2.4 Fish

Chikae et al. *(140)*, support not indicated, evaluated the hatching, survival, and sex ratio of Japanese medaka (*Oryzias latipes*) exposed to DEHP **[purity not given]** in ethanol at nominal DEHP concentrations of 0, 0.01, 0.1, 1.0, and 10.0 μg/L. Final ethanol concentrations were < 100 μg/L. Fish were exposed during the "embryo stage" **[exact time of exposure not indicated]**. Observations were made of hatching time, hatching success, mortality, body weight **[age not specified, but possibly at 5–6 months, when the experiment was terminated]**, sex ratio, and gonadosomic index **[not defined; other authors have used this term for relative gonad weight]**. There were significant delays in hatching among the eggs exposed to DEHP 0.1 and 1.0 μg/L but not among the eggs exposed to DEHP 10.0 μg/L. The percent of embryos showing eye development and the percent successfully hatching were not altered by DEHP exposure. Adult mortality was increased in the groups exposed to DEHP 0.01, 0.1, and 1 μg/L but not in the group exposed to DEHP 10.0 μg/L. Sex ratio was reduced (fewer males) in the 0.01 μg/L group only. Body weight was reduced in a DEHP concentration-dependent manner with significant difference from control at and above 0.1 μg/L. The authors concluded that DEHP exposure of Japanese medaka embryos "negatively affected some biological parameter[s] in both embryo and adulthood." They acknowledged that the effects were not necessarily dose-dependent.

Strengths/Weaknesses: Multiple exposure levels allowed for a dose-response evaluation. The quantitative presentation of data was fairly good. There were clear effects on some endpoints, but **c**hanges in many parameters appear random and unrelated to dose. Findings were not interpreted in light of postulated mode of action in the development of mammalian males. Evaluation of embryo levels of the active fish androgen (11-ketotestosterone) would have been helpful.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in the evaluation process.

3.2.5 Abstracts

CERHR retrieved several abstracts reporting developmental toxicity associated with DEHP exposure. Although information from abstracts is not considered by the Expert Panel in reaching final conclusions, the abstracts are briefly summarized for the sake of completeness.

Borch et al. *(141)* gavage dosed pregnant Wistar rats on GD 7–21 with DEHP 0 or 750 mg/kg bw/day or with a combination of DEHP 750 mg/kg bw/day plus di(2-ethylhexyl)adipate 400 mg/kg bw/day. Testosterone production by offspring testes over a 3-hour *ex vivo* incubation period was reduced from 2.9 ng in controls to 0.4 ng in the DEHP-exposed group and to 0.9 ng in the group exposed to DEHP plus di(2-ethylhexyl)adipate. Testicular testosterone was reduced from 1.8 ng/testis in controls to 0.4 ng/testis in the group exposed to DEHP and 0.3 ng/testis in the group exposed to DEHP plus di(2-ethylhexyl)adipate. Plasma testosterone was reduced in male offspring from 188 pg/mL in controls to 25 pg/mL in the group exposed to DEHP and 18 pg/mL in the group exposed to DEHP plus di(2-ethylhexyl)adipate. There were no significant differences between the effects of DEHP alone and DEHP plus di(2-ethylhexyl)adipate. Similar treatment of dams during gestation and lactation resulted in no significant alterations in serum testosterone in male offspring evaluated on PND 27.

Phokha et al. *(142)* evaluated the toxicokinetics of DEHP and MEHP in pregnant and nonpregnant Sprague-Dawley rats. Nearly all orally administered DEHP was hydrolyzed in the intestine to MEHP. The AUC of DEHP was 0.7% that of MEHP. The AUC did not change in non-pregnant rats with

repeated administration and was 950, 970, and 1070 nmol-h/L per mmol/kg dose on the first, fourth, and seventh consecutive days of treatment. In pregnant rats, administration on GD 14–19 resulted in AUC values (nmol-h/L per mmol/kg dose) of 1250 on GD 14 and 970 on GD 19.

Foster and Barlow *(143)* gavage dosed 10 pregnant Sprague Dawley rats per group on GD 12–21 with corn oil vehicle, 100 mg/kg bw/day DEHP, 100 mg/kg bw/day dibutyl phthalate, or 100 mg/kg bw/day DEHP in combination with 100 mg/kg bw/day dibutyl phthalate. Treatment with DEHP + dibutyl phthalate reduced anogenital distance on PND 1 and increased retained areolae on PND 13 in male offspring; there were no significant effects in rats treated with either phthalate alone. Only a small number of reproductive organ lesions were observed in the phthalate groups. There were no consistent changes in organ weights. The study authors concluded that the effects of the 2 phthalates were additive.

Lambright et al. *(144)* treated pregnant Sprague-Dawley rats with DEHP 1000 mg/kg bw/day on GD 14–18. Fetal testes were harvested on GD 18 and placed in culture for 3 hours. Testosterone and progesterone production in culture were decreased by gestational DEHP treatment.

Gray et al. *(145)* gavage dosed Sprague-Dawley rats with DEHP at 0, 11, 33, 100, or 300 mg/kg bw/day from GD 8 to PND 17. Dosing continued in half of the male offspring from PND 18 to 63–65, at which time they were necropsied. In those offspring necropsied on PND 63–65, liver and adrenal weights were affected at ≥ 11 mg/kg bw/day, puberty was affected at ≥ 100 mg/kg bw/day, and reproductive organ weights were affected at 300 mg/kg bw/day DEHP **[no further details were provided regarding effects]**. The other half of male offspring were not dosed further after PND 17 and were necropsied upon reaching full maturity. Permanent effects noted in those offspring treated with 300 mg/kg bw/day DEHP were reductions in anogenital distance, reduced reproductive organ weights, and increased incidence of nipples. Testicular and/or epididymal abnormalities were observed in 25% of offspring from the 300 mg/kg bw/day group; a low incidence of malformations was observed at the lower dose levels.

Gray et al. *(146)* treated pregnant and lactating Sprague-Dawley rats with DEHP from GD 8 to day 17 of lactation at dose levels of 0, 11, 33, 100, or 300 mg/kg bw/day, given by gavage. Dosing was continued in 2 or 3 males/litter from PND 18 until PND 63–65. Puberty was delayed and males were heavier at the time of preputial separation in the 100 and 300 mg/kg bw/day groups. The authors concluded that DEHP is similar to dibutyl and benzyl butyl phthalate in exerting anti-androgenic effects on the developing androgen-signaling pathway.

Wilson et al. *(147)* treated pregnant Sprague-Dawley rats with oral DEHP 0, 100, 300, 600, or 900 mg/kg bw/day on GD 8–18. GD-18 fetal testes were harvested and incubated for 3 hours in 500 µL medium, following which media were evaluated for hormone concentrations. Harvested testes were investigated using RT-PCR for assessment of gene expression. DEHP treatment resulted in a decrease in testicular testosterone production in the 300, 600, and 900 mg/kg bw/day groups and a decrease in testicular progesterone production in the 900 mg/kg bw/day group. Insl3 gene expression was decreased in a dose-dependent manner.

Hass et al. *(148)* treated pregnant and lactating Sprague-Dawley rats (n = 8/group) with gavage doses of DEHP at 0, 10, 30, 100, 300, 600, and 900 mg/kg bw/day from from GD 7 to PND 17. Anogenital

distance was decreased and nipple retention increased at all DEHP doses compared to the control, although the anogenital distance difference from control was not statistically significant in the groups exposed to less than 300 mg/kg bw/day. The authors suggested that nipple retention may be a more sensitive indicator of anti-androgenic effects on development.

Numtip et al. *(149)* evaluated the toxicokinetics of DEHP in pregnant (n = 4) and nonpregnant (n = 8) marmosets. Animals were treated orally with 30 or 500 mg/kg bw/day for 29 days (GD 96 – 124). The AUCs for DEHP were about 20 times less than the AUCs for MEHP. The concentration-time curve for MEHP showed an oscillating pattern that the authors concluded represented enterohepatic cycling of MEHP glucuronide, which is not encountered in rats. Pregnancy did not alter the AUC of MEHP after the low dose of DEHP, but some reduction in MEHP AUC occurred in late pregnancy at the high DEHP dose. Maximum MEHP concentrations and AUCs were lower in pregnant marmosets than has been reported for pregnant rats.

Regnier *(150)* incubated GD 9 rat embryos for 48 hours in serum obtained from CD rats treated with 1000 mg/kg bw DEHP on GD 6 – 11. The DEHP-exposed embryos had increased malformations and reduced crown-rump and head lengths, somite numbers, and morphological scores. In a second *in vitro* experiment comparing embryotoxicity of DEHP and its metabolites, the order of potency (highest to lowest) was MEHP oxidized metabolites > DEHP = MEHP = 2-ethylhexanol > 2-ethylhexanoic acid.

3.3 Utility of Developmental Toxicity Data

Since the initial CERHR evaluation of DEHP, 4 human studies were published that examined associations between *in vivo* DEHP or metabolite levels and adverse development outcome or premature thelarche. Another human study examined puberty in children who had received ECMO as neonates. Eight multiple dose-level animal toxicity studies have been published since the original DEHP evaluation. The studies focused on the effects of gestational or neonatal DEHP exposure on reproductive organ toxicity, pulmonary toxicity, and neurobehavioral endpoints. One study compared testicular toxicity following neonatal exposure to DEHP through the oral or IV route. Numerous studies focused on mechanistic aspects of DEHP-induced toxicity, such as effects on testosterone production, identification of target cells, interaction with the androgen receptor, and effects on zinc metabolism.

3.4 Summary of Developmental Toxicity Data

3.4.1 Human Data

Only 3 useful studies have been conducted among humans assessing developmental toxicity. Each study measured a different endpoint, and each had limitations. The Main et al. paper *(17)* suggested possible subtle effects in male infants associated with MEHP, and the Swan et al. paper *(108)* suggested subtle effects associated with the presence of MEHP metabolites. Replication of these studies with more extensive consideration of confounding and with larger sample sizes should be undertaken.

Swan et al. *(108)* measured MEHP and its oxidative metabolites in the urine of pregnant women. Anogenital distance was evaluated at 2 – 18 months of age in the male children born to these women. There was no significant association between maternal urinary MEHP concentration and infant anogenital index (anogenital distance adjusted for weight). The regression coefficients for 5-oxo- and 5-OH-MEHP, while not significant, were of similar magnitude to regression coefficients for other

phthalate monoesters (monobutyl, monoethyl, and mono-isobutyl phthalate) that were significantly associated with reduced anogenital index.

Main et al. *(17)* studied the association of breast milk levels of MEHP and other phthalates and cryptorchidism and blood levels of reproductive hormones in 3-month-old boys in Denmark and Finland. No association between phthalate exposure and cryptorchidism was found. Milk concentration of MEHP was observed to have a marginally significant correlation with free testosterone (Spearman $r = -0.169$, $P = 0.107$) and inhibin B ($r = 0.185$, $P = 0.075$). This conclusion is tempered by concern about possible contamination by use of breast pumps and limited evaluation of confounders and effect modifiers.

Rais-Bahrami et al. *(109)* examined onset of puberty and sexual maturity parameters in 14 – 16-year-old adolescents (13 males and 6 females) who had been potentially exposed to high DEHP levels as a result of receiving ECMO as neonates. Except for 1 female with Marfan syndrome, growth percentiles were normal for age and sex. Pubertal development was stated to be normal. Laboratory results indicated normal thyroid, liver, and renal function. LH, FSH, testosterone, and 17β-estradiol levels were normal for stage of pubertal development. No control children were evaluated, and exposure to phthalates was assumed rather than measured. While this study was supportive of no dramatic effect in ECMO-treated children, its small size and the wide range of normal values for adolescents limit the power to detect effects.

3.4.2 Experimental Animal Data

Experimental animal studies using multiple dose levels and thus providing dose-response information are summarized in Table 28.

In a number of studies, developing rats were exposed to DEHP and subsequently examined for effects on reproductive and endocrine systems. Five studies were notable, 4 that examined dose-related effects of DEHP exposure, and a fifth study that compared testicular toxicity in rats dosed by the oral versus the IV route. The remaining studies largely focused on mechanisms of developmental reproductive toxicity.

Moore et al. *(113)* orally dosed at least 8 pregnant Sprague-Dawley rats/group with DEHP at 0, 375, 750, or 1500 mg/kg bw/day from GD 3 (GD 1 = day after sperm detected) to PND 21. Parameters associated with sexual development were observed through puberty or adulthood in male and female offspring, and male offspring were tested for sexual behavior. DEHP treatment reduced prenatal maternal weight gain at the middle and high dose. There was no significant effect on implantation sites. Number of pups born was reduced at the high dose, and postnatal survival was decreased at the middle and high dose. The most sensitive DEHP effect on males was an increase in areolae or nipples, which occurred at all dose levels and persisted through adulthood. Incomplete preputial separation, non-descent of testes, and agenesis of anterior prostate and other accessory reproductive organs did not attain statistical significance at the lowest dose level, but the authors considered them to be biologically significant at all doses due to the rarity of the effects. Reproductive effects observed in males exposed to higher doses included reduced anogenital distance, agenesis of seminal vesicle and epididymis, decreased sperm count, and reduced testis, epididymis, glans penis, prostate, and seminal vesicle weights that often persisted through adulthood. In female pups, DEHP treatment had no effect on anogenital distance. Body weight of high-dose females was 68% that of control body weight on the day of vaginal opening, and the effect was statistically significant. The study authors attributed

the effect to DEHP-induced toxicity and not to an estrogenic effect. The study authors identified a LOAEL of 375 mg/kg bw/day for this study based on a significant decrease in anterior prostate weight and increase in permanent nipple retention.

Li et al. ***(119)*** examined dose-related effects of DEHP on neonatal rat gonocytes and Sertoli cells. Male Sprague-Dawley rat pups from 4–7 litters were pooled and randomly placed into groups of 4 or 5 pups. On PND 3 (day of birth = PND 1), the rats were gavaged with DEHP at 0, 20, 100, 200, or 500 mg/kg bw. Pups were killed 24 hours after dosing, and testes were collected for morphological examination and measurement of Sertoli cell proliferation through BrdU uptake. The time course of effects was examined in a second experiment in which rats were dosed with 0 or 200 mg/kg bw DEHP and examined between 6 and 48 hours following exposure. In rats treated with 100–500 mg/kg bw DEHP, there was a dose-related increase in abnormally large gonocytes containing 2–4 nuclei. Multinucleated gonocytes were first detected at 12 hours following exposure to DEHP 200 mg/kg bw, and multinucleated gonocyte numbers increased with time. Sertoli cell proliferation was reduced in rats treated with ≥100 mg/kg bw DEHP. Sertoli cell proliferation rebounded at 48 hours following treatment. DEHP did not affect serum FSH levels. **[Due to reporting deficiencies, only the Sertoli cell proliferation data were considered to be of utility.]**

Cammack et al. ***(120)*** examined reproductive development of Sprague-Dawley rats (n = 16/group) treated IV or orally with DEHP. Beginning at 3–5 days of age, rats were treated for 19–21 days with DEHP at 0, 60, 300, or 600 mg/kg bw/day by IV infusion or 0, 300, 600 mg/kg bw/day by oral gavage. Seven rats/group were scheduled to be killed following the dosing period, and 9/group were scheduled to be held for a recovery period until 90 days of age. Histopathological analyses of prostate, seminal, vesicle, and epididymis and an evaluation of sperm count, motility, and morphology were conducted in the 90-day-old rats. Body weight gain was decreased in rats given 600 mg/kg bw/day by IV infusion and oral gavage. In animals killed immediately after the dosing period, absolute testis weight was significantly reduced at IV and oral doses ≥300 mg/kg bw/day. Absolute liver weight was increased in rats given ≥300 mg/kg bw/day DEHP by IV infusion. Depletion of germinal epithelium and/or decreased seminiferous tubule diameter was noted in all animals from the 300 and 600 mg/kg bw/day oral and IV dosing groups. Germinal epithelium depletion was rated as moderate (51–75% reduction in thickness) in the 600 mg/kg bw/day oral group and mild (25–50% change) in all other groups given ≥300 mg/kg bw/day DEHP. Reduced tubule diameter was rated as mild (25–50% reduction in diameter) in the 600 mg/kg bw/day oral group and minimal (<25% change) in the other groups treated with ≥300 mg/kg bw/day DEHP. In animals killed at 90 days of age, reduced testicular weights persisted in IV and oral groups given ≥300 mg/kg bw/day. The only persisting testicular lesion was a minimal (<25%) decrease in seminiferous tubular diameter in 2 of 5 rats in the 300 mg/kg bw/day oral group and 3 of 7 rats in the 600 mg/kg bw/day oral group. No prostate, epididymis, or seminal vesicles lesions and no adverse effects on sperm count, motility, or morphology were observed.

A multigeneration DEHP toxicity study conducted in rats also provided some information on developmental toxicity *(151)*. The study is described in detail in Section 4. Briefly, offspring of rats that were fed DEHP in diet at 3000 and 9000 ppm (340 and 1088 mg/kg bw/day) during gestation and lactation experienced an increase in stillbirth, an increase in PND 0–4 pup mortality, retardation of F_2 pup body weight, altered male anogenital distance, and retained nipples/areolae. A delay in sexual maturation was also noted in F_1 offspring at the 9000 ppm exposure level.

The **NTP *(114)*** multigeneration continuous breeding study in rats evaluated effects of DEHP in feed at dose levels of 1.5 (control group exposed to background DEHP levels in feed), 10, 30, 100, 300, 1000, 7500, and 10,000 ppm. Ranges of DEHP intake in the F_0, F_1, and F_2 animals were estimated at 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day. **[Because developmental effects were reported, particularly on the male reproductive system, the study is included in this section. This summary with additional details concerning the reproductive effects is also presented in Section 4.2.2.2.]** The lowest dose level producing dose-related effects in breeding F_1 offspring was 7500 ppm, and those effects included decreases in number of live pups/litter, reduced male anogenital distance, and delays in vaginal opening, preputial separation, and age of testicular descent. Additional effects noted in the F_1 offspring from the 10,000 ppm group included decreased live pup weight at birth and during the lactation period and increased ratio of female anogenital distance to body weight. **[The Expert Panel carefully considered the finding of small reproductive organ sizes by gross observations in both F_1 and F_2 rats. The combined F_1 and F_2 data were reviewed to determine the occurrence of these alterations on a per animal and per litter basis across the dose range, as shown in Table 23. Based on the incidence of small reproductive organ size at necropsy, the Expert Panel considered 300 ppm (about 14–23 mg/kg bw/day) to be an effect level, giving a NOAEL of 100 ppm, about 3–5 mg/kg bw/day.]**

A multiple dose study in rats by **Shirota et al. *(112)*** was designed to evaluate testicular pathology after intrauterine exposure to DEHP. Pregnant Sprague-Dawley rats were given gavage doses of DEHP in corn oil on GD 7–18 at 0, 500, or 1000 mg/kg bw/day in 1 experiment and 0, 125, 250, or 500 mg/kg bw/day in a second experiment. Decreased fetal weight and increased intrauterine mortality were noted at 1000 mg/kg bw/day. Postnatal findings included changes in pup weight at 250 and 500 mg/kg bw/day and increased incidences of multinucleated germ cells at ≥125 mg/kg bw/day and interstitial hyperplasia at 250 and 500 mg/kg bw/day.

Jarfelt et al. *(117)* evaluated the effects of perinatal exposure of groups of 20 Wistar rats to DEHP with or without diethylhexyl adipate. Timed-mated pregnant animals were treated by gavage from GD 7 to PND 1 with vehicle control, DEHP 300 mg/kg bw/day, DEHP 750 mg/kg bw/day, or DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day (n = 20/group). Litters were raised by their dams until weaning on PND 21, after which 1 male and 1 female per litter were retained. Recorded endpoints included anogenital distance on PND3, retention of nipples/areolae on PND13, onset of vaginal opening and balano-preputial separation, and epididymal sperm parameters and testicular histopathology on PND 190. Non-retained pups and dams were killed on PND 22 and evaluated for macroscopic lesions, and 3–5 males/litter underwent histopathologic/immunocytochemical examination of the testes. Increased postimplantation loss, reduced anogenital distance, and increased incidence of retained nipples were significantly different at all levels of DEHP exposure. There was also evidence of increased incidence of abnormal testes histology at both levels of DEHP exposure.

Effects noted in numerous single dose-level studies were consistent to those observed in the multiple dose-level studies summarized above. Although testicular weight and histology were not affected in offspring of rats treated with 100 mg/kg bw/day DEHP during pregnancy or lactation *(111)*, treatment of rats with DEHP 750 mg/kg bw/day in late pregnancy and/or early lactation resulted in decreased testicular weights and testicular lesions in offspring *(121, 123)*. Nipples and reduced anogenital distance were repeatedly observed in male offspring exposed to 750 mg/kg bw/day DEHP during

gestation or lactation *(115, 121*). Additional observations in male offspring of rats dosed with 750 mg/kg bw/day during late pregnancy and early lactation were lack of testicular descent, agenesis of accessory reproductive organs, and incomplete preputial separation *(121*).

A number of studies examined mechanisms of DEHP toxicity. Single dose-level studies with exposures during gestation and/or lactation and examination of fetal or immature rats consistently demonstrated reductions in blood testosterone levels at ≥100 mg/kg bw/day DEHP *(111, 115*), Leydig cell testosterone production at ≥100 mg/kg bw/day *(111*), testicular testosterone content at ≥300 mg/kg bw/day *(115, 123*), and *ex vivo* testicular testosterone production at ≥750 mg/kg bw/day *(115, 123, 124*). One of the studies indicated that reductions in testosterone production observed shortly after exposure in neonatal or weanling rats were no longer present in adulthood *(111*).

Evidence that DEHP targets Leydig cells, gonocytes, and Sertoli cells was noted following gestational and lactational exposure of rats to ≥100 mg/kg bw/day DEHP *(111, 119, 123*). MEHP, but not 2-ethylhexanol, was found to cause increases in large multinucleated gonocytes and to inhibit Sertoli cell proliferation *(119*). An *in vitro* study demonstrated that gonocytes and Sertoli cells are susceptible to MEHP-induced toxicity during periods of proliferation *(130*). In 1 study, DEHP doses ≥750 mg/kg bw/day during gestation in rats reduced testicular expression of insulin-like hormone 3, a hormone produced by Leydig cells and possibly involved in development of the gubernaculum *(124*).

Liu et al. *(125*) evaluated gene expression profiles in the GD 19 fetal testis after GD 12 – 19 gavage treatment of dams with 1 of 7 phthalates (n = 5/group) or with corn oil vehicle (n = 10; vaginal sperm = GD 0). The phthalates were DEHP or diethyl, dimethyl, dioctyl tere-, dibutyl, dipentyl, or benzyl butyl phthalate at a dose level of 500 mg/kg bw/day. On GD19, pups were evaluated for anogenital distance and testes were processed for gene expression profiles for 3 pups/treatment group, each from a different litter. Anogenital distance was significantly reduced by pregnancy treatment with DEHP, dibutyl phthalate, benzyl butyl phthalate, and dipentyl phthalate. Dimethyl, diethyl, and dioctyl terephthalate did not affect anogenital distance. Of 391 significantly altered gene probe sets, there were 167 characterized sequences. Genes related to lipid, sterol, and cholesterol homeostasis accounted for 31 of these 167 genes. There were also 10 genes involved in lipid, sterol, and cholesterol transport, 12 genes involved in steroidogenesis, 9 transcription factor genes, 22 signal transduction genes, 11 genes involved in oxidative stress, and 13 genes related to the cytoskeleton. In general, there was a similar pattern of gene expression profile with those phthalates that altered anogenital distance as compared those that did not, suggesting that these phthalates operate by a common mode of action on the developing testes. Targeted pathways were directly or indirectly related to Leydig cell production of testosterone and pathways important for Sertoli cell-gonocyte interaction.

Table 28. Summary of DEHP Effects on Developmental Toxicity

Species and Strain	Treatment	Effect Levels (mg/kg bw/day)				Reference
		NOAEL	Maternal LOAEL	Developmental		
				LOAEL	BMDL [a]	
Sprague-Dawley Rat	Oral 0, 375, 750, or 1500 mg/kg bw/day GD 3 to PND 21.	*Maternal:* 375 *Developmental:* NA [b]	750 (↓prenatal weight gain)	375 (↑in areolae or nipples, incomplete preputial separation, non-descent of testes, and agenesis of anterior prostate)	*10% Level:* 167 (↓epididymal sperm) *1 SD Level:* 135 (PND 105 epididymis weight)	Moore et al. *(113)*
Sprague-Dawley Rat	Gavage 0, 20, 100, 200, or 500 mg/kg bw PND 3 (Day of birth=PND 1)	*Developmental:* 20	NA	100 (↓Sertoli cell proliferation)		Li et al. *(119)*
Wistar Rat	Feed 0, 1000, 3000, and 9000 ppm (0, 113, 340, and 1088 mg/kg bw/day) During gestation and lactation	*Maternal:* 340 *Developmental:* 113	1088 (↓feed intake and body weight gain)	340 (↑pre- and postnatal mortality, ↓body weight, altered male anogenital distance, and retained nipples/areolae)	*10% Level:* 231 (F_2 pup survival on PND 0–4)	Schilling et al. *(151)* Discussed in Section 4.
Sprague-Dawley Rat	Gavage 0, 300, or 600 mg/kg bw/day 19–21 days beginning at 3–5 days of age.	***Examination immediately after dosing:***				Cammack et al. *(120)*
		NA		300 (depletion of germinal epithelium and/or ↓seminiferous tubule diameter and ↓absolute testes weight)	*10% Level:* 77.4 *1 SD Level:* 628 (testis weight)	
		Examination at 90 days of age:				
		Developmental: <300		300 (↓in seminiferous tubular diameter, ↓absolute testis weight)	*10% Level:* 125 *1 SD Level:* 65	

Appendix II

Species and Strain	*Treatment*	*Effect Levels (mg/kg bw/day)*				
		NOAEL	*Maternal LOAEL*	*Developmental*		*Reference*
				LOAEL	*BMDL* [a]	
Sprague-Dawley Rat	IV 0, 60, 300, or 600 mg/kg bw/day 21 days beginning at 3–5 days of age.	***Examination immediately after dosing:***				Cammack et al. *(120)*
		Developmental: 60		300 (depletion of germinal epithelium and/or ↓seminiferous tubule diameter, ↓absolute testes weight, and ↑absolute liver weight)	*10% Level:* 106 *1 SD Level:* 125 (testis weight)	
		Examination at 90 days of age:				
		Developmental: 60		300 (↓absolute testis weight)	*10% Level:* 190 *1 SD Level:* 105 (testis weight)	
Sprague-Dawley Rat	Feed 1.5, 10, 30, 100, 300, 1000, 7500, or 10,000 ppm (0.09–0.12, 0.5–0.8, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day)	Study authors: *Maternal:* 46–77 *Development:* 46–77	Study authors: 392–592 (body weight changes)	Study authors: 392–592 (Pregnancy indices, litter data)	*10% Level:* 33–56 *1 SD Level:* 45–75 (F3 sperm/cauda)	The National Toxicology Program *(114)*
		Expert Panel: *Maternal:* 46–77 *Developmental:* 3–5	Expert Panel: 392–592 (↓body weight gain)	Expert Panel: 14–23 (combined F_1 and F_2 gross observations of small reproductive organs)		
Sprague-Dawley Rat	Gavage 500, and 1000 (Experiment 1) 0, or 0, 125, 250, and 500 mg/kg bw/day (Experiment 2) GD 7–18	*Maternal:* 500 *Developmental:* 500	1000 (↓maternal body weight)	1000 (↑intrauterine mortality, ↓live fetuses/litter).	*10% Level:* 334 *1 SD Level:* 490 (↓live fetuses)	Shirota et al. *(112)*

Species and Strain	*Treatment*	*Effect Levels (mg/kg bw/day)*				
		NOAEL	*Maternal LOAEL*	*Developmental*		*Reference*
				LOAEL	*BMDL*[a]	
Wistar Rat	Gavage GD 7–17 at 0, 300, or 750 mg/kg bw/day.	*Maternal:* ≥750 *Developmental:* NA	NA	300 (postnatal death, male anogenital distance, retained nipples, abnormal testis histology)	*10% Level:* 13 (postimplantation loss) *1 SD Level:* 179 (male anogenital distance)	Jarfelt et al. *(117)*
Wistar Rat	Gavage 0, 300, or 750 mg/kg bw/day GD 7–PND 17	*Maternal:* ND *Developmental:* 300	ND[c]	750 (plasma hormone changes)	Not calculable from data in study report.	Borch et al. *(115)*
	Gavage 0, 300, or 750 mg/kg bw/day GD 7–PND 17	*Maternal:* ND *Developmental:* <300	ND	300 (↑apoptosis in fetal tissue)	Not calculable from data in study report.	Borch et al. *(118)*
CD-1 Mouse	Feed 0, 17, 47, and 140 mg/kg bw/day during gestation, 0, 60, 172, and 493 mg/kg bw/day During lactation 0, 16–19, 48–56, and 145–171 mg/kg bw/day From weaning to 9 weeks of age in F_1 offspring.	*Maternal:* 140–493 *Developmental:* 47–172	NA	140–493 (↓survival during lactation period)		Tanaka *(133)*

[a] See the footnote to Table 20 for definitions and a discussion of the use of benchmark dose in this report.
[b] NA: Not applicable;
[c] ND: Not Determined

CONCLUSIONS BASED ONLY ON LITERATURE APPEARING SINCE THE FIRST EXPERT PANEL REPORT

There is insufficient evidence in humans that DEHP causes developmental toxicity when exposure is prenatal. While there was one human study *(108*) judged to be useful, it was not sufficient to draw conclusions regarding developmental toxicity following prenatal exposure. The study found no significant association between maternal prenatal urinary MEHP and anogenital index in male offspring, and the interpretation of this novel index as applied to humans has not been established.

There is insufficient evidence in humans that DEHP causes developmental toxicity when exposure is during childhood. While there were two human studies judged to be useful, they were not sufficient to draw conclusions regarding developmental toxicity. One study *(109*) had very small sample size with no measurement of exposure. The other study *(17*) was limited in size and by the possibility of contamination by breast pump use.

There is sufficient evidence that DEHP exposure in rats causes developmental toxicity with dietary exposure during gestation and/or early postnatal life at 14–23 mg/kg bw/day as manifested by small or absent male reproductive organs *(114*). There were multiple other studies supporting effects on the developing male reproductive tract at higher dose levels. The critical period for effects on the testes extends into the immediate postnatal period *(120*) with decreased Sertoli cell proliferation seen in male rats exposed by oral gavage to DEHP 100 mg/kg/day on PND 3 *(119*).

There is sufficient evidence that DEHP causes developmental toxicity with 21 days of IV exposure starting at PND 3–5 at 300 mg/kg/day as manifested by decreased testes weight, depletion of germinal epithelium, and decreased seminiferous tubule diameter *(120*). The reduced testicular weights persisted through at least 90 days of age. These findings are consistent with those observed after oral exposure.

These data are assumed relevant to assessment of human risk.

***NOTE**: The definitions of the term sufficient and the terms assumed relevant, relevant, and not relevant are in the CERHR guidelines at http://cerhr.niehs.nih.gov/news/guidelines.html.*

CONCLUSIONS FROM THE ORIGINAL EXPERT PANEL EVALUATION

The original Expert Panel report on DEHP contained conclusions about developmental toxicity in Section 5. These conclusions have been extracted and reproduced below, with the section numbering as found in the original document. The references listed in the conclusion are listed, and the table to which the conclusions refer is reproduced, numbered Table 71 as in the original.

5.1.3

There were no studies located on the developmental toxicity of DEHP or its metabolites in humans.

5.1.3.1

Developmental toxicity findings were remarkably consistent. DEHP was found to produce malformations, as well as intrauterine death and developmental delay. The pattern of malformations seen in fetuses is consistent across studies. It included morphological abnormalities of the axial skeleton (including tail), cardiovascular system (heart and aortic arch), appendicular skeleton (missing limb bones, finger abnormalities), eye (including open eye), and neural tube (exencephaly).

In general, across studies there was not a strong relationship between the type and amount of maternal toxicity and developmental toxicity.

In addition to the studies of developmental toxicity with post-conception exposure discussed above, developmental toxicity was also manifested in reproductive toxicity studies . . .. The database as a whole identified CD-1 mice as the most sensitive species for DEHP developmental toxicity via the oral route. The critical papers are [149,150,168,186]. LOAELs and NOAELs for some relevant developmental toxicity studies are presented in Table 71. Developmental effects in reproduction studies are listed in Table 76 **[See Section 4 of this report.]** Studies that address developmental toxicity are consistent in identifying the lower effective range of oral exposure, taking into account differences in duration of treatment.

The DEHP database contains four rat studies conducted by a route other than oral: an IV study, two IP studies, and an inhalation study. These studies provide valuable information but do not contain enough data for separate route-specific hazard identification and NOAEL/LOAEL selection.

The panel is not confident that the lowest dose has been established at which developmental toxicity (the development of the male reproductive system) occurs.

5.1.3.6

The database provides adequate information to identify DEHP as a developmental toxicant by the oral route and for identification of NOAELs and LOAELs for dose–response assessment.

The data are also sufficient to identify the metabolites (MEHP, 2-EH, 2-EHA) as developmental toxicants. However, there are not enough studies for independent hazard identification and dose–response assessment for the parenteral route. Because of the known role of intestinal lipase in DEHP metabolism, it is not possible to readily generalize dose–response assessment from the oral to intravenous route. Existing PBPK models do not include fetal compartments; and hence have limited use at present.

5.2

As will be discussed below, there are sufficient data in rodents to conclude confidently that oral exposure to DEHP can cause reproductive and developmental toxicity in rats and mice. Further, an effect observed in rats involves adverse effects on the development, structure, and function of the male reproductive tract. Thus, for DEHP, the effects on reproduction and development are intertwined.

The developmental toxicity database contains well-conducted and reported studies, many available as full GLP study reports, and additional, more restricted studies that provide supplemental and supportive information. The database is somewhat limited in that it consists almost entirely of studies in rats and mice orally exposed during gestation where effects are seen by examining physical development of rodent pups just prior to birth (i.e. prenatal assessment). These studies indicate that a range of effects may occur, including malformations (tail malformations, axial and appendicular skeletal abnormalities, cardiovascular malformations, and neural tube closure defects), developmental delays, and intrauterine death. The NOAEL based on malformations in rodents was ~40 mg/kg bw/day and a NOAEL of 3.7–14 mg/kg bw/day was identified for testicular development/effects in rodents. In contrast, functional reproductive endpoints that are evaluated through postnatal observation have not been adequately studied. This is a significant data limitation. There are a limited number of studies by the inhalation, dermal, and intravenous administration routes. It was noted that results are consistent across studies, taking into account doses, route, species, timing,

The examination of effects during the late gestational and neonatal periods is quite recent and incomplete. Despite the general belief among expert panel members that this represents a time of potentially high sensitivity to DEHP-induced disruption of the reproductive system, the dose–response relationships for reproductive effects following exposures in gestational versus postnatal ages are unknown. Low-dose studies examining sensitive endpoints following late gestational exposure are a critical data need.

There is a study that demonstrated the same spectrum of developmental toxicity (as seen in 'normal' mice) in mice that were genetically incapable of expressing peroxisome proliferation due to lack of PPAR-alpha.

DEHP data from rats and mice are assumed relevant to judging hazard to human reproduction and development; they are the standard mammalian test systems used.

REFERENCES FROM THE ORIGINAL REPORT CITED ABOVE

[146] Price CJ, Tyl RW, Marr MC, Myers CB, Sadler BM, Kimmel CA. Reproduction and fertility evaluation of diethylhexyl phthalate (CAS No. 117-81-7) in CD-1 mice exposed during gestation. Research Triangle Park, NC: National Toxicology Program, 1988.

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[186] Reel JR, Tyl RW, Lawton AD, Jamb JC. Diethylhexyl phthalate (DEHP): Reproduction and fertility assessment in CD-1 mice when administered in the feed. PB84-181734. Springfield, VA: available from: NTIS, Research Triangle Park: National Toxicology Program,1984.

Table 71. Summary of DEHP Effects in Developmental Toxicity Studies with Oral Exposure

Protocol & Doses	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)		Fetal Effects at Higher Doses
		Maternal	Developmental	
Prenatal feeding study in CD-1 mice. 30/group received 0, 44, 91, 191, or 293 mg/kg bw/day on GD 0–17. Dams and pups examined in late gestation. [*149, 185*]*	Maternal: 44 Developmental: 44	91 Clinical signs ↓Weight gain	91 ↑Skeletal, visceral, and external malformations	↑Skeletal, visceral, and external malformations ↑Prenatal mortality ↓Fetal weight
Prenatal gavage study in CD-1 mice. 14 /group received 0, 40, 200, or 1,000 mg/kg bw/day on GD 6–15. Dams and pups examined in late gestation. [*150*]*	Maternal: 200 Developmental: 40	1000 ↑Liver weight ↓Weight gain	200 ↑Visceral and external variations and malformations	↑Skeletal, visceral, and external variations and malformations ↑Prenatal mortality
Prenatal feeding study in Fischer 344 rats. 20/group received 0, 164, 313, or 573 mg/kg bw/day on GD 0–20. Pups evaluated postnatally. [*156*]*	Maternal: 164 Developmental: 164	313 ↓Food Intake	313 ↑Prenatal mortality	↑Prenatal mortality ↓Pup body weight on PND 1 only
Prenatal feeding study in CD-1 mice. 28/group received 0, 19, 48, or 95 mg/kg bw/day from GD 0–17. Pups evaluated postnatally. [*146*]*	Maternal: 95 Developmental: 48	No higher doses	95 ↑Prenatal mortality ↓Pup survival on pnd 4	No higher doses

*Doses calculated by study authors.

4.0 REPRODUCTIVE TOXICITY DATA

4.1 Human Data

Since the initial CERHR Expert Panel Report on DEHP, human studies have evaluated measures of male reproductive function and endometriosis in females in association with estimates of DEHP exposure.

Modigh et al. ***(152)***, supported by the Swedish Environmental Protection Agency and Swedish Work Environment Fund, evaluated time-to-pregnancy in the partners of men potentially exposed to DEHP. Men employed or the partners of women employed in 1 of 3 plants were invited to participate. Among the 284 men identified as eligible, 234 responded. The responders had produced 397 pregnancies. After excluding pregnancies for which information was unavailable, 326 pregnancies were available for analysis. Information on time-to-pregnancy was obtained in a telephone or written interview in which couples were asked how many months they had unprotected intercourse prior to achieving pregnancy. Pregnancies were counted if the couple was attempting to become pregnant or not attempting but not avoiding pregnancy. Only pregnancies ending in 1987 or later were counted. Information was accepted from either partner; the woman's answer was used if there was disagreement. Exposure was estimated from employed subjects' description of work tasks and measurements that had been made in each of the 3 workplaces during the general time period relevant for the pregnancies. Exposure categories were created as follows: *unexposed* pregnancies (n=182) were fathered by operators who were not exposed during the time leading up to pregnancy, by office staff, or unexposed male partners of female workers; *low-exposure* pregnancies (n=100) were fathered by men with estimated non-zero DEHP exposures <0.1 mg/m^3; and *high-exposure* pregnancies (n=44) included 25 pregnancies fathered by men exposed to DEHP 0.1–$<$0.2 mg/m^3, 15 pregnancies fathered by men exposed to DEHP 0.2–$<$0.5 mg/m^3, and 4 pregnancies fathered by men with DEHP exposures of 0.5 mg/m^3 or higher. The highest estimated mean DEHP exposure level was 1.9 mg/m^3. To account for possible effects at any time during the 70-day period of spermatogenesis, exposures were evaluated 1, 2, and 3 months prior to the month of attempted pregnancy. Fecundability ratios calculated from a Cox proportional hazards model were estimated using the unexposed pregnancies as the referent. A binomial regression model was used to control for potential confounders (father's age, mother's age, and length of time to recall). An additional analysis was conducted using only the first pregnancy from couples for which more than 1 pregnancy was available.

Median time-to-pregnancy was 3.0 months in the unexposed group, 2.25 months in the low-exposure group, and 2.0 months in the high-exposure group. The crude and adjusted fecundability ratios for the exposed pregnancies were all close to 1.0, and the 95% confidence intervals all overlapped unity. There was no significant effect of restricting the analysis to the first pregnancy of couples with more than 1 pregnancy or of excluding pregnancies conceived by employed women. Excluding couples with known fertility problems did not influence the findings. The results did not depend on whether exposure status was used for the month under consideration or lagged 1, 2, or 3 months. The authors concluded that there was no evidence of a DEHP-associated prolongation in time-to-pregnancy, although they recognized that there were few highly exposed men in their sample; the mean DEHP exposure level for men in the study was less than 0.5 mg/m^3.

Strengths/Weaknesses: Time-to-pregnancy can be a sensitive marker of reproductive impairment. The use of unexposed men from the same workplaces as referents is a strength. Measurements of exposure were objective and independent of self-reports of work tasks and locations. The study considered numerous

potential confounders. Weaknesses include the small number of highly exposed men and measurement error involved in the retrospective assessment of paternal exposure. The use of only men who fathered pregnancies is a limitation of the retrospective time-to-pregnancy assessment. Other weaknesses include the low response rates and the inability to mask participants to exposure and outcome status.

Utility (Adequacy) for CERHR Evaluation Process: This paper is useful in the evaluation process.

Rozati et al. *(153)*, support not indicated, measured phthalate esters in the seminal plasma of 21 men with unexplained infertility. The men were male partners in couples presenting for infertility evaluation **[not otherwise defined]**. All subjects had a sperm concentration <20 million/mL, rapidly progressive motility <25%, total progressive motility <50%, or <30% normal forms. Sperm concentration was assessed using a hemocytometer, and morphology was assessed after Papanicolaou staining. **[Details on motility evaluation were not provided. The number of semen samples per subject was not indicated.]** Additional testing included eosin-nigrosin staining to determine vitality, hypo-osmotic swelling test, chromatin decondensation after treatment with SDS and EDTA, and chromatin susceptibility to acid denaturation, determined with acridine orange staining and fluorescent microscopy. Seminal fluid concentrations of phthalate esters **[as a group]** were assessed by HPLC, using a commercial phthalate esters mixture as a standard. **[According to the manufacturer's web site, this mixture contains 0.2% each DEHP, di-*n*-octyl, dimethyl, diethyl, di-*n*-butyl, and benzyl butyl phthalate in hexane (http://www.sigmaaldrich.com/cgi-bin/hsrun/Suite7/Suite/HAHTpage/Suite.HsSigma AdvancedSearch.formAction, accessed April 27, 2005).]** Comparison was made to seminal plasma phthalate concentrations in a control group of 32 men with evidence of conception and normal semen analysis **[not otherwise characterized]** using Student *t* test. Correlation between seminal phthalate ester concentration and individual sperm test results was evaluated using linear regression analysis. **[Regression terms were not specified. Polychlorinated biphenyl concentration was also evaluated in seminal plasma. It is not stated whether the regression analysis adjusted for polychlorinated biphenyl concentration.]**

The mean ± SD seminal plasma phthalate ester concentration in the infertile group was 2.03 ± 0.214 μg/mL, compared to 0.06 ± 0.02 μg/mL in the control group ($P < 0.05$). There was a significant inverse correlation between seminal phthalate ester concentration and normal sperm morphology ($r = -0.769$, $P < 0.001$) and a positive correlation between seminal phthalate ester concentration and the percent acid-denaturable sperm chromatin ($r = 0.855$, $P < 0.001$). There was no significant correlation between seminal phthalate ester concentration and ejaculate volume, sperm concentration, progressive motility, sperm vitality, sperm osmoregulation, or sperm nuclear chromatin decondensation.

The authors concluded that adverse effects on fertility of phthalate esters, which they called xenoestrogens, were consistent with published data on male reproductive toxicity of these compounds.

Strengths/Weaknesses: There was extensive reproductive assessment of cases, but the sample size was small, and there was very little information on the selection of controls for infertile cases. There was very limited assessment of possible confounders (mean age, urban/rural, fish consumption) and no evidence that exposure assessment was carried out blind to case/control status of participants.

Utility (Adequacy) for CERHR Evaluation Process: This report is of limited usefulness in the evaluation process.

Duty et al. *(154)*, supported by NIEHS, evaluated urinary MEHP and semen analysis parameters. Subjects included 168 men being evaluated in a clinic as part of a fertility evaluation. A questionnaire was used to obtain information on lifestyle factors. A single semen sample was produced by masturbation after instructions to abstain from ejaculation for 48 hours. Sperm concentration and percent motility were assessed using computer-assisted sperm analysis, and morphology was evaluated by light microscopy of air-dried smears after application of a commercial stain (Diff-Quik). A single spot urine was collected on the same day as the semen sample and analyzed using HPLC with tandem MS for MEHP and for monoethyl, monomethyl, mono-*n*-butyl, monobenzyl, mono-*n*-octyl, mono-isononyl, and monocyclohexyl phthalates. Urine phthalate concentrations were adjusted based on urine specific gravity and were dichotomized as high or low based on median values. Sperm parameters were dichotomized based on published norms. Abnormal sperm concentration was <20 million/mL, abnormal motility was <50% motile, and abnormal morphology was <4% normal forms. Mantel-Haenszel chi-squared test was used to assess the relationship between high/low phthalate concentration in the urine and normal/abnormal semen parameter. Multivariate logistic regression was used to adjust for smoking status, age, race, body mass index, and abstinence time.

The study population included 28 men (17%) with low sperm concentration, 74 men (44%) with <50% motility, and 77 men (46%) with >4% normal forms. There were 77 men (46%) who were normal in all 3 domains. There were no significant associations between abnormal semen parameters and MEHP urine concentration above or below the group median. **[Associations were identified and explored with respect to monobutyl, monobenzyl, and monomethyl phthalate, which are not discussed here.]** The authors did not present conclusions relative to MEHP. In this paper and in the following papers from this group, the authors discussed limitations of their methods:

- Use of single spot urines. The authors indicated that because phthalates are rapidly eliminated, use of a single spot urine assumes steady state exposure from food and personal care products.
- Adjustment of urine phthalate concentrations using specific gravity. The authors acknowledge that creatinine is often used to adjust urine concentrations, but argue that use of creatinine may not be appropriate for compounds that are not excreted through glomerular filtration. They further note the dependence of creatinine secretion on muscle mass, physical activity, time of day, diet, urine flow, and disease states.
- Use of a fertility population. The authors expressed doubt that a fertility sample would include men that necessarily differ from men in the general population in their testicular response to phthalate exposure.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a limitation, and the use of only one semen sample per individual is also a limitation.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in the evaluation process.

Duty et al. *(30)*, supported by NIEHS, evaluated urinary MEHP and sperm motion parameters by computer-assisted sperm analysis. Subjects were the male partners in couples presenting for fertility evaluation, without regard to whether the male had a fertility problem. Of the 259 men who agreed to participate, 234 provided a urine sample and a semen sample. **[It is assumed, though not stated,**

that some of these subjects were also reported in Duty et al. *(154)*.] Thirteen semen samples did not contain motile sperm, and 1 semen sample was not submitted for computer-assisted analysis, leaving 220 subjects with motile sperm measurements and spot urine samples. After elimination of urine samples with specific gravities below 1.010 or above 1.030, 187 semen-urine pairs remained for evaluation. Urine samples were frozen for subsequent phthalate monoester analysis by HPLC and tandem MS. The phthalate monoesters included the monoethyl, monomethyl, mono-*n*-butyl, monobenzyl, mono-*n*-octyl, mono-isononyl, and monocyclohexyl, as well as MEHP. Urinary phthalate determinations were normalized using specific gravity. Multiple linear regression analysis was used to evaluate the association between tertile of normalized urinary MEHP concentration and sperm motion parameter. Covariates included in the model were smoking status, race, age, body mass index, and abstinence interval prior to collection of semen.

Subjects had a mean ± SD age of 36.3 ± 5.6 years. Mean ± SD sperm concentration was 115.6 ± 99.2 million/mL with 13.2% of samples having a sperm concentration < 20 million/mL. Mean ± SD percent sperm motility was 52.2 ± 22.6% with 41.8% having < 50% motile sperm. Mean ± SD percent normal morphology was 7.4 ± 4.6% with 22.3% having < 4% normal forms. **[The cut-offs representing the norms cited in Duty et al. *(154)*.]** The authors stated that there was evidence of a dose-response relationship with respect to MEHP and straight-line velocity, curvilinear velocity, and linearity with *P* values for trends of 0.1 – 0.3. They further stated that use of quartiles instead of tertiles, use of phthalate concentration as a continuous parameter, and use of actual phthalate concentrations rather than concentrations adjusted for specific gravity produced results that were consistent with the initial analysis. **[Data and analyses were not shown.]**

The authors concluded that there was an overall pattern of decline in straight-line velocity, curvilinear velocity, and linearity, which was identified with mono-*n*-butyl and monobenzyl phthalate as well as MEHP. They postulated that the lack of statistical significance may have reflected the relatively small sample size. They indicated that if phthalates were associated with sperm motion abnormalities, their study may have under-ascertained the effect because immotile sperm did not give rise to motility parameters.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a weakness. This report appears to include the same subjects as Duty et al. *(154)* and cannot be considered independent.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in the evaluation process.

Duty et al. *(155)*, supported by NIEHS, evaluated a possible association between urinary phthalate monoester concentrations and sperm DNA damage, assessed using the neutral comet assay **[so named because fragmented DNA streams away from the main cell body on electrophoresis, producing a visual image that looks like a comet]**. The subjects and samples were as described in Duty et al. *(30)*, although the number of samples tested was lower (n = 141). Semen samples were frozen prior to comet assay. Urinary phthalate monoester concentrations were adjusted for specific gravity and analyzed in quartiles using multiple linear regression adjusted for smoking status, race, age, body mass index, and abstinence interval prior to collection of semen. There were no significant associations between comet assay parameters and MEHP urinary concentrations. **[Significant associations were identified only for monoethyl phthalate.]** Inclusion of urine samples that had been excluded

based on specific gravities below 1.010 or above 1.030 did not change the results. The authors did not express conclusions relative to MEHP.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a weakness. This report appears to include the same subjects as Duty et al. *(154*) and cannot be considered independent.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in the evaluation process.

Duty et al. *(156*), supported by NIEHS, evaluated the relationship between serum concentrations of testosterone, sex hormone-binding globulin, inhibin B, FSH, and LH and phthalate monoester concentrations in spot urine samples. The subjects included 295 men attending a clinic as part of a fertility evaluation. **[It is not known how many of these men were also included in the previously discussed studies from this group *(30, 154, 155*).]** Blood, semen, and urine samples were collected. Serum was frozen until assayed using RIA (testosterone), enzyme immunometric assay (sex hormone-binding globulin), enzyme-linked immunosorbent assay (inhibin B), or microparticle enzyme immunoassay (LH, FSH). Urine concentrations of MEHP and monomethyl, monoethyl, mono-*n*-butyl, and monobenzyl phthalate were assayed using HPLC with tandem MS and were adjusted based on urine specific gravity. Spearman correlation coefficients were calculated in the exploratory analysis followed by multiple linear regression with adjustment for smoking status, age, race, body mass index, previous fertility evaluation, prior fathering of a pregnancy, season, and time of day.

In their primary analysis, using all urine samples, the authors identified a "negative non-significant association" between urine MEHP concentration and serum testosterone, with a change in serum testosterone of −0.47 nmol/mL (95% CI − 1.03 to 0.10, $P = 0.10$) for each quartile increase in MEHP concentration. In a secondary analysis, in which urine samples were excluded if they had a specific gravity < 1.010 and > 1.030, the association between MEHP concentration and testosterone was described as weaker (− 0.42 ng/mL for each quartile increase; 95% CI − 1.05 to 0.21; $P = 0.19$). **[Additional associations were identified between urinary mono-*n*-butyl and monobenzyl phthalate and serum concentrations of inhibin B and FSH.]**

The authors did not draw conclusions with respect to MEHP. They indicated that they could not tell whether the associations they identified represented physiologically relevant changes or were the result of conducting multiple comparisons.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a weakness. This report appears to include the same subjects as Duty et al. *(154*) and cannot be considered independent.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in the evaluation process.

Jönsson et al. *(85*), supported by the Swedish Research Council, AFA Foundation, the Swedish Government Funding for Clinical Research, the Crafoordska Fund, the Ove Tulefjords Fund, the Foundation for Urological Research, and the Medical Faculty of Lund University, studied semen parameters and urinary phthalate monoester levels in 234 military recruits. The subjects were 18 – 21 years old at the

time of examination. Combined testicular volume was estimated based on ultrasound measurements, semen was obtained by masturbation, and spot urine samples were collected for measurement of phthalic acid, MEHP, and monoethyl, monobutyl, and monobenzyl phthalate. The limit of detection for MEHP was 15 ng/mL **[µg/L]**. Seminal plasma was assayed for neutral α-glucosidase, zinc, prostate-specific antigen, and fructose. Blood samples were collected for determination of serum FSH, LH, sex hormone-binding globulin, testosterone, 17β-estradiol, and inhibin. Seminal sperm were assessed for concentration and motility, including computer-assisted parameters, and were subjected to the sperm chromatin structure assay. Subjects were categorized into quartiles by urine concentration of individual phthalate monoesters (uncorrected and creatinine-adjusted), and ratios with 95% confidence intervals were calculated for highest:lowest quartile groups.

The median urinary MEHP concentration was below the limit of detection. The 75th and 95th percentile values were 5.1 and 12 ng/mL **[µg/L]**, respectively. The maximum value was 25 ng/mL. There were no significant associations between highest versus lowest urinary MEHP quartile and any of the dependent variables. The authors found no evidence of interaction between phthalate metabolites and polychlorinated biphenyl (PCB)-153 on testicular function.

Strengths/Weaknesses: Population not selected based on fertility characteristics was a strength. However, because only 14% of men agreed to participate, they may not represent the source population. Although participants may have been more concerned with their fertility than non-participants, they were blind to their phthalate exposure, making it unlikely that participation was biased by exposure. Comprehensive and objective measurements of semen parameters (ultrasound assessment of testicular volume, computer assisted assessment of motility) were strengths. Assessment of confounding by abstinence time and smoking was a strength, but lack of assessment of confounding by age or BMI was a limitation.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful in the evaluation process.

Cobellis et al. ***(157)***, support not indicated, measured DEHP and MEHP concentrations in the plasma and peritoneal fluid of 35 women identified by laparoscopy as having endometriosis. The operations were performed for ovarian cysts, chronic pelvic pain, or dysmenorrhea. A comparison group consisted of 24 age-matched controls without known reproductive disease **[laparoscopy was not performed on these women, and it is not known how many of them may also have had endometriosis]**. Blood samples were collected from the women undergoing surgery either the day prior to the procedure or "immediately before anaesthesia for laparoscopy." **[The paper does not indicate how many, if any, of the patients were receiving IV infusions at the time of sampling. The Expert Panel notes that Section 1.2.4 of the original CERHR report contains information on the amount of DEHP that can be transferred by medical infusions.]** Blood samples were obtained from the age-matched controls at the same phases of the menstrual cycle as the surgical patients. Estimation of DEHP and MEHP concentration was by HPLC. The proportions of women in each group with detectable concentrations were compared using the Fisher exact test, and concentrations were compared using the Wilcoxon test. Correlation between stage of endometriosis (a semi-quantitative estimate of the extent of visible endometriosis implants) and DEHP/MEHP concentration was made by Spearman correlation coefficient. There was no difference in the proportion of women in either group with detectable DEHP or MEHP (91.4% of surgical patients compared to 92.6% of control women). The median concentration

(interquartile range) of DEHP in the patients was 0.57 (0.06 – 1.23) µg/mL compared to a control value of 0.18 (0 – 0.44) g/mL ($P = 0.0047$). The median concentration (interquartile range) of MEHP in the patients was 0.38 (0.1 – 0.97) µg/mL compared to a control value of 0.58 (0.34 – 0.71) g/mL ($P = 0.12$). There was no significant association between DEHP/MEHP concentration in plasma or peritoneal fluid and stage of endometriosis in women undergoing laparoscopy. The authors concluded that there could be a plausible causal relationship between DEHP exposure and endometriosis, but that, "further studies are needed in order to elucidate the mechanisms underlying the observed statistical association."

Strengths/Weaknesses: Weaknesses include possible exposure of cases due to medical procedures, very limited information on the selection of controls for endometriosis cases, very limited evaluation of confounding, and small sample size.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful in the evaluation process.

Hauser et al. *(158)*, supported by NIEHS, evaluated possible interactive effects of polychlorinated biphenyls and phthalates on sperm motility in male partners of couples seeking infertility evaluation. Phthalate exposure was estimated based on urinary monoester concentrations, corrected for specific gravity, and polychlorinated biphenyl exposure was estimated using measurements in blood samples. Both phthalate and polychlorinated biphenyl levels were dichotomized at the median as high or low, and sperm concentration was dichotomized as normal or abnormal based on World Health Organization criteria. Other sperm parameters were measured, but motility had the strongest association with phthalate monoesters and polychlorinated biphenyls, and only the motility data were presented. Multivariate logistic regression was used to explore interactions. A relative excess risk of the interaction was calculated as 1 plus the relative risk (of low motility) associated with high values for both chemical classes less the relative risk associated with a high value for 1 chemical class and a low value for the other chemical class. Men with low values for both chemical classes were the reference group. This calculation was made for the individual phthalate monoesters, including MEHP and monobutyl, monobenzyl, monoethyl, and monomethyl phthalate, and for individual and grouped polychlorinated biphenyls.

There were 303 men with urinary phthalate monoester levels. The median unadjusted MEHP concentration was 6.6 ng/mL **[µg/L]**, with a 95th percentile value of 112 ng/mL. Interactions were identified for monobutyl phthalate and different classes of polychlorinated biphenyls. There were no significant interactions between urinary MEHP and any of the polychlorinated biphenyls or groupings, either with regard to sperm motility or other sperm parameters.

The authors concluded that there were statistical interactions between some polychlorinated biphenyls and phthalates in relation to low sperm motility. They hypothesized that polychlorinated biphenyl metabolites could interfere with phthalate metabolism through inhibition of UDP-glucuronyl transferase.

Strengths/Weaknesses: This report contains additional analyses of the population in the Duty et al. studies, which were well-conducted and only suffered the limitation of being conducted on a subfertile population. The groupings of PCBs were made a priori based on structure-activity relationships and PCBs were expected to share metabolic pathways with phthalates.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process.

4.2 Experimental Animal Data

Since the initial CERHR Expert Panel Report on DEHP, studies in rodents have evaluated effects on ovarian follicles or their constituents. Additional studies in male rodents and marmosets have been performed to characterize testicular toxicity and to evaluate mechanisms of toxicity.

4.2.1 Female

Lovekamp and Davis *(159)*, supported by NIEHS, evaluated the effects of MEHP on F344 rat granulosa cells in culture. Four-week-old animals were stimulated with diethylstilbestrol or with pregnant mare serum gonadotropin (PMSG) following which ovaries were removed and granulosa cells harvested. Cells were cultured in the presence of 500 nM testosterone and 200 ng/mL FSH. MEHP **[purity not specified]** was added to cultures at concentrations of 0 – 200 μM **[0 – 55.6 mg/L]** in a comparison with other phthalate monoesters and with Wy-14,643, another peroxisome proliferator. After 48 hours, estradiol and progesterone were measured in media using commercial RIA kits. RNA was extracted from cells and RT-PCR performed to quantify mRNA for aromatase and for cholesterol side-chain cleavage enzyme. Aromatase protein was quantified by Western blot. Statistical comparisons were made using the Student *t* test or ANOVA, followed by least significant difference test.

A comparison with other phthalate monoesters showed a decrease in estradiol in the medium (controlled for protein content) with MEHP 100 or 200 μM **[27.8 or 55.6 mg/L]** but not with comparable molar concentrations of monomethyl, -ethyl, -propyl, -butyl, -pentyl, or -hexyl phthalate. Monopentyl phthalate was associated with a decrease in estradiol production at 400 μM. mRNA for aromatase was estimated in media after culture with MEHP 0, 25, 50, or 100 μM **[0, 7.0, 13.9, or 27.8 mg/L]**. Graphically, there appeared to be a concentration-dependent decrease in estradiol concentration and in aromatase mRNA; pair-wise comparisons with control were statistically significant for the 100 μM concentration for estradiol and for the 50 and 100 μM concentrations for aromatase mRNA. The peroxisome proliferator Wy-14,643 also decreased estradiol and aromatase mRNA. Cholesterol side-chain cleavage enzyme was not altered by MEHP, suggesting specificity of the effect on aromatase mRNA. Aromatase protein was decreased by MEHP at concentrations of 100 and 200 μM **[27.8 and 55.6 mg/L]**. In a final experiment, granulosa cells were incubated for 48 hours with MEHP 0 or 200 μM **[0 or 56 mg/L]** and 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) added for the last 24 hours. mRNA for aromatase and cholesterol side-chain cleavage enzyme and medium progesterone levels were increased by 8-Br-cAMP in the absence of MEHP. In the presence of MEHP, mRNA for aromatase and medium estradiol levels were suppressed, but there was no suppression of P450 side-chain cleavage enzyme mRNA or progesterone. The authors interpreted their results as consistent with transcriptional suppression of aromatase by MEHP independent of the FSH-cAMP pathway. They proposed a PPAR pathway as a candidate mechanism for MEHP suppression of granulosa cell steroidogenic function.

Strengths/Weaknesses: The strengths of this study include appropriate technical proficiency and appropriate study controls and statistics, as well as examination of a set of structurally diverse phthalates. The Panel has confidence in the veracity of these results. This study identified a probable main point of interference with steroid production, showed that it was most affected by MEHP and not other phthalates (thus identifying MEHP as the main concern), and proposed a possible mechanism by which this interference might occur (PPAR mediation). This *in vitro* study comes after several *in vivo*

studies that demonstrated low-estradiol-related changes in female rats, so relevance is another strength. Weakness includes examining cells from only 1 species and using MEHP of uncertain purity.

Utility (Adequacy) for CERHR Evaluation Process: The data point towards a subcellular site of action. Their utility in risk assessment would come in helping to identify vulnerable species as those having these target enzymes. The *in vitro* data are presumed relevant for *in vivo* protection when circulating blood levels of MEHP (or even better, tissue levels) are known.

Lovekamp-Swan et al. *(160)*, supported by NIEHS, evaluated the interaction of MEHP with PPAR pathways as a mechanism for modifying ovarian steroidogenic functions. Granulosa cells were harvested from 4-week-old Fisher rats 24 hours after injection of PMSG. Cells were cultured for 48 hours with 500 nM testosterone and 200 ng/mL FSH with or without MEHP **[purity not specified]** 50 μM **[13.9 mg/L]**. Culture media were assayed for estradiol using a commercial RIA kit. RNA was extracted from cells and specific RT-PCR probes used to amplify mRNA for quantification. The mRNAs were chosen to reflect the activity of genes involved in the PPAR pathways, steroidogenesis, or phthalate toxicity. The mRNAs included aromatase, 17β-hydroxysteroid dehydrogenase IV (which metabolizes estradiol to estrone), cholesterol side-chain cleavage enzyme, the aryl hydrocarbon (Ah) receptor, cytochrome P450 1B1 (CYP1B1), epoxide hydrolase, and heart-fatty acid binding protein (H-FABP, which is associated with luteal transformation of granulosa cells). mRNA results were normalized to glyceraldehyde 3-phosphate dehydrogenase as an internal control.

To test the hypothesis that MEHP inhibition of granulosa cell aromatase is mediated through different PPAR isoforms, the experiments included the coadministration with MEHP of troglitazone (a PPARγ ligand), selective PPARα and γ agonists, a selective PPARγ antagonist, a selective retinoic acid X receptor (RXR) agonist (because a PPARγ:RXR heterodimer is believed to decrease aromatase), 9-*cis*-retinoic acid (which binds both RXR and the retinoic acid receptor, RAR), and 15-deoxy-Δ12,14-prostaglandin J2 (a PPAR activator). Statistical comparisons were made using the Student *t* test or ANOVA, followed by the least significant difference test.

MEHP in culture reduced aromatase mRNA by >40% compared to control. The addition of a PPARγ antagonist partially reversed the decrease in aromatase mRNA. PPARα and γ agonists reduced aromatase mRNA to an extent similar to that after treatment with MEHP. To test the hypothesis that MEHP-activated PPARγ plus activated RXR results in aromatase suppression mediated by the PPARγ:RXR heterodimer, cells were treated with MEHP plus RXR or RAR ligands. All treatments decreased aromatase, with significant additional suppression by MEHP when it was added with 9-*cis*-retinoic acid. To demonstrate that MEHP activity could be mediated through PPARα, MEHP treatment was shown to increase mRNA for 17β-hydroxysteroid dehydrogenase IV, which is inducible by PPARα. The addition of a PPARγ antagonist did not alter the MEHP induction of 17β-hydroxysteroid dehydrogenase IV, suggesting MEHP activation of the α-isoform of PPAR. Both MEHP and a selective PPARα agonist, but not a selective PPARγ agonist, increased the expression of Ah receptor, CYP1B1, and epoxide hydrolase. Cholesterol side-chain cleavage enzyme was not induced by MEHP or by either PPAR isoform agonist. H-FABP was induced by MEHP and by each specific PPAR isoform agonist. The authors concluded that MEHP effects on granulosa cell gene expression, which would serve to decrease estrogen production, were mediated by both PPAR pathways.

Strengths/Weaknesses: These data further dissect the molecular pathways by which MEHP reduces estradiol production in granulosa cells. The experiments were well-conceived and executed, used appropriate statistics, and the Expert Panel has confidence in the veracity of these studies. Possible weaknesses for the evaluation process include limited species evaluation, and relying on the presumption that these *in vitro* data report processes that are relevant and active *in vivo*.

Utility (Adequacy) for CERHR Evaluation Process: These *in vitro* data are adequate for the evaluation process and are presumed relevant for *in vivo* biology. An additional assumption implicit in these experiments is that immature stimulated rodent granulosa cells are appropriate models for human granulosa cells, and that the agonists and antagonists employed are specific for their intended targets. The data are uniquely valuable in describing a mechanism of toxicity that may be tested in other species, and that is presumed relevant for other species sharing a similar biochemistry.

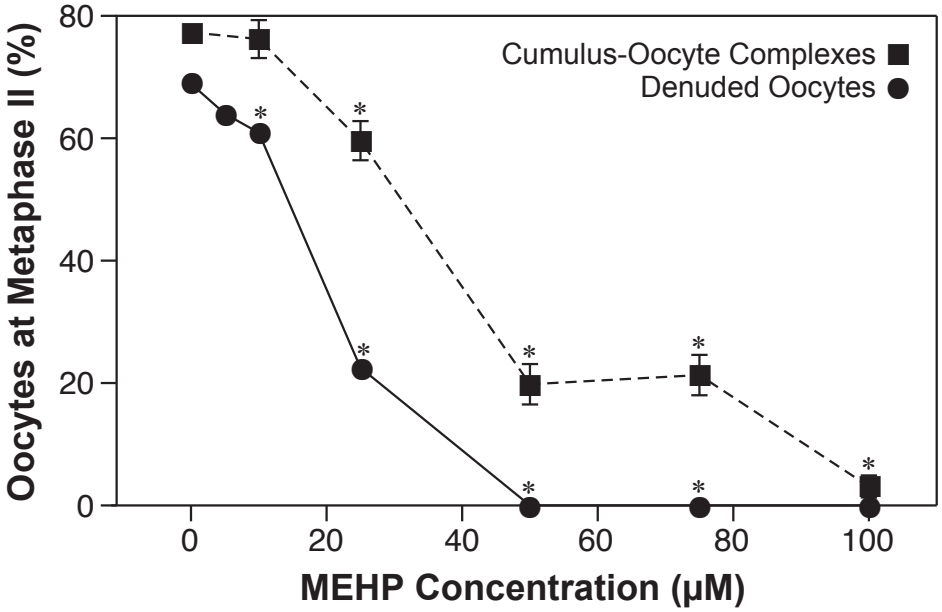
Anas et al. *(161)*, supported by the Japanese Ministry of Agriculture, Forestry, and Fisheries, evaluated the effect of MEHP on *in vitro* maturation of bovine oocytes. Cumulus-oocyte complexes were obtained by aspirating 2–5 mm follicles from slaughterhouse beef ovaries. Oocytes with unexpanded cumulus layers were selected for study. In experiment 1 (n=91–99 oocytes/group), cumulus–oocyte complexes were cultured for 24 hours with MEHP (90% purity) at 0, 10, 25, 50, 75, or 100 μM **[0, 2.8, 7.0, 13.9, 20.9, or 27.8 mg/L]**, after which they were evaluated for cumulus expansion and for stage of oocyte maturation. **[Maturation was assessed in ethanol-fixed orcein-stained oocytes using unspecified criteria.]** In experiment 2 (n=123–131 oocytes/group), denuded oocytes were cultured with MEHP at the same concentrations with the addition of a 5 μM **[1.4 mg/L]** concentration level, followed by assessment of oocyte maturation stage. In experiment 3, cumulus-oocyte complexes were cultured for 24 hours with MEHP 0, 50, or 100 μM **[0, 13.9, or 27.8 mg/L]**, following which some oocytes were evaluated for maturational stage. Other MEHP-treated oocyte cultures were continued in MEHP-free culture medium for an additional 24 hours or were continued at their original MEHP concentration (50 or 100 μM) for an additional 24 hours. The treatment groups (n=124–135 oocytes/group) consisted of MEHP exposure for 24 hours, MEHP exposure for 24 hours followed by 24-hour "recovery," and MEHP exposure for 48 hours. Statistical analysis was by ANOVA and Fisher protected least significance test. Experiments 1 and 2 showed a concentration-dependent decrease in progression through oocyte maturation stages, with a significant decrease in oocytes reaching metaphase-II beginning at MEHP 25 μM **[7.0 mg/L]** for cumulus-oocyte complex culture and at 10 μM **[2.8 mg/mL]** for denuded oocytes (Figure 6). Cumulus expansion was not impaired in experiment 1 **[suggesting no effect of MEHP on granulosa cell division and differentiation]**. In experiment 3, impairment of progression to metaphase II was seen with the initial 24-hour culture with MEHP, as expected. Culture in MEHP-free medium permitted the progression of oocyte maturation to metaphase II in 64.5–71.1% of oocytes, although the proportion of oocytes reaching metaphase II did not recover completely to control levels (83.2% at 24 hours). The recovery of maturation ability suggested that the MEHP-associated decrease in oocytes reaching metaphase II was not likely to be due to nonspecific cytotoxic or lethal effects. When oocytes were cultured in MEHP 50 μM for 48 hours, the proportion reaching metaphase II was higher at 48 hours (41.6%) than at 24 hours (26.9%), suggesting that MEHP delayed maturation rather than preventing maturation altogether.

Strengths/Weaknesses: The strengths of this study include very large sample sizes, reasonable statistics, a useful study design that included recovery, and a novel approach. It would have been more valuable

to have included additional measures of cytotoxicity and perhaps to have gone past simple description to the use of specific agonists or antagonists (see Lovekamp et al. *(160)*, above).

Utility (Adequacy) for CERHR Evaluation Process: The data are adequate for the evaluation process and are presumed relevant for humans. They show a direct effect on the ovary, although not a unique effect. They add little to mechanistic understanding. They do, however, expand the list of affected species.

Figure 6. Percent of Bovine Oocytes Reaching Metaphase II after 24-hour Culture with MEHP



* *Significant difference from 0 ppm group. Drawn from Anas et al. (161).*

Sekiguchi et al. ***(162)***, supported by the Cooperative System for Supporting Priority Research in Japan Science and Technology Corporation, evaluated number of ova ovulated in response to 15 or 30 international units (IU) equine chorionic gonadotropin as an assay of female reproductive toxicity in F344 rats. DEHP was used as an illustrative chemical in their system. The equine chorionic gonadotropin was injected intramuscularly (IM) at 25 days of age. The animals were killed 72 hours later, and uteri and ovaries were removed and weighed. Ova were flushed from the oviducts with saline, denuded with hyaluronidase, and counted under a microscope. Because the maximum number of ova recovered in spontaneously ovulating rats was 11, superovulation was defined as the presence of more than 11 ova in the oviducts. To evaluate the effects of the test chemical, DEHP **[purity not specified]** 0 or 500 mg/kg bw/day in olive oil was given by sc injection daily from 24 to 27 days of age. There were 12 rats treated with 15 IU equine chorionic gonadotropin, 6 of which received DEHP, and 7 rats treated with 30 IU equine chorionic gonadotropin, 3 of which received DEHP. Ovulation was induced in 4 of the 6 rats that received 15 IU equine chorionic gonadotropin in the presence or absence of DEHP treatment. In rats given 30 IU equine chorionic gonadotropin, ovulation occurred in 1 of 3 rats given DEHP and 4 of 4 rats given the vehicle. This difference was described by the authors as not statistically significant, possibly due to the small number of animals **[statistical methods not described; *P*=0.14, Fisher exact test by CERHR]**. None of the rats treated with DEHP demonstrated superovulation compared to 2 of 4 rats given 15 IU equine chorionic gonadotropin and 1 of 4 rats given 30 IU equine chorionic gonadotropin.

The mean number of recovered ova was described by the authors as having been reduced by DEHP treatment (mean ± SEM 12.7 ± 7.75 [control] compared to 2.50 ± 0.85 [DEHP] in animals given 15 IU equine chorionic gonadotropin and 8.00 ± 4.69 [control] compared to 0.33 ± 0.33 [DEHP] in animals given 30 IU equine chorionic gonadotropin **[*P* = 0.22 and 0.23, *t* test by CERHR]**). The authors concluded that DEHP may have suppressed ovulation due to disrupted ovarian steroidogenesis and/or follicle growth.

Strengths/Weaknesses: The use of intact animals allows the assessment of an integrated physiologic response. This is overshadowed by the small sample sizes, the lack of appropriate statistics, and the fact that the studies did not move past simple description, and thus, add little that is new or of value to our understanding.

Utility (Adequacy) for CERHR Evaluation Process: These data are inadequate for the evaluation process, based on small sample size and inadequate statistics.

A 65-week feeding study in marmosets performed at Mitsubishi Chemical Safety Institute, Ltd. *(92)* contained information on ovarian and uterine weight and histology. Because the focus of this study was on testicular effects, the study is discussed in Section 4.2.2.2.

4.2.2 Male

Studies on the male reproductive effects of DEHP in rodents often employ young animals because these animals are more sensitive to the testicular effects of phthalates. Although toxicity produced in prepubertal animals can be considered developmental toxicity, these studies attempt to characterize testicular effects that are not unique to the developing gonad and are included in the reproductive toxicity section. There is a single study in which prepubertal boars were treated, and this study is considered in this section as well.

4.2.2.1 Cultured cells and tissues

Dees et al. *(163)*, supported by NIEHS, reported the effects of MEHP on MA-10 cells in culture. The MA-10 cell is a mouse Leydig tumor cell that resembles the normal Leydig cell in ultrastructure and in steroid synthesis, except that it produces progesterone instead of testosterone. The cells were grown for 24 hours in the presence of MEHP **[purity not specified]** 0, 0.3, 1, 3, 10, 30, 100, 300, 1000, 3000, and 10,000 μM **[0, 0.08, 0.28, 0.84, 2.8, 8.4, 28, 84, 279, 838, and 2794 mg/L]**. After MEHP exposure, cells were washed and incubated for 2.5 hours with human chorionic gonadotropin (hCG) 50 ng/mL in the absence of MEHP. Media were assayed by RIA for progesterone. Cell protein was quantified, and cell viability was assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Electron microscopy was performed and cells photographed at 6000× for morphometric analysis based on nuclear and cytoplasmic volumes as a percent of total cell volume. Statistical analyses were performed by ANOVA with post hoc Tukey test. **[The methods state that cells were grown to confluence in 24-well plates, and that there were 2 experimental replicates. A figure legend indicates n = 16 from 5 different experiments for progesterone and protein levels and n = 9 from 3 different experiments for cell viability using MTT determination.]**

Progesterone production was stimulated by hCG in cells not exposed to MEHP. Significant reductions in progesterone production occurred after incubation with MEHP at 3, 10, 3000, and 10,000 μM but not at the intervening concentrations. Viability and protein were decreased at 3000 and 10,000 μM. MEHP

treatment was associated with an increase in cytoplasmic lipid droplets at MEHP concentrations of 1 μM and higher. Morphometric analysis did not show alterations in nuclear or cytoplasmic volumes up to an MEHP concentration of 1000 μM, the highest tested concentration at which viability was not affected. Abnormal mitochondria, including pale, swollen, and ring-form mitochondria and mitochondria with longitudinal or degenerating cristae, were seen at MEHP concentrations of 1 μM and higher. Rough endoplasmic reticulum was decreased at concentrations of 100 and 1000 μM. The authors concluded that these data were consistent with an MEHP effect on Leydig cell mitochondria and steroidogenesis, with consequent cholesterol accumulation resulting in an increase in lipid droplet accumulation.

Strengths/Weaknesses: This report is useful for having performed an in-depth analysis of structural alterations in an immortalized cell line that produces steroids and for confirming previous reports. A weakness is that only an integrated biochemical measure was taken (progesterone secretion), and no other probes were used to explore the site or nature of the biochemical disruption or to confirm possible mechanisms for the observed changes (mitochondrial changes, increased lipid droplets). The authors could offer no convincing explanation for the peculiar dose-response curve**.**

Utility (Adequacy) for CERHR Evaluation Process: This paper is largely confirmatory of other studies. The model is modestly relevant (all immortalized cell lines have some significant biochemical changes that confer immortality, and thus separate them from the rest of life) providing little new knowledge.

Kang et al. ***(164)***, supported by the Korean Ministry of Environment, evaluated the effect of DEHP on gap junctional intercellular communication in cultured mouse Sertoli cells obtained from normal 11 – 13-day-old mice. **[Most of the text indicates TM4 cells, but the abstract, a figure legend, and the discussion indicate TM5. The Expert Panel assumes based on the endpoints being studied that the TM4 cell was used. The study authors present this paper as primarily an investigation of altered gap-junctional communication as a possible mechanism of DEHP carcinogenicity but discuss possible importance of gap-junctional communication for Sertoli – germ cell interactions.]** A neutral-red uptake assay for cytotoxicity was performed using 24-hour exposures to DEHP concentrations of 0, 1, 10, 100, 250, 500 and 1000 μM **[0, 0.39, 3.91, 39.1, 97.7, 195.3, and 390.6 mg/L]**, showing the highest concentration tested not to be cytotoxic. DNA content of the lysed cultured cells increased after a 3-day exposure to DEHP 500 μM **[195.3 mg/L; the figure legend, however, says 100 μM (39.1 mg/L)]**, interpreted as an indicator of cell proliferation. Gap junctional communication was evaluated using the scrape-loading dye transfer technique in which Lucifer yellow was added to the culture medium and scrape-loaded into the cells using a single pass of a razor blade across the cells, thus injuring some cells and allowing dye entry. This procedure was performed on a monolayer of cultured cells that had been exposed for 24 hours to DEHP 0, 100, or 500 μM **[0, 39.1, or 195.3 mg/L]**. The distance the dye traveled in 3 minutes, observed using fluorescence microscopy, was evaluated as decreased at both concentrations of DEHP compared with the control **[data not shown; amount of decrease not specified]**. Morphologic nuclear apoptotic changes in response to 12 hours of serum deprivation were assessed using the stain Hoechst 33258. Cells pretreated with DEHP 500 μM **[195.3 mg/L]** for 24 hours showed less nuclear staining than control cells, implying a reduction in apoptosis **[data not shown]**. Western blot analysis was used to quantify phosphorylated connexin-43, a gap-junction protein, and poly(ADP-ribose) polymerase (PARP), a substrate for caspase in the apoptosis pathway. Evaluation of phosphorylated connexin-43 protein was performed in lysed cells after exposure to DEHP 500 μM **[195.3 mg/L]** for 1, 4, or 9 hours. The DEHP exposure was described

as reducing phosphorylated connexin-43 in a time-dependent manner **[graph shown without error bars or statistical analysis]**. Evaluation of PARP cleavage products was performed in response to 12 hours of serum deprivation in cells exposed to DEHP 500 μM **[195.3 mg/L]** for 9, 12, or 24 hours. Whereas control cells showed an increase in cleavage products with time, these products were largely absent in DEHP-exposed cells **[graph shown without error bars or statistical analysis]**. The study authors concluded that DEHP inhibited gap junctional communication, possibly associated with a decrease in phosphorylated connexin-43. A decrease in apoptosis associated with a decrease in PARP cleavage was believed possibly to be involved in the carcinogenicity of DEHP.

Strengths/Weaknesses: The weaknesses of this paper include numerous internal inconsistencies that make it difficult to ascertain what was actually done, a lack of statistical analysis, and uncertainty about experimental rationale and execution. Dye transfer experiments frequently require more than 3 minutes for the transfer of the dye to adjacent cells. The nuclear staining and early indicators of apoptosis are generally considered as issues separate from those relating to cell communication, so the combination here is perplexing. Sertoli cells are not targets of the tumorigenic activity of DEHP, so are a model of questionable relevance to the *in vivo* situation. The working dose most frequently used in this study was exceptionally high, and while perhaps useful as a biochemical tool, is of limited relevance.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful for the evaluation process, as there is considerable uncertainty about what was done, whether statistical analysis was performed, and the relevance of the findings to the *in vivo* situation.

Suominen et al. *(165)*, supported by the EU and the Academy of Finland, evaluated the effects of MEHP on segments of CD-1 mouse seminiferous tubules. Testes from 2–3-month-old mice were decapsulated and microdissected to provide 1 and 2 mm tubule segments at stages III–V, VII–VIII, and IX–XI (determined by transillumination and confirmed in squash preparations). Tubule segments at stages IX–XI were incubated for 24 hours with MEHP **[purity not specified]** at concentrations of 0, 0.01, 0.1, and 1.0 mM **[0, 2.8, 28, and 279 mg/L]** for 24 hours. Tritiated thymidine was added for the last 4 hours of incubation for estimation of DNA synthesis by thymidine incorporation. Graphic representation of the incorporated label at the end of the experiment suggested a concentration-related decrease in DNA synthesis, but none of the values were statistically different from the control by ANOVA. Incubation of 2 mm segments of tubules at stages III–V and VII–VIII with MEHP 0.1 mM **[28 mg/L]** also had no significant effect on thymidine incorporation. Stage IX–XI tubule segments were incubated for 8 hours with MEHP at 0, 0.01, 0.1, and 1.0 mM **[0, 2.8, 28, and 279 mg/L]**, following which squash preparations were evaluated for apoptosis using in situ 3' end-labeling followed by light microscopy to count positive cells. A subsequent time-course experiment was performed in which stage IX–XI tubules segments were incubated with MEHP 0.1 mM **[28 mg/L]** for 4, 8, or 24 hours and evaluated for apoptosis by *in situ* 3' end-labeling. Stage specificity of apoptosis induction was evaluated using segments of tubules at stages III–V and VII–VIII incubated with MEHP 0.1 mM **[28 mg/L]** for 8 hours. Statistical evaluation used ANOVA with post hoc Dunnett test for dose-response experiments and *t* test for comparison of stage-specific results with one another. The number of apoptotic cells per tubule segment was approximately doubled **[estimated from a graph]** by MEHP 0.01 and 0.1 mM **[2.8 and 28 mg/L]** compared to control but was not significantly altered by MEHP 1.0 mM **[279 mg/L; lack of effect attributed by the study authors to a high incidence of early cell death at this concentration]**. In the time-course experiment, MEHP 0.1 mM **[28 mg/L]** significantly increased the

number of apoptotic cells per tubule segment only at 8 hours. **[The numeric value appeared to be higher from a graph at 24 than at 4 or 8 hours, but the standard error was large, and statistical significance was not identified.]** In the stage-specificity experiment, only tubule segments at stages IX–XI showed an increase in number of apoptotic cells after MEHP exposure. The authors concluded that their finding of stage specificity in the mouse was "partly in agreement" with previous reports that stages XI–XIV and I–II were the most sensitive to phthalate testicular toxicity in the rat, associated with the reliance of these stages on FSH and with inhibition by phthalates of FSH binding.

Strengths/Weaknesses: One strength of these studies is that they were performed with technical competence and were not over-interpreted. No biochemical mediators of cell death or stage-specific biochemistry were evaluated in these studies, which is something uniquely available in this type of preparation.

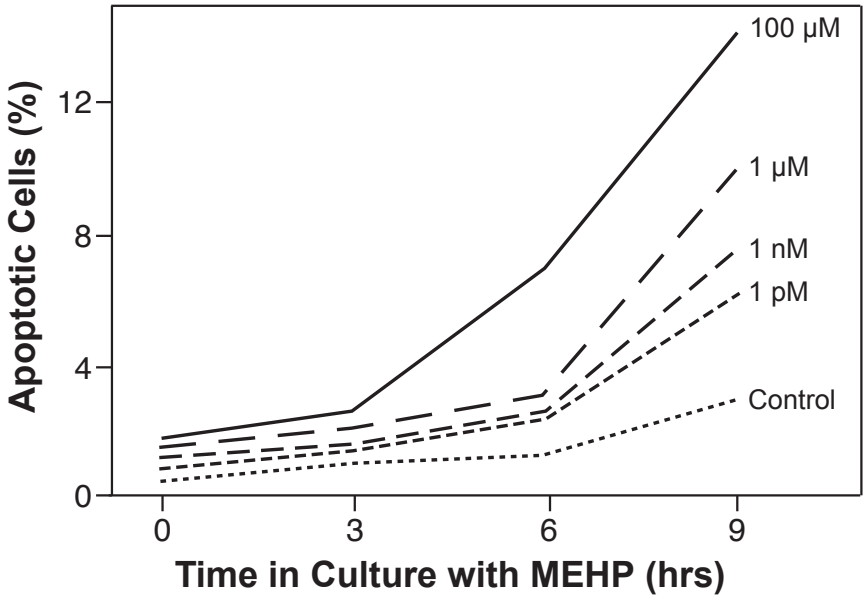
Utility (Adequacy) for CERHR Evaluation Process: These studies appear adequate for the evaluation process but add little that is new to our understanding of where and how DEHP/MEHP works in the testis.

Andriana et al. *(166)*, supported by the Japanese Ministry of Health, Labor, and Welfare, evaluated morphologic MEHP-associated alterations in spermatogenic cells in Sprague-Dawley rat testis culture. Testes were harvested from an unspecified number of 20-day-old animals and 1 mm^3 portions were cultured on filter paper. MEHP **[purity not specified]** was added to culture media at 0, 10^{-6}, 10^{-3}, 1, or 100 μM **[0, 0.00028, 0.28, 279, or 27,936 mg/L]**. Testis fragments exposed at each concentration were evaluated at 1, 3, 6, and 9 hours. TUNEL staining using a commercial kit was applied to 5 μm paraformaldehyde-fixed paraffin-embedded sections with methyl green counterstaining. Apoptotic cells were counted in 25 randomly selected round tubules from 20 tissue cultures, expressed as a percentage, and compared using ANOVA. Transmission electron microscopy was used to characterize ultrastructural evidence of apoptosis and necrosis. Tissues for electron microscopy were fixed in 2.5% glutaraldehyde/0.05 M cacodylate buffer, post-fixed in osmium tetroxide, and embedded in Araldite-M. **[No quantitative results were presented from the transmission electron microscopy portion of the study.]** In the control cultures, apoptosis increased after 9 hours of culture with 2.8% of cells positive. In the MEHP-treated cultures, apoptosis increased in a time- and concentration-dependent manner, with a maximum of 14% of cells affected in the 100 μM culture at 9 hours (Figure 7). At the highest concentration, there was evidence of Sertoli cell apoptosis at 1 hour, characterized by the authors as partial lysis of the nuclear membrane by transmission electron microscopy. Partial lysis of spermatogenic cell nuclear membranes was noted at 1 hour at the 1 and 100 μM MEHP concentrations. The authors concluded that "even a low concentration of MEHP caused permanent changes in testicular tissue cultures of rats."

Strengths/Weaknesses: One strength of this design was the novel exposure system, which allowed for normal cell-cell interactions to be maintained. Another unique aspect was the wide range of concentrations used. One weakness is the unspecified purity of the MEHP, and also the fact that all tubules were not affected equally by MEHP, so the selection of random tubules for assessment depended on truly random selection, which would almost certainly have diluted the effect of the MEHP. Another weakness is the uncertainty surrounding the use of electron microscopy-derived membrane structure to reach conclusions about cell death, and the fact that this finding is inconsistent with the published *in vivo* literature (i.e., Sertoli cell death is not reported after *in vivo* dosing and evaluation). The use of the word "permanent" when describing effects of a 9-hour exposure is questionable.

Utility (Adequacy) for CERHR Evaluation Process: This study appears adequate for the evaluation process, although it produces no new insights into the site or mechanism of MEHP toxicity, confirming what was known previously.

Figure 7. Percent Apoptotic Cells in Rat Testicular Culture with MEHP



Drawn from data in Andriana et al. (166). Standard error bars omitted for clarity. Statistical comparisons were not indicated in the original.

Andriana et al. *(167)*, supported by the Japanese Ministry of Health, Labour, and Welfare, evaluated the effects of MEHP **[purity not specified]** on cultured goat testis. Testes were harvested from 2-month-old Shiba goats, decapsulated, and cut into 1-mm^3 pieces for culture on filter paper floated in Dulbecco's Minimal Essential Medium. The medium contained antibiotics, DMSO, and ethanol. Some cultures included MEHP at 0.001, 1, or 100 nmol/mL **[0.0028, 2.8, or 279 mg/L (nmol/mL=µM); DMSO and ethanol concentrations, control culture conditions, number of animals, and number of cultures were not specified]**. Explants were harvested at 1, 3, 6, or 9 hours of culture and fixed in 2.5% glutaraldehyde/0.05 M cacodylate buffer. Specimens were prepared for light microscopy using toluidine blue staining or transmission electron microscopy using uranyl acetate and lead citrate. Results were expressed qualitatively. Light microscopy showed a concentration-related increase in apoptotic or necrotic Sertoli and spermatogenic cells with vacuolization and sloughing of germ cells beginning at 3 hours "at each concentration." Transmission electron microscopy showed infrequent distended mitochondria, abnormal nuclear vesicles, and ruptured mitochondrial membranes, which were apparent as early as after 1 hour of exposure to 1 µM MEHP **[the results are not specific about which cells were affected, but the figure legend and discussion suggest that the Sertoli cells were affected first]**. At 3 hours, vacuolization and abnormal vesicles were described as frequent, with damage in Sertoli and germ cells. At 6 hours, there were apoptotic spermatogonia. The lowest concentration (0.001 µM) produced marginated chromatin in Sertoli cells at 6 hours. The authors concluded that even the lowest MEHP concentration produced permanent testicular damage, and that their results support the Sertoli cell as the primary target of MEHP toxicity, with germ cell damage as a consequence of Sertoli cell alterations.

Strengths/Weaknesses: A strength of this paper is that it adds another species to the list of those shown to be affected by DEHP/MEHP, and it uses methods shown to have worked for the more commonly employed rat model. It avoids having to expose the whole goat, which saves compound and avoids unknown toxicokinetic issues. Significant weaknesses are the uncertainty about control culture conditions, number of animals used, and the number of experiments, not to mention the uncertainty introduced by including the solvents DMSO and ethanol in the medium. Paucity of experimental detail limits confidence in the relevance of these data, and confidence is further decreased by the solvent issues because DMSO can significantly enhance the toxicity of lipophilic compounds *in vitro*. There is uncertainty about the relevance of the findings at the lower dose levels because these concentrations *in vivo* have not been previously associated with the reported effect.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited relevance and utility to the evaluation process, based on the uncertainty about the methods, solvents, and the doubt around the results at the lowest concentrations, and the assertions of "permanent" damage after short-term *in vitro* exposure and assessment.

Awal et al. *(168)*, supported by the Japan Society for Promotion of Sciences and the Japanese Ministry of Health, Labour, and Welfare, evaluated the effects of MEHP on guinea pig seminiferous tubule culture. Tubules harvested from 28-day-old animals were exposed to MEHP **[purity not specified]** in corn oil at 0, 1, 10, and 100 μM **[0, 279, 2794, or 27,936 mg/L]** for 3, 6, or 9 hours. Cultured tissues were evaluated by light and transmission electron microscopy and by in situ TUNEL staining for apoptosis. Intact round seminiferous tubules (100 per dose group) were counted for apoptotic spermatogenic cells, expressed as a percent. Statistical analysis was by ANOVA with the Fisher least significant differences test. After 3 or more hours of MEHP exposure, germ cell detachment was evident by light microscopy and Sertoli cell vacuolation was identified by electron microscopy **[effect levels not given, but the figures show tubule sections from the 100 μM group, for which the response was said to be maximal]**. The number of apoptotic cells by TUNEL staining was said to increase with exposure level and time. **[The data figure does not have significant differences marked; the legend says that significance between treated and control cultures is recognized at $P < 0.05$.]** The authors concluded that "MEHP induces testicular toxicity in guinea pigs *in vitro*."

Strengths/Weaknesses: It is unclear whether the animals were treated with MEHP before donating tissues to culture or the tissues were treated *in vitro* and exposed to MEHP dissolved in corn oil and added to the culture. There is sufficient confusion about the wording to make the method quite unclear, although much of the description seems to indicate the latter (corn oil was added to the cultures). Weaknesses of this study include this confusion, the unusual exposure paradigm in the absence of any determination of MEHP levels in the medium, uncertainty in the paper about which effects were seen at which concentrations, and the implausibility of finding these effects in cultured tissue fragments due to highly lipophilic compounds being dosed into an aqueous medium in corn oil. The only light-level micrographs use different magnifications for the control and treated cultures, making it difficult for the reader to closely assess the adequacy (i.e., normal structure) of the control cultures. The study confirms an affected cell type but adds little that is detectably new to our understanding.

Utility (Adequacy) for CERHR Evaluation Process: This study is inadequate for the evaluation process due to the confusion about the exposure and the fact that the data are redundant of previous literature.

4.2.2.2 *In Vivo Studies With or Without Ex Vivo Component*

Richburg et al. *(169)*, supported by NIEHS, NIH, and the Burroughs Wellcome Fund, evaluated the effect of MEHP on testicular apoptosis in *gld* mice, which have a nonfunctional form of fas-ligand (fasL). The study was performed to explore the involvement of the fasL, a mediator of cell-cell death, and hypothesized to mediate germ cell death after Sertoli cell damage, in the development of the DEHP lesion in mice. **[The study also evaluated radiation-induced injury; only the MEHP results are presented here.]** Wild-type controls were C57BL/6 males at 28 days of age. Wild-type and *gld* mice were given MEHP (>94% purity) in corn oil at 0 or 1000 mg/kg as a single gavage treatment. The animals were killed 0, 3, 6, 12, or 24 hours later, and testes were harvested. One testis was fixed in 10% neutral buffered formalin embedded in glycol methacrylate, sectioned, and stained with periodic acid-Schiff/hematoxylin for light microscopy. The other testis was frozen until analyzed for apoptosis using a commercial TUNEL staining kit. Apoptosis was evaluated (3 animals/group) using the number of tubules with 0–3 or >3 apoptotic germ cells. Homogenization-resistent testicular sperm heads were also counted. Statistical comparisons were made using ANOVA with Fisher least significant difference test. Baseline testicular parameters (n=15 *gld* and 17 wild-type mice at time 0) showed the *gld* mice to be 15% heavier and to have 6% higher testis weights than wild-type mice. There were 17% more sperm heads/testis in the mutants, which also had 2.2 times the proportion of tubules with >3 apoptotic germ cells. After MEHP treatment, there was histologic evidence of injury (germ cell sloughing, increased tubule lumen, Sertoli vacuolation) in both *gld* and wild-type mice, but only the wild-type mice had histologic findings consistent with germ cell apoptosis (cell shrinkage, chromatin condensation). TUNEL labeling increased in wild-type mice 3–12 hours after MEHP treatment, with the proportion of tubules containing >3 apoptotic germ cells increasing 2.7-fold in that interval **[estimated from a graph]**. By contrast, there was only a 1.7-fold increase in tubules containing >3 apoptotic germ cells in the *gld* mice at 12 hours **[estimated from a graph]**. The proportion of tubules meeting these criteria was statistically higher in wild-type than mutant mice at 6, 12, and 24 hours. The authors concluded that the insensitivity of *gld* mice to MEHP-induced germ cell apoptosis "further underscores the participation of the [f]as system in the regulation of germ cell apoptosis after MEHP-induced Sertoli cell injury."

Strengths/Weaknesses: Strengths of this study are the technical competence with which it was performed and the creativity of the approach to address the experimental question. The data are internally consistent and support the proposed hypothesis. The study used MEHP as a tool to show the involvement of fasL in a lesion.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process in that they appear to define the mechanism by which the germ cells die. These data were not intended to shed any light on the means by which MEHP affects Sertoli cells.

Ichimura et al. *(170)*, support not indicated, evaluated the expression of fas, fasL, and caspase-3, which are 3 apoptosis-associated proteins, as well as TUNEL positivity and electrophoretic DNA laddering in the testes of DDY mice treated with DEHP. DEHP **[purity not specified]** in corn oil was given orally **[gavage assumed]** to 4-week-old male mice as a single dose of 0, 4, 40, 400, or 4000 mg/kg bw (n=3/dose group) **[doses given in the original in mg/g and converted by CERHR to mg/kg by multiplying × 1000]**. Testes were removed 12 hours after the treatment. One testis per animal was fixed in paraformaldehyde and sectioned for immunohistochemistry for fas, fasL, and caspase-3. TUNEL

labeling was evaluated in sections as an indicator of apoptosis. DNA extraction and electrophoresis was performed on homogenates of both testes from 3 other animals, with the density of electrophoretic bands estimated photographically using Adobe PhotoShop® software. FasL positivity was identified in a distribution consistent with Sertoli cell cytoplasmic processes and associated spermatocytes. Fas and caspase-3 co-localized in the middle to outer portion of the epithelium. **[Results are described for the group given DEHP 4000 mg/kg bw.]** TUNEL-positive nuclei were clustered in the middle layer of the epithelium. The maximum number of positive nuclei per section in the 0, 4, 40, 400, and 4000 mg/kg bw groups were 3, 3, 5, 7, and 22, respectively. **[Statistical comparisons were not presented, and numbers of total cells/section were not given.]** DNA laddering was identified in all dose groups, with band density estimated as 2.2 times the control in the high-dose group and 1.1 times the control in the other dose groups. **[Statistical comparisons were not presented.]** The authors interpreted the numeric increase in number of TUNEL-positive nuclei per section as possibly indicating a dose-response relationship, concluding that exposure to DEHP at as little as 40 mg/kg bw might produce an increase in apoptosis in mouse testis. Further, they concluded that the 10% increase in electrophoretic band density in the lower dose groups meant that DEHP might have an effect on mouse testis after a dose as low as 4 mg/kg bw.

Strengths/Weaknesses: The strengths of this study include the technical co-localization approach and the number of doses of DEHP used. Weaknesses include the means of quantifying the density of bands after electrophoresis.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate for the evaluation process, although it does not add significantly to our understanding of the early events in the production of the testis lesion, nor was it intended to. The point of this study was to examine the co-localization of factors involved in cell death after treatment with a compound that would predictably induce that death. The changes at the lowest two doses, although detectable, are probably of little biological consequence.

Giammona et al. *(171)*, supported by NIEHS, examined the role of MEHP in inducing apoptosis in pubertal rodent testicular germ cells and evaluated the role of fas-independent receptors in apoptosis. A single dose of 1000 mg/kg bw MEHP (> 97% purity) in corn oil was administered by gavage to 5-week-old Sprague-Dawley rats, 28-day-old wild-type C57CL/6 mice, or 28-day-old *gld* mice. The *gld* mice express a dysfunctional fasL protein, which cannot bind to the fas receptor to initiate apoptosis. A commercial TUNEL staining kit was used to identify apoptosis. Immunohistochemistry was used in sections of rat testis to identify fas and DR4, DR5, and DR6 receptors, which are fas-independent death receptors in the tumor necrosis factor (TNF) superfamily. Western blot was used to measure caspases, fas receptor, and DR receptors in homogenized mouse and rat testis. **[Statistical analysis methods were not described.]** Following MEHP exposure, apoptosis was found to occur primarily in spermatocytes in both wild-type and *gld* mice. In wild-type mice, germ cell apoptosis was significantly increased from 6 to 48 hours following MEHP exposure. Apoptotic activity peaked between 12 and 24 hours with ~5-fold increases compared to baseline levels. A significant ~2-fold increase in apoptosis compared to baseline levels was observed at 12 and 48 hours following MEHP exposure of *gld* mice. In both groups of mice, apoptotic activity returned to baseline levels by 96 hours following exposure. Western blot analyses revealed that fas expression significantly increased in wild-type mice (~3-fold) at 3 hours following MEHP exposure. There was no significant alteration in fas expression following MEHP exposure in *gld* mice. Expression of DR4, DR5, and DR6 proteins occurred in both wild-type and

gld mice, but MEHP exposure did not increase expression in either strain. DR5 but not DR4 expression significantly increased in Sprague-Dawley rat testes (~1.5-fold) at 1.5 and 3 hours following MEHP exposure. Procaspase 8 cleavage products, downstream receptor-mediated signals of apoptotic pathways, were detected in testes of wild-type and *gld* mice, but expression was significantly increased only in *gld* mice at 6 hours following MEHP exposure. Electrophoretic mobility shift assays demonstrated that DNA binding of NFκB, a receptor-mediated downstream signal possibly involved in cell death or survival, was generally reduced in wild-type mice but upregulated in *gld* mice following MEHP exposure. The study authors concluded that these findings demonstrate that germ-cell related death receptors and downstream signaling products appear to respond to MEHP-induced cell injury; it has not yet been determined if these factors are involved in testicular apoptosis following MEHP exposure.

Strengths/Weaknesses: The creative experimental approach and competence are both strengths of this paper. These studies used MEHP as a tool to explore the cell death mechanism in the rat and mouse testis and were effective in charting the expression of death-related genes in germ cells after exposure to a Sertoli cell toxicant.

Utility (Adequacy) for CERHR Evaluation Process: The data are considered adequate and useful for the evaluation process, mostly by way of identifying those factors involved in germ cell death.

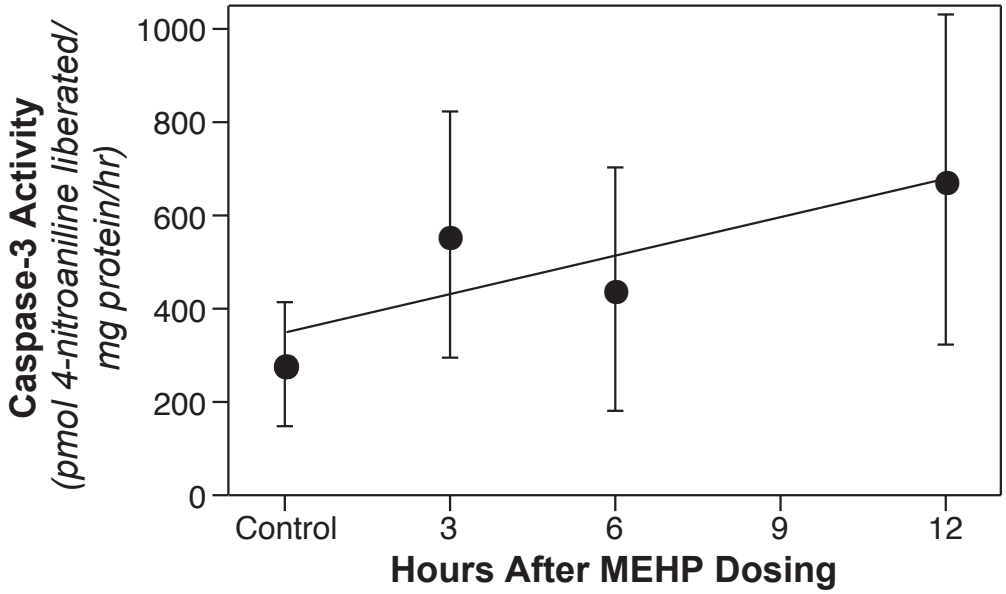
Dalgaard et al. ***(172)***, supported by the Danish Medical Research Council, evaluated the acute effects of MEHP on the testes of 28-day-old Wistar rats in an attempt to infer initial effects on the androgen receptor or intracellular levels of cyclic AMP. A single dose of MEHP **[purity not specified]** was given in corn oil by gavage at 0 or 400 mg/kg bw. Rats given corn oil were killed 2 hours later, and rats given MEHP were killed 3, 6, or 12 hours later (n = 12 per time point). Testis sections were stained with hematoxylin and eosin for light microscopy or were immunostained for vimentin and androgen receptor. Testis sections were also TUNEL stained for determination of apoptosis. Portions of testis and ventral prostate were processed with RT-PCR for quantification of *TRPM-2* gene message, and caspase-3 was estimated in testis samples based on its interaction with a colorimetric substrate. A commercial ligation-mediated PCR kit was used to detect nucleosomal ladders in testicular tissue, and TUNEL staining was performed using a commercial kit. Randomly selected tubules were evaluated for the presence of 0, 1 – 3, or >3 apoptotic germ cells/tubule. Statistical comparisons were made using ANOVA with post hoc Dunnett test. Light microscopy showed germ cell sloughing beginning 6 hours after MEHP treatment. Collapse of vimentin filaments in the Sertoli cell cytoplasm and perinuclear condensation were evident by immunostaining at 3 hours and persisted throughout the study. Androgen receptor staining was not altered by treatment at any time point. *TRPM-2* expression was increased at 3 hours after MEHP treatment but returned to control levels at 6 and 12 hours. Caspase-3 activity was increased by treatment, although the authors noted that there was considerable caspase-3 activity in the control and that the 6-hour value was not statistically different from the control (Figure 8).

There were no detectable treatment effects on number of apoptotic cells per tubule or DNA laddering patterns. The authors concluded that the collapse and perinuclear condensation of vimentin fibers beginning at 3 hours was an early and sensitive MEHP-associated change, occurring at an exposure level 5 times lower than previously reported. The increase in caspase-3 was consistent with MEHP initiation of apoptosis but could not be confirmed by later apoptotic events, perhaps due to the large variability in TUNEL staining and DNA laddering among individuals.

Strengths/Weaknesses: This study attempted to investigate potential early events in the lesion produced by MEHP and repeated some previous findings. A weakness is the inability to explain the discordance between some of the early events the authors report and the absence of expected later events.

Utility (Adequacy) for CERHR Evaluation Process: The data are adequate for the evaluation process and eliminate some possible early events (changes in androgen receptor events or cAMP levels) as possible mediators of MEHP toxicity.

Figure 8. Testicular Caspase-3 Activity After Administration of a Single Oral Dose of MEHP to Wistar Rats



Drawn from Dalgaard et al. (172). Mean ± SD, n = 8.
**Different from control, P < 0.05 by ANOVA with post hoc Dunnett test.*

Park et al. *(173)*, supported by NIH, evaluated the role of zinc depletion as a causative factor in the testicular toxicity of DEHP in young Sprague-Dawley rats. Animals were treated beginning at 25 days of age. Dosing was by gavage with DEHP in corn oil at 0 (n = 15) or 2000 (n = 35) mg/kg bw daily for up to 14 days. Five control animals per time point were decapitated after 2, 7, or 14 days of treatment, and 5 DEHP-treated animals per time point were decapitated after 1, 2, 3, 5, 7, 10, and 14 days of treatment. Testes were removed and weighed, following which 1 testis was frozen for subsequent estimation of *ZnT-1* mRNA by branched DNA signal amplification using a commercial kit. The other testis was fixed in neutral buffered formalin. One-half of the fixed testis was sectioned and stained with hematoxylin and eosin for histologic evaluation by light microscopy and for quantification of apoptosis using the TUNEL assay. An apoptotic index was calculated based on 1000 – 1500 cells per animal, evaluated at 400× for the percentage showing apoptosis. The other half of the testis was digested with nitric acid for zinc determination by atomic absorption spectrophotometry. Comparisons of testicular *ZnT-1* mRNA, zinc, and apoptotic index were made using ANOVA, pooling data from the 3 control time points, which did not differ from one another. Body weight comparisons, made using repeated measures ANOVA, showed a decrease in body weight gain after DEHP exposure beginning on the third day of treatment. Absolute testis weight was decreased in the DEHP group on treatment days

7 and 14 to, respectively, 45 and 25% of control **[estimated from a graph]**. Relative testis weight was also significantly decreased by DEHP at these time points. DEHP treatment was associated with histologic evidence of apoptosis, which occurred early and was most marked by treatment day 3, and necrosis, which was most severe from treatment day 7. Testicular zinc did not change over the 14-day treatment period in control animals but decreased in DEHP-treated animals beginning on treatment day 3 and declining to 73% of the control level by treatment day 14 **[estimated from a graph]**. The testis level of *ZnT-1* mRNA was not altered by DEHP until treatment day 14, when it was about 30% lower than the control level. The authors noted that the reduction in testicular zinc associated with DEHP treatment was consistent with other studies but concluded that this reduction was likely to be secondary to DEHP testicular toxicity rather than a cause of it. They noted that zinc depletion was not demonstrated prior to treatment day 3, whereas histologic abnormalities of the testis were evident within 1 day of initiating treatment. The maintenance of normal *ZnT-1* mRNA until treatment day 14, well after the earliest demonstration of testicular toxicity, suggested that this transporter protein does not play a role in mediating DEHP testicular toxicity.

Strengths/Weaknesses: The technical approach and competence are two strengths of this paper. This study explored the loss of zinc as a causative factor that might be unique to the DEHP lesion. The authors clearly showed a separation between germ cell death and loss (early) and changes in the zinc levels and transporter expression, which happened after the initial germ cell changes. The authors marshal considerable previous data in support of their contention that zinc changes are probably secondary and do not mediate the germ cell death after DEHP exposure.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate for the evaluation process and is useful in providing a summary of, and some recent data on, zinc changes in the testis and their relationship to the DEHP-induced lesion. Collectively, the data suggest that zinc loss is not a causative factor in this lesion.

Kasahara et al. *(174)*, supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology, evaluated the role of oxidative stress in DEHP-associated testicular toxicity in Wistar rats. In the first experiment, males at 4–5 weeks of age were given DEHP **[purity not specified]** in corn oil by gavage at 0, 1000, or 2000 mg/kg bw/day (n=8–15/group) for 7 days. Animals were killed, and body weight and weights of testis, liver, and kidneys were determined. Total glutathione, low-molecular-weight thiols, and ascorbic acid content of testis, liver, and kidney were determined, and in 5 animals/group, glutathione peroxidase and catalase were estimated in these tissues. Body and kidney weights were decreased by about 20% **[estimated from a graph]** by 2000 mg/kg bw/day DEHP. DEHP 1000 mg/kg bw/day increased liver weight by about 50% and decreased testis weight by about 20%, with an additional 50% decrease in testis weight at 2000 mg/kg bw/day **[estimated from a graph]**. Total glutathione, low-molecular-weight thiols, and ascorbic acid were decreased in testis after treatment with DEHP 1000 mg/kg bw/day. Glutathione and low-molecular-weight thiols were increased, and ascorbic acid was unchanged in liver and kidney at both DEHP dose levels. These antioxidants were unchanged in plasma and brain of treated animals **[data not shown]**. Glutathione and catalase were increased in testis at both doses of DEHP and unchanged or decreased in liver and kidney.

Testicular cells were obtained from minced testis, and differential centrifugation was used to obtain predominantly spermatocytes. These cells were incubated with a chemiluminescent probe and digitonin,

following which 5 mM NADH was added to initiate release of reactive oxygen species. The release of reactive oxygen species was measured as increased chemiluminescence that was reported to increase in a time-dependent manner in cells derived from animals treated with DEHP 2000 mg/kg bw/day. **[The data graph is not marked with significant differences.]** The increase in chemiluminescence was prevented by addition of superoxide dismutase, catalase, or sodium azide, suggesting to the authors that the superoxide radical and hydrogen peroxide were the reactive oxygen species responsible for the increased chemiluminescence. Reactive oxygen species generation was also evaluated in Sertoli cell and germ cell cultures obtained from 14-day-old rats. Cultures were grown in the presence of DEHP or MEHP **[the MEHP was synthesized and HPLC-confirmed to be minimally contaminated]** at concentrations of 0, 25, 50, 100, and 200 μM, using fluorescence of 2′,7′-dichlorofluorescein to estimate reactive oxygen species. Fluorescence in germ cells was increased by MEHP in a dose-dependent manner beginning at 50 μM **[19.6 mg/L]**. There was no increase in fluorescence at any concentration of DEHP in germ cells and no increase in fluorescence in Sertoli cells at any concentration of either chemical.

Testicular sections were stained using the TUNEL method 12 hours after administration of DEHP 2000 mg/kg bw **[it is not stated whether these were animals from the first experiment or if this experiment involved new animals given a single dose of DEHP]**. TUNEL-positive cells were reported to be markedly increased in the testes of DEHP-treated animals, and TUNEL-positive cells showed chromatin condensation after propidium iodide staining, confirming apoptosis **[no data were shown for TUNEL staining or propodium iodide staining]**.

In a final experiment, mitochondria were isolated from fresh testes **[age of animals not specified, n=3 indicated in data figure]** and incubated with DEHP or MEHP at concentrations of 0, 2, 10, or 50 μM for 5 minutes. Samples were centrifuged at 8000 *g* for 5 minutes, and supernatants were evaluated using SDS-PAGE and Western blotting for cytochrome *c*. The authors described an increase in cytochrome *c* release from mitochondria exposed to MEHP but not DEHP **[the data figure is not marked with significant differences]**.

The authors concluded that DEHP treatment of rats causes testicular atrophy through generation of reactive oxygen species and reduction in antioxidant enzymes. MEHP-associated apoptosis in germ cells was attributed, at least in part, to release of cytochrome *c* from mitochondria in response to oxidative stress.

Strengths/Weaknesses: The conclusions from these extensive studies are minimally but measurably reduced by the uncertainties noted above. The variety of approaches used for these studies is a clear strength. The report is weakened by the use of whole-testis mitochondria, which is a problem, given that the testis is composed of DEHP-responsive cells and DEHP-resistant cells, at least as far as oxidative response is concerned. Of greater significance are the data indicating that germ cells were affected but Sertoli cells were not; this finding contradicts the existing gestalt in the field, supported by numerous histologic studies that show changes in Sertoli structure prior to germ cell death. The localization data, combined with the time course of apoptosis *in vivo* and active oxygen generation (Figures 7 and 4B, respectively, of the report), make it difficult or impossible to conclude that the oxidative damage was a cause of the germ cell death. Additionally, several of the time-dose points had a great deal of cell death occurring, making this separation impossible. Thus, it is likely that oxidation occurs as part of the cell death process in dying germ cells.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process, but the uncertainties over whether the oxidative damage or the cell death comes first, limit their mechanistic utility. The data showing the presence of oxidative damage are clear and believable; the uncertainties lie with the assertion that this damage produces rather than results from the germ cell death.

Ablake et al. *(175)*, supported by the Japanese Ministry of Education, Science, Sports, and Culture, evaluated the effect of vitamins C and E in accelerating recovery from the testicular toxicity of DEHP in CD mice. Ten-week-old males were given 0 or 2% dietary DEHP (>98% purity) for 16 days, following which all animals were given untreated feed for an additional 50 days. A vitamin-supplemented group was given drinking water containing vitamin C 3.0 mg/mL and vitamin E powder 1.5 mg/mL during the 50-day recovery period. **[Based on the reported estimated mean feed consumption of 5 g/mouse/day and mean fluid consumption of 5 mL/mouse/day, and using the starting mean body weight of 35 g/mouse (estimated from a graph), CERHR calculated a mean DEHP intake during the treatment period of 2857 mg/kg bw/day. During the recovery period, the mean intake of vitamin C was calculated at 429 mg/kg bw/day and the mean intake of vitamin E at 214 mg/kg bw/day.]** Counting the first day of DEHP treatment as day 0, 4–7 animals/treatment group were killed on each of days 0, 3, 5, 7, 9, 12, 15, 25, 35, 45, 55, and 65. **[The Expert Panel notes that time points after day 15 did not include DEHP treatment and included supplemented vitamins C and E.]** Testes were harvested and samples prepared for light microscopy, electron microscopy, TUNEL staining, BrdU incorporation, or histochemical detection of lipid peroxidation. Light microscopy was performed on sections fixed in Bouin fluid, embedded in plastic, and stained with hematoxylin and eosin. Spermatogenic disturbance was graded in at least 100 tubule sections per mouse using a 10-point scale, and the ranks were averaged to produce an overall score per mouse. Electron microscopy, TUNEL staining (using a commercial kit), BrdU incorporation, and detection of lipid peroxidation were performed on samples obtained on days 0, 7, and 15 (before, in the middle, and at the end of the DEHP treatment period). Statistical analysis was by ANOVA.

Body weight decreased during the 16 days of DEHP exposure, recovering to control levels 10 days after DEHP was removed from the feed. Absolute testis weight increased on day 5 of DEHP treatment, and testes appeared grossly edematous. By day 12, testis weight was significantly decreased by DEHP treatment. Testis weight recovered toward control levels 20 days after the cessation of DEHP treatment, although at the end of the experiment on day 65, testis weight had not completely recovered. Testis weight was higher in the group given vitamin-supplementation than in the group not given vitamin supplementation during the recovery period **[although pair-wise statistical comparisons of these 2 groups were not reported]**. Relative testis weight showed a pattern similar to that of absolute testis weight. The mean histologic scores declined during the period of DEHP treatment from 10 to 2 **[estimated from a graph]**. The scores returned toward control levels after cessation of DEHP treatments with the vitamin supplemented group reaching nearly control levels by day 45 and the unsupplemented group reaching nearly control levels by day 55. There were significant differences between supplemented and unsupplemented DEHP-treated animals in histologic score on experiment days 25, 35, and 45. Electron microscopy during the DEHP treatment period showed vacuoles and dark droplets in Sertoli cell cytoplasm in contrast to light microscopic findings, which apparently did not show Sertoli cell damage. Lipid peroxidation was prominent in the basal compartment of the seminiferous epithelium and surrounding interstitial tissues on day 15 of treatment. A few cells with TUNEL staining and BrdU staining were noted on day 15, but these evaluations appeared limited due to the hypocellularity of

tubules in day 15 DEHP-treated animals. The authors concluded that the antioxidant effects of vitamins C and E accelerated the recovery of the testis from DEHP-induced injury.

Strengths/Weaknesses: The novel design of this study is a strength. This study really tests the ability of the testis to recover after DEHP-induced damage, and it is not clear that the DEHP damage is significantly different from any other toxicant-induced lesion. The findings are weakened by the lack of measurement of feed and water intake and the consequent inability to report actual DEHP or vitamin consumption, the lack of data on the levels of these two antioxidants in the mouse chow, and the lack of verification of water levels of these two vitamins (vitamin E is not inherently water-soluble, making this verification more important). The authors concluded that oxidative damage is the mechanism of the DEHP lesion, which is consistent with previous literature they cite, but cannot be a logical conclusion of this paper because they did not test for abrogation of the DEHP lesion by concurrent vitamin and DEHP administration. The DEHP-specific conclusion is also significantly limited by the fact that only a single toxicant was used. Had the authors employed another testicular toxicant that works by a presumably different mechanism, they might have seen a difference, in which case a DEHP-specific conclusion would be warranted. It would also have been very useful for the authors to have evaluated this finding in another species to address its extensibility.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process, as the study was conducted credibly. The data are of use in considering treatments for recovery but are of limited use in determining mechanisms of DEHP toxicity.

Kijima et al. ***(176)***, support not indicated, administered a single oral dose of DEHP (99% purity) in corn oil **[gavage assumed]** to 6-week-old male Crj:CD(SD)IGS rats at dose levels of 0, 20, or 2000 mg/kg bw. This study was conducted to evaluate expression changes in many genes, looking for new leads on mechanism. Rats were killed 3, 6, 24, or 72 hours after dosing and testes were excised. There were 6 rats per dose group and time point. The right testis of each rat was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and either stained with hematoxylin and eosin or used for TUNEL staining. The left testis was frozen at −80ºC for subsequent RNA extraction. RNA extracts were pooled within dose and time groups and analyzed using a microarray consisting of 3060 cDNA probes. Altered regulation was defined as expression at less than half or more than twice the control level for the microarrays. Real-time PCR was used to evaluate mRNA expression for the apoptosis-related genes caspase-2, -3, -6, -8, -9, and -11, *bcl-2*, *bax*, *fas*, and *fasL*, normalized to β-actin. Statistical analysis was performed using the Student *t* test. There were no treatment-related histologic changes, but apoptotic cells were identified by TUNEL staining near the seminiferous tubule basement membrane in testes from rats treated with DEHP 2000 mg/kg bw. At 3 and 6 hours, the apoptosis involved spermatogonia, and at 24 hours the apoptosis also involved spermatocytes and spermatids. At 72 hours after exposure, only spermatogonia and spermatocytes were affected. The proportion of tubules with at least 3 apoptotic cells was increased significantly compared to control at 24 and 72 hours after treatment with DEHP 2000 mg/kg bw, with maximal response **[almost 40%, estimated from a graph]** at 24 hours. The microarray analysis patterns showed only "slight" overlap for the response to the 20 and 2000 mg/kg bw DEHP treatments. The genes up-regulated by DEHP 2000 mg/kg bw included genes involved in apoptosis, cell proliferation, metabolism, stress response, cell adhesion, immune response, DNA repair, and expression sequence tags. The RT-PCR results for apoptosis-related genes showed no effect of treatment with DEHP 20 mg/kg bw except for a transient increase in *bcl-2* at 6 hours. Following treatment with DEHP 2000 mg/kg bw, significant

increases in message were seen as early as 3 hours for capsase-2, caspase-3, caspase-8, caspase-9, *bax*, *fas*, and *fasL*. These increases persisted through the 24-hour time point and, for the caspases, through the 72-hour time point. There was a significant decrease in *bcl-2* message in the 2000 mg/kg bw group at 3, 6, and 24 hours after the dose. The authors concluded that DEHP affected all stages of spermatogenesis with apoptosis peaking at 24 hours. They postulated that the decrease in identification of apoptosis at 72 hours may have represented Sertoli cell phagocytosis of apoptotic spermatogenic cells.

Strengths/Weaknesses: The scientific rigor of the approach and the combined use of histology and focused gene expression methods are strengths. The RT-PCR experiments were not described in adequate detail, and there is an apparent lack of adequate controls. Other weaknesses include lack of replicates and insufficient and inappropriate statistical analyses.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate for the evaluation process, although it does not fundamentally add to our knowledge of the initiating mechanisms of DEHP damage. These data may help identify the subcellular pathways of cell death used by the testis after DEHP exposure.

Kim et al. *(177)*, supported by the Japanese Ministry of Education, Science, Sports, Culture, and Technology, evaluated DEHP-associated changes in testosterone catabolism in 4-week-old male Wistar rats. Animals (6/group) were given DEHP orally **[purity not specified; gavage assumed]** in corn oil at 0, 100, or 1000 mg/kg bw/day for 5 days. Two weeks later, animals were killed and testes and livers were removed and weighed. The tissues were homogenized, and microsomal fractions were isolated by centrifugation. Microsomal protein was evaluated using SDS-PAGE and Western blot for CYP2C11 and CYP3A2. Blood was obtained for estimation of testosterone using a commercial RIA kit. The microsomal fraction was used for quantification of 17β-hydroxylation of androstenedione, 6β-(CYP3A2) and 16α-hydroxylation (CYP2C11) of testosterone, and 3-hydroxylation (CYP3A2) and N-desmethylation (CYP2C11) of diazepam. HPLC was used to quantify the reaction products. 5α-Reductase was quantified in the microsomal fraction **[method specified by reference to a 1979 article]**. Total RNA was extracted from testes, and RT- PCR was used to quantify aromatase (CYP19). Statistical testing was performed using ANOVA **[post hoc testing was not discussed]**. Results are summarized in Table 29. The authors proposed that the observed increase in 5α-reductase may have been a compensatory response to reduced testicular testosterone, and that the reduction in aromatase would be expected to result in decreased estrogen availability. The alterations in CYP isoforms were characterized by the authors as more sensitive markers of DEHP disruption of testicular steroid catabolism than testosterone synthesis from androstenedione.

Strengths/Weaknesses: The use of a recovery period after dosing ended complicates the interpretation of DEHP effects. The absence of histopathology renders the condition of the testis at the end of the experiment uncertain. The absence of measurements of the end products of each of these enzymatic reactions also reduces confidence in the biological importance of the findings. Not having a biological correlate or sequelae to these changes also reduces their value.

Utility (Adequacy) for CERHR Evaluation Process: The study is inadequate for the evaluation process. It adds slightly to our appreciation of testosterone catabolic pathways, although how and why the findings are important, particularly with the two-factored dosing scheme (dosing plus recovery), is not clear.

Table 29. Testosterone Metabolism after DEHP Treatment of 4 Week-old Rats

Parameter	*DEHP dose group (mg/kg bw/day × 5 days)*	
	100	*1000*
Testis weight	↔	↓62%
Serum testosterone	↔	↓34%
Androstenedione 17b-hydroxylation, testis	↔	↔
5α-Reductase, testis [a]	↑21%	↑36%
5α-Reductase, liver	↔	↔
6β-Hydroxylation of testosterone, testis [a]	↑1.2-fold	↑1.3-fold
16α-Hydroxylation of testosterone, testis [a]	↑1.2-fold	↑1.6 fold
3-Hydroxylation of diazepam, testis	↔	↑1.4-fold
N-Desmethylation of diazepam, testis	↔	↑4.6-fold
Microsomal CYP3A2, testis, Western blot	↔	↑2.3-fold
Microsomal CYP2C11, testis, Western blot	↔	↑2.6-fold
Aromatase mRNA expression, testis [b]	↓41%	↓53%

[a] Estimated from graph.

[b] **[Results in text state that aromatase was reduced by 59 and 47% compared to control. Figure suggests that the results should have stated that aromatase was reduced to 59 and 47% of control values.]**

↑,↓ Statistically significant increase, decrease in the parameter; ↔ no significant change in the parameter.

From Kim et al. *(177)*.

Kim et al. ***(178)***, supported by the Japanese Ministry of Education, Science, Sports, Culture, and Technology, performed a follow-up study to evaluate cytosolic phospholipase A2 activity in testes obtained from DEHP-treated rats. Four-week-old male Wistar rats were given DEHP **[purity not specified]** in corn oil at 0, 100, or 1000 mg/kg bw/day for 5 days. Testes were removed 2 weeks after the last treatment, and cytosol and microsomes were isolated by centrifugation of S-9 fractions. Phospholipase A2 activity was measured using a commercial kit. Phospholipase A2, cyclooxygenase-2, 12-lipoxygenase, and CYP4A1 were evaluated using SDS-PAGE with chemiluminescence detection. Arachidonic acid was measured using reverse-phase HPLC. Statistical analysis was by Dunnett test. DEHP treatment did not alter rat body weight, although serum testosterone and testicular weight were decreased by treatment with DEHP 1000 mg/kg bw/day. Cytosolic phospholipase A2 was decreased to 62% of control values by DEHP 1000 mg/kg bw/day. Arachidonic acid concentrations were decreased 18% by DEHP 100 mg/kg bw/day and 24% by DEHP 1000 mg/kg bw/day. The expression of lipooxygenase-12 and CYP4A1 was increased by DEHP **[quantification not given]**, but cyclooxygenase-2 was not altered by treatment. The authors postulated that a DEHP-associated decrease in arachidonic acid may be at least partly responsible for a decrease in testosterone synthesis. The decrease in arachidonic acid may be due to a decrease in formation by phospholipase A2 and a decrease in metabolism by lipooxygenase-12 and CYP4A1. The increase in CYP4A1 may have been due to PPARα activation by DEHP (not measured in this study) and would be expected to increase lipid oxidation. Lipoxygenase activity would also be expected to generate superoxide.

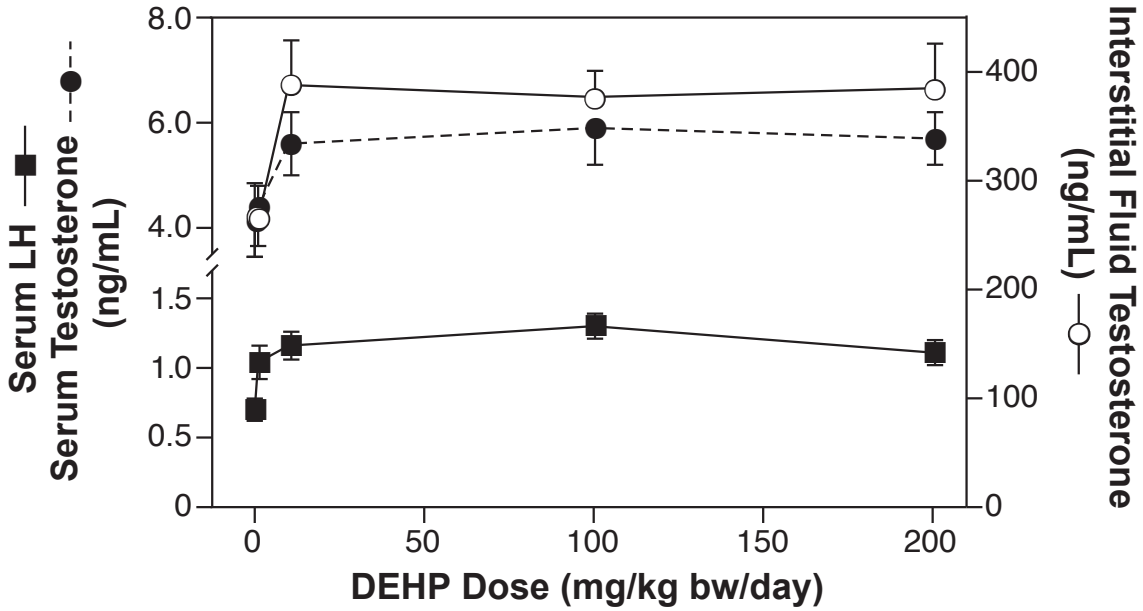
Strengths/Weaknesses: This study used the same confounding dosing-plus-recovery schedule did in the previous study *(177*). However, the evaluation of active fatty acids is a significant plus, as it broadens our appreciation of biological sequelae of DEHP exposure. The dosing sequence renders the results difficult to interpret.

Utility (Adequacy) for CERHR Evaluation Process: The dosing and recovery schedule make this study difficult to interpret and prevent it from being useful in the evaluation process. The focus on phospholipase A2 and arachidonic acid pathways may be a potentially important contribution to mechanistic understanding of DEHP toxicity but is not interpretable at this time based on the treatment schedule.

Akingbemi et al. *(111*), supported by NIEHS, evaluated the effect of DEHP on Leydig cell function in male Long-Evans rats exposed during pregnancy, lactation, or post-weaning stages (pregnancy and lactation results are presented in Section 3.2.1.1). Prepubertal rats were exposed to DEHP (>99% purity) on PND 21–34, 35–48, or 21–48, and young adult rats were exposed on PND 62–89. DEHP was administered by gavage in corn oil at 0, 1, 10, 100, or 200 mg/kg bw/day (n = 10/dose group). Animals were decapitated within 24 hours of the final dose, and trunk blood was collected for measurement of LH and testosterone by RIA. Testes and seminal vesicles were weighed, and testicular interstitial fluid was collected for measurement of fluid testosterone by RIA. Testicular histology was evaluated. Leydig cell cultures were prepared from testes by Percoll density gradient preceded in 49- and 90-day-old rats by centrifugal elutriation. The resulting Leydig cell preparations were 95–97% pure, as evaluated by staining for 17-hydroxysteroid dehydrogenase. Cultures were evaluated after 3 hours incubation with and without a maximally stimulating concentration of ovine LH. Testosterone was measured in the medium. The activity of different enzymes in the testosterone biosynthesis pathway were evaluated by incubating Leydig cells for 3 hours with saturating concentrations of substrate for the enzyme of interest: 22R-hydroxycholesterol for P450-cholesterol side-chain cleavage enzyme, pregnenolone for 3β-hydroxysteroid dehydrogenase, progesterone for P450-17α hydroxylase/17,20-lyase, and androstenedione for 17β-hydroxysteroid dehydrogenase. The steroid products were measured by HPLC with ultraviolet detection. Statistical analysis was by ANOVA and Duncan multiple range test.

There were no treatment-related effects on body weight gain or feed consumption. Treatment of prepubertal rats on PND 21–34 or PND 35–48 did not produce alterations of serum LH or testosterone, but longer treatment (on PND 21–48) produced a dose-related increase in serum LH and testosterone and in interstitial fluid testosterone that was statistically significant at a DEHP dose level of 10 mg/kg bw/day (Figure 9). Leydig cells isolated from rats after DEHP treatment on PND 21–34 showed a decrease in basal and LH-stimulated testosterone production *in vitro* (LOAEL 100 mg/kg bw/day). Leydig cells isolated from rats after DEHP treatment on PND 35–48 displayed decreased basal and LH-stimulated testosterone production *in vitro* (LOAEL 10 mg/kg bw/day) and decreased activities of enzymes in the testosterone synthesis pathway. All tested enzyme activities were affected, with the most sensitive being 17β-hydroxysteroid dehydrogenase (reduced 74% at 10 mg/kg bw/day compared to control; other enzyme activities were significantly reduced at DEHP dose levels of 100 or 200 mg/kg bw/day). Leydig cells isolated from prepubertal rats that had been treated on PND 21–48 showed an increase in basal and LH-stimulated testosterone production with a LOAEL of 10 mg/kg bw/day. There was no effect of DEHP treatment in adults at any tested dose on serum testosterone or LH or on *in vitro* Leydig cell steroidogenesis **[data not shown]**. There were no effects of any treatment on testicular histology.

Figure 9. Effect of DEHP Treatment in Male Rats on PND 21–48.



LOAEL = 10 mg/kg bw/day by pair-wise testing (drawn from data presented by Akingbemi et al. (111)).

The authors postulated that the decrease in *in vitro* steroidogenesis after treatment of rats on PND 35–48 without a decrease in evidence of *in vivo* testosterone synthesis may have represented enzyme inhibition that was not great enough to alter *in vivo* serum testosterone levels; i.e., the animals could compensate for the treatment effect on synthesis. The authors pointed out that the substrates were provided for the Leydig cell culture experiments in higher amounts than may typically be available *in vivo*. The authors believed that the increased testosterone and LH serum levels and the increase in *in vitro* testosterone biosynthetic ability of Leydig cells after 28 days of DEHP treatment represented a compensatory mechanism, with a disruption of the negative feedback mechanism that typically maintains homeostatic testosterone levels. They identified 1 mg/kg bw/day as a NOAEL in their experiments and 10 mg/kg bw/day as a LOAEL.

Strengths/Weaknesses: This study benefits from intelligent study design, technical expertise, and appropriate dosing intervals and quantities. Some of the groups have fewer animals than might be optimal. Appropriate statistics is a strength.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate and useful for the evaluation process because it helps identify a vulnerable window of exposure and during that more-vulnerable period, does a good job of parsing the dose-response curve. The study effectively separates inherent effects from compensatory change by the smart use of ex vivo cultures and identifies critical enzymatic steps in steroidogenesis.

Akingbemi et al. *(179)*, supported by NIEHS, evaluated Leydig cell hyperplasia and steroid production in response to DEHP treatment of male Long-Evans rats as a follow-up to the previous study *(111)* in which treatments were given on PND 21–48. Three experiments were described in the current paper, with comparisons performed using ANOVA and post hoc Dunnett multiple range test. In the first experiment, animals were gavaged on PND 21 to PND 90 or to PND 120 with DEHP **[purity**

not specified] in corn oil at 0, 10, or 100 mg/kg bw/day (n = 10/group). These treatments caused no significant change in body weight or paired testis weight at 120 days of age. At the end of the treatment periods, serum LH and testosterone were measured by RIA, and Leydig cells were harvested by collagenase digestion and Percoll density centrifugation **[methods obtained from the earlier paper *(111)*)]**. The Leydig cells were cultured for 3 hours with and without maximally stimulating concentrations of ovine LH. Medium testosterone was measured by RIA and normalized to cell number. In the animals treated until PND 90, serum LH and testosterone were increased in both DEHP dose groups compared to controls, and testosterone production in Leydig cell culture was significantly reduced, both with and without added LH. On PND 120, the increase in serum LH and testosterone and the decrease in basal and stimulated testosterone production in culture were identified only in the animals exposed to DEHP 100 mg/kg bw/day. The study authors believed the results were consistent with Leydig cell hyperplasia because serum testosterone was increased in the face of a reduction in testosterone production per Leydig cell.

In the second experiment, the possibility of Leydig cell hyperplasia was evaluated using markers of cell division (PCNA, cyclins D3 and G1, and p53, estimated by RT-PCR of steady-state mRNA levels in Leydig cells), incorporation of tritiated thymidine in cultured Leydig cells, and counting of Leydig cells in 10 sections/testis. The treatment regimens were the same as in the first experiment. mRNA levels were increased in both DEHP exposure groups for PCNA and both cyclins and in the high-dose group for p53. Leydig cell numbers were also increased in both DEHP dose groups. **[Data for these outcomes were only described for the animals treated until PND 90.]** Tritiated thymidine incorporation was increased in both DEHP exposure groups in animals treated until PND 120. **[Data not described or shown for animals treated until PND 90.]**

The third experiment evaluated the hypothesis that a DEHP-associated increase in LH induced estrogen synthesis. Male rats were gavaged with DEHP in corn oil at 0, 10, or 100 mg/kg bw/day from PND 21 to PND 48 or 90 at which time serum estradiol was measured by RIA. Leydig cells were cultured for 3 hours with or without exogenous LH, and estradiol in the medium was measured and normalized for cell number. Aromatase mRNA was measured in Leydig cells. Serum estradiol was increased on PND 48 in rats in both DEHP exposure groups. Basal estradiol production in cultured Leydig cells was increased in the 100 mg/kg bw group, and LH-stimulated estradiol production was increased in both DEHP dose groups on PND 48. Aromatase mRNA was also increased in Leydig cells from both DEHP-treated groups. On PND 90, there was no significant difference between control and DEHP-treated animals in serum estradiol level, in basal or LH-stimulated estradiol production in culture, or in aromatase mRNA. MEHP but not DEHP was detectable in serum by HPLC on PND 48 and 90, suggesting that MEHP mediated the testicular effects of DEHP treatment. Serum MEHP was more than 6-fold lower **[estimated from a graph]** on PND 90 than on PND 48.

The authors concluded that DEHP increases Leydig cell populations associated with chronically increased LH and testosterone levels, and that a decrease in testosterone and increase in estradiol synthesis (per cell) were consistent with induction of aromatase activity in Leydig cells. They further proposed that the increase in estradiol and LH could act together to increase cyclin proteins and thereby increase Leydig cell numbers. They concluded that the Leydig cell hyperplasia identified in their study might have implications in the production of testicular neoplasms, and that chronic exposure to DEHP had anti-androgenic and estrogenic activity.

Strengths/Weaknesses: The multiple technical approaches and good design of these studies are buttressed by the sequential hypothesis-testing approach to produce an excellent paper. This report extends our knowledge about the hormonal perturbations to include hyper-estrogenization, and deepens our appreciation of the complexity of this syndrome.

Utility (Adequacy) for CERHR Evaluation Process: The data are adequate for the evaluation process and increase the useable data-set by adding estrogen-induced effects to the spectrum of sequelae. The use of 100 mg/kg bw/day is not meant to plumb a NOAEL but to employ an effective dose level. This paper suggests that elevated levels of estradiol might be present in some of these animal models and human populations and should be considered in further evaluations.

Rasoulpour and Boekelheide *(180)*, support not indicated, evaluated MEHP activation of the transcription factor NF-κB, which is a mediator of the Fas signaling pathway. Fischer 344 rats were treated when 28 days old with single gavage doses of MEHP **[purity not given]** in corn oil at 0 or 1000 mg/kg bw. Testes were excised and processed 1, 3, 6, and 12 hours after treatment (n = 3 or 4 rats/treatment/time point). Sections were immunostained for the NF-κB subunits p50, p65, c-Rel, and Rel-B. TUNEL staining was performed on additional sections and seminiferous tubules evaluated for the presence of 0, 1 – 3, or > 3 apoptotic cells/tubule section. Nuclear protein was extracted from homogenized testes and used to assess NF-κB-binding by electrophoretic mobility shift assay. Statistical analysis was by ANOVA with Bonferroni correction.

Within 1 hour of MEHP treatment, p50 staining increased in spermatocytes. Distribution of the p65 subunit (Sertoli cells, Leydig cells, spermatogonia, and spermatids) was not altered by MEHP treatment at 1 hour, but at 3 hours some spermatocytes showed staining and Sertoli cell staining was intense. At 6 hours, all germ cells were positive for p50, p65, and c-Rel, and at 12 hours the interstitium was also involved. Rel-B staining was not influenced by treatment at any time point. TUNEL staining was decreased by MEHP at 6 hours post-treatment, but was increased at 12 hours with 37.4% of tubules showing >3 apoptotic cells compared to 4.1% of control tubules. Increased NF-κB-binding activity was shown by electrophoretic mobility shift, which peaked 1 hour after treatment, with p50 and c-Rel identified as the main subunits activated.

The authors considered the changes in NF-κB subunit localization in spermatocytes to be consistent with the sensitivity of these cells to MEHP-induced damage. They hypothesized that NF-κB through its anti-apoptotic effects may play a protective role in the MEHP-exposed testis, with a decrease in apoptosis at 6 hours that subsequently is overwhelmed.

Strengths/Weaknesses: This paper examined the testicular response of NF-κB to phthalate exposure with a focus on examining activation of this system as part of the injury response. Strengths include appropriate techniques for assessing a whole-testis response, including use of immunohistochemistry and electrophoretic mobility shift assay. The biological relevance of these proteins and their involvement in cell death is a strength as are the many time points examined. Weaknesses include the use of a single high dose of exposure and the uncertainty of how these changes relate to cell death since all cells showed the changes whether or not they were killed by phthalates.

Utility (Adequacy) for CERHR Evaluation Process: This paper may add to the mechanistic under-

standing of the response to phthalate treatment but does not contribute to the evaluation of risk in the CERHR process.

Awal et al. *(181)*, supported by the Japan Society for the Promotion of Sciences and the Japanese Ministry of Health, Labor, and Welfare, evaluated the testicular effects of MEHP given to pre-pubertal guinea pigs. The animals were treated at 5 weeks of age with a single gavage dose of MEHP **[purity not given]** in corn oil at 0 or 2000 mg/kg bw. Animals were killed 3, 6, or 9 hours after treatment for evaluation of testes by light and electron microscopy. **[The number of animals per group was not explicitly stated. If group sizes were equal, there were 2 guinea pigs/treatment/time point.]** For light microscopy, testes were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections were stained with hematoxylin and eosin or with hematoxylin and periodic acid Schiff stain. TUNEL staining was used for evaluation of apoptotic spermatogenic cells, expressed as a percentage of total spermatogenic cells, and analyzed with ANOVA and Fisher least significant difference test.

At 3 hours post-dosing, testicular morphology was described as normal by light microscopy, although the authors noted sloughed spermatogenic cells in the efferent ductules. There was "progressive detachment" of spermatogenic cells between 3 and 9 hours **[not otherwise quantified]**. Electron microscopy showed degenerative vacuolated Sertoli cells **[time course not described]**. TUNEL staining showed a time-dependent increase in the percentage of apoptotic spermatogenic cells beginning at 3 hours and reaching a value of ~9% at 9 hours **[estimated from a graph; control value ~0.7%]**.

The authors concluded that MEHP causes testicular toxicity in guinea pigs manifested as an increase in germ cell apoptosis mediated by direct effects on the apoptotic pathway or through altered Sertoli cell function.

Strengths/Weaknesses: This paper essentially replicates work from the early 1980s by re-demonstrating the known sensitivity of the guinea pig to phthalate exposure, and shows that germ cells rapidly die by apoptosis after high-dose exposure. The low number of animals and high dose level are weaknesses.

Utility (Adequacy) for CERHR Evaluation Process: Because of the single high dose used and because these studies have been done previously and incorporated into the first evaluation of DEHP, this paper adds nothing to the current evaluation.

Ljungvall et al. *(182)*, supported by the Swedish Environmental Protection Agency, treated pre-pubertal male pigs with DEHP (99.6% pure) 50 mg/kg bw/dose, estradiol benzoate 0.25 mg/kg bw/dose, or peanut oil vehicle. Treatments were given twice/week IM for 5 weeks, beginning 1 week after weaning, at 6 weeks of age **[DEHP 50 mg/kg bw/dose twice/week would be 100 mg/kg bw/week or about 14 mg/kg bw/day; the Expert Panel notes that this calculated daily dose after twice-weekly injection may not be comparable to a similar oral daily dose. The authors justified the IM route as the most practical route for treating young pigs.]** Evaluations were planned for 5 days after the last treatment (the immediately evaluated group) and for 4.5 months after the last treatment period (the delayed-evaluation group). The immediately evaluated group consisted of 1 male pig from each of 4 litters assigned to each treatment (4 male pigs/treatment group). The delayed-evaluation group consisted of 5 male pigs from 4 litters assigned to DEHP, an additional 5 male pigs from 4 litters assigned to the vehicle control, and 3 male pigs from 3 litters assigned to estradiol benzoate. In the immediately evaluated animals, blood

was sampled weekly during treatment for plasma testosterone, 17β-estradiol, and LH by RIA. Five days after the last treatment, immediately evaluated animals were necropsied and relative testicular weight was recorded. In the delayed-evaluation group, blood was collected for the plasma testosterone, 17β-estradiol, and LH before the first treatment, after the last treatment, and at the time of slaughter at 7.5 months of age. Necropsy was performed, and relative testicular and seminal vesicle weights were recorded. Hematoxylin and eosin-stained testis sections were evaluated by light microscopy, and immunohistochemistry for vimentin was performed. Digitized images of testis sections were evaluated for Leydig cell area and tubule diameter. Leydig cell area relative to tubule area, the proportion of testicular morphologic abnormalities, and the proportion of tubules in Stage VIII were compared between groups with the Wilcoxon-Mann-Whitney test. ANOVA was used for other comparisons.

In the immediately evaluated group, there were no significant differences in plasma testosterone concentration in DEHP- or estradiol benzoate-treated animals compared to control. At 7.5 months of age, DEHP treatment was associated with an increase in plasma testosterone, and estradiol benzoate treatment was associated with a decrease in plasma testosterone compared to control. There were no DEHP-related effects on plasma estradiol. Plasma LH was described by the authors as tending to be higher after 2 weeks of DEHP treatment ($P = 0.08$) in the immediately evaluated group. In the delayed-evaluation group, LH was lower in the DEHP group prior to treatment compared to the control group. There were no significant effects of DEHP treatment on relative weight of the testis or seminal vesicles. There were no significant differences in testis morphology in the DEHP-treated animals, although Leydig cell area relative to tubule area was increased by DEHP to 0.45 ± 0.026 (SEM) from a control value of 0.35 ± 0.033. The authors noted a "weak indication that spermatogenesis was affected by DEHP" based on an increase in proportion of Stage VIII tubules ($P = 0.1$). They indicated that although they had characterized DEHP toxicokinetics in male pigs after oral administration *(183)*, they did not have data on metabolism or kinetics after IM administration in male pigs. They noted that in rats, parenterally administered DEHP is metabolized to MEHP. The authors concluded that DEHP administered before puberty in male pigs increased plasma testosterone and testosterone-producing cells 4.5 months after the last exposure.

Strengths/Weaknesses: The variety of appropriate measures and time points and the examination of a new species make this a useful and relevant study. The IM injection route complicates the toxicokinetic profile somewhat but does not fundamentally detract from the utility of this study. The low numbers of animals used, while logistically justifiable, complicates the interpretation and reduces our confidence in the veracity of the data. The twice-weekly administration, in the absence of blood level measures of MEHP, also weakens the direct comparison of these data to other daily dosed lab animal data.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate and useful for the evaluation process in showing Leydig cell hypertrophy in young swine (in parallel with that shown in rats in other studies). The serum hormone changes previously documented in rats do not appear as consistently in these swine, perhaps because of the low numbers and twice-weekly dosing schedule. These data are sufficient to show that twice-weekly dosing with 50 mg/kg to young swine can cause Leydig cell hypertrophy 4.5 months after the end of exposure.

Ljungvall et al. *(184)*, supported by the Swedish Environmental Protection Agency, treated post-weaning male piglets with DEHP and evaluated post-pubertal sexual function. The pigs were Swedish Yorkshire × Swedish Landrace crossbreeds. Two male siblings in each of 10 litters were selected

to receive DEHP (99.5% pure) 300 mg/kg bw/dose or water. Treatments were by oral gavage and were given 3 times/week for 4 weeks beginning at weaning (3 weeks of age). During the exposure period, blood samples were taken weekly for measurement of testosterone, 17β-estradiol, and LH. Mating behavior was evaluated with an artificial sow when males were 6–9 months of age. Mating trials occurred twice/week for 14 weeks. Two pigs died of unrelated illnesses, and their siblings were removed from the trial, resulting in 8 sibling pairs for the mating evaluations. At 9 months of age, the male pigs had IV catheters placed while under anesthesia, following which the GnRH agonist buserelin was administered. Blood samples were drawn periodically from the catheters from 1 hour before administration of buserelin until 12 hours thereafter, and the response of LH and testosterone to the GnRH agonist was determined. Statistical analysis included paired *t*-tests, chi-squared, and the Wilcoxon nonparametric test.

During the second week of exposure to DEHP, control animals showed ~44% reduction in plasma LH **[estimated from a graph]** compared to pretreatment and week 1 values. DEHP-treated animals did not demonstrate the week 2 LH decrease. There were no other differences between DEHP-treated and control pigs in plasma LH, testosterone, or 17β-estradiol during the treatment period. The response of plasma LH and testosterone to buserelin treatment was similar in DEHP-treated and control pigs except for 1 time point of 36 (45 minutes), when plasma LH was lower in DEHP-treated animals than in controls. Sexual behavior, evaluated by number of trials to first mount, number of trials to first ejaculation, time to first mount, proportion of trials with mount, and proportion of trials with ejaculation, did not differ by treatment group.

The authors concluded that decreased LH response to GnRH stimulation occurred, although the significance of the finding was unknown. The hormonal effects of DEHP in young pigs were described as "minor," and the lack of effect on mating behavior was considered possibly due to species differences between rats and pigs. **[The Expert Panel disagrees with the authors' conclusions that the decreased LH at 45 minutes was meaningful, treatment-related, or represented a "lasting effect on the hypothalamus-pituitary-gonadal axis." Given that only 1 (the 45 min) time point out of 36 was significant and there was no supporting trend for depressed LH at other time points, this single change appears to be spurious. In addition, if each time point was analyzed with $\alpha = 0.05$, approximately 2 false positives out of the 36 evaluations would be expected. This single findings falls within the expected false positive rate.]**

Strengths/Weaknesses: Strengths include the use of another species, although no rationale was given for this particular species, and multiple timepoints for analysis. There was excellent statistical design and control for litter and sibling effect. A split-litter design was used, balancing littermates across the treatment and control groups. In addition, since the piglets and not the sows were dosed post-weaning, the individual piglet was the appropriate unit of analysis. Post-weaning dosing also diminished maternal and litter influence. The sample size was sufficient given the use of repeated measures and the inclusion of an animal factor in the statistical designs to account for repeated measure on the same animal. A robust evaluation of LH and testosterone was conducted following GnRH stimulation, utilizing repetitive blood sampling at short intervals. Weaknesses include the single dose level used. This dose did not produce significant adverse effects. It is not clear if the pig is a more or less relevant species (than the rat) for evaluating effects on human reproductive hormones and sexual behavior following exposure to DEHP.

Utility (Adequacy) for CERHR Evaluation Process: These data show that exposure to DEHP at 300 mg/kg, 3 times per week, between 3 and 7 weeks of age, did not produce adverse effects on LH, testosterone, or mating behavior of male pigs. Because only a single dose level was used, the data cannot be used in models to estimate NOAELs/LOAELs. It is not clear that the conclusion of the authors that different species (rats versus pigs) may respond differently to perinatal DEHP exposure is supported by their findings. Additionally, statements in the Discussion ("noted lower concentrations of LH in the DEHP-treated animals after GnRH stimulation.") appear to contradict the data in Figure 2 of the paper. These data are of limited utility in the evaluation process.

4.2.3 Male and Female

Kim et al. *(185)*, support not indicated, evaluated the effects of DEHP (99% pure) on gonad development and serum vitellogenin in Japanese medaka. DEHP was dissolved in acetone and added to aquarium water at up to 250 µL/L; controls received acetone. Serum vitellogenin was assessed after exposure for 5 days to DEHP-containing water at 0, 10, 50, and 100 µg/L, n = 10 fish/concentration **[both males and females; ratio not indicated]**. Serum was evaluated using SDS-PAGE, and a band at 200 kDa was taken to represent vitellogenin based on the finding that 17β-estradiol produced a prominent band in this location. The heaviness of the band on the gel was evaluated with the naked eye to compare the effects of treatments on vitellogenin content of serum, with the finding that DEHP exposure appeared to decrease the heaviness of the band in female fish **[the text says that this effect occurred at 1 µg/L, which was not listed in the Methods as 1 of the concentrations used in this experiment]**. A chronic experiment was performed using DEHP concentrations of 0, 1, 10, and 50 µg/L with exposure from 1 or 2 days after hatching until 3 months of age. Serum vitellogenin was evaluated in 1 male and 1 female per exposure level. The remaining fish were fixed in neutral buffered formalin, weighed, and measured; gonads were removed for weighing and histologic evaluation. After the chronic exposure, vitellogenin was again described by visual inspection as reduced in females **[effect level not given]**. Mean weight and length were not altered by treatment. Relative testicular weight and testicular histology were not altered by treatment, but relative ovarian weight was reduced at DEHP concentrations of 10 and 50 µg/L **[to 33 and 38% of control values]**. Histological evaluation revealed impaired maturation of oocytes, with the achievement of yolk deposition in 54% of control females, 37% of females exposed to DEHP 1 mg/L, no females exposed to DEHP 10 µg/L, and in 22% of females exposed to DEHP 50 µg/L. The authors concluded that the decrease in serum vitellogenin and the impairment of oocyte maturation associated with DEHP exposure were consistent with an anti-estrogenic effect of DEHP. **[Actual concentrations of DEHP were noted to be 88% of target at 0 hours and 57% of target at 72 hours. The authors calculated that their protocol of changing the DEHP solutions every 48–72 hours would have resulted in exposure to 73–78% of the nominal concentrations as a time-weighted average.]**

Strengths/Weaknesses: The strengths of this paper include the measurement of DEHP in the aquarium water. The paper is weakened both by the fact that vitellogenin was not determined by Western blotting or some other method of positive identification and by the extremely low numbers of animals used to collect the vitellogenin data after "chronic" exposure (1/group). A repeated *t*-test was not the correct statistic to use to compare multiple dose groups to controls. Consequently, it is difficult to believe the significance values for the relative gonadal weights, especially given that the mean and SD for the controls are the same approximate size (making a reduction by approximately 67% in the middle-dose group

of dubious significance). The bar graph representation of the oocyte progression data was useful. The non-dose-related changes increase our uncertainty of the veracity and replicability of these findings.

Utility (Adequacy) for CERHR Evaluation Process: This paper is adequate to show that water-based exposure to ~7 and ~35 μg/L DEHP was effective in reducing ovarian development, although the actual degree of this reduction is as difficult to ascertain. The relevance to mammalian reproductive processes of these data is uncertain.

Mitsubishi Chemical Safety Institute, Ltd. *(92)*, in an unpublished report, described a 65-week oral-dose toxicity study of DEHP in marmosets. **[Data from this report also appeared in an abstract *(186)*.]** The study was sponsored by the Japan Plasticizer Industry Association. DEHP (99.6% purity) was administered by gavage in corn oil to juvenile marmosets beginning at 90–115 days of age at dose levels of 0, 100, 500, and 2500 mg/kg bw/day (n=9 males and 6 females/dose group). The rationale for the age at onset of treatment was to start treatment as early "as technically possible." The treatment period was designed to extend to the age of sexual maturation at about 18 months. Wasting and death occurred in 1–3 males/group but was not treatment related, and these animals were replaced. Blood was drawn every 13 weeks for hematology, chemistry, and determination by RIA of testosterone, estradiol, triiodothyronine, and thyroxine. Animals were killed 1 day after the final DEHP dose. Six males and all 6 females in each dose group underwent gonad removal. The left testis and epididymis were fixed in Bouin fluid for light microscopy. The right testis and epididymis were frozen for sperm counting, and a portion of the frozen right testis was used for determination of zinc, sorbitol dehydrogenase, γ-glutamyl transpeptidase, total glutathione, glutathione-*S*-transferase, and glutathione peroxidase. Left ovaries were frozen for histochemistry for 3β-hydroxysteroid dehydrogenase determination. Livers from both sexes were analyzed for enzyme levels or activities. An additional 3 males per dose group were perfused with glutaraldehyde, and testes were used for 3β-hydroxysteroid dehydrogenase histochemistry and for electron microscopy. Data were analyzed using ANOVA with post hoc Dunnett or Scheffé test or Kruskal-Wallis test with post hoc Dunnett rank-sum or Scheffé test.

In analyzing their data, the study investigators excluded certain animals from inclusion in summary tables because they were considered to be "growing." **[Although exclusion was reportedly related to low body weight, this criterion appears to have been applied somewhat arbitrarily without a clearly stated rationale. Although it is recognized that sexually immature animals may reasonably be excluded from the summary analysis of certain parameters (i.e., sperm counts), the lack of a clear rationale makes the interpretation and independent assessment of the conclusions difficult.]** According to the summary tables reported, there were no treatment-related alterations in hematology, blood chemistry, or blood hormones. Body weights were not affected by treatment. Organ weights were not affected except for ovarian and uterine weights, which were significantly increased at DEHP dose levels of 500 and (for ovaries) 2500 mg/kg bw/day (Table 30). These increases in ovarian and uterine weight were associated with elevations, relative to controls, in serum 17β-estradiol at DEHP dose levels of 500 and 2500 mg/kg bw/day **[suggesting an accelerated onset of puberty with exposure]**. Mean serum testosterone levels were highly variable, but the data suggested the possibility of a delay in the onset of puberty with increasing DEHP dose. Testicular enzymes were not altered by treatment, although there was a 16–21% decrease in testicular zinc at the 100 and 500 mg/kg bw/day dose levels. There were no alterations in sperm counts; however, 1 animal in each of the DEHP groups was omitted from the analysis as having exceptional values due to these animals being diagnosed as "growing."

Hepatic cytochrome P450 content and testosterone 6β-hydroxylation were significantly increased in females at DEHP 500 mg/kg bw/day, but hepatic peroxidase enzymes were not altered by treatment. There were no gonadal histopathologic findings by light or electron microscopy that were attributable to DEHP treatment, although small growing animals had testicular findings consistent with immaturity. Degenerative testis changes similar to those described elsewhere for rodents were not identified in marmosets. Immunohistochemistry findings were not altered by treatment.

Table 30. Ovary and Uterine Weight Findings in a Marmoset 65-Week DEHP Feeding Study

Organ Weight[b]		*Dose Level (mg/kg bw/day)*			*Benchmark Dose*[a] *(mg/kg bw/day)*			
		100	*500*	*2500*	BMD_{10}	$BMDL_{10}$	$BMD_{1\ SD}$	$BMDL_{1\ SD}$
Ovary	Absolute	100	180*	169*	507	259	2063	1196
	Relative	106	167*	162*	572	303	1999	1173
Uterine	Absolute	106	188*	168	562	258	2545	1356
	Relative	100	167*	150	677	296	2759	1374

[a] Calculated by CERHR. See Table 22 for definitions and a discussion of the use of benchmark dose in this report.
[b] Data presented as percent of control.
*Significantly different from control, $P < 0.05$. From Mitsubishi Chemical Safety Institute, Ltd. *(92)*.

The authors concluded that in spite of demonstration of absorption of the compound, as manifested by adaptive hepatic changes, DEHP in marmosets did not exert testicular toxicity at a dose level as high as 2500 mg/kg bw/day. The authors suggested, based on an accompanying pharmacokinetic study (reviewed in Section 2.1), that the most likely explanation for the lack of testicular toxicity of DEHP in marmosets as opposed to rodents was the limited accumulation of DEHP and its metabolites in the marmoset testis. **[The Expert Panel noted the paper by Li et al. *(187)*, which evaluates the marmoset as a model for reproductive studies. Li et al. described the relatively high free levels of steroids in marmosets, which result in a generalized end-organ steroid insensitivity syndrome and differ from steroid levels in rats, humans, and other Old World primates (to which humans are most closely related). This insensitivity would be unremarkable except in the context of a toxicant the mechanism of action of which involves reduced steroid levels and downstream effects flowing from the absence of that steroid. An animal model that does not need gonadal steroids to the degree that humans do would not be an optimal model for this situation. In addition, Li et al. noted the short gut transit time (4–8 hours) and the propensity to diarrhea, which would combine to limit absorption and keep circulating levels of an orally dosed compound low. Another factor is that common marmosets require high levels of dietary vitamin C in their diet, and vitamin C is protective against the testicular effects of DEHP in rats and mice *(175, 188)*. In addition, the lack of LH (and concomitant use of chorionic gonadotropin to fulfill the general functions of LH) is another, arguably less serious, difference between marmosets and humans.]**

Strengths/Weaknesses: Strengths of this study are the use of a primate species, the length of exposure, the determination of DEHP and MEHP levels in numerous tissues, and the numerous endpoints relevant to the reproductive system. Limitations include concerns about the husbandry practices, because 1–3 animals per group had to be replaced during the treatment period due to "wasting;" the marked variability within groups for some of the endpoints (for example, serum testosterone); failure to

collect all appropriate data (for example, testis weights in the Group 2 animals); a somewhat arbitrary exclusion of certain animals from consideration in summary tables because of apparent immaturity; and a general lack of transparency in the study design and the intended use of the animals. Additional weaknesses are those of marmosets as noted above, which significantly limit our reliance on this species as a surrogate for humans.

Utility (Adequacy) for CERHR Evaluation Process: These data are useful for the evaluation process and suggest that marmosets, exposed to DEHP at up to 2500 mg/kg bw/day from ~3 months of age until ~18 months of age (sexual maturity), had no discernable effect on the testis, either at the ultrastructural, histochemical, or gross functional (sperm count) level, although there are significant limitations to the confidence that can be placed on these male data. In addition, it is important to note apparently unique features of endocrine responsivity in the developing male marmoset that limit the relevance of these data to the evaluation process. The data are adequate to show an effect on ovary and uterine weight in these young adults at necropsy at administered doses of 500 and (for ovary) 2500 mg/kg bw/day associated with increases in serum 17β-estradiol and suggestive of an earlier onset of puberty in the 2 high-dose groups relative to the control and low-dose groups.

Schilling et al. *(151)*, sponsored by the European Council for Plasticizers and Intermediates (a sector group of CEFIC, the European Chemical Industry Council), performed a 2-generation reproductive toxicity study of DEHP in Wistar rats. DEHP (99.7% pure) was administered in feed at 0, 1000, 3000, and 9000 ppm, resulting in estimated DEHP intakes of 0, 113, 340, and 1088 mg/kg bw/day. F_0 male and female animals were 36–38 days old when they were placed on test (n=25/group). Animals were mated at least 73 days after the beginning of treatment. Females littered and raised their own pups. On PND 4, litters were standardized to 8 (4 males and 4 females where possible). Pups were weaned on PND 21. Treated feed was provided throughout the gestation and lactation period, and pups were weaned to the diet assigned to their parents. During the rearing period, 25 males and 25 females per dose group (1/sex/litter where possible) were assigned to be F_1 parents. Mating (non-sibling) occurred at least 75 days after assignment. F_2 litters were standardized on PND 4 and weaned on PND 21. On PND 28, 10 F_2 offspring/sex/dose group were evaluated by functional observation battery and motor activity testing, and an additional 10 F_2 offspring/sex/dose group were evaluated in a water maze test of learning and memory. Estrous cycle was monitored daily for all F_0 and F_1 females for at least 3 weeks prior to mating. Cauda epididymal sperm were assessed for motility, morphology, and head count in F_0 and F_1 males at necropsy. Sperm head count was also performed in testes. Reproducing females were killed after weaning, and uteri were stained in 10% ammonium sulfide for calculation of implantation sites, used in the calculation of postimplantation loss. Male pups were evaluated on PND 12 and 13 for the presence of nipples and areolae. Age and weight at vaginal opening and preputial separation were assessed in pups. One pup/sex/litter was killed on or after PND 21, and brain, spleen, thymus, liver, kidneys, testes, ovaries, and uterus (with oviducts and cervix) were weighed. Gross necropsy was performed on pups culled on PND 4 and on those pups not selected as parents for the next generation or for neurobehavioral testing (on PND 21). Histologic examination was performed on reproductive organs and other selected organs, and follicle counts were performed in ovaries. Statistical comparisons were made using the Dunnett test for means and the Fisher exact test for proportions or the Kruskal-Wallis test with Wilcoxon test for proportions of affected pups/litter, pup organ weights, and several neurobehavioral endpoints. Selected results are given in Table 31.

Table 31. Results of 2-Generation Study of DEHP in Wistar Rats

Parameter	Dose in feed (ppm)			Benchmark dose (ppm)[a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_0 Feed Consumption							
During pregnancy	↔	↔	↔				
Laction day 1–4	↔	↔	↓18%	5433	4016	7244	5257
Laction day 4–7	↔	↔	↓21%	6886	5015	8578	5792
Laction day 7–14	↔	↔	↓33%	6928	4465	6986	4577
Other F_0 Parameters							
F_0 Body weight gain during Pregnancy	↔	↔	↓11%	8849	7167	8965	7894
F_1 Body weight on lactation day 21	↔	↔	↓14%	8741	7225	8547	6170
F_0 Males with confirmed mating	↔	↔	↔				
F_0 Males with confirmed fertility	↔	↔	"↓"12%[b]				
F_0 Sperm parameters	↔	↔	↔				
F_0 Females with stillborn pups	↔	↔	↑4-fold	6414	250		
F_0 Litter size (F_1 pups/litter)	↔	↔	↔				
F_1 Pups Surviving (pup basis)							
PND 0–4	↔	↓4%	↓7%	11,399	8541		
PND 4–21	↔	↔	↔				
Other F_1 Parameters							
Live F_1 pups/litter on PND 4, 7, 14, and 21	↔	↔	↔				
Sex ratio F_1 pups	↔	↔	↔				
Postimplantation loss per F_0 female	↔	↔	↑2.1-fold	7850	813	9070	7659
F_1 Pup Body Weight, Male							
PND 1	↔	↔	↓6%	9274	9034	9139	8389
PND 4	↔	↔	↔				
PND 7	↔	↔	↓6%	8780	5470	8919	6431
PND 14	↔	↔	↓26%	5448	3903	5516	3463
PND 21	↔	↔	↓31%	4661	3005	4615	2875
F_1 Pup Body Weight, Female							
PND 1	↔	↔	↔				
PND 4	↔	↔	↔				
PND 7	↔	↔	↓16%	7643	4684	7777	5202
PND 14	↔	↔	↓27%	5460	3818	5224	3451
PND 21	↔	↔	↓31%	4733	3386	4583	3084
F_1 PND 1 Anogenital Distance							
Male	↔	↔	↓14%	6943	5417	5514	4242
Female	↔	↔	↔				

Parameter	Dose in feed (ppm)			Benchmark dose (ppm) [a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Other F_1 Genital Parameters							
F_1 Males with nipples/areolae per litter	↔	↔	↑38-fold	6238	2222		
F_1 Days to vaginal opening	↔	↔	↑12%	7921	6534	5407	3631
F_1 Days to preputial separation	↔	↔	↑19% [c]	5780	4325	3986	2592
F_1 PND 21 Absolute Organ Weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↓7%	10,964	9118	6484	4136
Thymus	↔	↓12%	↓39%	2506	2056	3443	2728
Spleen	↓15%	↓13%	↓57%	2446	1265	3962	2578
Liver	↔	↑17%	↔	1271	805	2713	1817
Kidney	↔	↔	↓33%	5946	4458	8487	5214
Testis	↔	↔	↓37%	5357	2995	6122	3863
Ovary	↔	↔	↓32%	8472	2265	9013	6273
Uterus	↔	↔	↓22%	8537	2859	9235	8276
F_1 PND 21 Relative Organ Weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↑38%	5292	3104	5274	3095
Thymus	↔	↔	↓12%	2467	1541	9534	9379
Spleen	↓11%	↓8%	↓38%	3450	2224	4300	2801
Liver	↑8%	↑22%	↑30%	1138	911	1184	956
Kidney	↔	↑5%	↔				
Testis	↔	↔	↔				
Ovary	↔	↔	↔				
Uterus	↔	↔	↔				
F_1 Feed Consumption							
GD 0–7	↔	↑5%	↓7%	9107	8803	8893	7787
GD 7–14	↔	↔	↓9%	8989	8218	8782	7034
GD 14–20	↔	↔	↔				
Laction day 1–4	↔	↔	↓32%	4587	3079	6266	4328
Laction day 4–7	↔	↔	↓31%	4540	2715	5159	3040
Laction day 7–14	↔	↓12%	↓44%	2556	1848	3352	2193
Other F_1 Parameters							
Body weight gain during pregnancy	↔	↔	↓15%	6015	3920	7748	5283
Body weight on lactation day 21	↔	↔	↓21%	8539	5480	8501	5238
Males with confirmed mating	↔	↔	↔				
Males with confirmed fertility	↔	↔	"↓" 24% [b]				
Sperm count (testis, epididymis)	↔	↔	↔				
Percent abnormal sperm	↔	↔	↑27%	3061	1174	25,588	9256
Percent motile sperm	↓2%	↔	↔				
Females with stillborn pups	↔	↑3-fold	"↑" 2.8-fold				
Litter size (F_2 pups/litter)	↔	↔	↓19%	5790	3137	9657	6697

Parameter	Dose in feed (ppm)			Benchmark dose (ppm) [a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_2 Pups Surviving (Pup Basis)							
PND 0–4	↔	↓15%	↓13%	2325	2045		
PND 4–21	↔	↔	↔				
Other F_2 Parameters							
Live F_2 pups/litter on PND 4, 7, 14, 21	↔	↔	↔				
Sex ratio F_2 pups	↔	↔	↔				
Postimplantation loss per F_1 female	↔	↔	↔				
F_2 Pup Body Weight, Male							
PND 1	↔	↔	↔				
PND 4	↔	↔	↔				
PND 7	↔	↔	↓11%	7703	2940	9299	6072
PND 14	↔	↔	↓29%	4204	2940	3827	2521
PND 21	↔	↔	↓35%	4085	2790	4142	2787
F_2 Pup Body Weight, Female							
PND 1	↔	↔	↔				
PND 4	↔	↔	↔				
PND 7	↔	↔	↓11%	7894	5078	10,689	6703
PND 14	↔	↓8%	↓21%	3691	2724	3356	2476
PND 21	↔	↔	↓33%	4523	3094	3652	2318
F_2 PND 1 Anogenital Distance							
Male	↔	↓9%	↓10%	8810	6204	6597	3981
Female	↔	↔	↔				
F_2 Males with nipples/areolae per litter	↔	↑45-fold	↑54-fold	1610	Failed [d]		
F_2 PND 21 Absolute Organ Weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↓7%	11,077	9201	5432	3686
Thymus	↔	↔	↓39%	2872	1957	4185	2963
Spleen	↔	↔	↓53%	4059	2262	5555	3828
Liver	↑14%	↑20%	↔	↓1618	1096	2557	1480
Kidney	↔	↔	↓30%	6134	4155	6591	4700
Testis	↔	↔	↓38%	4063	2447	4905	3252
Ovary	↔	↔	↓26%	3157	2015	9484	6354
Uterus	↔	↔	↔				
F_2 PND 21 Relative Organ Weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↑39%	4598	3379	4307	3000
Thymus	↔	↔	↓12%	7302	4633	9860	6359
Spleen	↔	↔	↓32%	5423	3424	6230	4311
Liver	↑12%	↑24%	↑33%	939	636	1637	1149
Kidney	↑6%	↑6%	↔				
Testis	↔	↔	↓10%	9393	6427	8853	5832

Parameter	Dose in feed (ppm)			Benchmark dose (ppm)[a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Ovary	↔	↔	↔				
Uterus	↔	↔	↑28%	8469	3304	8913	6381

From Schilling et al. *(151)*.
↑,↓,↔ Increase, decrease, or no change in parameter compared to 0 ppm group by statistical testing. "↓" , "↑" refers to study author conclusion of a difference in the absence of statistical confirmation.
[a] Calculated by CERHR. See Table 22 for definitions and a discussion of the use of benchmark dose in this report. Benchmark dose was calculated only when a treatment effect was demonstrated by the study authors' analysis.
[b] Histologic changes in testes and/or epididymal sperm led the study authors to conclude that there was a treatment-related reduction in fertility in this dose group although statistical significance was not shown.
[c] Two males with hypospadias, penile hypoplasia, and cleft prepuce.
[d] BMDL calculation could not be performed by EPA program using Hill model, which gave the best fit.

In general, the high-dose level (9000 ppm) was associated with a decrease in feed consumption and weight gain at several intervals during the study. Clinical signs leading to unscheduled sacrifice were present in 1 high-dose F_0 female. Six high-dose adult F_1 females died or were killed in moribund condition during the pregnancy or lactation periods; 3 of these females were found to have liver necrosis. F_2 pups in the high-dose group were smaller and gained less weight from birth through the period of assessment of functional observation battery and water maze testing. Grip strength was reduced in males, and hind-limb splay was reduced in both sexes in the high-dose group. There were no other treatment-related findings in the functional observation battery or in the water maze. Differential ovarian follicle counts of F_0 and F_1 adults showed a deficit in growing follicles and corpora lutea in the high-dose group **[expressed per follicle]**.

The authors concluded that reproductive performance and fertility were affected at the 9000 ppm dose level with a NOAEL of 3000 ppm, and that developmental toxicity was noted at 3000 and 9000 ppm, with an increase in stillbirth, an increase in PND 0–4 pup mortality, retardation of F_2 pup body weight gain, reduced male anogenital distance, and increased retained nipples/areolae in males. A delay in sexual maturation was also noted in F_1 male and female offspring at 9000 ppm. The NOAEL for developmental toxicity was considered by the study authors to be 1000 ppm. The alterations in pup organ weights were noted, but the changes in spleen and thymus weight were assessed as causally related to the body weight alterations and not as primary effects of DEHP. The alterations in liver weight were considered likely to be due to peroxisome proliferation and were not representative of developmental toxicity. The NOAEL for systemic toxicity was considered by the study authors to be 1000 ppm. **[The Panel noted that focal tubular atrophy, the most subtle manifestation of the phthalate effect, was noted in the F_0 animals in 0, 1, 3, and 6 males, and in the F_1 generation, in 2, 7, 4, and 13 male rats. The Panel considers the increases in affected animals to be treatment-related at all doses, so that the lowest effective dose was 1000 ppm (~100 mg/kg bw/day). These effects are consistent with other reports in the literature and show a reasonable dose-response that becomes more severe in gestationally exposed animals. The lowest BMD_{10} for reproductive toxicity was 2325 ppm ($BMDL_{10}$: 2045 ppm) for F_2 pup survival on PND 0–4. Based on the dose regression provided by the authors, the corresponding DEHP intake is 263 mg/kg bw/day ($BMDL_{10}$: 231 mg/kg bw/day).]**

Strengths/Weaknesses: Strengths of this study include the completeness and widely accepted rigor (i.e., to GLP standards) with which it was performed, the number of animals, and the number of endpoints.

Utility (Adequacy) for CERHR Evaluation Process: This study was useful for the evaluation process and showed a LOAEL of 1000 ppm (~100 mg/kg bw/day) based on testicular histopathology in both generations. That this is a conclusion of the Expert Panel and not the authors is a cause for concern and limits the confidence that this conclusion can bear.

Tanaka et al. *(137)*, support not indicated, gave DEHP (>97% purity) to CD-1 mice in the diet from 5 weeks of age in the F_0 generation to 9 weeks of age in the F_1 generation. A single dietary dose level of 0.03% was used, with control animals receiving untreated basal feed (n=20/sex/treatment group). At 9 weeks of age, 10 DEHP-treated females were paired with DEHP-treated males, 10 DEHP-treated females were paired with control males, 10 control females were paired with DEHP-treated males, and 10 control females were paired with control males. The females' diet was available to males during the 5-day cohabitation phase. Females reared their own unadjusted litters, which were weaned at 4 weeks of age. One female and male from each litter were retained and fed their dam's diet until 9 weeks of age. Statistical analyses were performed using ANOVA or Kruskal-Wallis test, followed by Bonferroni multiple comparison test. Proportions were evaluated using chi-squared or Fisher test. Based on measured feed consumption, mean DEHP intake by treated males **[rounded by CERHR]** was 46 mg/kg bw/day. Treated females received 53–57 mg/kg bw/day during the preconception period, ~43 mg/kg bw/day during mating, 46–49 mg/kg bw/day during gestation, and 154–171 mg/kg bw/day during lactation. DEHP had no effect on feed consumption or dam body weight. There were no significant treatment effects on the number of pregnant females, number of litters, number of offspring, average litter size or weight, or sex ratio. The authors concluded that "DEHP caused few significant adverse effects on reproductive or neurobehavioral parameters." **[Neurobehavioral testing of the F_1 offspring, the focus of the study, was discussed in Section 3.2.2.]**
Strengths/Weaknesses: The crossover design of this study is a strength, as are the statistical evaluations and the presentation of the data. The use of a single dose level weakens the study for use in assessing reproductive risk, which admittedly was not the major focus of the study.

Utility (Adequacy) for CERHR Evaluation Process: The data are sufficient to conclude that DEHP dosing between 46 and 154 mg /kg bw/day was insufficient to materially change any reproductive parameter measured in this study in mice.

The National Toxicology Program *(114)* sponsored a multigeneration continuous breeding study in rats with the intent of evaluating whether responses seen at very low doses might be different from, or forerunners of, responses seen at higher doses. Task 1 of the study was conducted to determine the doses used in subsequent tasks. Sprague-Dawley rats (13 weeks old; 8/sex/group) were given feed containing 0, 5000, or 10,000 ppm DEHP from 7 days prior to mating through the cohabitation period, which extended until necropsy. **[The time period of cohabitation was not specified.]** DEHP intake was estimated by the study authors at 0, 321.42, and 643.95 mg/kg bw/day. In litters born during the cohabitation period, anogenital distance was measured on PND 1, and growth and mortality were monitored through PND 21. Signs of systemic toxicity included decreased feed and water intake during lactation in females from the 5000 and 10,000 ppm groups and a decrease in body weight gain in females from the 10,000 ppm group. Reproductive effects included a decrease in uterus, cervix, and vagina weights in PND 21 pups from the 5000 and 10,000 ppm groups. At 10,000 ppm, ratio of anogenital distance to pup weight was increased in female pups, and pup weights were decreased on PND 4 and 21.

In task 2 of the study, 17 Sprague-Dawley rats/sex/group were randomly assigned to groups and fed diets containing 1.5 (control group exposed to background DEHP levels in feed), 10, 30, 100, 300, 1000, or 7500 ppm DEHP (99.8% pure) from the first day of the study until the day of necropsy. Due to a lack of reproductive effects in the first litter produced, the study was repeated with 2 additional doses, 1.5 (control) and 10,000 ppm. **[It is not clear why the authors concluded there were no reproductive effects in the 7500 ppm group when several significant effects were observed in the first litter, as discussed below.]** Ranges of DEHP intake in the F_0, F_1, and F_2 animals were estimated at 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day. At about 5 weeks of age, F_0 rats were fed the DEHP-containing diets for 6 weeks prior to mating and were then cohabitated for 9 weeks. Concentrations of dosing solutions were verified. The first 2 litters delivered during the cohabitation period (F_{1a} and F_{1b}) were counted, weighed, assessed for anogenital distance, and then discarded. The third litter (F_{1c}) was raised by the dam until weaning on PND 21 **[designation for day of birth not specified]**. Following weaning of pups, vaginal cytology was monitored in F_0 females for 14 days. After completion of crossover studies described below, at least 10 F_0 rats sex/group were necropsied. Sperm analyses were conducted, and organs were collected for histopathological evaluation. Ovaries were preserved in Bouin fluid. Testes and epididymides were preserved in 2% paraformaldehyde/3% glutaraldehyde. F_1 pups were counted, weighed, and examined for anogenital distance and nipple retention during the lactation period. On PND 16, 1 female per litter was evaluated for vaginal opening, and a second was selected for F_1 mating. One male per litter was selected for mating, and 4 or 5 males per litter were evaluated for testicular descent and preputial separation; both groups of rats were necropsied. At weaning (PND 21), pups were given diets containing the same DEHP concentrations as their parents. On PND 81, the F_1 rats chosen for mating (17/sex/group) were randomly assigned to breeding pairs (preferably non-sibling) and cohabited for 9 weeks. The study conducted in F_0 parents and F_1 offspring was repeated in F_1 parents and F_2 offspring, except that the third F_3 litter born (F_{3c}) did not undergo the continuous-breeding protocol. Selected F_{3c} males were necropsied on PND 63–64 and selected females on PND 60–74. Statistical analyses included Jonckheere test to determine if data should be analyzed by Shirley or Dunn test. Shirley test was used to evaluate data that consistently increased or decreased according to dose. Dunn test was used to evaluate data with severe departures from monotonicity. Additional statistical analyses included Wilcoxon, Cochran-Armitage, and chi-squared tests.

Some systemic effects were consistent across all generations. During numerous time periods of the study and especially at necropsy, body weight gains were decreased in rats from the 7500 and 10,000 ppm groups. Dam body weights during delivery and lactation were decreased by 8–20% in the F_0 10,000 ppm group. Increases and decreases in feed intake were observed at most dose levels. In the F_0 7500 and 10,000 ppm groups, feed intake was decreased during lactation. The liver was identified as a target of toxicity, with increases in liver weight and hepatocellular hypertrophy observed at dose levels ≥1000 ppm. Changes in organ weights and lesions were also observed in kidney at ≥7500 ppm and adrenal gland at 10,000 ppm.

Reproductive toxicity findings in all generations of rats are summarized in Table 32.

Table 32. Results of Continuous Breeding Multigeneration Study of DEHP in Sprague-Dawley Rats

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_0 Females											
Cumulative days to deliver	↔	↔	↔	↔	↔	↔	↑ litter 1				
Estrous cycle effects	↔	↔	↔	↔	↔	↔	↔				
F_0 Males											
Spermatid/testis	↑16.5%	↔	↔	↔	↔	↔	↓31%				
Sperm velocity	↔	↔	↔	↔	↔	↔	↓11%				
Body weight at necropsy	↔	↔	↔	↔	↔	↔	↓6%				
Absolute organ weight											
Cauda epididymis	↔	↔	↔	↔	↔	↔	↓19%				
Epididymis	↔	↔	↔	↔	↔	↔	↓16%				
Testis	↔	↔	↔	↔	↔	↔	↓23%				
No. with small right testis	0	0	0	0	0	0	2/10				
F_0 Matings											
Live pups/litter (F_{1a})	↔	↔	↔	↔	↔	↓18%	↓21%	7008	3237	6771	2849
Live males/litter (F_{1a})	↔	↔	↔	↔	↔	↓20%	↓26%	3802	3303	2650	2227
Proportion liveborn pups	↔	↔	↔	↔	↔	↔	↓4%				
Pup body weight	↔	↔	↔	↔	↔	↔	↓9%				
Adjusted for litter size	↔	↔	↔	↔	↔	↔	↓10%				
Pup anogenital distance											
Male F_{1a}	↔	↔	↔	↔	↔	↓7%	↓14%	10,566	7631	3411	2382
Male F_{1b}	↔	↔	↔	↔	↔	↓8%	↓15%	8408	7465	2874	2127
Female, relative to bw	↔	↔	↔	↔	↔	↔	↑10 –17%				
Male pup retained nipples	↔	↔	↔	↔	↔	↔	↔				
Pup weights PND 1–21											
Male	↔	↔	↔	↔	↔	↔	↓30%				
Female	↔	↔	↔	↔	↔	↔	↓30%				
Pup survival, PND 4–21	↔	↔	↔	↔	↔	↔	↔				

Appendix II

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_1 Males and females											
Age at vaginal opening	↔	↔	↔	↔	↔	↑3 days	↑8 days				
Age at preputial separation	↔	↔	↔	↔	↔	↑4 days	↑11 days				
Age at testes descent	↔	↔	↔	↔	↔	↑3 days	↑6 days				
Pregnancy index, first 2 litters (control 76–100)	100	94	100	94	94	71	0				
	76	88	88	81	82	59	0				
Cumulative days to litter	↔	↔	↔	↔	↔	↔	no litters				
F_1 Females											
Estrous cycle effects	↔	↔	↔	↔	↔	↔	↑0.4 days				
Body weight at necropsy	↔	↔	↔	↔	↔	↔	↓19%				
Relative uterine weight	↔	↔	↔	↔	↔	↔	↑36%				
Relative ovarian weight	↔	↔	↔	↔	↔	↔	↑35%				
F_1 Breeder males											
Body weight at necropsy	↔	↔	↔	↔	↔	↔	↓16%				
Absolute organ weight											
Cauda epididymis	↔	↔	↔	↔	↔	↓37%	↓62%	6532	3576	5970	2224
Epididymis	↔	↔	↔	↔	↔	↓35%	↓55%	6764	3896	6184	2226
Testis	↔	↔	↔	↔	↔	↓51%	↓80%	6577	1693	5940	511
Seminal vesicles	↔	↔	↔	↔	↔	↔	↓29%				
Ventral prostate	↔	↔	↔	↔	↔	↓28%	↔	7056	3062	6998	2725
Dorsolateral prostate	↔	↔	↔	↔	↔	↔	↓29%				
No. with small testes	0	0	0	0	0	8/10	10/10				
No. with small epididymides	0	0	0	0	0	2/10	0				
F_1 Non-breeder males											
Body weight at necropsy	↔	↔	↔	↔	↔	↓9%	↓21%	6752	6408	1634	1229
Absolute organ weight											
Epididymis	↔	↔	↔	↔	↔	↓20%	↓54%	6845	3349	5787	797
Seminal vesicles	↔	↔	↑9%	↔	↔	↔	↔				

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Cauda epididymis	↔	↔	↔	↔	↔	↓20%	↓44%	6802	4188	5669	1450
Testis	↔	↔	↔	↔	↔	↓34%	↓80%	2945	2584	1221	946
Relative organ weight											
Epididymis	↔	↔	↔	↔	↔	↔	↓42%	6474	5814	3088	1965
Testis	↔	↔	↔	↔	↔	↓28%	↓75%	3895	3253	1903	1312
Cauda epididymis	↔	↔	↔	↔	↔	↔	↓33.8%				
No. with small:											
Testes	0	0	0	3/45	0	9/30	21/21				
Epididymides	0	0	0	2/45	0	0	21/21				
Seminal vesicles	0	0	0	2/45	0	0	1/21				
Ventral prostates	0	0	0	0	3/43	1/30	1/21				
Caudae epididymides	0	0	0	1/45	0	0	21/21				
Spermatid/testis	↔	↔	–	↔	–	↓69%	↓100%	5834	1329	5534	916
Sperm/cauda	↔	↔	↔	↔	↔	↓61%	↓99.8%	6771	2865	6663	2472
Epididymal sperm density	↔	↔	↔	↔	↔	↔	↓99.6%	6389	2819	6181	2310
F_1 Matings											
Live F2 pups/litter	↔	↔	↔	↔	↔	↔					
Live pup weight	↔	↔	↔	↔	↔	↓10%		7496	7119	5996	1762
Adjusted for litter size	↔	↔	↔	↔	↔	↓11%		7245	6708	2123	1247
Male F_{2a} anogenital distance	↔	↔	↔	↔	↔	↓13%		5749	4582	1093	963
Male F_{2c} anogenital distance	↔	↔	↔	↔	↔	↓18%		5375	3284	3702	2517
Male pup weight PND 1	↔	↔	↔	↔	↔	↓13%		7192	4135	6788	2649
Male pup weight PND 4	↔	↔	↔	↔	↔	↓22%		6960	2715	6362	2365
Female pup weight PND 1	↔	↔	↔	↔	↔	↓17%		5801	3505	6669	2036
Female pup weight PND 21	↔	↔	↔	↔	↔	↓28%		5097	2144	4638	2209
Male pup survival PND 1–21	↔	↔	↔	↔	↔	↓20%		7004	2992	7082	3504
Combined survival PND 1–21	↔	↔	↔	↔	↔	↓19%		7293	3057	6924	3130

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_2 Males and females											
Age at vaginal opening	↔	↔	↔	↔	↔	↑6 days					
Preputial separation (days)	↑1.9	↑1.0	↑1.4	↑0.7	↑6.5						
Testes descent (days)	↔	↑1.4	↑0.8	↑0.4	↑3.4						
Pregnancy index, 1st 2 litters (control 100)	100	100	100	100	100	53(↓)					
	100	100	100	100	94	47(↓)					
Cumulative days to deliver	↔	↔	↔	↔	↔	↔					
No. cycling rats	↔	↔	↔	↓29%	↔	↔					
No. litters/pair	↔	↔	↔	↔	↔	↓					
F_2 Males (breeders)											
Body weights at necropsy	↔	↔	↔	↔	↔	↓14%					
Absolute organ weight											
Epididymis	↔	↔	↔	↔	↔	↓36%		6593	1846	4490	678
Seminal vesicles	↔	↔	↔	↔	↔	↓24%		2916	2009	2209	1705
Testis	↔	↓10%	↔	↔	↔	↓60%		5874	1329	2408	1300
Cauda epididymis	↔	↔	↔	↔	↔	↓63%		6493	1641	6711	2432
Relative testis weight	↔	↔	↔	↔	↔	↓53%		2334	1224	1815	1065
No. with small:											
Testes	0	0	0	0	0	8/10					
Epididymides	0	0	0	0	0	8/10					
Caudae epididymides	0	0	0	0	0	8/10					
F_2 Males (non-breeders)											
Body weight at necropsy	↔	↔	↔	↔	↔	↓14.5%		7190	4446	6289	3153
Absolute organ weight											
Epididymis	↔	↔	↔	↔	↔	↓27%		4350	2269	2574	1270
Testis	↔	↔	↔	↔	↔	↓49%		1788	1362	987	758
Cauda epididymis	↔	↔	↔	↔	↔	↓32%		2532	2005	1132	895

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Relative organ weight											
Testis	↔	↔	↔	↔	↔	↓40%		1948	1470	1132	1003
Cauda epididymis	↔	↔	↔	↔	↔	↓20%		4187	2714	1718	1472
No. with small:											
Testes	0	0	0	1/21	1/25	11/20					
Epididymides	0	0	0	1/21	1/25	7/20					
Caudae epididymides	0	0	0	1/21	1/25	6/21					
Spermatid/testis	↔	↔	↔	↔	↔	↓74%		2885	1092	2043	1161
Sperm/cauda	↔	↔	↔	↔	↔	↓72%		1795	935	1374	854
Epididymal sperm density	↔	↔	↔	↔	↔	↓64%		1158	897	1830	1542
Percent motile sperm	↔	↔	↔	↔	↔	↓25%		3825	2038	554	412
F_2 Matings											
Live F_3 pups/litter	↔	↔	↔	↔	↔	↔					
Live pup weight	↔	↔	↔	↔	↔	↔					
Male anogenital distance, F_{3a}	↔	↔	↔	↔	↔	↓13%		7322	4638	6846	2661
Pup weight (PND 1–21)	↔	↔	↔	↔	↔	↔					
Pup survival (PND 1–21)	↔	↔	↔	↔	↔	↔					
F_3 Females											
Age at vaginal opening	↔	↔	↔	↔	↔	↑6					
F_3 Males											
Retained nipples	↔	↔	↔	↔	↔	↑11%					
Age at preputial separation	↔	↔	↔	↔	↔	↑5.1					
Age of testes descent	↔	↔	↔	↔	↔	↑2.5					
Body weights at necropsy	↔	↔	↔	↔	↔	↔					
Absolute organ weights											
Dorsolateral prostate	↔	↔	↔	↔	↔	↓41%		1926	1401	2204	1840
Testis	↔	↔	↔	↔	↔	↓45%		1960	1399	1060	851

Parameter	*Dose in feed (ppm)*							*Benchmark dose (ppm)*[a]			
	10	*30*	*100*	*300*	*1000*	*7500*	*10,000*	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Relative organ weights											
Testis	↔	↔	↔	↔	↔	↓48%		1870	1307	1330	1097
Epididymis	↔	↔	↔	↔	↔	↓35%		3223	1625	2332	1285
Spermatid/testis	↔	↔	↔	↔	↔	↓79%		1159	782	1100	695
Sperm/cauda	↔	↔	↔	↔	↔	↓95%		787	728	1188	970
Epididymal sperm density	↔	↔	↔	↔	↔	↓94%		797	750	1108	933

[a]Calculated by CERHR. See Table 22 for definitions and a discussion of the use of benchmark dose in this report. The 10,000 ppm dose group was not included in the benchmark dose calculations because it used a different control than the other dose groups. Benchmark dose was calculated only when there was a significant treatment effect according to the study authors' analysis.

↑,↓,↔ Increase, decrease, or no change in parameter compared to 0 ppm group by statistical testing.

From NTP *(114)*.

In the F_0 generation, there were no effects on fertility or estrous cycles. The only reproductive effects observed in the F_0 parents occurred at 10,000 ppm and included decreases in sperm counts and velocity, reductions in testis and epididymis weights, and increased numbers of rats with small testes. Histopathological findings associated with small testes included minimal-to-marked atrophy of seminiferous tubules characterized by loss of germ cells. The lowest dose level producing dose-related effects in F_1 offspring was 7500 ppm, and those effects included decreases in number of live pups/litter, reduced male anogenital distance, and delays in vaginal opening, preputial separation, and age of testicular descent. Additional effects noted in the F_1 offspring from the 10,000 ppm group included decreased live pup weight at birth and during the lactation period and increased ratio of female anogenital distance to body weight.

In contrast to findings in the F_0 generation, fertility was compromised in the F_1 rats from the 10,000 ppm group, which did not produce any viable litters. Other reproductive effects observed in F_1 parents were similar to those observed in F_0 parents but usually occurred at lower dose levels. In the non-mating F_1 adult males of the 300 ppm group, there was a small increase in the number of animals (3 of 45) with small testes and/or epididymides. The effects were not observed at the next higher dose (1000 ppm), but small testes were observed in 10 of 30 males of the 7500 ppm non-mating group. Small testes and epididymides were observed in 21 of 21 animals of the 10,000 ppm non-mating group. A small percentage (3 – 7%) of non-mating rats treated with ≥ 1000 ppm had small ventral prostates. In rats that were mated, the only decreases in reproductive organ size occurred in testes at 7500 and 10,000 ppm (8 of 10 and 10 of 10 affected at each dose) and epididymides at 7500 ppm (2 of 10). Histopathological findings observed in all animals of the 7500 and 10,000 ppm groups were consistent with those observed in the F_0 generation and included minimal-to-marked seminiferous tubule atrophy and occasional sperm release failure. Minimal seminiferous tubule atrophy was observed in 1 of 10 males in the 100 and 300 ppm groups. Reductions in numerous reproductive organ weights were observed in mating and non-mating F_1 males treated with ≥ 7500 ppm. Additional reproductive effects observed in F_1 rats were reduced sperm counts at 7500 ppm and higher and increased uterus and ovary weights at 10,000 ppm. Estrous cycle length was slightly increased at 10,000 ppm. In the F_2 pups, delays in preputial separation and testicular descent occurred at every dose level above the control. **[In no other generation did delays in preputial separation and testicular descent occur at such low doses, but the study authors did not offer any explanations for this observation. The Expert Panel believes these findings are consistent with a problem with the control group in that generation.]** All other effects occurred in F_2 pups of the 7500 ppm group and included delayed vaginal opening and reductions in live pup weight at birth and during the lactation period, male anogenital distance, and survival during the lactation period.

No F_2 adult rats from the 10,000 ppm group were available due to complete infertility in F_1 rats of that group. In F_2 adult rats, a decreased number of cycling females observed in the 300 ppm group was not observed at any other dose level. In non-mating males, rats with small testes and epididymides were observed at ≥ 300 ppm (1 of 21), 1000 ppm (1 of 25), and 7500 ppm (7 – 11 of 20). However, in males that were mated, small epididymides and testes were only observed at the 7500 ppm dose level (8 of 10). Seminiferous tubule atrophy was observed in 10 of 10 males of the 7500 ppm group. All other reproductive effects in F_2 adults occurred at 7500 ppm and included decreases in pregnancy index, the number of litters per pair, male reproductive organ weights, sperm counts, and sperm motility. In F_3 pups, a decrease in postnatal survival of females was observed only on PND 7 in the 300 ppm group

but was not observed on any other day or dose level. All other effects in F_3 pups occurred at 7500 ppm and included decreases in male anogenital distance, delayed vaginal opening, preputial separation, and testicular descent, and an increase in male pups with nipples. F_3 pups were the only generation of rats to experience an increase in males with nipples. At necropsy of adult F_3 rats, effects were only observed at the 7500 ppm dose level and included reduced sperm counts and weights of dorsolateral prostate, testis, and epididymis.

The study authors discussed the relevancy of small male reproductive organ sizes observed in both F_1 and F_2 rats of the 300 ppm groups. They noted that although incidences were low, the effects were consistent with phthalate-induced toxicity. The incidence of small testes and epididymides exceeded historical control data from the laboratory. Therefore, the study authors considered the effects as potentially treatment-related. However, the study authors concluded that the overall significance of the effects could not be determined due to lack of histopathological data and lack of adverse reproductive effects at 300 and 1000 ppm.

Two crossover breeding studies were conducted. The first was conducted to investigate the lack of litters in F_1 rats of the 10,000 ppm group. The second was conducted to investigate the decrease in F_3 pup body weight in the 7500 ppm group. The studies were conducted by mating the control and high-dose rats of each sex (n = 17/sex/group) with naïve animals for 7 days or until a vaginal plug was detected. Pups were counted, weighed, assessed for anogenital distance, and discarded. Implantation sites were examined in naïve females. The crossover studies (Table 33) demonstrated that pregnancy and fertility indices were reduced in males of the 7500 ppm and 10,000 ppm groups. Implantation sites were reduced in the naïve rats mated with DEHP-treated males. A decrease in pup weight and male anogenital distance was seen in offspring born to females treated with 7500 and 10,000 ppm DEHP and mated to naïve males.

Table 33. Reproductive Crossover Breeding Study

Parameter	*Dam × Sire*	*F_1 DEHP treatment Groups*		*F_2 DEHP Treatment Groups*	
		10,000 ppm × naïve	*Naïve × 10,000 ppm*	*7500 ppm × naïve*	*Naïve × 7500 ppm*
Pregnancy index		↑	↓ (0 Pregnant)	↔	↓
Fertility index		↑	↓ (0 Fertile)	↔	↓
Mating index		↔	↓ (0 Mated)	↔	↔
Implantation sites		Not Reported	↓97.9%	Not Reported	↓54.5%
Adjusted live male pup weight		↓13.6%	NA	↓8.2 – 12.3%	↔
Male pup anogenital distance		↓16.9%	NA	↓11.5%	↔
Female anogenital distance/bw		↑17.6%	NA	↔	↔
Live female pups/litter		↔	NA	↔	↓31.0%

↑,↓,↔ Increase, decrease, or no change in parameter
NA = Not Applicable
From NTP *(114)*.

The study authors concluded, "The findings obtained in this study indicate that DEHP is clearly a reproductive and developmental toxicant at 7500 and 10,000 ppm based upon changes in fertility and pregnancy indices, litter data, sperm parameters, sexual development, and/or histopathological changes in testes." Intake at 7500 ppm was estimated at 392 – 592 mg/kg bw/day, and intake at 10,000 ppm was estimated at 543 – 775 mg/kg bw/day. **[The lowest BMD_{10} was 787 ppm based on F_3 sperm/cauda. Extrapolating from the authors' estimates of intakes at 1000 mg/kg bw/day, this intake level is 36 – 61 mg/kg bw/day. The lowest $BMD_{1\ SD}$ is 554 ppm based on F_2 percent motile sperm. Extrapolating from the authors' estimates of intakes at 300 ppm, this intake level is 9 – 15 mg/kg bw/day. The Expert Panel notes that Sertoli cell vacuolation, which was the endpoint driving the LOAEL in the original CERHR Expert Panel evaluation of DEHP, was not increased by DEHP treatment in this study.]**

Strengths/Weaknesses: Clearly, a major strength of this study is the number of doses evaluated. The relatively small group sizes were compensated by the unusually high numbers of groups and the very low doses used. An additional strength is the fact that more offspring were evaluated early for alterations in the development of the reproductive system; a weakness might be that not all animals were so evaluated. The quality of the histology is another strength. The lack of vacuoles is perplexing, but not lethal to the study.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process and show that 10,000 and 7500 ppm are clearly toxic to the developing reproductive system in rats. The Expert Panel considers 300 ppm and 1000 ppm to represent the tail of the dose-response curve in this study, based on the incidence of testicular abnormalities, which would put the NOAEL for these developmental effects at 100 ppm, in the 3 – 5 mg/kg bw/day range.

4.2.4 Abstracts

CERHR retrieved several abstracts reporting reproductive toxicity associated with DEHP exposure. Although information from abstracts is not considered by the Expert Panel in reaching final conclusions, the abstracts are briefly summarized for the sake of completeness.

Boekelheide et al. *(189)* presented an abstract on the use of mutant mice to investigate the MEHP mechanism of testicular toxicity. Knockout mice deficient in fasL, p53, or both were protected against testicular toxicity after treatment with MEHP. The authors concluded that the apoptotic response of mouse testis to MEHP is at least partially dependent on fasL and p53. **[The Expert Panel notes that the results for fasL-deficient mice reported in this abstract appear similar to the results in Richburg et al. *(169)*, discussed above, from the same laboratory.]**

Noriega et al. *(190)* treated male Sprague-Dawley rats on PND 23 – 43 or 44 with DEHP 0, 100, 300, or 900 mg/kg bw/day **[route not specified]**. There were reductions in the 300 and 900 mg/kg bw/day groups in testis, seminal vesicle, Cowper gland, levator ani, and bulbocavernosus muscle weights compared to the controls. Testicular testosterone (basal and stimulated) was reduced in these 2 groups, and serum testosterone was reduced in the 900 mg/kg bw/day group. There was a reduction in the proportion of males with complete preputial separation in the 300 and 900 mg/kg bw/day groups compared to the controls. Female rats dosed from PND 23 concurrently with males showed no difference in time to vaginal opening in the 900 mg/kg bw/day group compared to the corn-oil control group.

Nakajima et al. *(191)* gave wild-type and PPARα-null mice DEHP in the diet for 4 weeks at exposure levels of 0 or 0.05%, following which the animals were mated within dose and genetic-strain groups. Two generations (F_1 and F_2), produced without direct exposure to DEHP, showed decreases in number of pups born and in pups surviving to 16 weeks of age. Only wild-type mice were affected. In another experiment, dietary DEHP exposures at 0, 0.01, and 0.05% were used, producing an increase in resorptions and a decrease in newborn survival among wild-type mice born to treated animals. There were no alterations in parental reproductive organs.

Kang and Lee *(192)* treated male Sprague-Dawley rats orally for 4 weeks with phthalate diesters at 500 mg/kg bw/day or phthalate monoesters at 250 mg/kg bw/day. Animals were evaluated for body weight, testicular and epididymal weights, epididymal sperm count, and sperm motion parameters. There were no significant effects of treatment on body or reproductive organ weight. The order (strongest to weakest) for effects on sperm motility for the diesters was DEHP > DnOP > DEP > DUP > DIDP > BBP, and for the monoesters was MBuP > MEP > MEHP. **[Abbreviations were not defined but are assumed to be: DnOP = di-n-octyl phthalate; DEP = diethyl phthalate; DUP = diundecyl phthalate; DIDP = diisodecyl phthalate; BBP = butyl benzyl phthalate; MBuP = monobutyl phthalate; MEP = monoethyl phthalate.]**

Jayes and Davis *(193)* cultured granulosa cells from PMSG-stimulated immature rats. Medium containing FSH and testosterone was treated with MEHP at 0, 0.001, 0.01, 0.1, 1, 2, or 5 μM. Estradiol was assayed in media at 51 hours, and aromatase was measured in cells exposed to 0, 1, or 5 μM MEHP. Estradiol and aromatase were decreased by MEHP at 1 or 5 μM compared to controls.

4.4 Summary of Reproductive Toxicity Data

4.4.1 Human Data

There are 7 useful studies of adult male exposure to MEHP and various reproductive endpoints. The studies are consistent in that they do not identify any significant associations between MEHP and adverse semen parameters, hormone levels, time-to-pregnancy, or infertility diagnosis. However, in 2 of these studies, there were suggestions of associations between MEHP and decreased sperm velocity and serum testosterone.

Modigh et al. *(152)* evaluated time-to-pregnancy in the partners of men potentially exposed to DEHP. Exposure was estimated from employed subjects' description of work tasks and classified as *unexposed* pregnancies (n = 182, fathered by operators who were not exposed), *low-exposure* pregnancies (n = 100, fathered by men with estimated non-zero DEHP exposures < 0.1 mg/m^3), and *high-exposure* pregnancies (n = 44, by men exposed to DEHP > 0.1 mg/m^3). There was no association found between time-to-pregnancy and exposure group (median time-to-pregnancy was 3.0 months in the unexposed group, 2.25 months in the low-exposure group, and 2.0 months in the high-exposure group). The crude and adjusted fecundability ratios for the exposed pregnancies were all close to 1.0, and the 95% confidence intervals all overlapped unity. The Expert Panel noted that the exposures were not assessed using biologic markers, and misclassification of exposure was possible, biasing the results toward the null.

Duty et al. *(154)* evaluated urinary MEHP and semen analysis parameters in 168 men being evaluated in a clinic as part of a fertility evaluation. A single semen sample and single spot urine were collected

on the same day. There were no significant associations between abnormal semen parameters and MEHP urine concentration above or below the group median.

Duty et al. *(30)* evaluated urinary MEHP and sperm motion parameters in 187 male partners of couples presenting for fertility evaluation. A single spot urine was collected on the same day as the semen sample. Multiple linear regression analysis was used to evaluate the association between tertile of normalized urinary MEHP concentration and sperm motion parameters. There were no significant relationships between MEHP and sperm motion parameters, although there was a suggestion of an association with straight line velocity ($P = 0.08$).

Duty et al. *(155)* evaluated the association between urinary phthalate monoester concentrations and sperm DNA damage, assessed using the neutral comet assay. The 141 subjects were a subset of those described in Duty et al. *(30)*. Urinary phthalate monoester concentrations were adjusted for specific gravity and analyzed in quartiles using multiple linear regression adjusted for smoking status, race, age, body mass index, and abstinence interval prior to collection of semen. There were no associations between comet assay parameters and MEHP urinary concentrations.

Duty et al. *(156)* evaluated the relationship between serum concentrations of testosterone, sex hormone-binding globulin, inhibin B, FSH, and LH and phthalate monoester concentrations in spot urine samples. The subjects included 295 men attending a clinic as part of a fertility evaluation. No significant associations between MEHP and hormone levels were reported although there was a suggestion of a negative association between MEHP and serum testosterone ($P = 0.10$).

Hauser et al. *(158)* evaluated possible interactive effects of polychlorinated biphenyls and phthalate on sperm motility in male partners of couples seeking infertility evaluation. Both phthalate and polychlorinated biphenyl levels were dichotomized at the median as high or low, and sperm concentration was dichotomized as normal or abnormal based on World Health Organization criteria. There were no significant interactions between urinary MEHP and any of the polychlorinated biphenyls or groupings, either with regard to sperm motility or other sperm parameters.

The **Duty et al. *(30, 154-156)* and Hauser *(158)*** papers represented an overlap of subjects in a special population of men presenting for fertility evaluation. The studies generally had good evaluation of and control for potential confounders. Although the studies found no statistically significant effects for MEHP with sperm abnormalities (concentration, motility and normal forms), sperm motion, sperm DNA damage, or hormone levels, there were suggestions of dose-response with reduced sperm motility measures ($P = 0.08$) and marginal significance for the association of MEHP with reduced serum testosterone ($P = 0.10$).

Jönsson et al. *(85)* studied semen parameters and urinary phthalate monoester levels in 234 military recruits aged 18–21 years. Combined testicular volume was estimated based on ultrasound measurements, semen was obtained by masturbation, and spot urine samples were collected for measurement of MEHP. Seminal sperm were assessed for concentration and motility, including computer-assisted parameters, and were subjected to the sperm chromatin structure assay. Subjects were categorized into quartiles by urine concentration of individual phthalate monoesters (uncorrected and creatinine-adjusted), and ratios with 95% confidence intervals were calculated for highest:lowest quartile groups. There were no significant associations between highest versus lowest urinary MEHP quartile and any

of the dependent variables. They also found no evidence of interaction between phthalate metabolites and PCB-153 on testicular function.

4.4.2 Experimental Animal Data

4.4.2.1 Female

Mitsubishi Chemical Safety Institute, Ltd. ***(92)***, in an unpublished report, described a 65-week oral-dose toxicity study of DEHP in marmosets. DEHP was administered by gavage in corn oil to juvenile marmosets beginning at 90 – 115 days of age until 18 months of age (young adulthood) at dose levels of 0, 100, 500, and 2500 mg/kg bw/day. Both males and females were assessed with a battery of in-life hormonal assays and with histopathology at necropsy. In the female marmosets, DEHP at 500 and 2500 mg/kg bw/day was associated with early increases in serum 17β-estradiol consistent with an early onset of puberty, manifested as a significant increase in ovarian and uterine weights at necropsy in these groups.

4.4.2.2 Male

Male reproductive toxicity data in experimental animals are summarized in Table 34.

At dose levels of 10 and 100 mg/kg bw/day, **Akingbemi et al.** ***(111, 179***) showed reduced Leydig cell testosterone production ex vivo, increased serum LH, testosterone, and 17β-estradiol, and Leydig cell hyperplasia. The authors concluded, and the Expert Panel concurs, that DEHP increases Leydig cell populations associated with chronically increased LH and testosterone levels, and that a decrease in testosterone and increase in estradiol synthesis (per cell) was consistent with induction of aromatase activity in Leydig cells. The authors identified 1 mg/kg bw/day as a NOAEL and 10 mg/kg bw/day as a LOAEL.

In another study, 10-week-old male mice were given 0 or 2% dietary DEHP for 16 days following which all animals were given untreated feed for an additional 50 days with vitamin supplementation *(175*). **[CERHR calculated a mean DEHP intake during the treatment period of 2857 mg/kg bw/day.]** Groups of animals were killed during and after the end of exposure and assessed for testicular effects by weight and histology. By day 12 of exposure, testis weights were decreased and recovered towards control levels 20 days after cessation of exposure. Similarly, testis histopathology abnormalities were seen during treatment and recovered after the end of exposure. Groups of animals were killed during and after the end of exposure and assessed for testicular effects by weight and histology. By day 12 of exposure, testis weights were decreased and recovered towards control levels 20 days after cessation of exposure. Similarly, testis histopathology abnormalities were seen during treatment and recovered after the end of exposure.

Table 34. Summary of Male Reproductive Toxicity Data from Studies in Rats and Mice

Species and Dosing	*Most Sensitive Outcome*	*Effect Levels (mg/kg bw/day)*	*Reference*
Single Dose-Level Studies (excluding control)			
Mice, C57Bl6 wild-type and gld mutant MEHP 1000 mg/kg bw × 1 by gavage	TUNEL labeling greater in wild-type than fasL-deficient mutant mice	1000	Richburg et al. *(169)*
Mice, C57Bl6 wild-type and gld mutant MEHP 1000 mg/kg bw × 1 by gavage	Apoptotic response decreased in fasL-deficient mutant compared to wild-type mice	1000	Giammona et al. *(171)*
Mice, CD DEHP 2% in the diet for 16 days	Decreased testis weight, abnormal histopathology findings	**[2857]**	Ablake et al. *(175)*
Rat, Sprague-Dawley MEHP 1000 mg/kg bw × 1 by gavage	Increased expression of death receptors	1000	Giammona et al. *(171)*
Rat, Wistar MEHP 400 mg/kg bw × 1 by gavage	Germ cell sloughing, collapse of Sertoli cell vimentin filaments 3 hours after treatment	400	Dalgaard et al. *(172)*
Rat, Sprague-Dawley DEHP 2 mg/rat/day **[19,000 – 29,000 mg/kg bw/day]** × 1 – 14 days	Decreased body and testis weight, testis histologic evidence of apoptosis beginning treatment day 3	**[19,000 – 29,000]**	Park et al. *(173)*
Multiple Dose Levels			
Rat, Wistar DEHP 0, 1000, or 2000 mg/kg bw/day by gavage × 7 days	Decreased total glutathione, low molecular weight thiols, and ascorbic acid in testis	LOAEL 1000	Kasahara et al. *(174)*
Rat, Wistar DEHP 0, 100, or 1000 mg/kg bw/day by gavage × 5 days	Decreased aromatase and increased CYP2C11 and CYP3A2 testosterone hydroxylation in testis	LOAEL 100[a]	Kim et al. *(177)*
Rat, Long-Evans DEHP 0, 1, 10, 100, or 200 mg/kg bw/day by gavage × 14 or 28 days	Decreased 17α-hydroxylase in testis, altered ex vivo Leydig cell testosterone synthesis	LOAEL 10 NOAEL 1	Akingbemi et al. *(111)*
Rat, Long-Evans DEHP 0, 10, or 100 mg/kg bw/day by gavage × 70 or 100 days	Increased serum LH and testosterone, decreased ex vivo Leydig cell testosterone synthesis (PND 90)	LOAEL 10	Akingbemi et al. *(179)*

[a]The use of a recovery period in the dosing schedule prevented the Expert Panel from being able to interpret the results.

4.4.2.3 Male and Female

Reproductive toxicity data in male and female rats are summarized in Table 35.

Schilling et al. *(151)* performed a 2-generation reproductive study of DEHP in Wistar rats. DEHP was administered in feed at 0, 1000, 3000, and 9000 ppm, resulting in estimated DEHP intakes of 0, 113, 340, and 1088 mg/kg bw/day. The high-dose level (9000 ppm) was associated with a decrease in feed consumption and weight gain at several intervals during the study. An extensive evaluation was conducted, including a functional observation battery, water maze, estrous cyclicity, cauda epididymal sperm evaluation, testicular spermatid head counts, presence of nipples and areolae in male pups, and day and weight at vaginal opening and preputial separation. F_2 pups in the high-dose group were smaller and gained less weight from birth through the assessment of functional observation battery and water maze testing. Grip strength was reduced in males and hind-limb splay was reduced in both sexes in the high-dose group. Differential ovarian follicle counts of F_0 and F_1 adults showed a deficit in growing follicles and corpora lutea in the high-dose group. **[The Expert Panel noted dose-related increases in the number of animals showing focal tubular atrophy in both generations, beginning at the lowest dose (1000 ppm, ~113 mg/kg bw/day). The LOAEL for reproductive toxicity was considered by the Expert Panel to be 1000 ppm (the lowest dose in the study), which was also the NOAEL for systemic toxicity.]**

Mitsubishi Chemical Safety Institute, Ltd. *(92)*, in an unpublished report, described a 65-week oral-dose toxicity study of DEHP in marmosets. DEHP was administered by gavage in corn oil to juvenile marmosets beginning at 90–115 days of age until 18 months of age (young adulthood) at dose levels of 0, 100, 500, and 2500 mg/kg bw/day. Both males and females were assessed with a battery of in-life hormonal assays and with histopathology at necropsy. Although there were significant limitations in the study and the reporting of findings, the results suggest little effect of DEHP exposure at 2500 mg/kg bw/day in marmosets on testicular structure and function. Mean serum testosterone levels were highly variable, but the data suggested the possibility of a delay in the onset of puberty in male marmosets with increasing DEHP dose. The authors suggested, based on an accompanying pharmacokinetic study (reviewed in Section 2.1), that the most likely explanation for the lack of testicular toxicity of DEHP in marmosets as opposed to rodents was the limited accumulation of DEHP and its metabolites in the marmoset testis. This limited accumulation may in part be a result of the short gut transit time and propensity to diarrhea. In addition, the male marmoset has an apparent relative end-organ steroid resistance compared to humans. **[Because of the limited blood levels achieved in this study, differences between marmosets and humans in terms of their steroid resistance, and the difficulties in ascertaining the exact disposition of animals and inclusion of animals in the final data set, the male data from the Mitsubishi study are of limited ultimate use in the evaluation process.]**

The NTP *(114)* sponsored a multigeneration continuous-breeding study in rats. Sprague-Dawley rats were fed diets containing 1.5 (control group exposed to background DEHP levels), 10, 30, 100, 300, 1000, or 7500 ppm DEHP from the first day of the study until the day of necropsy. Due to a described lack of reproductive effects in the first litter produced, the study was repeated with 2 additional doses, 1.5 (control) and 10,000 ppm. Ranges of DEHP intake in the F_0, F_1, and F_2 animals were estimated at 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day. At about 5 weeks of age, F0 rats were fed the DEHP-containing diets for 6 weeks prior to mating and were then cohabitated for 9 weeks. The first 2 litters delivered during the cohabitation period (F_{1a}

and F_{1b}) were counted, weighed, assessed for anogenital distance, and discarded. The third litter (F_{1c}) was raised by the dam. Following weaning of pups, vaginal cytology was monitored in F_0 females for 14 days. After completion of crossover studies described below, at least 10 F_0 rats sex/group were necropsied. Sperm analyses were conducted, and organs were collected for histopathological evaluation. F_1 pups were counted, weighed, and examined for anogenital distance and nipple retention during the lactation period. On PND 16, 1 female per litter was evaluated for vaginal opening, and a second was selected for F_1 mating. One male per litter was selected for mating, and 4 or 5 males per litter were evaluated for testicular descent and preputial separation. At weaning on PND 21, pups were given diets containing the same DEHP concentrations as their parents. On PND 81, the F_1 rats chosen for mating (17/sex/group) were randomly assigned to breeding pairs (preferably non-sibling) and cohabited for 9 weeks. The study conducted in F_0 parents and F_1 offspring was repeated in F_1 parents and F_2 offspring, except that no F3 offspring were mated. Selected F_{3c} males were necropsied on PND 63–64 and selected females on PND 60–74.

The liver was identified as a target of toxicity with increases in liver weight and hepatocellular hypertrophy observed at dose levels ≥ 1000 ppm. Changes in organ weights and lesions were also observed in the kidney at ≥ 7500 ppm and the adrenal gland at 10,000 ppm.

Reproductive effects observed in the F_0 parents occurred only at 10,000 ppm and included decreases in sperm counts and velocity, reductions in testis and epididymis weights, and increased numbers of rats with small testes. The lowest dose level producing dose-related effects in F_1 offspring was 7500 ppm, and those effects included decreases in number of live pups/litter, reduced male anogenital distance, and delays in vaginal opening, preputial separation, and age at testicular descent.

F_1 rats from the 10,000 ppm group produced no viable litters. At and above 7500 ppm, rats had reduced sperm counts, seminiferous tubular atrophy, and delayed preputial separation and testicular descent. In the F_3 offspring, reproductive toxicity was noted in numerous endpoints at 7500 ppm but not at lower dose levels. Effects included seminiferous tubule atrophy and decreases in pregnancy index, the number of litters per pair, male reproductive organ weights, sperm counts, and sperm motility.

[The Expert Panel carefully considered the finding of small reproductive organ sizes by gross observations in non-mating F_1 and F_2 rats. The combined F_1 and F_2 data were reviewed to determine the occurrence of these alterations on a per animal and per litter basis across the dose range, as shown in Table 23 in Section 3.2.1.1. Based on the incidence of small reproductive organ size at necropsy, the Expert Panel considered 300 ppm (about 14–23 mg/kg bw/day) to be an effect level, giving a NOAEL of 100 ppm, about 3–5 mg/kg bw/day. The Expert Panel notes that Sertoli cell vacuolation, which was the endpoint driving the LOAEL in the original CERHR Expert Panel evaluation of DEHP, was not increased by DEHP treatment in this study.]

Appendix II

Table 35. Summary of Reproductive Toxicity Studies Involving Male and Female Rats

Species and Dosing	*Most Sensitive Outcome*	*LOAEL, ppm*	*NOAEL, ppm*	*Lowest BMDL[a], ppm*	*Reference*
Rat, Wistar DEHP in feed 0, 1000, 3000, or 9000 ppm × 2 generations DEHP intakes estimated as 0, 113, 340, or 1088 mg/kg bw/day)	↑Abnormal sperm ↓Male fertility ↓Testis size ↓Litter size and viability Deficit in growing follicles and corpora lutea	*Study authors:* 9000 **[~1088 mg/kg bw/day]** *Expert Panel:* 1000 **[~100 mg/kg bw/day]**, based on testicular histopathology	*Study authors:* 3000 **[~340 mg/kg bw/day]** *Expert Panel:* Not applicable	*10% level:* 2045 ppm **[~231 mg/kg bw/day]** based on F_2 pup survival PND 0–4	Schilling et al. *(151)*
Rat, Sprague-Dawley DEHP in feed at 1.5, 10, 30, 100, 300, 1000, 7500, or 10,000 ppm (the 10,000 ppm dose was run separately with its own 1.5-ppm control). Rats were treated over 3 generations using a continuous-breeding protocol for up to 3 litters/pair. DEHP intake was estimated at 0.09–0.12 and 0.47–0.78 for the 2 controls, 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day	Fertility and pregnancy indices, litter data, sperm parameters, sexual development, and histopathological changes in testes	*Study authors:* 2 mg/kg bw/day; occasional effects at 300 ppm were considered possibly treatment-related.) *Expert Panel:* 300 ppm (14–23 mg/kg bw/day)	*Study authors:* 1000 ppm (46–77 mg/kg bw/day) *Expert Panel:* 100 ppm (3–5 mg/kg bw/day)	*10% level:* 728 **[33–56 mg/kg bw/day]** based on F_3 sperm/cauda *1 SD level:* 412 **[19–32 mg/kg bw/day]** based on F_2 percent motile sperm	National Toxicology Program *(114)*

[a]Calculated by CERHR. See Table 22 for definitions and a discussion of the use of benchmark dose in this report.

↑,↓ Increase or decrease in parameter

CONCLUSIONS BASED ONLY ON LITERATURE APPEARING SINCE THE FIRST EXPERT PANEL REPORT

There is insufficient evidence in humans that DEHP causes male or female reproductive toxicity. There were 7 human studies judged to be useful for evaluating male reproductive toxicity. One study addressed occupational exposure and 6 addressed non-occupational exposure. Five of the 6 studies were conducted in the same population of men seeking evaluation for infertility, which may limit the studies' generalizability. There were suggestions of associations between specific male reproductive parameters and urinary MEHP but none reached a conventional level of statistical significance ($P < 0.05$). In the one study of an occupationally exposed group of men, no association was found with time-to-pregnancy. This study was limited by small sample size and indirect exposure estimates. There were no studies judged to be useful for evaluating female reproductive toxicity.

There is sufficient evidence in female rats to conclude that DEHP causes reproductive toxicity (decreased numbers of corpora lutea and growing follicles) with dietary exposure at 1088 mg/kg bw/day for multiple generations *(151)*.

There is sufficient evidence in female marmosets to conclude that DEHP causes reproductive toxicity (increased ovary weight and uterine weight) when exposure is by oral gavage at 500 mg/kg bw/day for ~15 months in the peripubertal period *(92)*. The Expert Panel found these data consistent with precocious puberty in the 2 highest dose DEHP-exposed groups (500 and 2500 mg/kg bw/day).

There is sufficient evidence in male rats to conclude that DEHP causes reproductive toxicity when exposure is by oral gavage or in feed at 10–113 mg/kg bw/day for exposures that included gestational and/or peripubertal periods. The critical effects are small reproductive organ size (14–23 mg/kg bw/day *(114)*), focal tubular atrophy (113 mg/kg bw/day *(151)*), and Leydig cell hyperplasia and altered reproductive hormones (10 and 100 mg/kg bw/day *(111, 179)*). The Expert Panel found the data suggestive of male reproductive toxicity caused by changes in the gonadal hormonal response as well as direct effects on Sertoli cells.

There is sufficient evidence to conclude that DEHP causes reproductive toxicity in adult male mice at dietary exposure levels of 2857 mg/kg bw/day as manifested by decreased testis weight and histopathologic alterations *(175)*.

The experimental animal data are assumed relevant for consideration of human risk.

Note: The definitions of the term sufficient and the terms assumed relevant, relevant, and not relevant are in the CERHR guidelines at http://cerhr.niehs.nih.gov/news/guidelines.html.

CONCLUSIONS FROM THE ORIGINAL EXPERT PANEL EVALUATION

The original Expert Panel report on DEHP contained conclusions in Section 5. These conclusions have been extracted and reproduced below, with the section numbering as found in the original document. The references listed in the conclusion are listed, and the table to which the conclusions refer is reproduced, numbered 76 as in the original.

5.1.4 Reproductive Toxicity

There are no data on the reproductive toxicity of DEHP or its major metabolites in humans.

5.1.4.1.1 Females

There are data that indicate that oral exposure to DEHP can affect reproductive processes in rats and mice. Data presented in [168] clearly show adverse functional effects at a dietary dose of 425 mg/kg bw/day where complete infertility was observed, although the design did not allow conclusions as to whether males, females, or both sexes contributed to reduced number of pups and pup viability. Only the data from [219] come close to evaluating broadly the effects on the female tract, and this study examined only a high dose. Davis et al. [219] showed clear effects on estradiol synthesis and ovulation in rats at 2000 mg/kg bw/day. No histopathological structural changes were seen in the uterus or vagina. Further, there are no studies that have evaluated adult female reproductive structure and function after prenatal exposure. Current data are not adequate to confidently ascribe a NOAEL or LOAEL for female reproduction (Table 76).

5.1.4.1.2 Males

The oral exposure studies of Lamb [168] and Schilling et al. [169] are sufficient to conclude that DEHP is a reproductive toxicant in male rats and mice. In the Lamb study [168], only the control and high-dose F_0 mice were necropsied; thus, it is not known if the reduced fertility at ~141 mg/kg bw/day (the middle dose) is partly male-mediated resulting from testicular damage. Schilling et al. [169] reported effects (androgen mediated and testicular lesions) at 1060 mg/kg bw/day in rats, and no effects (i.e. a NOAEL) at ~339 mg/kg bw/day. The study of Lamb [168] has a NOAEL of ~14 mg/kg bw/day (Table 76)… it is clear from the existing data that testicular pathology and reduced sperm numbers are consistent effects. The data are sufficient to conclude that DEHP is a reproductive toxicant in male rats, mice, ferrets, and guinea pigs when administered orally. There is greater uncertainty when determining lowest-adverse and no-adverse effect levels of exposure. The two studies [75,169] that used peripubertal dosing (believed to be the most sensitive period for causing adverse effects) and evaluated the rats when at, or close to, maturity (believed to be the most sensitive period for observing adverse effects) present markedly different NOAELs. The expert panel could not confidently reconcile these differences (~339 versus 3.7 mg/kg bw/day). Confidence in results observed at a given dose in [169] is eroded slightly by the small group size. The study of Poon et al. [75] was thorough in its design and execution, including verification of dose. While there were 20 animals per dose group, only 10 were males; the study design did not incorporate measures of reproductive

function. The authors were clear in asserting that "…the mild Sertoli cell vacuolation at 500 ppm (~38 mg/kg bw/day) should be considered an adverse effect." The expert panel finds there is a reasonable basis for such a conclusion (i.e. a LOAEL). The NOAEL from this study is therefore 3.7 mg/kg bw/day. When comparing the NOAELs from [75,168], 3.7 mg/kg bw/day versus 14 mg/kg bw/day, it is reasonable to conclude that these values are indistinguishable given the wide dose spacing and inherent biological variability in the endpoints. It is the panel's view that the existing data support a NOAEL within the range of 3.7−14 mg/kg bw/day for oral exposure in rats.

REFERENCES FROM THE ORIGINAL REPORT CITED ABOVE

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Table 76. Summary of DEHP Reproductive Toxicity Studies with Oral Exposure

Protocol & Study	NOAEL (mg /kg bw/day)	LOAEL and Effects (mg/kg bw/day)		Reproductive Effects at Higher Doses
		Reproductive	Systemic	
Continuous breeding and cross-over mating study 11-week- old CD-1 mice 20 pairs of mice Fed diets with DEHP (0, 14, 141, and 425 mg/kg/day) for 7 days prior to mating and during a continuous 98-day mating period. *(168, 186)*	*Reproductive:* 14 *Systemic:* Not known since lower dose groups not examined	141 Reduced fertility ↓Live pups per litter	425 ↑Liver weight	Complete infertility in females and reduced fertility in males Seminiferous tubule damage with adverse effects on sperm numbers, motility, and morphology ↓Male reproductive organ weights
Dose-setting two-generation dietary study Wistar rats. 10 pairs per group Received 0, 110, 339, or 1,060 mg/kg/day for 70 days prior to mating through gestation and lactation. *(169)*	*Reproductive:* 339 *Systemic:* None	1,060 ↓Anogenital distance in F_2 males ↓Ovary weight in F_0 ↓Testes and epididymis weight in F_1 Testicular lesions ↑Nipple development in F_1 males Spermatocyte loss in F_1 ↓Postnatal survival in F_1 pups and prenatal survival in F_1 and F_2 pups	110 ↑Liver weight in F_0 females	No higher doses
90 Day repeat-dose dietary study Sprague-Dawley rats. 10 rats/sex/group (4−6 weeks old) Received doses of: M: 0, 0.4, 3.7, 38, 375 F: 0, 0.4, 4.2, 42, 419 *(75)*	*Reproductive:* 37 (M) *Systemic:* 37−42 (M and F)	38 (M) Mild Sertoli cell vacuolation	38(M)−42(F) Liver enzyme effects	Atrophy of seminiferous tubules, loss of spermatogenesis, and Sertoli cell vacuolation

Protocol & Study	*NOAEL (mg /kg bw/day)*	*LOAEL and Effects (mg/kg bw/day)*		*Reproductive Effects at Higher Doses*
		Reproductive	*Systemic*	
1 – 12 day repeat-dose gavage study Female Sprague-Dawley rats. 6 – 10 rats per group (60 – 70 days old) received doses of 0 or 2,000 mg/kg/day. *(219)*	*Reproductive:* Not known since only one dose administered *Systemic:* Not examined	2,000 ↓Estradiol levels and suppression of ovulation	Not examined	No higher doses

5.0 SUMMARY, CONCLUSIONS, AND CRITICAL DATA NEEDS

5.1 Developmental and Reproductive Toxicity

5.1.1 Developmental Toxicity (Update)

One human study was considered useful for evaluating the association between maternal prenatal urinary MEHP excretion and anogenital index in male offspring. No significant association was found. Two human studies that were considered useful for evaluating DEHP exposure and development during childhood suffered from small sample sizes and problems in accurate exposure characterization. The data are insufficient to evaluate the prenatal or childhood effects of DEHP exposure in humans.

Developmental toxicity has been assessed in rats following exposure to DEHP during gestation and postnatally to sexual maturation. Most of the relevant rat studies focused on effects on male offspring. Data from a multigeneration study indicated that dietary exposures in the range of 14–23 mg/kg bw/day result in small and/or absent components of the male urogenital tract. At higher levels of both dietary and gavage exposure, effects on *in utero* survival, reduced anogenital distances, undescended testes, retained nipples/areolae, incomplete preputial separation, and disruption in spermatogenesis were evident in postnatal animals. Based on two studies that exposed neonatal male rats to DEHP by oral gavage, the critical period for effects on the testis extends into the immediate postnatal period. In one of the studies, decreased Sertoli cell proliferation was seen in male rats exposed by oral gavage to DEHP 100 mg/kg bw/day on PND 3. In the other study, neonatal rats were exposed by the IV route starting on PND 3–5 and continuing for 21 days. Exposures to 300 mg/kg bw/day and higher resulted in decreased testis weight, depletion of germinal epithelium, and decreased seminiferous tubule diameter. The reduced testis weights persisted through at least 90 days of age. The NOAEL for this study was 60 mg/kg bw/day. The findings from the IV route were similar to those observed after oral exposure to the same dose levels.

These data are sufficient to conclude that DEHP is a developmental toxicant in rats by the dietary, oral gavage, and IV routes of administration at the indicated dose levels. These animal data are assumed relevant to the assessment of human risk.

5.1.2. Reproductive Toxicity (Update)

Several human studies have examined the relationship between adult male exposure to MEHP and various reproductive endpoints. While there were suggestions of associations between MEHP and decreased sperm velocity and serum testosterone, no significant associations were found between MEHP exposure and adverse semen parameters, hormone levels, time-to-pregnancy, or infertility diagnosis. There were no studies of adult female exposure that were judged to be useful. The data are insufficient to evaluate the reproductive effects of DEHP exposure in humans.

There are data to indicate that oral exposure to DEHP can affect reproductive processes in female marmosets. The Mitsubishi study found an increase in serum 17β-estradiol at weeks 52 and 65, and an increase in ovary and uterine weights at necropsy in the 500 and 2500 mg/kg bw/day groups *(92)*. There were no other new studies on female reproductive function.

The oral exposure studies of Akingbemi et al. *(111, 179)*, Schilling et al. *(151)*, and the NTP *(114)*

are sufficient to conclude that DEHP is a reproductive toxicant in male rats at the indicated dose levels. All of those data are assumed relevant. Despite some internal inconsistencies in the Akingbemi studies, the data provide a plausible explanation for the Leydig cell hyperplasia observed in other studies, with a LOAEL of 10 mg/kg bw/day and a NOAEL of 1 mg/kg bw/day. These data are assumed relevant for human reproduction. The Schilling et al. data show an effect of DEHP exposure (increased seminiferous epithelium vacuolation) at ~113 mg/kg bw/day in male rats in a multigeneration study. The NTP study found an increase in grossly small male reproductive tissues at necropsy in multiple generations, which was first seen at 300 ppm in feed (~14–23 mg/kg bw/day) and which increased in a dose-related manner at higher doses. These effects are in the range of effects observed by Poon et al. *(194)* and cited in the previous Expert Panel Report on DEHP, which found seminiferous epithelium vacuolation at 500 ppm (~38 mg/kg bw/day) in a multigeneration study in rats. Despite a less-than-thorough evaluation of all the animals born into the NTP study, the NOAEL in that study was 4.8–7.9 mg/kg bw/day, which is also below the LOAEL in Akingbemi et al. The convergence of data from the NTP study, Akingbemi, and Poon around the 10–30 mg/kg bw/day range gives added confidence that this is the range of the lowest effective dose level. It is the panel's view that the existing data support a NOAEL between 1 and 10 mg/kg bw/day for oral DEHP exposure in rats.

5.2 Human Exposure

The information in this report is an update of the first Expert Panel Report on DEHP, and the reader is referred to the Summary of Exposure in Section 5.1 of that report for background and context for the following remarks.

DEHP is ubiquitous in the environment. Humans can be exposed to DEHP through many routes including ingestion (food, infant formula, and breast milk), contact with contaminated household dust and consumer products (cosmetics and toys), inhalation, and through medical procedures. The largest source of general population exposure to DEHP is dietary. Food surveys show a range of DEHP content with fatty foods, including dairy, fish, meat, and oils, containing the most.

DEHP is currently the primary phthalate plasticizer used in PVC-containing medical devices. Medical exposures can be IV, oral, and inhalational, and the exposures can be either DEHP alone or mixtures of DEHP and MEHP.

Exposures to DEHP can be estimated using probabilistic calculations from measurements in environmental matrices or dose reconstruction from urinary metabolite measurements. Probabilistic estimates can accurately estimate exposures if all routes/pathways of exposure are accounted for and the environmental matrices in these pathways are well-characterized. Dose reconstruction from urinary measurements can also accurately estimate exposures if the toxicokinetics are well-defined and reasonably stable. For both exposure estimate methods, uncertainties exist. For example, for probabilistic estimates, other pathways of exposure may contribute significantly such as medical exposures, occupational exposures, some indoor air exposures and potentially exposure from mouthing of DEHP-containing objects. Similarly, dose reconstruction is limited because the toxicokinetics vary within and among persons and the proportion of any given metabolite attributable to the total fraction excreted may vary within and between persons as well. In addition, a steady state excretion is assumed whereas the urinary measurement may have captured either a peak or background exposure. The limitations listed above for both dose estimation methods lead to uncertainties in exposure estimates; however, both the

probabilistic and dose reconstruction approaches agree within an order of magnitude suggesting that both methods are appropriate for estimating dose ranges with a reasonable degree of certainty.

For the purposes of comparison in this report, the Expert Panel relied primarily on dose reconstruction from urinary metabolites to estimate DEHP daily intake. The Expert Panel used the full range of reported urinary MEHP concentrations in determining exposure ranges; however, the literature provided 2 separate approaches for dose reconstruction that differ primarily based upon the excretion fraction of MEHP. Both metabolite excretion fraction estimates were based upon limited data, and at this time, it is not possible to determine which excretion fraction estimate is most accurate. Thus, the Expert Panel chose to include both dose reconstruction approaches in determining a range of exposure for the US population and selected subpopulations. In determining exposure ranges from dose reconstruction, the Expert Panel used the median or geometric mean concentration of MEHP and the 95th percentile of the distribution of MEHP in each study to estimate the exposure range (Table 36).

Table 36. Estimated DEHP Dose Ranges for Selected US Population Groups

Population Group		***Estimated dose range (µg/kg bw/day)***
General population [a]	20+ years	1 – 30
	12 – 19 years	1 – 25
	6 – 11 years	1 – 30
	< 6 years	Unknown [c]
Medical exposures, neonates [b]		130 – 6000

Lower range estimate is median or geometric mean values estimated using the excretion fraction used by David (37). Upper range estimate is 95th percentile using the excretion fraction used by Koch (31, 36), which gives higher values.

a Based upon NHANES 2001 – 2002 data (n = 2782) (27).

b Based upon Calafat 2004 (n = 6) (5).

c US population-level urinary excretion data not available; the previous Expert Panel concluded that the level could be several-fold higher than in the general adult population.

The general population dose estimates based upon the most recent US urinary MEHP data are similar to those contained in the previous report. In addition, recently published probabilistic estimates for general population doses agree well with the ranges presented in Table 36, providing additional support for these estimates. However, dose estimates from medical procedures in neonates using both dose reconstruction and probabilistic estimates are highly variable, depending largely upon the medical treatments given and the duration of the treatments. Recent dose estimates are shown in Table 37.

Pregnant and lactating women represent a population of special concern because of the potential impact of their exposures on the fetus and nursing infant. For example, data suggest that metabolites cross the placenta and enter breast milk in free form. In addition, women undergoing certain medical procedures during pregnancy or lactation may increase their exposures significantly above the general population level, thus potentially resulting in higher exposures to the fetus and nursing infant. Another potential source of infant exposure is from expressed and stored breast milk that has been contaminated by DEHP-containing breast pumps.

Table 37. Dose Estimates for DEHP/MEHP Exposures from Medical Procedures in Neonates

Dose Estimation Method	*Estimate (µg/kg bw/day)*	*Reference*
Dose reconstruction	130 – 6000 [a]	Calafat et al. *(5)*
Dose reconstruction	1 – 170 [a,b]	Green et al. *(63)*
Probabilistic	7000	Loff et al. *(56)*
Probabilistic	2800	FDA *(2)*
Probabilistic	800 – 2000	Kambia et al. *(59)*

[a] Estimated by Expert Panel according to the methods used in Table 36.

[b] Upper bound is 75th percentile.

5.3 Overall Conclusions

The Expert Panel noted that DEHP and some other phthalates have been shown to act through the same mode of action and to induce similar effects in exposed animals. The combined effects of multiple phthalate exposures have implications for exposure and risk assessment. The conclusions in this report assume exposure only to DEHP.

5.3.1. General Adult Population

First Report:

DEHP conversion to active MEHP involves intestinal lipases that appear to be at significantly greater levels in rodents than in primates; adult rodents require 1 – 2 orders of magnitude more DEHP than is required in juvenile rodents to produce testicular effects; and adult marmosets (primates) showed no testis effects when exposed to DEHP at oral doses (2500 mg/kg bw/day) for 13 weeks, conditions that produce testicular toxicity in juvenile rodents. Based on these data, the panel has minimal concern that ambient human exposures adversely affect adult human reproduction. This level of concern is not appreciably altered for adults medically exposed to DEHP or MEHP.

Update:

Data not considered in the earlier report demonstrated that humans have ~2 – 3-fold lower levels of intestinal lipases than ferrets and rats. New information suggests that marmosets may be less susceptible to hormonal disruption, a key feature of DEHP toxicity, than most other species, including rats and humans. In the absence of significant new adult rodent toxicity data, the previous Lamb et al. *(195)* data remain informative: a LOAEL of 425 mg/kg bw/day, orally in the diet. Based on these data, the panel has minimal concern that general population exposures adversely affect adult human reproduction. In addition, there have been some, albeit insufficient, human studies addressing reproductive effects under general population exposures, and these do not increase our level of concern. This level of concern is not appreciably altered for adults medically exposed to DEHP or MEHP. This conclusion concurs with the conclusion of the first DEHP Expert Panel.

5.3.2 Healthy Infants and Toddlers

First Report:

DEHP produces testicular toxicity at lower doses in juvenile rodents than in adults; the reproductive system is (and specifically, the Sertoli cells are) still in proliferative mode until puberty, and reproductive system

development has been shown to be sensitive to MEHP in rodents; intestinal lipase activity is found at adult levels in babies older than 6 months of age. All of these points increase the level of concern. While adult marmosets showed no testicular toxicity at doses that produced toxicity in adult rats, no data are available for infant primates. If healthy human infant/toddler exposure is several-fold higher than adults, the panel has concern that exposure may adversely affect male reproductive tract development.

Update:
There are 2 new studies on the effects of DEHP following postnatal only exposure of rats. DEHP was shown to induce testicular toxicity following oral exposure of neonates, and the susceptibility of the testes of the young animal was confirmed in these studies to be greater than that for adults. In the first study *(120*), the BMDL10 for the effect on testicular weight was 77 mg/kg bw/day (there was no NOAEL in this study; the lowest dose was 300 mg/kg bw/day). In the second study *(119*), a NOAEL of 20 mg/kg bw/day was found for effects on Sertoli cell proliferation.

There are no data for exposure levels of healthy infants under the age of one year. Exposure of children aged 1–6 years has been estimated to be up to several-fold higher than the general population exposure estimate of 1–30 μg/kg bw/day. There are indications that younger children have elevated ratios of the metabolites of MEHP in their urine compared to older individuals, suggesting differential internal exposure to the metabolites of DEHP. The toxicity of these metabolites has not been studied in this age group.

If the level of exposure is at the high end of the estimated range, the Panel has some concern that exposure to DEHP can adversely impact reproductive development in male children older than 1 year. The Expert Panel has concern that DEHP exposure can adversely affect reproductive development in infants less than 1 year old because of their greater susceptibility and uncertainties regarding exposure. This conclusion is a refinement of the first Expert Panel's conclusion in distinguishing concern by age group within the infant-toddler category.

5.3.3 Critically Ill Infants

First Report:
Documented parenteral medical exposure to DEHP of critically ill infants can exceed general population exposures by several orders of magnitude; parenteral exposures to DEHP involving blood and blood products include concurrent exposure to MEHP; the most sensitive process (reproductive system development) is still occurring; human parenteral exposures can approach the rat parenteral NOAEL. On the other hand, concern is lowered by the fact that there is less conversion of DEHP to MEHP by the parenteral route of exposure, although the exact degree of reduction is not known. It is not known if primate Sertoli cells are more, equally, or less sensitive than rodent Sertoli cells to the effects of MEHP. The available reproductive and developmental toxicity data and the limited but suggestive human exposure data indicate that exposures of intensively-treated infants/children can approach toxic doses in rodents, which causes the panel serious concern that exposure may adversely affect male reproductive tract development. The panel recognizes that benefits of medical procedures can outweigh any risks.

Update:
New information on the mechanism of DEHP-induced developmental toxicity focuses on gonadal endocrine effects. Recent additional human studies confirm our previous assumptions and concerns that intensively medically treated infants are exposed to doses that are toxic in rodents. The Panel has

serious concerns that such exposures may adversely affect male reproductive tract development and function. The Panel believes that the benefits of medical procedures can be significant but that minimizing exposure to DEHP should be a goal. This conclusion concurs with that of the first Expert Panel.

5.3.4 Pregnancy and Lactation

First report:

In utero development is a life stage of particular vulnerability; exposures may be on the order of 3 – 30 µg/kg bw/day; the most relevant rodent data suggest a NOAEL for testis/developmental effects of 3.7 – 14 mg/kg bw/day; DEHP produces malformations in rodents, with a NOAEL of ~40 mg/kg bw/day; even time-limited exposures are effective at producing irreversible effects; the active toxicant MEHP passes into breast milk and crosses the placenta. On the other hand, absorption from the primate gut appears to be less effective than from the rodent gut, which reduces the level of concern for oral exposure. Given that oral exposure is < 30 µg/kg bw/day for humans and toxic effects are seen in rodents at > 3 mg/kg bw/day in rodents, even in the face of significant species differences in absorption, the panel has concern that ambient oral DEHP exposures to pregnant or lactating women may adversely affect the development of their offspring.

Update:

Since the last report, 4 useful studies have been conducted involving exposure of rats to DEHP during gestation, 2 of which are multigenerational. Based upon a constellation of effects on the developing male reproductive tract and accessory sex organs seen across studies, a mode of action involving anti-androgenicity has been confirmed. Numerous other mechanistic studies support these observations. The lowest LOAEL identified for effects on testicular tract development was 14 – 23 mg/kg bw/day, with a NOAEL of 5 – 8 mg/kg bw/day *(114*). This level was determined by the Expert Panel based upon an overall assessment of response across generations. Estimates of exposure to DEHP for the adult human population range from 1 to 30 µg/kg bw/day.

Based upon the projected level of exposure and the toxicity observed in the offspring of treated rats, the Expert Panel has some concern for the effects of DEHP on male offspring of humans exposed to general population levels during pregnancy. This reduction in level of concern from that of the first Expert Panel is due to greater confidence in population exposure levels and greater confidence in the effect level in experimental animals. The Expert Panel notes that it has concern for possible effects on male fetuses of women undergoing certain medical treatments where additional exposure to DEHP could occur. Lactation concerns are expressed in Section 5.3.2., above, with reference to children under 1 year of age.

5.4 Critical Data Needs

1. ***First report:***

   Identification and follow-up studies of human populations (e.g., premature infants) who were heavily exposed to DEHP. This would directly address the issue of whether there are functional effects in the most heavily and simultaneously the most vulnerable, human population. This would consist primarily of follow-up evaluation of reproductive system development and function.

   Update:

   There is one study by Rais-Bahrami et al. *(109*) that evaluated sexual development and serum hormone levels in adolescents that had been subjected to ECMO treatment. However, the panel

found this study insufficient to draw conclusions regarding human developmental toxicity. This data need continues.

2. ***First report:***
Obtain better medical exposure data. Common clinical research designs with unified analysis approaches across centers, as are often used in the large group cooperative studies of cancer therapy, would be one approach to acquiring better data. Potential toxicity from medical exposures could also be evaluated using the multi-center model to study DEHP/MEHP exposed neonates and adults longitudinally over decades to capture the reproductive, developmental, and other outcomes of concern based upon animal toxicity studies. Finally, discussions with the manufacturers of the medical devices used in these procedures would be helpful to determine whether and how much the formulations of PVC blood bags, ECMO circuits, hemodialysis machines, and other medical devices that contain DEHP or MEHP have changed over time.

Update:
There are additional data on DEHP release from medical devices (e.g., *55, 56, 59, 61*). The panel also identified 3 studies *(5, 63, 84*) that better characterized medical exposures in infants undergoing intensive medical procedures. In these studies, DEHP urinary metabolite levels were measured, thus providing information on DEHP exposures through multiple routes. Studies with larger numbers of subjects relating the nature of the procedures to exposure levels using measures of internal dose with multiple metabolites are needed.

3. Significance of perinatal exposure:

3.1 ***First report:***
Dose-response of male and female reproductive tract malformations. There is a need to gather dose-response data across a wider range of lower exposures in dam and pups in order to correlate blood levels of MEHP with reproductive effects.

Update:
An NTP continuous breeding study *(114*) examined male and female reproductive organs in rats fed DEHP in the diet at concentrations of 10 – 10,000 ppm (0.47 – 775 mg/kg bw/day). The doses address the range of estimated exposures in infants undergoing intensive medical procedures. The dose range included 3 dose levels below the LOAEL (300 ppm) at which developmental toxicity was observed. Information on MEHP blood levels and reproductive effects remains limited.

3.2 ***First report:***
Relevant animal model (*in utero* reproductive tract maturation) in guinea pig or non-human primate to correlate dose with effects, if any, and compare these doses with those of rodents where adverse effects do occur.

Update:
There are no new studies further addressing prenatal developmental toxicity in a non-rodent species. This is still a data need. A pre/postnatal study in a cynomolgus monkey would be most useful.

3.3 ***First report:***

Timing, PPAR, metabolism.

Update:

There are no new studies examining the effects of timing of exposure on developmental toxicity. There are no new studies examining the possible role of PPAR on developmental toxicity. The Expert Panel considers PPAR to be a less critical data need given studies showing that the mode of developmental toxicity appears to be independent of this class of receptors. There are 3 studies that examined metabolism and/or toxicokinetics in pregnant animals. Two studies were in rats *(86, 94*) and one in marmosets *(92*).

4. ***First report:***

Extension of PBPK model to

- Pregnancy, because this is the human group thought to be most at risk.
- Species (marmoset/human), as humans are the species of interest. The marmoset data provide a positive control to show that the PBPK model works as advertised.
- ADME, specifically phase l and phase II metabolism extended across species, and into pregnant humans.
- In order to acquire better data on primate/human toxicokinetics, including immature animals and humans, there is a need for a fetal compartment in the PBPK model and rate constants for fetuses and newborns for absorption, metabolism, and excretion.
- Model DEHP/MEHP dose for IV exposure. This is another route of exposure for a great many people who may have reduced capability to clear the compound.

Update:

There are no new relevant publications on PBPK modeling and this remains one of the most important data needs. As noted above, the most desirable study would include the cynomolgus monkey.

Additional data needs identified by the current Expert Panel include:

- *In vitro* and *in vivo* metabolic data including information across ages and species on lipase, cytochrome P450, glucuronyl transferase, and dehydrogenase enzyme kinetics. There is a critical lack of human *in vitro* data.
- Gene expression and enzyme induction studies on DEHP and its metabolites, particularly in DEHP target tissues and human cell types to better understand dose-response relationships.
- Comparison of C_{max} and AUC measurements of exposure to better understand the appropriate dose metric for toxicity extrapolation.
- Human studies evaluating endocrine-mediated effects in males with consideration of confounding effects and with larger sample size.
- Additional exposure and toxicokinetic information on pregnant women, infants, and children 1 – 6 years of age. This information should include exposure in breast milk, including potential contamination from breast pumps.
- Investigation of mode/mechanism of action.
- Effects of mixtures of phthalates including questions of additivity and interference.

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National Toxicology Program
U.S. Department of Health and Human Services

Center For The Evaluation Of Risks To Human Reproduction

PUBLIC COMMENTS ON THE EXPERT PANEL UPDATE ON DI(2-ETHYLHEXYL) PHTHALATE

Appendix III

Health Care Without Harm

the campaign for environmentally responsible health care

CAMPAIGN HEADQUARTERS

1901 N. MOORE STREET
SUITE 509
ARLINGTON, VA 22209
T: 703.243.0056
F: 703.243.4008
EMAIL: INFO@HCWH.ORG
WWW.NOHARM.ORG

Dr. Michael B. Shelby, Director
Center for Evaluation of Risks to Human Reproduction
NIEHS
P.O. Box 12233, MD EG-32
Research Triangle Park, NC 27709

February 2, 2006

Re: NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl)phthalate – November 2005

Dear Dr. Shelby:

On behalf of Health Care Without Harm (HCWH), we submit these comments on the NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl-phthalate, issued November 2005.

Health Care Without Harm agrees with the expert panel's conclusions that:

- DEHP is a reproductive and development toxicant in animal studies that are relevant to humans;
- Health care delivery can be a significant source of DEHP exposure;
- Levels of DEHP exposure in sick infants receiving medical care are of serious concern; and
- Because DEHP crosses the placenta, exposures in pregnant women receiving medical treatments are also of concern.

We also emphasize that the expert panel's experiences with the unpublished Mitsubishi and BASF (Schilling) studies confirm the importance of carefully reviewing study design, raw data, data handling, data interpretation, and authors' conclusions if such studies will continue to be used by future CERHR and NTP panels. As the Expert Panel noted, for unjustified reasons the authors of the marmoset study removed from data analysis specific animals that apparently showed adverse impacts of DEHP exposure. Moreover, the study authors failed to examine the histopathology of the testis of each animal as required in their study design. The Expert Panel also concluded that the BASF/Schilling study showed histopathologic impacts of DEHP in the testes of animals in all treatment groups and that no NOAEL was identified in this study. The authors of the original paper provided no justification for ignoring those pathologic changes in the lowest dose treatment group of animals.

Detailed comments follow and are tracked in the PDF document attached. (Our comments are in page order.)

Page iii: In the introductory section to the Panel's conclusions, the report indicates that there was not consensus around reducing the concern level for pregnant women. We agree with some of the panel members, that the level of concern for pregnant women identified in the 2000 expert panel report should NOT be reduced. It is our position that pregnant women's exposure remains a concern, as stated, because:

> 1) MEHP passes the placenta in free form where it may not be detoxified by the fetus, 2) exposure throughout pregnancy is not necessary to cause damage in animal models, and 3) current exposure estimates in women of child bearing age do not distinguish peak or episodic exposures from average exposures.

Page 4, Table 5: It does not look like the data are expressed as percentages but rather as microgms/kg/day. Which is correct? The later reference on page 26 that "more than 90% of the intake is from food" is not consistent with Table 5. Table 5 indicates that less than 90% intake *for infants* is from food.

Page 11, Figure 2: It is not clear what the "x" axis represents on the graph.

Page 26 – The statement that estimates the percentage intake of DEHP from food is not accurate for infants (See comment for page 4, Table 5 above).

Page 27: The report does not indicate what the expert panel thinks about the validity of David's argument and Koch's response. Is there any other research to support David's case? The expert panel report should explicitly note that David has a conflict of interest inasmuch as he has financial ties to the phthalate manufacturing industry.

Page 55: In reference to the Rais Bahrami, et al. study, the panel identifies the utility of the study as minimally significant due to the small sample size and the lack of a comparison group. A major limitation of the study is the lack of measurements of phthalate exposure. The panel report should explicitly note this important limitation of the study.

Page 98, Table 28: In reference to the developmental LOAEL, the panel's conclusion for the LOAEL differs from the author's conclusion, which is apparently quoted in the Table. It is important to note that the Table reflects the authors' conclusion and does not reflect the panel's conclusion, especially so that the subsequent panel discussion of Schilling in Section 4 does not appear to be in contradiction to what is noted here.

Page 143: In reference to the discussion of the Schilling study, the note in the "utility" section states that the study was useful in the evaluation process, showing a LOAEL of 1000 ppm (~100 mg/kg bw/day). This was the lowest dose tested. The panel, however, goes on to say that since this is a conclusion of the Expert Panel and not the authors', it is a cause for concern and limits the confidence that this conclusion can bear. We disagree that the panel's conclusion bears

limited confidence. A review of the original paper clearly shows histological impacts that the authors simply ignored when deriving their conclusion. Adverse impacts of DEHP exposure are discernable at every dose tested.

Page 169: In the section updating the science on reproductive toxicity, the report should make it clear that in the Schilling study ~113 mg/kg/day was the lowest dose tested.

Page 171: In reference to the Schilling study, the "update" states, "Data not considered in the earlier report demonstrated that humans have ~2–3-fold lower levels of intestinal lipases than ferrets and rats." We are unable to identify a reference for this in the draft report. The summary of Ito et al. (page 41) discusses rodents and marmosets, but *not* humans. The 2001 FDA "Safety Assessment of DEHP Released from Medical Devices" states (page 31):

> "Consequently, individuals with high rates of lipase activity and/or low rates of glucuronidation activity could be at higher risk of DEHP-induced adverse effects than the rest of the population. Polymorphisms in genes coding for pancreatic (Hegele et al., 2001) and hepatic (Cohen et al., 1999) lipase in humans are known to exist and these polymorphisms can result in lipase deficiency. Low lipase activity would be expected to exert a protective effect in these individuals with regard to DEHP-mediated effects. Conversely, pancreatic lipase activity is increased by heparin administered to patients on hemodialysis (Montalto et al., 1997) and plasma lipoprotein lipase activity is increased by erythropoietin (Goto et al., 1999), which is also administered to patients on hemodialysis. Increased lipase activity would facilitate the conversion of DEHP to its active metabolite. Smoking is also known to increase lipase activity (Kong et al., 2001) and DEHP itself induces lipase activity in rodents (Mocchiutti and Bernal, 1997). Consequently, some individuals in the DEHP-exposed population can convert DEHP to MEHP more efficiently than others. This variability is evidenced, to some extent, by the variability in the rate at which intestinal mucosal cell preparations obtained from two humans hydrolyzed a number of di-n-alkyl phthalates (Lake et al., 1977). The metabolic rates between these two individuals differed by around 3- to 6-fold. Presumably, the degree of variability would increase with a larger sample size."

We also note that ref 96 in the Expert Panel report discusses differences in phthalate hydrolysis among non-human primates.

> It is also important to keep in mind the absorption and serum levels of DEHP metabolites reported by Koch after he ingested labeled DEHP. Levels were comparable to those in marmosets given far higher doses. This suggests that there may be differences between humans and marmosets with respect to intestinal or pancreatic lipase levels and marmosets should not be assumed to accurately reflect levels in humans without empirical data supporting that conclusion. Moreover, intestinal and pancreatic lipase is inducible and in vitro assays will not accurately portray in vivo circumstances.

Page 175: In the section identifying additional data needs, we also suggest noting a need for human *in vivo* data. Enzymes are inducible and vary with diet and co-exposure to xenobiotics

capable of enzyme induction. These variables may influence individual susceptibility to DEHP exposure.

Thank you for considering our comments. We look forward to seeing the CERHR/NTP monograph on DEHP.

Sincerely,

Ted Schettler, MD, MPH
Science and Environmental Health Network
(978) 462-4092

Anna Gilmore Hall, RN
Executive Director, Health Care Without Harm
(703) 505-3239

Julie Silas, JD
Co-Chair, Safer Materials Workgroup, Health Care Without Harm
(510) 594-8270

B BRAUN
SHARING EXPERTISE

February 2, 2006

Dear Dr. Shelby:

B. Braun Medical Inc., a global leader in safety-minded and environmentally-sound hospital products, commends the findings of the expert panel convened by the National Toxicology Program (NTP), Center for the Evaluation of Risks to Human Reproduction (CERHR). We share the panel's concerns regarding the potential impact of DEHP on reproductive development in critically ill infants, and in women pregnant with male fetuses who are undergoing medical treatments where high exposure to DEHP could occur. B. Braun applauds the NTP/CERHR for convening this second independent panel of experts to review and update its previous report.

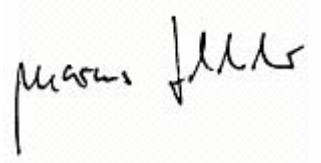
At B. Braun, our commitment to patient and environmental safety is evident in our products, which provide clinicians the ability to administer their infusion therapy needs with devices that are manufactured with DEHP- and PVC-free alternatives. B. Braun encourages all efforts to reduce and, wherever possible, eliminate actual or potential toxic substances such as DEHP in all medical products.

Today, B. Braun is the only full-line manufacturer of basic IV solutions in PVC-free and DEHP-free containers, and a leader in patient-safety technology and policy. The two major critical-care products B. Braun markets – EXCEL® and PAB® IV Containers – are manufactured with nontoxic, biologically inert copolymers of ethylene and propylene, and provide the most drug-compatible IV containers available.

In 2003, B. Braun recommended that all medical product companies minimize DEHP use. B. Braun urged the U.S. Food and Drug Administration to draft and support a regulation to mandate labeling for all medical devices plasticized with DEHP, allowing clinicians to make informed decisions when using products containing DEHP in clinical practice.

Until there are revised national standards for DEHP leachate levels or requirements to label products that leach DEHP, healthcare professionals will need to evaluate the data and make their own determination of the risks involved. As these caregivers consider available options, and the possible impact on patients' long-term health, B. Braun will continue to be at the forefront of evaluating and producing products that are DEHP-free.

Sincerely,



Marcus Schabacker, MD, PhD
Corporate Vice President
Research and Development and Medical and Clinical Affairs

American Chemistry Council
Good Chemistry Makes It Possible

February 3, 2006

VIA EMAIL: shelby@niehs.nih.gov

Dr. Michael D. Shelby
CERHR Director
CERHR
79 T.W. Alexander Drive
Building 4401, Room 103
Research Triangle Park, NC 27709.

Dear Dr. Shelby:

The American Chemistry Council Phthalate Esters Panel (PE Panel) is submitting the attached comments on the November 2005 *Expert Panel Update on the Reproductive and Developmental Toxicity of Di(2-ethylhexyl) Phthalate* (Final Update) to assist the NTP-CERHR in its review of the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate (DEHP) and in response to NTP's request for comments on the Final Update. 70 Fed. Reg. 69567 (Nov. 16, 2005). The PE Panel includes the major domestic manufacturers of phthalate esters and some users.

The PE Panel appreciates the Expert Panel's work in preparing the Final Update and believes that the Final Update, in general, provides a reasonable summary of the new information that has become available on DEHP since the first Expert Panel review in 2000. The PE Panel believes that the Final Update suffers from several shortcomings due to some aspects of the process by which the Final Update was produced, and that some of the scientific conclusions in the Final Update are not supported by the scientific data for DEHP. In particular, the PE Panel has obtained opinions from two experts in marmoset toxicology, which indicate the Final Report understates the value of recent marmoset data for evaluating human male testicular development, while it overstates the value of the marmoset data for evaluating human female reproductive development.

For the reasons discussed in the PE Panel's comments on the August 2005 Draft Update, and the comments presented here, the PE Panel believes that the available information for DEHP supports a conclusion that the overall concern for risk to human reproduction from DEHP exposure is minimal.

If you have any questions, or if you need any further information, please call Marian K. Stanley, Senior Director and Manager of the Phthalate Esters Panel, at (703) 741-5623, email her at marian_stanley@americanchemistry.com, or write her at the address below.

Sincerely yours,

Hasmukh Shah

Hasmukh C. Shah
Acting Vice President, CHEMSTAR

Attachments

Responsible Care®

Comments of The
American Chemistry Council Phthalate Esters Panel
On the Final NTP-CERHR Expert Panel Update On The
Reproductive And Developmental Toxicity Of Di(2-Ethylhexyl) Phthalate

February 3, 2005

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INTRODUCTION AND SUMMARY

The American Chemistry Council Phthalate Esters Panel (PE Panel) submits these comments on the November 2005 *Expert Panel Update on the Reproductive and Developmental Toxicity of Di(2-ethylhexyl) Phthalate* (Final Update) to assist the NTP-CERHR in its review of the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate (DEHP) and in response to NTP's request for comments on the Final Update. 70 Fed. Reg. 69567 (Nov. 16, 2005).[1] The PE Panel includes the major domestic manufacturers of phthalate esters and some users.[2]

The PE Panel believes that the Final Update, in general, provides a good summary of the new information that has become available on DEHP since the first Expert Panel review in 1999-2000. However, the PE Panel also believes that the Final Update suffers from several shortcomings that NTP-CERHR should take into account while reviewing the reproductive and developmental toxicity of DEHP. The following comments and suggestions are intended to assist NTP-CERHR in its review and use of the Final Update and to enhance the robustness of NTP-CERHR's conclusions. Attached to these comments are expert opinions by Professor Stefan Schlatt and Dr. Suzette Tardif, both scientists with extensive experience in the use of marmosets for toxicology. Those opinions provide perspectives that differ from those of the Final Update and which should be seriously considered by the NTP-CERHR.

These comments make the following points:

- The Expert Panel review process, while well-executed in many respects, suffered in several critical aspects, such that the Final Update contains some scientific conclusions which have not been fully deliberated or subjected to public comment. Key process concerns include:

    - The Expert Panel relied heavily on a study that was published on the last day of the public comment period, and there was no prior public notice that the study would even be discussed at the public meeting. The study was highly important to the deliberations, because it questioned the suitability of marmoset studies for assessing potential human health hazards, and was used by the Expert Panel as a primary rationale for largely disregarding a key DEHP study in marmosets that showed no male reproductive effects following very high exposure. Stakeholders were given inadequate opportunity to provide scientific comment on this important publication. As a consequence, the Expert Panel appeared to adopt the critical positions in this publication with no meaningful reflection on other information supporting the use of marmosets as an experimental model for testicular toxicity. The PE Panel is providing with these comments the opinions of two experts with extensive experience in marmoset research. These opinions rebut many of the hasty conclusions adopted during the Expert Panel deliberations.

[1] The Final Update is available at http://cerhr.niehs.nih.gov/chemicals/dehp/DEHP__Report_final.pdf.

[2] The Panel members are: BASF Corporation, Eastman Chemical Company, ExxonMobil Chemical Company, Ferro Corporation and Teknor Apex Inc.

- The Update was conducted in a manner that prevented reaching conclusions based on an integrated view of the entire database for DEHP. For example, the Expert Panel discussed the marmoset data in the Mitsubishi (2003) study virtually independent of several other studies which also indicate primate insensitivity to testicular effects from DEHP. As a consequence, the Expert Panel's conclusions regarding concern for male reproductive toxicity were unduly influenced by its unfavorable view of the Mitsubishi marmoset study, and insufficiently influenced by the total weight of the evidence from all primate studies.

- Several significant scientific issues were raised by the Expert Panel for the first time in the final minutes of the public meeting, and thus were not fully deliberated, but were nonetheless included in the Final Update. This, combined with the inability of the public to comment on these issues at the meeting, led to several hastily- and scantily-considered conclusions being adopted as the consensus opinion of the Expert Panel.

- The PE Panel believes that these procedural flaws compromised the ability of the Expert Panel to render an objective and thoughtful opinion as to the reproductive toxicity of DEHP. The PE Panel makes recommendations to enhance the Expert Panel review process in the future, including:

  - Being more cautious about relying on information that becomes available at the last minute, and where the information is significant to the deliberations, adjusting the process to allow adequate scientific input from stakeholders, including allowing additional comments and expert opinions after the close of the public meeting for consideration by the Expert Panel, and even reconvening the Expert Panel where necessary; and

  - Being more flexible about accepting stakeholder scientific input during the Expert Panel deliberations, as critical issues arise.

- The marmoset, despite some unique aspects of its biology, is a valuable model for male human reproductive toxicity – particularly for the evaluation of developmental and toxicological aspects of the testis and spermatogenesis.

  - The review of the Mitsubishi study relied upon by the Expert Panel exhibits an unwarranted bias against the marmoset as a model, focusing on negative information and ignoring data that indicate the marmoset is a good model.

  - The marmoset has several similarities to human reproductive biology, in particular Sertoli cell development – a primary concern of the Expert Panel – that makes it an excellent model for male reproductive toxicity.

  - There is no evidence that the generalized steroid hormone resistance in the marmoset applies to sex steroids.

- As discussed in previous comments to NTP-CERHR, the marmosets' requirement of dietary Vitamin C and lack of luteinizing hormone are not significant disadvantages of the marmoset model.

- Additional criticisms of the marmoset model have been addressed in other comments, which are provided as an attachment to these comments.

- The Mitsubishi marmoset study, while presenting some concerns about the health of the animals, nonetheless provides valuable information regarding the sensitivity of primates to DEHP. The study provides strong evidence that DEHP had no major effect on testicular development even after long-term DEHP exposure at very high concentrations that would have profound adverse effects on rodent testes.

- In its comments on the August 2005 Draft Update, the PE Panel summarized an extensive body of literature indicating that differences between rodents and primates in the absorption, distribution, metabolism and excretion (ADME) of DEHP can explain in large part the lower sensitivity of primates to the reproductive effects of DEHP. Because most, but not all, of the primate ADME data came from the marmoset, the Expert Panel apparently chose to disregard these data due to perceived significant limitations with the Mitsubishi study. At the very least, the ADME data should provide the Expert Panel with sufficient information to acknowledge that the use of rat data for human risk assessment is likely to be very conservative (i.e., health protective).

- While the PE Panel applauds the Expert Panel for considering the PE Panel's recommendation in its comments on the Draft Update to calculate exposures based on available biomonitoring data, the Final Update also could have used available biomonitoring data to show that DEHP exposures to children ages 12-18 months are *not* several fold higher than adults.

- The Final Update erroneously indicated that cosmetics and breast milk pumps are significant sources of human exposure to DEHP. To the contrary, the data on DEHP in cosmetics and pumped breast milk indicate that DEHP exposures from these sources are minute or non-existent.

- The data reviewed in the final update do not support an oral developmental NOAEL as low as 3-5 mg/kg/day. The Expert Panel failed to include a significant number of individuals (F1 and F2 breeding males and F2 non-breeding males) in the overall number of individuals examined in the NTP multi-generation study (NTP 2004), which caused the Expert Panel to significantly overstate the incidence rate of "small" organs and, in turn, significantly overstate the magnitude of the effect. In addition, no laboratory historical control data were made available for review in these studies, which makes it difficult to adequately evaluate the statistical significance of the very low reported incidence rate of treatment effects.

- The data from the Mitsubishi study showing increased uterine and ovarian weights in female marmosets do not support the conclusion that DEHP exposure resulted in precocious puberty in marmosets. The increased uterine and ovarian weights correlate to

body weights which were higher in the higher dose females, versus lower body weights in controls and low dose females (probably the result of an unhealthy marmoset colony). Those higher body weights likely reflected healthier animals that were able to reach sexual maturity, with its accompanying increase in ovarian weight, rather than an effect of DEHP.

- The studies by Akingbemi et al. (2001; 2004) reviewed in the Final Update are flawed and do not support a NOEL of 1 mg/kg/day. The studies: 1) rely on single-point measures of serum hormones to support the report of abnormal changes in testosterone production in response to DEHP exposure; and 2) fail to account for differences in Leydig cell density between treatments and controls, which leads to the authors analyzing only a subset of the Leydig cells present in the treatment group.

Based on the complete database for DEHP, for the reasons discussed in the PE Panel's comments on the August 2005 Draft Update and the comments presented here, the PE Panel believes that the available information for DEHP supports a conclusion that the overall concern for risk to human reproduction from DEHP exposure is minimal.

I. THE FINAL UPDATE CONTAINS SCIENTIFIC CONCLUSIONS THAT WERE NOT FULLY DELIBERATED OR SUBJECT TO PUBLIC COMMENT

The NTP-CERHR intended that the Expert Panel's review "provide objective and scientifically thorough assessments of the scientific evidence that adverse reproductive/ developmental health effects may be associated with [DEHP] exposures."[3] Toward that end, CERHR solicited scientific information on DEHP and nominations for the Expert Panel (*see* 70 Fed. Reg. 6024 (Feb. 4, 2005)), provided a draft of the Report for public comment, and held a public meeting for the Expert Panel deliberations. Given the considerable amount of information that had become available after the first Expert Panel Report was published in October 2000,[4] the PE Panel agrees that an "objective and scientifically thorough" update is needed. However, we believe that in several respects the Final Update falls short of the mark, and that the shortcomings reflect at least in part inadequacies in the assessment process.

A. Stakeholders Were Not Given Adequate Time to Analyze and Comment on Late-Arising Information

Public comments on the Draft DEHP Update were due September 28, 2005.[5] At the public meeting on October 11-12, 2005, the Expert Panel relied heavily on a paper by Li et al. (2005) that only became available as an online preprint on September 28 – the day public comments were due. Even assuming the PE Panel became aware of that paper the day it was released, it clearly could not have commented on the paper within the comment deadline. Input

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3 Final Update Preface, page ii.

4 NTP-CERHR Expert Panel Report on Di(2-ethylhexyl) Phthalate, October 2000. Available at: http://cerhr.niehs.nih.gov/chemicals/dehp/DEHP-final.pdf.

5 *See* 70 Fed. Reg. 43870 (Jul. 29, 2005).

is now being provided with these comments, but it is too late to be reflected in the Expert Panel's deliberations.

The Li et al. paper critiqued the suitability of the marmoset as a human reproductive model. The Li paper was critical to the Expert Panel's deliberations, because it raised doubts about reliance on a key study for DEHP in marmosets that showed no effects on male reproductive development, even at very high doses (Mitsubishi, 2003). The PE Panel was not made aware that the Expert Panel would be discussing the Li et al. paper and relying on it to critique the DEHP study until the second day of the public meeting. When the lack of opportunity for public comment was pointed out, an opportunity for brief oral comment to the Expert Panel was provided, but this clearly was an unsatisfactory situation. Stakeholders had a very limited amount of time to review and comment on the study, and the Expert Panel had extremely limited time in which to consider those undoubtedly incomplete comments.

As detailed in Section II.A below, a critique of Li et al. by an expert in marmoset reproductive biology shows that the Li et al. paper overstates the case against using marmosets as a model, and fails to acknowledge factors that make marmosets good models for human testes development. Without the benefit of that input from a scientist who has done extensive research in marmosets and is expert in marmoset reproductive biology, the Expert Panel adopted the positions in Li et al. with little or no discussion. The net result, as explained more fully later in these comments, is that the Expert Panel received a one-sided and overly-critical view of the scientific value of DEHP marmoset studies, and inappropriately discounted the results of those studies.

The PE Panel realizes that its more robust comments on the Li et al. review will now be considered by the NTP-CERHR as its prepares its final brief on DEHP. However, the Expert Panel report, as a consensus document of an independent group of experts, carries great weight. Further, until the NTP-CERHR brief is public, the Expert Panel report is the "last word" on DEHP reproductive toxicity. Therefore, it is important that the Expert Panel report reflect truly fair and robust consideration of all key studies cited in its report. That did not happen in the case of the paper by Li et al., because the process did not allow it to occur.

There were other instances of the Expert Panel considering last minute information for which the public had no meaningful opportunity for comment. These include a summary of raw data from a key multi-generation study (see Section IV.A) and speculation on the potential contribution to exposure from use of breast milk pumps (see Section III.B).

In the future, the PE Panel strongly recommends that NTP-CERHR exercise caution in using or relying on last minute information, and that it adjust its process so that stakeholders have adequate opportunity to provide comment and scientific input concerning the late-arising information, including statements by independent experts where appropriate. For highly significant information, NTP-CERHR should allow a post-public meeting comment period with an opportunity for the Expert Panel to review and consider those comments. In some cases, it may be necessary to reconvene the Expert Panel to entertain other expert scientific input relevant to the new information. Otherwise, there is a risk that the Expert Panel report will appear up-to-date but in fact be the product of hasty and premature conclusions, as the PE Panel believes occurred in this case.

B. The Update Report Does Not Adequately Reflect the Complete Weight of Evidence for DEHP, Because the Expert Panel Reviewed Post-2000 Data in Isolation from Other DEHP Data

The PE Panel considers that a major failing of the update process was that the post-2000 data was reviewed almost in complete isolation from the pre-existing database for DEHP. Thus, the Update Report conclusions essentially reflect a view of DEHP based on the post-2000 set of data, rather than on the complete weight of evidence for DEHP.

A prime example of the imbalance created by this approach is the Expert Panel's consideration of and conclusions relating to primate data. In drawing its conclusions about the significance of primate data for assessing potential risks of DEHP, the Expert Panel focused only on the recent Mitsubishi (2003) marmoset study and its limitations. However, as discussed in Section II.C, below, several pre-2001 studies in both old world and new world primates show that primates are generally much less sensitive to testicular effects from DEHP exposure than are rodents. Consideration of these data in addition to the Mitsubishi study might well have modified the Expert Panel's conclusions. To complete a comprehensive review, the Expert Panel should have considered the post-2000 data as an integrated package with previous data, both while drafting the Final Update and during the public meeting. However, there was no mechanism in the review process for doing so. For its final brief of DEHP, NTP-CERHR should itself consider the full body of data for DEHP. The PE Panel also urges that the process for future updates to provide a mechanism for integrating previous and update information.

C. Several Significant Issues Were Raised for the First Time in the Final Minutes of the Public Meeting, and Thus Were Not Fully Deliberated, but Were Nonetheless Included in the Final Update

Several significant issues were raised in the final minutes of the public meeting and, despite not being fully deliberated, were included in the Final Update and represented to be the Expert Panel's consensus opinion. One such issue, as pointed out in an October 12, 2005 letter from the PE Panel to NTP-CERHR,[6] concerns the first paragraph of Section 5.3 (Overall Conclusions) of the Final Update. This paragraph states that "[t]he combined effects of multiple phthalate exposures have implications for exposure and risk assessment." [7] There were absolutely no deliberations concerning additive effects of DEHP and any other chemical by the Expert Panel, and no opportunity for public comment on this paragraph, which was written at the very end of the meeting. As stated in the PE Panel's letter, the only study available on this issue, Foster et al. (2002), found no additive effect from a combination of DBP and DEHP. Foster et al. concluded "[t]his study did not indicate an additivity of response or an interaction of the two phthalates in combination. Aggregation of risk of these doses would not be appropriate."[8] Thus, the paragraph suggesting a concern for additivity of effects is presented as the consensus of the Expert Panel after receiving no deliberation or opportunity for comment, and in direct opposition

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6 Letter from Marian K. Stanley, Manager PE Panel, to Dr. Michael Shelby, CERHR Director, Re: DEHP Update Expert Panel Report, dated October 12, 2005.

7 Final Update at page 171.

8 *Id.*

to the only available evidence about additivity. For these reasons, this paragraph should not have been included in the Final Update.

In addition, the Final Update concludes that "[t]here is sufficient evidence in female marmosets to conclude that DEHP causes reproductive toxicity (increased ovary weight and uterine weight) when exposure is by oral gavage at 500 mg/kg bw/day for ~15 months in the peripubertal period . . . ."[9] The Final Report interprets this observation by stating that "[t]he Expert Panel found these data consistent with precocious puberty . . . ."[10] However, this conclusion was not the result of any true deliberation by the Expert Panel. In the closing minutes of the public meeting, a comment was made that the observation of increase in ovarian weight should be accompanied by a statement about the implications of that observation. A single panel member, almost off-handedly, asserted that the data were indicative of precocious puberty. This interpretation was accepted by the Expert Panel with no discussion and no opportunity for public comment. For this assertion, which has very significant implications for concern about potential DEHP effects,[11] there was absolutely no deliberation by the Expert Panel or opportunity for public comment about the interpretation of the data. In fact, as discussed in Section V.A, below, the increased ovarian and uterine weights may simply correlate with increased female body weights, and therefore represent no adverse effect at all. Yet as a result of there being no deliberation or opportunity for public comment, the conclusion that DEHP causes precocious puberty in marmosets is now represented as the consensus opinion of the Expert Panel.

As a final example, Table 23 of the Final Update, which was compiled at the last minute with no real deliberation or quality checking, arguably contains errors in the total numbers of animals observed at each dose (see Section IV.A, below). These errors likely affected the Panel's interpretation of the data, and consequently its conclusions regarding the toxicity of DEHP.

Due to the lack of any deliberation or opportunity for public comment, the PE Panel believes it is highly inappropriate that the above statements are included in the Final Update as the Expert Panel's consensus opinion. In denying the PE Panel's request that the paragraph suggesting additivity of effects be removed from the Final Update, NTP-CERHR disclaimed that "the conclusions reached by CERHR expert panels are their own" and "should not be construed to represent the views of the National Toxicology Program."[12] Despite this disclaimer, it is likely that the Final Update will nonetheless be pointed to by interested parties as the consensus view of an ostensibly objective "eleven-member panel of government and non-

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9 Final Update at page 163.

10 *Id.*

11 Given current debate about endocrine disruption, "precocious puberty" is a highly charged term.

12 Letter from Dr. Michael D. Shelby, Director CERHR, to Marian K. Stanley, Manager PE Panel, dated October 28, 2005. NTP-CERHR also stated that "because the expert panel report is not a government document, the NTP is unable to [remove the paragraph]." *Id.* It seems somewhat disingenuous, however, to suggest that NTP-CERHR is powerless to correct a documented flaw in the report. If nothing else, NTP-CERHR could have shared the PE Panel's letter with the Expert Panel to receive direction on whether to remove the offending paragraph.

government scientists."[13] Consequently, it is not sufficient merely to state that the conclusions of the Expert Panel are their own, and not those of NTP-CERHR. NTP-CERHR should take a hard look at its Expert Panel process and implement safeguards to ensure that the "consensus opinions" put forth in panel reports are indeed the fully-deliberated consensus view of the panel, and not last-minute, hastily accepted but substantively important add-ons.

The need for such a deliberative period is illustrated by the note included in the Preface to the Final Update which states that "[w]hile the expert panel reached consensus on all conclusions during the panel meeting, following the meeting . . . three panel members reconsidered their position on one conclusion. Upon reconsideration, they did not concur with [the conclusion arrived at the public meeting]."[14] The mere existence of this note shows that a hastily-arrived-at "consensus" is really no consensus at all. The Final Update should reflect a true consensus, in which case there would be no need of a note such as this. Allowing an additional period of reflection and comment would help ensure that panel reports reflect a true consensus of the expert panel.

D. NTP-CERHR Should Be More Flexible In Allowing Audience Participation

The negative impact of the process flaws identified above might have been *partially* mitigated had the public meeting been run in a similar manner as the first phthalate Expert Panel meetings in 1999 and 2000. At those meetings, members of the public were allowed to raise their hands during the meeting to comment and provide clarification on various scientific issues or specific data. These public comments were presented in a respectful and orderly fashion, by highly qualified scientists, and contributed to, rather than detracted from, the Expert Panel's deliberations. Indeed, the value of that scientific input was acknowledged by many participants in the public meetings. In the 2005 meeting, however, public comments appeared to be more discouraged than encouraged. They were limited to 15 minute presentations and there was no opportunity to comment during the Expert Panel's discussion.[15] Without adequate opportunity for the public to comment during the deliberations, there was no effective way to address or even identify many of the issues described above. Indeed, there was effectively little or no way to address issues that had not first surfaced in the draft Expert Panel report.

The issues that are addressed by an NTP-CERHR Expert Panel frequently are cutting-edge scientific issues that benefit from robust discussion. That discussion, of course, is centered around the Expert Panel, which hopefully will have the requisite expertise and be free of conflict or bias. However, industry scientists or other stakeholder scientists often will have important points to contribute, and may in some cases be able to offer the perspective of expertise that is lacking on the Expert Panel. The PE Panel believes NTP-CERHR should not

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13 *See* Final Update at page ii.

14 *Id.* at page iii.

15 Comments could be given to the CERHR director to be passed on to the Expert Panel chair, but this was very ineffective. It is not clear that all comments were given to the chair, or that all comments were presented to the full Expert Panel, and even when comments were passed on, they often "lost something in the translation."

manage the deliberations in a way that shuts out that input, but instead should encourage participation in a reasonable and respectful manner, as occurred with the initial phthalate Expert Panel assessments.

If NTP-CERHR's goal truly is an "objective and scientifically thorough assessment," then NTP-CERHR should be flexible in the way it manages the public meetings, and give scientists in the audience some credit for being able to exercise discretion in offering scientific input during the deliberations. An overly rigid approach can only serve to deny Expert Panel members access to relevant scientific input. A more flexible approach can only enhance the quality and objectivity of the final work product.

II. THE MARMOSET IS A VALUABLE MODEL FOR MALE HUMAN REPRODUCTIVE TOXICITY

The Final Update lists several aspects of marmoset biology that differ from human biology and, based largely on a review of marmoset reproductive biology by Li et al. (2005), ultimately concludes that these differences "significantly limit our reliance on this species as a surrogate for humans."[16] While the marmoset, like all animal models, is not a perfect model for all aspects of human toxicity, the PE Panel believes strongly that marmoset is a good model for male reproductive development. In particular, the marmoset is a particularly good model for the reproductive endpoints investigated in the Mitsubishi (2003) study.

To assess the usefulness of the marmoset as a model for human reproductive toxicity, the PE Panel engaged Professor Stefan Schlatt to render his opinion on the value of the marmoset model. Dr. Schlatt is an Assistant Professor in the Department of Cell Biology and Physiology at the University of Pittsburgh School of Medicine, and is an expert in mammalian reproductive biology. Dr. Schlatt has more than 13 years of experience researching reproductive function and endocrine activities in animal models, including rats, hamsters, and various nonhuman primates, including marmosets, and has authored more than 50 refereed articles on various aspects of this field. In Dr. Schlatt's opinion (provided as Attachment 1 to these comments), "there is no doubt that the marmoset is a useful model to explore developmental and toxicological aspects of the testis," and "many similarities in regard to testicular organization, general developmental pattern and hormonal regulation render the marmoset a much more useful model when compared to rodents."[17]

A. The Review Relied Upon by the Expert Panel, Li et al. (2005), Exhibits an Unwarranted Bias Against the Marmoset as a Model for Male Reproductive Toxicity

In discussing the validity of the marmoset model, the Final Update relies heavily on the review by Li et al. (2005), which points out several differences between marmoset and human reproductive biology. This review, however, ignores information or data that indicate the marmoset is a good model, and focuses on negative information. As stated by Dr. Schlatt, the Li

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[16] *Id.* at page 141.

[17] Opinion of Dr. Schlatt, Attachment 1, at page 4.

et al. "interpretation of the suitability and validity of the [marmoset] model carries an unjustified negative bias."[18] As discussed above, the merits of the Li et al. criticisms were not sufficiently evaluated by the Expert Panel, due to the timing of its publishing versus the public comment period.

B. The Marmoset Has Similarities to Human Reproductive Biology that Make it a Valuable Model

As discussed in Dr. Schlatt's opinion (Attachment 1), several features of marmoset reproductive biology make it a valuable model for human reproductive toxicity.

1. The Similarity of Human and Marmoset Sertoli Cell Development Make the Marmoset a Good Model for Studying Effects on Germ Cell Development – a Primary Concern of the Expert Panel

One of the key concerns of the Expert Panel was the effect of DEHP exposure on Sertoli cells (vacuolation and reduced proliferation) in developing animals. This concern is based on data from rats, in which Sertoli cell effects are seen at relatively low oral doses. However, the data in Mitsubishi (2003) indicate that extremely high oral doses of DEHP, up to 2500 mg/kg/day, had no effects on marmoset testes even at the cellular level. Because of this disparity in effects, the value of the marmoset as a model of human reproduction becomes an essential question.

As stated by Dr. Schlatt, there are "striking similarities of organization of the marmoset and human spermatogenic epithelium (both species have a multistage organization of spermatogenic stages per tubular crossection)," and "quite similar mechanisms of germ cell development and clonal expansion of germ cells in marmosets and man." [19] Also, while Li et al. point out an unusual uniformity of Sertoli cell morphology throughout the marmoset spermatogenic cycle, Dr. Schlatt explains that it is not yet known whether humans and marmosets differ with respect to this observation, and that "it appears likely that the human and marmoset Sertoli cell show a high degree of similarity in this respect".[20] Dr. Schlatt concludes:

> With choice of the correct timepoints [the marmoset] should be highly useful and informative for exploring the effects on Sertoli cell differentiation, testicular growth and effects of FSH on the testis. The striking similarities to man make it an excellent model for studying effects on germ cell development, the organization of the seminiferous epithelium and changes to the kinetics of spermatogenesis.[21]

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18 *Id.*

19 Opinion of Dr. Schlatt, Attachment 1, at page 2.

20 *Id.*

21 *Id.* at page 4.

Thus, similarities in biology indicate that the marmoset is a good model for determining the potential effects of DEHP on human Sertoli cells, and available data indicate that the marmoset is far less sensitive to oral DEHP exposure than rodents. Despite concerns with the health of the animals in the Mitsubishi study (see discussion in Section II.C below), the extreme insensitivity of marmosets to oral DEHP exposure, at the very least, provides information that puts the rodent model into perspective as being much more sensitive to testicular effects of DEHP than primates. Therefore, the marmoset data should not have been completely disregarded for purposes of evaluating male reproductive toxicity.

2. There Is No Evidence that the Generalized Steroid Hormone Resistance in the Marmoset Mentioned by Li et al. Applies to Sex Steroids

Based primarily on the review of Li et al. (2005), the Expert Panel concludes that marmosets have a general end-organ steroid resistance relative to humans, as indicated by their high serum levels of steroids, which limits their value as a human reproductive model. However, as explained by Dr. Schlatt (see Attachment 1), while a general insensitivity has been proposed for the mineralocorticoid and glucocorticoid hormones, it is unknown whether such steroid resistance exists for the gonadal (sex) steroids. Marmosets, but not mice, have sex hormone binding globulin that separates the serum testosterone into active free (biologically active) and bound (biologically inactive) fractions. Due to the binding affinities of these proteins, marmosets appear to have high levels of unbound testosterone in the circulation which is similar to their unusually high levels of glucocorticoids. However, these higher testosterone levels may have little or no biological significance since the kinetics of testosterone-receptor binding and post-receptor binding events is unknown. Therefore, high levels of serum testosterone in marmosets are not necessarily indicative of sex steroid resistance. As stated by Dr. Schlatt, "in the absence of solid data on sex steroids it appears poorly justified and premature to transfer the conclusion of high sex steroid resistance from the glucocorticoid and mineralocorticoid system to the sex steroid system."[22]

3. The Marmoset's Requirement of Dietary Vitamin C and Lack of Luteinizing Hormone Are Not Significant Disadvantages of the Marmoset Model

The Final Update, citing the review of Li et al. (2005), mentions, as additional disadvantages of the marmoset model, the potentially protective action of Vitamin C in the marmosets' diet and the marmosets' lack of Luteinizing Hormone (LH). Each of these concerns was addressed in the PE Panel's April 2005 submission to NTP-CERHR[23] and the Vitamin C issue again in the PE Panel's September 2005 Comments on the Draft Update,[24] and has been

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22 *Id.* at page 3.

23 Recent Information on Exposure to and Toxicology of Di(2-ethylhexyl) Phthalate (DEHP), American Chemistry Council Phthalate Esters Panel, submitted to NTP-CERHR April 21, 2005. (April 2005 Comments.)

24 See Section IV.B. of: Comments of The American Chemistry Council Phthalate Esters Panel On the Draft NTP-CERHR Expert Panel Update On The Reproductive And Developmental Toxicity

shown to be of no substantial importance in assessing the validity of the marmoset model. As discussed in the PE Panel's previous submissions, the marmosets' dietary Vitamin C requirement should not be of concern because: 1) the levels of Vitamin C used in the Mitsubishi study are not high relative to the marmoset's requirements, and 2) based on the available science, it is not clear that Vitamin C affords any protection to primates from DEHP exposure. Moreover, if the level of Vitamin C in the marmosets' diet in the Mitsubishi study in fact provided the degree of protection necessary to be responsible for the observed lack of effects, then the level of Vitamin C in the average human diet would be protective of any likely exposure to DEHP. In other words, the Vitamin C levels in the marmoset diet in the Mitsubishi study were similar to normal levels in the human diet and, consequently, whether Vitamin C had a protective effect in this study is not directly relevant to a risk assessment.

As for the marmoset's lack of LH, the data cited in the PE Panel's earlier submission show that this difference in the hormone that initiates testosterone synthesis between the common marmoset and humans does not provide a sufficient basis for rejecting the marmoset as a model for human testicular development and function.[25] This opinion is shared by Dr. Schlatt who states: "Despite . . . the exchange of LH by CG the marmoset shows many similarities to man. The function and regulation of FSH and CG and their feedback mechanisms resemble other primates."[26] Even with this hormonal difference, Dr. Schlatt concludes that the marmoset is a valuable model, particularly for the investigation of effects of DEHP exposure on Sertoli cell proliferation, which is regulated primarily by Follicle Stimulating Hormone in marmosets and other primates, but not rodents.

4. Other Criticisms in the Li at al. Review Are Insufficient to Invalidate Use of the Marmoset Model to Evaluate Potential Effects of DEHP on Human Reproductive Development

The Li et al. review includes some additional criticisms of the marmoset model, not explicitly discussed in the Final Update. Both these and some of the foregoing criticisms have been addressed in the context of comments by the PE Panel to the California Office of Environmental Health Hazard Assessment (OEHHA). A copy of those comments is provided as Attachment 2. As NTP-CERHR prepares the final brief for DEHP, to the extent it considers the Li et al. review paper, it also should consider Section I of the comments to OEHHA.

C. The Mitsubishi Study Provides Valuable Information Regarding the Sensitivity of Primates to DEHP

The Final Update notes concern about the body weights of some of the animals in the Mitsubishi marmoset study, as does Dr. Tardif in her independent review (See Attachment 3). However, as pointed out in the opinion of Dr. Schlatt, notwithstanding these concerns, the Mitsubishi study nonetheless provides "strong evidence that DEHP had **no major** effect on

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Of Di(2-Ethylhexyl) Phthalate, Submitted to NTP-CERHR September 28, 2005. (Comments on Draft Update.)

[25] See Section II.B.2.d.2. of April 2005 submission.

[26] Opinion of Dr. Schlatt, Attachment 1, at page 3.

testicular development even after very long and intense DEHP exposure."[27] Dr. Schlatt concludes that the Mitsubishi data "should be carefully and critically considered for evaluating the risk of gonadotoxic effects in humans after exposure to DEHP."

Indeed, the Mitsubishi data should not be completely disregarded with respect to implications for the degree of concern for male reproductive effects in humans. The shortcomings of the study might limit its usefulness on a quantitative level, but it still provides important qualitative information about the relative toxicity of DEHP to primates versus rodents, specifically that *very high concentrations of DEHP that would have **profound** adverse effects on rodent testes had **no** adverse effects on the testes of marmosets*. Moreover, despite it being considered essentially in isolation in the Final Update, the Mitsubishi study results are supported by several other primate studies. In other studies of both old world and new world primates, no testicular effects have been observed at doses up to 2500 mg/kg/day (Pugh et al., 2000; Kurata et al., 1998; Rhodes et al., 1986; Short et al., 1987). Combined with the Mitsubishi study, the clear conclusion is that primates are much less sensitive than rodents to the effects of DEHP. Even if primates are not used to establish the NOAEL for male reproductive effects, the PE Panel strongly believes that these data should be used to modify the degree of concern for effects in human testicular development from DEHP.

Due to the limitations in the process discussed above, the Expert Panel essentially ignored these older studies and instead focused only on the Mitsubishi data and its limitations. The Panel should consider the Mitsubishi data in light of these other primate studies, which together provide substantial evidence that primates are less sensitive to DEHP than rodents. The Expert Panel's lack of consideration of Mitsubishi in the context of this additional primate data might have been avoided had the Public Meeting allowed for public comments beyond the 15 minute presentations allowed.

In summary, substantial evidence, not all of which appears to have been adequately considered by the Expert Panel, indicates that marmosets are a valuable model for the investigation of human reproductive toxicity. Like any other non-human model, the marmoset is useful for some comparisons but not others. In this case, the parameters measured in the Mitsubishi study, particularly the lack of Sertoli cell effects, were those for which a marmoset model would be appropriate, and in fact superior to the rodent model. The use of these data should not be precluded by the fact that the marmoset may not be a good model in other respects not germane to the issues at hand.

D. Toxicokinetic Data Support the Lower Sensitivity of Primates to the Effects of DEHP

In Section II of its comments on the August 2005 Draft Update, the PE Panel summarized an extensive body of literature indicating that differences between rodents and primates in the absorption, distribution, metabolism and excretion (ADME) of DEHP can explain in large part the lower sensitivity of primates to the reproductive effects of DEHP. The ADME data provide a consistent explanation both among mammals (i.e., rodents vs. primates) and within primates (i.e., marmoset vs. cynomolgus monkey) as to why primates absorb less of the

[27] *Id.* at page 6 (emphasis in original).

toxicologically relevant metabolite (MEHP) and exhibit lower MEHP / DEHP levels in the blood and target tissues than rodents. The Expert Panel does acknowledge some of the ADME data that have appeared since the first Expert Panel review in 2000 but in its conclusions seems unwilling to make the connection between lower systemic doses of MEHP in primates and the lower sensitivity of primates to DEHP toxicity. Because most, but not all, of the primate ADME data came from the marmoset, the Expert Panel apparently chose to disregard these data due to perceived significant limitations with the Mitsubishi study. At the very least, however, the ADME data should provide the Expert Panel with sufficient information to acknowledge that the use of rat data for human risk assessment is likely to be very conservative (i.e., health protective).

III. COMMENTS ON SECTION I: USE AND HUMAN EXPOSURE

A. The Final Update Could Have Used Available Biomonitoring Data to Show That DEHP Exposures to Children Ages 12-18 Months Are Not Several Fold Higher than Adults

The PE Panel applauds the Expert Panel for considering the PE Panel's recommendation in its comments on the Draft Update to calculate exposures based on available biomonitoring data. It was the PE Panel's recommendation that "[t]he section. . .on human exposures to DEHP could be significantly improved by including conversions of urinary metabolite levels to estimates of environmental exposure," and that "[t]he CDC biomonitoring data are the most comprehensive and accurate estimates available of exposures of the U.S. population, including children, to DEHP."[28] Using this approach, the PE Panel developed exposure estimates from the biomonitoring data that were consistent with probabilistic estimates based on sources of exposure. Although the Expert Panel articulated the uncertainties associated with each method, the PE Panel agrees that combining, or at least comparing, the two methods, as done in the Final Update, provides the best overall approach.

However, the Expert Panel did not extend the process of exposure estimation from biomonitoring data to children ages 12-18 months (Brock et al., 2002), a subpopulation that was considered at potentially greater risk. Instead, the Expert Panel indicated that children 1-6 years old could have exposures several-fold higher than the population estimates based on the study by Koch et al. (2004) of German children ages 2.5-6.5, which showed higher levels of excreted phthalate per gram creatinine than adults in the same household.[29] However, the Expert Panel could instead have used the same approach of calculating exposures on the Koch et al. study, and combined it with the study by Brock et al., to show that exposures of this age bracket are in fact not several fold higher than adults (see Table 1 below). The PE Panel urges NTP-CERHR to take this approach in assessing DEHP exposures for its final brief.

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[28] Comments on Draft Update at page 7.

[29] As stated in the PE Panel's September 2005 comments on the Draft Update, there are significant differences in creatinine excretion between adults and children – children generally excreting half the level of creatinine excreted by adults).

Table 1: DEHP Exposures to Children Calculated from Biomonitoring Data in Koch et al. (2004) and Brock et al. (2002).

Source	Mean/median DEHP metabolite/g creatinine	Exposure estimate (µg/kg/d)
Koch et al.	55.8 (5-OH-MEHP)[a]	5.52
Brock et al.	26.14 (MEHP)[b]	2.57

a = calculated using the method of David (2000) and the excretion ratio of Koch et al. (2004).
b = calculated using the method of David (2000) and the excretion ratio of Anderson et al. (2001).

B. The Final Update Erroneously Indicates that Cosmetics and Breast Milk Pumps Are Significant Sources of Human Exposure to DEHP

Section 1.0 of the Final Update states: "Phthalates are used in a variety of products including . . . perfumes, hairsprays, and cosmetics . . . ", implying that DEHP is so used.[30] The Final Update conclusions also imply that cosmetics are a significant source of human exposure to DEHP.[31] However, information in the Final Update indicates that DEHP use in cosmetics and personal products is extremely limited or non-existent. As discussed in Section 1.1.1, a survey of 42 perfumes, 8 deodorants, 21 nail polishes, and 31 hair care products marketed in Korea found DEHP in only 2 of the perfumes and 2 of the nail polishes, and none of the deodorants or hair products (Koo and Lee, 2004). The maximum level of DEHP detected was 25 mg/L (25 ppm). Similarly, a 2002 Environmental Working Group report found only 3 products out of 72 tested that contained DEHP, again with a maximum concentration of 25 ppm.[32] That is a concentration of only 0.0025%. It is unlikely that DEHP would have functionality and therefore be intentionally added to a formulation at such a low level. More likely, the DEHP was a trace contaminant in the formulation or was an artifact of laboratory contamination. In fact, the Cosmetic, Toiletry and Fragrance Association has indicated that no cosmetic products currently manufactured in the US contain DEHP.

In an exceedingly large number of places, often particularly prominent places such as boxed language, the Final Update speculates that breast milk expressed into breast pumps may be a source of DEHP exposure, and uses concern about the potential for such exposure as a basis for asserting uncertainty about exposures of infants to DEHP. See pages 7, 33-34, 54, 55, 93, 97, 171, and 175 of the Final Update. Yet the Final Update cites not one analytical result demonstrating that breast milk pumps contribute any DEHP to breast milk, much less significant amounts that would warrant the great deal of attention given to this

30 Final Update at 2.

31 *See* Final Update at page 169, which states: "DEHP is ubiquitous in the environment. Humans can be exposed to DEHP through many routes including ingestion (food, infant formula, and breast milk), contact with contaminated household dust and consumer products (cosmetics and toys). . . ."

32 Environmental Working Group (2002). Not too pretty: Phthalates, beauty products and the FDA., page 2. Available at: http://www.ewg.org/reports_content/nottoopretty/nottoopretty_final.pdf

speculative source. In fact, the available data cited in the Final Update suggests breast milk pumps do not affect DEHP levels in breast milk.[33]

The PE Panel's knowledge of breast pumps indicates that it is highly unlikely breast milk pumps would be a significant contributor to DEHP exposures. To the PE Panel's knowledge, no milk container portion of any pump is made of vinyl that would contain DEHP (or any other phthalate), and therefore there would be no DEHP available to migrate into the milk during storage. Some breast milk pumps have flexible vinyl tubing that may contain DEHP; however, the tubing is used to pull air away from the container (creating the vacuum that pumps the milk); the milk does not come into contact with the tubing. The cup that is placed against the breast might be made of flexible vinyl that might contain DEHP, and it is possible some breast milk might come into contact with the cup, but any such contact would be fleeting and would allow for very little migration of DEHP into the milk.[34]

For these reasons, the rather extreme concern about potential infant DEHP exposures to DEHP from breast milk pumps is spurious. The PE Panel requests that, in its final brief, the NTP-CERHR clarify the potential role of breast milk pumps in DEHP exposures. To the extent that the Expert Panel expressed concern due to uncertainty over infant exposures because of the speculative contribution of breast milk pumps, NTP-CERHR should express a lower level of concern.

IV. COMMENTS ON SECTION 3: DEVELOPMENTAL TOXICITY DATA

A. The Data Reviewed in the Final Update Do Not Support an Oral Developmental NOAEL of 3-5 mg/kg/day

The first CERHR Expert Panel Report did not identify a firm NOAEL for developmental toxicity on the developing male reproductive tract.[35] In the Final Update, the Expert Panel cited the multigeneration continuous breeding study conducted by NTP (2004) as providing a developmental NOAEL of 3-5 mg/kg/day. This NOAEL is based on the incidence of gross observations of small reproductive organs (with no change in organ weights) observed in a few animals in the 14-23 mg/kg/day and the 46-77 mg/kg/day groups. In deriving this NOAEL, the Expert Panel stated that it evaluated the combined incidence of small male

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33 "The authors tested milk samples in 1 common Danish pump system and found no effect on phthalate monoester levels." Final Update at 7. "Women who used a breast pump in Denmark had significantly higher levels of monoethyl and monobutyl phthalate. Breast pump-associated levels of other phthalates were not significantly different . . . ." Final Update at 55.

34 Like the Li et al. paper, the concern about breast milk pumps is an example of information newly raised at the Expert Panel meeting for which there was no adequate opportunity for public comment (see Section I.A, above). The PE Panel attempted to provide an explanation such as given herein to the Expert Panel, but it is not clear that the explanation was conveyed to the Expert Panel Chair, much less the rest of the Expert Panel, a failing which could have been overcome by a more flexible approach to audience participation (see Section I.D., above).

35 The Expert Panel stated "The Panel is not confident that the lowest dose has been established at which developmental toxicity (the development of the male reproductive system) occurs." First Expert Panel Report at 88.

reproductive organs in the F1 and F2 non-breeding males.[36] However, the total number of examined organs listed in Table 23 of the Final Update does not reflect this, as the table contains only the number the F1 non-breeding males examined and omits the number of F2 non-breeding males.

Counting the F2 non-breeding males, the total combined number or organs examined should have been 59, 61, 64, 61, 66, 68, 50 for the 1.5, 10, 30, 100, 300, 1000, and 7500 ppm dose levels, respectively. In addition, the PE Panel believes that the number of breeding males in the F1 and F2 generations should also be included in the combined incidence. While the breeding males were older than non-breeding males at the time of sacrifice, the effect of small reproductive organs, if present, would not be expected to disappear over this time period. Including the breeding males would increase the total number of each group by 20. By not including in the total number of examined organs the number of F2 non-breeding males and breeding males, the Expert Panel significantly overstates the incidence rate of small organs, which in turn significantly overstates the magnitude of the effect. The PE Panel provides below what it believes to be the correct version of Table 23.

Table 23. Reproductive Organ Abnormalities in Combined $F_1 + F_2$ Non-breeding Males in NTP Multigeneration Study

	DEHP dose level, ppm in feed (number of organs examined)						
	1.5	10	30	100	300	1000	7500
Organ	(79)	(81)	(84)	(81)	(86)	(88)	(70)
Testis	0	0	0	0	4	3	21
Epididymis	0	0	0	0	3	3	7
Seminal vesicles	0	1	0	0	2	0	0
Prostate	0	0	0	0	0	4	1
Any reproductive organ	0	1(1)*	0	0	5(4)*	7(5)*	22(14)*

*Data expressed as number of animals (litters) affected. From NTP (*114*)

In addition, although the NTP study reported that the finding of small reproductive organs in the 300 and 1000 ppm groups was at a significantly higher incidence rate than laboratory historical control data, the historical data were not available for review. The PE Panel questions whether sufficient historical control data for small reproductive organs have been evaluated to ascertain whether the limited incidence of small organs seen in the NTP study can be definitively determined to be treatment related. For the low incidence of small organs observed in the treatment groups to be statistically significant, the control incidence of this effect would have to be zero, or very close to zero. As mentioned in the PE Panel's comments to the Draft Update, historical data from contract laboratories indicate that there is a 2-3% incidence of testicular atrophy at necropsy in control populations of sexually mature Sprague-Dawley rats from Charles River Laboratories. In the absence of reduced organ weight (individual or group mean) or evidence of lowered reproductive success, which cannot be assessed because the effects were only reported for non-mating males, the small organs reported at 1000 and 300 ppm should

[36] Final Update at 66 and 94.

not be considered toxicologically significant. Therefore, the PE Panel believes it is necessary to evaluate historical control data for this strain of rat to evaluate the statistical significance of the incidence of the small reproductive organs. Without such information, the PE Panel questions the validity of making the determination of a NOAEL in this study based on this finding.

Moreover, at the Expert Panel public meeting, Dr. Robert Chapin, the Expert Panel member who most closely reviewed the NTP data, stated that the 300 ppm (about 14-23 mg/kg/day) LOAEL was at the "very tail end of the response"[37] and that the Expert Panel was "flying along in the weeds" at this level. This raises the issue of whether the effects reported in the NTP study at the 300 ppm LOAEL were in fact treatment related, or were non-treatment related noise. In other words, if 14 mg/kg/day is the LOAEL, then the NOAEL is likely much closer to this value than the value chosen by the Expert Panel, the lowest tested dose of 100 ppm (about 3-5 mg/kg/day).

Thus, the PE Panel believes that the NTP (2004) data as reviewed by the Expert Panel do not support a NOAEL as low as 3-5 mg/kg/day. For the foregoing reasons, the PE Panel believes NTP-CERHR should consider 14-23 mg/kg/day as a NOAEL, or at the least should find the NOAEL to be near that level (e.g., 12 mg/kg/day).

V. SECTION 4: REPRODUCTIVE TOXICITY DATA

A. The Data From the Mitsubishi Study Showing Increased Uterine and Ovarian Weights in Female Marmosets Do Not Support the Conclusion that DEHP Exposure Resulted in Precocious Puberty in Marmosets

After rejecting the Mitsubishi (2003) data for assessing male reproductive toxicity, due in part to concerns about the reliability of the study, the Expert Panel used data from that same study to conclude that increased uterine and ovarian weights in female marmosets were "consistent with precocious puberty in the 2 highest dose DEHP-exposed groups (500 and 2500 mg/kg bw/day)."[38] However, a review of the Mitsubishi study by Dr. Suzette Tardif, the Associate Director of the Southwest National Primate Research Center and an expert in female marmoset reproductive biology with over twenty years experience raising primates, determined that these data are inconclusive and do not support the conclusion that DEHP exposure causes precocious puberty in female marmosets.

Dr. Tardif's analysis of Mitsubishi (2003) (see Attachment 3 to these comments) shows that the increased uterine and ovarian weights correlate to body weights that were higher in the higher dose females, versus lower body weights in controls and low dose females. As Dr. Tardif explains, the females in the Mitsubishi study, particularly the controls and the low dose group, were of extremely low weight for their age at the end of the study (about 17 months), compared to a healthy marmoset colony. Dr. Tardif attributed this to the study procedures, which involved daily gavage for many weeks in a row and resulted in many "basically unhealthy

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[37] This comment is acknowledged in the Final Update, which states at page 151: "The Expert Panel considers 300 ppm and 1000 ppm to represent the tail of the dose-response curve in this study...."

[38] Final Update at page 163.

animals with impaired growth."[39] While there was no significant difference in average weight across the treatment groups, there was a trend for the females in the two highest dose groups to have the highest body weights, and these were the groups which also had increased uterine and ovarian weights. Specifically, only three out of six subjects in the control and low dose groups had a 17-month-old body weight that Dr. Tardif would consider suitable for research (275 g), five of six and six of six of the animals in the higher dose groups would have been suitable.

Moreover, based on measured estradiol concentrations, many of the animals in fact appeared to be pre-pubescent throughout the length of the study, which was terminated when the animals were at an age at which all should have been adults. The failure of many individuals to enter puberty was correlated with the abnormally low body weights.

Dr. Tardif found that the higher ovarian weights in the higher dose groups were generally associated with higher body weights and the occurrence of ovulation and corpus luteum formation that accompanies sexual maturity. In Dr. Tardif's opinion, the higher ovarian weights seen in the highest dose group were simply due to the higher weight group having more normal weight animals which were more likely to have ovulated. Normal ovarian function in marmosets includes the development and maintenance of a large, steroidogenic interstitial gland, and the persistent presence of this gland, along with the cyclical presence of corpus lutea, leads to the heavier, sexually mature females having higher ovarian weights. Thus, the higher ovarian and uterine weights in the two high dose groups is an artifact of those groups having higher weight females, and is not an effect of DEHP dose. Accordingly, the PE Panel disagrees with the Expert Panel's conclusion that the increased female ovarian and uterine weights in the Mitsubishi study are indicative of precocious puberty.[40]

B. The Studies by Akingbemi et al. Reviewed in the Final Update Are Flawed and Do Not Support a NOEL of 1 mg/kg/day

The Expert Panel concluded that the results from two Akingbemi et al. (2001; 2004) studies reporting a NOEL of 1 mg/kg/day based on data obtained from purified Leydig cells were adequate for the evaluation process.[41] The effects reported by Akingbemi et al. at the LOEL (10 mg/kg/day) were abnormal changes in testosterone production and altered Leydig cell proliferation in the testes of prepubertal rats. However, because these studies suffer from a methodological deficiency – they rely on single-point measures of serum LH and testosterone – the PE Panel has significant concerns regarding the suitability of these studies for deriving the NOEL.

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39 Opinion of Dr. Tardif, Attachment 2, at page 1.

40 An equally plausible interpretation of the data is that high doses of DEHP enhance the health of females, thus enabling high dose females to mature in the usual time frame, with resulting increases in ovarian weights over the less healthy controls and lose dose females. The PE Panel dose not advance this hypothesis – it would be far too speculative a conclusion based on this one study. The PE Panel believes that the assertion of precocious puberty is equally speculative.

41 Final Update at 168.

Several papers and texts (Culler, 1998; Levine and Duffy, 1988; Creasy, 1999) provide excellent descriptions as to why single point measures of isolated or combined gonadotropin and gonadosteroid measurements do not allow for identifying toxicity from chemical exposures. The methodological problem arises from the fact that pulsatile releases of gonadotropin releasing hormone (GnRH) control LH release and pulsatile LH levels control testosterone production and release from Leydig cells. These pulsatile releases are superimposed on a circadian rhythm pattern for the secretion of these releasing factors and hormones. If multiple samples are not collected and analyzed from each animal over several hours during the peak phase of the circadian cycle, then the pulsatile releases may be missed. To ensure accurate comparisons among individuals, care must be taken to collect samples from all of the animals at precisely the same time relative to the circadian pattern described above.

The Akingbemi et al. studies do not meet these criteria. The Materials and Methods Sections in both Akingbemi et al. papers simply state that the blood was taken within 24 hours of the last DEHP exposure; there is no mention of any attempt on the part of the authors to control for the time of day (circadian pattern) between treatment groups. The lack of control for these circadian patterns and pulsatile releases of these factors and hormones is a critical flaw in the study design. For example, if all the control animals were sacrificed first, followed by the low dose, mid dose, and high dose animals, the investigators would be introducing a systematic sampling bias to their measurements because the animals may have been in different stages of the circadian pattern and have been experiencing varied pulsatile release of these factors/hormones.

Another significant flaw in the Akingbemi et al. studies lies in the method by which the authors isolated the Leydig cells from these animals and how they interpreted the data from endpoints derived from these cells. Leydig cells in immature rats are relatively quiescent and small and will elute/migrate through a Percoll gradient with a different density than mature Leydig cells. As the authors describe in the 2001 paper, PND 21 (progenitor) Leydig cells elute/migrate within the Percoll gradient at a band representing a density of 1.062 – 1.070 g/ml. PND 35 control rats have immature Leydig cells which elute/migrate through the same gel at a band representing a density of 1.070 to 1.088 g/ml and the Leydig cells obtained from PND 49 and 90 (mature) animals have densities greater than 1.070 g/ml. In addition, the authors used an enzyme critical for testosterone biosynthesis, 3β hydroxysteroid dehydrogenase (3β-HSD), as a biomarker for whether these cells were able to produce testosterone. PND 21 Leydig cells stain lightly or not at all for 3β-HSD indicating that they are quiescent in terms of testosterone biosynthesis. The Leydig cells from PND 35 and greater stain intensely for 3β-HSD indicating that testosterone biosynthesis can occur in those cells. Therefore, in control rat testes, as the Leydig cells mature and gain the ability to synthesize testosterone, their cellular density (a product of cell mass and size) changes and, correspondingly, the distance they elute/migrate within the Percoll gradient changes.

Typically, one of the first alterations noted in cells undergoing abnormal cell division (e.g., hyperplasia) or reacting to a toxic insult, is a change in cell mass and size. These changes, which are well described in textbooks of toxicological pathology, are changes that pathologists look for microscopically. However, measuring changes in cell mass or size (i.e., density) is very difficult using histological techniques, as a three dimensional object (the cell) is

only represented in two dimensions under a microscope. Consequently, it is not difficult to miss changes in cell density using microscopic histological techniques.

In mature rat testes, Leydig cells make up a significant portion of the total weight, about 20 – 25% (Creasy, 1999), and any change in Leydig cell number should cause a change in that percentage. However, in the Akingbemi et al. papers, the authors note a lack of change in either testes weight or size. If there was Leydig cell hyperplasia (as proposed by these authors), but no corresponding increase in testicular mass, then it stands to reason that the Leydig cells present are of different size, shape, and density than those found in control testes. The authors, therefore, have made a fundamental error in these experiments; they assumed that the Leydig cells from the treated animals would have the same density as those from the control animals. Because they assumed that the control and treated animals would have the same Leydig cell density, the authors only collected the Leydig cells that migrated within the band of the Percoll gradient that matched the control Leydig cells' migration. They did not consider that the Leydig cells from the treated animals may have a different density and therefore would migrate into a different band within the Percoll gradient.

As a consequence, all of the reported measures from the isolated fraction of Leydig cells simply represent the remaining Leydig cells within the treated testes that have the same density as those of control Leydig cell populations at that developmental stage. The authors did not collect other density bands within the Percoll gradient and examine whether the Leydig cells within those bands could also synthesize testosterone or have other characteristics of mature Leydig cells. As described above, cells that are hyperplasic or that have undergone toxic insult can have a different density. These cells were never detected or collected in these experiments.

Therefore, all the endpoints in Akingbemi et al. that make use of isolated Leydig cell preparations, (the ability of the Leydig cells to produce testosterone at either a basal level or in response to LH stimulation, the indicators of cell cycle stage used to investigate hyperplasia, the counting of the cells, aromatase levels, etc.) are flawed due to the assumption made by the investigators that the Leydig cells from the DEHP treated animals would migrate/elute in the same density band as those from the control animals.

In addition to the above flaws, at least one member of the Expert Panel questioned the reliability of the data in the Akingbemi et al. studies at the Public Meeting, stating that the data were "messy and perplexing" and that "I would never ask this group to do toxicological work again." However, rather than finding these data unsuitable for its review, the Expert Panel instead cherry-picked results from the studies which suggested DEHP toxicity. This is yet another example of procedural inequities in the review process described above, and is unacceptable in a review process that is supposed to be objective and deliberative.

Because of the serious flaws in the Akingbemi et al. studies described above, the PE Panel does not agree with the Expert Panel that these studies "are sufficient to conclude that DEHP is a reproductive toxicant in male rats at the indicated dose levels."[42]

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[42] *Id.*

CONCLUSION

For the reasons stated herein, the PE Panel has significant concerns regarding both the process by which the Final Update was produced and some of the scientific conclusions present in the Final Update. The PE Panel believes that because of significant procedural flaws, the Final Update does not adequately represent an objective and fully-deliberated consensus opinion. In addition, the PE Panel believes that the Expert Panel incorrectly disregarded scientific data which show that very high concentrations of DEHP that would cause profound adverse effects in rodent testes had no adverse effects on the testes of marmosets. The marmoset is a useful model for human male reproductive toxicity and the Mitsubishi study, while not perfect, provides valuable information about the toxicity of DEHP to primates, with importance implications for the degree of concern for human health. In addition, several aspects of the Expert Panel's review of reproductive and developmental toxicity do not, in fact, support the low NOAELs chosen, and there is less uncertainty about infant exposures than indicated by the Final Report. The PE Panel requests that the NTP-CERHR keep these points in mind as it considers the conclusions of the Expert Panel in the Final Update.

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ATTACHMENTS

1. Curriculum Vitae and Opinion of Dr. Stefan Schlatt entitled: Evaluation of the marmoset (Callithrix jacchus) as a model for reproductive toxicity.

2. Comments of the Phthalate Esters Panel of the American Chemistry Council on Notice of Intent to List Chemicals, submitted to the California Office of Environmental Health Hazard Assessment, May 24, 2005.

3. Curriculum Vitae and Opinion of Dr. Suzette Tardif entitled: Findings regarding female reproductive physiology from the Mitsubishi Study #B000496, "Sixty-five week repeated oral dose toxicity study of DEHP in juvenile common marmosets."

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University of Pittsburgh

School of Medicine
Department of Cell Biology and Physiology
Center for Research in Reproductive Physiology

W952 Biomedical Science Tower
3500 Terrace Street
Pittsburgh, PA 15261
412-648-8316
Fax: 412-648-8315

Evaluation of the marmoset (*Callithrix jacchus*) as a model for reproductive toxicity

Author: Stefan Schlatt, Ph.D.

e-mail: schlatt@pitt.edu

Date: January 30, 2006

1) Information on the Author

I obtained a PhD from the University Münster, Germany and was awarded a number of postgraduate fellowships including a Welcome Trust Training Fellowship in Reproductive Research and a Heisenberg-Fellowship which is the most prestigeous German Senior Research Fellowship granted by the Deutsche Forschungsgemeinschaft. At present, I am an Assistant Professor at the University of Pittsburgh School of Medicine, PA, USA. In recent years I have been employed and trained at several Centers of Excellence in Reproductive Sciences including the Institute of Reproductive Medicine of the University Münster, Germany; the Institute of Reproduction and Development of Monash University, Clayton, Australia and the Center for Animal Transgenesis and Germ Cell Research of the University of Pennsylvania, Kennett Square, PA, USA. I have explored a wide spectrum of research in reproductive biology including such diverse topics as the photoperiod-dependent regulation of female and male reproductive organs in Djungarian hamsters as well as preclinical research using macaque and marmoset monkeys to analyse the role of inhibin in the testis, the effects of hormones on prepubertal testis development and the development of male contraceptive regimens. I and my colleagues established several milestones for the development of germ cell transplantation as a clinical tool performing the first successful germ cell transplant into a testis of non-human primates and men. Recently, a novel strategy to use testicular xenografting as a tool to generate fertile sperm was described and applied on marmosets, macaques and men. We were also able to describe xenografting as a new tool for exploration of toxic effects during testicular development in rhesus monkeys.

This document has been compiled in response to a request by the American Chemistry Council Phthalate Esters Panel. I have been asked to critique a recent review by Li et al. (2005) and to render my opinion of the general value of the marmoset as a model for human testis development and its use for risk assessments of gonadotoxic exposures in the male. In addition I was asked to express my opinion on the validity and relevance of the Mitsubishi study.

2) Introduction

The marmoset has been intensely used for many years as a non-human primate model to describe the physiology of testicular development and function. In contrast to rodent animal models, this small new world primate shows many similarities with old world primates and man in regard to the general developmental pattern. Like human and old world primates the marmoset enters three periods of development with an active postnatal phase of the hypothalamus-pituitary-gonadal axis, a prepubertal period of quiescence and a re-awakening of the hormonal axis and final differentiation of the male reproductive system during puberty. This pattern is quite different from that in mice and rats which do not show a marked prepubertal period of no or slow gonadal growth and differentiation. Since the marmoset is easy to maintain and to reproduce and – as a primate - appears to be evolutionarily close to man, it has been considered a valuable and clinically relevant animal model to analyze toxic effects on the developing male reproductive system. However, the marmoset also exhibits some interesting and unique features in regard to hormonal regulation of its reproductive functions. A detailed review by Li et al. (2005) that summarizes similarities and differences concludes that interpretation of experimental findings on the testicular effects in marmosets should be made with caution. The present short report revisits the pro- and contra-arguments of using the marmoset as a model for testicular developmental toxicity and concludes that the marmoset presents a useful and valid model to explore several aspects of testicular development. I have also been asked to critically review an unpublished study from the Mitsubishi Chemical Safety Institute using the marmoset as a model to explore the testicular toxicity of DEHP. As discussed below, I conclude that the results of this study should be carefully and critically considered for the evaluation of risks associated with exposure to DEHP.

3) The value of the marmoset as a model for male testis development and evaluation of bias in Li review

3.1 Physiological characteristics of the male marmoset endocrine and reproductive systems

The recent review by Li et al. (2005) is a comprehensive review of the testicular development and function in the marmoset. The authors present the current knowledge on embryonic testicular development and describe the changes occurring during the postnatal period (2-3 weeks) and the pubertal period (6-12 months). They show the striking similarities of organization of the marmoset and human spermatogenic epithelium (both species have a multistage organization of spermatogenic stages per tubular crossection). Although nine stages of the seminiferous epithelial wave have been distinguished originally, many authors have adapted the human staging system for the marmoset indicating quite similar mechanisms of germ cell development and clonal expansion of germ cells in marmosets and man. This organizational similarity has prompted scientists to promote the marmoset as a "suitable model for studies relevant to human testicular function" (Millar et al., 2000).

Li et al. (2005) point out an unusual uniformity of Sertoli cell morphology throughout the marmoset spermatogenic cycle. In rodents and macaques Sertoli cells show specific morphological features related to specific stages of spermatogenesis. However, it is not clear how stage-specific changes of Sertoli cells can be identified in seminiferous tubules of men and marmosets which show a mixed pattern of spermatogenic stages. Although there are insufficient human data on Sertoli cell morphology to know whether such uniformity occurs in humans, it appears likely that the human and marmoset Sertoli cell show a high degree of similarity in this respect.

Li et al. (2005) quote studies reporting a high efficiency of marmoset spermatogenesis although this finding appears slightly controversial in the literature. They indicate that FSH is the prime regulator of Sertoli cell proliferation which correlates well with the role of FSH in other primates but differs from its less significant role in rodents.

One of the most interesting marmoset specific adaptations is the absence of the LH hormone. In the marmoset and other new world monkeys a CG type hormone is expressed in the pituitary and is responsible for regulation of steroid secretion in the ovary and the testis. This change is accompanied by a deletion of the LH receptor which lost exon 10 and can therefore not bind to LH, but has high affinity for CG. Despite of the exchange of LH by CG the marmoset shows many similarities to man. The function and regulation of FSH and CG and their feedback mechanisms resemble other primates. It is quite interesting to note that the LH receptor deletion in the marmoset mimics the situation in a hypogonadal patient (Gromoll et al., 2000). The availability of the marmoset as a model opened a new research field to explore the molecular mechanisms of hormone specificity of the LH receptor (Gromoll et al., 2003).

Li et al. (2005) propose a generalized steroid hormone resistance in the marmoset. This phenomenon is indeed quite striking and has been proposed for the mineralocorticoid and glucocorticoid hormones. However, whether or not this resistance also exists for gonadal steroids is poorly understood. Although some evidence exists that the response to sex steroids is rather poor, these observations should be balanced with the facts that many aspects of the marmoset in regard to sex steroids are strikingly similar to humans and differ from those in the rodent.

The authors mention that there is little information on marmoset testosterone synthesis and testosterone metabolism. The marmoset, but not the mouse, has sex hormone binding globulin separating the serum testosterone into active free and bound fractions. Due to affinities of these proteins the marmoset appears to have high levels of unbound testosterone in the circulation which is similar to the unusually high levels of glucocorticoids. However, in the absence of solid data on sex steroids it appears poorly justified and premature to transfer the conclusion of high sex steroid resistance from the glucocorticoid and mineralocorticoid system to the sex steroid system.

Some other curiosities exist which – in my opinion- do not impact the use of the marmoset as a model. Intact male marmosets respond with positive feedback to estradiol. Such a response is usually observed in orchidectomized rats or macaques and many homosexual men. The development of the testis relies on the presence of FSH and CG. A delay in testicular development does not have serious consequences, similar to other primates. Our own studies using xenografting as a model system showed the expected outcome that mouse LH can not stimulate the androgen-dependent differentiation of grafted marmoset tissue due to its deleted LH-receptor (Schlatt et al., 2002). Co-grafting with hamster tissue was not sufficient to induce testicular development (Wistuba et al., 2004). Li et al (2005) interpreted our data as indicative of unique factors responsible for marmoset spermatogenesis. However, the more likely explanation is that CG is needed to stimulate organogenesis of the immature testicular fragments and that the testosterone supplied by the hamster graft is insufficient. This explanation is supported by very recent data showing that autografted marmoset testes can develop through puberty (Wistuba et al., 2005), an indication that their hormonal system can drive spermatogenesis in autografted tissue.

In conclusion, the review by Li et al. (2005) presents a useful resource for any scientist interested in the marmoset model. However, the authors' interpretation of the suitability and validity of the model carries an unjustified negative bias.

3.2 Personal judgement of the value of the marmoset as research model for assessment of male reproductive toxicity.

In my opinion there is no doubt that the marmoset is a useful model to explore developmental and toxicological aspects of the testis. Certainly, old world primates appear to be favorable as they maintained a closer physiological relation to humans. However, many similarities in regard to testicular organization, general developmental pattern and hormonal regulation render the marmoset a much more useful model when compared to rodents. The marmoset therefore has its valid place when it comes to the analysis of toxic effects on testicular development. With choice of the correct timepoints this species should be highly useful and informative for exploring the effects on Sertoli cell differentiation, testicular growth and effects of FSH on the testis. The striking similarities to man make it an excellent model for studying effects on germ cell development, the organization of the seminiferous epithelium and changes to the kinetics of spermatogenesis. Due to its unusual gonadotropic and sex steroid regulation it is certainly an interesting biological model for exploring steroid feedback and the functions of the CG-molecule with less relevance for humans in these specific aspects.

4) The value of the Mitsubishi study to determine male reproductive toxicity

4.1 Validity of the results of the Mitsubishi study in respect to pitfalls and shortcomings

This study is a unique effort to explore the effects of DEHP on testicular development in a non-human primate model. In the oral dose toxicity part of the study marmoset monkeys of 90-110 days of age were exposed to three doses of DEHP (100, 500, 2500 mg/kg) and vehicle for 65 weeks. The monkeys received a daily oral dose in corn oil by gavage. The size of the male treatment groups finishing the exposure was 8 (vehicle), 9 (100mg/kg), 10 (500 mg/kg) and 9 (2500 mg/kg). 3 animals in each group were subjected to perfusion allowing electron microscopic analysis of the testes. At the time of sacrifice an impressive number of endpoints were analyzed: 10 hematological parameters, 21 blood chemical parameters, levels of testosterone, estradiol and T3/T4 as endocrine parameters, body weights and organ weights of pituitary, thyroids, liver, pancreas, spleen, kidneys, testes, prostate, seminal vesicles and epididymides. Histological analysis of all organs was performed and additional histochemical (3betaHSD) and ultrastructural analysis was performed in the testes. Testicular homogenates were used to determine counts of spermatids (heads per g testicular tissue) and 6 testicular enzymes were analyzed biochemically. The activities of nine hepatic enzymes were determined in liver homogenates.

The results of the study reveal no statistical changes in clinical parameters and body weights, hematology, blood chemistry, hormone levels and testicular and hepatic enzymes for the male monkey groups. In each DEHP treatment group and in the control group one monkey showed low testis and reproductive organ weights. The authors report that this usually correlated with low body weight and that these three monkeys were therefore considered to be immature. This implies that they consider each other monkey in the study to be fully adult at the time of sacrifice. However, for reasons discussed below, this may not be the case. When summing up the age at the start of the study and the duration of the study, the time point of sacrifice was around 20 months of age which corresponds to the late phase

marmosets go through puberty. It is characteristic for this species that the variability to sexually mature is extremely wide (12-20 months). Therefore, a subgroup of four (or 5, see below) late pubertal monkeys among 36 monkeys is not unusual at this age.

The apparent occurrence of different developmental stages could have been avoided by delaying the time point of sacrifice by several months. While the period of exposure was wisely selected and covered the full period of testis development after weaning, the period of sacrifice means that both adult and late pubertal monkeys were analyzed. The exclusion of some of the treated animals from sperm count analysis may be an indication that poor definition of developmental stages at the time of analysis may have influenced the outcome of the study and might have induced high variability in the data.

I have some questions in regard to the interpretation of testicular histology. Photographs 1 and 2 show the histological patterns of qualitatively and quantitatively normal testes of fully adult marmosets. If these testicular crossections are – as indicated - representative of the control and high dose DEHP groups, these micrographs clearly reveal that the exposure to DEHP had no impact on testis development and spermatogenic induction in marmosets. Photographs 3 and 6 depict a rather unusual pattern of testicular development for a late pubertal monkey and are potentially showing some testicular damage. The degree of vacuolization is quite high and the number of germ cells is rather low for the late stage of pubertal testicular development. It is therefore difficult to determine whether animals 10208 (Fig. 6) and 10204 (Fig. 3) are showing an immature or damaged testicular phenotype. Surprisingly an underdeveloped testis as shown for Mk 10208 has not been reported elsewhere in the study. It might be useful to send all testicular histological samples for a blinded re-analysis to a scientist who has abundant expertise in primate testicular development. Together with the results on testis weight and reproductive organ weights this might enable analysis of subtle effects and confirmation whether the proposed “growing” monkeys are indeed immature or whether their testes are damaged.

There was a significant loss of monkeys during the study. About 15% of the experimental animals died during the exposure period and were replaced. Marmosets are highly sensitive and show high mortality rates when exposed to poor hygiene conditions or poor diet. The main reason for this high death rate (also known as wasting syndrome) is intestinal infection. Marmosets have a short gut transit time, easily and frequently develop diarrhea, and eventually develop colitis leading to a rapid loss of body weight and death. The animal husbandry conditions in the study which exposed isolated single monkeys to a solid chow without supplementation of additional fruit or additional protein components were suboptimal to maintain the animals in good health. These monkeys live in social family groups and isolation might have been an additional stressor throughout the experiment. In addition, the effect of daily exposure to approximately 0.2-0.5 ml of corn oil must have had additional negative effects on their digestive tract. The amendments and deviations from the study protocol as outlined in the report (see 4.4.3: addition of milk powder to the diet and 5.4: discontinuation of oral exposure in 6.6% of applications) show that the animal care staff and veterinarians experienced serious problems in respect to the health and the nutritional status of the monkeys.

Given these factors, it is possible that the lack of statistical significance in this study might be due to high inter-animal variations in many of the analyzed parameters. Reasons for the high variation could be the different developmental status of the animals at the time of sacrifice, poor health of some of the monkeys, and/or a high individual variability known for marmosets.

4.2 Personal judgement of the value of the Mitsubishi study for risk assessment

Despite of the above described shortcomings this study presents strong evidence that DEHP had **no major** effect on testicular development even after very long and intense DEHP exposure. The study analyzed many different parameters of which hardly any showed a significant change. Unfortunately, the poor design of the study and suboptimal animal husbandry render this study not fully conclusive.

The oral dose toxicity study has generated a unique and wide set of data in a primate species which – as outlined above – has been used and should be considered in the future as a valuable model for the study of testicular development. Although the marmoset has an unusual androgen-CG feedback loop, the most striking effect of DEHP in other animal models is related to direct effects on Sertoli cells during testicular growth. In regard to this parameter the marmoset should be considered an excellent animal model. The analysis of the histological testicular parameters in the Mitsubishi study are therefore valid and impressively confirm a minor – if any – negative effect of DEHP exposure on Sertoli cell development and induction of spermatogenesis.

In my opinion the Mitsubishi study can therefore not be fully neglected and should be carefully and critically considered for evaluating the risk of gonadotoxic effects in humans after exposure to DEHP.

Yours sincerely,

(Stefan Schlatt, Ph.D.)

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May 24, 2005

Via Facsimile: (916) 323-8803
Via Email: coshita@oehha.ca.gov

Ms. Cynthia Oshita
Office of Environmental Health Hazard Assessment
P.O. Box 4010
Sacramento, CA 95812-4010

Dear Ms. Oshita:

The American Chemistry Council Phthalate Esters Panel (Panel) submits these comments in response to the California Office of Environmental Health Hazard Assessment (OEHHA) Notice of Intent to List Chemicals of March 4, 2005. The Panel includes the major U.S. producers and some processors of phthalate esters. These comments pertain to the four phthalates for which OEHHA provided notice that it intends to list as chemicals known to the state to cause reproductive toxicity under the Authoritative Bodies Mechanism of Proposition 65 – dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-n-hexyl phthalate (DnHP), and diisodecyl phthalate (DIDP). These comments will show that these four phthalates fail to meet the listing criteria of Proposition 65.

The Panel renews in whole the comments it submitted to OEHHA on August 26, 2004. These comments reiterate and expand upon those earlier comments, and address some of OEHHA's responses to those comments. These comments make the following points: 1) The marmoset is a scientifically sound model for investigating potential effects of phthalates on human reproduction, as pointed out in the Panel's previous comments, and OEHHA's responses to those comments are inadequate to disqualify the marmoset as such a model; 2) the marmoset data comprise scientifically valid data, not considered by NTP-CERHR, which establish that the phthalates do not meet the criteria for identification "as causing reproductive toxicity;" 3) NTP-CERHR found minimal to negligible risk of human reproductive toxicity and, therefore, the Proposition 65 listing criteria are not met for these phthalates; and 4) OEHHA has no statutory mandate to list chemicals such as these phthalates, which pose no significant risk to public health, and the Panel believes it would be poor public policy to list such low-risk chemicals under Proposition 65.

For the reasons given in these comments, and in its earlier comments, the Panel believes that DBP, BBP, DnHP and DIDP do not meet the criteria for listing under Proposition 65 pursuant to the authoritative bodies mechanism, and that OEHHA should not list these four phthalates under Proposition 65.

If you have any questions, please call Marian K. Stanley, Manager of the Phthalate Esters Panel, at (703) 741-5623 or email her at Marian_Stanley@americanchemistry.com.

Sincerely yours,

Courtney M. Price
Vice President, CHEMSTAR

Enclosure

Before the
California Environmental Protection Agency
Office of Environmental Health Hazard Assessment

COMMENTS OF THE
PHTHALATE ESTERS PANEL OF THE AMERICAN CHEMISTRY COUNCIL
ON NOTICE OF INTENT TO LIST CHEMICALS

Notice of Intent to List Chemicals California Regulatory Notice Register 05, No. 9-Z, pp. 289-290 (March 4, 2005)

Courtney M. Price
Vice President
CHEMSTAR

Dell E. Perelman, Esq.
Vice President and
General Counsel

Marian K. Stanley
Manager, Phthalate Esters Panel

Karyn M. Schmidt, Esq.
Counsel, CHEMSTAR

Of Counsel:
William K. Rawson, Esq.
Ann Claassen, Esq.
Latham & Watkins LLP
555 Eleventh Street N.W.
Suite 1000
Washington, D.C. 20004

May 24, 2005

AMERICAN CHEMISTRY COUNCIL
1300 Wilson Blvd.
Arlington, VA 22209
(703) 741-5000

EXECUTIVE SUMMARY

The American Chemistry Council Phthalate Esters Panel (Panel) submits these comments in response to the California Office of Environmental Health Hazard Assessment (OEHHA) Notice of Intent to List Chemicals of March 4, 2005 (California Regulatory Notice Register 05, No. 9-Z, pp. 289-290 (March 4, 2005)). The Panel includes the major U.S. producers and some processors of phthalate esters. These comments reiterate and expand upon the Panel's comments of August 26, 2004, which were submitted in response to OEHHA's May 28, 2004 Request for Information on Chemicals Under Consideration for Possible Listing via the Authoritative Bodies Mechanism. In addition, these comments address portions of OEHHA's response to those earlier comments, contained in a March 1, 2005 letter from Dr. George V. Alexeeff, OEHHA Deputy Director of Scientific Affairs, to Ms. Courtney M. Price, Vice President of CHEMSTAR. These comments pertain to the four phthalates for which OEHHA has provided notice that it intends to list as chemicals known to the state to cause reproductive toxicity under the Authoritative Bodies Mechanism of Proposition 65 – dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-n-hexyl phthalate (DnHP), and diisodecyl phthalate (DIDP). For the reasons presented in both the August 2004 comments and these comments, the Panel strongly believes that these phthalates should not be listed under Proposition 65. These comments renew in whole the comments submitted by the Panel in August 2004, and make the following points:

- OEHHA's responses to the Panel's earlier comments that new marmoset data strongly suggest the effects observed in rodents are not relevant to humans are not sufficient to invalidate the marmoset as a model for investigating the potential effects of phthalates on human reproduction. In general, a primate is considered to be a more relevant species than rats for human risk assessment, since humans are themselves primates.

- OEHHA's stated basis for listing these phthalates under the Authoritative Bodies Mechanism is the monographs published in 2003 by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR). OEHHA acknowledged in its response that the marmoset data were "relevant for DBP, BBP, DnHP, and DIDP" and thus considered by OEHHA to be "'new data' that were not considered by the authoritative body." Because the marmoset data were not considered by the authoritative body (NTP-CERHR), and those data clearly establish that the association between adverse reproductive effects in humans and the phthalates is not "biologically plausible," these four phthalates fail to meet the listing criteria of Proposition 65.

- In its representation of NTP-CERHR's conclusions, OEHHA fails to acknowledge that NTP-CERHR found minimal to negligible risk of human reproductive toxicity for these phthalates. Because NTP-CERHR did not clearly conclude that the phthalates cause reproductive toxicity in humans, the Proposition 65 listing criteria are not met for these four phthalates.

- OEHHA has no statutory mandate to list chemicals such as these phthalates, which pose no significant risk to public health. Therefore, listing these phthalates under Proposition

65 makes little sense from a public policy perspective, as it would likely to lead to public and regulatory concern about these substances that is not warranted in light of the data for them. Proposition 65 listing also is likely to lead to reformulation of products away from the listed phthalates, and toward other chemicals about which less reproductive toxicity information may be known. The Panel strongly believes that sound public policy would avoid promoting such consequences through Proposition 65 listing, where human exposures to substances – such as DBP, BBP DnHP and DIDP – have been shown to pose a very low risk of reproductive or developmental toxicity.

The Panel believes that DBP, BBP, DnHP and DIDP do not meet the criteria for listing under Proposition 65 pursuant to the authoritative bodies mechanism, and that in any event would be poor public policy to list such low-risk chemicals. Therefore, OEHHA should not list these four phthalates under Proposition 65.

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INTRODUCTION

The American Chemistry Council Phthalate Esters Panel (Panel) submits these comments in response to the California Office of Environmental Health Hazard Assessment (OEHHA) Notice of Intent to List Chemicals of March 4, 2005.[1] The Panel includes the major U.S. producers and some processors of phthalate esters.[2] These comments pertain to the four phthalates that OEHHA has stated it intends to list as chemicals known to the state to cause reproductive toxicity under the Authoritative Bodies Mechanism of Proposition 65[3] – dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), di-n-hexyl phthalate (DnHP), and diisodecyl phthalate (DIDP). OEHHA states that the basis for these listings is the monographs published in 2003 by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR, 2003a,b,c,d).

At the outset, the Panel reaffirms in whole its August 26, 2004 comments, which were submitted in response to OEHHA's May 28, 2004 Request for Information on Chemicals Under Consideration for Possible Listing via the Authoritative Bodies Mechanism.[4] In addition, these comments reiterate and expand upon the Panel's earlier comments, and address portions of OEHHA's response to those earlier comments, which are contained in a March 1, 2005 letter from Dr. George V. Alexeeff, OEHHA Deputy Director of Scientific Affairs, to Ms. Courtney M. Price, Vice President of CHEMSTAR.

Part I of these comments addresses OEHHA's responses to the Panel's previous submission of marmoset data (not considered by NTP-CERHR), which strongly suggest that the effects of phthalates observed in rodents are not relevant to humans. In its response, OEHHA listed four features of marmoset reproductive physiology, based upon which it concluded that the marmoset is not a suitable model for evaluating the potential reproductive effects of phthalates on humans. Because these features have not been shown to be relevant to the mechanism by which phthalates affect reproduction in the species most sensitive to phthalate perturbation (i.e. rodents), and do not appear to interfere with other phthalate effects known to manifest in marmosets (e.g., liver enzyme induction), they do not invalidate the marmoset as a model for investigating the potential effects of phthalates on human reproduction. The Panel continues to believe that the marmoset data strongly suggest that phthalates do not pose a reproductive toxicity hazard for humans.

OEHHA acknowledges in its response that the marmoset data were "relevant for DBP, BBP, DnHP, and DIDP" and thus considered by OEHHA to be "'new data' that were not considered by the authoritative body." Part II of these comments explains that, because the marmoset data were not considered by the authoritative body (NTP-CERHR), and those data

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1 California Regulatory Notice Register 05, No. 9-Z, pp. 289-290 (March 4, 2005); http://www.oehha.ca.gov/prop65/CRNR_notices/admin_listing/intent_to_list/noilpkg21.html.

2 The Panel's members include BASF Corporation, Eastman Chemical Corporation, ExxonMobil Chemical Company, Ferro Corporation, and Teknor Apex Company.

3 *See* CAL. HEALTH & SAFETY CODE § 25249.8(b); CAL. CODE REGS. tit. 22, § 12306.

4 http://www.oehha.ca.gov/prop65/CRNR_notices/admin_listing/requests_info/dcallin21.html#get

clearly establish that the association between adverse reproductive effects in humans and the phthalates is not "biologically plausible," these four phthalates fail to meet the listing criteria of Proposition 65.

Part III of these comments points out that, even assuming that the rodent data cited by NTP-CERHR and relied upon by OEHHA are relevant to humans, NTP-CERHR generally found that these phthalates posed minimal to negligible risk of reproductive or developmental effects in humans. Nevertheless, in its representation of NTP-CERHR's conclusions, OEHHA fails to acknowledge qualifying language, the result of which is an overstatement of NTP-CERHR's findings of phthalate reproductive toxicity. Because NTP-CERHR did not clearly conclude that the phthalates cause reproductive toxicity, the Proposition 65 listing criteria are not met for these four phthalates.

Part IV of these comments makes the point that OEHHA has no statutory mandate to list chemicals such as these phthalates, which pose no significant risk to public health. Therefore, the Panel believes that listing these phthalates under Proposition 65 makes little sense from a public policy perspective as it would likely to lead to public and regulatory concern about these substances that is not warranted in light of the data for them, and would also likely lead to reformulation of products away from the listed phthalates, and toward other chemicals about which less reproductive toxicity information may be known.

For the reasons given in these comments, and in its August 2004 comments, the Panel believes that DBP, BBP, DnHP and DIDP do not meet the criteria for listing under Proposition 65 pursuant to the authoritative bodies mechanism, and that in any event would be poor public policy to list such low-risk chemicals. Therefore, OEHHA should not list these four phthalates under Proposition 65.

I. OEHHA'S RESPONSES TO THE PANEL'S EARLIER COMMENTS ARE NOT SUFFICIENT TO INVALIDATE THE MARMOSET AS A SUITABLE MODEL OF HUMAN REPRODUCTIVE PHYSIOLOGY

A. The Features of Marmoset Reproductive Physiology Listed by OEHHA in its Response Letter Are Insufficient to Invalidate the Marmoset as a Model for Investigating the Potential Effects of Phthalates on Human Reproduction

In its August 26, 2004 comments, the Panel stressed that a recent study on marmosets (MCSI, 2003; Tomonari, 2004), which had not been evaluated by NTP-CERHR, strongly suggests that DEHP and other phthalates do not pose a reproductive toxicity hazard for humans. This study demonstrated that daily administration of 2500 mg DEHP/kg/day from weaning through maturity did not affect male reproductive tract development in the marmoset. In its response to this comment, OEHHA stated:

> OEHHA agrees with [the Panel] that these data are relevant for DBP, BBP, DnHP, and DIDP. . .and thus considers this study as "new data" that were not considered by the authoritative body.

However, OEHHA then listed four features of marmoset male reproductive physiology, based upon which it concluded that the marmoset is not a suitable species for investigating potential

reproductive effects of DEHP (and by implication other phthalates) in humans. The comments below demonstrate that these four features of marmoset reproductive physiology do not invalidate the marmoset as a model for investigating potential human reproductive effects of phthalates.

Initially, it is reasonable to presume that unique features of any non-human animal model can be cited that might affect the comparison of that model to humans. For example:

- rhesus macaque males display seasonal variation in gonadal function and testosterone production that is not mirrored in humans, marmosets or rats;
- macaques exhibit very low levels of inhibin B neonatally, relative to rats and humans; and
- rats and macaques show a segmental/radial distribution of stages of spermatogenesis within the testis, while humans and marmoset exhibit a semi-helical organization (Sharpe, et al., 2000).

Because all animal models will be different from humans in some respects, merely pointing out those differences does not provide justification sufficient to reject a particular model. Rather, it is critical to determine whether the differences between the animal model and humans are pertinent to the specific comparison, which in turn depends on whether the mechanism that produces the effects of a given compound is relevant to the particular species differences described. Absent strong evidence to the contrary, a primate is considered to be a more relevant species than rats for human risk assessment, since humans are themselves primates.

With this in mind, what follows is an examination of the four features of marmoset reproductive physiology cited by OEHHA in its response to the Panel's comments, and an analysis of whether the differences between marmoset and human male reproductive physiology are pertinent to a determination of whether the marmoset is a valid model of human male reproduction. This analysis shows that there is no evidence that the features of marmoset reproductive physiology listed in OEHHA's response letter are related to the mechanism by which DEHP, or other phthalates, affect male reproduction. This analysis (and that of Section I.B.) is based on a review of relevant research by Dr. Suzette Tardif, Associate Director of the Southwest Foundation for Biomedical Research, who is a leading authority on marmoset reproduction.[5]

1. Marmoset Sertoli Cell Morphological Uniformity Does Not Invalidate the Marmoset as a Model for Human Reproductive Toxicity

OEHHA's Response 1 to Comment 1: "*There is no morphological variation in the spermatogenic epithelium . . . [indicating that] marmosets are totally different from most other mammals studied, including rodents and humans*."

[5] Dr. Tardif's comments reflect her opinions and are not to be interpreted as necessarily the opinions of the Southwest Foundation for Biomedical Research.

This statement appears to be based on the conclusions of Rune, et al. (1992) that there was no indication of "a dependent relationship between the spermatogenic stages and Sertoli cell morphology" and that "[a]s far as this finding is concerned, marmoset Sertoli cells differ from those of other species." While Sertoli cells appear to be an initial target of DEHP (and MEHP) in rodents, there is no evidence that this difference (Sertoli cell morphological uniformity) is in any way related to the mechanism by which DEHP affects the Sertoli cells. In fact, despite this difference, Rune, et al. conclude that, because marmoset Sertoli cells appear and behave similarly in vitro and in vivo, "the adult marmoset monkey could provide a primate model for mature Sertoli cells in culture, since there is [also] a close similarity to human adult Sertoli cells in vitro and in vivo."

2. **The Marmoset's Pituitary Production of Chorionic Gonadotropin, Rather Than Luteinizing Hormone Does Not Invalidate the Marmoset as a Model for Human Reproductive Toxicity**

OEHHA's Response 2 to Comment 1: *[T]he pituitary of common marmosets does not produce [luteinizing hormone] LH. Instead, it produces chorionic gonadotropin (CG), which is only produced in the placenta of humans or rodents. . . ."*

The evidence does support the conclusion that the primary luteotrophic gonadotropin produced by the marmoset pituitary is CG, and not LH. However, this distinction may be inconsequential, as both CG and LH bind to the same receptors and CG essentially acts like LH in tissues such as the luteal cells of the ovary. Whether the difference in molecular structure of the pituitary gonadotropin is significant is unknown, and will depend upon the phthalates' not yet established mechanism of action. On the other hand, it is clear that the basic hypothalamic-pituitary-gonad control mechanisms present in other primates are also present in marmosets. For example, release of pituitary gonadotropins and, subsequently, of testosterone production in male marmosets are affected by GnRH analogues in a fashion identical to that of other primates, including humans (e.g., Prince, et al., 1998).

3. **The Inability to Co-Transplant Marmoset and Hamster Testicular Tissue into Nude Mice Does Not Invalidate the Marmoset as a Model for Human Reproductive Toxicity**

OEHHA's Response 3 to Comment 1: *"Recent studies using transplanting techniques have shown that the conditions needed for initiation of spermatogenesis in the marmoset are remarkably different from those present in other mammals."*

This comment appears to be based on the work of Wistuba et al. (2004) in which the authors co-grafted marmoset and hamster testicular tissue into nude mice, but were unable to get marmoset spermatogenesis to proceed beyond the spermatogonial stage. The failure of the grafting techniques described by Wistuba et al. is likely related to Response No. 2 above, i.e., the difference in gonadotropin structure in New World monkeys, such as marmosets, compared to Old World monkeys and rodents. Witsuba et al. acknowledge that although they attempted to circumvent this difference by administering human CG to the host hamsters, "it might be that the exogenous administration was not sufficient to achieve a microenvironment in the mouse recipient that mimics the situation in the marmoset." This hypothesis would best be tested by

attempting a similar grafting with testicular tissue from another New World monkey, such as a squirrel monkey, since all New World monkeys are thought to share the same LH receptor changes that likely drive the difference in pituitary gonadotropin. Such an experiment has not yet been attempted, and until is has been, no valid basis exists to conclude that the failed transplant experiments of Wistuba et al. indicate that marmosets are not a suitable human reproductive model.

4. Marmoset Twin Germ Cells Likely Are Not Chimeric, and Therefore Marmoset Chimerism Does Not Invalidate the Marmoset as a Model for Human Reproductive Toxicity

OEHHA's Response 4 to Comment 1: *"XX germ cells have been reported from the testes of male marmosets with a female twin . . . [t]he chimeric feature of [marmoset] twins is rare in most mammals including rodents and humans. . . ."*

The chimeric nature of marmoset twin germ cells cited by OEHHA appears to be based on two older studies, Benirschke and Brownhill (1963) and Hampton (1973). However, the results of these older studies have not been replicated and OEHHA acknowledged in its response letter that the occurrence of germ cell chimeras in marmosets has been questioned (e.g., Ford and Evans, 1977) and that more study is needed to clarify this issue. Indeed, most investigators working with marmosets today agree that it is highly unlikely that germ cell chimerism occurs in marmosets (Gengozian et al., 1980; Ford and Evans, 1997). Moreover, given the presence of hematopoietic cells (which are known to be chimeric) in most marmoset tissues, it is difficult to definitively demonstrate that chimerism occurs in germ cells as opposed to supporting hematopoietic cells within the gonad. Consequently, this purported difference between marmosets and other mammals is unverified and should not be relied upon to conclude that marmosets are not a viable model for human reproduction studies.

B. The Conclusions of Zuhkle and Weinbauer Regarding the Use of Marmosets as a Model for Human Reproductive Toxicity are Incorrect, and Possibly Biased

OEHHA's Response: *"Because of the fundamental differences in the testis between common marmosets and humans, it has recently been suggested that 'the use of this animal model cannot be recommended for reproductive toxicology assessment' (Zuhkle and Weinbauer, 2003)."*

Most of the points raised in Zuhkle and Weinbauer (2003) are the same as those enumerated by OEHHA in Responses 1 – 4 above. Some of the additional points raised by the authors (i.e., that high interindividual fluctuations of steroid hormone levels makes monitoring of ovarian cycle based upon serum concentrations difficult or not feasible; and that marmosets require a complex diet in captivity) are simply incorrect. In relation to the ability to monitor ovarian cyclicity based on serum hormone concentrations, numerous studies have used circulating estradiol and progesterone concentrations to track ovarian cyclicity, so this is not an issue.

Moreover, the U.S. Food and Drug Administration (FDA), one of the five authoritative bodies specifically identified in Proposition 65 for the purposes of identifying chemicals as causing reproductive toxicity,[6] has proposed that the marmoset is an appropriate model for human health assessment, and may be a more appropriate model than the rat for evaluation of reproductive toxicity hazard to humans. For example, the FDA Safety Assessment of DEHP states that:

> Spermatogenesis in the marmoset is organizationally similar to the process that occurs in humans, with regard to length of the spermatogenic cycle, duration of spermatogenesis, and number of mitotic divisions (Millar et al., 2000; Weinbauer et al., 2001). Consequently, the marmoset has been described as an appropriate model for experimental studies of human spermatogenesis. By analogy, it can be assumed that DEHP-induced effects on this process seen in marmosets would be applicable for humans.

(FDA, 2001, p. 35.)

Finally, there is a possibility of bias of Zuhkle and Weinbauer in favor of the cynomolgus macaque as a model for reproductive toxicology, given that both authors work for Covance, a company that sells cynomolgus macaques (but not marmosets) to pharmaceutical firms and biomedical institutions.

C. Vitamin C Levels in the Marmoset Do Not Negate the Relevance of the Marmoset Study to Human Risk Assessment

OEHHA's Response: *"[V]itamins C and E are protective against the testicular effects of DEHP in rats or mice (Ishihara et al., 2000; Ablake et al., 2004). Common marmosets require high levels of dietary vitamin C so regular diets for this species usually contain high levels of vitamin C supplements (e.g., MCSI, 2003). Serum levels of vitamin C in common marmosets are markedly higher (2.56 mg/100ml in average; Flurer and Zucker, 1987; 1989) than most other mammals (0.63 mg/100ml in average in humans; Hampl et al., 2004), suggesting a possibility of reduced sensitivity to DEHP in this species."*

As indicated by the above response, OEHHA is concerned that the lack of observed effects of DEHP on marmoset reproduction (e.g., MCSI, 2003; Tomonari et al., 2004) may be due to the protective action of high doses of vitamin C, rather than a difference in the effects of DEHP between rodents and primates. These concerns, however, are not well founded because: 1) the levels of vitamin C used in Tomonari et al. (2004) are not high relative to the marmoset's requirements and 2) based on the available science (discussed below), it is not clear that vitamin C affords any protection to primates from DEHP exposure. Moreover, if the level of vitamin C in the marmosets' diet in Tomonari et al. in fact provided the degree of protection necessary to be responsible for the observed lack of effects at doses of 2500 mg/kg/day, then the level of vitamin C in the average human diet would be protective of similar exposures to DEHP (test exposures were more than 100,000-fold higher than CDC data demonstrate actually occur in humans). In other words, the vitamin C levels in the marmoset diet in the Tomonari et al. study

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[6] CAL. CODE REGS. tit. 22, § 12306(i)(5).

were similar to normal levels in the human diet and, consequently, whether vitamin C had a protective effect is not directly relevant to a risk assessment.

Marmosets, like all primates, require that their diet be supplemented with vitamin C (ascorbic acid) (NRC, 2003). Flurer et al. (1987) reported that marmosets need more vitamin C than do humans, suggesting that a minimum of 20 mg/kg/day (the same amount cited by NRC, 2003) should be provided in the diet. Flurer et al. also stated that they consider the optimal vitamin C content in the diet of the marmoset to be 2,000 ppm. The diet used in Tomonari et al. provided 1g vitamin C per 1,000 g feed (0.1%, or 1,000 ppm, or about 80 mg/day), an amount recommended in the published literature (Layne and Power, 2003), and only one-half that recommended by Flurer et al. Thus, the amount of vitamin C used in the Tomonari et al. study was not excessive relative to the marmoset's dietary requirements and any potential protection conferred by the vitamin C would not be out of line with the degree of protection afforded the marmoset by its natural diet.

Moreover, it is not clear whether a vitamin C-supplemented diet even impacts DEHP-induced testicular effects. Ishihara et al. (2000) demonstrated that rats given vitamins C and E in drinking water (about 450-500 mg/kg/day vitamin C) exhibited reduced testicular effects, relative to animals not receiving vitamins, from exposure to 20,000 ppm (1,000 – 1,500 mg/kg/day) DEHP in the diet. The absolute testes weights of DEHP/vitamin treated animals were significantly lower than controls (although testes-to-body weight ratios were comparable to controls), but significantly higher than DEHP-exposed rats that did not receive vitamins C and E. In addition, testicular pathology of DEHP/vitamin rats was improved relative to DEHP rats, though not entirely normal (spermatogenesis was present, but not at control levels; severe aspermatogenesis was not observed in DEHP/vitamin animals). Thus, the combination of vitamins C and E afforded some protection to the rats against the reproductive toxicity of high doses of DEHP to rats.

Similarly, in Ablake et al. (2004), CD-1 male mice were fed a diet containing 2% DEHP for 15 days and then fed a DEHP-free diet with or without supplementation of 3.0 mg/mL vitamin C and 1.5 mg/ML vitamin E in drinking water for another 50 days. The results showed that the DEHP-treatment induced aspermatogenesis, but that the damaged seminiferous epithelium spontaneously recovered whether the vitamins were provided or not, indicating that the DEHP-induced aspermatogenesis was reversible. In addition, the supplementation of vitamins C and E in the diet significantly accelerated regeneration of the injured seminiferous epithelium, suggesting that the vitamins have a therapeutic effect on DEHP-induced aspermatogenesis.

However, the potential protective effect of vitamin C in Ishitara et al. and Ablake et al. cannot be distinguished from that of vitamin E because, in both studies, the two vitamins were provided together. Verma and Nair (2001) showed that mice pretreated with vitamin E showed little or no signs of testicular toxicity following treatment with aflatoxin. On the other hand, Cave and Foster (1990) reported that very high levels of vitamin C (2 mM) were required for any protective effect against *m*-dinitrobenzene or *m*-nitrosonitrobenzene toxicity on Sertoli cells *in vitro*. Hence, it is possible that vitamin C had little impact on testicular toxicity, and that vitamin E played the larger role in the protective effect observed by Ishihara et al. in rats and Ablake et al. in mice.

Even if vitamin C does protect rats and mice against the effects of DEHP exposure, because rodents produce their own vitamin C the protective effect of dietary vitamin C in primates would have to be much greater than in rodents to account for the results of Tomonari et al. For example, since rats produce about 150 mg/kg/day of their own vitamin C (Chatterjee, 1973), the rats in the Ishihara et al. study were effectively exposed to a total vitamin C dose of about 600 – 650 mg/kg/day. Comparing the results of Ishihara et al. to Tomonari et al., rats given about 600 mg/kg vitamin C (plus 225 mg/kg vitamin E) exhibited smaller testes and reduced spermatogenesis after exposure to 1,000 mg/kg/day DEHP whereas marmosets given only about 360 mg/kg/day vitamin C had normal-sized testes and comparable spermatogenesis to controls (based on sperm counts) when ingesting 2,500 mg/kg/day DEHP. Thus, if the hypothesis is that dietary vitamin C accounted for the lack of effects seen in Tomonari et al., as opposed to a difference in the marmosets' sensitivity to DEHP, then a much smaller dose of vitamin C (50 – 66% of the amount given to the rats) would have to have protected the marmosets against 2 – 2.5 times the amount of DEHP given to rats. Put another way, vitamin C would have to be about 3 – 5 times more protective in primates than rodents to account for the results of Tomonari et al.

Indeed, if such a small amount of vitamin C in the diet had a complete protective effect against the high doses of DEHP given the marmosets, one might question the possible impact of DEHP exposure on human health. The RDA for vitamin C is 75 mg/person/day for women and 90 mg/person/day for men (NRC, 2003), although the mean daily intake is about 100 mg/day based on NHANES III and CSF II surveys (NRC, 2003). If 80 mg/day was as protective to primates as suggested, then the risk to humans would appear quite low since human exposures to DEHP are at least 100,000 times lower than the amount received by the marmosets (McKee et al., 2004), and the human diet contains higher levels of vitamin C. Even if one were to calculate the protective potential of that much vitamin C on a mg/kg body weight basis, the 360 mg/kg/day dose of vitamin C (hypothetically) protected the marmosets from testicular effects at 2,500 mg/kg DEHP (roughly a 7-fold protection factor). Applying this protection factor to an average human intake of 1.3 – 1.4 mg/kg/day vitamin C (90 – 100 mg/day for a 70 kg person), humans would be at no risk of testicular effects from DEHP exposures up to 6 mg/kg/day or roughly 10,000 times the mean exposures as determined by the CDC (Blount et al., 2000; CDC, 2001; CDC, 2003).

Thus, it seems unlikely that the amount of vitamin C provided the marmosets in Tomonari et al. invalidates the study's findings of no effect. Further, even if vitamin C had a protective effect, it is unlikely that any human other than one severely deficient in vitamin C would be at risk of adverse effects from exposure to the amounts of DEHP found in the environment.

In addition, contrary to OEHHA's statement that "[s]erum levels of vitamin C in common marmosets are markedly higher (2.56 mg/100 ml in average; Flurer and Zucker, 1987; 1989) than most other mammals (0.63 mg/100ml in average in humans; Hampl et al., 2004)," human and marmoset serum levels of vitamin C are not that different. Hampl et al. (2004) indicate that mean vitamin C levels in human plasma range from about 0.64 mg/dL (36.3 μM) to 0.97 mg/dL (55 μM), with an average of about 0.8 mg/dL (44 μM). The serum vitamin C level of 2.6 mg/dL cited as "average" by OEHHA is derived from marmosets that were given 2,000 ppm dietary vitamin C (Flurer et al., 1987). This level is four times the minimum requirement cited by Flurer

et al., and twice the recommended level of Layne and Power (2003). The marmosets in the Tomonari DEHP study were fed only 1,000 ppm vitamin C, with no reported vitamin C-related ill effects. Visual inspection of the Figure 2 in the Flurer et al. study (1987) indicates that, for marmosets, a 1,000 ppm diet results in a plasma vitamin C level of about 1.9 mg/dL. Therefore, it is equally, if not more, appropriate to conclude that average marmoset serum vitamin C levels are about 1.9 mg/DL, which is only a 2-fold difference from humans, not 4-fold as indicated by OEHHA. Even this small difference may not be statistically significant as the HPLC methodology used by the two different groups of investigators incorporated different detection systems, electrochemical detection (Hampl et al., 2005) and spectrophotometry (Flurer et al., 1987).

In any event, intracellular levels, not plasma levels, are probably responsible for any protective effect that vitamin C may afford. Intracellular ascorbate levels are about 100-fold greater than those found in plasma (Tsukaguchi et al., 1999). Intracellularly, vitamin C serves to maintain prosthetic ions in their reduced forms (e.g., Fe^{++}), scavenges free radicals to protect tissues from oxidative damage, and functions as a cofactor in a number of enzyme systems involved in the synthesis of collagen, microsomal drug metabolism, and the processing of certain neurotransmitters and peptide hormones (Marcus and Coulston, 1990; Tsukaguchi et al., 1999). As summarized by the National Research Council (1989), vitamin C is absorbed in the intestine by a sodium-dependent transport system and distributed to body tissues via blood as an unbound anion. From the blood, vitamin C is taken up by cells via a saturable, high affinity, sodium-dependent, transport system that results in intracellular vitamin C levels in the mM range. This transport system has been identified in a variety of cell types including leukocytes (Moser, 1987), endothelial cells (May and Qu, 2005), lung cells (Castranova et al., 1993), and Leydig cells (Moger, 1987).

Because intracellular levels are what matter, it is the kinetic parameters (e.g., Km, Vmax) of the vitamin C transport systems in marmosets and humans, not the absolute plasma levels, that will determine whether plasma vitamin C levels provide any protection from DEHP-induced testicular toxicity. While these kinetic differences are not known, evolutionary pressures typically result in enzyme systems that operate most efficiently under typical biological conditions, which may vary significantly among species. Thus, the average plasma vitamin C levels in marmosets (1.9 mg/dL) and humans (0.8 mg/dL) noted at required dietary levels for each species (NRC, 1989; Layne and Power, 2003) probably afford each a comparable degree of protection, if any. In other words, directly comparing plasma vitamin C levels across species is probably not a reliable indicator of the relative degree of protection those plasma levels might afford each species.

In summary, the need for supplemental vitamin C in primate and human diets reinforces the similarity between the two primate species. Since the amount of vitamin C administered in Tomonari et al. was in line with dietary recommendations, and since there is no reliable way to compare serum vitamin C levels across species, there is no reason to question the results of the study, and no reason to consider the results not relevant to assessing potential health effects in humans. The administration of medically appropriate amounts of vitamin C to the marmosets certainly would not appear to provide any scientific reason to prefer rodent data over the primate data for human hazard and risk assessment. Further, one might question whether it would have been scientifically appropriate, or even ethical, to withhold vitamin C from the marmosets.

Indeed, had vitamin C been withheld or administered in artificially low doses, interpretation of any adverse findings would be difficult at best.

D. Based on Pharmacokinetic Differences, Marmosets Are Less Susceptible Than Rodents to Developmental Toxicity from Phthalate Exposure

OEHHA Comment: "*In general, findings from [several studies cited by the Panel] clearly indicate that pharmacokinetic features of DEHP are qualitatively similar between marmosets and rats.*"

OEHHA concludes that data from several studies indicate that there are no DEHP pharmacokinetic differences between marmosets and rats, and that these studies do not support the Panel's statement in its earlier comments that "primates are less susceptible than rodents for developmental toxicity based on metabolism, absorption and elimination." On the contrary, several studies do support the conclusion that, based on pharmacokinetic differences, marmosets are less susceptible than rodents to developmental toxicity from phthalate exposure.

Rhodes et al. (1986) reported that marmosets dosed with dietary DEHP at 2,500 mg/kg/day achieved a maximum absorbed dose that was 10 to 25-fold lower than that of equally dosed rats. Similar results were obtained in studies in cynomologus monkeys (Astill, 1989). Both findings are supported by results of a recent study (Kurata et al., 2005) in which juvenile rats and marmosets were gavaged with 100 mg/kg DEHP. Plasma radioactivity measurements taken up to 24 hr post-dosing indicated that rats absorbed 20 to 100-fold more DEHP than marmosets. While this radiolabel study could not differentiate between DEHP and its metabolites, the results of Kessler et al. (2004) bear on this issue. In Kessler et al., pregnant and nonpregnant rats and marmosets were given oral doses of 30 or 500 mg/kg/day DEHP. In both species, MEHP was present in the blood at much higher levels than DEHP. In rats, the normalized areas under the concentration-time curves (AUCs) for MEHP were 100-fold higher than the normalized AUCs for DEHP; in marmosets, however, this difference was only about 10-fold. There was also a significant interspecies difference in plasma MEHP levels. Peak blood levels of MEHP in rats were 2 to 4-fold higher than those in marmosets, while AUC measurements indicated that MEHP levels in rats were 4 to 12-fold higher than those of marmosets. Thus, current evidence indicates that, when exposed to similar levels of DEHP, rats experience much higher levels of the toxicologically relevant metabolite, MEHP, than do marmosets. This indicates that marmosets, and other primates, are less susceptible than rodents to developmental toxicity from phthalate exposure based on pharmacokinetic differences.

II. THE MARMOSET DATA COMPRISE SCIENTIFICALLY VALID DATA, NOT CONSIDERED BY NTP-CERHR, WHICH ESTABLISH THAT THE PHTHALATES DO NOT MEET THE CRITERIA FOR LISTING UNDER PROPOSITION 65

As discussed above, OEHHA stated in its response to the Panel's earlier comments that the marmoset data in Tomonari et al. (2004) were "relevant for DBP, BBP, DnHP, and DIDP" and thus considered by OEHHA to be "'new data' that were not considered by the authoritative body." After acknowledging this fact, OEHHA rejected the marmoset data, stating that several of the marmoset's reproductive features make marmosets an unacceptable model for

investigating developmental toxicity in humans. However, as the preceding section makes clear, OEHHA's rejection of the marmoset study is unfounded; the marmoset is a suitable model for investigating the potential developmental toxicity of phthalates to humans. Because the marmoset study provides new data that were not considered by the authoritative body, and those data clearly establish that the association between adverse reproductive effects in humans and the phthalates is not "biologically plausible," OEHHA's decision to list these phthalates fails to meet the listing requirements of Proposition 65.

Under Proposition 65 Section 12306(h), to list the phthalates OEHHA must first determine that an authoritative body has "formally identified" the phthalates as causing reproductive toxicity.[7] OEHHA must further determine that the studies considered by NTP-CERHR satisfy the Section 12306(g) criteria for "as causing reproductive toxicity."[8] According to regulations, a chemical is identified "as causing reproductive toxicity" when:

> (1) Studies in humans indicate that there is a causal relationship between the chemical and reproductive toxicity; or
>
> (2) Studies in experimental animals indicate that there are sufficient data, taking into account the adequacy of the experimental design and other parameters such as, but not limited to, route of administration, frequency and duration of exposure, numbers of test animals, choice of species, choice of dose levels, and consideration of maternal toxicity, indicating that an association between adverse reproductive effects in humans and the toxic agent in question is biologically plausible.[9]

NTP-CERHR has not concluded that studies in humans indicate a causal relationship between these phthalates and reproductive toxicity (the first criterion). Rather, as the OEHHA listing package recognizes, NTP-CERHR relied upon studies in rodents in reaching its ostensible conclusions. Therefore, in this case only Section 12306(g)(2) is relevant. As such, the phthalates should be listed only if the data from experimental animals indicate that an association between adverse reproductive effects and the phthalates is "biologically plausible."

Proposition 65 also contains a provision, Section 12306(h), which states:

> The lead agency [OEHHA] shall find that a chemical does not satisfy the definition of "as causing reproductive toxicity" if scientifically valid data which were not considered by the authoritative body clearly establish that the chemical does not meet the criteria of subsection (g), paragraph (1) or subsection (g), paragraph (2). [10]

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7 *See* CAL. CODE REGS. tit. 22, § 12306(d).

8 *See id. at* § 12306(g)(1)-(2).

9 *Id.*

10 *Id. at* 12306(h).

Thus, if scientifically valid data from experimental animals which were not considered by NTP-CERHR clearly establish that an association between adverse reproductive effects and the phthalates is not "biologically plausible," OEHHA must find that the phthalates do not satisfy the definition of "as causing reproductive toxicity." The marmoset data in Tomonari et al., which were acknowledged by OEHHA to be new data that were not considered by NTP-CERHR, clearly establish that an association between adverse reproductive effects and the phthalates is not "biologically plausible."

Tomonari et al. (2004; and MCSI, 2003) conducted a repeated oral dose study of the effects of DEHP treatment on the development of the male reproductive tract in common marmoset monkeys (*Callithrix jacchus*). The animals were administered 0, 100, 500 or 2500 mg/kg/day by gavage on a daily basis for 65 weeks, from weaning (about three months) until about 18 months of age. This exposure period covered the entire sexual maturation phase as marmosets reach sexual maturity at about 400 to 450 days (57-65 weeks). During the treatment period, the testosterone levels in all treated groups were similar to those of control groups. At the end of the treatment period, the animals were examined for gross and histologic evaluation of principal organs. The testes and accessory organs were subjected to light and electron microscopic examination, and measurements of hormone levels and sperm counts were carried out.

No treatment-related abnormalities were observed in microscopic and functional examinations of the marmosets' testes, and there were no treatment-related effects on sperm count. In addition, histochemical examination after 3β hydroxysteroid dehydrogenase staining did not reveal any alteration in steroid synthesis. The only significant effect observed, a dose-dependent increase in P450 content, was considered to be an adaptive change and not an adverse affect. Thus, this study demonstrated that daily administration of high doses of DEHP (up to 2,500 mg/kg/day) spanning the entire period of sexual maturation had no effect on male reproductive tract development in the marmoset.

Therefore, the empirical data from marmosets, which were shown in the preceding section to be valid for assessing human reproductive toxicity, indicate that primates are at least much less sensitive to the effects of phthalates than are rodents and may in fact be refractory, as there was no evidence of effects at the highest levels tested. A similar lack of effect was noted by Kurata et al. (1998) in adult marmosets treated with 2,500 mg/kg DEHP for 13 weeks, and by Pugh et al. (2000) in adolescent cynomolgus monkeys treated with 500 mg/kg DEHP for 14 days.

Humans are primates, and therefore data from primate studies are likely much more indicative of what effect can be anticipated in humans than data from rats. The recent marmoset data, along with the data of Kurata et al. and Pugh et al., demonstrate that an association between phthalates and adverse reproductive effects in humans is not biologically plausible. Thus, scientifically valid data from experimental animals which were not considered by NTP-CERHR clearly establish that of the criteria for "as causing reproductive toxicity" are not met. Therefore, the phthalates fail to meet the Proposition 65 listing criteria.

III. NTP-CERHR FOUND MINIMAL TO NEGLIGIBLE RISK OF HUMAN REPRODUCTIVE TOXICITY FOR THESE PHTHALATES; THEREFORE THE PROPOSITION 65 LISTING CRITERIA ARE NOT MET

OEHHA's stated basis for listing these phthalates is the monographs published in 2003 by NTP-CERHR. (NTP-CERHR, 2003a,b,c,d). However, in representing NTP-CERHR's conclusions in these monographs, OEHHA fails to acknowledge qualifying language, which results in an overstatement of NTP-CERHR's findings of phthalate toxicity. For example, for DBP, BBP and DIDP, respectively, NTP-CERHR stated:

> In this case, recognizing the lack of human data and the clear evidence of effects in laboratory animals . . ., the NTP judges the scientific evidence sufficient to conclude that DBP may adversely affect human reproduction or development *if exposures are sufficiently high*.

(NTP-CERHR, 2003b, emphasis added); and

> The NTP believes it is reasonable and prudent to conclude that the results reported in laboratory animals indicate a potential for similar or other adverse effects in human populations *if exposures are sufficiently high.*

(NTP-CERHR, 2003a, emphasis added); and

> In this case, recognizing the lack of human data and the evidence of effects in laboratory animals, the NTP judges the scientific evidence sufficient to conclude that DIDP is a developmental toxicant and could adversely affect human development *if the levels of exposure were sufficiently high.*

(NTP-CERHR, 2003d, emphasis added).

Thus, for each case in which NTP-CERHR made a determination of concern about a phthalate's potential reproductive or developmental toxicity (it made no concern determination for DnHP), it qualified its determination by indicating the potential for toxicity only at "sufficiently high" exposure levels. Because of these qualifications, NTP-CERHR's findings of potential toxicity are inextricably tied to exposure levels, which, as explained at length by the Panel in its earlier comments (Section III, Table 1), are not "sufficiently high" to indicate a potential for human effects. OEHHA fails to acknowledge the significance of NTP-CERHR's use of this qualifying language.

In addition to failing to acknowledge language linking risk to exposure, OEHHA ignores the fact that NTP-CERHR found minimal or negligible concern for human developmental or reproductive toxicity for DBP, BBP and DIDP. About DBP, NTP-CERHR stated:

> The NTP concurs with the CERHR Phthalates Expert Panel that there is *minimal concern for developmental effects* when pregnant

> women are exposed to DBP levels estimated by the Panel (2-10 µg/kg bw/day);[11]

and

> "The NTP concurs with the CERHR Phthalates Expert Panel that there is *negligible concern for reproductive toxicity* in exposed adults.

(NTP-CERHR, 2003b, emphasis added).

About BBP, NTP-CERHR stated:

> The NTP concludes that there is *minimal concern for developmental effects* in fetuses and children;

and

> The NTP concurs with the CERHR Phthalates Expert Panel that there is *negligible concern for adverse reproductive effects* in exposed men.

(NTP-CERHR, 2003a, emphasis added).

About DIDP, NTP-CERHR stated:

> "The NTP concurs with the CERHR Phthalates Expert Panel that there is *minimal concern for developmental effects* in fetuses and children ;

and

> "The NTP concurs with the CERHR Expert Panel that there is *negligible concern for reproductive toxicity* in exposed adults (emphasis added).

(NTP-CERHR, 2003d, emphasis added).[12]

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[11] Based upon estimated DBP exposures among some women of reproductive age, the NTP did have "some concern" for DBP causing adverse effects to development to fetus of women so exposed. However, the exposure estimates causing "some concern" were based on preliminary urinary metabolite data from the CDC for a small, nonrepresentative sample of women (Blount et al., 2000; Kohn et al., 2000). CDC scientists subsequently analyzed data for women of childbearing age in a much larger and statistically representative sample (Manori et al., 2004). Those results showed that women of reproductive age had DBP exposure levels the same as or lower than other age groups of women.

Moreover, regarding the toxicity of DnHP, NTP-CERHR stated:

> The NTP judges the scientific evidence insufficient to reach a conclusion regarding the potential for DnHP to adversely affect human development or reproduction.

(NTP-CERHR, 2003c). Because NTP-CERHR reached no conclusion with regard to the potential of DnHP to adversely affect human reproduction or development, OEHHA's justified its listing decision by relying on "the generally accepted assumption that 'an agent that produces an adverse developmental effect in experimental animal studies will potentially pose a hazard to humans *following sufficient exposure during development* . . . .'" (citing EPA, 1991, emphasis added). In so doing, OEHHA both ignores the authoritative body's explicit failure to conclude that DnHP adversely affects human development or reproduction and, again, fails to acknowledge qualifying language necessarily linking adverse affects to "sufficient exposure." Therefore, even more so than for DBP, BBP and DIDP, OEHHA relies on an overstatement of the authoritative body's assessment of DnHP's toxicity to justify its listing.

These statements by NTP-CERHR do not satisfy the prong of the first regulatory criterion that the authoritative body's report must "conclude[] that the chemical causes reproductive toxicity" because for purposes of Proposition 65, the reproductive toxicity must plausibly be in humans.[13] As discussed in Section II of these comments, the Proposition 65 regulations define a conclusion of "as causing reproductive toxicity" as one that satisfies the requirement that studies in experimental animals indicate that there are sufficient data to show that an association between adverse reproductive effects in humans and exposure to the chemical in question is "biologically plausible."[14] NTP-CERHR does not "conclude" that the phthalates cause reproductive toxicity in humans under this definition. Instead, their statements conclude only that such effects occur in rodents. There is no finding that an association between the phthalates and adverse reproductive effects is biologically plausible. As such, the suggestion to treat the phthalates as potentially reproductively toxic in humans is merely a default assumption, not a conclusion of biological plausibility.

As a result, OEHHA's decision to list these four phthalates under Proposition 65 is based on its incomplete, and therefore overstated, representation of NTP-CERHR's conclusions as to the phthalates' potential toxicity. NTP-CERHR found only that these phthalates have the "potential" to adversely affect humans if concentrations are "sufficiently high," and stated that it had minimal or negligible concern for developmental or reproductive effects in humans. By

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12 The NTP-CERHR stated that "[t]hese conclusions are based on the assumption that the general US population is exposed to DIDP at less than 30 µg/kg bw/day." In its August 2004 comments, the Panel explained that, based on analogy to DINP exposures, urinary metabolite data indicated that the best estimate for ambient exposure to DIDP is ≤ 1 µg/kg/day (McKee et al., 2004). This exceedingly low exposure level is supported by another study which found that urinary levels of DIDP following exposure from the use of personal hygiene products were below detectable limits. (Stock et al., 2001; Stock personal communication). Thus, the NTP-CERHR's overall conclusions of minimal to negligible concern from DIDP exposures are well supported.

13 *See* CAL. CODE REGS. tit. 22, § 12306(d)(1).

14 *Id.* at § 12306(g)(2).

leaving out this language, OEHHA changes fundamentally the nature of NTP-CERHR's conclusions. Because NTP-CERHR did not conclude that the phthalates cause reproductive toxicity, the Proposition 65 listing criteria are not met for these four phthalates.

IV. OEHHA HAS NO STATUTORY MANDATE TO LIST CHEMICALS SUCH AS THESE PHTHALATES, WHICH POSE NO SIGNIFICANT RISK TO HUMAN HEALTH; THEREFORE, LISTING OF THESE PHTHALATES MAKES LITTLE SENSE FROM A PUBLIC POLICY PERSPECTIVE

As stated in its August 2004 comments, the Panel believes it makes little sense to list these phthalates under Proposition 65, as it would be poor public policy to list chemicals for which the data clearly demonstrate no significant risk to public health. The Panel's earlier comments demonstrated that low risk by showing that exposures to phthalates from all sources are well below what are likely to be Maximum Allowable Dose Levels (MADLs) for DBP, BBP, DnHP and DIDP. OEHHA's response to those comments states that OEHHA has not calculated MADLs for these chemicals and so has no basis to agree or disagree with the Panel's comment. This is a somewhat disingenuous response. The procedure for calculating a MADL is straightforward – select the most sensitive relevant study of sufficient quality, divide its no observed effect level by 1000, and multiply by either 70 or 58 kilograms, depending on whether the applicable reproductive effect is upon the male or upon the female or conceptus.[15] This is precisely what the Panel did to generate likely MADL values. The Panel knows of no study OEHHA could select that would give a substantially lower MADL value. However, even if OEHHA were to calculate MADLs an order of magnitude below those calculated by the Panel, average exposures to the phthalates – from all sources – would still be well below the MADL. The Panel's primary point, therefore, remains valid: the risks from phthalates are so low that it is highly unlikely that a Proposition 65 warning would be necessary for any product containing these phthalates.

OEHHA also states that the question of whether exposures are below the MADL has no bearing on an authoritative bodies listing – that it is a question for consideration when and if the phthalates are listed. The Panel disagrees. There is no statutory mandate that OEHHA list each and every substance which an authoritative body has concluded to cause reproductive toxicity in animals. The statute states:

> On or before March 1, 1987, the Governor shall cause to be published a list of those chemicals known to the state to cause cancer or reproductive toxicity within the meaning of this chapter, and he shall cause such list to be revised and republished *in light of additional knowledge at least once per year thereafter*. (CAL. HEALTH & SAFETY CODE § 25249.8, emphasis added)

Part of the additional knowledge OEHHA could apply is knowledge that a given chemical is unlikely ever to pose a risk to human reproduction due the large gap between effect levels in test animals and human exposures. In addition, as discussed in Section III of these comments, the very authoritative body on which OEHHA relies for its proposed listings of DBP, BBP, DnHP and DIDP has found minimal to negligible concern that these phthalates will cause reproductive

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[15] *See* CAL. CODE REGS. tit. 22, § 12803.

effects in humans. In such a case, the Panel believes that good policy judgment dictates that the chemicals not be listed.

OEHHA, in its response to the Panel's earlier comments, describes Proposition 65 as a "public right-to-know statute" and an "informational resource" for the public about chemicals "known to cause reproductive toxicity." Yet, as discussed at length in the Panel's earlier comments, and expanded upon here, the toxicity and exposure data indicate it is highly unlikely that human exposures to these phthalates will in fact cause such effects. Thus, rather than the Proposition 65 list serving as a reliable "informational resource" about risks from phthalates, it would be misleading with respect to these substances.

Moreover, inclusion of chemicals on the Proposition 65 list inevitably leads to public and regulatory concern about the chemicals thus listed. As a result, even though a company's product may result in phthalate exposures below the maximum allowable dose level, exempting the product from warning requirements, [16] the stigma associated with using a chemical listed as "known to the State" to cause reproductive/developmental toxicity often forces companies to eliminate use of the listed chemical. Yet, where a chemical is well studied, such that its risks are well characterized, as for phthalates, use of an unlisted substitute chemical will not necessarily result in a public health benefit. The substitute may be unlisted because it is not as well-studied, so that its own hazards have not yet been discovered. It makes little sense to drive companies to make such a substitution where the data show that risks from the chemical are extremely low, as is the case for the phthalates.

The use of a given chemical in products results from a balancing of safety, performance and cost. Reformulation away from the chemical is likely to cause degradation in at least one of those factors. The Panel strongly reiterates that sound public policy would avoid promoting such consequences through Proposition 65 listing, where human exposures to substances – such as DBP, BBP DnHP and DIDP – have been shown to pose a very low risk of reproductive or developmental toxicity.

CONCLUSION

The data presented in the Panel's earlier comments, and expanded upon here, support a conclusion that DBP, BBP, DnHP and DIDP do not pose a significant risk of reproductive or developmental toxicity in humans. The recent marmoset data and NTP-CERHR's statements demonstrate that the Proposition 65 listing criteria are not met for these four phthalates. Further, it would not be good public policy to list such low-risk chemicals. The Panel therefore believes that OEHHA should not list these phthalates under Proposition 65.

[16] See CAL. HEALTH & SAFETY CODE § 25249.10(c); 22 CCR. § 12801.

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January 19, 2006

To: Marian Stanley, ACC

From: Suzette Tardif, Ph.D.

Subject: Findings regarding female reproductive physiology from the Mitsubishi Study #B000496, "Sixty-five week repeated oral dose toxicity study of DEHP in juvenile common marmosets"

I have reviewed the findings from the above-referenced study relative to female reproductive physiology – specifically the findings relative to ovarian weight at the end of the study and to circulating estradiol concentrations throughout the study. I do not believe that these findings can be used to support or refute the conclusion that DEHP treatment resulted in precocious puberty in female marmosets, primarily due to the extremely low body weights of the majority of the subjects. My reasoning is as follows:

1. The weights of female subjects at the end of the study (week 66 of study, when the animals would be approximately 78 weeks, or 17 months, old) is extremely low, particularly for the control group and for females receiving the lowest DEHP dose (100) – see Figure 1. The average weights for females in the four groups are all well below the average for a healthy marmoset at 17 months of age (350-370 grams). I believe this reflects the fact that the study procedures, involving daily gavage for many weeks in a row, resulted in many basically unhealthy animals with impaired growth.

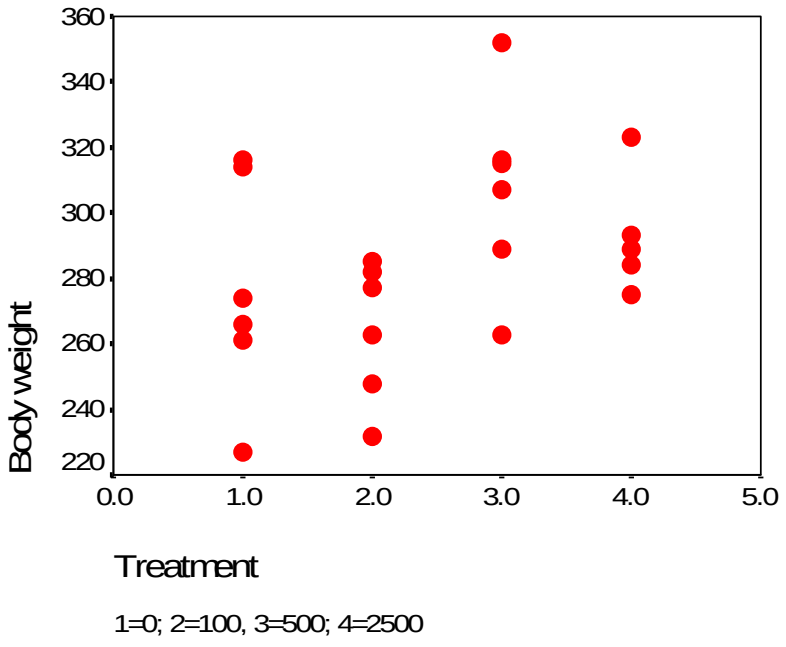

FIGURE 1.

2. While the difference in average weights across the treatment groups is not significant at $p<0.05$ ($F= 2.849$, $p= 0.065$), there is a trend for females in the two highest DEHP doses to have higher weights (see Figure 1). In our colony, we would consider any 17 month old animal below 275 grams as unsuitable for research use. If we use this criterion on the Mitsubishi study population, then only 3 out of 6 subjects in the control and low dose groups would be suitable, while 5/6 and 5/5 of the animals in the higher dose groups would be suitable.

3. Estradiol concentrations appear to be bimodally distributed, with most under 50pg/ml and a few from 130-1300 pg/ml. I believe this bimodal distribution reflects the difference between pre-pubertal and post-pubertal state. The range for earliest age at which subjects displayed estradiol concentrations reflective of likely post-pubertal state is from around 51 weeks (or 12 months) of age to 65 weeks (or 17 months) of age. The lower limit on this range (12 months of age) is well within the norm for age of puberty for female marmosets. Animals who appear to remain prepubescent up to 17 months of age (n=5) and who appear to be pre-pubescent throughout the length of the study (n=11) are abnormal, displaying what I would call delayed puberty. This condition appears to be related to body weight – see Figure 2

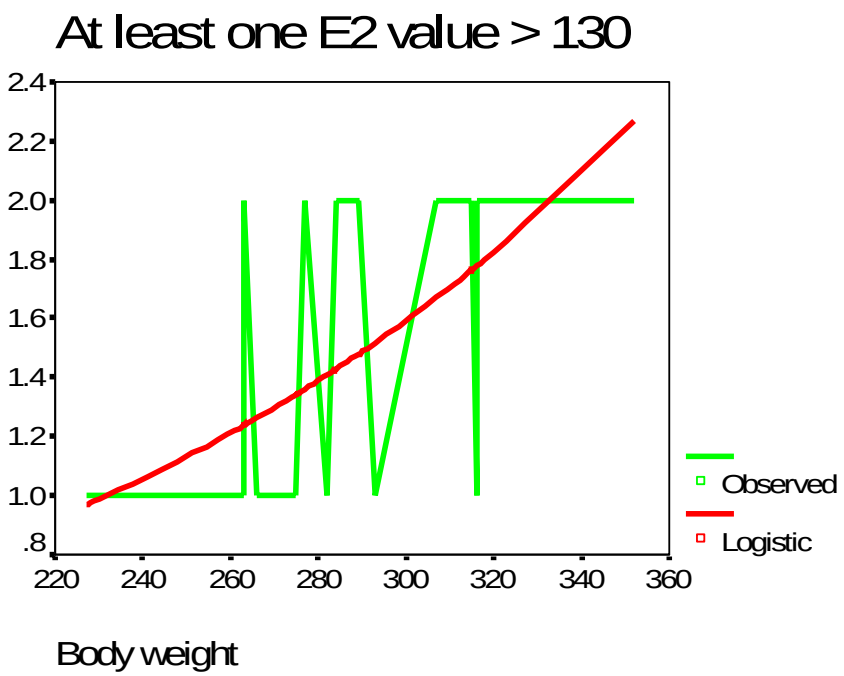

Figure 2. Logistics regression of puberty occurrence (as assessed by at least one estradiol concentration over 130 pg/ml; 1=no; 2=yes) versus body weight. Body weight was a significant determinant of puberty occurrence ($F = 10.64$, $df= 21$, $p=0.004$).

4. Ovarian weight is largely reflective of body weight. Relative ovarian weight (ovarian weight/body weight) is also still largely reflective of body weight – see Figure 3.

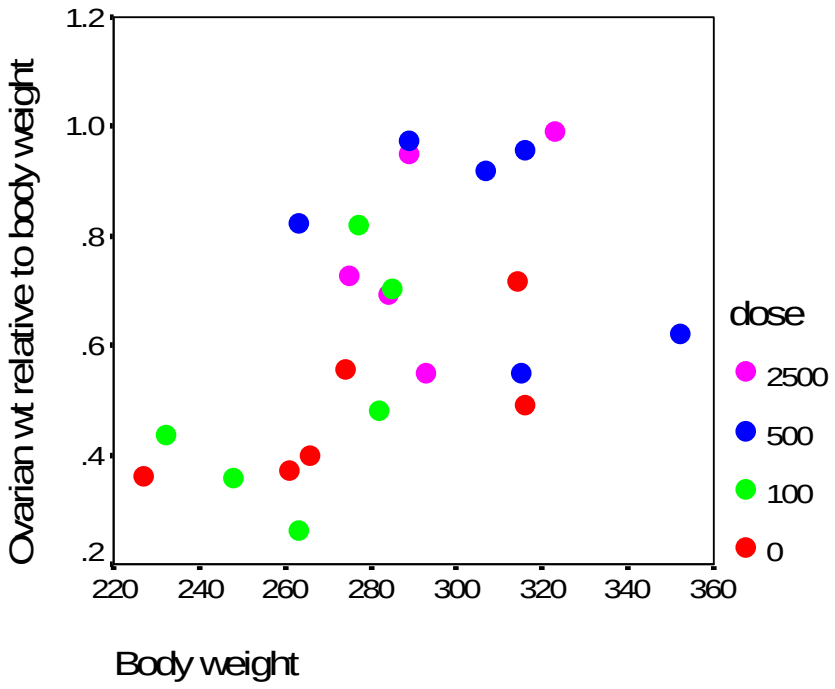

Figure 3.

In a general linear model analysis of relative ovarian weight x treatment, with body weight as a covariate, there is a marginal relationship between treatment and relative ovarian weight ($F=2.787$, $df=3$, $p=0.070$). If those subjects below 275 grams are removed (see #2), then there is no indication of a relation between treatment and relative ovarian weight ($F=1.097$, $df=3$, $p=0.391$), however the sample size is so small for the control and low dose group that the validity of the comparison becomes questionable.

The higher ovarian weights were generally associated with higher body weights and occurrence of ovulation/corpus luteum formation – i.e., they are normal in a sexually mature female. The fact that these higher ovarian weights were seen in the group with the highest dose exposure is, I believe, simply due to the fact that this group contained more normal weight animals who, therefore, were more likely to have ovulated. Normal ovarian function in marmosets includes the development and maintenance of a large, steroidogenic interstitial gland. The persistent presence of this gland, along with the cyclical presence of corpus lutea, means that sexually mature females will have higher ovarian weights. I disagree with the study conclusion that such features are "usually observed in more mature females." At the age at which these females were sacrificed, I would have expected most, if not all, of them to display evidence of sexual maturity.

PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

HEADQUARTERS
501 FRONT STREET
NORFOLK, VA 23510
TEL 757-622-PETA
FAX 757-622-0457

February 3, 2006

Dr. Michael D. Shelby, CERHR Director
NIEHS
P.O. Box 12233
MD EC–32 Research Triangle Park,
North Carolina 27709

Submitted electronically to shelby@niehs.nih.gov

Dear Dr. Shelby,

The following comments are submitted on behalf of the more than one million members and supporters of People for the Ethical Treatment of Animals (PETA), in response to the NTP-CERHR expert panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate (DEHP) which was prepared in November, 2005. PETA is the world's largest animal rights organization and is committed to using the best available science to protect animals from suffering and to promote the acceptance of alternatives to animal testing.

Summary of comments

Recommendations for several of the data needs listed in the update's summaries and conclusions section call for additional studies on animals, including primates. However, as noted in the American Chemistry Council (ACC) comments to the draft update (2005), these data needs have been sufficiently met by existing studies, including a number of recent studies that do not appear to have been fully considered by the expert panel. Existing data clearly demonstrate that primates, including humans, are much less sensitive than rats to the developmental and reproductive effects of DEHP. Considering that estimates of human exposures calculated by the ACC (2005) from recent CDC biomonitoring data are 1,000 to 10,000-fold lower than NOELs determined in rats, the existing weight of evidence is clearly sufficient to establish conservative NOELs for all relevant human populations and exposures to safeguard the public health without subjecting additional animals to suffering and death.

Low level dose-response data exist for DEHP and MEHP

Under the heading "Significance of Perinatal Exposure", section 3.1, the need for additional dose-response data correlating mono-(2-ethylhexyl) phthalate (MEHP) levels and developmental reproductive effects in rats is identified. However, the dose-responses of rats to DEHP and its toxicologically active metabolite MEHP at low levels are well-characterized and sufficient to establish a developmental NOEL of 46 mg/kg. Li et al. (2000) investigated the effects of low doses of DEHP and MEHP on the testicular development of rat pups. DEHP doses of 20, 100, 300 and 500 mg/kg were administered to 3-day-old rat pups as a single dose by oral gavage. The investigators found that the lowest dose of DEHP that produced changes in neonatal testicular cells was 100 mg/kg.

The results of a recent NTP continuous breeding study (2005) are consistent with these findings. Although the purpose of this study was to assess potential reproductive effects, developmental effects were also measured. DEHP doses of 10, 30, 100, 300, 1,000, 7,500, and 10,000 ppm were administered in feed to groups of 17 male and 17 female rats. Reproductive effects were noted in the 7500 ppm and 10,000 ppm groups. Developmental effects, such as decreases in the weights of sex organs and histological abnormalities, were also observed at these concentrations. While mention is made of small sex organs in several rat pups in the 300 and 1000 ppm groups, the authors viewed the toxicological significance of these findings as questionable since organ weights were normal, no other reproductive effects were noted, and the incidence of these findings was low. As a result, the developmental NOEL, as suggested by the expert panel, is most likely 1000 ppm, calculated to be no more than 46 mg/kg/day based on feed consumption.

The effect of intravenous exposure to DEHP on the development of reproductive organs in male rat pups was investigated by Cammack et al. (2003). No effects of any kind were observed in animals treated with 60 mg/kg/day, while testicular changes were noted in the 300 and 600 mg/kg/day dose groups. Other groups were dosed daily by oral gavage at 300 and 600 mg/kg/day. The investigators noted that testes changes were generally more severe among animals dosed orally than intravenously. These consistent findings across studies support a conservative developmental reproductive NOEL of 46 mg/kg/day for oral exposure to DEHP and 60 mg/kg/day for intravenous exposure.

ADME data in primates explains reduced sensitivity

Under the heading "Extension of PBPK Model", section 4, the need to extend ADME data across species into primates is identified and under the "Additional Data Needs" heading *in vivo* metabolic data on lipase across species is listed. There is ample data to conclude that primates are much less sensitive to the developmental and reproductive effects of DEHP than are rodents. In addition, the mechanisms responsible for this lower sensitivity can be understood on the basis of existing ADME data. A recent study by Tomonari et al. (2004) found that exposure to very high levels of DEHP – 2500 mg/kg/day – resulted in no observed effects on testicular development in marmosets. In addition, a recent human study found no developmental effects of DEHP in adolescents who had been exposed to high medical treatment related levels as neonates (Rais-Bahrami et al., 2004).

Studies by Rhodes et al. (1986), Astill (1989) and Kurata et al. (2005) demonstrated that DEHP is absorbed 10 to 100-fold less efficiently in marmosets and cynomolgous monkeys than in rats. Furthermore, at higher doses absorption efficiency decreased with the peak blood level for MEHP in marmosets leveling off at 20 mg/L. Lipase is the enzyme that catalyzes the hydrolysis of DEHP to its toxicologically active metabolite MEHP. Ito et al. (2005) found that lipase activity in rats was more than 10-fold higher than in marmosets, and the Vmax/Km ratio was nearly 200-fold greater. In addition, MEHP and its metabolites remained in their more active, free forms in rodents, while in primates they were conjugated with glucuronide (Silva et al., 2003; Kato et al., 2004; Kurata et al., 2005). Glucuronide conjugation decreases their toxicological activity and increases their water

solubility resulting in faster excretion in the urine. This conclusion is supported by the results of Kessler et al. (2004) who demonstrated that while the area under the plasma concentration versus time curves (AUCs) for MEHP was 3 to 10-fold higher in rats than in marmosets, the peak blood level was only 1 to 3-fold higher, a result of faster excretion of MEHP in marmosets. The observed lower sensitivity of primates to the developmental and reproductive effects of DEHP can therefore be explained by less efficient absorption, lower activity of lipase, and increased glucuronidation of MEHP and its metabolites resulting in faster excretion. This explanation is consistent with the observation that the AUC for MEHP was 100-fold higher than the AUC for DEHP in rats but only 10-fold higher in marmosets (Kessler, et al., 2004). Also, a much higher proportion of oral DEHP doses was excreted as DEHP in the feces in marmosets than in rats.

Estimated human exposures are 1,000 to 10,000-fold lower than the experimental NOEL

In its comments to the draft update, the ACC calculated human exposure to DEHP based on recent CDC urinary metabolite biomonitoring data. The range of exposures for all U.S. populations was conservatively estimated to be 3-30 µg/kg/day. This range is 1,000 to 10,000-fold lower than the experimentally determined rat oral NOEL of 46mg/kg/day.

The need for additional dose-response data correlating MEHP levels and developmental reproductive effects in rats has been met by studies of Li et al. (2000), Cammack et al. (2003) and NTP (2005). The results of each of these studies are consistent with establishing a conservative developmental reproductive NOEL in rats of 46 mg/kg/day for oral exposure to DEHP and 60 mg/kg/day for intravenous exposure. Developmental reproductive effects of DEHP exposure were not observed in humans (Rais-Bahrami et al., 2004) or other primates (Tomonari et al., 2004) even at much higher exposure levels, such as those that have been found in medical treatment-related exposures.

This lower sensitivity is adequately explained by species differences in the absorption, metabolism and excretion of DEHP and its active metabolites (Rhodes et al., 1986; Astill, 1989; Silva et al., 2003; Kato et al., 2004; Kurata et al., 2005). Considering that estimates of human exposures calculated from recent CDC biomonitoring data are 1,000 to 10,000-fold lower than NOELs determined in rats, the NTP can reasonably conclude that more animal data will not increase public safety and that the developmental NOELs based on existing data are sufficient.

Please feel free to contact me at 610-586-3975 or via e-mail at JosephM@peta.org if you have any questions.

Sincerely,

Joseph Manuppello
Research Associate, Research & Investigations
People for the Ethical Treatment of Animals

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