EVIDENCE ON THE DEVELOPMENTAL AND
REPRODUCTIVE TOXICITY OF

Bisphenol A

October 2009

Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

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PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code Section 25249.5 *et seq.*) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. This document by OEHHA, and related documents by other bodies incorporated by reference, address the reproductive toxicity of bisphenol-A (BPA). They provide information on whether this compound should be identified as known to cause reproductive toxicity under Proposition 65.

There are several mechanisms for listing chemicals under Proposition 65. One mechanism is that in the opinion of the "state's qualified experts" the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity" (Health and Safety Code Section 25249.8(b)). The state's qualified experts regarding reproductive toxicity are the members of the Developmental and Reproductive Toxicant (DART) Identification Committee of OEHHA's Science Advisory Board (Title 27, California Code of Regulations, section 25302) [1].

Another mechanism for listing chemicals is where a body identified as authoritative by the DART IC has formally identified the chemical as causing reproductive toxicity. The National Toxicology Program is designated as an authoritative body for reproductive toxicity under Proposition 65, "solely as to final reports of the National Toxicology Program's Center for the Evaluation of Risks to Human Reproduction [NTP-CERHR]" (Title 27, California Code of Regulations, section 25306(l)). In 2008, the NTP-CERHR published a final report identifying clear evidence of "high" dose developmental toxicity of BPA in laboratory animals. As noted in OEHHA's "Process for Prioritizing Chemicals for Consideration under Proposition 65 by the 'State's Qualified Experts,'" chemicals that have been recently reviewed by an authoritative body generally will not be proposed for review by the Developmental and Reproductive Toxicant Identification Committee (DART IC). However, the document also notes that exceptions to this generalization may occur. For example, an authoritative body may have evaluated a chemical but failed to review all relevant data, or compelling new data may have become available since the evaluation.

OEHHA had selected BPA through its prioritization process as a candidate for consideration by the DART IC, and substantial staff work on preparation of hazard identification materials had already occurred, before the NTP-CERHR Monograph was published. Additionally, a considerable number of relevant studies currently available were not considered by NTP-CERHR. OEHHA determined that, under these specific circumstances, the most efficient, timely and appropriate mechanism for consideration of BPA for listing under Proposition 65 was to bring it forward for consideration by the DART IC.

[1] Formerly Title 22 California Code of Regulations, section 12302

This document provided the DART IC with information relevant to the reproductive toxicity of BPA. The document does not provide dose-response evaluation, exposure assessment, or determination of allowable or safe exposure levels, but does provide information which may be useful in such appraisals.

A public meeting of the Committee was held on July 15, 2009, in Oakland, California. Following discussion and Committee deliberation, the Committee did not determine that BPA "has been clearly shown through scientifically valid testing according to generally accepted principles" to cause reproductive toxicity. Consequently, BPA was not listed under Proposition 65 for reproductive toxicity.

Executive Summary

Bisphenol A (BPA; 4,4'-dihydroxy-2,2-diphenyl propane) is an organic compound with two phenol functional groups. It is produced in large quantities for use primarily in the production of polycarbonate plastics and epoxy resins. Polycarbonate plastics are used in certain food and drink packaging, e.g., water and infant bottles, compact discs, impact-resistant safety equipment, and medical devices, and can be blended with other materials to create molded parts for use in mobile phone housings, household items, and automobiles. Epoxy resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. Some polymers used in dental sealants or composites contain BPA-derived materials. Bisphenol A is known to leach from dental composites as well as food containers such as cans and polycarbonate plastic water bottles. Detectable levels of BPA have been found in the general population.

This document reviews the evidence on the developmental and reproductive toxicity (DART) of BPA. It evaluates DART data from epidemiologic and toxicologic studies, as well as relevant pharmacokinetic and mechanistic data.

There are few epidemiologic studies, mostly of cross-sectional design. The human BPA studies are thus of limited usefulness for evaluating causal relationships. In contrast, there are extensive data from animal studies on the developmental, female reproductive and male reproductive toxicity of BPA. Interpretation of the animal data is complicated by the variety of species, strains, dosing regimens, endpoints evaluated and techniques used for their evaluation, and analytical methodologies used. The main mechanisms of action by which BPA is hypothesized to exert adverse effects involve disruption of the endocrine system. Studies reporting adverse reproductive effects of BPA and studies reporting no such effects are both numerous. Overall, studies that used sensitive methodologies to assess appropriate endpoints consistently reported developmental, female-reproductive and male-reproductive effects.

Possible DART effects at low levels of exposure can be consistent with endocrine disruption, and may be contrasted with the generally low systemic toxicity of BPA. The chemical has low acute toxicity following oral, inhalation and dermal exposure. Its acute oral LD_{50} in the most reliable experiments falls above 2000 mg/kg in mice and rats. With regard to non-DART effects, repeat-dose animal studies with oral dosing identified effects on intestine, liver and kidney in the dose range of 25 to ≥500 mg/kg bw/day. Effects in subchronic inhalation studies in rats included cecal enlargement resulting from distention by food and transient, slight hyperplasia and inflammation of epithelium in the anterior nasal cavity at ≥50 mg/m^3.

Developmental Toxicity

Epidemiologic studies of exposure to BPA and developmental outcomes consist of two recent studies. One study found no association between measurements of BPA in 404 women in the third trimester and gestational age or infant body size at birth. A second study collected blood from 40 women in hospital for delivery and found no association between BPA concentration

and birth weight or gestational age. However, certain aspects of these studies, such as small sample size, may have limited their ability to detect developmental effects.

The literature on BPA developmental toxicity in animals consists of guideline developmental and multigenerational studies and a large number of investigator-initiated studies. The main topics of the investigator-initiated studies are BPA action on gene expression during embryo/fetal development; behavioral effects after BPA exposure during the period of sex-differentiation of the brain; and BPA effects on immune system development. The studies vary in design and methodology used to address these topics. Many of the studies administer BPA orally to rats or mice at doses <1 mg/kg-d. They often include coordinated mechanism investigations and are oriented toward providing information relevant to human health.

The following effects to offspring were reported across the range of studies where BPA was administered during pregnancy:

- Offspring viability.
- Sex-differentiation of exploratory and affective behavior.
- Immune hyper-responsiveness.

In interpreting these results, it should be noted that:

- None of the developmental toxicity studies has been replicated either by the original researchers or by independent researchers.
- Comprehensive screening of BPA for developmental neurotoxicity has not yet been conducted.

Female Reproductive Toxicity

Seven epidemiologic studies, six of which were of cross-sectional design, report on associations between blood or urine concentrations of BPA and certain female reproductive outcomes. One case-control study found an association between blood concentration of BPA and recurrent miscarriage. In two different cross-sectional studies by the same researchers, BPA blood concentration was associated with polycystic ovary syndrome. In both studies, BPA blood levels were also positively correlated with free and total testosterone concentrations. In two studies, BPA concentrations were lower in patients with endometrial cancer and in women with complex endometrial hyperplasia. One study reported no associations between urinary BPA concentrations and self-diagnosed endocrine disorders. Another study reported no association with urinary BPA levels and endometriosis, and another study did not find BPA associated with pubertal status (breast development and pubic hair development) in 9 year-old girls. The cross-sectional studies have limited usefulness for evaluating the potential effects of BPA on the female reproductive system.

Numerous animal studies have examined the effects of BPA on the female reproductive system. The study designs vary by dose regimen, body status (pregnant or non-pregnant) at time of exposure, and age at exposure. Animals were exposed to BPA orally or via subcutaneous (s.c.) injection. Key endpoints reported in these studies are:

- Uterine effects:
    - Alterations in number of implantation sites.
    - Changes in weight.
    - Cell morphology – proliferation of endometrial lining.
    - Protein expression.
    - Gravid uteri.
- Ovarian effects:
    - Formation of cysts (cystic ovaries).
    - Differences in treated animal ovarian weights compared with controls.
- Ovarian follicle and oocyte effects:
    - Cystic follicles.
    - Problems with oocyte maturation (meiotic maturation).
- Estrous cycle effects:
    - Earlier (younger) age for first estrous cycle.
    - Altered cycle lengths.
- Fertility effects:
    - Reduced number of pups/litter.
- Vaginal effects:
    - Keratinization of the vaginal epithelium.
    - Earlier (younger) age when vaginal opening occurs.
- Mammary gland effects:
    - Earlier onset (younger age) for mammary gland development.
    - Variations in prolactin levels.
    - Increased proliferation/apoptosis ratio in both the epithelial and stromal compartments (TEB, TD, and alveolar buds).
    - Gene expression alterations.

In interpreting these results, it should be noted that:

- Many different study designs were used, with variations in parameters such as species and strains tested, and routes, levels and periods of exposure.
- No studies have exactly replicated each other, so no reported effects have been exactly replicated.
- Some study designs used may not be optimal for the female reproductive endpoint evaluated.

Male Reproductive Toxicity

Epidemiologic studies of male reproductive outcomes and BPA exposure consist of two small occupational studies of cross-sectional design. Both report associations between urinary levels of BPA and hormone levels. One found an association with lower levels of follicle-stimulating hormone (FSH) and no significant association with luteinizing hormone (LH) or testosterone. The other found an association with higher levels of LH, but no significant association with FSH

or testosterone. These studies are limited by factors such as their cross-sectional design and small number of study subjects (42 exposed in one, 25 in the other).

Evidence in laboratory studies on the male reproductive toxicity of BPA comes from nearly 100 studies, including both *in vivo* and *in vitro* studies. The designs for the animal studies vary substantially in many aspects including strain, age or developmental stages at exposure or final observation, route of exposures, numbers of animals per group, biological or toxicological endpoints evaluated, and methods of histopathological evaluations. Nearly all studies were in rats or mice.

In vitro studies consistently show effects of BPA on cultured cells or tissues from the testis (Sertoli or Leydig cells) or the prostate.

The major observations from the *in vivo* animal studies are as follows:

- Most studies that treated the animals by s.c. (injection or implantation) or intraperitoneal (i.p.) injection reported that BPA caused testicular or prostate effects.
    - The s.c. and i.p. studies that did not observe these effects did not use more sensitive methods than traditional ones (e.g., routine histopathological evaluation would not necessarily detect subtle changes in the testis).
- Most oral studies that used advanced approaches (e.g., immunostaining for structural or functional proteins in the testis or prostate) reported that BPA affected the testis or the prostate, regardless of the dosing period.
- The studies that observed changes in the accessory glands (e.g., epididymis, seminal vesicles) or in the anogenital distance or preputial separation (common indicators for sexual development) also observed effects in the testis.
- Several comprehensive reproductive toxicity studies reported that BPA caused a reduction in the number of live pups per litter, possibly resulting (at least in part) from male-mediated reproductive effects.
    - The reduction varied from noticeable but not statistically significant to obvious with statistical significance.
    - All the studies used the oral route of BPA administration.

In interpreting these results, it should be noted that:

- Sub-cutaneous and i.p. injection studies that used routine histopathological evaluation typically did not report effects on the testis or prostate.
- Only a few oral studies that used only traditional approaches to evaluate the testis or prostate have found significant effects resulting from BPA treatment.
- Effects on pup viability in many studies could have been the result of both male and female toxicity or direct effects on the concepti.

Endocrine Activity

As noted earlier, mechanisms by which BPA may affect reproductive function is likely due to the chemical's endocrine-disrupting potential. Several studies provide data on the disruptive effects of BPA on the endocrine system. In these studies BPA was observed to:

- Activate human and rat estrogen receptor (ER) (α and β).
- Inhibit human androgen receptor.
- Interact with plasma steroid binding protein.
- Bind to other receptors such as human estrogen-related receptor gamma and the aryl hydrocarbon receptor.
- Affect ERα and progesterone receptor expression in rats.
- Mimic E_2-driven cytosolic second messenger activation.
- Affect steroidogenesis by:
  - Decreasing aromatase activity.
  - Altering steroid metabolism.
- Affect other hormones by:
  - Reducing mean LH concentration, pulse amplitude, and LH frequency in lambs.
  - Stimulating of production of prolactin in rats.
  - Increasing the production of insulin in mice.
  - Interfering with thyroid hormone (triiodothyronine, T_3) action and acting as a receptor antagonist in rats.
  - Reducing the activity of thyroid hormone in an in vivo amphibian bioass

Acronyms and Abbreviations

ADHD	attention-deficit hyperactivity disorder
ADME	absorption, distribution, metabolism, and excretion
AGD	anogenital distance
AhR	aryl hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AO	arachis oil
ArKO	aromatase knock out
ARC	arcuate nucleus
ARE	autoregulatory element
ARNT	AhR nuclear translocator
AUC	area under (serum concentration) curve
$AUC_{0–24}$	area under the curve for the 24 h after administration
$AUC_{0\rightarrow\infty}$	area under the curve extrapolated to infinity
AVPV	anteroventral periventricular nucleus
BMI	body mass index
BPA	bisphenol A
BrdU	bromodeoxyuridine
BST	bed nucleus of the stria terminalis
bw	body weight
^{14}C-BPA	^{14}C-labelled (radioactively labeled) BPA
Ca^{+2}	calcium ion
CAS	Chemical Abstracts Service
$CD4^{+}CD25^{+}$	regulatory T cells
CEH	cystic endometrial hyperplasia
CGI	short segment of DNA with high frequency of cytosine guanine sequences
CHO	Chinese hamster ovary
CK-10	cytokeratin 10
CL	corpus luteum (plural: corpora lutea)
Cmax	maximum blood concentration
COCs	cumulus oocyte complexes
CRH	corticotropin-releasing hormone
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
d	day(s)
d_{16}-BPA	isotopic labeled bisphenol A-d_{16}
DART	developmental and reproductive toxicity
DART IC	Developmental and Reproductive Toxicant Identification Committee
DEHP	di(2-ethylhexyl) phthalate
DES	diethylstilbesterol
DHT	dihydrotestosterone
DMAB	3,2'-dimethyl-4-aminobiphenyl
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
dpc	days post conception
DRD1	dopamine receptor D1
DSP	daily sperm production (expressed as 10^6 sperm produced/d)
E_2	estradiol (estrogen)
EC_{50}	half-maximal effective concentration
EDs	endocrine disruptors
EE	17α-ethynyl estradiol
ELISA	enzyme linked immunosorbant assay
ERE	estrogen response element
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERαKO	estrogen receptor alpha knock out
ERβKO	estrogen receptor beta knock out
ERR γ	estrogen-related receptor gamma
EFSA	European Food Safety Authority
EU	European Union
F_1	first filial generation, produced by crossing two parental lines
F_2	second filial generation, produced by intercrossing the F_1
F_3	third filial generation, produced by intercrossing the F_2
FDA	Food and Drug Administration
fM	femtomolar; 10^{-12} M
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
GABAα	alpha receptor for the inhibitory neurotransmitter GABA
GC	granulosa cells
GC/MS	gas chromatography/mass spectrometry
GC/MS-NCI	gas chromatography/mass spectrometry–negative ion chemical ionization detection
GD	gestation day
GP30	a specific membrane estrogen receptor
GPx	glutathione peroxidase
GSH	glutathione
GT	glucuronyl transferases
GVBD	germinal vesicle breakdown
^{3}H-BPA	tritium-labeled BPA
h	hour(s)
hSHBG	human sex hormone binding globulin
HPLC	high-pressure liquid chromatography
HOXA10	human gene is necessary for uterine development, specifically normal decidualization and pregnancy
IC_{50}	concentration that causes 50% inhibition
ICI	ER antagonist ICI 182,780
i.m.	intramuscular
i.p.	intraperitoneal

i.v.	intravenous (-ly)
kDa	kilodaltons
kg	kilogram
kg bw	kilogram of body weight
K_a	association constant
K_D	dissociation constant
K_{init}	initial rate constant
K_{term}	terminal phase elimination rate constant
Lac	lactating
LC	liquid chromatography
LC-TMS	liquid chromatography-tandem mass spectrometry
LH	luteinizing hormone
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantitation
M	molar
μg	micrograms
min	minute(s)
μM	micromolar; 10^{-6} M
mg	milligrams
MPA	medial preoptic area
mRNA	messenger RNA (ribonucleic acid)
MS	mass spectrometry
MXC	methoxychlor
NC	not calculated
ND	no data
NHANES	National Health and Nutrition Examination Survey
NICU	neonatal intensive care unit
ncmER	non-classical membrane bound form of the estrogen receptor
ng	nanogram
NIOSH	National Institute for Occupational Safety and Health
NL	non-lactating
nM	nanomolar; 10^{-9} M
NOAEL	no observed adverse effect level
NTP-A	NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008
NTP-B	NTP Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008
NTP-CERHR	National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction
OEHHA	Office of Environmental Health Hazard Assessment
o.i.	oral instillation
OVX	ovariectomized

PAP	prostatic acid phosphatase
PBPK	physiologically-based pharmacokinetics
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pM	picomolar; 10^{-12} M
PMSG	pregnant mare serum gonadotropin
PND	postnatal day
PNW	postnatal week
POA:	preoptic area
ppb	parts per billion
PPG	pseudopregnancy
ppm	parts per million
PPL	progressive proliferative lesion
PPS	preputial separation
PRF	prolactin releasing factor
PRL	prolactin
PVC	polyvinyl chloride
RACB	reproductive assessment by continuous breeding
rAFP	rat α-fetoprotein
RARα	retinoic acid receptor α
RFU	relative fluorescent units
RLGS	restriction landmark genomic scanning
RTI	Research Triangle Institute
RT-PCR	reverse transcriptase polymerase chain reaction
RXRα	retinoid X receptor
s.c.	subcutaneous (-ly)
SD	Sprague Dawley
SDN-POA	sexually dimorphic nucleus of the preoptic area
SOD	superoxide dismutase
sst3	somatostatin receptor subtype 3
SV	seminal vesicle
T	testosterone
$T_{½}$	half-life
T_3	triiodothyronine
T_4	thyroxine
TBARS	thiobarbituric acid-reactive substance
TD	terminal ducts
TE	terminal ends
TEB	terminal end buds
TEB/area	number of TEB per ductal area
TEB area/area	area of all TEB per ductal area
TI	theca interstitial (cells)
TH	thyroid hormone
T_{max}	time to maximum plasma concentration
TMS	tandem mass spectrometry

TR	thyroid hormone receptor(s)
TSCO	tocopherol-stripped corn oil
UDP	uridine diphosphate
UGT	uridine diphosphate glucuronosyltransferase
VMH	ventromedial nucleus of the hypothalamus
VO	vaginal opening
wt	weight

A. Introduction

This report reviews information relevant to whether bisphenol-A (BPA) causes developmental and reproductive toxicity (DART). This high production volume chemical has a wide range of uses in industrial and consumer products. Several reports on the DART of this compound are available to the general public. These reports include:

- Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A. National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (CERHR, 2008)
- European Union Risk Assessment Report, 4,4'-isopropylidenediphenol (bisphenol-A) (EU, 2003)
- European Union Updated Risk Assessment of 4,4'-isopropylidenediphenol (Bisphenol-A) (EU, 2008)

These three documents are attached to this report as part of the hazard identification materials for BPA. Detailed study summaries contained in these documents are referenced in this report, and are not repeated here. Toxicity studies discussed in this report that were not included in those documents are summarized in Appendix 1.

A.1. Chemical and physical characteristics

Bisphenol A (BPA; 4,4'-dihydroxy-2,2-diphenyl propane) is an organic compound with two phenol functional groups. At ambient temperature it is a white solid with a mild phenolic odor. It has a molecular formula of $C_{15}H_{16}0_2$ and a molecular mass of 228.29 g/M. The Chemical Abstracts Service (CAS) number for BPA is 80-05-7.

The chemical structure of BPA is shown in Figure 1, and its physicochemical characteristics are summarized in Table B1. Additional information on the physicochemical characteristics of BPA can be found in the EU risk assessment document (EU, 2003) or the NTP-CERHR Expert Panel Report (CERHR, 2008).

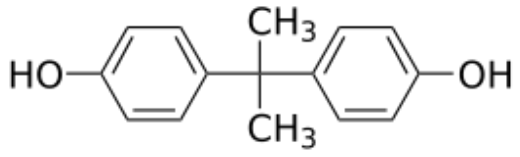

Figure A1. Molecular structure of BPA

Table A1. Chemo-physical properties of BPA

Property	Value
Odor threshold	No data found
Boiling point	220°C at 4 mm Hg; 398°C at 760 mm Hg
Melting point	150-157°C
Specific gravity	1.060–1.195 g/ml at 20–25°C
Solubility in water	120–300 mg/l at 20–25°C
Vapor pressure	8.7×10^{-10}–3.96×10^{-7} mm Hg at 20–25°C
Stability/reactivity	No data found
Log K_{ow}	2.20–3.82
Henry constant	1.0×10^{-10} atm•m^3/mol

A.2. Use and exposure

Detailed information on the general use, production, and human exposure levels of BPA has been reviewed by the EU (EC, 2002; EU, 2003) and the NTP-CERHR (CERHR, 2008). However, no data specific to California are available to OEHHA. As noted by NTP-CERHR:

> "Bisphenol A (BPA) is a chemical produced in large quantities for use primarily in the production of polycarbonate plastics and epoxy resins. …. Polycarbonate plastics have many applications including use in certain food and drink packaging, e.g., water and infant bottles, compact discs, impact-resistant safety equipment, and medical devices. … Polycarbonate plastic can also be blended with other materials to create molded parts for use in mobile phone housings, household items, and automobiles. Epoxy resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. Some polymers used in dental sealants or composites contain bisphenol A-derived materials. In 2004, the estimated production of bisphenol A in the United States was approximately 2.3 billion pounds, most of which was used in polycarbonate plastics and resins."

The most common route of BPA exposure is oral. Bisphenol A is known to leach from dental composites as well as food containers such as cans and polycarbonate plastic water bottles. Detectable levels of BPA have been found in the general population. Exposure to BPA was measured in a one-third random subset of the 2,517 participants in National Health and Nutrition Examination Survey (NHANES) 2003–2004 (Calafat et al., 2008). BPA, measured as urinary BPA (free plus conjugated species), was detected in 92.6% of persons ≥ 6 years of age with total concentrations ranging from 0.4 μg/L to 149 μg/L (geometric mean = 2.6 μg/L (2.6 μg/g creatinine)). Children had significantly higher concentrations than adolescents, who had significantly higher concentrations than adults. Least square geometric mean concentrations of BPA of participants in households with incomes < $20,000 were significantly higher compared with participants in households with incomes > $45,000. Least square geometric mean concentrations of BPA were significantly lower in Mexican Americans than in non-Hispanic blacks and non-Hispanic whites. In a recent study of 54 premature infants in neonatal intensive care units mean total urinary BPA concentrations (free and conjugated) (28.6 μg/L) were almost

10 times higher than concentrations in 6- to 11-year old children measured in NHANES 2003–2004 (Calafat et al., 2009).

A.3. General toxicity

A.3.1. Acute toxicity

Reviews of the acute toxicity of BPA other than DART effects have concluded that BPA is a chemical of low toxicity. For example, the EU (EU, 2003) concluded that:

> "No useful information is available on the effects of single exposure to BPA in humans. Oral LD_{50} values beyond 2,000 mg/kg are indicated in the rat and mouse, and dermal LD_{50} values above 2,000 mg/kg are evident in the rabbit. Few details exist of the toxic signs observed or of target organs. For inhalation, a 6-hour exposure to 170 mg/m^3 (the highest attainable concentration) produced no deaths in rats; slight and transient slight nasal tract epithelial damage was observed. These data indicate that BPA is of low acute toxicity by all routes of exposure relevant to human health."

There was no additional information on acute toxicity from the NTP-CERHR Monograph (CERHR, 2008) or the EU Risk Assessment Update (EU, 2008).

A.3.2. Repeated inhalation exposure

The NTP-CERHR Monograph (CERHR, 2008) reports that acute effects of inhalation exposure in rats included transient and slight inflammation of nasal epithelium and ulceration of the oronasal duct following exposure to 170 mg/m^3 BPA dust for 6 hr.

A.3.3. Repeated oral treatment

The NTP-CERHR Monograph (CERHR, 2008) reports that possible target organs or systems of toxicity identified in repeat-dose animal studies with oral dosing included intestine, liver, kidney, and male, and female reproductive systems. Intestinal findings (effect levels) in rats included cecal enlargement (≥25 mg/kg bw/day) and cecal mucosal hyperplasia (≥200 mg/kg bw/day). Hepatic effects included prominent hepatocyte nuclei or inflammation in rats (≥500 mg/kg bw/day), multinucleated giant hepatocytes in mice (≥120 mg/kg bw/day), and increased weight with no evidence of histopathology in dogs (≥270 mg/kg bw/day). Renal tubule degeneration or necrosis was observed in rats dosed with ≥500 mg/kg bw/day. Effects in subchronic inhalation studies in rats included cecal enlargement resulting from distention by food and transient, slight hyperplasia and inflammation of epithelium in the anterior nasal cavity; both effects occurred at (≥50 mg/m^3).

With regard to carcinogenicity, the NTP-CERHR Monograph (CERHR, 2008) reports that the carcinogenic potential of BPA was evaluated in rats and mice, and that the NTP concluded that under the conditions of the study, there was no convincing evidence that BPA was carcinogenic in F344 rats or B6C3F1 mice. However, NTP stated that there was suggestive evidence of increased cancer in the hematopoietic system based on marginally significant increases in leukemia in male rats, non-statistically significant increases in leukemia in female rats, and a marginally significant increase in combined incidence of lymphoma and leukemia in male mice. A statistically significant increase in testicular interstitial cell tumors in aging F344 rats was also considered suggestive evidence of carcinogenesis. The effect was not considered conclusive evidence because of the high incidence of the testicular neoplasm in aging F344 rats (88% incidence in historical controls). The European Union (EU, 2003) stated that the evidence does not suggest carcinogenic activity of BPA in rats or mice.

There are a number of recent scientific publications on BPA as a possible modulator of or contributor to certain sex hormone sensitive cancers. A full review of this body of evidence is beyond the scope of the present document.

A.3.4. Dermal exposure

There are no data available on the general toxicity of BPA following dermal exposure beyond a reported LD_{50} value above 2,000 mg/kg in the rabbit (EU, 2003).

B. Developmental Toxicity

This section reviews the two epidemiologic studies and numerous laboratory animal studies relevant to BPA developmental toxicity. There is also a large literature concerning effects on development in wildlife, including aquatic species (Crain et al., 2007; European Union, 2008), which is not reviewed here.

We focus on studies with prenatal BPA exposure. However, animal studies with combined prenatal and postnatal exposure are also reviewed.

The review of animal studies is divided by endpoint:

- Embryo,fetal, and neonatal endpoints
- Postnatal growth
- Immune responsiveness
- Effects on sex differentiation of the brain and behavior
- Developmental neurobehavioral toxicity

Research programs in individual laboratories sometimes use the same animal model (species, strain, route, and dose) in a series of hypothesis-testing studies. In this section, individual studies are presented in the context of the other information from the same model. A similar approach was used in the European Union review of BPA (EU, 2008).

Because of BPA's potential estrogenic activity (Section E.1), comparison estrogens are often used as a "positive control" in animal studies of BPA. Positive controls are useful in evaluating the sensitivity of the study design and endpoints. Although all these agents can be classified as "estrogenic" they differ in their effectiveness at the many different estrogen receptors. The following briefly describes the estrogenic agents used as positive controls in the studies reviewed in this section:

- 17 β estradiol (E_2): Prominent endogenous estrogen; low bioavailability; benzoate form often used
- Ethinyl estradiol (EE): Ethinyl group substitution of estradiol enhances oral bioavailability
- Diethylstilbestrol (DES): Therapeutic nonsteroidal estrogen; minimal binding to α fetoprotein enhances bioavailability
- Methoxychlor (MXC): Estrogenic pesticide
- Genistein: Phytoestrogen found in soy products
- Nonylphenol: Industrial chemical with estrogenic effects

Information from comparison estrogens are provided when available throughout this section.

B.1. Developmental toxicity studies in humans

Two recent studies of birth outcomes in humans have become available. A cross-sectional study compares maternal BPA levels in blood collected from 40 women in hospital for delivery to birth weight and gestational length (Padmanabhan et al., 2008). A prospective study compares birth weight, birth length, head circumference and gestational age to mothers' BPA levels measured in the third trimester, for 404 mother/newborn pairs (Wolff et al., 2008b). Neither study reported an association between BPA and birth outcomes; however, certain aspects of the studies limited their ability to detect potential developmental effects, including small sample size, cross-sectional study design, a single measure of exposure and low exposure levels. There are no other epidemiologic studies of developmental outcomes for BPA (NTP, 2008).

B.2. Developmental toxicity studies in laboratory animals

B.2.1. Embryo and fetal endpoints and pregnancy outcome in animal models

B.2.1.1. Regulatory guideline studies

The U.S. EPA, the EU and other governmental agencies have established guidelines for routine testing for DART in laboratory animals.

Routine guideline-type studies are outlined in Table B1. There are two developmental toxicity studies, one study with several subdivisions using the Reproductive Assessment by Continuous Breeding (RACB) protocol, two rat multigeneration studies and one mouse multigeneration study. Many of these studies (Morrissey et al., 1987; Tyl et al., 2002a; Tyl et al., 2002b; Tyl, 2003; Tyl et al., 2005; Tyl et al., 2006; Tyl et al., 2008a; Tyl et al., 2008b; Tyl et al., 2008c) have been conducted by the Research Triangle Institute (RTI), a commercial research organization that offers DART testing services.

Table B1. Routine guideline type studies with data on pregnancy outcome after prenatal BPA exposure.

Study	Design	General toxicity	Pregnancy outcome
female exposure (developmental toxicity studies)			
Hardin et al., 1981 EU 2003	SD rats GD 1–15 i.p. 0, 85, 125 mg/kg-d	25% preg/inseminated, 125 mg/kg-d 100% pregnancy completion N=4.	↓ live fetuses per litter ↓ fetal body weight ↓ fetal body length Both doses
Morrissey et al., 1987 NTP-A EU 2003	CD-1 mice GD 6–15 gavage 0, 500, 750, 1000, 1250 mg/kg-d N=21–26	dam mortality 18% , 1250 mg/kg-d corrected maternal weight gain 71% of control, 1250 mg/kg-d	↑ % resorptions/litter, 1250 mg/kg-d ↓ fetal body weight, 1250 mg/kg-d Linear trend tests significant
	CD rats GD 6–15 gavage 0, 160, 320, 640 mg/kg-d N=25–29/group	No dam mortality 640 corrected maternal weight gain 66% of control	No effects
Kim et al., 2001b NTP-A	SD rats GD 1–20 0, 100, 300, 1000 mg/kg-d, gavage N=14–20	↓ dam body weight ↓ dam body weight gain ↓ corrected dam body weight gain	↓ live pups/litter ↓ male pup weight/litter ↓ female pup weight/litter ↓ skeletal ossification
Male and/or female exposure (RACB studies)			
Morrissey et al., 1989 NTP-A EU 2003 (cited as NTP 1985b)	Swiss mice 14 wk, begin 1 wk prior to mating Diet 0, 0.25, 0.5, 1% diet 470, 900, 1880 mg/kg-d Male and female exposure	↓ dam weight at delivery, ↑ days to litter	↓ litters/pair, ↓live pups/pair, ↓live males/females/litter, % pups born alive ↑ live pup weight/litter ↑ live male pup weight/litter ↑ live female pup weight/litter
Morrissey et al., 1989 NTP-A EU 2003 (cited as NTP 1985b)	Swiss mice 14 wk, begin 1 wk prior to mating Diet 1920 mg/kg-d Female exposure only	↓dam weight; ↑dam adjusted liver and kidney weights	↓ live pups/litter ↓ live male pups/litter ↓ live female pups/litter ↑ live pup weight/litter ↑ live male pup weight/litter ↑ live female pup weight/litter adjusted

Table B1. Routine guideline type studies with data on pregnancy outcome after prenatal BPA exposure (continued).

Study	Design	General toxicity	Pregnancy outcome
Male and female exposure (multigeneration studies)			
Ema et al., 2001 NTP-A EU 2008	SD rats 2-generation Gavage 0, 0.2, 2, 20, 200 µg/kg-d N=19–24	No mortality or effects on dam weight; No weight gain data presented	No effects : pups/litter, sex ratio; pup weight PND 0
Tyl et al., 2002b NTP-A (cited as Tyl et al. 2000a, 2000b, 2002b) EU 2003	SD rats 3-generation Diet 0, 0.001, 0.02, 0.3,5,50,500 mg/kg-d Male and female exposure	↓ dam body weights and weight gain gestation and lactation	↓ live pups/litter ↓ pups/litter ↓ implantation sites Comparison estrogen, estradiol 2.5 mg/kg No effect M,F body weight Trend across doses live pups; no trend analysis; no weight trend ↓ live pups/litter ↓ pups/litter ↓ implantation sites
Tyl, 2008 NTP-A (as Tyl et al., 2006) EU 2008	CD-1 mice 2-generation Diet 0, 0.003, 0.03, 0.3,5, 50, 600 mg/kg-d N=55(control), 19–25 (BPA)	No effects on dam weight or dam weight gain; ↑ rel liver weight; ↑ kidney weight	Significant ANOVA; No pairwise effects ↓ live pups/litter ↓ total pups/litter ↓ stillbirth index
Tyl et al., 2002a NTP-A (cited as Tyl et al. 2002)	CD-1 mice, 1-generation 0, 437,875, 1750 mg/kg-d during gestation	↓ dam body weights and pregnancy weight gain; ↑ liver kidney weights, ↑ gestation length	↓ live pups/litter ↓ total pups/litter Significant dose trend ↓ female PND0 bw
Tyl et al., 2006 EU 2008 (as abstract) (E_2 study)	CD-1 mice 2-generation 0, 0.001,.005, .05, .5 ppm E_2 0, 0.2, 1.0, 1, 30, 100 mg E_2/kg -d	No effects on maternal weight, maternal weight gain, liver weight or kidney weight	↓ live pups/litter ↓ total pups/litter ↑ pup weight

Table B1. Routine guideline type studies with data on pregnancy outcome after prenatal BPA exposure (continued).

Study	Design	General toxicity	Pregnancy outcome
Other studies with pregnancy outcome data			
Tinwell et al., 2002 NTP-A EU 2008	SD, Wistar rats GD 6–21 20, 100 µg/kg, 50 mg/kg, gavage N=7/group	No effects maternal weight	No effects litter size, sex ratio, birth weight
Negishi et al., 2003 NTP-A EU 2008	F344 rats 0, 4, 40, 400 mg/kg-d GD 10–PND 20 Gavage N=8,9/group	40, 400 ↓ maternal body weight during pregnancy	No effect: total pups/litter, sex ratio at birth; no birthweight measure

*based on author estimate

NTP-A: study description in the NTP-CERHR Expert Panel Report within the "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

NTP-B: study description in the NTP Brief within the "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008

EU 2003: study description in "European Union Risk Assessment Report: 4,4'-isopropylidenediphenol (bisphenol-A)", 2003

EU 2008: study description in "Updated European Risk Assessment Report 4,4'-Isopropylidenediphenol (bisphenol-A), April 2008.

One of the three available developmental toxicity studies is a small study with intraperitoneal (i.p.) administration of BPA to rats. This study was sponsored by the National Institute of Occupational Safety and Health (NIOSH) and briefly reported in a paper that included studies of 18 other chemicals (Hardin et al., 1981). Number of live fetuses per litter and fetal body weight and length were adversely affected at both doses used. Maternal toxicity was not reported. Morrissey et al. (Morrissey et al., 1987) reported more comprehensive studies in both mice and rats with gavage administration during embryogenesis. No developmental or maternal toxicity was reported in the rat study. The mouse study reported a significant linear trend for increased resorption and decreased fetal body weight as well as a significant difference from controls at the highest dose (1250 mg/kg-d). This dose also caused 18% mortality in the dams. A more recent rat study with gavage administration (Kim et al., 2001) used a more extended BPA administration from gestation day (GD) 1 to GD 20 and also a wider dose range than the Morrissey et al. study. This study found BPA effects on pregnancy completion ratio, pre- and post- implantation loss, number of live fetuses/litter, fetal weights, and delayed ossification, with most effects occurring at the highest dose tested (1000 mg/kg-d) and no significant effects at the lowest dose (100 mg/kg-d). As regards maternal toxicity, maternal weight, weight gain and corrected body weight gain were affected at the two higher doses.

Although no comparison estrogens were used in the guideline developmental toxicity studies, effects on fetal viability and weight are consistent with hypothesis-testing studies of the effects of estradiol on pregnancy in the rat (Bartholomeusz et al., 1999; Matsuura et al., 2004). For instance, Matsuura et al. administered estradiol benzoate (E_2) to Wistar rats on GD 12–19 and found a dose-dependent reduction in fetal survival and fetal weight on GD 20. Smaller placentas, degeneration of trophoblasts and reduction of fetal vessels in the placental labyrinth

were also observed after the estradiol treatment, thus implicating placental mediation of fetal effects. The lowest effective dose (10 μg E_2/day by injection) produced a nonsignificant 47% increase in maternal plasma estradiol.

In addition to the developmental toxicity studies, a larger number of recent routine guideline reproductive toxicity studies are available for BPA. Some measures in these studies can reflect developmental toxicity. For example, litter size, pup weight and mortality at birth in the F_0 generation (dams exposed to BPA before and during pregnancy only) may reflect developmental toxicity. Pregnancy outcome in later generations may be influenced by effects on reproductive tract maturation in the breeders.

Some of these routine guideline studies include a comparison estrogen as a positive control. Separate studies of estrogenic agents using these study designs are also available for comparison (Biegel et al., 1998a; Biegel et al., 1998b; Tyl et al., 2008c).

Multigeneration studies of BPA in both rats and mice have been performed at RTI. Reports of these studies are published in the peer-reviewed literature (Tyl et al., 2002b; Tyl et al., 2008b) and were also made available for this review as the original study reports (Tyl et al., 2000; Tyl et al., 2007). Additional work from RTI relevant to this review include a one-generation BPA study in mice, a two generation study of E_2 in mice, and a 13-week BPA dose range-finding study in mice (Tyl et al., 2002a; Tyl et al., 2005; Tyl et al., 2006). Details of these studies are presented in Table B1. The multigeneration studies are also reviewed in Table C6 and Section D.2.1 because the data are potentially relevant to female and male reproductive toxicity as well as developmental toxicity.

In terms of pregnancy outcome in the first generation, the RTI studies were consistent in finding BPA and E_2 effects on pup survival. The BPA study in rats (Tyl et al., 2000) found treatment effects on two indices related to pup survival:

- Total number of pups on postnatal day 0.
- Number of live pups on postnatal day 0.

There was also a significant linear trend across doses for these parameters, and a significant difference from control in the high dose group for both measures in the rat study. The BPA study in mice (Tyl et al., 2007) also found significant treatment analysis of variance (ANOVAs) for a number of variables reflecting pup survival:

- Number of live pups on postnatal day 0.
- Total number of pups on postnatal day 0.
- Number of dead pups on postnatal day 0.
- Stillbirth index on PND 0.
- Live birth index on PND 0.

None of the pairwise comparisons of a BPA-treated group with the combined control group was significant in the mouse study using the statistical approaches of the study. These pup survival

measures in mice were also affected by the comparison estrogen E_2 at a dose of 0.5 mg/kg-d (Tyl et al., 2007).

There were no effects on pups' birth weight in either the rat or the mouse study. However, it is important to note that BPA led to increased gestation length in the mouse study and calculations of birth weight were not corrected for gestational age. Pups will weigh more at birth if born later. Also pup weights were not corrected for the number of pups in the litter. Pups in smaller litters can weigh more. Pup weights in the high dose group began to fall behind after birth and were significantly lower than controls by PND 7. In the rat study, there was no effect on pup birth weights but a significant treatment effect and a linear dose trend were seen for pup body weights by PND 4. As was the case in the mouse study, pup birthweights were not corrected for gestation age or litter size.

Although there was no comparison estrogen in the rat three-generation study, pregnancy outcome in the first generation was very similar to that in an estradiol one-generation study (Biegel et al., 1998b) in finding decreased pups/litter, live pups per litter and implantation sites. Using litter size and sex ratio as covariates in the analysis, the Biegel study also found an effect on birthweight. Gestation length was not reported.

In addition to the multi-generation RTI studies, Ema et al. (Ema et al., 2001) conducted a multigeneration study in rats based on Japanese and international guidelines, and characterized by gavage administration of BPA and a low dose range (≤0.2 mg/kg-d). This study found no effects on litter size or pup birth weight in the first generation.

Another type of routine guideline study is the RACB, in which dosing begins shortly before mating in both parents and continues through the birth of several litters over a 90-day period. There are three relevant RACB guideline studies using BPA:

- A study using subcutaneous administration via minipump to the dam.
- A study using dietary exposure of both parents.
- A study using dietary exposure of the dam only (Morrissey et al., 1989) (Table B1).

Notably, the implanted minipump study reported increased litter sizes and pup birth weights in the BPA-exposed groups. However, the study report cited problems with retention of the implants which led the investigators to disregard the data. The diet studies, with either both parents or dam only exposed, reported decreased litter sizes but increased pup birth weights. It is possible that the greater birth weights were secondary to the smaller litter sizes in the BPA group. Corrections for litter size were not performed in the statistical analysis. BPA effects in the RACB studies occurred at doses >900 mg/kg-d. Maternal/paternal toxicity data were not included in the study report.

In order to provide information on maternal/paternal toxicity at the dose levels used in the RACB studies, an "abbreviated" one-generation study was conducted at RTI (Tyl et al., 2002a) using the effective doses from the RACB study and the same mouse model (CD-1). In this design, only one litter was produced by each mating pair and the duration of exposure prior to mating was two weeks. The study identified maternal/paternal toxicity as increased liver and kidney

weights and pathology (reflected in histopathology and clinical chemistry values). Reproductive toxicity included prolonged gestation length and decreased litter size (total and live pups per litter). Increased liver and kidney weights in the parents, first identified in the one-generation study, were seen in the later RTI multigeneration study of BPA.

In general these studies suggest that fetal viability is sensitive to BPA at doses >500 mg/kg-d by the oral route in these experimental paradigms. Based on limited data, malformations and variations do not seem affected by BPA. Fetal/newborn weights were variably affected but evaluation of this endpoint is complicated by BPA-induced increase in gestation length and decrease in litter size, both of which would tend to increase fetal/newborn weights.

Table B2 demonstrates the varying statistical approaches to evaluation of key pregnancy outcome variables in the routine guideline-style multigeneration studies. Different tests used (ANOVA, trend test), as well as different statistical programs and approaches to data summary, prevent a simple agree/disagree conclusion when comparing two studies. This is the case throughout the BPA literature.

In addition to choice of statistical tests, the recent multigeneration studies from RTI differed from other studies reviewed here in several aspects of the approach to statistical analysis. In these studies "statistical outliers" were eliminated from the data sets of some variables prior to analysis and not included in the tables of individual animal data. The criteria for determination of "statistical outlier" and the reasons that some variables but not others were selected were not stated. In addition "unrealistic" values were excluded from the data analysis and reports of individual data. The RTI statistical analysis also used one-sided rather than two-sided group mean comparisons for some variables. Additionally, the RTI statistical analysis used "robust regression" analyses when homogeneity of variance assumptions were not met for ANOVA (Levene's test). Other studies reviewed here used nonparametric statistical analyses under these conditions. Finally, sex differences were not evaluated statistically for any endpoint in these studies. Although the RTI studies were fairly consistent in their statistical approach, dose trend tests were included for some studies (mouse developmental toxicity, rat three-generation, mouse one-generation) but not others (mouse BPA and E_2 two-generation).

Table B2. Statistical approaches used to evaluate the same endpoints in different in guideline studies of BPA and E_2.

Reference Type of study	Implantation #	Pups/litter	Live pups/litter	Fetal weight
Morrisey et al., 1987 BPA rat dev tox	ANOVA\Dunnett's or Williams post hoc; one tailed; Dose trend test	Measure not reported	ANOVA\Dunnett's or Williams post hoc; one tailed; Dose trend test	ANOVA\Dunnett's or Williams post hoc; two- tailed; Dose trend test; not adjusted
Kim, 2001 BPA rat dev tox	Kruskal-Wallis ANOVA\Mann-Whitney post hoc tests	Measure not reported	ANOVA\post hoc Scheffe test	ANOVA\post hoc Scheffe test; not adjusted
Biegel et al. 1998 E_2[a] E_2 rat multigen	Dose trend test (Jonckheere)	Dose trend test (Jonckheere)	Dose trend test (Jonckheere)	Dose trend test (Jonckheere) adjusted for litter size, sex ratio
Tyl et al., 2002b BPA rat multigen	ANOVA\Dunnett's post hoc test one-tailed Dose trend test	ANOVA\Dunnett's post hoc test, one-tailed Dose trend test	ANOVA\Dunnett's post hoc test, one-tailed Dose trend test	ANOVA\Dunnett's post hoc test, two-tailed Dose trend test Not adjusted
Morrisey, 1987 BPA mouse dev tox	ANOVA\Dunnett's or Williams post hoc; one-tailed; Linear trend test	Measure not reported	ANOVA\Dunnett's or Williams post hoc; one-tailed; Linear trend test	ANOVA\Dunnett's or Williams post hoc; two- tailed; Linear trend test; not adjusted
Tyl et al., 2008c E_2 mouse multigen	ANOVA\Dunnett's post hoc test one-tailed	ANOVA\Dunnett's post hoc test, one-tailed	ANOVA\Dunnett's post hoc test, one-tailed	ANOVA\Dunnett's post hoc test, two-tailed Not adjusted
Tyl et al., 2002a BPA mouse one gen	ANOVA\Dunnett's post hoc test one-tailed Dose trend test	ANOVA\Dunnett's post hoc test, one-tailed Dose trend test	ANOVA\Dunnett's post hoc test, one-tailed Dose trend test	ANOVA\Dunnett's post hoc test, two-tailed Dose trend test Not adjusted
Tyl et al., 2008b BPA mouse multigen	ANOVA\Dunnett's post hoc test one-tailed	ANOVA\Dunnett's post hoc test, one-tailed	ANOVA\Dunnett's post hoc test, one-tailed	ANOVA\Dunnett's post hoc test, two-tailed Not adjusted
Ema et al., 2004	ANOVA\Dunnett's Post hoc tests One/two tailed not stated	ANOVA\Dunnett's Post hoc tests One/two tailed not stated	ANOVA\Dunnett's Post hoc tests One/two tailed not stated	ANOVA\Dunnett's Post hoc tests Not adjusted

[a] ANOVA was not mentioned in the statistical procedures for these endpoints in this study.

[b] trend tests were not mentioned in statistical procedures sections for the mouse E_2 and BPA studies (Tyl 2008b, c).

B.2.1.2. Hypothesis-testing studies

Many studies of BPA and embryo/fetal development have focused on possible estrogenic effects at the early stages of development. Several potential targets of estrogenic action are known to be critical during embryo/fetal development:

- Estrogen receptor is expressed at various stages of embryo/fetal development both prior to and after differentiation of the fetal gonads and fetal production of steroid hormones.
- Nuclear estrogen receptors act as transcription factors.
- Estrogen receptor forms heterodimers with other members of the nuclear receptor family such as retinoic acid and thyroid hormone to influence gene transcription during embryonic/fetal development.

Independent studies reviewed in Table C6 have documented the distribution of BPA and BPA metabolites from mother to conceptus during pregnancy.

B.2.1.2.1. Preimplantation effects

Estrogen receptor mRNA is expressed in the early 2-cell embryo as well as in the blastocyst prior to maturation (Hou and Gorski, 1993; Hiroi et al., 1999). To determine the potential impact of BPA on preimplantation embryos, 2-cell mouse embryos were cultured in medium containing BPA with or without the estrogen receptor antagonist tamoxifen (Takai et al., 2000). At lower concentrations (1 and 3 nM) BPA accelerated embryonic development, increasing the percentage of 2-cell embryos advancing to blastocysts after 48 h in culture. At a higher concentration (100 μM) blastocyst formation was suppressed. Tamoxifen blocked both of these effects, indicating that they could be estrogen-receptor mediated. Blastocyst morphology and cell counts did not differ from controls (Takai et al., 2001). When BPA-exposed blastocysts were transferred to the uterus of pseudopregnant mice (Takai et al., 2001), pregnancy and birth parameters did not differ from control, but offspring were heavier at weaning (see Table B4). Acceleration of embryonic development at low doses of BPA has also been shown in a fish model (Ramakrishnan and Wayne, 2008).

B.2.1.2.2. Gene expression in the embryo/fetus

Some studies used "upstream" indicators such as gene expression and proteomics to demonstrate biological actions of BPA relevant to *in utero* development (Table B3). Estrogen receptor activation by BPA was demonstrated in GD 13.5 transgenic mouse fetuses carrying a luciferase reporter gene with an estrogen response element (Lemmen et al., 2004). DES was also active in this assay at similar concentrations to BPA. Using an *in vitro* system with the luciferase reporter, BPA had 3–4 orders of magnitude lower potency than DES, suggesting that fetal estrogenic sensitivity *in utero* is greater than indicated by isolated cell assays.

Other studies compared gene activation patterns of BPA to those of 17α-ethinyl estradiol (EE) and genistein in the reproductive organs of GD 20 rat fetuses (Naciff et al., 2002; Naciff et al., 2005). The BPA exposure was via s.c. injection of the dam on GD 11–20 and fetuses were collected 2 h after the last dose. For ovary and uterus, EE and BPA had similar

expression profiles as reflected in microarray data and confirmed by polymerase chain reaction (PCR), but at higher doses (400 mg BPA/kg vs. 10 μg EE/kg) (Naciff et al., 2002). In testis/epididymis, higher doses of all three agents (BPA 400 mg/kg-d, EE 10 μg/kg-d and genistein 100 mg/kg-d) showed common activation of 50 genes (Naciff et al., 2005). These studies indicate that BPA can activate estrogen-mediated gene transcription in the embryo/fetus. At doses greater than 1 mg/kg-d by injection, the BPA effects were weaker than those of the comparison estrogen. The injection route avoids first pass glucuronidation, providing a better comparison between the parent compounds.

Links between altered gene expression in fetal reproductive organs and abnormal development of these organs have not yet been made. However, BPA effects on fetal chromosomes have been shown to carry over to the pubertal ovary. In a study of fetal ovary (Susiarjo et al., 2007), chromosome abnormalities were detected in oocytes of GD 18 mouse fetuses who had been exposed to 20 μg/kg-d BPA from 11.5 days post conception (dpc). Specifically, oocytes entering meiosis had abnormal chromosome alignment and a greater frequency of recombination. When oocytes were examined postnatally at 4–5 weeks of age, higher rates of aneuploidy were found in prenatally BPA exposed mice (21.4%) as compared to controls (1.8%). A comparison estrogen was not used in this study, but the authors suggest an anti-estrogenic mechanism based on the finding of similar oocyte abnormalities in estrogen receptor beta knock out (ERβ (-/-)) mice. ERα (-/-) mice did not show these chromosome abnormalities in oocytes.

Other studies have looked at the gene expression and pathology in adult reproductive organs after prenatal BPA. These are discussed under Female Reproduction (Section C) and Male Reproduction (Section D).

Table B3. Effects of BPA on gene expression in the embryo/fetus.

Reference	Species Route	Dose - time of exposure	Endpoints - time of assessment	Findings
Nishizawa et al. 2003 NTP-A EU 2008	ICR mice oral	2 μg/kg-d GD 6.5–17.5	RARα RXRα mRNA Cerebrum, cerebellum, Gonads, Liver 12.5, 14.5 18.5 dpc	General decrease in RAR and RXR expression, age and sex dependent. ↑ RARα cerebella, 12.5 dpc No comparison estrogen
Nishizawa et al. 2005a NTP-A	ICR mice oral	0, 0.02, 2, 200, 20,000 μg/kg-d GD 6.5–13.5 GD 6.5–17.5	AhR, RAR,RXR mRNA 14.5, 18.5 dpc	↑AhR, RAR, RXR mRNA, brain and gonads, depending on dose, sex and age No comparison estrogen
Nishizawa et al. 2005b NTP-A	ICR mice oral	0, 0.02, 2, 200, 20,000 μg/kg-d GD 6.5–13.5 GD 6.5–17.5	AhR ARNT AhRR GST Cyp1A1	General increase in expression, dose and age dependent; Comparison estrogen E_2

Imanishi et al. 2003 NTP-A EU 2008	ICR mice "oral "	2µg/kg-d GD 6.5–17.5	Placenta; microarray of 20 nuclear receptor mRNA dpc 18.5	Sex dependent pattern of activation/repression No comparison estrogen
Lemmen et al. 2004 NTP-A	transgenic mice; C57BI/6J x CBA F_1 injection (i.p.)	13.5 days post conception (dpc) 10–10,000 µg/kg	Luciferase with ERE 8, 24 h after treatment	ERE activation at 1,000 and 10,000 µg/kg-d in embryos 8 h after dosing; Comparison estrogens E_2 propionate, DES
Susiarjo et al. 2007 NTP-A EU 2008	pregnant C57BL/6 mice implanted time-release BPA pellets ERα KO mice	GD 11.5 (for one week) 20 µg/kg-day (pellets released 400 ng BPA daily)	Chromosome analysis of oocytes of GD 18 fetuses	Chromosome abnormalities in oocytes during meiosis 20 µg/kg-d No comparison estrogen
Naciff et al. 2002 NTP-A EU 2008	SD rats injection (s.c.)	GD 11–20 0, 5, 50, or 400 mg/kg-day (1 ml/kg bw of dose solution, controls received DMSO)	Gene activation in ovaries and uterus of GD 20 fetuses	Gene activation pattern similar to EE 400 mg/kg-d Comparison estrogens genistein and EE
Naciff et al. 2005 NTP-B EU 2008	SD rats injection (s.c.)	GD 11–20 0, 5, 50, or 400 mg/kg-day (1 ml/kg bw of dose solution, controls received DMSO)	Gene activation in testes and epididymis of GD 20 fetuses	50 genes showed common activation for all three agents Comparison estrogens genistein and EE
Smith and Taylor 2007	CD-1 mice Injection (i.p.) n≥group	0.5, 1.0,5.0, 50, 200 mg/kg-d GD 9–16	Uterine stromal cell HOXA10 expression 2–6 wk postnatal	↑ HOXA10
Moriyama et al. 2002 NTP-B EU 2008	*in vitro* studies	100 µM–1 nM	TR binding and activation	↓ T_3 binding to TR ↓ TR activation of gene transcription
Yaoi et al. 2008	ICR/Jc mice Injection (s.c.)	20 µg/kg-d GD 0–12.5 or 14.5	Mouse embryo forebrain methylation assay with follow-up cloning	↑ and ↓ in methylation target sites in CGI and promoter regions of genes relevant to brain development No comparison estrogen
Dolinoy et al. 2007 NTP-B	Mice a/a (Avy/a embryos)	50 mg/kg diet 2 weeks premating, pregnancy, lactation	Offspring coat color, methylation at relevant sites of the Agouti gene promoter	Shift in distribution of offspring coat color; 30% hypomethylation; reversible by feeding methyl donors

Embryonic, fetal and placental gene expression after BPA exposure were studied in a series of papers that focused on retinoic acid receptors (RAR, RXR) and aryl hydrocarbon receptors (AhR). Retinoic acid (RA) is a prominent regulator of development during organogenesis. Exogenous RA is known to cause malformation in both humans and experimental animals. RA acts by binding to retinoic acid receptors RAR and RXR which dimerize to form transcription factors. Metabolism of retinol (vitamin A) to RA involves enzymes regulated by the AhR receptor. AhR is also involved in degradation of the estrogen receptor ERα. In cell culture studies, BPA has been shown to both increase and decrease AhR gene transactivation depending on dose (Bonefeld-Jorgensen et al., 2007).

In a series of papers, Nishizawa et al. (Imanishi et al., 2003; Nishizawa et al., 2003; Nishizawa et al., 2004; Nishizawa et al., 2005) advance the hypothesis that BPA can activate AhR expression in embryos, thus subsequently altering RA action during embryogenesis. BPA was administered orally during organogenesis to mice and embryos were collected for analysis of mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR). Specifically the brain (cerebrum and cerebellum) and gonads (ovaries, testes) were assessed on GD 12.5, 14.5 and 18.5 embryos 24 h after discontinuation of treatment begun on GD 6.5. Across the dose range examined (0.02–20,000 μg/kg-d), BPA generally increased AhR expression in cerebrum, cerebellum and gonads of the embryos, an effect similar to the positive control E_2 (5 μg/kg-d). In further support of AhR activation, expression of two other genes in this pathway was also increased. CYP1A1 and GST (two enzymes regulated by AhR) mRNA and protein expression were also increased at the higher doses of BPA and with E_2. Curiously, the 2 μg/kg dose of BPA did not increase AhR and related protein expression, although both lower (0.02 μg/kg-d) and higher (200 and 20,000 μg/kg-d) doses were effective.

In a separate paper with no comparison estrogen, BPA was generally found to increase RAR and RXR expression over the dose range. A third paper reported that BPA generally decreased RAR and RXR expression in brain and gonads at the 2 μg/kg-d dose (Nishizawa et al., 2003). Using this same dose, nuclear hormone receptors in placentas were also examined at GD 18.5. The hypothesis centered on possible changes in nuclear receptors using a microarray panel of 20 nuclear receptors and 7 non-nuclear receptors. A sex-dependent pattern of up and down regulation was seen for several receptors, including progesterone receptor, estrogen receptor beta, steroidogenic factor 1, and alpha-fetoprotein mRNA. This work demonstrates that important developmental pathways in the embryo and placenta are impacted by BPA after oral administration to the dam at doses less than 1 mg/kg-d. The exact series of events involved and the consequences for embryonic development have not been explored.

Important gene expression changes were also indicated in a study by Smith and Taylor (Smith and Taylor, 2007). BPA injected i.p. in mice during organogenesis led to higher expression of the Hoxa10 protein in the uterus of immature (2 week old) and mature (6 week old) female offspring. This effect persisted in a small group of mice that were ovariectomized prior to examination of Hoxa10, indicating that the enhanced gene expression was not secondary to higher estrogen stimulation of the uterus. Hoxa10 is a gene that is critical for reproductive tract maturation. E_2, DES, MXC and a number of other xenoestrogens have been shown to alter Hoxa10 regulation during development.

Three papers have looked at changes in embryo/fetal DNA methylation due to BPA exposure. DNA methylation status is an important regulator of gene expression. For example, changes in DNA methylation of the Agouti gene during embryogenesis produces different coat colors in adults. Dolinoy et al. (Dolinoy et al., 2007) showed that BPA treatment (50 mg/kg diet) resulted in a larger percent of offspring with yellow coat color. In another study, mouse embryonic forebrain was examined for methylation after maternal BPA exposure (Yaoi et al., 2008). Age specific (GD 12.5, 14.5) changes in methylation status were identified. Follow-up cloning showed that DNA with altered methylation was located in CGI in gene promoter regions, a common site for alteration of gene expression. A change in gene expression was confirmed for two of these genes, Vps52 and LOC7235, two proteins involved in membrane protein transport. This same BPA treatment was shown to alter forebrain morphology in other papers from this group (Nakamura et al., 2006; Nakamura et al., 2007b). Studies of DNA methylation in reproductive organs are discussed under Male Reproductive Toxicity (Section D).

B.2.2. Postnatal Endpoints

B.2.2.1. Postnatal growth as indexed by body weight

Low level (< 4 mg/kg-d) prenatal BPA exposures have been associated with greater postnatal weights in immature offspring while higher BPA exposures (>20 mg/kg-d) can produce growth retardation (Table B4). Greater postnatal weight gain at low doses may be relevant to underlying metabolic disorders.

As mentioned previously, when blastocysts exposed *in vitro* to BPA were transferred to the uterus of pseudopregnant mice, pregnancy and birth parameters did not differ from controls, but offspring were heavier at weaning (Takai et al., 2001). The weight differential was 39% for 1 nM exposure and 34% for the 100 μM exposure. This finding can be taken to suggest that the effects of developmental BPA on postnatal weight are not necessarily mediated by BPA effects on the maternal system, since the blastocysts were exposed *ex utero*.

This finding of significantly greater weaning weights after BPA exposure of preimplantation embryos is similar to a finding by Howdeshall et al. (Howdeshell et al., 1999; Howdeshell and vom Saal, 2000) after exposure of postimplantation embryos (GD 11–17) to low doses (2.4 μg/kg-d) of BPA *in utero*. In this experiment the weight differential was about 10% but varied depending on uterine location (proximity to female fetus). Previous research has shown that estrogen/testosterone levels in fetuses depend to an extent on whether they are positioned next to male or female fetuses in the uterus.

Two other studies examined postnatal body weight after longer periods of developmental exposure to low dose BPA via drinking water (Table B4). Rubin et al. (Rubin et al., 2001) exposed rats from GD 8 to PND 16 and reported higher weights in the BPA-exposed group beginning at birth. The higher weights were more persistent in female as compared to male offspring extending though PND 110, and were similar or less in the high than the low dose group. Another study (Miyawaki et al., 2007) exposed mice from GD 10 to PND 30 and found greater body weights in the BPA-treated juvenile mice compared to controls. This effect was sex-dependent in that females were more affected at lower doses. Body weights were positively

correlated with adipose tissue weight in both sexes, suggesting that greater weights were secondary to greater fat depots. Adipose tissue weight was in turn correlated positively with serum leptin, serum lipids and glucose in females but not males. Statistical conclusions of these studies (Rubin et al., 2001; Miyawaki et al., 2007) are limited by the lack of litter-based statistics, the small number of dams and the large litter sizes. However, the general pattern of results is consistent with the findings of Takai and Howdeshall using prenatal exposure and litter-based statistics. There is a recent study with early neonatal BPA exposure (PND 0–3) in rats which found greater weights in treated than control males on PND 68 (Patisaul and Bateman, 2008). That study used a dose of 50 μg/kg-d by s.c. injection.

Table B4. Studies reporting changes in offspring weight after in utero bisphenol A with or without postnatal exposure.

Reference	species	route	time	dose	findings
Low dose studies					
Takai et al 2001 NTP-A EU 2003	B6C3F1 mouse embryos ICR mouse dams	Embryo culture	GD 1–3	1 nM 100 µM	↑ body weight PND 21, both doses
Howdeshell et al. 1999 Howdeshell and vom Saal 2000 NTP-A	CF-1 mouse	Oral (dam)	GD 11–17	2.4 µg/kg-d	↑ body weight PND 21 in females positioned next to one or two other females in utero
Rubin et al. 2001 NTP-A EU 2003	SD rats	Drinking water	GD 6 through lactation	0.1, 1.2 mg/kg-d	↑ body weight PND 4, 7, 11 males and females, both doses; ↑ body weight PND 28–110 females low dose
Miyawaki et al. 2007 NTP-B	ICR mice	Drinking water	GD 10–PND 30	0.3, 3 mg/kg-d	↑ body weight PND 31, females both doses, males high dose
Higher dose studies					
Negishi 2003 NTP-A EU 2008	F344 rats	Oral	GD 10–PND 20	0, 4, 40, 400 mg/kg-d	↓ body weight 40 , 400 mg/kg-d, PND 7, 21, 28, (PND 56 males only 400 mg/kg-d)
Takagi et al. 2004 NTP-A EU 2008	SD rats	Diet	GD 15–PND 10	0, 60, 600, 3000 ppm 0, 5, 49, 232 mg/kg-d	↓ body weight gain neonates, PND 2–10, 232 mg/kg-d
Hardin 1981 EU 2003	SD rats	i.p.	GD 1–15	0, 85, 125 mg/kg-d	↓ fetal body weight, 125 mg/kg-d
Matsumoto et al. 2004 NTP-A	ddY mice	Diet	GD 14–PND 7	0, 10,000 ppm 0, 1,000 mg/kg-d	↓ body weight PND 1–7
Morrissey 1987 NTP-A EU 2003	CD-1 mice	p.o.	GD 6–15	0, 500, 1250 mg/kg-d	↓ fetal body weight, 1250 mg/kg-d

Consideration should be given to possible contradictory findings in other studies with BPA exposure. Although a large number of studies have administered BPA during pregnancy to rats and mice, some used higher doses (>10 mg/kg-d) and many others did not report on weight growth. Table B4 includes studies with doses >10 mg/kg-d which reported lower postnatal body weight in BPA exposed groups. Some studies used only prenatal exposure while some used combined prenatal/postnatal exposures.

B.2.2.2. Immune system

Immunotoxicity is a recently established research area of BPA developmental toxicity. Two studies with prenatal exposure and two studies with combined prenatal and postnatal exposure are available for review (Table B5). All studies used mice and evaluated immune function after weaning or in adults. The studies used oral dosing and the lowest effective doses were in the <50µg/kg-d range.

Hypotheses concerning BPA developmental immunotoxicity were based on:

- The known immune-modulating effects of estrogen.
- The demonstration that BPA could influence immune response endpoints in adult rodents (Richter et al., 2007; Willhite et al., 2008).

Mice exposed to BPA either as adults or *in utero* demonstrate exaggerated immune response. Mice exposed during early embryogenesis to BPA in drinking water had an enhanced cytokine and inflammatory response to antigen after infection with the protozoan Leishmania major (Yan et al., 2008). The number of specialized T-cells that regulate immune responses ($CD4^{+}CD25^{+}$ T-cells) was reduced, a finding that is potentially relevant to hypersensitivity disorders like allergy and asthma. A similar hypersensitivity after immunization with protein antigen has been shown after prenatal BPA exposure by another laboratory (Yoshino et al., 2004). This study also presented data indicating no effect of the BPA exposure on litter size, sex ratio or adult body weight. A third study using extended developmental exposure (prenatal and postnatal) similarly found reduced numbers of the specialized regulatory T-cells and increased antibody production in mice challenged as adults (Ohshima et al., 2007). This study measured serum BPA in dams and offspring at the end of the exposure period and reported that they were approximately 10 times the concentrations seen in humans.

Linking these immune system findings to possible epigenetic mechanisms, proteomic evaluation of spleen and thymus from 3- and 7- week old mice exposed to BPA during development showed significant dose-dependent up or down regulation of 7 proteins, one of which (apo-A1) also showed both increased protein and mRNA expression on follow-up (Yang et al., 2008). Apo-A1 is involved in tissue cholesterol efflux and may have a role in regulating cytokine production. However, the proteomics study was carried out in a higher dose range than the immune response studies.

Table B5. BPA developmental immunotoxicity studies.

Reference	Species, sex	Route	Exposure Period	Dose LOAEL	Findings
Prenatal					
Yoshino et al. 2004 NTP-A	DBA mice Male and female	Oral, oral instillation	17 days, beginning 1 day before mating	0, 3, 30, 300, 3000 µg/kg-d LOAEL 30 µg/kg-d	↑ antibody production; ↑ splenic cytokine response;
Yan et al. 2008	BALB/C, C57Bl/6J mice male	Oral, drinking water	3 weeks beginning 2 weeks premating	0, 1, 10, 100 nM 0, 0.03, 0.3, 3 µg/kg-d LOAEL 0.3 µg/kg-d	↑ foot pad swelling; ↑ splenic cytokine response; ↓ $CD4^+CD25^+$ splenic lymphocytes.
Prenatal and postnatal					
Ohshima et al. 2007	Mice, WT and Tg, BalbC background, male	Oral, diet	Gestation and lactation	0, 0.1, 1.0 ppm 0, 10, 100 µg/kg-d* LOAEL 10 µg/kg-d	↑ cell proliferation; ↑ antibody production; ↓ $CD4^+CD25^+$ splenic lymphocytes
Yang et al. 2008	ICR mice	Oral, drinking water	GD 7–PND 21	0, 9, 171 mg/kg-d No statistics reported	↑ ApoA1, DPP111 and VAT1 protein; ↑ ApoA1 mRNA in liver, spleen and thymus

WT=wildtype, Tg=transgenic
*estimated by OEHHA

B.2.2.2. Sex differentiation of genital morphology

BPA effects on maturation of the reproductive tract are discussed separately for females and males under sections C and D. Some of the studies of sex differentiation of genital morphology (Table B6a) used exclusively prenatal exposure and are also reviewed here including data for both sexes.

Anogenital distance (AGD) has most often been studied as a marker of secondary sex differentiation after prenatal bisphenol A exposure of laboratory rodents. AGD is defined as the distance between the anus and genital papilla (penis or clitoris). AGD is greater in males than females beginning in the fetal period due to the action of endogenous male reproductive hormones, specifically dihydrotestosterone (DHT). However, exogenous estrogenic agents can also affect AGD. Estrogen (E_2, 2 mg/kg, s.c. injection) during organogenesis (GD 11–14) produced a dramatic of decrease of AGD in male mouse fetuses at term (Gupta and Goldman, 1986). E_2 induces androgen receptor expression in the genital tubercle, which is typically higher in females than males in this area (Agras et al., 2006).

AGD measurement can be operationalized somewhat differently depending on how the animal is restrained for the measurement, the measuring tool, etc. (Vandenbergh and Huggett, 1995). AGD can also be measured internally at necropsy. Newborn AGD is around 5 mm in rats and 1 mm in mice, making accuracy an important issue.

Table B6a. Effects on anogenital distance (AGD) after in utero exposure to BPA.

Reference	Species Time of exposure Route of exposure Doses Group size	Age at assessment Measure Sexes studied Comparison estrogen	Findings
Rat studies			
Talsness et al. 2000 NTP-A	SD rats GD 6–21 oral, gavage 0, 0.1, 50 mg/kg-d N=18, 20	PND 3, 15, 21 AGD, AGD/BW$^{1/3}$ Male and female EE	Shorter, males, 50 mg/kg-d, PND 15, 21; adjusted and unadjusted 0.1 mg/kg-d shorter PND 15, 21. unadjusted; EE longer PND 15, 21
Tinwell et al. 2002 NTP-A EU 2008	SD, Wistar rats GD 6–21 oral, gavage 0, 0.02, 0.1, 50 mg/kg N=7	24 h after birth AGD Male and female EE	No effect; not adjusted; no effect of EE
Tyl et al. 2002b NTP-A EU 2008	SD rats, 3-generation Premating-birth oral, diet 0, 0.001, 0.02, 0.3,5, 50, 500 mg/kg-d 1, 20,300µg/kg-d 5, 50, 500 mg/kg-d N=26–30	PND 0 AGD Male and female No comparison estrogen	Longer in F_2 females; 4 doses; Not adjusted No effect males
Mouse studies			
Gupta 2000 NTP-A NTP-B EU 2003	CD-1 mice GD 16–18 "fed", probably o.i. 0, 50 µg/kg-d N=15/group	PND 3, 21 AGD/BW Male DES	Longer in males DES, longer at low dose, shorter at high dose
Honma et al. 2002 NTP-A EU 2008	ICR mice GD 11–17 injection, s.c. 2, 20 µg/kg-d N=10	PND 22, 60 AGD/BW: Male and female DES	Longer in females PND 22; longer in males PND 60; similar trends DES
Tyl et al. 2008b NTP-A EU 2008 (cited as Tyl et al. 2007)	CD-1 mice, 3-gen Premating-birth oral, diet 0, 0.003, 0.03,.3.5 50, 600 mg/kg-day N=55 control, 19–25 BPA	PND 0 AGD, AGD adjusted Male and female E_2	Longer F_2 females 0.003 mg/kg-d dose, unadjusted and adjusted by covariance No E_2 effect

o.i. = oral instillation

Anogenital distance is highly correlated with body size within sexes. Thus, a corrected version of AGD is usually reported as a ratio (AGD/bw, AGD/$bw^{1/3}$), or body weight is used as a covariate.

Four studies reported a *longer* AGD (masculinized) in offspring after prenatal BPA exposure. (Although the multigeneration studies included prenatal and postnatal exposure, changes in the AGD on PND 0 could only be attributed to prenatal exposure). One study reported *shorter* AGD in male rats after prenatal BPA (Talsness et al., 2000b), while a second study (Tinwell et al., 2002) using similar doses and time of exposure, but with a smaller sample size, found no effect. The Talsness et al. study used a 50 mg/kg-d dose, higher than those in the Gupta and Honma et al. studies. Further, the administration was by gavage, potentially producing a higher peak exposure than in the multigeneration studies. In the Gupta study, the comparison estrogen DES produced *longer* AGDs at lower doses and *shorter* AGDs at higher doses.

Additional measures of morphological sex maturation (preputial separation, PPS and vaginal opening, VO) were measured in some of the studies with exclusively prenatal exposure (Table B6b). The Honma et al. and Tinwell et al. studies used litter-based statistics, but the Talsness et al. study was not obviously litter-based.

Table B6b. Other findings on morphological sex maturation in studies with prenatal BPA.

Reference	Findings
Talsness et al. 2000 NTP-A	AGD shorter PPS later 0.1 mg/kg-d Testes descent later 0.1 mg/kg
Honma et al. 2002 NTP-A EU 2008	AGD longer VO earlier 20 mg/kg
Tinwell et al. 2002 NTP-A EU 2008	AGD no BPA effects VO Wistar rats later 50 mg/kg-d); no effect in SD rats PPS no BPA effect; Testes descent not studied; VO earlier EE

B.2.2.3. Brain and behavior

B.2.2.3.1. Sex-differentiated brain and behavioral endpoints

Exposures during the late fetal and early postnatal period are known to be most relevant for disruption of sexual differentiation of the brain in rodents. Thus, few BPA animal studies in this area use the exclusively prenatal exposures. Division of exposure into prenatal and postnatal periods is meaningful in terms of maternal mediation of effects that might occur, e.g. by maternal physiology in the gestational period, and maternal behavior and nutritional support via lactation

in the postnatal period. However, it is less relevant to brain development. Brain development that takes place prenatally in humans largely occurs postnatally in rats and mice, which are precocial (immature) at birth. Sexual differentiation of the brain occurs primarily in the second/third trimester in humans and nonhuman primates, but primarily in the neonatal period in rats and mice. In this section, studies with prenatal exposure are integrated along with closely related studies with pre and postnatal exposure since all such studies may be relevant to potential risks for adverse effects on brain development in humans from *in utero* exposure.

Sex differentiation of rodent brain morphology focuses on specific structures. Some sex-differentiated structures of rodent brain studied in connection with BPA are:

- AVPV, anterior periventricular nucleus of the hypothalamus, regulates gonadotropin releasing hormone (GNRH) which in turn controls gonadal function. Th (tyrosine hydroxylase), is a marker for dopaminergic neurons, and Th-ERα neurons are seen only before puberty and are thought to regulate GNRH at this time. Size: ♀>♂
- BST, bed nucleus of the stria terminalis, is implicated in gender identity in humans and in stress response in rodents, associated with neurons expressing corticotrophin releasing hormone. The stria terminalis connects the amygdala with hypothalamic and thalamic areas. Size: ♂>♀
- Locus coeruleus is a structure in the brainstem that is the origin of noradrenergic neurons of the brain and is a target of human pharmacotherapy agents such as Modafanil and Vestra for anxiety and panic disorders. Size: ♀>♂
- SDN-POA, sexually dimorphic nucleus of the preoptic area of the hypothalamus, regulates male reproductive behaviors. Calbindin influences apoptosis of cells in this area in males, which produces the sexual differentiation in size. Regulates steroid hormones and sexual behavior. Size: ♂>♀

Sex differentiation of rodent behavior focuses on non-reproductive behaviors as well as reproductive behaviors. In the most strictly defined framework, behavior is sex-differentiated if it is "fundamentally and permanently" different between sexes and is known to be determined developmentally by gonadal hormones (McCarthy and Konkle, 2005).

Some sex-differentiated behaviors in rodents are:

- Mating behavior.
- Parental behavior.
- Social interaction (including aggression and play).
- Affective behavior (anxiety).
- Spatial learning and memory.

There are two basic approaches to identifying an effect on sex differentiation of brain or behavior:

- Sex differences occur in controls, but not in treated groups.
- Treatment effects occur in one sex but not the other.

In the latter approach, a sex-differentiated behavior is usually studied. Some studies reviewed below used the former approach (compare sexes within treatment groups; sections B.2.2.3.1.4. and B.2.2.3.1.3.), and some used the latter approach (compare treatment groups within sexes; section B.2.2.3.1.2.), making findings difficult to compare on an agree/disagree basis.

In other scenarios, BPA effects on a behavioral measure may be seen in both sexes after prenatal exposure (Section B.2.2.3.2.). These scenarios are relevant to developmental neurobehavioral toxicity although they do not support hypotheses concerning BPA effects on sex differentiation of the brain and behavior.

B.2.2.3.1.1. Studies with 15-1500 µg/kg-d BPA exposures in Wistar rats

BPA doses of 1.5 mg/kg-d during prenatal/postnatal development did not affect sexual differentiation of Wistar rats as reflected in estrus cycles, sperm count, AGD, vaginal opening, serum hormones, the volume of the brain area associated with reproductive tract differentiation (SDN-POA), or mating behavior (Kubo et al., 2001; Kubo et al., 2003) (Table B7). However, the volume of the locus coeruleus, another sex-differentiated brain area associated with sex differences in affective behavior (anxiety, depression), was larger in control females than males, but smaller in BPA-treated females than males. This finding was consistent with the absence of sex difference in the BPA-treated offspring for measures taken in a novel environment and in response to shock. Female rodents typically show less emotional response in these tests. Because sexual differentiation of the locus coeruleus, which occurs prenatally in the rat, appeared to be the endpoint most sensitive to BPA, a third study was undertaken looking specifically at prenatal exposure and at an expanded set of behavioral tests that depend on this brain area (Fujimoto et al., 2006).

The volume and neuron number of the locus coeruleus is sexually dimorphic in rodents, being larger in females than males. This sexual dimorphism occurs under the influence of prenatal hormones, and androgen receptor is an important mediator (Guillamon et al., 1988; Garcia-Falgueras et al., 2005). In contrast, the sexual differentiation of the SDN-POA occurs later, on GD 18 through PND 5 (Rhees et al., 1990a, b). The locus coeruleus is a structure in the brainstem that is the origin of noradrenergic neurons of the brain and is a target of human pharmacotherapy by anti-noradrenergic drugs for depression, anxiety and panic disorders. Behavioral assessments used in rats to assess locus coeruleus function include activity in a novel open field and avoidance of shock. Female rats typically show less anxiety and depression in these tests. At higher doses with prenatal and postnatal BPA exposure (Kubo et al., 2001; Kubo et al., 2003), exploratory behavior in an open field and passive avoidance of shock failed to show anticipated sex differentiation. With the lower dose and exclusively prenatal BPA exposure (Funabishi et al. 2006), sex differentiation did not occur on rearing, one of the open field measures which was sex-differentiated in controls. Passive avoidance behavior was sex differentiated after the low dose BPA treatment. Two additional tests in the low dose prenatal experiment were persistence of swimming in inescapable water, time spent in open vs. enclosed areas of an elevated plus maze. Significant sex differentiation of elevated plus maze was not demonstrated in either control or BPA groups, while sex-differentiation of struggling behavior in the forced swim test seen in controls (more struggling in females) was eliminated in the BPA

group. Immobility in the forced swim test was increased by BPA, and this effect was also significant in males only, although immobility was not sex-differentiated in controls.

Table B7. Studies of sexual differentiation of the brain and behavior in Wistar rats exposed via drinking water to low doses of BPA (≤1.5 mg/kg-d).

Study	Exposure time/ dose	endpoint	BPA effect
Kubo et al. 2001 NTP-A EU 2008	GD 0–PND 21 0, 1.5 mg/kg-d	Reproductive organ weights Hormones (LH, FSH, E_2/T)	No effect No effect
		Brain: SDN-POA, Volume	Cont: sex diff BPA: sex diff
		Brain: Locus Coeruleus Volume, cell #	Cont: sex diff BPA: no sex diff
		Behavior: Exploratory	Cont: sex diff BPA: no sex diff
		Behavior: Passive avoidance	Cont: sex diff BPA: no sex diff
Kubo et al. 2003 NTP-A EU 2008	GD 0–PND 21 0, 30, 300 µg/kg-d Comparison estrogens: Resveratrol, DES	Reproductive organ weights Hormones (LH, FSH, E_2/T) Sperm parameters Estrus cycles Anogenital distance Testes descent Vaginal opening Behavior: male mating	No effect No effect No effect No effect No effect No effect No effect No effect
		Brain: SDN-POA, volume	Cont: sex diff BPA: sex diff
		Brain: Locus Coeruleus, volume, cell#	Cont: sex diff BPA: no sex diff 30, 300 µg/kg-d
		Behavior: Exploratory	Cont: sex diff BPA: no sex diff 30, 300 µg/kg-d
Fujimoto et al. 2006 NTP-A EU 2008	GD 13–PND 0 0, 15 µg/kg-d	Behavior: Exploratory	Cont: sex diff BPA: no sex diff
		Behavior: Passive avoidance	Cont: sex diff BPA: sex diff
		Behavior: Forced Swim	Cont: sex diff BPA: no sex diff
		Behavior: Elevated Plus maze	Cont: no sex diff BPA: no sex diff

A number of other studies have studied sexual differentiation of the brain using either postnatal or combined prenatal and postnatal BPA administration (Table B8). Some of these studies compared sexes within treatment groups and some compared treatment groups within sexes. They suggest that size of brain areas that are typically sex differentiated in size in rodents (SDN-POA, AVPV) is not influenced by BPA, but sex differentiation in terms of number of phenotypically sex-differentiated neurons can be affected. An exception is the volume of the

locus coeruleus, which has been shown to be affected by developmental BPA exposure as discussed above.

Table B8. BPA effects on sex differentiation of the rat brain. None of these studies used exclusively prenatal exposure.

Study **Exposure period (dam/pup)** **Dose** **Comparison estrogen**	**Structures/measures** **Age at assessment**	**Findings**
Funabashi et al. 2004 Wistar rats Pregnancy/lactation (dam) 2.5 mg/kg bw/day NTP-B EU 2008	SDN-POA: CRH immunoreactive neurons	Control: sex diff BPA: sex diff
	BST: CRH immunoreactive neurons Assessed at 4-7 months of age	Control: sex diff BPA: no sex diff
Kubo et al. 2003 Wistar rats GD 0–PND 21 30, 300 μg/kg-d (dam) DES comparison NTP-A EU 2008	SDN-POA, volume, cell #	Control: sex diff BPA: sex diff
	Locus coeruleus, volume, cell # Assessed at 14 weeks of age	Control: sex diff BPA: no sex diff
Kwon et al. 2000 SD rats GD 11–PND 10; 320 mg/kg-d, (dam) DES comparison NTP-A EU 2008	SDN-POA volume, Assessed at 10 days of age	Females: no BPA effect
Nagao et al. 1999 SD rats PND 1–5 (pup) 300 μg/g sc= 3 mg/kg bw NTP-A	SDN-POA volume, Assessed at 14 weeks of age , males only	Males: no BPA effect
Takagi et al. 2004 SD rats GD 15–PND 10 (dam) 0, 60, 600, 3000 ppm, 0, 6, 65, 308 mg/kg-d* NTP-A EU 2008	SDN-POA volume, adult males and females Assessed at 21 days of age and 11 weeks of age	Males: no BPA effect Females: no BPA effect

Table B8. BPA effects on sex differentiation of the rat brain. None of these studies used exclusively prenatal exposure (continued).

Study **Exposure period (dam/pup)** **Dose** **Comparison estrogen**	**Structures/measures** **Age at assessment**	**Findings**
Patisaul et al. 2006 SD rats PND 1, 2 (pup) 250 µg/d, 4 injections =100 mg/kg-d Genistein comparison E_2 comparison NTP-A EU 2008	AVPV. Volume	Males: no BPA effect
	AVPV: Th-immunoreactive cells	Males: BPA ↑ (feminized) Females: no effect
	AVPV:Th-immunoreactive/ERαcells Assessed at 19 days of age	Males: no effect Females BPA ↓ (masculinized)
Patisaul et al. 2007 SD rats Postnatal, PND 1, 2 (pup) 250 µg/d, 4 injections =100 mg/kg-d Genistein comparison NTP-B EU 2008	AVPV: volume	Males, no BPA effect
	SDN-POA, volume,	Males, no BPA effect
	SDN-POA, calbindin-ir cells Assessed at 85 days of age, males only	Males, ↑ (masculinized) ?
Facciolo et al. 2002, 2005 SD rats 8 days before mating through lactation (dam) 0, 40, 400 µg/kg-d NTP-A EU 2008	Somatostatin receptor sst3 mRNA in limbic and hypothalamic regions of the brain Assessed at 10, 23 or 55 days of age, females only	Females: Up or down regulation of sst3 mRNA in different brain regions

B.2.2.3.1.2. Studies with 40 µg/kg-d BPA exposure in SD rats

This series of studies includes six papers using perinatal (pre and postnatal combined) exposures (Farabollini et al., 1999; Dessi-Fulgheri et al., 2002; Adriani et al., 2003; Della Seta et al., 2005; Porrini et al., 2005), two papers which used cross fostering to identify exclusively prenatal effects (Aloisi et al., 2002; Farabollini et al., 2002) and one paper that used only postnatal exposure (Della Seta et al., 2006) (Table B9). In this model, BPA was administered by micropipette into the mouth (oral instillation, o.i.) to Sprague Dawley rats. The initial studies used a short term higher dose, as well as a long term lower dose of 40 µg/kg-d. Some studies included a comparison estrogen (Della Seta et al., 2006). Two other papers were directed at examination of estrogen-related brain mechanisms (Facciolo et al., 2002; Facciolo et al., 2005).

The research focused on BPA effects on social and reproductive behaviors, using a variety of potentially sensitive assays at the same dose level. Sex-differentiated nonreproductive behaviors were also studied. In these experiments, sex differentiation was assessed by examining treatment

effects within sexes. Behavioral evaluations occurred at different times in the life cycle including:

- Postnatal day (PND) 0–21 (preweaning) infancy
- PND 21–28 juvenile
- PND 29–60 pubertal/adolescent
- PND 60 + adult

The investigators used a dose level thought to be relevant to human exposures, below the dose of 50 µg/kg-d considered to be without effect in several human risk assessments. They did not provide detailed information on pregnancy outcome, or examine reproductive tract morphology. Two studies from the series (Adriani et al., 2003; Della Seta et al., 2005) provided information on body weight. Using the mating-through-weaning dosing period, no effects were found on pup body weight on PND 2, 7, or 21 or on adult offspring weight (>70 days of age).

In the first paper in the series, social interaction was studied in juvenile/adolescent offspring (Dessi-Fulgheri et al., 2002). The BPA exposure was to the dam during pregnancy and lactation and interactions between treatment and offspring sex were considered. Juvenile social interaction endpoints sensitive to the BPA exposure included play directed to females, immature reproductive behavior, sociosexual exploration and social interest. As regards sex-differentiation of the treatment effect, immature reproductive behavior and social interest were less sex differentiated in controls and BPA had similar effects in both sexes, namely a decrease in the frequency of these behaviors. For sociosexual exploration, the behaviors were more highly sex-differentiated and BPA affected males and females differently, increasing the frequency of behaviors at the low dose in females and decreasing the frequency of behaviors at the high dose in males. Play directed toward females was also highly sex-differentiated. BPA increased these behaviors at the low dose in females and at the high dose in males.

Further information on sociosexual behavior (aggression, mating) in adult offspring was provided in a second paper (Farabollini et al., 2002) which used fostering to identify exclusively prenatal effects. This study used 72 offspring from 20 pregnancies, suggesting 3–4 rats per litter. Data were analyzed within sex. In males, the aggression test demonstrated significantly more defensive and less aggressive behavior during agonistic encounters in the prenatally treated BPA group. The same pattern was seen for the aggression test in females, but comparisons were not statistically significant. No BPA effects were seen in the sexual orientation test (time spent near rat of the opposite or same sex). The latency to intromission (insertion of penis into the vagina during mating) was increased in prenatally exposed males, while the number of intromissions was increased in postnatally exposed males. Additionally, the duration of genital sniffing was increased in prenatally BPA-treated males compared to controls. In females, the latency to approach males and the frequency of lordosis were greater in BPA-treated females tested during the proestrous/estrous stage of the estrous cycle (when mating behaviors usually occurs). Pre- and postnatal BPA groups were combined for the analysis since they did not differ statistically; thus, time of exposure was not a factor in the analysis and exclusively prenatal effects could not be identified for the estrous cycle endpoint.

Although not part of this series of studies, a recent investigation of aggression in male Long Evans rats exposed developmentally to BPA has been conducted (Patisaul and Bateman, 2008). This study used a BPA dose of 50 µg/kg-d similar to the 40 µg/kg-d dose of the Farabollini study. However, a brief postnatal exposure by injection to the pups was used (PND 0–3) and no effect on aggression was found when the males were adults.

Table B9. Studies of BPA effects on sex-differentiation and BPA sex-specific effects on behavior using 40µg/kg-d dose in SD rats.

Study	**Exposure Period Dose**	**Endpoint Age at assessment**	**Findings**
Prenatal or postnatal exposure			
Farabollini et al. 2002 NTP-A EU 2008	Pregnancy or lactation (cross fostered) 40 µg/kg-d	Socio sexual behavior, PND 100	↑ latency to intromission (pre) ↑ number of intromissions (post) ↑ genital sniffing (pre)
Aloisi et al. 2002 NTP-A EU 2008	Pregnancy or lactation (cross fostered) 40 µg/kg-d	Pain response. PNW 22	↑ limb flexion (pre) ↓ paw jerk (post)
Prenatal and postnatal exposure			
Farabollini et al. 1999 NTP-A EU 2008	Pregnancy and lactation 40 µg/kg-d; or GD 14–PND 6 400 µg/kg-d	Exploration, (holeboard) anxiety (elevated plus maze) PND 85	↓ head dipping ↓ activity ↓ anxiety
Dessi-Fulgheri et al. 2002 NTP-A EU 2008	Pregnancy and lactation 40 µg/kg-d; or GD 14–PND 6 400 µg/kg-d	Juvenile social interaction, 8 factors derived from PCA of behavior scoring PND 25, 35, 45	↑ play directed to females ↓ low intensity mating behavior ↓ sociosexual exploration males ↑ sociosexual exploration females ↓ social interest
Della Seta et al. 2005 NTP-A	Pregnancy and lactation; 40 µg/kg-d	Maternal behavior PND 3, 4; PND 8, 9	↓ duration licking grooming pups
Porrini et al. 2005 EU 2008 NTP-A	Pregnancy and lactation; 40 µg/kg-d	Social behavior of female offspring PND 35,45, 55	↑ social/nonsoc explore ↓ play with males ↓ social grooming
Adriani et al. 2005 NTP-A EU 2008	Pregnancy and lactation 40 µg/kg-d	Novelty preference PND 30–45	↓ females
		Activity PND 35–45	↑ males and females
		Impulsive behavior "adult"	↓ males and females
		Amphetamine response "adult"	↓ males
Della Seta et al. 2006 NTP-A EU 2008	PND 23–30 40 µg/kg-d	Male social and sexual behavior PND 45, 90	↓ latency to intromission

PCA= principal components analysis

Further studies of sexual behavior were also conducted with exposures in juveniles prior to puberty (PND 23–30) (Della Seta et al., 2006). In this study, ethinyl estradiol (EE, 0.4 µg/kg-d) was used as a positive control. When tested as adults (> 90 days of age) the direction of mean differences from control were similar for the BPA and EE treated groups, although statistical significance in post hoc tests was usually reached only in the EE group. Latency to intromission was the only endpoint significant for the BPA group in post hoc comparisons. EE/BPA groups had a shorter latency to mount and to intromission (not all rats ejaculated), and a higher frequency of intromissions and intromissions per mount. Duration of genital sniffing, however, was lower in the EE/BPA groups. In separate group, plasma testosterone and estradiol were measured both during the pubertal treatment (PND 37) and in adults (PND 105) (groups of 5 to 8). E_2 was not influenced by treatment but T was lower than control in both the EE and BPA groups at both ages, with the BPA group having the lowest mean, a major finding of the study.

A final reproductive behavior study in this model was maternal behavior. Using the exposure period beginning premating through lactation, maternal behavior was evaluated at PND 3, 4 and PND 8, 9 (Della Seta et al., 2005). The analysis reported generally reduced behavior in terms of duration and frequency of licking-grooming, anogenital licking and arched-back posture; the change in duration of licking-grooming was significant by ANOVA at $P<0.05$. Nest building was not affected. This study is not relevant to developmental toxicity but adds information on BPA effects on sex-differentiated behaviors. The study is also discussed in section C.2.9.

Several papers in this series looked at sex-differentiated non-reproductive behavior. A sex-differentiated behavior, response to pain, was assessed in BPA-treated adult offspring (Aloisi et al., 2002). Prenatal/postnatal dosing and cross fostering were used as in Farabollini et al. (2002). In the test, formalin was injected into the paw resulting in three responses known to be sex-differentiated and influenced by exogenous estrogen (Aloisi et al., 1994; Aloisi et al., 1997; Aloisi and Ceccarelli, 2000; Aloisi, 2003)

- Withdrawal of the paw (limb flexion); increased by estrogen.
- Licking of the paw; increased by estrogen.
- Shaking the paw (paw jerk); decreased by estrogen.

The prenatal BPA-treated groups showed greater pain response on variables that increase in response to exogenous estrogen (licking and flexing of the affected limb). Additionally, postnatal BPA decreased paw jerk response, which is also consistent with an estrogenic effect. Measures of general exploratory behavior were also obtained in this study, most of which were sex-differentiated. The data generally indicated reduced exploration in the prenatally exposed groups, consistent with the earlier study (Farabollini et al., 1999), but the BPA effect was not statistically significant.

In other studies, behaviors reflecting emotionality were considered. Adult offspring exploratory endpoints sensitive to developmental BPA in the first study (Farabollini et al., 1999) included head dipping in the hole board test (↓), and square crossing in the holeboard exploration test (↓) as well as in the plus maze test (↓). There were some sex-specific effects. BPA-exposed males (but not females) had less indication of anxiety (greater percent entries into the open arm of the

maze) compared to controls. In the second study (Adriani et al., 2003), behaviors reflecting serotonergic and dopaminergic systems were targeted: novelty preference (dopamine), impulsivity/reward discounting (serotonin) and response to amphetamine (dopamine). Most of the measures taken during the test were sex-differentiated, and showed sex by treatment interactions. Novelty preference was reduced in BPA-treated females only, and response to amphetamine in males only. The impulsivity measure was not sex-differentiated and showed an effect of BPA with the sexes combined (less impulsivity in the BPA group). Responding during the intertrial interval of the impulsivity task was sex-differentiated and showed a BPA effect (increased responding) in males only.

Although not part of this series of studies, a recent investigation in male Long Evans rats exposed developmentally to BPA also found reduced anxiety in the elevated plus maze (Patisaul and Bateman, 2008). This study used a BPA dose of 50 µg/kg-d, similar to the 40 µg/kg-d dose of the Farabollini study. Here a brief postnatal exposure by injection to the pups was used (PND 0–3).

Changes in brain have also been studied in this model. These studies focused on somatostatin receptors, neuropeptide receptors that develop a sex-differentiated pattern of expression in the brain under the influence of estrogen (Table B9). Somatostatin inhibits the secretion of various hormones that promote growth such as growth hormone, glucagon, insulin, thyrotropin, and gastrin. Somatostatin receptors colocalize on the cell membrane with the membrane estrogen receptor (GP30), as well with a receptor for the inhibitory neurotransmitter GABA (GABAα receptor). Somatostatin receptor genes have an estrogen response element and BPA was shown to induce somatostatin receptor subtype 3 (sst3) expression in hypothalamic areas in a manner that interacted with the effect of specific GABA agonists (Facciolo et al., 2005). In other studies, BPA was found to bind to sst2 in limbic areas of the brain of PND 10 and PND 23 rats (Facciolo et al., 2002). In both studies, dams were given the BPA treatments (40 or 400 µg/k/d) beginning before mating and continuing through lactation. Brain somatostatin receptors have also been shown to be influenced by treatment with atrazine, an estrogenic pesticide (Giusi et al., 2006).

Thus, in this group of studies, prenatal BPA exposure at 40 µg/kg dose in Sprague Dawley rats was reported to affect mating and aggressive behavior in male rat offspring (both decreased relative to controls) and alter pain response in both male and female offspring (increased relative to controls). These effects resulting from prenatal exposure occur in the context of more extensive changes in exploratory, social and reproductive behavior and changes in brain after prenatal/postnatal exposure at the same dose.

B.2.2.3.1.3. Studies with F344 rats

There are three studies of developmental BPA effects in F344 rat models, none of which used exclusively prenatal exposure (Table B10). These studies are valuable in investigating effects of developmental BPA exposure on behavior in a rat strain other than Sprague Dawley. Research has shown that inbred mouse and rat strains differ in their response to exogenous estrogen and estrogenic agents (Spearow et al., 1999; Spearow and Barkley, 2001; Spearow et al., 2001) and that the F344 strain is more sensitive to estrogen than the Sprague Dawley strain in some assays.

One of the studies used the approach of assessing treatment effects within sexes (Negishi et al., 2003). A follow-up study at a lower dose tested only males (Negishi et al., 2004). One study (Carr et al., 2003) examined a well-known sex-differentiated behavior, spatial learning and memory, and assessed sex differences within treatment groups.

Table B10. Studies in Fischer 344 rats with prenatal/postnatal exposure.

Study	Exposure	Endpoint	Effects
Negishi et al., 2003 NTP-AEU-2003	0, 4, 40, 400 mg/kg-d GD 10–PND 20 Gavage N=8–18/group	Spontaneous motor activity Exploratory activity Active avoidance learning	↑ % time immobile in dark phase, ♀40 mg/kg-d, test prior to puberty ↓ % grooming, ♂, 4 mg/kg-d, no sex effect ↓ avoidance , 4 mg/kg-d, 8 weeks old, no sex effect
Negishi et al., 2004 NTP-AEU-2003	100 µg/kg-d; GD 3–PND 20 Gavage N=9, 10/group	Males only tested Spontaneous motor activity Exploratory activity Elevated plus maze Passive avoidance learning Active avoidance learning	 No effect No effect No effect No effect ↓ avoidance , 15 weeks of age
Carr et al., 2003 NTP-AEU-2008	100, 250 µg/kg-d PND 1–14 Gavage N=10/group	Spatial learning	Cont: sex diff BPA100 µg/kg/d: no sex diff E_2: 72 µg/kg/d: no sex diff

In the first study (Negishi et al., 2003), both juvenile (PND 28–34) and adult (PND 56–62) behavior were studied. BPA was reported to affect the behavior of males (but not females) on several endpoints: shock avoidance performance at both ages, grooming behavior in the adults, and time spent immobile during the dark phase of the daily diurnal cycle in juveniles. None of the behaviors affected by BPA were sex-differentiated behaviors in this study (no effect of sex in the ANOVA). In addition to different effects on males and females, low (4 mg/kg-d), intermediate (40 mg/kg-d), and higher (400 mg/kg-d) doses of BPA had some qualitatively different effects. The low dose was most effective in inducing higher duration of grooming in males and lower avoidance response of adult males. The intermediate and high doses affected avoidance response of juvenile males and the intermediate dose affected immobility in females.

Data on general toxicity were provided in this study. BPA influenced maternal body weight during pregnancy. Maternal organ weights at weaning were not affected with the exception of the thymus, which was lighter at the highest dose. Litter size was not affected but postnatal offspring weights were lower in some BPA-treated groups (see discussion under body weight above). The group size was N=8–9 dams with litters culled to 8 at birth and N=8–27 per group

for offspring behavior tests. The figure captions suggest that all offspring were used for the juvenile activity test, then some were selected for the avoidance test and others for the open field test. It was not clear whether litter-based statistics were used.

A follow-up study by this group (Negishi et al., 2004) with a lower dose exposure (100 µg/kg-day, GD 3–PND 20) used avoidance tasks shown to be sensitive at higher doses and a comparison estrogenic agent (nonylphenol). These tests were conducted in adult male offspring. The extended test battery included the elevated plus maze, an anxiety test. This test, as well as tests of exploratory behavior and spontaneous motor activity, were not found to be affected by the low dose BPA. Similarly, passive avoidance was not affected, but the BPA exposure led to poorer performance of the active avoidance task. This was similar to result at the lowest previously tested dose (4 mg/kg-d) but dissimilar to higher doses (40, 400 mg/kg-d) which increased avoidance performance. Also, BPA treated males failed to show the hyperactivity in response to monoamine oxidase inhibitor injection shown by controls. Similar results were reported for nonylphenol. Notably, BPA exposures are not limited to the prenatal period. This study demonstrates effects of BPA in a third rat strain (in addition to Wistar and Sprague Dawley, Tables B7 and B9), different effects of high and low doses, connection to monoamine systems in brain, and effects at doses less than 1 mg/kg-d by the oral route.

The Carr et al. study in F344 rats focused specifically on spatial learning and memory, a cognitive behavior known to be sex-differentiated and influenced by developmental hormone exposures in both rodents and humans. Offspring were tested as adolescents (PND 34–38). Learning of this test was found to be sex-differentiated, with male controls performing significantly better than females on the last day of training. However, this sex difference in performance did not appear in the low dose BPA-treated offspring. At the higher dose (250 µg/kg) the sex difference was clearly seen. This study reported that BPA effects sex-differentiated behavior, and that effects differ depending on dose. E_2, the comparison estrogen, also led to an absence of sex difference in acquisition of the spatial task.

B.2.2.3.1.4. Studies with 10 µg/kg-d BPA in CD-1 mice

Investigators who had previously studied the effects of other estrogenic agents (DES, MXC, and o,p-DDT) on sexual differentiation of brain and behavior in mice (Palanza et al., 1999; Panzica et al., 2007) undertook similar studies with BPA. They used the oral instillation technique of administration and corn oil as a vehicle (Palanza et al., 2008). The 10 µg/kg dose was selected for use because it is the tolerable daily intake established for humans by the EU (European Commission Scientific Committee on Food 2002). The focus of the studies was reproductive behavior (maternal behavior) as well as sex-differentiated exploratory and affective behaviors. The studies are outlined in Table B11.

Two studies used prenatal exposure (GD 11–18, GD 14–18) (Palanza et al., 2002b; Laviola et al., 2005) and one continued the exposure to the early postnatal period (GD 11–PND 8) (Gioiosa et al., 2007). General toxicity data were limited but no effects on dams' gestational weight, litter size, offspring weights at PND 3–15, or pup motor development (righting and cliff aversion) were seen in the first study (Palanza et al., 2002b).

The first study examined maternal behavior (Palanza et al., 2002b). There was no positive control (estrogenic agent) in this study, but a previous study by this group found a similar effect with MXC-treated females (Palanza et al., 2002a). BPA was found to decrease nursing behavior in mice that were treated with BPA either *in utero* or during pregnancy as adults. This is in general agreement with a study of BPA effects on maternal behavior in rats (Della Seta et al., 2005). This study used 2–3 pups per litter per group in a split litter design with a total of 51 control F_1 females and 31 BPA treated F_1 females (each treatment group was further subdivided in two for the adult pregnancy treatment, exact group sizes for the 4 resulting groups were not given). This study is also discussed in Section C.2.9.

The second study looked at amphetamine-conditioned place preference in offspring of treated mice treated during gestation (Laviola et al., 2005). In this paradigm, an amphetamine injection at one of three doses was administered to adult mice in one of two distinct locations. Research has shown that mice will prefer the location where they experience the consequences of the amphetamine injection. Conditioned place difference is a sex-differentiated behavior, with males having a somewhat higher preference for the drug-related location than females. Female offspring of BPA treated dams did not show conditioned place preference, indicating that the rewarding effects of amphetamine were not experienced. A similar lack of preference was seen in the MXC-treated females included as a positive control. BPA did not affect the place preference of males. This study used one male and one female pup per litter per dose, thus providing a litter-based statistical analysis.

The third study looked at sex-differentiated exploratory and affective behavior (Gioiosa et al., 2007). This study extended the exposure period into the first week postnatal and tested the offspring as juveniles and adults. Tests were selected that commonly show sex-differentiation. The majority of the endpoints measured in the three tests (novelty, open field, elevated plus maze) demonstrated sex by treatment interactions, with comparisons in most cases supporting sex difference in the control but not the BPA group. An MXC-treated control group showed a similar pattern of effects.

Table B11. Studies in CD-1 mice with 10 µg/kg BPA administered by oral instillation.

Study	exposure	Endpoint	Findings
Palanza et al. 2002 NTP-A EU 2008	GD 14–18 ; treatment *in utero* and/or when pregnant	Maternal and nonmaternal behavior PND 2–15	↓ nursing behavior ↑ behavior away from nest In utero or when pregnant; in utero and pregnant not affected
Laviola et al. 2005 NTP-A EU 2008	GD 11–18; n=10–12 dams/group; MXC comparison (20 µg/kg-d)	Amphetamine conditioned place preference, PND 60	↓ place preference BPA and MXC females (not males)
Gioiosa et al. 2007 NTP-B	GD 11– PND 8	Exploratory behavior:: Time in open field Time in light area Time in center Time near home cage Elevated plus maze: Time in closed arms, entries into open arms Novelty preference test: Time in novel area Activity in novel area Risk assessment	Control: sex diff BPA: no sex diff Control: sex diff BPA: no sex diff Control: sex diff BPA: no sex diff

The results of this most recent study (Gioiosa et al., 2007) are consistent with previously reviewed studies in which developmental BPA at various doses was found to eliminate sex differences in open field exploratory behavior:

1500 µg/kg-d (Kubo et al., 2001)
300 µg/kg-d (Kubo et al., 2003)
30 µg/kg-d (Kubo et al., 2003)
15 µg/kg-d (Fujimoto et al., 2006)
10 µg/kg-d (Gioiosa et al., 2007)

On the other hand, findings of Gioiosa et al. from the Elevated Plus Maze test are not readily compared to previously reviewed studies with the Elevated Plus Maze because the previous studies did not evaluate sex differences:

- Only one sex was tested (Negishi et al., 2004; Ryan and Vandenbergh, 2006; Patisaul and Bateman, 2008).
- No sex differences were detected in controls (Negishi et al., 2004).
- Sex differences were not evaluated within groups (Farabollini et al., 1999).

However, the study by Farabollini et al. (1999) using the Elevated Plus Maze did find an overall sex difference in behavior in the Elevated Plus Maze along with a significant interaction between

treatment and sex. This analysis generally supports the findings of Gioiosa et al. with the Elevated Plus Maze. There were no other studies using the Novelty Preference test.

The altered response to amphetamine in the Laviola et al. study is also consistent with studies in other models:

- Lack of hyperactivity response to MAO inhibitor in male F344 rats (Negishi et al., 2004).
- Reduced hyperactivity response to amphetamine in male SD rats (Adriani et al., 2003).

B.2.2.3.2. Studies unrelated to sexual differentiation

Because of the known estrogenic action of BPA, most brain/behavior studies have focused on sexual differentiation, but some look more generally at neurobehavioral toxicity. Many of these studies were based on known biological actions of BPA other than its estrogenic action.

B.2.2.3.2.1. Thyroid-related effects

A series of studies from a laboratory in Japan (Table B12) were based on the observation that BPA is a thyroid hormone receptor antagonist (Zoeller et al., 2005; Zoeller, 2007) and can counteract the effects of thyroid hormone (TH) on gene expression (Moriyama et al., 2002).

Table B12. Studies of BPA effects on prenatal mouse brain development.

Study	Species	Dose, route, time	Assays	Findings
Nakamura et al. 2006 NTP-A EU 2008	ICR/J mouse	20 µg/kg s.c. GD 0–14.5 or 16.5	Fetal neocortex GD 14.5, 16.5: Neurogenesis and migration; TH binding protein in neurons; genes regulated by TH and involved in neurogenesis	Accelerated neural differentiation and migration; upregulation of relevant TH regulated genes GD 14.5 including TH-binding protein disulfide isomerase
Nakamura et al. 2007 NTP-B	ICR/J mouse	20 µg/kg s.c. BrdU injection on GD 12.5, 14.5 or 16.5	BrdU labeled cells in offspring cortex 3, 4, 5, 7, 8, 12 weeks of age	Abnormal cortical location of GD 14.5 labeled neurons at 3 weeks of age; abnormal thalamocortical connections at 3 and 12 weeks of age
Yaoi et al. 2008	ICR/J mouse	20 µg/kg s.c. G 0–12.5 or 14.5	DNA methylation in offspring cortex GD 12.5, 14.5	Changes in methylation status and mRNA expression

Bromodeoxyuridine (BrdU) can be used to label neural progenitor cells early in development and to track their migration and differentiation in the brain. Two studies used this technique to study prenatal BPA effects on cortical development (Nakamura et al., 2006; Nakamura et al., 2007a)

(Table B12). An altered pattern of distribution of the labeled neurons in both the fetus and in weanling offspring was seen if pregnant dams were injected with 20 μg/kg BPA. Their hypothesis was that BPA might bind protein disulfide isomerase which acts as a storage protein for TH, increasing free TH. Gene expression of thyroid hormone regulated genes, including protein disulfide isomerase, was upregulated. Visualization of methylation of DNA fragments by the restriction landmark genomic scanning (RLGS) method showed that methylation status of a small specific set of sites was influenced by BPA treatment in a gestational age dependent (GD 12.5, 14.5) manner. Identification of the location of the affected sites showed they were CGI (CpG islands) in gene promoter regions. mRNA expression was examined at two of these sites and found to be altered in the GD 12.5 brains. These changes occurred in the absence of gross morphological or cytoarchitectural abnormalities. The dose for all studies was 20 μg/kg by s.c. injection.

The most recent advance using this model (20 μg/kg by s.c. injection during brain development) extended the treatment from GD 8 to PND 21 in rats and looked at functional changes in brain by examining electrophysiology in brain slices of cortex and striatum (Zhou et al., 2009). The authors reported failure of the normal progression of response to high frequency stimulation in this pathway which converts from long term potentiation to long term depression during the third week of postnatal life. This finding was linked to dopaminergic systems and the work of other investigators on these systems as described in Section D.3.2.2. An action on dopamine systems through membrane estrogen receptor was discussed.

B.2.2.3.2.2. Effects on dopamine system and interaction with drugs of abuse

These hypothesis-testing studies (Table B13) were based on known influences of estrogen on dopamine systems in brain which underlie reward and addiction processes. Dopamine is a monoamine neurotransmitter.

Developmental BPA effects in response to drugs of abuse (morphine, methamphetamine) were studied in connection with BPA administered in diet to mice. Most of the studies used exposure from mating through gestation and lactation to weaning, but the most recent study (Narita et al., 2007) identified the prenatal period as the sensitive period for inducing this effect.

Table B13. BPA studies related to dopamine and drugs of abuse.

Study	Species	Exposure	Endpoint	Findings
Suzuki et al. 2003 NTP-A EU 2008	ddY mice, male	0, 2, 500, 2000 mg BPA/g diet 0, 0.2, 50, 200 mg/kg bodyweight-d GD 0– PND21	Methamphetamine conditioned place preference, methamphetamine stimulated activity Dopamine stimulated ^{35}SGTPγS; Dopamine membrane and vesicle transporter proteins, dopamine receptor D1	↑ preference ↑ activity ↑ activation No effect on DAT ↑ DRD1 mRNA
Mizuo et al. 2004a NTP-A EU 2008	ddY mice, male	0, 2, 500, 2000 mg BPA/g diet 0, 0.2, 50, 200 mg/kg bodyweight-d GD 0–PND 21	Morphine conditioned place preference, Morphine stimulated activity Dopamine stimulated ^{35}SGTPγS	↑ preference ↑ activity No effects
Narita et al. 2006 NTP-A EU 2008	ddY mice male, 7 weeks old	0, 0.03, 0.3, 3, 500, 2000 μg BPA/g diet 0, 3, 30 μg/kg-d 3, 50, 200 mg/kg-d GD 0– PND 21	Morphine conditioned place preference, Morphine stimulated activity Dopamine stimulated ^{35}SGTPγS	↑ preference ↑ activity ↑ activation Effects at 0.03 and 2000 μg BPA/g diet
Narita et al. 2007 EU 2008	ddY mice, male	0, 2000 μg/g diet GD 0–7 or GD 7–14 or GD 14–21 or PND 0–21	Morphine conditioned place preference, Morphine stimulated activity Dopamine stimulated ^{35}SGTPγS	Effects with dosing at GD 0–7 or PND 0–21
Tando et al. 2007 NTP-A EU 2008	ddY mouse, male and female	3 μg/g, 8 mg/g Diet GD 0–PND 1	Dopamine neurons of substantia nigra and cortical neurons expressing calcium binding proteins 8–11 weeks of age	No effect on distribution of calcium binding proteins in cortical layers; Females: fewer Th (tyrosine hydroxylase) immunoreactive (ir) neurons in substantia nigra, low dose. Males: no effect on Th-ir neurons in SN

These studies found an enhancement of the effects of morphine and methamphetamine in male offspring of mice that had been treated with BPA during pregnancy and lactation. Activity and conditioned place preference measures were used to assess enhanced morphine and methamphetamine effect. The effects were demonstrated at a dose of 200 mg BPA/kg-d for methamphetamine and 50 and 200 mg BPA/kg-d for morphine (Suzuki et al., 2003; Mizuo et al., 2004a). Additionally, lower doses of BPA (0.003, 0.03, 0.3, 50, 200 mg BPA/kg) were investigated in connection with the morphine effect (Narita et al., 2006). The 200 mg/kg-d dose was effective as was the lowest dose (3 µg/kg-d), but intermediate doses had weaker or no effect thus indicating a "U-Shape" dose response curve. A final study looked for sensitive periods for the effect using morphine (Narita et al., 2007). Four periods were considered, preimplantation GD 0–7, organogenesis GD 7–14, "parturition" GD 14–20 and lactation PND 0–20. The two periods when exposures were effective were organogenesis and lactation using hyperactivity and conditioned place preference as the endpoints. This study used the 200 mg/kg-d dose, a higher dose than used in the studies of sex differentiation described above.

General toxicity data were not shown but it was stated that there were no effects on weight or maternal behavior. One paper also reported an absence of effects on pup growth and birth rate (Narita et al., 2006). The number of dams treated and the number of male pups per litter used in each test were not stated. The group sizes ranged from 6 to 14. Age at testing was 7 weeks. Consistency in results across studies increases confidence in the results, although it is not clear that litter-based statistics were used.

The studies also directly assessed the dopamine systems in brain. The first study looked at dopamine receptors, dopamine transporters and dopamine stimulated activation of G-protein signaling (Suzuki et al., 2003). The authors hypothesize that BPA does not act through classic estrogen receptors to produce low dose effect, but rather through the G-protein coupled membrane receptor which can modify the actions of neurotransmitter receptors in the cell membrane. Effects of BPA on dopamine mediated G-protein activation were confirmed in later studies (Mizuo et al., 2004a; Narita et al., 2006, 2007). This was also supported by a study reporting changes in D3 receptor binding, without changes in expression, in the brains of developmentally treated mice (Mizuo et al., 2004) and by *in vitro* studies (Miyatake et al., 2006). The most recent studies from this group examine the development of dopaminergic neurons (Miyagawa et al., 2007b).

Most of the studies from this group, as outlined in Table B13, used prenatal/postnatal exposure. However, one study demonstrated effects with exposure only during organogenesis (Narita et al. 2007) thus establishing the relevance of the research to hazard identification for *in utero* exposure. None of the studies provided information on litter distribution or litter-based statistics.

A study from a different research group looked only at neurons that synthesize dopamine (Tando et al., 2007). This study was based on the observation that BPA binds to the gamma-noradrenergic receptor (Nadal et al., 2000), a receptor for the monoamine neurotransmitters epinephrine, norepinephrine and dopamine. The authors explored the populations of dopaminergic neurons in the substantia nigra of mice exposed developmentally to BPA. Specifically they counted the number of neurons immunoreactive for tyrosine hydroxylase, a key enzyme in catecholamine synthesis. In addition, the distribution of cortical neurons expressing

calcium-binding proteins was studied because ERβ colocalizes with these proteins. A prenatal/postnatal exposure was used. There were fewer dopaminergic neurons in substantia nigra of BPA-treated mice than controls, although the difference was significant only in females. The distribution of calcium binding proteins that colocalize with ERβ and dopamine receptors was not affected.

B.2.2.3.2.3. Studies screening with a behavioral test battery

These three studies were not based on any particular hypothesis concerning BPA effects on the brain or development. They used behavioral test batteries to more generally assess potential developmental neurobehavioral toxicity. All of these studies used prenatal/postnatal exposures.

A laboratory that had previously studied BPA effects on dopamine systems and drugs of abuse (Suzuki et al., 2003; Narita et al., 2006, 2007) undertook a broader evaluation of behavior using the C57Bl/6 mouse (Miyagawa et al., 2007a). As previously, only male mice were tested. The test battery included elevated plus maze, rotarod, light-dark box and passive avoidance. In this study the number of dams per group was stated (n=10) and the authors indicated that the pups from each litter were distributed across the 4 tests with 5–11 male offspring per group per test. There were 3 groups, control and 2 BPA doses previously studied (30 ng/g and 2 mg/g diet, corresponding to 3 μg/kg/ body weight and 200 mg/kg body weight). The only treatment effect identified by statistical analysis was on retention of the passive avoidance response as assessed by time to enter 48 h after training. This was lower in both treated groups. A previous study (Kubo et al., 2001) used the passive avoidance test and found a lack of sex differentiation after developmental exposures to a 1.5 mg/kg-d dose.

A behavioral study in mice was also conducted using only female C57Bl/6 mice (Ryan and Vandenbergh, 2006). Developmental BPA (2 or 200 μg/kg-d, GD 3–PND 21) and also EE (5 μg/kg-d) were administered by oral instillation on GD 3–PND 21. The offspring were ovariectomized prior to puberty. This would ensure that behavioral effects were mediated by BPA actions on brain, rather than BPA actions on ovary that indirectly affected behavior by changing hormone production. EE was effective in accelerating puberty, increasing anxiety (elevated plus maze, light dark box) and improving spatial memory (radial arm maze, Barnes maze). The high BPA dose had similar effect on puberty and anxiety as did EE but not on spatial learning and memory, while the lower dose was not effective. This study can be distinguished from the other two in this section (Ema et al., 2001; Ryan and Vandenbergh, 2006) in that the BPA was administered by oral instillation rather than in food or by gavage.

A routine guideline style multigeneration study (Ema et al., 2001) in SD rats also evaluated reflex development prior to weaning, and exploratory activity and learning and memory in a water maze during puberty (5–7 weeks of age). Remarkably, gastric intubation was used for this long-term dosing regimen. For reflex development, males in the F_2 generation were delayed in acquisition of the negative geotaxis reflex at 0.2, 2 and 20 μg/kg-d dose, and both male and female F_1 pups had earlier onset of air righting at 20 μg/kg-d. There were no BPA effects on the pubertal tests. Litter based statistics were not discussed for the behavioral tests. As in other

studies in this dose range, no effects on fertility, pregnancy outcome or postnatal mortality and weight gain were recorded.

B.3. Summary and human health relevance

The literature on BPA developmental toxicity contains both commercial safety-testing studies and investigator-initiated research. None of the studies has been replicated either by the original researchers or by independent researchers. Although screening studies can uncover effects of BPA not anticipated by what is known of its mechanisms of action, comprehensive screening of BPA for developmental neurotoxicity has not yet been conducted. Routine guidelines are available for developmental neurotoxicity screening but have not yet been used for BPA. BPA interaction with neurological syndromes, neurodegenerative syndromes and childhood behavior disorders have also not been explored.

However, key findings that have appeared across the range of studies within and between laboratories when BPA was administered during pregnancy and offspring were evaluated. The effects of BPA include:

- Effects on offspring viability in the higher range of doses tested.
- Effects on sex-differentiation of exploratory and affective behavior at lower doses.
- Effects on immune hyperresponsiveness at lower doses.
- Effects on gender-differentiated morphology such as AGD.

Particularly important for Proposition 65 are the studies demonstrating altered sex differentiated behavior (Table B11) and immune function (Table B5) because:

- They include studies with exposure limited to pregnancy.
- They are consistent with known estrogenic actions.
- The design and statistical analysis are clear and appropriate.
- Doses are within the range of human exposures.

Potential mechanisms of action of BPA are reviewed in Sections C.3 and E.1. Of the variety of biological actions of BPA, direct regulation of gene expression in the embryo, action at membrane estrogen receptor sites, and modulation of second messenger systems are most often discussed as relevant to developmental toxicity endpoints reviewed here.

Human health relevance. The large number of studies considered here have intended to provide information relevant to human health issues including:

- Hazard identification for human health risk assessment.
- Effects on gene expression pathways critical for embryonic and fetal development.
- Early induction of metabolic disorders like obesity.
- Developmental influences that predispose to allergy.
- Alteration in social and affective behaviors known to be shaped by hormones during development.
- Differentiation of genitalia.
- Effects on dopamine systems that underlie syndromes such as ADHD, Parkinson's disease drug abuse and schizophrenia.

Effects demonstrated in *routine guideline DART studies* are relevant to human health because they are used to evaluate pesticides and chemicals for potential human health hazard and to develop estimates of acceptable levels of exposure. They are among the study designs for evaluating endocrine disruption that have been developed by U.S. EPA's Endocrine Disruptor Screening Program.

Even short term changes in *gene expression* are known to be significant for developmental trajectories because of the need for synchronized and coordinated gene expression for cell differentiation. More information is accumulating that altered DNA methylation, either as a result of genetic or environmental factors, can underlie changes in gene expression relevant to developmental disorders like autism and obesity. Increasing attention is being focused on the origins of obesity and diabetes in developmental exposures to chemicals (obesogen hypothesis, (Grun and Blumberg, 2006)).

Excess postnatal *weight* in rodents exposed prenatally to low doses of BPA may be relevant to this research topic. Research has demonstrated effects of BPA on adipocyte differentiation (Masuno et al., 2002; Masuno et al., 2005) a possible mediating mechanism. Low birthweight, as seen after higher BPA doses in rodents, is associated with later incidence of disease in humans (Godfrey and Barker, 2001).

Immune hyperreponsiveness in offspring of BPA-treated rodents is potentially relevant to human hypersensitivity disorders like asthma and allergy.

Sexual differentiation of behavior in rodents is one index of the appropriate action of hormones on brain development, and lack of sexual differentiation can indicate inappropriate brain development.

In humans, *anogenital distance* (as measured by anterior anoscrotal distance) was shorter in boys with hypospadias or cryptorchidism as compared to boys with normal genitalia (Hsieh et al., 2008). Investigators have also demonstrated induction of hypospadia in mice exposed to the estrogenic chemical benzophenone (Hsieh et al., 2007). Similarly, girls androgenized due to congenital adrenal hyperplasia have longer AGD than controls (Bongiovanni, 1962). This

suggests that anogenital distance can be an index of a more pervasive disruption of sexual differentiation in humans.

Effects of bisphenol A on *dopamine systems* are important because dopamine regulates hypothalamic-pituitary releasing hormones and also brainstem reward systems and motor systems. Human brain pathologies such as ADHD, Parkinson's disease and schizophrenia are known to involve disrupted dopamine function and are treated with drugs that affect the dopamine system. Dopamine is known to have an important role in mediating estrogen effects on sex-differentiated cognitive behavior and estrogen is known to affect neurodegeneration of dopaminergic neurons of the striatum (Quinlan et al., 2008).

C. Female Reproductive Toxicity

Numerous studies have been published on the effects of BPA on the female reproductive system. Animal studies have been conducted predominantly on rats and mice of varying strains. Different strains and species are known to have different estrogenic sensitivities, and this may translate to different study findings. Dose, route, period of exposure to BPA, and days of age at examination also vary widely across animal studies. These parameters may influence the likelihood that BPA will affect female reproductive outcome in any particular study. For example, exposure of a laboratory animal perinatally is likely to have a significantly different outcome on female reproductive endpoints than if that same laboratory animal was exposed as an adult. Dosing from gestation day (GD) 11 onwards encompasses the critical period of reproductive development in which most organogenesis occurs and a stage at which the developing fetus is more susceptible to endocrine disruption (McLachlan and Newbold, 1987; Cooper and Kavlock, 1997). In addition, the development of the uterus occurs during the fetal stage but that of the vagina continues into the early postnatal days (Suzuki et al., 2002). Effects resulting from exposure during organ development may result in persistent, irreversible alterations; these effects are termed "organizational" effects. On the other hand, effects resulting from exposure during adulthood are generally reversible, and are called "activational" effects.

Human studies examining the effects of BPA on reproduction are of limited study design and correspondingly limited in their findings. However, laboratory animal data on the female reproductive toxicological effects of BPA provide useful information on possible effects in humans. In rats, the uterus at birth corresponds developmentally to the human fetal uterus at GD 100 (Maekawa et al., 2004). The major findings relevant to female reproductive toxicity of BPA in laboratory animals are discussed by the following endpoints: uterus, ovary, follicles and oocytes, estrous cycle, fertility, vagina, mammary gland, and maternal-fetal transfer. In humans, a limited number of studies have examined female reproductive outcomes including recurrent miscarriage, chromosomal defects, endometriosis and endometrial hyperplasia, ovarian dysfunction, hormone levels, endocrine related disorders and pubertal status.

C.1. Female reproductive studies in humans

Female reproductive toxicity of BPA in humans as discussed in the NTP-CERHR report included studies examining:

1) Miscarriage (Sugiura-Ogasawara et al., 2005).
2) Chromosomal defects (Yamada et al., 2002).
3) Endometriosis and endometrial hyperplasia (Hiroi et al., 2004; Itoh et al., 2007).
4) Ovarian dysfunction (Takeuchi et al., 2004).
5) Hormone levels (Takeuchi and Tsutsumi, 2002).
6) Endocrine related disorders (Yang et al., 2006).

Since the NTP-CERHR report was released, a study of puberty in inner city girls has been published (Wolff et al., 2008a). This study is reviewed in Appendix 1. Also reviewed is the

publication by Itoh et al., 2007 that was included in the NTP Brief, but not reviewed by the NTP-CERHR Expert Committee.

In all, there are seven epidemiologic studies, six of cross-sectional design and one case control. These are shown in Table C1. These studies report on associations between blood or urine concentrations of BPA and certain female reproductive outcomes. One case-control study found an association between blood concentration of BPA and recurrent miscarriage. In two different cross-sectional studies by the same researchers, BPA blood concentration was associated with polycystic ovary syndrome. In both studies, BPA blood levels were also positively correlated with free and total testosterone concentrations. In two studies, BPA concentrations were lower in patients with endometrial cancer and in women with complex endometrial hyperplasia. One study reported no associations between urinary BPA concentrations and self diagnosed endocrine disorders. Another study reported no association with urinary BPA levels and endometriosis, and another study did not find BPA associated with pubertal status (breast development and pubic hair development) in 9 year old girls. The cross-sectional studies have limited usefulness for evaluating the potential effects of BPA on the female reproductive system.

Table C1. Summary table of female reproductive studies in humans.

Reference	*Study Type*	*Population*	*Method*	*Exposure Measures*	*Findings*
Takeuchi and Tsutsumi, 2002 NTP-A	Cross-sectional	14 healthy women, 11 health males, 16 women w/ PCOS	ELISA	Blood conc. BPA Total and free testosterone, 17β-estradiol, androstenedione, dehydroepiandrosterone sulfate, LH, FSH, prolactin	↑ BPA in normal men vs. normal women ↑ BPA in PCOS group vs. normal women Positive correlation between BPA and free and total testosterone
Takeuchi, et al., 2004 NTP-A	Cross-sectional	26 healthy women (19 non-obese, 7 obese), 19 women w/ PCOS (13 non-obese and 6 obese), 7 women w/ hyperprolactinemia, 21 women w/ hypothalamic amenorrhea	ELISA	Blood conc. BPA Total and free testosterone, 17β-estradiol, androstenedione, dehydroepiandrosterone sulfate, LH, FSH, prolactin, insulin	↑ BPA in non-obese and obese women w/ PCOS, and obese healthy women Positive correlation between BPA and free and total testosterone, androstenedione, and dehydroepidandrosterone sulfate
Hiroi et al., 2004 NTP-A	Cross-sectional	11 women controls, 19 women w/ endometrial hyperplasia, 7 women w/ endometrial carcinoma	ELISA	Blood conc. BPA	↓ BPA in women w/ endometrial cancer or complex endometrial hyperplasia compared w/ controls
Sugiura-Ogasawara et al., 2005 NTP-A	Case-control	45 patients w/ history of recurrent miscarriage, 32 controls - hospital employees	ELISA	Blood conc. BPA	↑ BPA in women w/ recurrent miscarriages
Yang et al., 2006 NTP-A	Cross-sectional	172 men and women	HPLC	Urinary conc. BPA Self diagnosed endocrine disorders	No association w/ BPA and endocrine-related disorders
Itoh et al., 2007 NTP-B	Cross-sectional	140 women, 20–45 years old. Hospital based population of infertile women	HPLC-MS	Urinary conc. BPA	No association between BPA and endometriosis
Wolff et al., 2008	Cross-sectional	192 healthy, 9-year-old girls	HPLC-MS	Urinary conc. BPA	No association between BPA and pubertal status (breast development and pubic hair development)

C.2. Female reproductive toxicology in laboratory rodents

C.2.1. Effects on the uterus

C.2.1.1. *In vitro* exposure

Rat uteri have uridine diphosphate (UDP)-glucuronosyltransferase (UGT), which is a necessary enzyme for the metabolism of BPA. The isoforms of UGT expressed in rat uteri include UGT1A1, 1A5, 1A6, and 1A7 (Matsumoto et al., 2007). In an *in vitro* assay, when the inner or outer side of a Wistar rat uterine sac was exposed to BPA, the concentration of the parent chemical was decreased in the buffer solution and BPA-glucuronide was observed on the outer side (maternal side) suggesting that the uterus metabolizes some BPA (Matsumoto et al., 2007). Expression of UGT in the uterus also suggests the uterus is capable of metabolizing BPA. Until uterine metabolism of BPA is complete, the uterus (and potential fetuses) may be adversely affected by BPA exposure.

C.2.1.2. *In vivo* exposure

C.2.1.2.1. Uterine weight effects

The uterotrophic assay is an established *in vivo* assay often used to test compounds for estrogenicity (Evans et al., 1941). The assay is based on the principle that the growth phase of the uterus (in the natural estrus cycle) is under the control of estrogens. When the natural source of estrogen is unavailable – either because of physical immaturity or because a female has been ovariectomized (OVX) – then the growth of the uterus becomes sensitive to external sources of estrogen. When exposed to such xenoestrogens, the immature or OVX female's uterus will increase in weight due to the absorption of fluid and cell proliferation initiated by the estrogen. Therefore, the primary endpoint in this assay is uterine weight, measured using dry or wet weight. Chemicals that act as estrogen agonists are expected to cause a statistically significant increase in uterine weight, while estrogen antagonists, when co-administered with a potent reference estrogen, would be expected to decrease uterine growth.

Laboratory mice and rats treated with μg to mg doses of BPA via oral and injection routes had significant increases in uterine weight (Ashby and Tinwell, 1998; Ashby et al., 2000; Laws et al., 2000; Markey et al., 2001b; Al-Hiyasat et al., 2004; Ashby and Odum, 2004). Immature female CD-1 mice exposed to BPA in concentrations ranging from 0.1 to 100 mg/kg body weight for 3 days via s.c. implanted Alzet osmotic pumps. A uterotrophic response (increase in uterine wet weight) was induced by 100 mg/kg BPA (Markey et al., 2001b). Howdeshell et al. demonstrated BPA released from used polycarbonate animal cages into water at room temperature could alter uterine weights in CD-1 mice. By housing some pre-pubertal females (PND 19–26) in used polycarbonate cages, a 16% increase in uterine wet weight (20.56 ± 1.13 mg) was observed

relative to uterine wet weight from females housed in used polypropylene cages (17.25 ± 0.70 mg) (Howdeshell et al., 2003). However, the difference was not statistically significant (P=0.31) (Howdeshell et al., 2003). In a two-generation study of CD-1 mice – although not directly comparable because the uterus, cervix, and vagina were weighed together – trends suggest BPA treatment increased uterine weight in F_0 females (Tyl et al., 2008b).

Yamasaki et al. examined the time-course changes of uterine weight in the immature rat uterotrophic assay using milligram (mg) amounts of BPA. Immature Crj:CD (SD) rats were injected subcutaneously (s.c.) with BPA, or BPA was administered orally via stomach tubes for 3 days (d) beginning on postnatal day (PND) 18 (Yamasaki et al., 2000). This study demonstrated that rats given 0, 8, 40, and 160 mg BPA/kg/day s.c. or 0, 40, 160, and 800 mg BPA/kg/day of BPA orally show evidence of an endocrine-disrupting effect, and that uterotrophic activity was more sensitive to s.c. injection than oral administration. Immature Alpk:AP rats (21–22 d old) given 3 daily doses via oral gavage or s.c. injection of 400 mg BPA/kg or 600 mg BPA/kg had significant increases in mean uterine weight compared with controls (Ashby and Tinwell, 1998).

In addition to reports of increases in uterine weight, BPA has also been reported to have no effect on uterine weight or to decrease uterine weight in the uterotropic assay. Adult OVX Sprague Dawley rats treated with BPA (1 mg/L, 10 mg/L, and 100 mg/L) in drinking water for 3 d did not have mean uterine wet weights that were significantly different from those of controls (Rubin et al., 2001). Also, Kato et al. reported a decrease in uterine weights of Sprague-Dawley rats exposed to 4 mg BPA/kg by injection on PND 0–9 (Kato et al., 2003) In a study by Talsness et al., pregnant Sprague-Dawley rats were given 0.1 mg BPA/kg-d orally on GD 6–21. The absolute mean uterine weight of female offspring from the 0.1 mg/kg-d treatment group was significantly reduced compared with controls during the diestrus and estrus phases (Talsness et al., 2000b).

These differing effects of seemingly similar treatments may result from differences in periods of exposure, strains of animals, or route of exposure. The results produced by Markey et al. (Markey et al., 2001b) showed that BPA-induced changes in the mouse uterus differ depending on dose and the end point measured, and that certain tissue effects show a non-monotonic relationship with dose.

C.2.1.2.2. Uterine cell morphology

Cellular alterations resulting from exposure to BPA have been shown, such as an increase in luminal epithelial cell height (Steinmetz et al., 1998; Fukumori et al., 2001). A study on pseudopregnant Sprague-Dawley rats demonstrated the uterus responds to BPA before and after decidual induction. Bisphenol A exposure was generally stimulatory on uterine proliferation during pre-decidual induction (d 14 of pseudopregnancy), while growth indices were markedly inhibited by BPA during post-decidual induction (pseudopregnant d 5–9) (Spencer et al., 2002). Morphological changes were noted in the uteri of Sprague-Dawley female offspring who were exposed to 0.1 or 50 mg BPA/kg-d on GD 6–21 (Schönfelder et al., 2004). Differentiation and stratification of the uterine epithelium were noted during estrus. The thickness of the total epithelium was significantly decreased after in utero exposure to 50 mg BPA/kg-d, full-length

estrogen receptor (ER)α expression in the uterus at 64 kilodaltons (kDa; the relative molecular mass) was increased during estrus, and ERβ expression in the uterus at 53 kDa was decreased during estrus at the protein level of all female offspring exposed to BPA (Schönfelder et al., 2004). Female CD-1 mice offspring exposed to nanogram (ng) concentrations of BPA in utero had a decrease in volume of the endometrial lamina propria, increased bromodeoxyuridine (BrdU; used to indicate cell proliferation) incorporation in the DNA of endometrial gland epithelial cells, and increased expression of ERα and progesterone receptor in the luminal epithelium of the endometrium and subepithelial stroma (Markey et al., 2005).

Fetal Sprague-Dawley rats (GD 20) exposed to mg amounts of BPA for 9 d *in utero* had uteri (as well as ovaries, and oviducts) which demonstrated essentially no changes or gross abnormalities in micromorphology (Naciff et al., 2002). Although Naciff et al. demonstrated no changes in micromorphology, in utero exposure to BPA altered gene-expression in these estrogen-sensitive tissues, but only at the medium- to high-dose ranges. Yoshida et al. administered BPA via oral gavage to pregnant Donryu rats from GD 2 to the day before weaning (PND 21). Even at the highest dose (6 mg BPA/kg-d), female offspring showed no alterations in the uterine expression of ERα (Yoshida et al., 2004). On the contrary, an *in vitro* study examining the effects of BPA on human Ishikawa cells (an endometrial carcinoma cell line) and *in vivo* on CD-1 mice demonstrated that HOXA10 gene expression increased (Smith and Taylor, 2007). *In vitro* exposure of human Ishikawa cells to 0.1 nM–25 μM BPA resulted in an increase in HOXA10 gene expression. The HOXA10 gene is necessary for uterine development, specifically normal decidualization and pregnancy. *In utero* exposure of mice to 0.5 to 1.0 mg BPA/kg increased Hoxa10 (mice) expression resulting in altered endometrial pinopods and microvilli, and increased litter size (Bagot et al., 2001; Daftary and Taylor, 2004; Smith and Taylor, 2007). Changes in expression levels of three estrogen responsive uterine genes have also been demonstrated in immature Alpk:ApfSD (Wistar derived) rats (Ashby and Odum, 2004).

C.2.1.2.3. Uterine protein expression

Calbindin-D_{9k} (CaBP-9k), a cytosolic calcium binding protein mainly expressed in the uterus, placenta and intestine, carries an estrogen response element that is involved in the steroid hormone regulation of the gene during the estrus cycle and gestation. The presence of estrogenic compounds can alter CaBP-9k expression. Treatment of immature rats with BPA results in a significant increase in uterine CaBP-9k protein at an injected dose of 500 mg BPA/kg bw-d in immature rats (An et al., 2003). In addition to BPA inducing alterations in uterine CaBP-9k expression in treated females, alterations can be seen in uterine CaBP-9k expression of female neonates from treated dams (Hong et al., 2003; Hong et al., 2004). Injection of a high dose (600 mg/kg bw-d) of BPA to pregnant Sprague-Dawley rats on GD 17–19 resulted in an increase in CaBP-9k protein in maternal uterus, and a significant increase in CaBP-9k mRNA in the fetal uterus (Hong et al., 2003). Similarly, CaBP-9k mRNA increased significantly in uteri of neonates when dams were treated with doses of 400 and 600 mg BPA/kg-d for the first 5 days after parturition (lactation d 1–5) (Hong et al., 2004). However, CaBP-9k protein in the uteri of neonates was undetectable despite the increase in CaBP-9k mRNA (Hong et al., 2004).

C.2.1.2.4. Effects on gravid uteri

Intrauterine implantation can also be adversely impacted by treatment with BPA (Berger et al., 2007; Berger et al., 2008). CF-1 dams injected s.c. with 10.125 mg/animal-d on d 1–4 of pregnancy had a significant reduction in the number of uterine implantation sites when sacrificed at d 6 (Berger et al., 2007). CF-1 rats treated on GD 0 had a significant decrease in the number of implantation sites following a single administration of 10.125 mg BPA, and rats treated on d 1 of pregnancy with 6.75 mg and 10.125 mg BPA showed a significant reduction in number of implantation sites (Berger et al., 2008). Pregnant ICR mice exposed to BPA on GD 0–7 had lighter uteri compared with pregnant control dams on GD 10 (0.542 g ± 0.063 g vs. 2.184 g ± 0.109 g, respectively), and GD 12 (1.144 g ± 0.038 g vs. 5.706 g ± 0.657 g, respectively) (Tachibana et al., 2007). Placentation and intervillous spaces were also altered by exposure to BPA. The placentae of controls were larger than those of BPA mice on GD 12, and the intervillous spaces (through which maternal blood flows) were narrowed in the BPA-treated dams on GD 10 and GD 12 (Tachibana et al., 2007).

C.2.1.2.5. Long-term uterine effects of neonatal exposure

Pre- and perinatal exposure to BPA may also have long-term adverse effects. Newbold et al. exposed pregnant CD-1 mice to 0.1, 1, 10, 100 or 1000 μg BPA/kg-d via s.c. injections on GD 9–16 (Newbold et al., 2009). After delivery on GD 19 (PND 0) pups were held for 18 months, at which time reproductive tissues were evaluated. Observed uterine alterations included cystic endometrial hyperplasia (CEH), prominent mesonephric duct remnants in the uterus similar to those seen in the ovary and oviduct, and endometrial polyps (Newbold et al., 2009). In a study which exposed female CD-1 mice via s.c. injections to 10–1000 μg BPA/kg-d on PND 1–5, long-term adverse effects included severe uterine pathologies such as adenomyosis, leiomyomas, CEH, polyps, and mesonephric (Wolffian) duct remnants (Newbold et al., 2007). The incidence of uterine CEH was increased in all BPA groups, but statistically significant in the 100 μg BPA/kg-d group compared with controls, indicating excessive estrogen stimulation (Newbold et al., 2007). Stromal polyps were seen in all groups, but there was a high incidence in the 100 μg BPA/kg-d group; enlarged mesonephric duct remnants were also found in BPA-treated mice (Newbold et al., 2007).

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations.

Reference	Species	Exposure	Dose	Findings
Prenatal Exposure				
Berger et al., 2007 NTP-A	CF-1 mice	injection (d 1–4 of pregnancy) or ingestion (d 1–5 of pregnancy)	injections – 0, 0.0005, 0.0015, 0.0046, 0.0143, 0.0416, 0.125, 0.375, 1.125, 3.375, or 10.125 mg/animal-d ingestion – concentrations of BPA/peanut butter 0%, 0.11 %, 1%, 3%, or 9 % BPA	• Significantly ↓ number of implantation sites in the uterine lining of the 10.125 mg/animal-d group, and significantly ↓ percent parturient. • A significant ↓ in the number of pups born for the 3.375 and 10.125 mg/animal dose • An average daily intake of 68.84 mg BPA terminated all pregnancies.
Berger et al., 2008	CF-1 ♀ (3–6 months old, sexually naïve) mice	**Exp. #1:** on d 1–4 of pregnancy, dams received s.c. injections **Exp. #2:** a single s.c. injection was administered on d 0, 1, or 2 of pregnancy	**Exp. #1:** 0, 0.0005, 0.0045, 0.05, 0.125, 1.125, 3.375, 6.75 or 10.125 mg BPA/animal-d (approx. 0, 0.01, 0.1, 1.5 3.5, 30, 100, 200 and 300 mg BPA/kg bw) **Exp. #2:** 0, 6.75, or 10.125 mg BPA/animal-d	• **Exp. #1:** The # of implantation sites significantly ↓ in the 6.75 and 10.125 mg/day groups compared with the control group. • **Exp. #2:** Rats treated on GD 0 had a significant ↓ in the number of implantation sites following a single administration of 10.125 mg BPA. Rats treated with 6.75 mg and 10.125 mg BPA on GD 1 showed a significant ↓ in number of implantation sites.
Hong et al., 2003 NTP-A	Pregnant Sprague-Dawley rats	s.c. injection on d 17–19 of pregnancy	200, 400, or 600 mg/kg bw-d	• 600 mg BPA/kg bw-d resulted in an ↑ of CaBP-9k protein in maternal uterus. • BPA induced a significant ↑ of CaBP-9k mRNA in the fetal uterus.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Markey et al. 2005 NTP-A, EU 2008	CD-1 mice	*in utero* exposure for 14 d (dams were s.c. implanted with osmotic pumps from d 9 of pregnancy until PND 4)	25 and 250 ng/kg bw-d	• ♀ offspring exposed to 250 ng/kg bw-d BPA had ↓ volume of the endometrial lamina propria, ↑ incorporation of BrdU into the DNA of endometrial gland epithelial cells, and ↑ expression of ERα and progesterone receptor in the luminal epithelium of the endometrium and subepithelial stroma.
Naciff et al. 2002 NTP-A, EU 2008	pregnant Sprague-Dawley rats	s.c. injection on GD 11–20	0, 5, 50, or 400 mg/kg-d (1 ml/kg bw of dose solution, controls received DMSO)	• Histological examination of fetal ovaries, oviducts, and uteri demonstrated essentially no changes or gross abnormalities in micromorphology. • The highest dose of BPA induced vaginal bleeding and early parturition in 1 of 8 dams, and prominent nipples/areolas in both ♀ and ♂ fetuses. • The genes showing the most robust estrogen-like response to transplacental exposure to BPA include intestinal calcium-binding protein (InCaBP), progesterone receptor (PrgR), 11-β-hydroxylsteroid dehydrogenase type 2 (11β-HSD), and vascular alfa actin (VaACTIN), granted only at medium- to high-dose ranges (50 to 400 mg/kg).
Newbold et al. 2009	pregnant adult ♀ CD-1 mice	daily s.c. injections on GD 9–16	corn oil (control), 0.1, 1, 10, 100, or 1000 μg BPA/kg-d	• Cystic endometrial hyperplasia were seen in all groups except the 0.1 μg/kg-d group [13% Control (2/16); 38% BPA-1 (5/13); 7% BPA-10 (1/14); 36% BPA-100 (5/14); and 8% BPA-1000 (1/13)]. • Prominent Wolffian (mesonephric) remnants in the uterus similar to those seen in the ovary and oviduct were observed in all of the BPA groups except the 100 μg/kg-d group. • Endometrial polyps in the 0.1, 1, and 10 μg/kg-d groups.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Rubin et al. 2001 NTP-A NTP-B EU 2003 EU 2008	**group 1:** pregnant ♀, **group 2:** ovariectomi-zed young adult ♀	**group 1**: BPA in drinking water from GD 6 to through the period of lactation (pups supplied with unadulterated water at weaning), **group 2**: BPA in drinking water for 3 days	1 mg/L (low dose; approx. 0.1 mg/kg bw-d consumed), 10 mg/L (high dose; approx. 1.2 mg/kg bw-d consumed), and 100 mg/L (only for the uterotrophic assay (group 2 ♀))	• Mean uterine wet weight (mg ± SEM) of the 1 mg BPA/L group was 78.2 ± 5.0, the 10 mg BPA/L group was 89.9 ± 2.8, and the 100 mg BPA/L group was 82.9 ± 8.9 compared with 78.2 ± 6.1 for controls (not statistically significant).
Schonfelder et al. 2004 NTP-A NTP-B EU 2008	gravid Sprague-Dawley dams	oral gavage of dams on GD 6–21	0.1 or 50 mg/kg-d	• Morphological changes were noted in the differentiation and stratification of the uterine epithelium during estrus in the *in utero* BPA-treated animals. • The thickness of the total epithelium was significantly ↓ after exposure to 50 mg/kg-d. • The full-length ERα expression at 64 kDa was ↑ during estrus in the uterus of all ♀ offspring exposed to the 50 mg BPA group. • ERβ expression at 53 kDa was ↓ during estrus at the protein level in the uterus of all ♀ offspring exposed to 0.1 and 50 mg BPA/kg-d compared with controls.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Smith et al., 2007	CD-1 mice & Human Ishikawa cells (a well different-iated endometrial adenocarcin-oma cell line)	i.p. injection GD 9–16 & *in vitro* cell culture for 24 h	0.5, 1.0, 5.0, 50, or 200 mg/kg & 0.1 nM to 25 μM	• *In utero* exposure to a 50 mg/kg dose resulted in one stillbirth followed by death and one death w/o parturition. • 200 mg/kg resulted in death of all pregnant mice, 0.5 mg/kg to 1.0 mg/kg resulted in a dose responsive ↑ in uterine stromal Hoxa10 (mouse) expression. • A 5-, 7-, and 10-fold ↑ in Hoxa10 protein expression was seen in the 0.5, 1.0, and 5.0 mg/kg treatments compared with controls at the 2-week time point. • A 5-, 9-, and 12-fold ↑ in Hoxa10 protein expression was seen in the 0.5, 1.0, and 5.0 mg/kg treatments compared with controls at the 6-week time point. • *In vitro*, an ↑ in HOXA10 (human Ishikawa cells) gene expression was seen with ↑ concentration of BPA treatment. • An ↑ in Hoxa10 gene expression was seen with ↑ concentration of BPA treatment.
Spencer et al., 2002 NTP-A	Pseudopreg-nant Sprague-Dawley rats	s.c. injection daily for 4 d (pseudopregnancy (PPG) d 1–4, or 5–8)	200 mg/kg	• BPA exposure was generally stimulatory on uterine proliferation during pre-decidual induction (PPG d 1–4), growth indices were markedly inhibited by BPA during post-decidual induction (PPG d 5–9). • BPA treatment consistently ↓ the ER mRNA levels throughout PPG on d 5–9.
Tachibana et al., 2007 NTP-B	ICR mice (10–12 wks old)	s.c. injection from GD 0–7 (8 d)	10 mg BPA/kg-d	• Mean uterine weight of pregnant control dams are significantly heavier than the mean uterine weight of BPA treated dams on GD 10 (2.184 g ± 0.109 g vs. 0.542 g ± 0.063 g, respectively) and GD 12 (5.706 g ± 0.657 g vs. 1.144 g ± 0.038 g, respectively). • Control dams had significantly more embryos than BPA treated dams. The intervillous spaces (through which maternal blood flows) were narrowed in the BPA mice on GD 10 and 12.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Talsness et al., 2000 NTP-A NTP-B	gravid Sprague-Dawley rats	oral gavage on GD 6–21	0.1 mg/kg-d (low) and 50 mg/kg-d (high)	• The absolute mean uterine weight of the 0.1 mg/kg-d treatment group was significantly ↓ compared with controls during diestrus (0.383 ± 0.060 vs. 0.497 ± 0.061, respectively) and estrus phases (0.518 ± 0.117 vs. 0.637 ± 0.165, respectively).
Yoshida et al., 2004 NTP-A NTP-B EU 2008	pregnant Donryu rats (Crj:Donryu rats)	oral gavage from GD 2 to the day before weaning (PND 21)	0, 0.006 mg/kg and 6 mg/kg	• No significant differences among the groups in all parameters: gestation period, the number of implantation sites, the average number of offspring per litter, and the body weights of offspring at birth. • No obvious morphological changes, including expression of ERα and the labeling index for cell proliferation activity in the uterus were observed in either of the BPA-treated groups before puberty.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Perinatal/Adolescent Exposure				
Ashby et al., 2004 NTP-A EU 2008	immature Alpk:APfSD (Wistar derived) rats	gavage, 3 daily doses starting on PND 19–20	0.002–800 mg/kg-d	• 800 mg/kg BPA gave a 2.6-fold ↑ in uterine weight when administered orally to immature rats for 3 d. • Expression levels of three estrogen responsive uterine genes – Complement component 3 (C3), lipocalin 2 (lipocalin), and PR – were ↑ after 2 μg–800 mg BPA/kg-d. • BPA gave maximal increases for PR, lipocalin, and C3 of 3-, 9-, and 730-fold, respectively. • Administration of BPA over the dose range of 2 μg/kg gave an ↑ in uterine weight 4 h after a single dose of between 2 μg–800 mg BPA/kg.
Ashby et al., 1998 NTP-A EU 2003	immature Alpk:AP rats (21–22 d old)	3 daily doses via oral gavage or s.c. injection	400 mg/kg, 600 mg/kg, or 800 mg/kg	• Treatment with BPA resulted in a positive uterotrophic assay response via both routes. • Statistically significant ↑ in both wet and dry uterine weights.
Fukumori et al., 2001	suckling ♀ mice	s.c. injection 5 d/week from PND 1–21	0, 0.8, 4, and 20 μg/kg-d or 500 μg/kg-d	• In the uterus, luminal epithelial cell height ↑ in the 4, 20 and 500 μg/kg-d groups compared with the control.
Hong et al., 2004 NTP-A	Sprague-Dawley rats (10 week old) – mated → pregnant	maternal injections for 5 d after delivery	200, 400, and 600 mg/kg bw-d	• A significant ↑ in CaBP-9k mRNA in the maternal uterus when the dams were treated with 600 mg BPA/kg-d, an ↑ in CaBP-9k protein was observed in the maternal rat uterus at all doses of BPA for 5 d. • CaBP-9k mRNA increased significantly in uteri of neonates when dams were treated with doses of 400 and 600 mg BPA/kg-d for 5 d. • CaBP-9k protein in the uteri of neonates was undetectable despite the increase in CaBP-9k mRNA.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Howdeshell et al., 2003	CD-1 mice (3 replicates of approximate-ly 6 litters per cage type for a total of 57 animals per cage type)	From PND 19–26 ♀ were housed in (1) used polycarbonate cages with water from used polycarbonate bottles, or (2) polypropylene cages with water from glass bottles	Up to 310 μg BPA/L was released from used polycarbonate animal cages, up to 0.3 μg BPA/L was released from new polycarbonate cages, and up to 1.5 μg BPA/L was released from new polysulfone cages	• On PND 19, there was no difference in body weight at weaning for ♀ placed in the two different cage types. • Bisphenol A from polycarbonate cages produced a 16% increase in uterine wet weight in prepubertal ♀ mice (20.56 ± 1.13 mg) relative to uterine wet weight from ♀ housed in used polypropylene cages (17.25 ± 0.70 mg), although the difference was not statistically significant (p=0.31).
Kato et al., 2003 NTP-A NTP-B EU 2008	Sprague-Dawley (Crj: CD (IGS)) neonates	injections once a day for 10 d from PND 0–9	0, 0.25, 1, 4 mg BPA; [12.5-, 50-, and 200-mg/ml (BPA and ethanol mixed with corn oil)]	• ↓ uterine weight (absolute and relative) in 4 mg BPA group compared with controls (P<0.01).
Markey et al., 2001 NTP-A NTP-B	CD-1 mice (23 d old)	s.c. implanted Alzet osmotic pumps for 3 days	0.1, 0.5, 1, 5, 50, 75, 100 mg/kg bw-d	• There was a 53% ↑ in uterine wet weight in response to 100 mg BPA/kg bw. • The uterus exhibited an ↑ in epithelial cell height in response to BPA at concentrations of 5, 75, and 100 mg BPA/kg bw.
Newbold et al., 2007 NTP-B EU 2008	Outbred CD-1 ♀ mice	daily s.c. injections on d 1–5 of age	10, 100, or 1000 μg/kg-d corn oil alone for control	• Assessed at 18 months of age. The incidence of uterine cystic endometrial hyperplasia (CEH) was ↑ in all BPA groups (but statistically significant in the BPA-100 group compared with controls) indicating excessive estrogen stimulation, stromal polyps were seen in all groups but there was a high incidence in the BPA-100 group. • Enlarged mesonephric duct remnants were also found in BPA-treated mice.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Yamasaki et al., 2000 NTP-A NTP-B EU 2003	Crj:CD Sprague-Dawley rats	s.c. injection and oral via stomach tube for 3 d beginning on PND 18	s.c.: 0, 8, 40, or 160 mg/kg-d; orally: 0, 40, 160, 800 mg/kg-d	• (study #1) - Uterine wet, blotted, and relative weights were increased in all groups given BPA s.c.; oral BPA resulted in ↑ uterine wet and blotted weights in the 800 mg/kg group, relative weight ↑ in the 160 and 800 mg/kg groups. • (study #2) Wet and blotted uterine weights ↑ in the 40 and 160 mg/kg groups, whereas relative weights ↑ in all groups given BPA. With oral administration, uterine wet, blotted, and relative weights ↑ in groups given 160 and 800 mg/kg BPA. • (study #3) Uterine wet, blotted, and relative weights ↑ in all BPA groups at 6 or 24 h after the last administration; weights also increased in the 40 and 160 mg/kg groups at 12 h. At 18 h, uterine wet, blotted, and relative weights were ↑ in all groups; relative weight ↑ in 40 and 160 mg/kg groups.
Adult Exposure				
Al-Hiyasat et al., 2004 NTP-B EU 2008	♀ Swiss mice, 60 d old	intragastric administration; daily for 28 d	5 μg/kg bw, 25 μg/kg bw, 100 μg/kg bw	• Mice exposed to the 25 and 100 μg/kg groups showed a statistically significant ↑ in relative uterine weights.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
Laws et al., 2000 NTP-A EU 2003	Longs Evans rats (prepubertal (21 d) and ovariectomi-zed adults (60 d))	For 3 d uterotrophic assays, oral gavage or s.c. injections were given once a day for 3 d. For age at vaginal opening, oral gavage treatment was given from 21–35 d of age. For examination of vaginal cytology, cycling animals were dosed for 25 d by oral gavage to 100 mg BPA/kg.	50, 100, 200, or 400 mg/kg	• Uterine wet weight 6 h following the last of three doses was significantly ↑ compared with control. • The magnitude of the uterotrophic response to 200 mg/kg BPA was greater following exposure via a s.c. injection as compared with exposure by oral gavage.
Steinmetz et al., 1998 NTP-A EU 2003	ovariectom-ized F344 and Sprague-Dawley rats (9–10 weeks of age)	i.p. injection once [F344 rats], and s.c. silastic implants for 3 d [F344 and SD rats]	0, 18.75, 37.5, 50, 75, 150, or 200 mg BPA/kg [injections], approximately 50 μg BPA [silastic capsules]	• Uterine cell height increased 2.5-fold in F344 rats implanted with BPA containing silastic capsules. • 37.5 mg BPA/kg caused a significant ↑ in cell proliferation in the uterus (and vagina). • Within 2 h after treatment with BPA, uterine *c-fos* mRNA increased 14–17-fold above control values.

Table C2. Prenatal, perinatal/adolescent, and adult exposure to BPA in vivo and in vitro produce uterine alterations (continued).

Reference	Species	Exposure	Dose	Findings
In Vitro Exposure				
Ashby et al., 2000 NTP-A	uterine post-microsomal supernatant, isolated from the tissue of immature ♀ Alpk:APfSD (Wistar derived) rats	s.c.; for 3 d	100 mg/rat (total volume of 3 ml sesame oil; administered twice daily (0.5 ml/dose)). 16.7 mg/dose, twice daily for 3 d.	• Uterine wet weight was significantly ↑ compared with control (135.6 ± 24.4 mg vs. 70.9 ± 12.9 mg), uterine dry weight was significantly ↑ (26.8 ± 5.9 mg vs. 15.8 ± 3.1 mg). • Vaginal cornification in the BPA group was also significantly ↑ compared with the control.
Bredhult et al., 2007	human endometrial endothelial cells	*in vitro* cell culture for 2–3 d	0.01 μM (low), 1 μM (medium), 100 μM (high)	• Significant ↓ in proliferation of human endothelial cells after exposure to low, medium, and high BPA. • BPA concentration at 100 μM decreased the cell viability and increased necrosis compared with control.

kDa: kilodaltons
PPG: pseudopregnancy
ER: estrogen receptor
PR: progesterone receptor
NTP-A: NTP 2008a.
NTP-B: NTP, 2008b.
EU 2003: EU (2003)
EU 2008: EU (2008)

C.2.2. Effects on the ovary

Xenoestrogens are believed to interact with endogenous estrogen through binding to estrogen receptors in target tissues such as the ovary *in vivo*. An *in vivo* BPA study conducted on mice lacking aromatase activity (aromatase knockout, ArKO) demonstrated that BPA has estrogenic properties (Toda et al., 2002). Specifically, a diet of 1% BPA (w/w) completely protected ArKO mice from hemorrhage formation and follicular loss in the ovaries (Toda et al., 2002). Exposure of mice to BPA has also resulted in the formation of cysts and lesions in female reproductive tissues. Newbold et al. administered μg amounts of BPA to CD-1 mice. There was a statistically significant increase in cystic ovaries and cystic endometrial hyperplasia (CEH) in the mid-dose BPA group as compared to controls (Newbold et al., 2007, 2009). Progressive proliferative lesion (PPL) of the oviduct and cystic mesonephric (Wolffian) duct remnants were also seen in all of the BPA groups (Newbold et al., 2007, 2009).

A more common ovarian effect of BPA exposure is a change in weight. Adult Swiss mice given 100 μg BPA/kg intragastrically for 28 d had a statistically significant 142% increase in relative ovarian weight compared with the control group (Al-Hiyasat et al., 2004). A two-generation CD-1 mouse study, showed paired ovarian weights tended to increase with higher concentration treatments of BPA, although this result was not statistically significant when individual treatments were compared with the negative control in F_0, and F_1 females (Tyl et al., 2008b).

Bisphenol A exposure may also reduce ovarian weight. In female F_1 rats who were born from females treated with 0.2 μg BPA/kg-d from before mating through lactation and treated with 0.2 μg BPA/kg from PND 23, ovarian weight was significantly reduced compared with controls (110 ± 15 mg vs. 123 ± 14 mg, respectively) when measured as adults at the time of necropsy (Ema et al., 2001). No significant differences were observed in relative organ weight of the ovaries from F_1 females treated with 0.2 μg BPA/kg-d vs. control females (32.8 ± 4.3 mg vs. 36.0 ± 3.8 mg, respectively) (Ema et al., 2001). In a study examining neonatal exposure of Sprague-Dawley rats on PND 0–9, a decrease in the area occupied by the corpora lutea (CL) in the ovary, and a decrease in ovarian weight in the 4 mg BPA group was noted (Kato et al., 2003).

Table C3. Female reproductive toxicology studies with ovarian endpoints and BPA exposures.

Reference	Species	Exposure	Dose	Findings
Histological alterations				
Newbold et al., 2007 NTP-B, EU 2008	Outbred CD-1 ♀ mice	daily s.c. injections on d 1–5 of age	10, 100, or 1000 μg/kg-d (corn oil alone for control)	• Assessed at 18 months of age. Cystic ovaries were common in all treatment groups (39% controls, 35% BPA-10, 70% BPA-100, 38% BPA-1000; but the BPA-100 was the only group statistically different from the controls). • PPL in the oviduct were seen in all groups of BPA treated mice (histologically resembles DES lesions).
Newbold et al., 2009	pregnant adult ♀ CD-1 mice	daily s.c. injections on GD 9–16	corn oil (control), 0.1, 1, 10, 100, or 1000 μg/kg-d	• Cystic ovaries were common in offspring from all groups, but only the 1 μg/kg-d group showed statistical significance compared with controls ($P<0.05$). • Neoplastic lesions in the ovary (included cystadenomas) were seen in the offspring from the 10, 100, and 1000 μg/kg-d groups, but not in controls. • PPL of the oviduct was seen in all offspring of BPA groups, but not in controls.

Table C3. Female reproductive toxicology studies with ovarian endpoints and BPA exposures (continued).

Weight alterations				
Ema et al., 2001 NTP-A, NTP-B, EU 2008	Crj: CD(SD) IGS rats	gastric intubation for 10 weeks (F_0 ♂) and 2 weeks (F_0 ♀) before mating, during the mating, gestation, and lactation periods; F_1 animals received BPA starting on PND 23; F_2 animals received BPA starting on PND 22 for 4 weeks (♂) and 11 weeks (♀)	0, 0.2, 2, 20, 200 μg/kg-d	• Ovarian weight was significantly ↓ in female F_1 adults who were born from rats treated with 0.2 μg BPA/kg and treated with 0.2 μg BPA/kg from PND 23 (110 ± 15 mg vs. 123 ± 14 mg, respectively). • No significant difference in relative organ weight.
Kato et al., 2003 NTP-A, NTP-B, EU 2008	Sprague-Dawley (Crj: CD (IGS)) neonates	injections once a d for 10 d from PND 0–9	0, 0.25, 1, 4 mg BPA/pup; [0, 12.5, 50, 200 mg/ml (BPA and ethanol mixed with corn oil)]	• In the 1 and 4 mg BPA groups there were ↓ in the area occupied by the CL in the ovary, cystic follicles, and ↓ ovarian weight in 4 mg BPA group.
Al-Hiyasat et al., 2004 NTP-B	♀ Swiss mice, aged 60 d	intragastrically, daily for 28 d	5 μg/kg, 25 μg/kg, 100 μg/kg	• The 100 μg/kg group had a statistically significant ↑ of 142% in relative ovary weights compared with the control group.

DES: diethylstilbesterol
PPL: progressive proliferative lesion

C.2.3. Effects on the ovarian follicles and oocytes

The relationship between female fertility and ovarian follicle development is well-recognized, but ovarian follicle development may be the more sensitive parameter for assessing female reproductive toxicity. Oocytes are contained within ovarian follicles. The cells of an ovarian follicle include an oocyte, granulosa cells, and the cells of the internal and external theca layers. "Bi-directional communication" between granulosa cells and an oocyte is necessary for oocyte maturation. Follicular somatic cells, which include granulosa cells, regulate the progression of meiosis. However, an oocyte orchestrates granulosa cell proliferation, differentiation, and function.

There is growing evidence that exposure to BPA has the potential to impact at least three different stages of oocyte development (Hunt and Hassold, 2008). The three stages of oocyte development that may be impacted by BPA exposure are as follows:

I. Meiotic initiation in the fetal ovary,
II. Follicle formation in the perinatal period, and
III. Oocyte growth and maturation in the adult.

Evidence from studies in humans and mice suggest the genetic quality of the oocyte may be influenced by events at each of the aforementioned stages.

In an *in vitro* study, Xu et al. exposed murine ovarian granulosa cells to BPA, and showed a decrease in granulosa cell viability and increased apoptosis of the granulosa cells (Xu et al., 2002). Ovarian granulosa cells were cultured in a range of BPA concentrations from 100 femtomolar (fM; 10^{-15} M)–100 μM for 24 to 72 hours. Bisphenol A decreased granulosa cell viability in a dose and time-dependent manner. Cultures of 100 picomolar (pM) BPA or more resulted in markedly decreased cell viability of granulosa cells in a dose-dependent manner as compared with control (Xu et al., 2002). Cultures of 100 μM BPA, decreased cell viability in a time-dependent manner and the difference was significant (Xu et al., 2002). Apoptosis of granulosa cells is a well-known mechanism involved in follicular atresia.

Spontaneous calcium (Ca^{+2}) oscillations are necessary for oocyte maturation and for the induction of various enzymatic responses by the oocyte to fertilization. *In vitro* exposure of immature CD-1/ICR mouse oocytes to 100 μM BPA results in irregular Ca^{+2} oscillations and shortens the duration of Ca^{+2} oscillations (Mohri and Yoshida, 2005).

Eichenlaub-Ritter et al. investigated:

1. The effects of continuous exposure of MF1 mouse follicular cell-denuded oocytes to BPA during *in vitro* maturation.
2. The effects sub-chronic *in vivo* exposure of C57Bl x CBA/Ca F_1 hybrid mice to BPA by oral gavage from PND 22–28.

On the afternoon of PND 28, animals were sacrificed and oocytes from large antral follicles were matured *in vitro*. In experiment #1, there was a significant increase in oocytes with germinal vesicle breakdown (GVBD) failing to emit a polar body, and an increase in the percentage of oocytes containing bivalent chromosomes in the 10 μg BPA/ml group during maturation (Eichenlaub-Ritter et al., 2008). Polyploidy was also significantly increased to 16% in the 10 μg BPA/ml group compared with 2.6% in the control group and 4.1% in the solvent control group (Eichenlaub-Ritter et al., 2008). In experiment #2, there was no evidence that BPA exposure affected the competence of oocytes to resume nuclear maturation. There was an increasing trend in chromosome congression failure in the 40 and 100 ng BPA/g bw groups (although this was not statistically significant), and no significant increase in hyperploidy rate (Eichenlaub-Ritter et al., 2008).

At a cellular level, other studies suggest that BPA interacts with microtubules and the organization of the meiotic spindle (Hunt et al., 2003; Can et al., 2005; Lenie et al., 2008). A

study by Hunt et al. showed meiotic maturation was altered in mouse oocytes (chromosome misalignment on the first meiotic spindle) when mice were inadvertently exposed to nM concentrations of BPA from damaged polycarbonate plastic (Hunt et al., 2003). In an *in vitro* study by Can et al., a higher ratio of chromosome misalignment was noted compared with the study by Hunt et al. (Hunt et al., 2003; Can et al., 2005). Exposure of maturing mouse cumulus-oocyte complexes (COCs; the oocyte surrounded by tightly packed layers of cumulus cells) to 10 and 30 µM BPA caused a dose-dependent retardation of meiotic progression compared with control oocytes (Can et al., 2005). The difference in magnitude of effect may be a result of Can et al. using BPA doses approximately 100-times greater than Hunt and colleagues' *in vivo* doses. Lenie et al. showed mouse follicles matured *in vitro* in BPA concentrations of 3 nM to 3 µM were generally morphologically normal, but 30 µM BPA exposure slightly reduced granulosa cell proliferation, lowered total estrogen production, and significantly increased meiosis I-arrested oocytes with unaligned chromosomes and spindle aberrations (Lenie et al., 2008). In cell culture systems, higher than necessary physiological concentrations of xenoestrogens may be used to invoke a cell response similar to what is expected *in vivo*.

A recently published study by the Hunt research group demonstrated a significant diet-related variation in both the frequency of abnormalities in oocytes from untreated females and in response to BPA (Muhlhauser et al., 2009). This study is described in detail in Appendix 1. The authors suggest their data support the idea that low doses of BPA have a normalizing effect on the oocytes of females on the soy diet.

In vivo, the effect of BPA on oocytes appears less pronounced. Pacchierotti et al. showed that female C57Bl/6 mice orally treated with various doses of BPA (7 daily administrations of 0.04 mg/kg and a concentration of 0.5 mg/L in drinking water for 7 weeks) had no significant induction of hyperploidy or polyploidy in metaphase II oocytes (Pacchierotti et al., 2008). However, in mice chronically exposed to BPA (0.5 mg BPA/L for 7 weeks) there was a statistically significant increase ($P<0.025$) in metaphase II oocytes showing premature centromere separation in more than 2 dyads (Pacchierotti et al., 2008).

Ovaries from the National Toxicology Program (NTP) Reproductive Assessment by Continuous Breeding (RACB) bioassays were used by Bolon et al. in a retrospective study to compare differential follicle counts and reproductive performance in laboratory mice. Bolon et al. showed follicle counts in CD-1 mice were not affected by exposure to BPA. However, Bolon et al. stated counts were not affected by toxicants such as BPA for which the susceptible sex could not be determined (Bolon et al., 1997). In multi-generation studies of CD-1 mice and Sprague-Dawley rats, follicular counts in the high dose groups and controls (0 parts per million (ppm)) did differ, but counts at mid-level doses in both studies were not made (Tyl et al., 2002b; Tyl et al., 2008b). In the two-generation CD-1 mouse study, paired ovarian follicle counts were not statistically different (the control F_0 generation was 92.1 ± 5.0 compared with 92.0 ± 7.0 in the 3500 ppm treatment group) (Tyl et al., 2008b). Similarly, paired ovarian follicle counts were not statistically different in the control F_1 generation (95.4 ± 5.1 compared with 91.0 ± 6.8 in the 3500 ppm treatment group) (Tyl et al., 2008b). However, in the three-generation Sprague-Dawley rat study, paired ovarian follicle counts were statistically different (the control F_0 generation was 315.9 ± 41.6 compared with 453.2 ± 26.3 in the 7500 ppm treatment group, $P<0.05$) (Tyl et al., 2002b).

Researchers have reported that exposure to BPA could also lead to abnormal follicular outcome, such as abnormal CL and cystic follicles. A study by Kato et al. reported that neonatal treatment of Sprague-Dawley rats resulted in the formation of multiple cystic follicles in the ovaries of treated animals. In the 1 mg BPA group, 4 of 8 females had cystic follicles, and all females in the 4 mg BPA group had cystic follicles (Kato et al., 2003).

It has also been reported that BPA exposure in utero can also disrupt early oogenesis in the mouse. The effect of BPA exposure on pregnant mice may be considered a "grandmaternal" effect in that the oocytes of female offspring are altered. Pregnant C57BL/6 mice were implanted on GD 11.5 with time-release BPA pellets that released approximately 20 µg/kg bw-d for one week (Susiarjo et al., 2007). A highly significant increase in synaptic abnormalities in oocytes from BPA-exposed females compared with controls was found (52.0% vs. 16.0%, respectively, $P<0.0001$) (Susiarjo et al., 2007). When these exposed females reached adulthood, oocytes from exposed female fetuses displayed gross aberrations in meiotic prophase, including synaptic defects and increased levels of recombination (aberrations were translated into an increase in aneuploid eggs and embryos) (Susiarjo et al., 2007).

Table C4. A comparison of the effects of BPA on ovarian follicles and oocytes.

Reference	Species	Exposure	Dose	Findings
Bolon et al., 1997 NTP-A, EU 2003	CD-1 mice	A retrospective study of previous RACB bioassays (BPA by s.c. implant and by feed: exposed for a 7-day premating period, paired and co-habitated and treated continuously for 98 d, mother was dosed through weaning and F_1 mice were dosed until mated at 74 ± 10 d of age)	implant: 25.0, 50.0, 100.0 mg/mouse; feed: 0.25%, 0.50%, 1.00%	• Small and growing follicles were 10- to 20-fold more numerous than antral follicles. • The mean number of small follicles ranged from 30–560 while counts of growing follicles ranged from 20–134. Follicle counts were not affected by toxicants (BPA) for which the susceptible sex could not be determined.
Can et al., 2005 NTP-A	Balb/c mice, 19–21 d old superovulated – COCs exposed to BPA	*In vitro*. Cell culture for 0–8 hrs (during germinal vesicle stage and M-I), 0–18 hrs (during germinal vesicle stage to M-II), 8–18 hrs (during M-I and M-II)	10 and 30 μM	• 10 μM BPA caused a delay in progression, oocytes mostly reached M-I, only 26% of cells remained in prometaphase. • In the 30 μM group, 61% of cells reached M-I while 37% remained in prometaphase I. • 2% of all cells treated with 30 μM BPA died. • BPA interferes with centrosomes and perturbs meiotic spindle formation during meiosis I and II.

Table C4. A comparison of the effects of BPA on ovarian follicles and oocytes (continued).

Reference	Species	Exposure	Dose	Findings
Eichenlaub-Ritter et al., 2008 NTP-B	MF1, stocks of C57Bl/6 and CBA/Ca, and (C57Bl/6 x CBA/Ca) F_1 hybrid mice	**Exp.1:** continuous *in vitro* exposure of MF1 mouse oocytes to BPA for 16 h during maturation. **Exp. 2:** oral, sub-chronic exposure to BPA followed by *in vitro* oocyte maturation (pups exposed PND 22–28 for 7 d)	**Exp. 1:** control, solvent control, 50 (0.22mM), 100 (0.44mM), 200 (0.88mM), 400 (1.75mM), 800 ng/ml (3.50mM), 4 (17.5mM), or 10 μg/ml (43.8mM). **Exp. 2:** corn oil, corn oil with 20, 40, or 100 ng BPA/g bw	• **Exp. 1:** a significant ↑ in oocytes with GVBD failing to emit a polar body, and an increase in the percentage of oocytes containing bivalent chromosomes in the 10 μg/ml group during maturation. Spindle formation, distribution of pericentriolar material and chromosome alignment on the spindle were altered in the 10 μg/ml group. • **Exp. 2:** no evidence of altered competence of oocytes to resume nuclear maturation, ↑ trend in chromosome congression failure in the 40 and 100 ng BPA/g bw groups (although this was not statistically significant), and no significant ↑ in hyperploidy rate.
Hunt et al., 2003 NTP-A, NTP-B, EU 2008	28 d old (for oocytes); 20–22 d old ♀ mice	6–8 d preceding oocyte collection; 3, 5, 7 d prior to oocyte analysis. Given orally (BPA in corn oil).	20, 40, 100 ng/g bw; 20ng/g bw	• Defects in the alignment of chromosomes on the first meiotic spindle (congression failure).
Kato et al., 2003 NTP-A, NTP-B, EU 2008	Sprague-Dawley (Crj: CD (IGS)) neonates	injections once a day for 10 d from PND 0–9	0, 0.25, 1, 4 mg BPA; [0, 12.5, 50, and 200 mg/ml (BPA and ethanol mixed with corn oil)]	• In the 1 mg BPA group, 4 of 8 ♀ had cystic follicles, and all 6 ♀ in the 4 mg BPA group had cystic follicles.

Table C4. A comparison of the effects of BPA on ovarian follicles and oocytes (continued).

Reference	Species	Exposure	Dose	Findings
Lenie et al., 2008	♀ F_1 hybrid (C57Bl/6j x CBA/Ca) mice	*In vitro* culture of preantral follicles between 100–130 μm containing an immature oocyte for 12 d	3, 30, 300 nM, or 3, 30 μM	• Follicles grown in BPA concentrations of 3 nM–3 μM were generally morphologically normal. • 30 μM BPA slightly ↓ granulosa cell proliferation and ↓ total estrogen production. • 18% of oocytes cultured in the presence of 30 μM BPA were unable to resume M after stimulation of oocyte maturation compared with controls, 37% arrested after GVBD, only 45% of the oocytes extruded a first PB. • 30 μM BPA led to a significant ↑ in MI-arrested oocytes with unaligned chromosomes and spindle aberrations.
Mohri et al., 2005 NTP-A	CD-1/ICR mice (fully grown, immature oocytes with intact germinal vesicles from 8–12 week olds)	60 minutes (incubation *in vitro*)	10 nM, 100 nM, 10 μM, 100 μM	• *In vitro* exposure of mouse oocytes to 100 μM BPA produced an irregular pattern of Ca^{+2} oscillations. • No significant differences were seen in BPA-treated oocytes at concentrations < 10 μM, but these oocytes showed a tendency to oscillate in irregular patterns (50% of oocytes exposed to 10 nM, 44% of oocytes exposed to 100 nM, and 60% of oocytes exposed to 10 μM).

Table C4. A comparison of the effects of BPA on ovarian follicles and oocytes (continued).

Reference	Species	Exposure	Dose	Findings
Muhlhauser et al., 2009	C57BL/6J mice	Daily oral doses for 7 d prior to oocyte collection, except adult (6–11 week old) ♀ used for oocyte analysis. Some animals were on a casein diet, and others were on a soy-based diet.	20, 40, 100, 200, or 500 μg BPA/kg bw	• Abnormal MII eggs were identified in 2% of eggs from control ♀ on the casein diet, but the abnormality rate increased to nearly 8% for the soy diet (P=0.002). • The casein diet produced an apparent linear dose response with a significant ↑ in spindle/chromosome alignment abnormalities at the 200 μg BPA/kg bw exposure level. • Baseline and vehicle categories yielded higher abnormality rates than did the 20 or 100 μg/kg exposure levels. • 200 and 500 μg/kg doses had elevated rates of abnormality over both the baseline and vehicle values. • 7.1% of metaphase II arrested eggs from adult ♀ (6–11 weeks old) exhibited severe aberrations in chromosome alignment or spindle formation. The abnormality rate observed in eggs from juvenile ♀ (28 d old) was 6.2%.
Pacchierotti et al., 2008 NTP-A, NTP-B, EU 2008	C57Bl/6 mice (4 or 9 weeks old at the time of treatment – prepubertal or adult)	Single dose, 7 daily administrations, or 7-weeks in drinking water. Given orally.	♀ germ cells: 0.2 and 20 mg/kg (via gavage), 7 daily administrations of 0.04 mg/kg and a concentration of 0.5 mg/L in drinking water for 7 weeks. For sub-chronic studies in ♂ germ cells and bone marrow: 0.002, 0.02, and 0.2 mg/kg for 6 d.	• ♀ C57Bl/6 mice orally treated with various doses of BPA had no significant induction of hyperploidy or polyploidy in metaphase II oocytes. • Mice chronically exposed to BPA (0.5 mg BPA/L for 7-weeks) had a statistically significant ↑ (P<0.025) in metaphase II oocytes showing premature centromere separation in more than 2 dyads.

Table C4. A comparison of the effects of BPA on ovarian follicles and oocytes (continued).

Reference	Species	Exposure	Dose	Findings
Susiarjo et al., 2007 NTP-A, NTP-B, EU 2008	pregnant C57BL/6 mice	Implanted time-release BPA pellets on GD 11.5 (for one week).	20 μg/kg-d (pellets released 400 ng BPA daily)	• Oocytes from exposed ♀ fetuses displayed gross aberrations in meiotic prophase, including synaptic defects and ↑ levels of recombination. • A significant ↑ in the level of hyperploid eggs in the BPA group (1.8% of the cells had more than the expected 20 chromosomes in the placebo group compared with 21.4% in the BPA group.)
Xu et al., 2002 NTP-A	murine [B6C3F1] ovarian granulosa cells	24, 48, or 72-hour *in vitro* cell culture.	0, 100 fM, 100 pM, 100 nM, and 100 μM	• BPA decreased granulosa cell viability in a dose and time-dependent manner. • BPA at 100 pM or more resulted in markedly ↓ cell viability of granulosa cells in a dose-dependent manner as compared with control. • BPA at 100 μM ↓ cell viability in a time-dependent manner and the difference was significant. • Treatment of granulosa cells with 100 μM BPA resulted in an ↑ of G2 to M arrest that reached a maximum after 48 h of treatment.

COCs: cumulus-oocyte complexes
d: days
GVBD: germinal vesicle breakdown
M: Meiosis
MI: Meiosis-I
MII: Meiosis-II
PB: polar body
fM: femtomolar
pM: picomolar

C.2.4. Effects on the estrous cycle

Stages of the estrous cycle are differentiated by the cell types that are present in the vagina. A frequently employed approach to summarize cyclicity is to determine the percentage of days in estrus or diestrus within a treatment group over a given period of time. If the effect of the toxicant exposure on the cycle is consistent within a group, then useful summary data are obtainable. However, if exposure results in some animals showing prolonged diestrus while others showed persistent estrus, then such group summaries may not reflect these changes and the differential effects in aggregate could result in an overall impression that cyclicity has not been affected (Goldman et al., 2007). Xenobiotic treatment can disrupt estrous cycles and cause a persistent estrus, a persistent diestrus, or cause an irregular pattern with cycles of extended duration (Goldman et al., 2007). In a rodent, a single cycle with a diestrus period of 4 days or longer and/or an estrus period of 3 days or longer has – for the purposes of toxicological assessment – been considered abnormal (Cooper and Goldman, 1999). Other periods have been used for characterizing abnormal cycles. For example, mice in estrus for four or more days were considered to have abnormal cycles; meanwhile, estrus for zero days (not detected) through three days were considered normal cycles (Tyl et al., 2006).

C.2.4.1. Altered estrous cycle patterns and lengths

Perinatal exposure of female laboratory rodents to BPA may have effects on the estrous cycle, which are evident later in life. The percentage of F_1 female offspring with normal estrous cycles from Crj: CD (SD) IGS rats treated with 20 µg BPA/kg-d during gestation and lactation was significantly lower than the percentage of female offspring having normal estrous cycles from control rats (Ema et al., 2001). A similar effect of irregular estrous cycles was seen in Sprague-Dawley rats treated from PND 0–9 with 1 and 4 mg BPA via injections (Kato et al., 2003) and female Sprague-Dawley offspring exposed to 50 mg BPA/kg-d on GD 6–21 (Talsness et al., 2000b). However, Sprague-Dawley females exposed to 250 µg BPA via 4 s.c. injections between PND 1–2 had normal, regular estrous cycles (Patisaul et al., 2006, 2007; Patisaul and Polston, 2008).

Rubin et al. examined the effect of BPA exposure on pregnant Sprague-Dawley rats and ovariectomized young adult females. Offspring of BPA-treated females exposed perinatally to 10 mg BPA/L (approx. 1.2 mg/kg bw-d consumed) from GD 6 through lactation exhibited altered patterns of estrous cyclicity. Some animals had intermittent extended periods of diestrus, while others exhibited extended periods of proestrus and/or estrus (Rubin et al., 2001).

Alterations in estrous cycles were also documented in female CD-1 mice offspring exposed in utero to BPA on GD 15–18. Dams were given four daily s.c. injections of 0.5 or 10 mg BPA/kg-d. In female offspring, vaginal smears were taken from 9–11 weeks of age, and estrous cycles were monitored. An average cycle length lasted 8.0 ± 0.4 d (0.5 mg/kg-d group) and 8.2 ± 0.3 d (10 mg/kg-d group) compared to 5.2 ± 0.1 d in untreated controls (Nikaido et al., 2004). Specifically, the time spent in diestrus phase was significantly longer in BPA-exposed offspring than in untreated controls (the 0.5 mg/kg group spent 38.9% ± 2.0% of the time in diestrus, and

the 10 mg/kg group spent 40.5% ± 1.2% of the time in diestrus compared with 24.2% ± 2.1% of the time for controls) (Nikaido et al., 2004). Similarly, pregnant Sprague-Dawley rats given 50 mg BPA/kg-d orally on GD 6–21 had female offspring with altered estrous cycles (Talsness et al., 2000b). Exposure to 50 mg/kg-d increased the proportion of total estrous cycles with estrus phases greater than one day in length, and increased the cycle length (Talsness et al., 2000b).

Studies have also reported that exposure to BPA later in life alters estrous cyclicity. Thirty percent of ICR/Jcl mice exposed on PND 0–5 via s.c. injections to 15 and 150 µg BPA/pup exhibited persistent diestrus compared with controls; however, this was not a statistically significant difference (Suzuki et al., 2002). In a study of Long Evans females (with normal 4–5 day estrous cycles) given 100 mg BPA/kg by oral gavage, Laws et al. showed oral BPA significantly reduced the total number of 4- to 5-day estrous cycles (Laws et al., 2000). The number of complete 4- or 5-day cycles during a 25 day treatment period in BPA-treated females was 3.7 ± 0.3 compared with 5.2 ± 0.2 in the control group (Laws et al., 2000).

Another study examined the effects of maternal dietary exposure to BPA during the critical period for brain sexual differentiation, and reported some effects on the reproductive and endocrine systems. Using pregnant Sprague-Dawley rats that were exposed to 0, 60, 600, and 3000 ppm BPA mixed with the diet during GD 15–PND 10, some female offspring from BPA-exposed dams exhibited extended diestrus, but there was no significant increase in the incidence compared to the corresponding controls (Takagi et al., 2004). Of the total of eight females that were examined during postnatal weeks 8–11 for estrous cyclicity (2 per treatment group), the 0 ppm group had one female that had extended diestrus, and the 60 ppm group had two females that had extended diestrus (Takagi et al., 2004).

Estrous cyclicity of parental (F_0 and F_1) CD-1 female mice was also evaluated in a two-generation reproductive toxicity study (Tyl et al., 2008b). Mice were fed 0, 0.018, 0.18, 1.8, 30, 300 or 3500 ppm BPA in their diets, which equates to intakes of 0, 0.003, 0.03, 0.3, 5, 50, or 600 mg/kg-d, respectively. Tyl et al. evaluated daily vaginal smears of F_0 and F_1 females for the last 3 weeks of their pre-breed exposure period. After identifying the stage of each smear, the duration of estrous cycles were calculated by determining the mean number of days between the end of one stage and the start of the same stage during the 21-day period. Control F_0 and F_1 females had mean cycle lengths of 5.3 ± 0.2 d and 5.3 ± 0.3 d, respectively. Group mean cycle lengths of treated F_0 females ranged from 4.6 ± 0.1 d to 5.4 ± 0.3 d. Mean cycle lengths of treated F_1 females ranged from 4.6 ± 0.2 d to 5.5 ± 0.3 d. Thus, the study authors concluded the cycle length was biologically and statistically equivalent across all BPA groups (Tyl et al., 2008b). However, it must be noted the authors calculated cycle lengths in different manner compared to most. Mice in estrus for four or more days were considered to have abnormal cycles; meanwhile, estrus for zero days (not detected) through three days were considered normal cycles (Tyl et al., 2006).

C.2.4.2. Alteration of estrous cycle onset

In addition to changes in estrous cycle length, studies reported that the onset of cyclicity can be altered by exposure to BPA. Female offspring from BPA-treated ICR/Jcl mouse dams had a significantly earlier (younger age) first vaginal estrous, and the total number of days in estrus was greater compared with controls (Honma and Iguchi, 2001; Honma et al., 2002). Female offspring from dams treated with 2 and 20 µg BPA/kg bw-d on GD 11–17 had estrous cycle lengths that lasted a mean of 5.8 ± 0.4 d and 5.5 ± 0.4 d, respectively, compared with controls that had a mean cycle length of 4.5 ± 0.4 d (Honma et al., 2002).

Table C5. Alterations in estrus cycle length and onset of vaginal estrus.

Reference	Species	Exposure	Dose	Findings
Honma et al., 2001; Honma et al., 2002 NTP-A, NTP-B, EU 2008	pregnant ICR/Jcl mice	s.c. injection on GD 11–17	2 or 20 μg/kg	• Age of ♀ offspring at first vaginal estrus was significantly earlier in the 20 μg BPA/kg group compared with controls. • Total days in estrus were longer in the BPA-treated groups compared with controls.
Patisaul et al., 2006; Patisaul et al., 2007* NTP-A, NTP-B, EU 2008	Sprague-Dawley rats	s.c. injection, every 12 h from PND 1–2; 4 injections total	250 μg	• BPA-treated ♀ had normal, regular estrus cycles.
Laws et al., 2000 NTP-A, EU 2003	Longs Evans rats (prepubertal (21 d) and ovariectomized adults (60 d))	For 3 d uterotrophic assays – oral gavage or s.c. injections were given once a d for 3 d. For age at vaginal opening – oral gavage treatment was given from 21–35 d of age. For examination of vaginal cytology – cycling animals were dosed for 25 d by oral gavage with 100 mg BPA/kg	50, 100, 200, or 400 mg/kg	• Oral exposure to BPA with doses up to 400 mg/kg-d were ineffective in altering the time of vaginal opening in Long Evans rats. • 100 mg/kg, oral BPA significantly reduced the total number of 4- to 5-d estrus cycles.

Table C5. Alterations in estrus cycle length and onset of vaginal estrus (continued).

Reference	Species	Exposure	Dose	Findings
Ema et al., 2001 NTP-A, NTP-B, EU 2008	Crj: CD(SD) IGS rats	gastric intubation for 10 weeks (F_0 ♂) and 2 weeks (F_0 ♀) before mating, during the mating, gestation, and lactation periods; F_1 animals received BPA starting on PND 23; F_2 animals received BPA starting on PND 22 for 4 weeks (♂) and 11 weeks (♀)	0, 0.2, 2, 20, 200 µg/kg-d	• F_1 ♀ from Crj: CD (SD) IGS rats treated with 20 µg BPA/kg-d during gestation and lactation had a significantly reduced percentage of normal estrus cycles compared with control F_1 rats (76.0% vs. 96.0%, respectively). (Diestrus was extended in treated F_1 females.)
Nikaido et al., 2004 NTP-A, NTP-B, EU 2008	Outbred Crj:CD-1 (ICR) timed pregnant mice	4 daily, s.c. injections beginning on GD 15	0.5 or 10 mg/kg-d	• In BPA-groups, the % of time spent in diestrus phase was significantly longer than in untreated controls (38.9 ± 2.0% for the 0.5 mg BPA/kg-d group, and 40.5 ± 1.2 % in the 10 mg BPA/kg-d group vs. 24.2 ± 2.1 % for the controls). • At 4-weeks of age CL were absent in both low- and high-BPA groups. CL were present in BPA-treated mice sacrificed at 8-, 12-, or 16-weeks of age.
Takagi et al., 2004 NTP-A, NTP-B, EU 2008	Crj: CD (Sprague-Dawley) IGS rats	oral BPA mixed with diet given from GD 15–PND 10	0, 60, 600, or 3000 ppm	• Onset of puberty (vaginal opening and preputial separation) was not affected by any dose of BPA. • Some BPA-exposed animals exhibited extended diestrus, but there was no increase in the incidence compared to the corresponding controls (0 ppm group had 1 ♀ that had extended diestrus, 60 ppm group had 2 ♀ that had extended diestrus; however, n=8 total for adulthood examination (2 per treatment group)).

Table C5. Alterations in estrus cycle length and onset of vaginal estrus (continued).

Reference	Species	Exposure	Dose	Findings
Kato et al., 2003 NTP-A, NTP-B, EU 2008	Sprague-Dawley (Crj: CD (IGS)) neonates	injections once a day for 10 d from PND 0–9	0, 0.25, 1, 4 mg BPA; [0, 12.5, 50, and 200 mg/ml (BPA and ethanol mixed with corn oil)]	• In the 1 and 4 mg BPA groups, there were irregular/persistent estrus cycles (6 of 8 in the 1 mg BPA group and 4 ♀ in the 4 mg BPA group had irregular estrus, and 2 ♀ in the 4 mg BPA group had persistent estrus).
Talsness et al., 2000 NTP-A, NTP-B	gravid Sprague-Dawley rats	oral gavage on GD 6–21	0.1 mg/kg-d (low) and 50 mg/kg-d (high)	• Exposure to 50 mg/kg-d ↑ the proportion of total estrus cycles with estrus phases greater than 1 d in length. • Cycle length was also increased following exposure to 50 mg/kg of BPA. • The percentage of ♀ exhibiting 3 consecutive cycles of 4 d in length was ↓ for both BPA doses, but not significant.
Rubin et al., 2001 NTP-A, NTP-B, EU 2003, EU 2008	**group 1:** pregnant ♀, **group 2:** ovariectomized young adult ♀	**group 1**: BPA in drinking water from GD 6 through the period of lactation (pups supplied with unadulterated water at weaning), **group 2**: BPA in drinking water for 3 d	1 mg/L (low dose; approx. 0.1 mg/kg bw-d consumed), 10 mg/L (high dose; approx. 1.2 mg/kg bw-d consumed), and 100 mg/L only for the uterotrophic assay (group 2 ♀)	• Offspring of BPA-treated ♀ exhibited an ↑ in body weight compared with controls. • ♀ exposed perinatally to the high dose of BPA exhibited altered patterns of estrous cyclicity (some animals had intermittent extended periods of diestrus, whereas others exhibited extended periods of proestrus and/or estrus), and ↓ levels of plasma luteinizing hormone (LH) in adulthood compared with control ♀ after long-term ovariectomy.

d: day(s)
CL: corpora lutea
LH: luteinizing hormone
*: Not in NTP-A

C.2.5. Effects on fertility

Fertility can be one of the least sensitive endpoints in laboratory animal studies (Schwetz et al., 1980). Some multi-generation and continuous breeding studies report effects on fertility; however, fertility assessments drawn from these studies may be less precise compared with studies specifically designed to assess fertility. For example, assessing oocyte fertility by *in vivo* mating trials is imprecise because the normal rat ejaculate has approximately ten-fold more sperm than needed for maximum fertilization (Aafjes et al., 1980; Working, 1988). It has been suggested that a more fertile male may compensate for a less fertile female (Smith et al., 1977; Steinberger et al., 1979). Despite the relative insensitivity of this measure, the multi-generation and continuous breeding studies discussed below show general trends of reduced female fertility as a result of BPA treatment.

C.2.5.1. Multi-generation studies

In a three-generation reproductive toxicology study of dietary BPA in Sprague-Dawley rats, the results suggest fertility is altered. Target dietary concentrations of 0, 0.015, 0.3, 4.5, 75, 750, or 7500 ppm BPA were given to Sprague-Dawley rats to provide BPA intakes of approximately 0, 0.001, 0.02, 0.3, 5, 50, or 500 mg/kg-d. The F_0 generation was fed BPA in diet for a 10-week pre-breeding exposure period, during mating, during gestation, and females through lactation until weaning. The F_1 and F_2 generations were exposed to BPA in diet for the same periods as the F_0 generation, but also received indirect gestational, lactational, and weaning exposures. The F_3 generation was exposed for 10 weeks during gestation, lactation, weaning, and post-weaning periods. The mean number of implantation sites/dam in the F_1 and F_2 generations, and the number of total pups/litter in the F_2 and F_3 generations of the 7500 ppm BPA group was significantly lower compared with untreated controls. There was a similar reduction trend in the mean number of implantation sites/dam in the F_1 and F_2 generations and the number of total pups/litter in the F_2 and F_3 generations of the 0.3, 75 and 750 ppm BPA groups, although this trend was not statistically significant (Tyl et al., 2002b).

In a two-generation reproductive toxicology study of dietary BPA in CD-1 (Swiss) mice, the results also suggest altered fertility. Mice were given BPA in their rodent chow at 0, 0.018, 0.18, 1.8, 30, 300, or 3500 ppm. Approximate BPA intake was 0, 0.003, 0.03, 0.3, 5, 50, or 600 mg/kg-d. The mice had a lengthy mating period of 14 days. Results included the following:

- The gestational index [(# females with live litters/# pregnant females) x 100] had a apparent declining trend with increasing dose up to 300 ppm, and the 3500 ppm dose is comparable with the negative control (0 ppm) (Tyl et al., 2008b).
- The still birth index tended to increase up to the 3500 ppm dose, with an exception at the 30 ppm dose, which was still greater compared with the control group (Tyl et al., 2008b).
- The live birth index had an apparent declining trend with BPA treatment at the 0.18 to 3500 ppm dose range (Tyl et al., 2008b).
- The percentage of post-implantation loss per litter with BPA treatment tended to decline except for the 300 ppm group; the 300 ppm group had more loss compared with the control group (Tyl et al., 2008b).

Table C6. Fertility trends in multi-generation reproductive toxicity studies.

Three Generation Sprague-Dawley Rat Study (Tyl et al., 2002b)							
Dose in feed (ppm)	0	0.015	0.3	4.5	75	750	7500
# implantation sites/dam							
F_0	14.23 ± 0.62	15.04 ± 0.51	14.93 ± 0.49	13.93 ± 0.61	14.74 ± 0.64	14.04 ± 0.48	11.89 ± 0.52 **
F_1	15.86 ± 0.44	16.33 ± 0.46	15.13 ± 0.64	14.85 ± 0.79	15.33 ± 0.39	16.00 ± 0.38	11.93 ± 0.43 ***
F_2	15.25 ± 0.33	15.03 ± 0.38	14.03 ± 0.53	14.19 ± 0.73	15.11± 0.39	14.44 ± 0.33	12.44 ± 0.29 ***
# total pups/litter							
F_1	14.4 ± 0.6	14.9 ± 0.7	14.3 ± 0.5	13.5 ± 0.6	14.0 ± 0.5	13.1 ± 0.6	11.8 ± 0.4 **
F_2	14.9 ± 0.6	15.1 ± 0.5	14.5 ± 0.7	14.7 ± 0.7	14.5 ± 0.5	15.0 ± 0.5	11.1 ± 0.5 ***
F_3	14.9 ± 0.4	14.3 ± 0.4	13.3 ± 0.5 *	13.8 ± 0.6	14.1 ± 0.4	13.8 ± 0.4	11.2 ± 0.4 ***
Two Generation CD-1 Mouse Study (Tyl et al., 2008b)							
Dose in feed (ppm)	0	0.018	0.18	1.8	30	300	3500
Gestational Index (%)							
F_0	92.7	100.0	96.2	96.4	96.4	81.5	96.4
F_1	100.0	96.2	100.0	100.0	96.4	92.3	100.0
Still Birth Index (%) on PND 0							
F_1	0.4 ± 0.4	0.9 ± 0.7	6.2 ± 3.9	6.0 ± 3.9	2.9 ± 2.6	9.5 ± 5.7	9.1 ± 4.5
F_2	1.6 ± 0.7	0.9 ± 0.7	1.6 ± 0.8	1.4 ± 0.8	0.9 ± 0.6	2.2 ± 1.4	0.0 ± 0.0
Live Birth Index (%)							
F_1	99.6 ± 0.4	99.1 ± 0.7	93.8 ± 3.9	94.0 ± 3.9	97.1 ± 2.6	90.5 ± 5.7	90.9 ± 4.5
F_2	98.4 ± 0.7	99.1 ± 0.7	98.4 ± 0.8	98.6 ± 0.8	99.1 ± 0.6	97.8 ± 1.4	100.0 ± 0.0
% Post-implantation loss/litter							
F_1	11.7 ± 3.6	2.9 ± 1.0	8.5 ± 3.0	8.4 ± 3.2	6.8 ± 3.6	17.9 ± 6.5	5.6 ± 1.5
F_2	6.0 ± 1.5	6.2 ± 3.8	4.2 ± 1.2	5.3 ± 1.6	9.7 ± 3.8	15.3 ± 5.5	9.4 ± 3.3

* $P<0.05$; statistically significant difference as compared to control values; data presented as mean ± SEM.

** $P<0.01$; statistically significant difference as compared to control values; data presented as mean ± SEM.

*** $P<0.001$; statistically significant difference as compared to control values; data presented as mean ± SEM.

C.2.5.2. Reproductive Assessment by Continuous Breeding (RACB) biosassay

In CD-rats given 0, 160, 320, and 640 mg BPA/kg-d on GD 6–15, there was a noticeable, although not statistically significant, decrease in the number of live fetuses per litter compared with the number of implantation sites per litter in the 320 and 640 mg BPA/kg-d groups (Morrissey et al., 1987). In the 0 mg BPA/kg-d group, there was no difference in the number of live fetuses per litter compared with the number of implantation sites per litter (Morrissey et al., 1987).

In CD-1 mice given 0, 500, 750, 1000, and 1250 mg BPA/kg-d on GD 6–15, there was a decrease in the number of live fetuses per litter compared with the number of implantation sites per litter. This trend was consistent in mice exposed to 0, 500, 750, 1000, and 1250 mg BPA/kg-d (Morrissey et al., 1987).

In another continuous breeding study, BPA was administered via implant and feed to 6 week old COBS Crl:CD-1 (ICR) BR outbred Swiss albino mice. The mid-dose BPA implant (50 mg/mouse; an estimated daily dose of 0.02 g/kg body weight) significantly increased the mean number of live pups per pair (Morrissey et al., 1989). The mid- and high-doses of 0.50 and 1.00% in feed (estimated daily doses of 0.90 and 1.88 g/kg body weight, respectively) significantly reduced the mean number of litters per pair and the mean number of live pups per pair, while there was a significant increase in the mean live pup weight per litter (Morrissey et al., 1989). The high-dose group also had a significant reduction in the proportion of pups born alive (Morrissey et al., 1989).

C.2.6. Effects on the vagina

Studies have found that different species and strains of laboratory rodents are likely to exhibit different vaginal effects due to different sensitivities to BPA. The F344 and Sprague-Dawley rat strains exhibit different levels of cell proliferation in the vaginal epithelium after exposure to BPA (Long et al., 2000). Sprague-Dawley rats exposed to 0.1 mg BPA/kg-d or 50 mg BPA/kg-d *in utero* on GD 6–21 showed morphological changes in differentiation, stratification, and cornification of the vagina during estrus (Talsness et al., 2000b; Schönfelder et al., 2002a). More specifically, keratinization of the vaginal epithelium was reduced and full length ERα was not expressed during estrus in the vagina of female offspring exposed to 0.1 or 50 mg BPA/kg-d when compared with controls. ERα expression did not differ from the control group during the diestrus stage (Schönfelder et al., 2002a). The down-regulation of ERα was the suggested reason for the altered vaginal morphology. Wistar derived (Alpk:APfSD) rats also had an increase in vaginal cornification compared with controls when exposed subcutaneously to 16.7 mg BPA/dose twice daily for 3 days (Ashby et al., 2000). CD-1 mice are one of the least sensitive strains to natural estrogens; however, female CD-1 mice exposed in utero to BPA had a decrease vagina weight compared with controls. At 3-months of age, female offspring exposed to 250 ng BPA/kg bw-d had significantly reduced absolute and relative vagina weights compared with controls (Markey et al., 2005). In ICR/Jcl mice exposed to 150 µg BPA/pup daily for the first 5 days of life, ovary-independent vaginal epithelial stratification was noted (Suzuki et al., 2002).

Exposure of suckling mice on PND 1–21 resulted in an increased number of vaginal epithelial cell layers in the 4, 20, and 500 µg BPA/kg-d groups compared with the controls (Fukumori et al., 2001).

Vaginal opening is a generally accepted signal of the onset of puberty in rodents. Female rats exposed *in utero* and neonatally to BPA exhibited vaginal opening at a younger age in treated compared with control rats (Talsness et al., 2000b; Kato et al., 2003; Durando et al., 2007). Similarly, female offspring exposed to BPA *in utero* via maternal s.c. injections are significantly younger compared with controls when vaginal opening occurs (Honma et al., 2002; Nikaido et al., 2004). Howdeshell et al. demonstrated in CF-1 mouse offspring that oral, prenatal treatment of dams with 2.4 µg BPA/kg significantly reduced the number of days between vaginal opening and first vaginal estrus, two events highly correlated with first post-pubertal ovulation (Howdeshell et al., 1999). Eight of 14 immature Alpk:AP rats (age 21–22 days old) exposed to 600 mg BPA/kg or 800 mg BPA/kg via 3 daily s.c. injections had premature vaginal opening (Ashby and Tinwell, 1998).

In a three-generation Sprague Dawley rat study, vaginal opening in F_1 females was significantly later in the 7500 ppm group compared with controls (Tyl et al., 2002b). The 7500 ppm BPA group exhibited vaginal opening on day 33.0 ± 0.6 vs. 30.5 ± 0.3 d by the control group (Tyl et al., 2002b). The later vaginal opening by F_1 females treated with 7500 ppm BPA compared with controls may be attributable to their lower body weights (92.32 ± 2.54 g vs. 102.52 ± 2.08 g, respectively) at acquisition of pubertal characteristics (vaginal patency), which Tyl and colleagues designated PND 22 (Tyl et al., 2002b).

Earlier vaginal opening did not occur in Donryu rat offspring exposed to BPA prenatally and postnatally. No significant inter-group difference for vaginal opening was found for control, 0.006 mg BPA/kg-d, and 6 mg BPA/kg-d groups. Vaginal opening for control female offspring occurred on day 29.4 ± 1.9, for offspring of dams exposed to 0.006 mg BPA/kg-d vaginal opening occurred on day 29.5 ± 1.4, and for female offspring of dams exposed to 6 mg BPA/kg-d vaginal opening occurred on day 30.0 ± 1.4 (Yoshida et al., 2004).

Table C7. Changes in vaginal epithelia, timing of vaginal opening, and weight resulting from exposure to BPA.

Reference	Species	Exposure	Dose	Findings
Vaginal Epithelia				
Ashby et al., 2000 NTP-A	ovariectomized rat model, assays (cytosolic ER from immature ♀Alpk:APfSD Wistar derived rats)	s.c. injection for 3 d	100 mg/rat (total volume of 3 ml; administered twice daily (0.5 ml/dose))	• Vaginal cornification was significantly ↑ (100 ± 0% cornified cells in BPA-treated vs. 8.2 ± 3.0% cornified cells in sesame oil controls, P<0.01).
Fukumori et al., 2001	sucking ♀ mice	s.c. injection 5 d/week from PND 1–21	0, 0.8, 4, and 20 µg/kg-d or 500 µg/kg-d	• ↑ # of epithelial cell layers in the 4, 20 and 500 µg/kg-d groups (5–7 layers vs. 3–4 layers in the controls).
Long et al., 2000 NTP-A, EU 2003	F344 and Sprague-Dawley rats (10–12 weeks of age)	one i.p. injection	0.2–150 mg/kg bw in 50 ml of solution [2.9 mg/kg bw for BPA clearance assessment given i.v.]	• F344 rats show a statistically significant ↑ in vaginal DNA synthesis at doses of 37.5 mg/kg and greater; Sprague-Dawley rats showed no effect of BPA on vaginal DNA synthesis.
Schonfelder et al., 2002 NTP-A, EU 2008	Sprague-Dawley rats (gravid dams)	oral gavage on GD 6–21	0.1 or 50 mg/kg-d	• Morphological changes in differentiation, stratification, and cornification of the vagina were demonstrated during estrus in post-pubertal offspring of treated dams compared with controls. • Keratinization of the vaginal epithelium was ↓. • Full length ERα was not expressed during estrus in the vagina of ♀ offspring exposed to either dose of BPA when compared with controls; ERα expression did not differ from the control group during the diestrus stage.

Table C7. Changes in vaginal epithelia, timing of vaginal opening, and weight resulting from exposure to BPA (continued).

Reference	Species	Exposure	Dose	Findings
Steinmetz et al., 1998 NTP-A, EU 2003	F344 and Sprague-Dawley rats (9–10 weeks of age)	i.p. injection and s.c. implants (silastic) for 3 d	i.p. – 0, 18.75, 37.5, 50, 75, 150, or 200 mg BPA/kg	• 37.5 mg BPA/kg caused a significant ↑ in cell proliferation in the uterus and vagina. • Within 2 h after treatment w/ BPA, levels of *c-fos* mRNA in the vagina were 7–9-fold above controls. • The thickness of the vaginal epithelium increased from 2–3 cell layers to 6–8 cell layers. • BPA ↑ keratinization of the vaginal epithelium and sloughing of surface cells.
Suzuki et al., 2002 NTP-A, NTP-B, EU 2008	ICR/Jcl mice	pregnant dams – s.c injections GD 10–18; prenatally exposed – PND 22–PND 40 (some OVX at PND 30, some mated); postnatally exposed offspring – s.c. injections for 5 d from the day of birth	pregnant dams – 10 or 100 mg BPA/kg bw (prenatally exposed offspring – 10 and 100 mg/kg); postnatally exposed offspring – 15 or 150 µg/pup	• Mice exposed prenatally to BPA did not show ovary-independent vaginal and uterine changes. • In the 150 µg BPA postnatal treatment group, ovary-independent vaginal epithelial stratification was noted as were polyovular follicles having more than one oocyte in a follicle.

Table C7. Changes in vaginal epithelia, timing of vaginal opening, and weight resulting from exposure to BPA (continued).

Reference	Species	Exposure	Dose	Findings
Earlier Vaginal Opening				
Howdeshell et al., 1999 NTP-A, NTP-B	pregnant CF-1 mice	dams fed BPA in oil during GD 11–17	2.4 µg/kg	• Prenatal exposure to BPA and intrauterine position ↓ number of days between vaginal opening and first vaginal estrus.
Ashby et al., 1998 NTP-A, EU 2003	immature Alpk:AP rats (21–22 days old)	3 daily doses via oral gavage or s.c. injection	400 mg/kg, 600 mg/kg, or 800 mg/kg	• Premature vaginal opening was seen in 8 of 14 animals exposed to 600 and 800 mg/kg by s.c. injection.
Durando et al., 2007 NTP-A, NTP-B, EU 2008	Wistar-derived rats, sexually mature ♀ (pregnant)	in utero exposure (GD 8–23) via s.c. implant with a miniature osmotic pump	25 µg/kg-d (0.25 mL/hr)	• ♀ offspring exposed in utero to 25 µg/kg-d exhibited earlier age at vaginal opening (39 ± 3 d for controls compared with 34 ± 1 d for 25 µg BPA/kg-d treated rats).
Honma et al., 2001; Honma et al., 2002 NTP-A, NTP-B, EU 2008	pregnant ICR/Jcl mice	s.c injection on GD 11–17	2 and 20 µg/kg	• Age of vaginal opening was significantly earlier (younger age) with 20 µg BPA/kg compared with controls.
Kato et al., 2003 NTP-A, NTP-B, EU 2008	Sprague-Dawley (Crj: CD (IGS)) neonates	injections once a day for 10 d from PND 0–9	0, 0.25, 1, 4 mg BPA; [0, 12.5, 50, and 200 mg/ml (BPA and ethanol mixed with corn oil)]	• In the 1 and 4 mg BPA groups, vaginal opening occurred 3 to 4 d earlier compared with controls (29.9 ± 1.2 d and 28.7 ± 1.0 d vs. 32.8 ± 1.0 d, respectively, $P<0.01$).

Table C7. Changes in vaginal epithelia, timing of vaginal opening, and weight resulting from exposure to BPA (continued).

Reference	Species	Exposure	Dose	Findings
Nikaido et al., 2004 NTP-A, NTP-B, EU 2008	Outbred Crj:CD-1 (ICR) timed pregnant mice	4 daily, s.c. injections beginning on GD 15	0.5 or 10 mg/kg-d	• Vaginal opening was significantly earlier in 10 mg/kg-d group compared with controls (24.8 ± 0.2 d vs. 26.0 ± 0.2 d, respectively).
Delayed Vaginal Opening				
Talsness et al., 2000 NTP-A, NTP-B	gravid Sprague-Dawley rats	oral gavage on GD 6–21	0.1 mg/kg-d (low) and 50 mg/kg-d (high)	• Low dose BPA caused a delay in vaginal opening of approximately 5.6 d; high dose resulted in a somewhat earlier vaginal opening (approximately 1.9 d). • Cornification of the vaginal epithelium was the main histological feature at estrus. • The cornified layer and the width of the total epithelium was thinner following exposure to 0.1 mg BPA/kg-d.
Yoshida et al., 2004 NTP-A, NTP-B, EU 2008	pregnant Donryu rats (Crj:Donryu rats)	oral gavage from GD 2 to the day before weaning (PND 21)	0, 0.006 mg/kg or 6 mg/kg	• No significant inter-group differences in days of vaginal opening were found. • Vaginal opening for ♀ offspring born from control dams occurred on d 29.4 ± 1.9, for ♀ offspring of dams exposed to 0.006 mg BPA/kg-d vaginal opening occurred on d 29.5 ± 1, and for ♀ offspring of dams exposed to 6 mg BPA/kg-d vaginal opening occurred on d 30.0 ± 1.4.
Vaginal Weight Alteration				
Markey et al., 2005 NTP-A, EU 2008	CD-1 mice	in utero exposure for 14 d (dams were s.c. implanted with osmotic pumps from GD 9 until PND 4)	25 or 250 ng/kg bw/d	• At 3-months of age, ♀ offspring exposed to 250 ng/kg bw-d BPA had ↓ absolute and relative vagina weights compared with controls (63.38 ± 2.77 mg vs. 83.11 ± 5.91 mg, and 0.227 ± 0.011 vs. 0.306 ± 0.025, respectively).

d: days OVX: ovariectomized

C.2.7. Effects on the mammary gland

Exposure to BPA has been reported to perturb the cell cycle of the epithelial cells of the mammary gland. Cell cycle alteration in the mammary gland is typically associated with carcinogenesis. Terminal end buds (TEB) are the structures in which mammary cancer originates in both rodents and humans. An increase in the number of TEB, terminal ends (TE), ductal density, and sensitivity to estradiol have commonly been noted after BPA exposure.

Moral et al. reported that pregnant Sprague-Dawley rats exposed to 250 µg BPA/kg bw had female offspring that at 21 d of age had significantly more TEB as the main epithelial structure compared with the low dose group (25 µg BPA/kg bw). Terminal end buds decreased in number as rats got older, and numbers of terminal ducts (TD) increased with age (Moral et al., 2008).

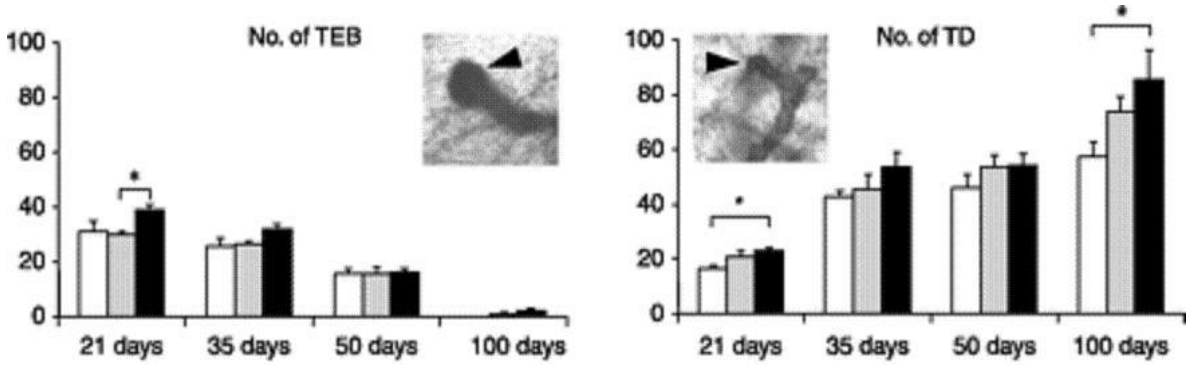

Figure F1. Morphological analysis of the mammary gland. Total number (mean ± SEM) of TEB and TD in the mammary glands from control (white bars) and BPA-exposed (grey bars: low dose [25 µg BPA/kg bw], black bars: high dose [250 µg BPA/kg bw]) rats at different ages of development. * significantly different ($P<0.05$) (Moral et al., 2008).

The 250 µg BPA/kg bw dose induced changes in genes related to differentiation suggesting alterations in normal development of the mammary gland. At 100 d of age, the 250 µg BPA/kg bw group exhibited 330 up-modulated genes (114 known) and 91 down-modulated expression sequences (42 known genes). Among the up-regulated genes, Moral et al. found an important cluster related to immune response (Cd3d, Ctse, Cd5, Ltb, Cxcl10, Ccl5, Mefv, Cd2, A2m, and Il1b) (Moral et al., 2008). Pregnant CD-1 mice treated with environmentally relevant doses of BPA (ng amounts) produced female offspring who at 30 days of age had a significant increase in the number of TEB relative to the area occupied by the ductal tree compared with controls (Muñoz-de-Toro et al., 2005). The increased number and area of TEB relative to the ductal area in the BPA-exposed animals suggested that ductal growth was impaired. A significant decrease in the number of apoptotic cells in TEB of treated groups relative to controls was also noted (Muñoz-de-Toro et al., 2005). The decreased apoptotic activity suggests impaired ductal growth and may explain the increased number of TEB/ductal area. Colerangle and Roy demonstrated the mammary cells of 4–5 week old female Noble rats exposed to BPA for 11 days via subcutaneously implanted alzet osmotic minipumps had their mammary epithelial cell cycle altered. Specifically, epithelial cells of the mammary gland had an increase in proliferation and a perturbation of cell cycle kinetics (Colerangle and Roy, 1997). In utero exposure on GD 8–23 of

Wistar-derived rats to 25 µg/BPA/kg bw-d BPA induced mammary gland alterations in female offspring at puberty (Durando et al., 2007). The mammary gland stroma of BPA-treated animals exhibited morphologic changes in the extracellular matrix. A dense stroma layer formed around mammary epithelial structures, and a fibroblastic stroma replaced the normal adipose tissue of the mammary gland exhibited by controls (Durando et al., 2007).

Markey et al. noted that in utero exposure to BPA induced changes in the timing of developmental events within the epithelium and stroma of the mammary gland, resulting in mammary tissue resembling that of early pregnancy (Markey et al., 2001a; Vandenberg et al., 2008). Mammary glands of control and BPA-treated 10 d old mice were not significantly different (Markey et al., 2001a). At one month of age, mice exposed in utero to 25 µg BPA/kg showed a greater ductal elongation beyond the edge of the lymph node, whereas those exposed to 250 µg BPA/kg showed retarded growth relative to the control group (Markey et al., 2001a). By 6 months of age, both 25 and 250 µg BPA/kg groups showed a significant increase in all ductal and alveolar structures relative to the control group (Markey et al., 2001a). The sensitivity of the mammary gland to estrogen (E_2) can also be increased in perinatally BPA-exposed CD-1 female mice that were ovariectomized before puberty (Wadia et al., 2007).

In another study using CD-1 mice, exposure of dams to 250 ng BPA/kg bw-day from GD 8–18 significantly increased ductal area and ductal extension in the mammary glands of exposed fetuses (assessed on GD 18), and reduced the effects of intrauterine positional differences (Vandenberg et al., 2007b). Intrauterine positional effects are attributable to the location of the fetus in the uterus with respect to the sex of the fetuses in close proximity (Howdeshell and vom Saal, 2000). Altered growth, cell size, and lumen formation was also observed in the epithelial compartment of the mammary gland. In control animals, epithelial cells were more rounded or oval-shaped along the outer border of the epithelial cord and arranged in a "tight manner" (Vandenberg et al., 2007b). The epithelial cells of BPA-exposed animals were more spindle-shaped and evenly spaced within the epithelial cord (Vandenberg et al., 2007b). In outbred Crj:CD-1 (ICR) mice exposed to 0.5 or 10 mg BPA/kg via s.c. injections on GD 15–18, mammary gland development was altered when female offspring were examined. Thoracic mammary glands were arbitrarily scored from 1 to 4 using the following criteria:

- Score 1 – low degree of differentiation, TEB in the periphery with lateral buds but no alveolar development;
- Score 2 – small number of alveoli in poorly developed ductal tree;
- Score 3 – intermediate development of ductal and alveolar structure;
- Score 4 – high degree of development, and lobulo-alveolar formation in the gland.

At 4 weeks of age, 2 out of 3 high-dose (10 mg BPA/kg) treated mice with CL showed alveolar differentiation with some alveoli showing secretory activity (score 3) (Nikaido et al., 2004).

Studies report that BPA treatment during pregnancy may also alter milk production in mice. A few studies have shown prolactin levels can be altered by exposure to BPA. Prolactin is a hormone known to positively regulate the secretion of breast milk in maternal mice. From GD 14 until delivery, ddY mice were fed 1% BPA (w/w) in feed. Subsequent results showed maternal serum prolactin levels were significantly less compared with controls, and offspring weighed significantly less compared with controls (Matsumoto et al., 2004). On the contrary, Fisher 344 rats exposed to 100 or 500 µg BPA/day on PND 1–5 had increased levels of

prepubertal serum prolactin when assessed up to day 30 of life compared with controls (Khurana et al., 2000). Prolactin levels in controls increased from PND 15–20 and remained unchanged from PND 20–30, whereas all females treated with BPA became hyperprolactinemic (serum prolactin increased from PND 20–30) (Khurana et al., 2000). Hyperprolactinemia is associated with infertility in women. The data showing different effects of BPA exposure on the mammary gland further demonstrate that the time of exposure is crucial, and can result in effects on the mother and female offspring.

Table C8. Alterations in mammary gland development resulting from perinatal and postnatal BPA exposure.

Reference	Species	Exposure	Dose	Findings
Prenatal Exposure				
Durando et al., 2007 NTP-A, NTP-B	Wistar-derived rats, sexually mature ♀ (preg-nant)	*in utero* exposure (GD 8–23) via s.c. implant with a miniature osmotic pump	25 µg/kg bw-d (0.25 mL/hr)	• ↑ proliferation/apoptosis ratio in both the epithelial and stromal compartments. • ↓ age at vaginal opening after prenatal exposure to BPA compared with controls. • During adulthood, ♀ offspring showed an ↑ number of hyperplastic duct and augmented stromal nuclear density.
Markey et al., 2001 NTP-A, NTP-B	CD-1 mice (8 weeks old)	*in utero* exposure on GD 9–20 via s.c. osmotic pumps in dams	25 or 250 µg/kg bw	• Mice showed differences in the rate of ductal migration into the stroma at 1-month of age, and a significant ↑ in the percentage of ducts, TD, TEB, and alveolar buds at 6-months of age. • The percentage of cells that incorporated BrdU (indicator of DNA synthesis) was significantly ↓ within the epithelium at 10 d of age and ↑ within the stroma at 6-months of age.
Matsumoto et al., 2004 NTP-A	ddY strain (preg-nant) mice	1% (w/w) in chow, which is equivalent to 1000 mg BPA/kg	526 mg/14 d/mouse	• Growth of newborn pups was markedly suppressed when maternal mice were fed BPA in their diet. • On PND 0, body weight of pups from dams fed BPA was similar to that of control pups. • On PND 1, the average weight of a BPA pup's stomach was 28.13 ± 3.33 mg compared with 46.54 ± 8.41 mg for controls. • On PND 4, the mean serum prolactin level of dams fed BPA was 137.65 ± 9.45 ng/ml compared with 254.60 ± 30.0 ng/ml in controls. • By PND 7, 30% of pups in the BPA group died.

Table C8. Alterations in mammary gland development resulting from perinatal and postnatal BPA exposure (continued).

Reference	Species	Exposure	Dose	Findings
Moral et al., 2008 NTP-B	Sprague-Dawley CD rats	oral gavage on d 10–21 post conception	25 µg/kg BW (low dose); 250 µg/kg BW (high dose)	• 25 µg/kg and 250 µg/kg exposures changed the gene expression signature of the mammary gland (low dose had the highest effect by 50 d, high dose had the highest influence on gene expression by 100 d). • At 21 d of age, the main epithelial structure was TEB, and its # ↓ over time in all groups; the # of TD ↑ with age in all groups; and lobules type 1 were significantly ↑ in the high-dose group in comparison with low-dose and control groups by 35 d of age. • The # of genes with changes in the gene expression was low at 21 d and lower at 35 d. At 21 d, the low-dose group had 31 genes ↑-modulated, the high-dose group had 65 ↑-regulated genes.
Nikaido et al., 2004 NTP-A, NTP-B, EU 2008	Outbred Crj:CD-1 (ICR) timed pregnant mice	4 daily, s.c. injections beginning on GD 15	0.5 or 10 mg/kg-d	• At 4 weeks of age, 2 out of 6 high-dose BPA-treated ♀ showed accelerated mammary gland differentiation (score 3). • At 8 weeks of age, 4 out of 6 low-dose BPA-treated ♀ had a mammary differentiation score of 3, and 1 had a score of 2; whereas 4 out of 6 high-dose BPA-treated ♀ had a mammary differentiation score of 3, and 1 had a score of 4.
Vandenberg et al., 2007 NTP-A, NTP-B	CD-1 dams (mice)	implanted with Alzet osmotic pumps from GD 8–18	250 ng/kg bw-d	• BPA-exposure significantly ↑ ductal area (0.098 ± 0.004 mm^2 in controls vs. 0.116 ± 0.08 mm^2 in BPA-exposed) and ductal extension (0.741 ± 0.018 mm in controls vs. 0.835 ± 0.030 mm in BPA-exposed) in mammary glands. • Intrauterine position affected mammary gland development: ♀ positioned between 2♀ had fewer branching points and TE than ♀ positioned between 1♂ and 1♀, and ♀ positioned between 2♂. • In BPA-exposed animals, 0♂ females had a significant ↑ in branching points compared with 0♂ control females, and exposure to BPA caused an ↑ in ductal extension in 1♂ and 2♂ females compared with control counterparts. • BPA exposure ↑ maturation of the fat pad (↓ density of fat pad) and altered the localization of collagen (↓ density of collagen deposits). • BPA led to a ↓ in cell size and delayed lumen formation.

Table C8. Alterations in mammary gland development resulting from perinatal and postnatal BPA exposure (continued).

Reference	Species	Exposure	Dose	Findings
Perinatal Exposure				
Khurana et al., 2000 NTP-A	Fisher 344 rats	s.c. injection on PND 1–5	100, or 500 µg/d	• BPA induced delayed, but progressive, ↑ in serum prolactin levels up to 3-fold above control levels. • Prolactin levels in controls increased from PND 15–20 and remained unchanged from PND 20–30, whereas all ♀ treated with BPA became hyperprolactinemic. • Serum prolactin levels progressively increased from PND 20–30 in response to treatment with BPA.
Munoz-de-Toto et al., 2005 NTP-A, NTP-B	pregnant CD-1 ♀ mice	s.c. implanted osmotic pumps from GD 9–PND 4 (14 d)	25 or 250 ng/BPA/kg bw-d	• ♀ offspring exposed to 250 ng BPA/kg bw-d had a significant ↑ in the number of TEB relative to the area occupied by the ductal tree compared with controls at PND 30. • The ↑ in the number of TEB relative to the area occupied by the ductal tree in the 25 ng BPA/kg bw-d group approached significance (P=0.054) compared with controls. • *In utero* exposure to BPA resulted in a significant ↓ in the number of apoptotic cells in TEB of both treated groups relative to controls.
Vandenberg et al., 2008	sexually mature CD-1 mice	s.c. implanted Alzet osmotic pumps from GD 8–PND 16	0, 0.25, 2.5 or 25 µg BPA/kg bw-d	• BPA-exposed ♀ had altered mammary phenotypes including appearance of alveolar buds and intraductal hyperplasia. • 3-month old ♀ exposed to 0.25 µg/kg bw-d (0.25BPA) had a significantly ↑ volume fraction of alveolar buds compared with controls. By 9-months of age, 0.25BPA had a significantly ↓ volume fraction ducts compared with controls, and 2.5BPA had a significantly ↑ volume faction alveolar buds compared with controls. • At 9-months, the incidence of beaded ducts was significantly higher in 0.25BPA, 2.5BPA, and 25BPA compared with controls. • By 12–15 months, the incidence of beaded ducts was significantly higher in 0.25BPA compared with controls.

Table C8. Alterations in mammary gland development resulting from perinatal and postnatal BPA exposure (continued).

Reference	Species	Exposure	Dose	Findings
Wadia et al., 2007 NTP-B	CD-1 and C57Bl6 mice	s.c. Alzet osmotic pumps (implants) GD 8–PND 2	0 or 250 ng BPA/kg-d	• Perinatal BPA exposure altered responses to E_2 at puberty for several parameters in both strains, although the effects in CD-1 was slightly more pronounced. • Number of TEB in 250BPA mice ↑ significantly over that observed in 0BPA mice after administration of 0.5 mg E_2/day. • TEB/area and TEB area/area were significantly ↓ in the 250BPA mice treated with 1 mg E_2/kg-d compared with 0BPA in C57Bl6 mice. • ↑ uterine wet weight in both strains with ↑ E_2. • Perinatal exposure to BPA significantly altered the response to E_2 later in life in both strains.
Juvenile / Adult Exposure				
Colerangle et al., 1997 EU 2003	Noble rats (5–6 weeks of age)	alzet osmotic minipumps implanted s.c. in the dorsal side of the cervical region of the rat for 11 d	0.1 mg/kg-d (low), 54 mg/kg-d (high), control received DMSO (vehicle)	• Conversion of immature structures to mature structures was significantly ↑ in response to low (proliferative activity ↑ 143%) and high (proliferative activity ↑ 220%) dose of BPA compared with controls.

BrdU: bromodeoxyuridine
2♀: 2 females
1♂: 1 male
1♀: 1 female
2 ♂: 2 males
0 ♂: no males
TEB: terminal end buds
TD: terminal ducts
E_2: estrogen
TEB/area: number of TEB per ductal area
TEB area/area: area of all TEB per ductal area

C.2.8. Maternal-fetal transfer of BPA

Some animal literature indicates BPA can traverse the maternal-fetal unit. In pregnant Sprague-Dawley rats injected intravenously with 2 mg BPA/kg on GD 17–19, the mean systemic clearance was 40.1 ± 14.8 ml/kg/min (Shin et al., 2002). Bisphenol A was rapidly distributed to the placenta, fetus, and amniotic fluid. Fetal tissue levels of BPA exceeded the maternal serum levels during most of the sampling period. The maximum concentrations in the placenta and fetus were 1399.2 and 794.0 ng/ml, respectively, and the maximum concentration of BPA in maternal serum was 927.3 ± 194.3 mg/ml (Shin et al., 2002). Pregnant female F344/DuCrj (Fisher) rats given 1g BPA/kg orally (25% wt/v in propylene glycol) on GD 18 demonstrated similar maternal-fetal transfer of BPA (Takahashi and Oishi, 2000). Maternal blood BPA levels reached a maximal concentration (14.7 µg/g) 20 minutes after dosing; the maximal level was 0.007% of the administered dose per gram of blood (Takahashi and Oishi, 2000). The BPA concentration in whole fetuses reached maximal concentration 20 minutes after administration (9.22 µg/g) (Takahashi and Oishi, 2000). In pregnant Wistar rats administered 10 mg BPA/kg on GD 19, concentrations of BPA peaked in maternal blood plasma and fetuses within 1 hour to 34 parts per billion (ppb) and 11 ppb, respectively (Miyakoda et al., 1999). Concentrations of BPA continued to fluctuate (down then up) in Wistar fetuses over 24 hours following oral administration, while maternal blood plasma BPA continued to decline. In pregnant ICR mice given 10 mg ^{14}C-BPA/kg on GD 15, many tissues (such as the ovary, uterus, and mammary gland) achieved peak concentration twice (at 20 minutes and 6 hours post-dosing). The concentration of radioactive ^{14}C-BPA in whole fetuses at 24 h was almost half of the one in maternal blood. No obvious difference was observed between the concentration in male and female fetuses (Kawamoto et al., 2005).

Toxicokinetic data in pregnant DA/Han rats administered BPA on GD 18 via i.v. injection also demonstrate that BPA reaches the fetal tissue and placenta (Moors et al., 2006). Levels of BPA in maternal tissues 30 minutes after i.v. injection include 6.2 µg/g for the uterus and 4.0 µg/g for placenta (Moors et al., 2006). Total BPA levels in the fetal liver at 20 and 30 minutes post-injection were 3.4 and 3.3 µg/g, respectively (Moors et al., 2006). These results suggest that the absorption and distribution of BPA in maternal organs and in fetuses are extremely rapid and that BPA can easily pass through the placenta. Maternal exposure of CF-1 mice to BPA via diet can transfer to fetuses and consequently alter postnatal development and sexual maturity at doses typically found in the environment (2.4 µg/kg) (Howdeshell et al., 1999).

An *in vitro* study of blastocysts exposed to BPA supports *in vivo* data that shows maternal exposure to BPA can result in reproductive changes of female offspring. Blastocysts from B6C3F1 female mice were cell cultured in the presence of 0 (control), 1 nM or 100 µM BPA for 48 hours and subsequently implanted into untreated ICR female mice. Pups were born spontaneously and at weaning (PND 21), pups that developed from blastocysts treated with 1 nM and 100 µM BPA were significantly heavier compared with controls (13.5 ± 1.6 g and 13.0 ± 1.6 g vs. 9.7 ± 2.8 g, respectively) (Takai et al., 2001). Increased body weight controlled for age has been associated with precocious puberty (Nikaido et al., 2004; Golub et al., 2008).

Evidence also suggests maternal exposure to BPA can be transferred to offspring via lactation. Vandenberg et al. exposed CD-1 female mice to 0.25, 2.5 or 25 µg BPA/kg bw-d from GD 8–PND 16 then examined mammary gland development in female offspring. At 3 months of age, female offspring exposed to 0.25µg BPA/kg bw-d showed a significant increase in the number of alveolar buds compared with controls (Vandenberg et al., 2008). In animals exposed to BPA through PND 2, these alterations were not observed until six months of age (Markey et al., 2001a).

In a two-generation CD-1 mouse study, F_1 offspring from dams treated with 3500 ppm BPA throughout lactation weighed significantly less compared with untreated controls on PND 7, 14, and 21 (Tyl et al., 2008b). These results suggest the dams may have had altered milk quality, milk quantity, or nursing behavior. Nursing behavior is reduced after exposure of pregnant dams to BPA (Palanza et al., 2002b). Dams exposed to BPA either as fetuses or during adulthood spent less time nursing their pups and more time out of the nest compared with the control group (Palanza et al., 2002b). Additional information on the Palanza et al. study is available in the developmental toxicity section B.2.2.3.1.4 "Studies with 10 µg/kg-d BPA in CD-1 mice".

Table C9. Maternal-fetal transfer of BPA.

Reference	Species	Exposure	Dose	Findings
Kawamoto et al., 2005	pregnant ICR mice (8 weeks of age)	orally on GD 15	1, 10, or 100 mg ^{14}C-BPA/5ml/kg	• In the 10 mg/kg bw group, many tissues (such as the ovary, uterus, and mammary gland) achieved peak concentration twice (at 20 minutes and 6 hours post-dosing). • The concentration of radioactive ^{14}C-BPA in whole fetuses at 24 h was almost half of the one in maternal blood. • No obvious difference was observed between the concentration in ♂ and ♀ fetuses. • Radioactivity appeared in fetuses soon after dosing. • Repeated administration of ^{14}C-BPA appeared to accumulate in maternal blood – ex: concentration at 24 h following second dosing was higher than the one at 24 h following first dosing.
Miyakoda et al., 1999 NTP-A, EU 2003	pregnant Wistar rats	oral dose on GD 19	10 mg/kg	• The concentration of BPA in both maternal blood plasma and fetuses peaked within 1 hour of administration to 34 ppb and 11 ppb, respectively. • At 3 h, concentration of BPA in maternal blood plasma had ↓ to 10% of peak value, and in fetuses was only about 40% of peak value, by 24 h the fetal concentration had ↑ to nearly 70% of peak value.
Moors et al., 2006 NTP-A	DA/Han rats	i.v. injection on GD 18	10 mg/kg bw	• Total BPA levels in maternal plasma – 3.8 μg/ml measured shortly after injection, ↓ to 0.7 μg/ml after 2 hr. • Total BPA in maternal tissues – 9.7 and 9.3 μg/g in the maternal liver 20 and 30 minutes after injection, respectively. • Total BPA in maternal kidney 20 and 30 minutes after injection were 7.6 and 8.6 μg/g, respectively. BPA levels in other maternal tissues include 6.2 μg/g for the uterus, 4.0 μg/g for placenta (observed at the 30-minute time point). • Total BPA in the fetal liver at 20 and 30 minutes post-injection were 3.4 and 3.3 μg/g.

Table C9. Maternal-fetal transfer of BPA (continued).

Reference	Species	Exposure	Dose	Findings
Shin et al., 2002 NTP-A, EU 2008	Pregnant Sprague-Dawley rats	injected (i.v.) into the jugular vein on GD 17–19	2 mg/kg (dissolved 20:80 (v/v) in polyethylene glycol)	• The mean systemic clearance was 40.1 ± 14.8 ml/min/kg. • BPA was rapidly distributed to the placenta, fetus, and amniotic fluid, the maximum concentrations achieved within 0.6 hours after i.v. injection. • The maximum concentration in the placenta and fetus were 1399.2 and 794.0 ng/ml; the maximum maternal serum concentration was 927.3 ± 194.3 ng/ml immediately after i.v. injection.
Takahashi et al., 2000 NTP-A, EU 2003	Pregnant ♀ F344/DuCrj (Fisher) rats	oral (dissolved in propylene glycol) on GD 18	1 g/kg	• BPA in maternal blood reached a maximal concentration (14.7 μg/g) 20 min after dosing; the maximal level was 0.007% of the administered dose per gram of blood; the concentration was 2% of maximum after 6 hours. • BPA in whole fetuses reached maximal concentration after 20 min (9.22 μg/g). The fetal maximal level of BPA was 0.0004% of the dose per gram of fetus.
Tyl et al., 2008	VAF Crl:CD-1 (ICR) BR mice (also known as CD-1 Swiss mice)	diet	0, 0.018, 0.18, 1.8, 30, 300, 3500 ppm BPA in feed	• F_1 offspring from dams treated with 3500 ppm BPA throughout lactation weighed significantly less compared with untreated controls on PND 7, 14, and 21.

ppb: parts per billion
min: minutes

C.2.9. Maternal behavior

Using an exposure period beginning premating through lactation, maternal behavior in rats exposed orally to 40 µg/kg-d BPA was evaluated at PND 3, 4 and PND 8, 9 (Della Seta et al., 2005). The analysis reported generally reduced behavior in terms of duration and frequency of licking-grooming, anogenital licking and arched-back posture; the change in duration of licking-grooming was significant by ANOVA at $P<0.05$. Nest building was not affected.

A study using prenatal exposure (GD 11–18, GD 14–18) to 10 µg/kg BPA in CD-1 mice examined maternal behavior (Palanza et al., 2002b). Bisphenol A was found to decrease nursing behavior in mice that were treated with BPA either *in utero* or during pregnancy as adults. This is in general agreement with the study of BPA effects on maternal behavior in rats by Della Sella et al. (Della Seta et al., 2005).

C.3. Mechanism of toxicity overview

As discussed at length in Section E, exposure to low doses of BPA has been observed to produce effects in endocrine organs including the androgen or estrogen responsive tissues, the immune system, thyroid hormone function, and the developing nervous system (Richter et al., 2007; Vandenberg et al., 2007a; Wetherill et al., 2007). Much of the attention directed toward BPA as a female reproductive toxicant is based on its ability to bind to estrogen receptors (ERα and ERβ). The highest expression of ERβ mRNA has been detected in the ovary of rats, with modest expression in the uterus (Kuiper et al., 1997). The ERα mRNA was highly expressed in the uterus (Kuiper et al., 1997). Bisphenol A binds to ERα and ERβ with relatively low affinity. It was also found that the binding affinity relative to 17β-estradiol for BPA at ERβ was 6.6-fold higher than at ERα; 0.33 and 0.05, respectively (Kuiper et al., 1997). The fact that BPA binds to ERα and ERβ implicates these receptors in the mode of action for female reproductive toxicity; however, it does not exclude additional pharmacological activities of the compound contributing to the effects observed and described above (Andersen and Barton, 1999).

Evidence suggests that BPA can stimulate cellular responses at doses of at least 25 µg/kg through genomic (nuclear estrogen receptor) or non-genomic (membrane-associated or intracellular transduction) mechanisms. Nuclear estrogen receptors regulate transcription, while estrogen receptors associated with the cell membrane promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA compared with the nuclear receptors. BPA also interacts with a variety of other cellular targets such as binding to a non-classical membrane-bound form of the estrogen receptor (ncmER), the estrogen-related receptor gamma ERR-γ, a seven-transmembrane estrogen receptor called GPR30, the aryl hydrocarbon receptor (AhR), and thyroid hormone receptors (TRs) (Nadal et al., 2000; Nadal et al., 2004; Alonso-Magdalena et al., 2005; Takayanagi et al., 2006; Bonefeld-Jorgensen et al., 2007; Liu et al., 2007; Matsushima et al., 2007; Abad et al., 2008; Kruger et al., 2008; Okada et al., 2008). Relatively higher doses are required for BPA to interact with androgen and thyroid hormone receptors compared with estrogen receptors.

Changes in gene expression, which may not necessarily be adverse changes in and of themselves, also indicate biological alterations that are reported to result from BPA exposure. A few studies showed alterations in the expression of genes in the uterus and mammary gland. *In utero* exposure to BPA altered (up-or down-regulated) gene expression in the developing uterus and ovaries of Sprague-Dawley rats at the 50 to 400 mg BPA/kg-d dose ranges (Naciff et al., 2002). Moral et al. demonstrated 250 µg BPA/kg bw-d induced changes in genes related to differentiation suggesting alterations in normal development of the mammary gland. In addition to genes related to differentiation, an important cluster of altered genes were related to immune response (Moral et al., 2008).

C.4. Summary and human health relevance

Multiple studies report that exposure of female laboratory rodents to BPA during gestation, lactation, adolescence, and adult reproductive age has effects on the uterus, ovary, follicles and oocytes, estrous cycle, fertility, vagina, mammary gland. The onset of puberty (as determined by vaginal opening and estrous cyclicity), estrous cycle length, and mammary gland development appear to be the most profoundly affected.

Data from female laboratory rodents exposed to BPA suggests female reproductive toxicity concerns are applicable to humans. Girls exhibiting early onset puberty may be more at risk for the development of reproductive tract cancers later in life. For example, an early age of menarche is a risk factor for breast cancer. An early onset of puberty is also a clear indicator of increased risk for the development of metabolic syndrome and/or ovarian hyperandrogenism/PCOS in adulthood (Golub et al., 2008). Polycystic ovarian syndrome is a disorder that is characterized by infertility, hirsutism, obesity, and menstrual alterations. Estrogens are also critical to the proliferation of breast tissue and development of mammary gland. Exposure to a weak estrogen, such as BPA, may be related to earlier breast development, as well as elevating the risk of breast cancer.

The findings of some epidemiologic studies suggest an association between BPA and hormone levels, polycystic ovarian syndrome, and endometrial changes. Observed effects from *in vitro* studies of BPA's ability to displace hormones from hSHBG may also support some of these findings. However, the number of epidemiologic studies examining BPA and reproductive outcomes are relatively few. Most of the epidemiologic studies are cross-sectional studies with significant limitations such that their usefulness in determining human health effects of exposure to BPA is also limited. Many of the studies employed an ELISA method to assess exposure to BPA, which may have resulted in an overestimation of the measured BPA levels. Some studies lacked a sufficient sample size, contained inadequate description of the study participants and how they were selected, conducted inappropriate statistical analyses, or did not consider potentially important covariates or confounding factors.

Table C10. Summary of laboratory rodent BPA studies with female reproductive system endpoints.

Organ/ Endpoint	Key Findings
Uterus	• Alterations in # of implantation sites, endometrial lining (proliferation), uterine weight, and gene expression
Ovary	• Formation of cysts (cystic ovaries) • Differences in treated animal ovarian weights compared with controls
Ovarian Follicles / Oocytes	• Cystic follicles • Problems with oocyte maturation (meiotic maturation)
Estrous cycle	• Earlier (younger) age for first estrous cycle • Altered (abnormal) cycle lengths
Vagina	• Keratinization of the vaginal epithelium • Earlier (younger) age when vaginal opening occurs
Mammary gland	• Earlier onset (younger age) for mammary gland development • Variations in prolactin levels • ↑ proliferation/apoptosis ratio in both the epithelial and stromal compartments (TEB, TD, and alveolar buds) • Gene expression alterations

D. Male Reproductive Toxicity

This section presents integrative evaluations of the evidence on male reproductive toxicity of BPA. There are limited data on the male reproductive effects of BPA in men, as presented below.

There are nearly 100 studies of male reproductive effects of BPA in laboratory animals, using *in vivo* or *in vitro* approaches. An integrative evaluation of the major findings from the *in vivo* studies is presented in Section D.2. below. Detailed summaries of the major findings from the animal studies are presented in Appendix 2.

D.1. Male reproductive studies in humans

Six studies were identified that examined potential male reproductive effects of exposure to BPA in humans (Milligan et al., 1998; Dechaud et al., 1999; Luconi et al., 2001; Hanaoka et al., 2002; Bennetts et al., 2008; Cha et al., 2008), four of which were *in vitro* studies. Two of these six studies, an occupational study (Hanaoka et al., 2002) and an *in vitro* study (Luconi et al., 2001) were summarized in the NTP-CERHR report (CERHR, 2008). The occupational study reported higher concentrations of urinary BPA in 42 epoxy resin sprayers exposed to bisphenol A diglycidyl ether (BADGE) than in 42 workers with no known exposure to BADGE. No significant differences were observed in testosterone (T) or luteinizing hormone (LH) levels between exposed and unexposed workers. However, follicle stimulating hormone (FSH) concentrations were significantly lower in exposed workers (median = 5.3 IU/L) than in controls (median = 7.6 IU/L) ($p = 0.022$). All values were within the normal range. Multiple regression analysis showed a significant inverse association between urinary BPA and plasma FSH concentrations, adjusted for age and alcohol drinking habits. In contrast, a recent occupational study (Cha et al., 2008) reported a significant association between urinary BPA and higher LH concentations using multiple regression analysis, while there was no association with FSH concentrations. Significant associations were reported between work duration and lower T and higher FSH concentrations, as well as between the exposure index and lower T concentrations. The concentration of FSH observed in the exposed workers in this study (geometric mean ± geometric standard deviation = 7.68 IU/L ± 2.54) was similar to the concentration in the control group reported by Hanaoka et al. (Table D1).

Table D1. Concentration (IU/L) of FSH and LH in workers in two occupational studies.

	Exposed Workers		**Controls**	
	FSH	LH	FSH	LH
Hanaoka et al., 2002*	**5.3** (4.0–8.3)	**4.0** (4.0–5.0)	**7.6** (5.4–11.0)	**4.0** (3.0–6.0)
Cha et al., 2008**	**7.68** ± 2.54	**5.34** ± 1.68	**5.53** ± 2.11	**3.16** ± 1.40

* Median values (interquartile range)
** Geometric mean ± geometric standard deviation

Two of the four *in vitro* studies presented here investigated the direct effects of estrogenic compounds, including BPA, on human sperm. Luconi et al., (2001), as reviewed in the NTP-CERHR report (CERHR, 2008), reported no effect of BPA on the calcium response to 17 β-

estradiol (E_2) or progesterone in spermatozoa from healthy donors. The results suggested that BPA does not interact with membrane receptors for either of these compounds. In addition, no effect was seen on acrosome reaction. The sperm samples in this study were obtained from men undergoing semen analysis for couple infertility. The study by Bennetts et al., (2008) examined the ability of various estrogenic compounds including natural, synthetic, and environmental estrogens to create oxidative stress and DNA damage in human spermatozoa *in vitro*. Although there was evidence that certain estrogenic compounds, such as catechol estrogens and quercetin, stimulated redox activity and produced DNA damage, other estrogens, such as BPA and E_2, showed no effects on these parameters. The compounds that exhibited high redox activity induced a complete loss of sperm motility. However, BPA did not show an effect on any outcome measure.

The two other *in vitro* studies observed that BPA can interact with human sex hormone-binding proteins. Dechaud et al. (1999) reported that BPA was a potent hSHBG-ligand, binding hSBHG with a reversible and competitive binding activity for T and E_2. As the authors state, the data suggest that hSHBG binding may transport some contaminant xenoestrogens into the plasma and modulate their bioavailability to cell tissues. Mulligan et al. (1998) examined the competitive binding ability of BPA to sex steroid binding proteins in humans using plasma from three pregnant women. The relative binding affinity of BPA in human plasma was very weak, being <0.01%. The results suggested that BPA is not likely to produce biological effects by displacing endogenous steroids from plasma steroid binding proteins unless it is present in very high concentrations.

Additionally, in one related study by Inoue et al. (2002), two analytical methods were compared for quantifying BPA in biological samples. Using human semen samples, liquid chromatography-mass spectrometry was determined to be a more accurate and more sensitive method than the enzyme-linked immunosorbent assay, which may give erroneously high values possibly due to the non-specific binding to the antibody.

Table D2. Summary table of male reproductive studies in humans.

Reference	Study Type	Population	Exposure Measures/Methods	Findings
Hanaoka et al., 2002 NTP-A NTP-B	Occupational, cross-sectional	42 workers using epoxy resin, 42 matched controls	Urinary BPA - HPLC Exposure to BADGE and mixed organic solvents Plasma T, LH and FSH	↑ BPA in exposed vs control grp ↓ FSH in exposed grp, and inversely associated with BPA in regression analysis No significant difference between grps for T or LH
Cha et al., 2008	Occupational, cross-sectional	25 epoxy resin painters, 25 controls, non-painters	Urinary BPA - HPLC Plasma T, LH and FSH	↑ BPA in exposed vs control grp ↑ LH associated with BPA in regression analysis No significant association between BPA and T or FSH
Dechaud et al., 1999	In vitro	Plasma from healthy men and women	Ammonium sulfate precipitation assay assessed ability of BPA to displace E_2 and T Solid phase binding assay assessed binding affinity of BPA to hSHBG	BPA was a potent hSHBG-ligand
Luconi et al., 2001 NTP-A	In vitro	Semen from normozoospermic men undergoing analysis for couple infertility	Measured intracellular calcium concentration Used acrosome reaction assay	No effect of BPA on calcium response for E_2 or progesterone, on acrosome reaction
Bennetts et al., 2008	In vitro	Semen from students, whose fertility was unknown	Dihydroethidium assay, Comet and TUNEL assay, and quantitative polymerase chain reaction used to assess redox activity and DNA integrity	No effect of BPA on redox activity or DNA integrity or lesion frequency
Milligan et al., 1998	In vitro	Plasma from 3 pregnant women	Filter assay technique to determine effect of BPA on binding of $[^3H]E_2$ or $[^3H]$DHT to steroid binding proteins	BPA unlikely to displace endogenous steroids from steroid binding proteins, except at high concentrations
Related Study				
Inoue et al., 2002 NTP-A	Analytic methods	Semen from 41 healthy males aged 18-38 years	Compared two analytic methods—LC-MS and ELISA—to quantify BPA	LC-MS - no detectable BPA ELISA – BPA ranged from no detection to 12 ng/ml. LC-MS is the analytic method of choice

D.2. Male reproductive toxicity in laboratory animals

The majority of the large number of studies in laboratory animals on the potential effects of BPA on the development and/or function of the male reproductive system have been reviewed and summarized by the NTP-CERHR (CERHR, 2008) and the EU (EU, 2003, 2008).

Many studies address the impact of BPA on the development of the male reproductive system of mice and rats. In rodents, male reproductive system development begins around gestation day (GD) 12 (Magre and Jost, 1991). It continues through the neonatal (first week) and infantile (second week) period, until postnatal week (PNW) 7, when sexual maturation of the whole male reproductive system is basically complete (Flickinger, 1971; Sun and Flickinger, 1979; Nazian and Mahesh, 1980; Vergouwen et al., 1993; Marty et al., 2003; Sharpe et al., 2003a).

The organs of the male reproductive system mainly develop from three embryonic structures: undifferentiated gonads (for the testis), Wolffian ducts (for the internal genital organs, such as seminal vesicles, epididymides, and external genitalia), and the urogenital sinus (for the prostate). Development of these organs is under the active and balanced control of numerous hormones, such as androgens (including testosterone (T) and dihydrotestosterone (DHT)), estrogens, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The exact biological effects of a hormone on the development and function of a particular organ depends on many factors. For example, the level of 17β-estradiol (E_2) in free (active) form, the level and function of its receptors estrogen α (ERα) and ERβ, and the ratio of these two receptors in the testis or prostate determine the exact effects of E_2 on these two organs at different stages of development. In addition, the androgens T and DHT can also significantly influence the effects of estrogens (O'Donnell et al., 2001; Akingbemi, 2005; Morani et al., 2008; Prins and Korach, 2008). Therefore, evaluation of the potential effects of an endocrine-modulating chemical requires careful consideration of all the potential compounding factors, including the pharmacokinetic characteristics of the dosing regimen and the timing of exposure.

This section reviews the major findings from all toxicological studies found with information relevant to the evaluation of the male reproductive toxicity of BPA. Each study has its own scientific merits and limitations in terms of, for example, the animal model and dosing regimen employed, the level of histopathology performed, and the time points of observation. Such design features must be kept in mind when interpreting the results of the study.

Appendix 2 summarizes the major findings from all studies in laboratory animals (using *in vivo* or *in vitro* models) with relevant information on male reproductive toxicity. The review summaries in this appendix should aid the reader of this section, which provides an overview of key findings regarding the potential male reproductive toxicity of BPA.

The relevant data on the potential male reproductive toxicity of BPA in animal models will be discussed in terms of:

- Fertility and reproductive outcome.
- Testicular effects.

- Prostate effects.
- Epididymal and seminal vesicle effects.
- Sexual maturation.
- Hormonal effects.

D.2.1. Fertility and reproductive outcome

Male-mediated effects of developmental exposure to BPA on adult fertility or reproductive outcome were evaluated in eight studies: two studies in neonatal rats (Nagao et al., 1999; Kato et al., 2006), three NTP-sponsored RACB studies, and three multi-generation reproduction studies (Ema et al., 2001; Tyl et al., 2002b; Tyl et al., 2008b). Details of the study design and the major findings from these studies are presented in Appendix 2. It should be noted that both male and female animals were treated in the RACB or multi-generation reproductive studies. Therefore, effects on fertility or reproductive outcome can be mediated either through male or female reproductive effects. The RACB study design includes a cross-over mating trial (Task 3), in which treated males are mated with control females. Effects observed in Task 3 experiments are thus indicative of male reproductive effects.

Impaired reproductive outcome, indicated by reduced number of live pups per litter, was observed in the dietary RACB study in CD-1 mice at doses of 437 mg/kg-d or higher, but not in the s.c. implant study (NTP, 1984, 1985; Morrissey et al., 1989; Tyl et al., 2002a). In the two-generation study in the same strain of mice, dietary exposure to BPA at 0.18–3500 ppm (0.03–600 mg/kg-d) caused an apparent increase in the still birth index (Tyl et al., 2007; Tyl et al., 2008b). The increase in the still birth index was not statistically significant, was higher in the 300 and 3500 ppm than in the lower doses, but had no clear linear dose-response relationship.

In rats, neonatal s.c. injection of BPA at doses ranging from 0.024 µg/rat (about 2 µg/kg-d; (Kato et al., 2006)) to 300 mg/kg-d (Nagao et al., 1999) did not cause apparent effects on the treated animals in adulthood. The two-generation study by Ema et al. (2001) found no apparent effect on reproductive outcome in SD rats, but there were no detailed data on total births or number of live pups per litter. In the three-generation study, rats in all three generations in the 7500 ppm group (434–1823 mg/kg-d) had significantly reduced number of total pups and live pups per litter. At lower doses, reduced total number of pups and live pups per litter was noticeable in the 4.5–750 ppm groups in F_1 and in the 0.3–750ppm groups in F_3, but the change was only statistically significant at 0.3 ppm in F_3 males. Therefore, the data on the number of live pups per litter in three-generation study in rats are to some extent consistent with similar observations in CD-1 mice in the RACB and two-generation reproduction study (NTP, 1985; Tyl et al., 2008b).

Fertility is a collective measurement of the function of the male reproductive system. It is one of the most sensitive endpoints in the RACB studies for detecting male reproductive toxicity of chemicals. However, in any other types of studies, including the multi-generation studies, it is a less sensitive endpoint (Schwetz et al., 1980; Morrissey et al., 1988; Morrissey et al., 1989; Chapin et al., 1997; Mangelsdorf et al., 2003). Continuous breeding in the RACB study design may reduce the high sperm reserve in the epididymis and thus increase the likelihood that

reproductive toxicity would be manifested as an effect on fertility. RACB studies also include up to 5 litters for statistical analysis and thus increase statistical power. The adverse reproductive effects of BPA in the RACB studies are consistent with these factors. For example, the reduced number of live pups per litter as observed in the dietary RACB study was more obvious in litters 4 and 5 than in the first three litters (NTP, 1985). When all five litters were included in the analysis, the average number of live pups per litter was low at all three doses, even though only the reduction in the middle and high dose groups was statistically significant. On the other hand, when only one litter per pair was used for testing, the statistically significant reduction was only observed at the high dose, 1.0% in diet or 1750 mg/kg-d (Tyl et al., 2002b). All the other studies, including the two- or three-generation reproduction studies, only included one litter per mating pair for analysis. Consequently, the effect on reproductive outcome in these studies may not be as obvious as was it in the RACB study. Therefore, to the extent they can be compared, the data on fertility and reproductive outcome in the RACB and multi-generation studies are largely consistent. It should be noted that BPA at lower doses (e.g., <300 ppm in mice or <75 ppm in rats) in the two- or three-generation studies caused no apparent general toxicity, indicating that the reduced number of live pups may not be associated with apparent general toxicity. The reduced mean number of live pups in BPA-treated males mated to control females, as observed in Task 3 of the NTP RACB study, together with the evidence on the potential epigenetic or testicular effects of BPA, suggests that the effect of BPA on reproductive performance may be, at least in part, male-mediated.

D.2.2. Testicular effects

There are about 80 studies that evaluated the testicular effects of BPA in laboratory animals. These studies used many endpoints such as testis weight, histopathology, sperm production, production of T by the Leydig cells and molecular or biochemical analysis of testicular tissue, but no single study measured all the endpoints. Numerous factors make the integrative evaluation of this dataset extremely challenging. The summary below attempts to point out the consistency among the key observations. Studies that included at least one of three major endpoints (testis weight, sperm parameters, and histopathology/other testicular evaluation) are highlighted in Table D3 (mice) and Table D4 (rats).

It should be clarified that the majority of the studies that performed histopathological evaluation of the testis used commonly used methods such as identification of degenerating germ cells in paraffin sections of testicular tissues. These methods are generally referred to in this document as “routine.” While these methods have been widely used in toxicological studies, especially in comprehensive studies that follow GLP guidelines, they may not be as sensitive as other methods, such as immunostaining for structural or functional proteins in the seminiferous epithelium.

Table D3. Testicular Effects of BPA in mice.

References	Strain	Exposure	Testis Weight and Effective Doses	Sperm Parameters and Dose Ranges	Histopathological changes and dose-ranges (bold = significant)
		Prenatal Exposure			
vom Saal et al., 1998	CF-1	Oral, oral instillation, GD 11-17. 2, 20 µg/kg-d.	No effect.	Reduction in DSP/g testis. **20 µg/kg-d.**	No Data (ND)
Cagen et al., 1999a	CF-1	Oral, oral instillation, GD 11-17. 0.2, 2, 20 µg/kg-d.	No effect.	Reduction in DSP/ g testis, but not significant.	No effect (routine).
Ashby et al., 1999	CF-1	Oral, oral instillation, GD 11-17. 2, 20 µg/kg-d.	No effect.	No effect.	ND.
Nagao et al., 2002	C57BL/ 6N	Oral gavage, GD 11-17. 2, 20, 200 µg/kg-d.	No effect.	No effect on epididymal sperm count.	No effect (routine).
Iida et al., 2002	ddY	Oral gavage GD 10-17. 1, 10, 100 mg/kg-d.	ND	ND	Increased number of seminiferous tubules with abnormal morphology. **1, 10, 100 mg/kg-d.**
Kawai et al., 2003	CD-1	Oral gavage. GD 11-17. 2, 20 ng/kg-d.	Relative. Reduced at **2 ng/kg-d** at PNW 8 and 12. **20 ng/kg-d** at PNW 12.	ND	ND
		Neonatal Exposure			
Aikawa et al., 2004	SHN	s.c. injection to newborn for 5 d. 0.5, 50 µg/mouse-d.	ND	ND on sperm count. Reduced motility. Poor morphology. **0.5, 50 µg/mouse-d.**	No effect (routine).
Toyama and Yuasa, 2004	ICR	s.c. injection to ICR mice for 12 days. 0.1-10 µg/mouse.	ND	ND	Multinucleated giant cells and other abnormal changes. **1-10 µg/mouse**.
		Perinatal Exposure			
NTP, 1984; Morrissey, 1989	CD-1	s.c. implants. RACB Task 2. 25, 50, and 100 mg/mouse (estimated release: about 11.65, 20.05, and 38.60 mg/mouse).	No effect.	ND	No effect.
Kabuto et al., 2004	ICR	Drinking water. 1 wk premating – PND 28. 5, 10 µg/ml in water.	Reduced. **5 and 10 µg/ml**.	ND.	Increased levels of oxidative stress. **5 and 10 µg/ml**.
Okada and Kai, 2008	ICR	s.c. implants. 3 days premating – PND 28. 0.1, 5 mg/mouse.	No effect on the relative weight.	ND	Decreased % of seminiferous tubules with spermatids. **5mg/mouse**.

Table D3. Testicular Effects of BPA in mice (continued).

References	Strain	Exposure	Testis Weight and Effective Doses	Sperm Parameters and Dose Ranges	Histopathological changes and dose-ranges (bold = significant)
		Pubertal Exposure			
Takao et al., 1999	C57BL/6	Drinking water. 4 or 8 wks from PNW 5. 0.5, 50 µg/ml in water.	No effect.	ND	Increased number of multinucleated giant cells. **50 µg/ml.**
Takao et al., 2003	C57BL/6	Drinking water. 8 wks from PNW 3. 0.5, 50 µg/ml in drinking water.	No effect.	ND	Increased expression of ERα, reduced expression of ERβ. **50 µg/ml.**
Nagao et al., 2002	C57BL/6N	Oral gavage. 3 wks from PNW 3. 2, 20, or 200 µg/kg-d.	No effect at PNW 6.	No effect on epididymal sperm count.	No effect (routine).
Takahashi and Oishi, 2001	C57BL/6	Feed. 0.25, 0.5, 1.0% % in diets for 44 days from PNW 4.	No effect.	No effect.	No effect (routine).
		Two-gen. Study			
NTP, 1985	CD-1	Feed. RACB study design. 0.25, 0.5, and 1.0% in diets (437, 875, 1750 mg/kg-d).	Reduced. **0.5 % in diets.**	Reduced sperm count and motility. **0.5, 1.0% in diets.**	ND
Tyl et al., 2007; Tyl et al., 2008b	CD-1	Feed. 2-gen. study design. 0.018 – 3500ppm (3µg/kg-d–600 mg/kg-d).	Reduced. **3500 ppm.**	Reduced sperm count. **3500 ppm.**	Increased hypoplasia of the seminiferous tubules. **3500 ppm.**
		Adult Exposure			
Nagao et al., 2002	C57BL/6N	Oral gavage. 2, 20, 200 µg/kg-d for 6 days.	No effect 6 wks after dosing.	No effect on epididymal sperm count.	No effect (routine).
Al-Hiyasat et al., 2002	Swiss	Oral gavage. 5, 25, 100 µg/kg-d for 30 d.	Reduced. **5 µg/kg-d, but not 25 or 100 µg/kg-d.**	Reduced total number of sperm per epididymis. **25, 100 µg/kg-d**	ND
Toyama et al., 2004	ICR	s.c. injection. 20, 200 µg/kg-d for 6 d.	ND	ND	Multinucleated giant germ cells, deformed spermatids, and abnormal changes under the electron microscope. **20, 200 µg/kg-d**
Anahara et al., 2006	ICR	s.c. injection. 2.4 µg/kg-d for 5 d.	ND	ND	Reduced expression of cortactin, a structural protein in the apical ectoplasmic specialization in the testis. **2.4 µg/kg-d**
Liu et al., 2006	Kuming (Swiss)	i.p. injection. 250, 500, 1000 µmol/kg-d (57, 114, 228 mg/kg-d)	No effect on relative weight.	ND	Increased level of oxidative stress and increased number of apoptotic cells. **57, 114, 228 mg/kg-d**

Table D4. Testicular Effects of BPA in rats.

References	Strain	Exposure	Testis weight	Sperm parameters and dose ranges (bold = significant)	Histopathological changes and dose-ranges (bold = significant)
		Prenatal Exposure			
Talsness et al., 2000b	SD	Oral gavage. GD 6 – 21. 0.1, 50 mg/kg-d.	Reduced. **0.1 mg/kg-d**, No effect at 50 mg/kg-d.	Reduced sperm production at **0.1 mg/kg-d** on PND 170, but not on PND 70. Reduced sperm production at **50 mg/kg-d** on PND 70, but not 170.	Minimal histological changes (routine).
Tinwell et al., 2002	SD	Oral gavage. GD 6 – 21. 0.02 – 50 mg/kg-d.	No effect.	No effect.	No effect (routine).
Tinwell et al., 2002	AP (Wistar)	Oral gavage. GD 6 – 21. 0.02 – 50 mg/kg-d.	No effect.	Reduced sperm production. **50 mg/kg-d.**	No effect (routine).
Wistuba et al., 2003	SD	Oral gavage. GD 6 -21. 0.1, 50 mg/kg-d.	ND	ND	Increased number of Sertoli cells per testis, but not per gram testis. **0.1, 50 mg/kg-d.**
Thuillier et al., 2003	SD	Oral gavage. GD 14 –birth. 0.1 – 200 mg/kg-d.	ND	ND	Increased expression of PDGF receptor α and β in the testis. **0.1 – 200 mg/kg-d**.
Wang et al., 2004	SD	Oral gavage. GD 14 – birth. 0.1 – 200 mg/kg-d.	ND	ND	Alterations in the expression of ER-associated proteins in germ cells. **0.1 – 200 mg/kg-d.**
Naciff et al., 2005	SD	s.c. injection. GD 11 to 20. 0.002 – 400 mg/kg-d.	ND	ND	No histopathological changes (routine). Altered expression of genes. No striking difference between low and high dose ranges. **2 µg/kg-d – 400 mg/kg-d.**
Saito et al., 2003a	Wistar	s.c. injection. GD 12-19. 50 µg/dam.	No effect.	ND	No data.
		Neonatal Exposure			
Saunders et al., 1997	Wistar	s.c. injection to newborn for 12 d. 0.5 mg/rat.	No effect.	ND	No effect on the diameter of seminiferous tubules.
Fisher et al., 1999	Wistar	s.c. injection to newborn for 12 d. 20 mg/rat (37 mg/kg-d).	No effect.	ND	No effect on the level of testicular aquaporin (water channel).
Nagao et al., 1999	SD	s.c. injection to newborn for 5 d. 300 mg/kg-d.	No effect.	ND	No effect (routine).

Table D4. Testicular Effects of BPA in rats (continued).

References	Strain	Exposure	Testis weight	Sperm parameters and dose ranges (bold = significant)	Histopathological changes and dose-ranges (bold = significant)
Atanassova et al., 2000	Wistar	s.c. injection to newborn for 12 days. 0.5 mg/rat-d.	Increased weight on PND 18. No effect on PND 25 or 90.	ND	Accelerated differentiation of the testis. **0.5 mg/rat-d.**
Sharpe et al., 2003b	Wistar	s.c. injection to newborn for 12 days. 0.5 mg/rat-d.	No effect on PND 18, 25 or 90.	ND	No effect on the number of Leydig cells. Increased blood level of T on PND 18, but not 25 or 90. **0.5 mg/rat-d.**
Toyama and Yuasa, 2004	Wistar	s.c. injection to newborn for 11 days. 1, 10, 100 µg/rat.	ND	ND	Multinucleated giant cells and other abnormal changes. **10, 100 µg/rat**.
Kato et al., 2006	SD	s.c. injection to newborn for 9 days. 0.024- 1000 µg/rat-d (0.002-97 mg/kg-d).	No effect.	No effect.	No effect on PND 10, 35, or 150.
		Perinatal Exposure			
GE, 1976	SD	Oral, feed. 17 weeks with no data on the beginning time. Dosing in F1 stop at PNW 13. 1000, 3000, or 9000 ppm in diets (70, 200, or 650 mg/kg-d).	No effect.	ND	No effect (routine).
GE, 1978	SD	Oral, feed. 18 weeks with no data on the beginning time. Dosing in F1 until PNW 13. 100 – 1000 ppm in diets (5- 60 mg/kg-d in males).	No effect.	ND	No effect (routine).
Cagen et al., 1999	Wistar	Oral, drinking water. 2 wks premating – PND 22. 0.01 – 10 ppm in drinking water (est. 0.001-0.004, 0.008-0.038, 0.1-0.391, 0.775-4.022 mg/kg-d).	No effect on PND 90.	No effect on sperm production.	No effect (routine)
Kwon et al., 2000	SD	Oral gavage. GD 11- PND 20. 3.2, 32, 320 mg/kg-d. necropsied on PND 180.	No effect on PND 180.	ND	No effect (routine).

Table D4. Testicular Effects of BPA in rats (continued).

References	Strain	Exposure	Testis weight	Sperm parameters and dose ranges (bold = significant)	Histopathological changes and dose-ranges (bold = significant)
Akingbemi et al., 2004	LE	Oral, gavage. GD 12-21. 2.4 µg/kg-d.	No effect on PND 90.	Reduced rate of T production by Leydig cells and reduced levels of T in testicular fluids. **2.4 µg/kg-d.**	ND
Yoshino et al., 2002	F344	Oral gavage. GD 0 to PND 21. 7.5, 120 mg/kg-d.	No effect on the relative testis weight.	Reduced number of sperm per testis at 120 mg/kg-d in the 1st experiment, but not observed in the 2nd experiment.	No effect (routine)
Ichihara et al., 2003	F344	Oral gavage. GD 0 to PND 21. 0.05, 7.5, 30 or 120 mg/kg-d.	No effect on the relative testis weight.	ND	ND
Kobayashi et al., 2002	SD	Oral gavage. GD 6- PND 20. 4, 40 mg/kg-d (400 mg/kg lethal to the dams).	No effect.	ND	ND
Watanabe et al., 2003	IGS (SD)	Oral gavage. GD 6 and PND 20. 4, 40 mg/kg-d (400 mg/kg lethal to the dams).	Stated no effect.	ND	No effect on levels of T in testicular tissues.
Howdeshell et al., 2008	LE	Oral gavage. GD 7 – PND 18. 2, 20, or 200 µg/kg-d in Block 1 and 20 or 200 µg/kg-d in Block 2 experiment.	No effect.	No effect.	No effect (routine).
		Pubertal Exposure			
Ashby and Lefevre, 2000	AP (Wistar)	Oral, gavage. 200 mg/kg-d for 14 days from PND 22; 100 mg/kg-d for 14 days from PND 35; 100, 150, or 200 mg/kg-d for 20 days from PND 35.	No effect.	ND	ND
Chitra et al., 2003a; Chitra et al., 2003b	Wistar	Oral gavage. 0.2, 2, 20 µg/kg-d for 60 days from PNW 7.	ND	Reduced epididymal sperm count and motility. No effect on sperm viability. Increased oxidative stress. **0.2, 2, 20 µg/kg-d**	ND
Tan et al., 2003	SD	Oral gavage. 100 mg/kg-d for 30 days from PNW 3.	No effect.	ND	ND

Table D4. Testicular Effects of BPA in rats (continued).

References	Strain	Exposure	Testis weight	Sperm parameters and dose ranges (bold = significant)	Histopathological changes and dose-ranges (bold = significant)
Takahashi and Oishi, 2001	F344	Feed. 0.25, 0.5, or 1.0% in diets (235, 466, or 950 mg/kg-d) for 44 days from PNW 4.	No effect.	ND	Degenerative changes. **466, or 950 mg/kg-d.**
Takahashi and Oishi, 2003 NTP-A	Wistar and Holtzman (SD)	Feed. 0.25% in diets for 2 months from PNW 4.	No effect.	No effect.	No effect (routine).
Akingbemi et al., 2004	LE	Oral, gavage. 2.4, 10 µg/kg-d or 100, 200 mg/kg-d for 15 days from PNW 3.	ND	ND	Reduced testicular T production and altered expression of ER in the testis. **2.4, 10 µg/kg-d.** No effect at 100 or 200 mg/kg-d.
Akingbemi et al., 2004	LE	Oral, gavage. 2.4 µg/kg-d for 90 days from PNW 3.	No effect.	ND	ND
Takahashi and Oishi, 2003	Wistar	s.c. injection. 200 mg/kg-d for one month from PNW 4.	Reduced. **200 mg/kg-d**	Reduced sperm production. **200 mg/kg-d**	Degenerative changes. **200 mg/kg-d**
Takahashi and Oishi, 2003	Wistar	Intraperitoneal injection. 2, 20 mg/kg-d for one month from PNW 4.	Reduced. **20 mg/kg-d.**	No effect.	No effect (routine).
Saito et al., 2003a	Wistar	s.c. injection. 5 µg/d or 5 mg/d for 8 wks from PNW 3.	No effect.	ND	ND
		Two-gen. Study			
Ema et al., 2001	SD	Oral gavage. Two-gen. study design. 0.2, 2, 20, 200 µg/kg-d.	No effect.	No effect.	No effect (routine).
Tyl et al., 2000; 2002b	SD	Feed. Three-gen. study design. 0.015, 0.3, 4.5, 75, 750, and 7500 ppm (0.001 – 500 mg/kg-d).	Reduced in F3 males at **all doses**, but not statistically significant at 4.5 and 75ppm.	Reduced DSP in F3 males at all doses, but only significant at **7500 ppm.**	No effect (routine).

Table D4. Testicular Effects of BPA in rats (continued).

References	Strain	Exposure	Testis weight	Sperm parameters and dose ranges (bold = significant)	Histopathological changes and dose-ranges (bold = significant)
		Adult Exposure			
Sakaue et al., 2001	SD	oral gavage. 2 ng/kg-d – 200 mg/kg-d for 6 d.	No effect.	Reduced sperm production. **≥ 20 µg/kg-d**.	ND on histopathology. Changes in testicular protein profile on 2-dimensional gel electrophoresis. **20 µg/kg-d**
Ashby et al., 2003	SD	oral gavage. 0.02, 2, 200 mg/kg-d for 6 d.	No effect.	No effect.	No effect (routine). ND on testicular biochemistry.
Deng et al., 2004	SD	Feed. 1, 5 g/kg in diets (50 or 250 mg/kg-d) for 2 wks.	Reduced. **250 mg/kg-d**.	ND	Reduced spermatids and spermatocytes per Sertoli cell; altered immunostaining for structural proteins. **50 and 250 mg/kg-d.**
Herath et al., 2004	Wistar	s.c. injection. 3 mg/kg-d for 5 wks.	No effect.	Reduced epididymal sperm count. **3 mg/kg-d.**	ND
Toyama et al., 2004	Wistar	s.c. injection. 20, 200 µg/kg-d for 6 d.	ND	ND	Deformed spermatids and multinucleated giant germ cells. Ultrastructural changes. **20, 200 µg/kg-d.**

D.2.2.1. Testis weight in mice

The majority of studies that measured this endpoint did not find any significant reduction, regardless of the strain of animals, exposure period, doses, or dosing methods (Table D3).

At doses < 50 mg/kg-d, Kawai et al. (2003) reported a significant reduction (by approximately 15%) in relative testis weight following prenatal exposure by oral gavage to 2 ng/kg-d of BPA at 8 wks of age, but not at 20 ng/kg-d at this age. At 12 wks of age, the reduction was significant at 2 ng/kg-d (by about 15%) and 20 ng/kg-d (by about 10%). The authors did not include histopathological evaluation of the testis or measure sperm parameters. None of the other prenatal studies in mice (e.g., (vom Saal et al., 1998, 1999b; Nagao et al., 2002)) found obvious weight reduction of the testis, although the doses (0.2–200 µg/kg-d) used in these studies were much higher than those (ng/kg-d) used by Kawai et al. (2003). It should be noted that absolute testis weight, not relative testis weight, is generally accepted as the appropriate metric for analysis of this endpoint (U.S.EPA, 1996; Bailey et al., 2004).

Perinatal exposure to 5 or 10 µg/ml of BPA in drinking water caused reduction in testis weight in ICR mice by about 19% at 5 µg/ml and 13% at 10 µg/ml (Kabuto et al., 2004). Increased levels of oxidative stress were also reported. However, perinatal treatment with BPA through s.c. implants at either low (0.1 or 5 mg/mouse; (Okada and Kai, 2008)) or high (25–100 mg/mouse; (NTP, 1984)) did not produce significant changes in testis weight. In the adult, Al-Hiyasat et al. (2002) found that BPA at 5 µg/kg-d by oral gavage, but not at 25 or 100 µg/kg-d, reduced testis weight by about 15%. Sperm count in the 25- or 100- µg/kg-d groups was reduced, whereas no change in the sperm count was observed at 5 µg/kg-d.

At very high doses (600 mg/kg-d or higher), BPA caused significantly decreased testis weights and sperm counts in the parent males and their offspring (NTP, 1985; Tyl et al., 2008b).

It appears that BPA at low doses caused a relatively small reduction in testis weight in mice in three studies, but not in any of other studies that used low doses of BPA. At high doses and extended exposure period (RACB or two-generation reproduction study), testis weight was significantly reduced.

D.2.2.2. Testis weight in rats

As was the case with studies in mice, most studies in rats either did not report testis weight or found no effect on it (Table D4). Talsness et al. observed reduced testis weight (by about 9%) in 70-day old SD rats exposed to 0.1 mg/kg-d of BPA by oral gavage of the mother on GD 6–21 (Talsness et al., 2000b). By 170 days of age, there was no apparent difference in testis weight between the controls and the animals treated with 0.1 mg/kg-d of BPA prenatally. There was no effect on testis weight at 50 mg/kg-d. Using the same study design as that used by Talsness et al. (2000b), Tinwell et al. (2002) did not find any obvious effect on testis weight.

In the three-generation reproduction study by Tyl et al. (2000; 2002b), testis weight in the F_3 males was reduced at all doses (by about 5–9%), but the reductions in the 4.5 and 75ppm (0.27 and 4.5 mg/kg-d, respectively) groups were not statistically significant. The testis weights in the

F_1 and F_2 generations of the BPA-treated groups were also lower than those in the control group, but the differences were only significant in F_2 males at 0.3 and 750 ppm. In addition, it has been shown that BPA reduced testis weight in adult SD rats following exposure through feed for two weeks at 250 mg/kg-d (Deng et al., 2004) or in pubertal rats treated by s.c. injection at 200 mg/kg-d for one month (Takahashi and Oishi, 2003). BPA was much more toxic by i.p. injection than by s.c. injection, and at 20 mg/kg-d, i.p. injection caused significant reduction in testis weight in pubertal rats. Although reduced testis weight was only observed in a few studies in rats, the findings appear to be consistent (relatively small reduction and presence through three generations in the multi-generation study).

Taken together, it appears that BPA at both low and high doses caused slight (around 5–15%), but statistically significant, decreases in testis weight under certain experimental conditions in rats. In mice the data are less clear at low doses, but the reduction is more obvious at high doses.

D.2.2.3. Sperm parameters

Measurements commonly used to evaluate sperm production and quality in laboratory animals include testicular sperm count and evaluation of sperm quality (number, motility, and morphology) from the cauda epididymis. Largely due to the technical difficulties in preparing tissue samples and evaluating the samples, background values in sperm parameters between different studies can vary greatly (e.g., (Ashby et al., 2003; Ashby et al., 2004; Choi et al., 2008)). This large inter-study variation must be considered when comparing data from different studies, even among studies from the same laboratory. It also emphasizes the importance of concurrent control as compared to historical controls.

In mice, 10 studies included measurement of some sperm parameters (Table D3). At very high doses, significant decreases in sperm count were obvious in CD-1 mice in the studies by NTP (1985) or Tyl et al. (2007; 2008b). At doses lower than 50 mg/kg-d, the results from different laboratories (eight studies reported from five different laboratories) are not consistent with each other. Detailed discussion and comparison of the prenatal studies by vom Saal et al. (1998), Ashby et al. (Ashby et al., 1999), and Cagen et al. (1999a) are presented on pages 9–10 in Appendix 2. Although Ashby et al. (1999) and Cagen et al. (1999) found no significantly reduced sperm count, their findings do not provide a basis for rejecting the findings by vom Saal et al. (1998). In fact, to some extent, slight (but not statistically significant) reduction in sperm count in the study by Cagen et al. (1999) and significantly reduced sperm count in the studies by Al-Hiysat et al. (2002), NTP (1985), and Tyl et al. (2008b) are consistent with the findings by vom Saal et al. (1998).

The study by Nagao et al. (2002) compared the effect of BPA among three different exposure scenarios in C57BL/6N mice. Adult mice received six days of treatment. This exposure may not be long enough to induce any detectable effect on epididymal sperm count. Juvenile mice (21-day old) received 21 days of treatment. At age 21 days, the epididymis is reaching the early stage of maturity and only has spermatozoa from the first wave of spermatogenesis (Sun and Flickinger, 1979). Depending on the targeted population of germ cells, examination of the epididymis at this age may not detect any effect on sperm production. For the 7-day gestational exposure, the cesarean surgery on GD 18 made interpretation of the data from this experiment

difficult. Therefore, there is no basis for using the findings on sperm count from this study to reject the findings from other studies. The lowest dose that has been reported to cause a significant reduction in sperm count in adult mice is 20 µg/kg-d administered during gestation (vom Saal et al., 1998).

In rats, a total of 16 (out of more than 40) rodent studies measured one or multiple sperm parameters. Table D5 lists these 16 studies.

Table D5. Evaluation of sperm parameters in rats.

References	Strain	Exposure	Sperm parameters and dose ranges (bold = significant)
		Prenatal Exposure	
Talsness et al., 2000b	SD	Oral gavage. GD 6 – 21. 0.1, 50 mg/kg-d.	Reduced sperm production at **0.1 mg/kg-d** on PND 170, but not on PND 70. Reduced sperm production at **50 mg/kg-d** on PND 70, but not 170.
Tinwell et al., 2002	SD	Oral gavage. GD 6 – 21. 0.02 – 50 mg/kg-d.	No effect.
Tinwell et al., 2002	Wistar	Oral gavage. GD 6 – 21. 0.02 – 50 mg/kg-d.	Reduced sperm production. **50 mg/kg-d.**
		Neonatal Exposure	
Kato et al., 2006	SD	s.c. injection to newborn for 9 days. 0.024-1000 µg/rat-d (0.002-97 mg/kg-d).	No effect.
		Perinatal Exposure	
Cagen et al., 1999b	Wistar	Oral, drinking water. 2 wks premating – PND 22. 0.01 – 10 ppm in drinking water (est. 0.001-0.004, 0.008-0.038, 0.1-0.391, 0.775-4.022 mg/kg-d).	No effect.
Yoshino et al., 2002	F344	Oral gavage. GD 0 to PND 21. 7.5, 120 mg/kg-d.	Reduced number of sperm per testis at 120 mg/kg-d in the 1st experiment, but not observed in the 2nd experiment.
Howdeshell et al., 2008	LE	Oral gavage. GD 7 – PND 18. 2, 20, or 200 µg/kg-d in Block 1 and 20 or 200 µg/kg-d in Block 2 experiment.	No effect (see discussions on pages A1-22-23).

Table D5. Evaluation of sperm parameters in rats (continued).

References	Strain	Exposure	Sperm parameters and dose ranges (bold = significant)
		Pubertal Exposure	
Chitra et al., 2003a; 2003b	Wistar	Oral gavage. 0.2, 2, 20 µg/kg-d for 60 days from PNW 7.	Reduced epididymal sperm count and motility. No effect on sperm viability. Increased level of oxidative stress.
Takahashi and Oishi, 2003	Wistar and SD	Feed. 0.25, 0.5, 1.0% in diets for 44 days from PNW 4.	No effect.
Takahashi and Oishi, 2003	Wistar	s.c. injection. 200 mg/kg-d for one month from PNW 4.	Reduced sperm production. **200 mg/kg-d**
Takahashi and Oishi, 2003	Wistar	i.p injection. 2, 20 mg/kg-d for one month from PNW 4.	No effect.
		Two-gen. Study	
Ema et al., 2001	SD	Oral gavage. Two-gen. study design. 0.2, 2, 20, 200 µg/kg-d.	No effect.
Tyl et al., 2000; 2002b	SD	Feed. Three-gen. study design. 0.015, 0.3, 4.5, 75, 750, and 7500 ppm (0.001 – 500 mg/kg-d).	Reduced DSP at all doses in F_3 males, but only statistically significant at **7500 ppm**.
		Adult Exposure	
Sakaue et al., 2001	SD	Oral gavage. 2 ng/kg-d – 200 mg/kg-d for 6 d.	Reduced sperm production. **≥ 20 µg/kg-d**.
Ashby et al., 2003	SD	Oral gavage. 0.02, 2, 200 mg/kg-d for 6 days.	No effect.
Herath et al., 2004	Wistar	s.c. injection. 3 mg/kg-d for 5 wks.	Reduced epididymal sperm count. **3 mg/kg-d.**

At doses >200 mg/kg-d, there was a significant reduction in sperm production (Tyl et al., 2000; Tyl et al., 2002b; Takahashi and Oishi, 2003). The noticeable but not statistically significant reduction in DSP in F_3 males from the three-generation reproduction study in SD rats (Tyl et al., 2000; Tyl et al., 2002b) is consistent with the observation in SD rats by Talseness et al. (2000b) or in AP (Wistar) rats by Tinwell et al. (2002). However, no effect on sperm count was reported in studies that included either low or moderately high (e.g., 50 mg/kg-d) doses (e.g., (Ema et al., 2001; Tinwell et al., 2002; Howdeshell et al., 2008a)). In studies that observed reduced sperm count or production, the reduction was relatively small in most cases. Considering the technical difficulties in evaluating sperm parameters and the small changes reported in such parameters, it would be difficult to replicate any effect that was observed in a different study. Interestingly, the lowest dose that caused reduced sperm count in rats is exactly the same as that in mice, 20 µg/kg-d (vom Saal et al., 1998; Sakaue et al., 2001). The low doses in the three-generation study included 1, 20, and 300 µg/kg-d (0.015, 0.3, 4.5ppm, respectively; (Tyl et al., 2000; Tyl et al., 2002b)). However, it should be noted that the study in mice treated the animals prenatally, while the study by Sakaue et al. used adult SD rats.

Thus, there are a number of studies that observed reduced sperm count in mice or rats, but also other studies in which no such effect was observed using similar doses or routes of exposure in the same strain or species. Slight changes resulting from BPA exposure, technical difficulties in assessing sperm parameters in rodent, and other factors (e.g., different effects of BPA at different developmental stages, see mechanistic discussions below) make an integrative evaluation of this dataset on sperm parameters very difficult. However, the data appear to indicate that BPA at doses >200 mg/kg-d causes a significant reduction in sperm production.

D.2.2.4. Histopathology and other endpoints

Histopathology is a sensitive endpoint for the analysis of reproductive toxicity. However, poor fixation of the testis and lack of appropriate evaluation by trained pathologists may cause slight morphological changes in the testis to be overlooked (Mangelsdorf et al., 2003). The most commonly used histopathological method, referred to in this document as a routine method, is identification of germ cell degeneration and other apparent deformations of the seminiferous epithelium or interstitial tissues in paraffin sections. While this is the method generally recommended in guideline studies, it may not be able to detect subtle changes in the testis, such as subtle reduction in germ cells of certain developmental stages or changes in the expression of certain structural or functional proteins. Detection of these subtle changes usually requires carefully designed experiments or molecular tools (e.g., immunostaining).

The majority of the studies listed in Tables D3 and D4 included histopathological or biochemical evaluation of the testis. In mice (Table D3), all of the eight studies that used routine methods and evaluated for common histopathological changes (e.g., degeneration in the seminiferous epithelium) did not find any significant effect. In contrast, the studies that utilized alternative methods observed abnormal changes in the testis. Examples of alternative evaluations include the number of seminiferous tubules with abnormal morphology (Iida et al., 2002), the number of multinucleated giant cells (Takao et al., 1999) immunohistostaining for structural or functional

proteins (Takao et al., 2003; Anahara et al., 2006), or the number of apoptotic cells (Liu et al., 2006). Increased levels of oxidative stress in testicular tissues of BPA-treated mice has also been reported by Kabuto et al. (2004) or Liu et al. (2006). The doses used in these studies include 2.4 µg/kg-d (Anahara et al., 2006), 20 µg/kg-d (Toyama and Yuasa, 2004) and 228 mg/kg-d (Liu et al., 2006). Therefore, it appears that BPA induced certain subtle, but still abnormal, histopathological, histochemical or biochemical changes in mouse testis at a large range of doses in these studies.

The histopathological findings in rats are similar to those in mice (Table D4). Clear degenerative changes in the seminiferous epithelium of rats treated with high doses of BPA (generally > 50 mg/kg-d) were reported in several studies (Tyl et al., 2000; Takahashi and Oishi, 2001; Deng et al., 2004). However, several studies that conducted routine histopathological evaluation did not find any obvious morphological changes in the testis (e.g., (GE, 1976a, 1978; Cagen et al., 1999b; Ema et al., 2001; Tinwell et al., 2002)). At lower doses, most studies that used routine methods failed to detect any obvious BPA-induced histopathological changes. However, studies that counted the number of cells, immunostained for certain proteins, or measured T production by Leydig cells observed effects. It should be noted that stimulatory effects of BPA in the fetal or neonatal testis reported by Wistuba et al. (Wistuba et al., 2003) and Atanassova et al. (Atanassova et al., 2000), respectively, are similar to the effects resulting from short exposure to low levels of exogenous estrogens (e.g., E_2 or DES) at the same developmental periods (Sharpe et al., 1998; Atanassova et al., 1999).

In summary, BPA at doses > 50 mg/kg-d has been reported to cause degenerative changes in testis in mice and rats. At doses in the range 2–20 µg/kg-d, it has been reported to cause subtle but noticeable histopathological or biochemical changes in testis of mice and rats, regardless of dosing methods and developmental periods. However, using routine methods to evaluate commonly observed morphological changes, few studies found any obvious effects. These histopathological findings, together with the data on sperm parameters, clearly demonstrate the complexity of the potential actions of BPA and the technical difficulties in evaluating the subtle testicular effects of chemicals.

D.2.3. Prostate effects

Table D6 lists all the studies that evaluated effects on prostate in mice, and Table D7 lists studies in rats. There were no neonatal studies in mice.

Table D6. Effects of BPA on prostate in mice.

References	Strain	Exposure	Weight and dose ranges (bold = significant)	Histopathology/other changes and dose ranges (bold = significant)
		Prenatal Exposure		
Nagel et al., 1997	CF-1	Oral instillation, GD 11-17. 2, 20 µg/kg-d. 2, 20 µg/kg-d.	Increased (absolute and relative) at 6 months of age. **2 and 20 µg/kg-d.**	ND
Cagen et al., 1999a	CF-1	Oral instillation, GD 11-17. 0.2-200 µg/kg-d.	No effect.	Tissue fixed, but no data reported.
Ashby et al., 1999	CF-1	Oral instillation, GD 11-17. 2, 20 µg/kg-d.	No effect.	ND
Timms et al., 2005	CF-1	Oral, oral instillation, GD 11-17. 20 µg/kg-d.	ND	Increased volume of ducts, proliferation/enlargement in the dorsal and dorsolateral pre-lobes on GD 19. **20** µg/kg-d).
		Perinatal Exposure		
NTP, 1984	CD-1	s.c. implant. RACB Task 2 study. 25, 50, 100 mg/mouse.	No effect.	ND
		Pubertal Exposure		
Takahashi and Oishi, 2003	C57BL/6	Feed. 0.25, 0.5, 1.0% in diets for 44 days.	No effect.	ND
		Two-gen. Study		
NTP, 1985	CD-1	Feed. RACB study design. 0.25, 0.5, and 1.0% in diets (437, 875, 1750 mg/kg-d).	Increased in F1 males at 0.5 and 1.0%, but not significant.	ND
Tyl et al., 2007; 2008b	CD-1	Feed. 2-gen. study design. 0.018 – 3500 ppm (3µg/kg-d – 600 mg/kg-d).	No effect.	No effect (routine).
		Adult Exposure		
Ogura et al., 2007	Balb/c	s.c. implants. 0.2 – 200 mg/mouse for 3 wks.	No effect.	Increased immunostaining for cytokeratin 10 in the basal epithelial cells, indicative of abnormal differentiation. **2-200 mg/mouse in the dorsolateral; 200 mg/mouse in the ventral prostate.**

D.2.3.1. Studies in mice

Other than in the study by Timms et al. (2005), all studies weighed either the whole prostate or just the ventral prostate. A statistically significantly increase in prostate weight was observed by Nagel et al. (1998) in adult CF-1 mice treated prenatally with 2 or 20 µg/kg-d of BPA. Using the same study design, Cagen et al. (1999) and Ashby et al. (1999) failed to replicate the findings by Nagel et al. (1998). A detailed discussion of these studies is presented on pages 9–10 in Appendix 2. A slight, not statistically significant, increase in prostate weight was observed in the F_2 males of CD-1 mice in the RACB study by NTP (1985). No other study found any significant effect on prostate weight.

Three out of nine studies in Table D6 conducted histopathological evaluation of the prostate. Using standard guideline histopathology protocols, Tyl et al. (2007; 2008b) observed no apparent morphological changes in the prostate of adult mice treated through two generations. In contrast, using a carefully designed morphometric method, Timms et al. (2005) observed significant increases in the volume of the prostate ducts and enlargement and proliferation of the dorsal and dorsolateral pre-lobes of the prostate in GD 19 fetal mice treated with 20 µg/kg-d of BPA from GD 11 to 17. In addition, Ogura et al. (2007) reported increased immunostaining for cytokeratin 10 in the basal epithelial cells of the prostate, indicative of abnormal differentiation of this cell type, in adult mice treated by s.c. implants of BPA at 0.2 or 200 mg/mouse for three weeks. Assuming complete absorption of BPA from the implants, the doses used in this study are approximately equivalent to 1 or 1000 mg/kg-d (implanting dose in 10 g mice divided by 20 days). It appears that prenatal or adult exposure to BPA altered the proliferation and/or differentiation of prostate epithelial cells without obvious alteration in prostate weight in mice.

D.2.3.2. Studies in rats

Similar to the observations in mice, only four out of numerous studies in rats reported a significant change in prostate weight. Significantly reduced prostate weight was found in pubertal or adult rats exposed to very high doses of BPA either during the pubertal growth period (Takahashi and Oishi, 2001, 2003) or through three generations (Tyl et al., 2000; Tyl et al., 2002b). In rats 50 days of age, s.c. injection of 3 mg/kg-d for 5 weeks caused a significant increase in weight of the ventral prostate (Herath et al., 2004). The blood level of T in BPA-treated animals was also significantly lower than that of controls, indicating a possible reduction in T production in the testis. While this observation of a reduced T level is supported by data from the studies by Akingbemi et al. (2004), it is not consistent with the increased weight of the prostate, since the rapid prostate growth at this age requires increasing circulating T levels in blood (Nazian and Mahesh, 1980).

As had been the case in mice, studies in rats that used routine histopathological evaluation of the prostate (e.g., (GE, 1976b; Kwon et al., 2000; Ema et al., 2001; Tyl et al., 2002b; Howdeshell et al., 2008b)) did not find any obvious effects. However, the findings by Ramos et al. (2001; 2003) indicate that prenatal exposure to BPA via s.c. implants caused altered proliferation and differentiation of the stromal cells, which in turn may result in disruption of the development of the epithelial cells in the prostate (Cunha et al., 2001; Ricke et al., 2007; Cunha, 2008). Using a well-established animal model to study the effects of estrogens on prostate development, Ho et al.

(2006) found that neonatal exposure to BPA at 10 µg/kg-d for 5 days predisposed the prostate to estrogen-induced neoplasia in the adult. The authors also demonstrated that the "estrogenizing effect" of BPA was mediated via an epigenetic mechanism (see detailed summaries on pages 22–23, Appendix 2).

Taken together, the data in mice and rats indicate that developmental exposure to BPA causes abnormal development of the prostate in these species. These effects can be detected with advanced molecular and/or cellular approaches, but not by traditional methods such as organ weight or routine histopathological evaluation.

Table D7. Effects of BPA on the prostate in rats.

References	Strain	Exposure	Weight (abs. or rel.) and dose ranges (bold = significant)	Histopathology/other changes and dose ranges (bold = significant)
Prenatal Exposure				
Tinwell et al., 2002	SD	Oral gavage. GD 6 – 21. 0.02 – 50 mg/kg-d.	No effect.	ND.
Tinwell et al., 2002	AP (Wistar)	Oral gavage. GD 6 – 21. 0.02 – 50 mg/kg-d.	No effect.	ND
Ramos et al., 2001	Wistar	s.c. implant. GD 8 to birth. 25, 250 µg/kg.	ND	Reduced expression of AR and prostatic acid phosphatase. Altered development of stromal cells.
Ramos et al., 2003	Wistar	s.c. implant. GD 8 to birth. 25, 250 µg/kg.	No effect on the ventral prostate.	Altered differentiation and proliferation of stromal cells.
Neonatal Exposure				
Kato et al., 2006	SD	s.c. injection to newborn for 9 day. 0.0024 -1000 µg/rat (0.002 – 97 mg/kg-d).	No effect on the ventral prostate.	ND
Ho et al., 2006	SD	s.c. injection to newborn on PND 1,3, and 5. 0 .1 µg/mouse (10 µg/kg).	No effect.	Increased incidence and severity of prostate neoplastic lesions induced by adult exposure to E_2 and T. Epigenetic changes.

Table D7. Effects of BPA on the prostate in rats (continued).

References	Strain	Exposure	Weight (abs. or rel.) and dose ranges (bold = significant)	Histopathology/other changes and dose ranges (bold = significant)
Perinatal Exposure				
GE, 1976	SD	Oral, feed. 17 weeks. Dosing in F_1 stop at PNW 13. 1000, 3000, or 9000 ppm in diets.	No effect.	No effect (routine)
GE,1978	SD	Oral, feed. 18 weeks. Dosing in F_1 until PNW 13. 100 – 1000 ppm in diets.	No effect.	No effect (routine)
Cagen et al., 1999b	Wistar	Oral, drinking water. 2 wks premating – PND 22. 0.01 – 10 ppm in drinking water (est. 0.001-0.004, 0.008-0.038, 0.1-0.391, 0.775-4.022 mg/kg-d).	No effect on PND 90.	ND
Kwon et al., 2000	SD	Oral gavage. GD 11-PND 20. 3.2, 32, 320 mg/kg-d.	No effect on PND 180.	No histopathological changes in the ventral prostate.
Akingbemi et al., 2004	LE	Oral, gavage. GD 12-21. 2.4 µg/kg-d.	No effect on PND 90.	ND
Yoshino et al., 2002	F344	Oral gavage. GD 0 to PND 21. 7.5, 120 mg/kg-d.	No effect on the relative weight.	No effect (routine).
Ichihara et al., 2003	F344	Oral gavage. GD 0 to PND 21. 0.05, 7.5, 30 or 120 mg/kg-d.	No effect on the relative weight.	No effect on preneoplastic or neoplastic lesions in the prostate induced by 3,2'-dimethyl-4-aminobiphenyl.
Howdeshell et al., 2008	LE	Oral gavage. GD 7 – PND 18. 2, 20, or 200 µg/kg-d.	No effect.	No effect (routine).

Table D7. Effects of BPA on the prostate in rats (continued).

References	Strain	Exposure	Weight (abs. or rel.) and dose ranges (bold = significant)	Histopathology/other changes and dose ranges (bold = significant)
Pubertal Exposure				
Akingbemi et al., 2004 NTP-A	LE	Oral, gavage. 2.4 µg/kg-d for 90 d from PNW 21.	No effect.	ND
Ashby and Lefevre, 2000	AP (Wistar)	Oral, gavage. 200 mg/kg-d for 14 d from PND 22; 100 mg/kg-d for 14 d from PND 35; 100, 150, or 200 mg/kg-d for 20 d from PND 35.	No effect.	ND
Kim et al., 2002	SD	Oral gavage. 10-1000 mg/kg-d or 50-500 mg/kg-d for 7 d in castrated rats.	Hershberger assay. No effect.	ND
Takahashi and Oishi, 2001	F344	Feed. 0.25, 0.5, 1.0 % in diets for 44 d from PNW 4.	Reduced in dorsolateral, but not in ventral prostate. 1.0 % (**950 mg/kg-d)**	ND
Takahashi and Oishi, 2003	Wistar	s.c. injection. 200 mg/kg-d for one month from PNW 4.	Reduced weights of all three lobes of the prostate. **200 mg/kg-d**	ND
Saito et al., 2003a	Wistar	s.c. injection. 5 µg/d or 5 mg/d for 8 wks from PNW 3.	No effect.	ND
Two-generation Study				
Ema et al., 2001	SD	Oral gavage. Two-gen. study design. 0.2, 2, 20, 200 µg/kg-d.	No effect.	No effect (routine)
Tyl et al., 2000; 2002b	SD	Feed. Three-gen. study design. 0.015, 0.3, 4.5, 75, 750, and 7500 ppm (0.001 – 500 mg/kg-d).	Reduced. **750, 7500 ppm**.	No effect (routine).
Adult Exposure				
Yamasaki et al., 2003	Wistar	Oral gavage. Hershberger assay. 50, 200, 500 mg/kg-d for 10 days.	No effect in castrated rats with or without T injection.	ND
Herath et al., 2004	Wistar	s.c. injection. 3 mg/kg-d for 5 wks.	Increased weight. **3 mg/kg-d.**	ND

D.2.4. Epididymal and seminal vesicle effects

Weights of the epididymis and seminal vesicles were measured in many studies as part of the overall assessment of the male reproductive system. Many studies that measured the weights of the epididymis and seminal vesicles also evaluated the effects on the testis or prostate. Comparing the effects on the epididymis or seminal vesicles to the testicular or prostate effects, it appears that the studies that observed changes in the weights of the epididymis or seminal vesicles also saw adverse effects in the testis or the prostate (e.g., (vom Saal et al., 1998; Chitra et al., 2003a; Akingbemi et al., 2004)). On the other hand, studies that found no effect on the weights of these two organs also did not observe obvious effects on the testis or the prostate. This indicates that the epididymis and seminal vesicles are either less than or at least not more sensitive to BPA than are the testis or prostate. In addition, the data on the epididymis and seminal vesicles are limited, compared to those on the testis or the prostate. Therefore, relevant data on the epididymis and seminal vesicles are not further integrated in this subsection. Detailed summaries regarding the effects of BPA on the epididymis and seminal vesicles resulting from exposure during different stages of development can be found in Appendix 2.

D.2.5. Sexual maturation

AGD, PPS, nipple retention, and testicular descent have been used as external indicators of sexual maturation in males. Exposure to exogenous androgens or anti-androgens can cause abnormal changes in these measurements, such as reduced AGD or delayed PPS, through mechanisms of reduced production of T and/or DHT or disruption of the androgen-AR interaction (Gallavan et al., 1999; Foster and McIntyre, 2002). Estrogens (e.g., E_2) can also reduce the AGD in males, similar to anti-androgens (Yamasaki et al., 2001; Wisniewski et al., 2003; Tyl et al., 2008c). Although measurements of these endpoints appear to be straightforward, there are many technical difficulties (e.g., accurate measurement, appropriate adjustment to body weight, etc.) that have sometimes made appropriate interpretation of the data on these endpoints difficult (Gallavan et al., 1999). In addition, there are no data to indicate that these endpoints are more sensitive than testicular evaluation as a means of detecting male reproductive toxicity. Among the studies that evaluated sexual maturation based on AGD or PPS, the studies that observed alterations in these endpoints also found significant effects in the testis (e.g., the prenatal study by (Talsness et al., 2000b); the two-generation study in mice by (Tyl et al., 2008b)). Therefore, major findings on AGD or PPS are not further integrated. Detailed summaries of the effects of BPA on indices of sexual maturation resulting from exposure during different stages of development can be found in Appendix 2.

D.2.6. Hormonal effects

Detailed summaries on the hormonal effects of BPA are presented in Appendix 2. Although many studies measured blood levels of sex hormones to provide evidence supportive of potential effects on hormone-producing glands (e.g., testis or pituitary), there are no consistent findings among these studies. Therefore, the hormonal data are not further integrated.

D.2.7. *In vitro* studies

Detailed summaries of the findings from a number of *in vitro* studies are presented in pages 56–59 of Appendix 2. These studies focused on three cell types from two organs: Sertoli cells and Leydig cells in the testis, and prostate cells. Most studies used tumor cell lines to investigate the molecular mechanisms underlying the actions of BPA on the testis or prostate. All the studies found that BPA altered the function or morphology of the cultured cells or tissues. Overall, the findings from different studies using the same cell types are consistent with each other.

D.2.8. Summary

Numerous studies on the endocrine-modulating effect of BPA have shown that BPA has weak estrogenic effects by binding to ERα and ERβ in many *in vitro* systems (see summaries in Section E, as well as reviews by the NTP-CERHR and Weatherill et al. (Wetherill et al., 2007; CERHR, 2008). Findings from *in vitro* studies using testicular or prostate cells (Appendix 2) indicate that BPA at relatively low concentrations can cause abnormal changes in Sertoli and Leydig cells, as well as in prostate cells. Although the data from *in vivo* studies are not consistent in many aspects and are difficult to integrate, it appears that the following four lines of evidence are generally consistent across the studies:

- BPA exposure was associated with significant reduction in the mean number of live pups per litter. This effect was statistically significant at doses generally >500 mg/kg-d. It was also present at relatively low doses (ranging from 0.2 to 200 mg/kg-d), although the change was not large enough to be statistically significant.
- At doses >500 mg/kg-d, reduced mean number of live pups per litter, reduced testis weights, sperm count, and prostate weights in males had been observed in mice and rats.
- A number of studies used non-traditional histopathological and functional methods to evaluate the testis. These studies had found subtle morphological or functional changes in the testis of mice and rats treated with BPA at doses as low as 2–20 µg/kg-d. The effects were observed in multiple strains of mice and rats. There is considerable variation in the levels and durations of exposure, methods of evaluation, and developmental periods used among these studies. Generally there was no clear dose-response curve. Most of these studies were mechanistic studies conducted in academic research laboratories and did not use as many animals as those used in traditional guideline studies. However, all of them were published in peer-reviewed journals. Some studies used established animal models that had been repeatedly used for many years. Although in some studies where a relatively small numbers of animals was used, statistical significance was attained indicating that the group size was large enough to detect the effects with sufficient statistical power (Festing and Altman, 2002; Whitley and Ball, 2002; Case and Ambrosius, 2007; Lenth, 2007).
- Similar to the testicular effects, BPA at low or intermediate doses administered at different developmental periods did not show a consistent association with effects on

prostate weight. However, histopathological evaluation with carefully designed approaches or immunostaining analysis of the prostate revealed a clear association between BPA exposure and effects on the prostate in mice and rats.

E. Other Relevant Data

E.1. Endocrine Activity

E.1.1. Introduction

Potential endocrine disrupting effects of BPA could be modulated through one or more of the following mechanisms:

- Interaction with a hormone receptor (plasma membrane or nuclear receptor) modifying the action of its natural ligand.
- Interaction with estrogen receptor (ER).
- Interaction with androgen receptor (AR).
- Interaction with plasma binding protein.
- Interaction with other receptors.
- BPA interaction with the signal transduction pathway and modification of the response of a hormone after its receptor is activated.
- Changes in ER expression.
- Changes in progesterone receptor (PR) expression.
- Cytoplasm biochemical alterations: enzyme activation/deactivation.
- Interaction with steroid metabolism.
- Steroidogenesis.
- Steroid metabolism, excretion.
- Other endocrine effects.
- Effects on gonadotropins.
- Effect on other hormones.

OEHHA identified 35 original articles that address one or more of the elements on this list. Seventeen of these studies were included in the documents by NTP-CERHR (CERHR, 2008) and the European Union (EU, 2003, 2008). Eighteen relevant publications not discussed in these earlier reports are summarized here by OEHHA.

As reviewed by NTP-CERHR (CERHR, 2008), there is evidence that BPA can disrupt endocrine function by interacting with two reproductive hormone receptors: the estrogen receptor (ER) and the androgen receptor (AR). There is also evidence that BPA can modify the binding capacities of plasma binding proteins and thus modify the bioavailability of sex hormones. In addition, there are data indicating that BPA can interact with signal transduction pathways and modify the response after a hormone receptor is activated.

In addition, there is evidence that BPA affects steroid metabolism. Data on the effects of BPA changing progesterone (P) levels in cultured granulosa cells and decreasing aromatase activity in human cells are discussed below, as are data on BPA altering the expression of metabolic enzymes and transporters.

In addition to these effects, there are data on BPA reducing mean luteinizing hormone (LH) concentration and LH pulse amplitude and frequency in lambs. Data show that BPA stimulates production of prolactin (PRL) in rats.

Lastly, studies report that BPA increases the production of insulin in mice and mice pancreatic β-cells, can interfere with thyroid hormone (triiodothyronine, T_3) action and can act as a thyroid receptor antagonist in rats.

E.1.2. Hormone receptor interaction

E.1.2.1. Interaction of BPA with the estrogen receptor (ER)

BPA interacts with steroid hormone receptors such as the ER. Studies reporting on BPA's interaction with ER are summarized below, and are listed in Table E1.

Binding and activation of BPA to ERα in both rat and human receptors in an *in vitro* bioassay was reported bu Sun et al. in 2008 (Sun et al., 2008). Human breast cancer cell line MCF-7 and African green monkey kidney cell line CV-1 were transfected with human (hERα) and rat (rERα) plasmids, respectively. Cells were also cotransfected with a luciferase reporter gene containing the estrogen response element (ERE) in the encoding region. The estrogen agonists estradiol-17β (E_2) and diethylstilbestrol (DES), the ERα antagonist ICI 182,780 (ICI), 4 alkyl phenols, and BPA were incubated with cells for 24 h before determination of reporter gene expression. E_2 activated the reporter gene in MCF-7 cells with a half-maximal effective concentration (EC_{50}) of 0.16 nM while DES activated the gene with an EC_{50} of $28x10^{-5}$ μM. Co-incubation of MCF-7 cells with 10 μM of the ER antagonist ICI completely inhibited E_2-induced luciferase expression. BPA treatment (log concentration 0.01 to 10 μM) stimulated luciferase activity and luciferase induction in a dose dependent fashion in the MCF-7 and CV-1 cell lines, respectively. In the hERα reporter assay, BPA had a maximum luciferase induction of 59.5 ± 4.4% (E_2 luciferase induction = 100%) and the EC_{50} for BPA was 0.34 μM. In the rERα reporter assay, BPA had a maximum induction of 142.8 ± 9.3% (E_2 luciferase induction = 100%) and an EC_{50} of 0.63 μM. These data suggest that BPA binds to ERα and transduces a signal in these cell lines.

In a study by Tse Sum Bui et al. (2008), BPA exhibited low binding to a synthetic ER structure. In a yeast bioassay for the hER, a low hER activation was observed (EC_{50} = 24 μM, compared to E_2 value of $13x10^{-5}$ μM in the same assay), suggesting low if any ER mediated effect for BPA (Tse Sum Bui et al., 2008).

BPA (from 12.5 to 75 μM) activated expression of a reporter gene (luciferase) in two breast cancer cell lines (MCF-7 and MDA-MB-231 cells) cotransfected with the ERα gene. The estimated EC_{50} was 50 μM for the MCF-7 cells (Wu et al., 2008). Similar results were obtained from two breast cancer cell lines: MELN (derived from MCF-7) and MELP. These cell lines express ERα and the ERE-luciferase construct. Among several xenoestrogens studied in this model, BPA (from 10^{-5} μM to 1 μM) was the second most potent compound (after genistein) for activating ERα and expressing the reporter gene. The estimated EC_{50} for BPA was 0.1 μM in the MELN cells and 0.5 μM in the MELP cells. The maximum stimulation of the reporter gene was

110% and 95% for the MELN and MELP cells, respectively (100% activity is the obtained with 10^{-3} μM E_2 in this model) (Buteau-Lozano et al., 2008).

BPA stimulates ER and reporter gene (luciferase) in the stable transfected MVLN cell line (this cell line expresses both ERα and ERβ) (Bonefeld-Jorgensen et al., 2007). In this *in vitro* model, BPA elicited a dose-dependent luciferase activity from 0.01 μM to 50 μM. The maximum activity was 75% of the maximum obtained by E_2 in the same system and the EC_{50} for BPA was 3.9 μM.

BPA also stimulated ER and a reporter gene (luciferase) in stable transfected HeLa cells with hERα or β and the ER-responsive reporter gene (Paris et al., 2002). Compared to controls, BPA increased the luciferase activity from 0.1 μM to 10 μM in both ERα and ERβ cells. The EC_{50} for BPA was 0.2 μM and 0.3 μM for ERα and ERβ cells, respectively while the EC_{50} for E_2 (positive control) was $8x10^{-6}$ μM for ERα-responsive cells and $2x10^{-5}$ μM for ERβ-responsive cells (Paris et al., 2002).

BPA from 0.01 μM to 1 μM and E_2 from 10^{-6} μM to 10^{-3} μM activated prolactin (PRL) secretion in primary cultured pituitary cells from F344 rats (Steinmetz et al., 1997). The EC_{50} for BPA, estimated from the dose response curve, was 0.02 μM and had a similar maximum stimulation to E_2, less than a 2.5-fold increase (Steinmetz et al., 1997) .

BPA binds and activates human ER in HepG2 hepatoma cells transiently cotransfected with the human ERα gene and a luciferase reporter gene (Gould et al., 1998). The EC_{50} was 0.0099 μM for E_2 and 0.218 μM for BPA. In addition, BPA's maximal activity was 70% of that of E_2. BPA activity was absent in cells transfected with a ER-null gene and inhibited by co-incubation with 1 uM of ICI. BPA activated rat ER cotransfected with luciferase into the HepG2 cells. Maximum activation of ER by BPA was 29% greater than that of E_2. However, the EC_{50}s were 0.0065 μM and 0.210 μM for E_2 and BPA respectively, similar to the calculated EC_{50} for human ERα (Gould et al., 1998).

BPA in the range 0.01 μM – 10 μM activates ERα of MCF-7wt and MCF-7SH cells (Vivacqua et al., 2003) transfected with luciferase. The calculated EC_{50} for BPA in this model was near 0.01 μM in MCF-7SH and between 0.01 – 0.1 μM in MCF-7wt cells. Co-incubation with 10 μM OH-tamoxifen inhibited the BPA activation of the reporter gene in both cell types (Vivacqua et al., 2003).

BPA competed with E_2 for binding the hERα and E_2 antibody in an enzyme-linked immunosorbant assay. The calculated dissociation constant (K_D) for BPA is about 1 μM (relatively high if compared to $K_{D,DES}$ at 10^{-3} μM in the same system) (Kwon et al., 2007).

Table E1. Studies reporting interaction of BPA with estrogen receptor (ER).

Reference	Species/type	model	EC_{50}	Max response (%)*
Sun et al., 2008	Human ERα	MCF7	0.34 μM	59
Sun et al., 2008	Rat ERα	ERα-1	0.63 μM	142
Tse Sum Bui et al., 2008	Human ER	yeast	24 μM	-
Wu et al., 2008	Human ERα	MCF7	50** μM	-
Wu et al., 2008	Human ERα	MDA-MB-231	-	-
Buteau-Lozano et al., 2008	Human ERα	MELN	0.1** μM	110
Buteau-Lozano et al., 2008	Human ERα	MELP	0.5** μM	95
Bonefeld-Jorgensen et al., 2007 NTP-B	Human ERα; β	MVLN	3.9 μM	75
Paris et al., 2002 NTP-A NTP-B	Human ERα	HeLa	0.2 μM	-
Paris et al., 2002 NTP-A NTP-B	Human ERβ	HeLa	0.3 μM	-
Steinmetz et al., 1997 NTP-A EU 2003	Rat F344	Anterior pituitary	0.02 μM	100 PRL production
Gould et al., 1998 NTP-A EU 2003	Human ERα	HepG2	0.22 μM	70
Gould et al., 1998 NTP-A EU 2003	Rat ERα	HepG2	0.21 μM	129
Vivacqua et al., 2003 NTP-A	Human ERα	MCF7-SH	0.01 μM	50
Vivacqua et al., 2003 NTP-A EU 2003	Human ERα	MCF7 wt	0.01-0.1 μM	50

EC_{50}: half-maximal effective concentration; * Percent of maximum response reporter gene activation relative to stimulation with the natural receptor ligand, i.e., E_2 for ER; MCF-7/ MDA-MB-231: human breast cancer cell lines; MELN: breast cancer cell line, derived from MCF7; MELP: breast cancer cell line, derived from MB-231; MVLN: luciferase stable transfected cell line derived from MCF-7; HeLa: human cervical carcinoma cell line; HepG2: hepatoma cell line; MCF-7SH: hormone independent variant of MCF-7 cell line; ** Value estimated from the original graph; - No data; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008; NTP-B: NTP Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

E.1.2.2. Interaction of BPA with the androgen receptor (AR)

Androgenic activity of BPA was assessed in human prostate adenocarcinoma PC-3 cells stably transfected with the human androgen receptor (hAR) and AR-responsive luciferase gene (Paris et al., 2002). BPA from 10^{-6} to $3x10^{-5}$ M decreased the observed activity of 10^{-10} M of the androgen agonist methyltrienolone (R-1881). The concentration of BPA that caused 50% inhibition (IC_{50}) under these conditions was 7 μM (Paris et al., 2002).

BPA interacts with the AR in Chinese hamster ovary (CHO) cells transiently transfected with the hAR gene and luciferase reporter gene (Bonefeld-Jorgensen et al., 2007; Vinggaard et al., 2008). BPA (from 0.6–20 μM) decreased the AR activation of 10^{-4} μM R-1881 with an IC_{50} of 1 μM and maximum inhibition of 90% (Bonefeld-Jorgensen et al., 2007). In a similar model for hAR activity, an IC_{25} for BPA between 1 and 3 μM was calculated (Vinggaard et al., 2008).

Table E2. Studies reporting interaction of BPA with the AR.

Reference	species	model	IC_{50}	Max %*
Paris et al., 2002 NTP-A NTP-B	Human	PC-3	7 μM	-
Bonefeld-Jorgensen et al., 2007 NTP-B	Human	CHO	1 μM	90
Vinggaard et al., 2008	Human	CHO	1–3 μM (IC_{25})	-

IC_{50}: concentration that causes 50% inhibition; *: Percent of maximum response relative to a 100% inhibition obtained after incubation with 0.1 nM R1881 (an AR antagonist); -: no data; CHO: Chinese hamster ovary; PC-3: Human prostate adenocarcinoma cell line; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008; NTP-B: NTP Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

E.1.2.3. Interaction with plasma binding proteins

Bioavailability of sex hormones is a consequence of a well-controlled balance between hormone bound to a binding protein and free hormone concentration. Several steroids compete for binding to a limited number of binding sites on sex hormone binding globulins (SHBG). Similarly, environmental steroid mimics such as BPA could alter this balance if they bind to and therefore compete for SHBG.

Milligan et al. described a weak or absent BPA interaction with two sex hormone binding proteins, rat α-fetoprotein (rAFP) and human sex hormone binding globulin (hSHBG) (Milligan

et al., 1998). In this study, binding proteins were obtained from rat amniotic fluid (for rAFP) and plasma from 4 pregnant women (for hSHBG). BPA had <0.01% displacement capacity against dihydrotestosterone (DHT) for hSHBG and did not compete with E_2 for rAFP (Milligan et al., 1998). Déchaud et al. (Dechaud et al., 1999) demonstrated that BPA can compete with endogenous hormones for binding to hSHBG with an affinity constant (K_a) of $0.41x10^5$ for T and $0.72x10^5$ for E_2 (Dechaud et al., 1999). The IC_{50} of BPA for displacing T was 51 µmol/l and 13.6 µmol/l for displacing E_2.

Furthermore, the amount of free steroid (not bound to hSHBG) increased when plasma samples were incubated with increasing doses of BPA. With BPA at 100 µmol/l, the free T and E_2 increased 30% and 16%, respectively (Dechaud et al., 1999).

Table E3. Studies reporting interaction of BPA with plasma sex hormone binding protein.

Reference	species	Competition	IC_{50}
Milligan et al., 1998	Human SHBG	<0.01% for DHT	-
Milligan et al., 1998	Rat AFP	0% for E_2	-
Dechaud et al., 1999	Human SHBG	K_a for T = 4 µM K_a for E_2 = 7.2 µM	51 µM 13.6 µM

SHBG: Sex hormone binding globulin; AFP: Rat α-fetoprotein; K_a: association constant; IC_{50}: concentration that causes 50% inhibition; T: testosterone; E_2: estradiol; DHT: dihydrotestosterone; -: no data.

E.1.2.4. Interaction with other receptors

BPA binds to human estrogen-related receptor gamma (ERR γ) (Matsushima et al., 2008; Nose and Shimohigashi, 2008). This receptor is highly expressed in the brain and placenta. It is activated without ligand and does not bind E_2, but makes a strong complex with BPA, with a K_D of 5.5 nM and an IC_{50} of 13.1 nM for the ERR γ-ligand binding domain (Matsushima et al., 2008).

BPA binds and interacts with the aryl hydrocarbon receptor (AhR) (Bonefeld-Jorgensen et al., 2007; Kruger et al., 2008). The interaction of BPA with the AhR was tested in a bioassay using a mouse hepatoma cell line (Hepa1.12cR) carrying the reporter gene luciferase (AhR CALUX assay). BPA decreased AhR activation from concentrations of 50 µM upwards, with a maximum inhibition of 54% at 100 µM (Bonefeld-Jorgensen et al., 2007).

Table E4. Studies reporting interaction of BPA with ERRγ and AhR.

Reference	Species/model	Receptor*	IC_{50}	K_D
Matsushima et al., 2008	Human	ERRγ	0.013 uM	5.5 nM
Bonefeld-Jorgensen et al., 2007 NTP-B	Mouse Hepa 1,12CR	AhR	10^4 μM	-

*ERRγ: estrogen related receptor γ; AhR: aryl hydrocarbon receptor; $IC_{50:}$ concentration that causes 50% inhibition; K_D: dissociation constant; - no data; NTP-B: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

E.1.3. BPA interaction with signal transduction pathway and modification of hormone response after receptor activation

E.1.3.1. Changes in ER expression

A study in F344 rats reported that BPA alters ERα expression. Animals were exposed by oral gavage to 4, 40 or 400 mg/kg-d (low, medium and high dose groups, respectively) for two generations. The effect of BPA on ER expression was determined by Western blot analysis. In the F_0 generation, BPA increased the expression of ERα in female rats at the high dose while decreasing ERα expression in males at the same dose (Miao et al., 2008). In the F_1 generation, similar results were observed in the medium and high dose groups.

Table E5. Study reporting effect of BPA on ERα gene expression.

Reference	species	Treatment	F_0*	F_1**
Miao et al., 2008	F344 Rat	Oral; 4; 40; 400 mg/kg-d for 2 generations	↑♀ ↓♂	↑♀ ↓♂

* At the high dose

** At the medium and high doses

BPA was given orally at 40 mg/kg-d for 42 days (from mating to weaning) to lactating or non-lactating female SD rats to determine the expression of ER in the medial preoptic area (MPA), ventromedial nucleus of the hypothalamus (VMH) and the arcuate nucleus (ARC) of the rat brain (results are shown in Table E6). BPA increased the number of ER-immunoreactive cells (neurons) in the MPA of both groups. However, BPA decreased the number of ER-immunoreactive neurons in the ARC of lactating rats, with no effect in non-lactating animals. BPA caused no significant changes in the other two areas studied (Aloisi et al., 2001).

Table E6. Study reporting effect of BPA on ERα gene expression in the brain*.

Reference	species	Treatment	MPA		VMH		ARC	
			Lac	NL	Lac	NL	Lac	NL
Aloisi et al., 2001	SD Rat	Oral; 40 mg/kg-d for 42 days (mating to weaning)	↑	↑	0	0	↓	0

SD: Sprague Dawley; Lac: lactating; NL: non-lactating; 0: no significant change.* MPA: Medial preoptic area; VMH: ventromedial nucleus of the hypothalamus; ARC: arcuate nucleus.

BPA can alter ER expression in areas of the brain specifically related to reproduction (Ceccarelli et al., 2007). Male and female SD rats were treated from postnatal day (PND) 23–30 with an oral dose of 40 μg/kg-d BPA or peanut oil control. Animals were sacrificed at PND 37 and PND 90. This BPA treatment induced the following changes in the number of ERα-positive neurons of hypothalamic areas studied (Ceccarelli et al., 2007):

- ARC: Increased in both genders at PND 37; no differences at PND 90
- VMH: Increased in females at PND 37; no differences at PND 90
- MPA: No statistically significant differences but, a non-significant increase at PND 37

BPA also decreased testosterone (T) levels in male rats at PND 37. There was a trend to high E_2 levels in male rats at PND 90. No other significant changes were observed for T or E_2 in this experiment (Ceccarelli et al., 2007).

Table E7. Study reporting effect of BPA on ERα gene expression in the brain.

Reference	Species	Treatment	MPA		VMH		ARC	
			Lac ♀	NL ♂	Lac ♀	NL ♂	Lac ♀	NL ♂
Ceccarelli et al., 2007 NTP-A NTP-B EU 2008	SD Rat	Oral; 40 mg/kg-d PND 23–30	↑NS	0	↑	-	↑	↑

* All changes were observed at PND 37, no significant changes were observed at PND 90; SD: Sprague Dawley; Lac: lactating; NL: no lactating; MPA: Medial preoptic area; VMH: ventromedial nucleus of the hypothalamus; ARC: arcuate nucleus. Lac: lactating and NL: non-lactating; 0: no significant change.; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008; NTP-B: NTP Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

In 2007, Monje et al. (Monje et al., 2007) studied the effect of BPA on the expression of ERα mRNA and protein in the anteroventral periventricular (AVPV) region of the POA. Female Wistar rats were treated with s.c. injection of BPA at 0, 0.05, or 20 mg/kg every 2 days from PND 1–7. Male siblings were used as controls. Determinations of ERα mRNA and ERα protein were made at PND 8 and 21. Female control rats showed higher expression of ERα mRNA than untreated males at PND 8, but were similar at PND 21. ERα mRNA was decreased by BPA treatment at 20 mg/kg and increased at 0.05 mg/kg at PND 8. At PND 21, female and male ERα mRNA expression were similar. At PND 21, BPA increased ERα mRNA expression at both concentrations used. BPA also modified ERα protein expression in the POA with similar results at PND 8 and 21. The 20 mg/kg BPA dose decreased ERα protein in female rat AVPV. Female rats showed an increase in the ERα protein expression at the lower BPA dose (0.05 mg/kg). No differences in E_2 serum levels were observed in PND 21 rats (Monje et al., 2007).

Table E8. Study reporting effect of BPA on ERα gene expression in the brain III (AVPV of POA)*.

Reference	**Species**	**Treatment** s.c.; mg/kg-2 days PND 1–7	**PND 8** **mRNA**	**Prot**	**PND 21** **mRNA**	**Prot**
Monje et al., 2007 NTP-B	Wistar Rat	0.05	↑	↑	↑	-
		20	↓	↓	↑	↓

* AVPV: anteroventral periventricular; POA: preoptic area; PND: postnatal day; NTP-B: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

E.1.3.2. Changes in progesterone receptor (PR) expression

Observations of estrogenic effect were made *in vivo* after treating immature SD rats with an oral dose of 0, 5, 10, 25, 50, 100 or 150 mg BPA/kg-d for three days. In a standard uterotrophic assay model, BPA had no effect on uterine weight. However, at all doses, BPA elevated the PR content from 50% to 90%, and increased peroxidase activity at the two highest doses by 60% and 100%, respectively (Gould et al., 1998). In contrast, BPA at the lowest dose decreased peroxidase activity. The observed effects of BPA on PR and peroxidase activity were lower than those obtained with $5x10^{-4}$ mg E_2/kg-d. BPA given together with E_2 at the same doses had no effect on the increase in uterine weight caused by E_2, but decreased E_2's effect on peroxidase by 43% (at the two higher doses of BPA) and reduced stimulation of PR levels by E_2 by 55% at all doses of BPA (Gould et al., 1998).

The effect of BPA on PR was confirmed by Ashby and Odum (Ashby and Odum, 2004). Alpk:APfSD (Wistar derived) rats were treated with an oral dose of BPA at 0.002–800 mg /kg-d for three days. In this model, BPA (at 200, 400, and 800 mg/kg-d) elevated the PR mRNA content with a maximum of a 3-fold increase with respect to controls (Ashby and Odum, 2004). No effects were observed at the lower doses (from 0.002 to 20 mg/kg-d) . The PR gene expression was followed by an increase in PR protein at the highest dose.

Table E9. Studies reporting effect of BPA on progesterone receptor gene expression.

Reference	Species/model	Treatment	PR	E_2-stimulated PR
Gould et al., 1998 NTP-A EU 2003	SD immature rat	Oral; 5–150 mg/kg-d x 3 days	↑	↓
Ashby and Odum, 2004 NTP-A EU 2008	Wistar Rat (Alpk:APfSD)	Oral; 0.02–800 mg/kg-d x 3 days	↑*	-

SD: Sprague Dawley; PR: progesterone receptor; * At 200, 400, and 800 mg/kg-d doses; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

E.1.3.3. Cytoplasmic biochemical alterations

Kabil et al. (Kabil et al., 2008) demonstrated that BPA and E_2 induced micronucleus formation (believe to be a cancer marker). These effects are mediated by the activation of cytoplasm second messenger signaling *in vitro*. MCF-7 cells treated with 5 μM BPA showed a two-fold increase in micronucleus formation; similar results were obtained with 1 nM E_2. This E_2 effect was not blocked by co-incubation with the ER agonist tamoxifen or ICI. The activation of the ER by E_2 or BPA rapidly triggers a cytosolic second messenger cascade, the phosphorylation of kinases Erk1 and Erk2. These kinases are involved in the formation of micronuclei. When cells were co-incubated with E_2 and BPA in the presence of 25 μM Erk1/Erk2 inhibitors, the observed number of micronuclei decreased. These data support the idea that BPA mimics E_2-driven cytosolic second messenger activation. This particular endpoint leads to DNA instability that may mediate cancer formation in the mammary gland.

E.1.4. Interaction with Steroid Metabolism

Steroidogenesis is an important biochemical step that could be altered by BPA and possibly change steroid hormone levels in the body. The metabolism and excretion of steroid hormones plays an important role in maintaining the body's endocrine balance, and could also potentially be disrupted by BPA.

E.1.4.1. Effects on steroidogenesis

BPA treatment (0.01 to 100 μM) for 72 h, modified the P production of porcine granulosa cells (GC) in culture (Mlynarcíková et al., 2005). GC from antral follicles were isolated and cultured

for 72 h with various concentrations of BPA. BPA at 1 μM stimulated P production while an inhibition of P production was observed at 100 μM. In another experiment, granulosa cells were incubated with 1 μg/ml of FSH or LH. Both gonadotropin treatments induced a 40 to 50-fold increase in P levels. BPA at 1 μM increased FSH-stimulated P production, while inhibition of FSH-stimulated P production was observed at 100 μM BPA. Treatment with BPA from 0.01 to 10 μM did not alter LH-stimulated P production. However, BPA at 100 μM M decreased LH-induced P production. FSH stimulated production of E_2 by GC by 50%. BPA in the concentration range of 1 μM to 100 μM significantly antagonized FSH-stimulated E_2 production (Mlynarcíková et al., 2005).

Table E10. Studies reporting effect of BPA on steroid production.

Reference	Species/model	Treatment	P	E_2	P + FSH*	E_2 + FSH*	Aromatase expression
Mlynarcíková et al., 2005 NTP-A	Pig granulosa cells	0.01–100 µM/72 h	↑ 10 µM ↓ 100 µM	- -	↑ 1 µM ↓	↓ 1 µM ↓	-
Kwintkiewicz and Giudice, 2008b	Human granulosa cell line KGN	20 to 100 µM/48 h	-	-		↓**	↓**
Akgul et al., 2008	SD rat: Theca interstitial Granulosa cells	10–1000 nM/48 h 20 and 100 nM	↑ 0	-	-	-	-

P: progesterone; E_2: estradiol; FSH: follicle stimulating hormone; * in the presence of 1 µg/ml FSH; SD: Sprague Dawley; ** previously stimulate aromatase expression with 100 ng/ml FSH. Maximum inhibition about 90% at100 µM BPA; 0: no significant change; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

BPA at 100 μM inhibited aromatase activity of human JEG-3 choriocarcinoma cells by 59% relative to a 100% inhibition achieved by 0.01 μM of a known aromatase inhibitor, 4-androsten-4-ol-3,17-dione (Bonefeld-Jorgensen et al., 2007).

BPA (500 μM) decreased aromatase activity by 30% in a human placental microsomal fraction incubated for 15 min (Benachour et al., 2007). P_{450} aromatase (aromatase) requires NADPH cytochrome P_{450} reductase (reductase). Aromatase and reductase enzymes were purified from equine testis. These enzymes were incubated with increasing doses of BPA (range 10 to 1000 μM). The calculated IC_{50} for aromatase was 435 μM and for reductase was 1250 μM. These data indicate that the inhibitory effect of BPA on aromatase was directly attributable to aromatase rather than the redox partner reductase.

Table E11. Studies reporting effect of BPA on aromatase activity.

Reference	Species/model	Treatment	Aromatase activity	E_2	Reductase Activity
Bonefeld-Jorgensen et al., 2007 NTP-B	Human JEG-3 cells	100 μM /48 h	↓	-	-
Benachour et al., 2007 NTP-B	Human placenta microsomes	500 μM/15 min	↓	-	-
Benachour et al., 2007 NTP-B	Equine testis purified enzyme	10–1000 μM/15 min	↓ IC_{50}: 435 μM	-	↓ IC_{50}: 1250 μM

E_2: estradiol; JEG-3: human choriocarcinoma cells; IC_{50}: concentration that causes 50% inhibition; NTP-B: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

BPA contributed to down regulation of FSH stimulated aromatase expression and estradiol production in human GC (KGN cell line) *in vitro* (Kwintkiewicz and Giudice, 2008a; Kwintkiewicz and Giudice, 2008b). FSH treatment for 48 h stimulated aromatase expression by 10-fold in KGN cells. BPA, at concentrations from 20 to 100 μM, decreased the aromatase gene expression induced by FSH in a concentration-dependent fashion with the maximum effect (91% inhibition) at 100 μM (Kwintkiewicz and Giudice, 2008b). Similarly, BPA decreased E_2 production in this culture by 90% at 100 μM treatment.

Cultured theca interstitial (TI) cells and GC from immature SD rats primed with pregnant mare serum gonadotropin (PMSG) were incubated for 8h with BPA from 0.01 to 1 μM. BPA treatment produced a near 20% increased in P production at 0.05 and 0.1 μM dose in TI cells with no effect on GC at any BPA dose (Akgul et al., 2008).

E.1.4.2. Steroid metabolism, excretion

Hanet et al. (Hanet et al., 2008) studied *in vitro* the effect of BPA on hepatic metabolic enzymes and E_2 conjugated transporter proteins using a human hepatic cell line (HepG2). BPA may affect the metabolism of E_2 in this system by modifying hepatic metabolic enzymes and/or transporter proteins.

Treatment with 80 μM BPA for 24 and 48 h altered the mRNA expression of three metabolic enzymes involves in E_2 metabolism (UGT1A1, UGT2B7 and ST1E1). The same BPA treatment increased the mRNA expression of two phase III transporters (MRP2 and MRP3) involved in E_2 metabolism in this system (Hanet et al., 2008).

In a separate experiment, increasing doses of BPA (2.5, 8, 25, and 80 μM) for 24 h were used. Under these conditions, the effects of BPA treatment on mRNA enzyme expression were:

- Increased UGT1A1 at 8 and 25 μM
- Decreased UGT2B7 at 80 μM
- Decreased ST1E1 at 25 and 80 μM

Similarly, the effect of BPA treatment on mRNA expression of conjugated E_2 transporters were:

- Decreased MDR1 at 25 μM and increased at 80 μM
- Increased MRP2 and MRP3 at 80 μM

BPA at 80 μM/24 h decreased the protein expression of ST1E1 and increased the protein expression of transporters MRP2 and MRP3. The HepG2 cells express a relatively low number of ER – the mRNA for ER was undetectable by PCR in this study. Therefore, the observed BPA effect on E_2 metabolism may not be related to the traditional ER pathway (Hanet et al., 2008).

To study E_2 flow, HepG2 cells were incubated for 24h with 80 μM BPA. Then, cells were exposed to 1 nM radiolabelled-E_2 ($[^3H]$-E_2) and the intracellular label was determined at different times up to 4h. BPA, at the same doses that changed the E_2 metabolic enzymes and transporters, decreased the intracellular radiolabel E_2, indicating a faster E_2 metabolic rate relative to control cells (Hanet et al., 2008).

Table E12. Study reporting effect of BPA on E_2 metabolism.

Reference	Species/model	Treatment	Metabolic enzymes mRNA	Metabolic enzymes protein	Transporters mRNA	Transporters protein
Hanet et al., 2008	Human HepG2 cells	80 μM/24 h*	↓	↓	↑	↑
		8, 25 μM/24 h	↑	0		

E_2: estradiol; HepG2: human hepatic cell line; * At this treatment there was a net decrease in E_2 flow.

E.1.5. Other endocrine effects of BPA

E.1.5.1. Effects on gonadotropins

BPA altered the pattern of LH secretion in lambs (Evans et al., 2004). Lambs at four weeks of age were treated with 3.5 mg/kg BPA intramuscularly (i.m.) twice weekly for 7 weeks. Animals were ovariectomized (OVX) two weeks prior to the end of the experiment. Blood samples were collected twice a week and every 15 min prior to euthanasia. BPA treatment significantly reduced mean LH concentration from 7.7 to 4.0 ng/ml ($P<0.05$), LH pulse amplitude from 7.1 to 1.6 ng/ml ($P<0.005$), and LH frequency from 6.7 to 2.3 pulses per 6 h ($P<0.005$).

E.1.5.2. Effect on other hormones

BPA stimulated production of PRL *in vivo* and *in vitro* in F344 and SD rats (Steinmetz et al., 1997). In the *in vitro* component of the study, pituitary cells from OVX rats were incubated for 3 days with E_2 or BPA. BPA caused increased PRL production, but at a 1000 to 5000-fold lower potency than E_2. In the *in vivo* component, animals were treated with silastic implants containing crystalline E_2 or BPA. The estimated rate of delivery was 40–45 μg/d and 1.2–1.5 μg/d for E_2 and BPA, respectively. Both rat strain had increased PRL concentrations in serum in response to E_2 treatment but only F344 rats had a positive response to BPA. The same E_2 and BPA treatments increased production of PRL releasing factor (PRF) as measured in a bioassay for PRF activity in F344, but not SD, rats. E_2 and BPA stimulated PRF activity from posterior pituitary cells from F344 but not from SD rats. Treatment with E_2 (0.01 μM) and BPA (1 μM) stimulated ERE-driven reporter gene (luciferase) production/activity in anterior and posterior pituitary cells from F344 rats (Steinmetz et al., 1997).

Table E13. Studies reporting effect of BPA on pituitary hormones.

Reference	Species/model	Treatment	LH			PRL/PRF
			Conc	Amp	freq	
Evans et al., 2004 NTP-A	Lamb 4 wks old	3.5 mg/kg i.m. twice daily/7 wks	↓	↓	↓	-
Steinmetz et al., 1997 NTP-A EU 2003	Pituitary cell culture: F344 Sprague Dawley	1.2–1.5 μg-d/ 3 days		- -		↑ 0

LH: luteinizing hormone; PRL: prolactin; PRF: PRL releasing factor; FSH: follicle stimulating hormone; ↑: incease; 0: no change; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

BPA affected glucose metabolism and insulin production in mice (Alonso-Magdalena et al., 2006). Swiss albino OF1 male mice (8–10 weeks of age) were treated with BPA or E_2 subcutaneously at 1, 10, or 100 μg/kg. BPA and E_2 treatments at 10 and 100 μg/kg induced a decrease in blood glucose concentration after 30 min of injection. This change in glycemia was accompanied by an increase in insulin concentration in response to a 10 μg/kg treatment with BPA and E_2. These results showed that BPA is mimicking the E_2 effect on glucose and insulin plasma concentration (Alonso-Magdalena et al., 2006). Pre-treating mice with ICI at 500 μg/kg-d for three days did not change the response to E_2 or BPA. Since ICI is a known inhibitor of estrogenic activity by specific interaction with the ER, these data suggest that the observed effects may be mediated by a non-classic ER-mediated response of E_2.

BPA and E_2 treatment at 10 and 100 μg/kg-d for four days increased the β-cell insulin content with a greater response at the higher dose (Alonso-Magdalena et al., 2006), This effect of E_2 and BPA was blocked by co-treatment with 500 μg/kg-d of ICI for four days, suggesting that the effect of E_2 and BPA on β-cells insulin content is also mediated through interaction with the ER.

BPA stimulates insulin production from pancreatic cells *in vitro* (Alonso-Magdalena et al., 2008). Pancreatic islets were isolated from male mice and treated with various doses of BPA (10^{-10} to 10^{-6} M) for 48 h. BPA stimulated insulin production in this system with an inverted U-shape dose response curve. The maximum stimulation was observed at BPA concentrations of 10^{-9} and 10^{-8} M. E_2 has been shown to stimulate insulin production with a similar profile to that observed with BPA treatment. Swiss albino OF1 male mice were s.c. injected with BPA at 100 μg or 1 mg/kg-d for four days. Insulin content was measured in pancreatic islets. BPA at the

lower dose, but not at the high dose, stimulated production of insulin by the pancreatic cells of treated animals. To investigate if this effect was mediated by ER, pancreatic cells were co-incubated *in vitro* with BPA and E_2 in the presence of ICI. Both, E_2 and BPA at 10^{-9} M failed to increased insulin when co-incubated with ICI at 10^{-6} or 10^{-5} M. In addition, E_2 or BPA did not stimulate insulin production from islets coming from ERα knock out (ERαKO) mice. On the contrary, when cells were obtained from ERβ knock out (ERβKO) mice, both E_2 and BPA stimulated insulin production. These data indicate that E_2 and BPA effects on insulin production are both mediated by ERα (Alonso-Magdalena et al., 2008).

BPA can also interfere with glucose metabolism by interacting with plasma membrane receptors in pancreatic α-cells (Alonso-Magdalena et al., 2005). Pancreatic α-cells were obtained from albino Swiss mice (8–10 weeks old). Cells were loaded with the Ca^{+2}-sensitive dye Fluo-3 and incubated with E_2, E_2-HRP (a membrane impermeable form of E_2) and BPA. These cells present a high oscillatory calcium wave that decreases in the presence of high glucose concentrations. Incubation of the cells with E_2-HRP and BPA (at 1 nM) reduced the oscillatory calcium movement after 5 min of stimuli with complete blockage in 50% of the cells. This observation suggests that there is a plasma membrane compartment triggering the specific estrogenic effect on the calcium movement. Furthermore, the observed effects of BPA and E_2-HRP were not sensitive to pre-incubation with 1 μM ICI (Alonso-Magdalena et al., 2005), suggesting the lack of participation of the classical ER in this effect. However, the BPA effect on Ca^{+2} oscillation was mimicked by 8-bromo cyclic guanidyl mono phosphate (8Br-cGMP), a cytosolic second messenger, and was sensitive to a protein kinase G (PKG) inhibitor (KT-5823). Since the E_2-HRP cannot cross the plasma membrane and the estrogenic effect is not blocked by ICI, it is suggested that the effects of E_2 and BPA in this model are mediated by a plasma membrane receptor associated with the cGMP/PKG second messenger pathway (Alonso-Magdalena et al., 2005).

In women, plasma BPA concentration correlates with high androgens levels and obesity (as measure by high body mass index). BPA levels are elevated in obese or non-obese women with polycystic ovary syndrome (PCOS) (Takeuchi et al., 2004).

Table E14. Studies reporting effect of BPA on insulin and glucose metabolism.

Reference	Species/model	Treatment	Insulin Plasma/β-cells	Glucose
Alonso-Magdalena et al., 2006 NTP-A NTP-B	Swiss albino mice 8–10 wks old	μg/kg s.c. 1 10 100	plasma 0 ↑ -	0 ↓ ↓
Alonso-Magdalena et al., 2006 NTP-A NTP-B	Swiss albino mice 8–10 wks old	μg/kg-d/4 days 10000 100000	β-cells ↑ ↑	-
Alonso-Magdalena et al., 2008	♂ Mice/β-cells	0.0001 μM – 1μM/48h	↑ max at 1–10nM	-
		s.c./kg-d/4 days	β-cells	-
	Swiss albino	100 μg	↑	-
		1000 μg	0	
			0	
	ERαKO		↑	
	ERβKO			

↑: increase; ↓: decrease; 0: no change; -: no available; ERαKO: estrogen receptor alpha knock out; ERβKO: estrogen receptor beta knock out; NTP-A: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008; NTP-B: NTP Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

BPA can interfere with thyroid hormone (triiodothyronine, T_3) action and act as a receptor antagonist (Moriyama et al., 2002). Nuclear T_3 receptors (TR) were isolated from SD rat livers and incubated with radio-labeled T_3 ($[^{125}I]T_3$) and increasing doses of unlabeled T_3 (0.001 to 1 μM) or BPA (1 to 1000 μM). BPA was found to displace T_3 from binding to TR with a Ki of 200 μM (Moriyama et al., 2002).

Recently, Fini et al. (Fini et al., 2007) used an *in vivo* amphibian bioassay to demonstrate that BPA (10 μM) reduced the activity of thyroid hormone. Transgenic tadpoles of *X. laevis* that expressed a TH-sensitive reporter gene (fluorescent, TH/bZip-eGFP) were used as a model to test the effect of BPA on T_3. Tadpoles were exposed, at larval stage NF45 (5 days after hatching) to BPA, alone or in combination with T_3. T_3 activity was determined by increasing number of relative fluorescent units (RFU) in the assay. BPA treatment for 72 h alone caused no changes in the RFU. However, when co-incubated with 0.005 μM of T_3, BPA from 1 to 10 μM decreased (by 20–30%) the T_3-augmented RFU (Fini et al., 2007).

Table E15. Studies reporting effect of BPA on thyroid hormone T_3.

Reference	Species/model	Treatment	Binding to TR Ki	T_3 activity
Moriyama et al., 2002 NTP-B	SD rat liver Nuclear T_3 receptors	1 μM–1000 μM	200 μM	-
Fini et al., 2007 NTP-B	*X. leavis* tadpoles	1 μM–10 μM / 72 h	-	↓*

T_3: triiodothyronine;TR: thyroid hormone receptor Ki: Inhibition constant; * decrease the observed activity of T_3 at 5 nM; NTP-B: National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR) Brief on Bisphenol A in "NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A", September 2008.

E.2. Pharmacokinetics

This section briefly describes absorption, distribution, metabolism, and excretion of BPA in humans and laboratory animals. A number of factors can influence the pharmacokinetics of the compound, including route, species, sex, dose level, pregnancy and age (Pottenger et al., 2000; Matsumoto et al., 2002; Domoradzki et al., 2004; Tominaga et al., 2006; Taylor et al., 2008). These features of BPA pharmacokinetics are also briefly discussed. A more detailed discussion can be found in the NTP-CERHR Monograph (CERHR, 2008).

E.2.1. Absorption

NTP-CERHR (CERHR, 2008) and EU (EU, 2003) conclude that BPA is extensively absorbed after exposure via the oral route. No published studies of absorption after inhalation or dermal exposure were identified, although toxic effects have been reported after acute and chronic inhalation exposures to BPA (Sections A.3.2 and A.3.3).

As discussed below, a study in rats and non-human primates indicated that BPA is less bioavailable via oral exposure than by s.c. injection (Tominaga et al., 2006). In both rats and non-human primates, (Tominaga et al., 2006) found oral administration of BPA resulted in lower area under the serum concentration curve (AUC) than by s.c. administration.

In contrast, a study in three day old CD-1 mice conducted at lower doses reported similar bioavailability by both s.c. and oral routes (Taylor et al., 2008). Plasma concentrations of unconjugated ^{3}H-BPA were the same 24 h after administration of 35 or 395 μg ^{3}H-BPA/kg orally or via s.c. injection. At each dose, there were also no significant difference across routes in the following calculated parameters: Cmax, initial rate constant (K_{init}), terminal phase elimination rate constant (K_{term}), AUC $_{0–24hr}$, AUC$_{0 \rightarrow \infty}$. These findings suggest that oral absorption may be nearly complete at the dose levels studied.

E.2.2. Distribution

Studies in both humans and laboratory animals indicate that BPA is widely distributed. The circulatory system is the major transporter of BPA within the body. Bisphenol A is rapidly absorbed into the blood and metabolized primarily in the liver following i.p. or s.c. injection, or oral exposure. In rats, most orally administered BPA circulates in plasma as BPA-glucuronide (Pottenger et al., 2000). After s.c. administration, most BPA is absorbed into systemic circulation without being metabolized in the subcutaneous tissue.

Laboratory animals. Yoo et al. administered BPA to adult rats at doses of 0.2, 0.5, 1, or 2 mg/kg. It was given by simultaneous i.v. bolus injection plus infusion to steady state, and levels were measured in serum and various organs. When expressed in concentration terms (e.g., amount accumulated per gram organ weight), BPA was found predominantly in the lung, followed by kidneys, thyroid, stomach, heart, spleen, testes, liver, and brain (Yoo et al., 2000). Ratios of the organ to serum bisphenol A concentrations exceeded unity for all the organs

examined (ratio range 2.0–5.8) except for brain (ratio 0.75). The authors suggest given the high systemic clearance and short elimination half-life, BPA is unlikely to accumulate significantly in the rat (Yoo et al., 2000).

A number of studies have looked at distribution of BPA after a single exposure. In one study, pregnant mice were injected with 100 mg/kg/day BPA. Within 0.5 hour of exposure, BPA was found in placenta, maternal and fetal serum, liver, and brain and fetal uterus and testis (Uchida et al., 2002). BPA was also given to pregnant Japanese monkeys (*Macaca fuscata*) and measured in fetuses (removed by cesarean section one hour after maternal exposure (50 mg/kg bw, s.c. injection). In the fetuses, the highest concentrations of BPA were found in fetal heart, intestine, liver, spleen, kidney, thymus, muscle, cerebrum, pons and cerebellum. The study authors concluded that the placenta does not protect the fetus from BPA exposure (Uchida et al., 2002).

A drinking water study in which mice were exposed to 5 or 10 μg BPA (both gestational and neonatal exposure) looked for BPA in the brain, kidney, liver and testes of 4 week old male pups. The study detected the highest concentration of BPA in the kidney, followed by testis, brain and then liver (Kabuto et al., 2004).

Humans. BPA has been detected in amniotic fluid, in umbilical cord blood and placenta (CERHR, 2008). One study found BPA levels in amniotic fluid samples were approximately 5-fold higher during early pregnancy compared to samples collected during late pregnancy (Ikezuki et al., 2002 as cited in CERHR, 2008). Another study compared BPA concentrations in maternal and umbilical blood (n= 37) and found that, in general, BPA concentrations were higher in maternal blood (4.4 ± 3.9 μg/L) than in fetal blood (2.9 ± 2.5μg/L). However, in 14 (of 37) cases fetal BPA was higher. The study also looked at BPA concentrations in placenta samples and found levels of 1.0–104.9 ng/kg (Schönfelder et al., 2002b).

BPA has also been detected in breast milk. Calafat et al. (2006) reported that free (unconjugated) BPA was found in breast milk samples from 12 of 20 women tested (median concentration, 1.1 μg/L, high, 7.3 μg/L).

Enterohepatic circulation of BPA is reported to occurs in laboratory rodents, but not in humans (Volkel et al., 2002). It is unclear the extent to which some enterohepatic cycling may occur in some human subpopulations.

E.2.3. Metabolism

Bisphenol A is conjugated and deconjugated by enzymatic processes. In laboratory rodents, BPA is glucuronidated in the liver and intestine by uridine diphosphate glucuronosyltransferase (UGT). There are numerous isoforms of UGT. The primary isoform of UGT responsible for BPA glucuronidation in rat liver is UGT2B1. UGT2B1 has similar homology to human UGT2B7 and UGT2B17. Both human isozymes are expressed in liver, but unlike UGT2B1 in rats, are also expressed in several steroid target tissues, such as brain, uterus, mammary gland, placenta, and testis (Belanger et al., 1998; Collier et al., 2002).

An *in vitro* study of human hepatocytes incubated with BPA found the major metabolites of BPA were BPA-glucuronide, BPA-glucuronide/sulfate diconjugate, and BPA-sulfate conjugate (Pritchett et al., 2002). The primary metabolite produced was BPA-glucuronide (Matthews et al., 2001; Pritchett et al., 2002; Inoue et al., 2004).

Volkel et al. investigated the metabolism and toxicokinetics of BPA in humans exposed to low doses of BPA (Volkel et al., 2002). After orally administering 5 mg of labeled bisphenol A-d_{16} (d_{16}-BPA) to 6 male (aged 28–54 years) and 3 female (24–31 years) subjects they detected only the d_{16}-BPA-glucuronide in urine and blood samples.

In a study conducted in healthy Koreans, ~10–33% parent compound, ~5–34% BPA-sulfate conjugate, and ~33–70% BPA-glucuronide were the urinary metabolite profiles from 15 men and 15 women (Kim et al., 2003; Ye et al., 2005). Gender-related differences were seen. In men, mean urinary levels were 29.1% BPA, 66.2% BPA-glucuronide, and 4.78% BPA-sulfate conjugate (Kim et al., 2003). For women, they were 33.4% BPA, 33.1% BPA-glucuronide, and 33.5% BPA-sulfate conjugate (Kim et al., 2003). Thus, there were significant levels of parent compound in samples from both men and women. Also, the authors concluded that women had a greater ability for sulfation compared with men.

E.2.4. Excretion

In rats, BPA is primarily eliminated through bile and feces, and a smaller percentage of the dose is eliminated through urine. Bisphenol A is the major compound detected in feces, and BPA-glucuronide is the major compound detected in bile and urine. In humans, most BPA is eliminated through urine as BPA-glucuronide. In the 10 human subjects investigated by Volkel et al., the administered dose of d_{16}-BPA was completely (118 ± 21%) recovered in urine; $T_{1/2}$ of urinary elimination was less than 6 h (Volkel et al., 2002).

In rats, fecal elimination of ^{14}C-BPA-derived radioactivity was the major elimination pathway for all dose groups, comprising 52–83% of the administered dose (Pottenger et al., 2000). The urinary elimination of ^{14}C-BPA derived radioactivity was consistently about 2-fold greater in females compared with males, across all doses and routes, representing 13–16% and 21–34% of the administered dose in males and females, respectively (Pottenger et al., 2000).

E.2.5. Age dependent pharmacokinetics (humans)

Calafat et al. evaluated 42 premature infants' urinary concentrations of several phenols, including BPA. At least one urine sample per infant was collected in 2003. Urine samples were stored under controlled conditions in vials at -40^{o}C until secondary analysis in 2008. Calafat et al. detected BPA in all of the first set of urine samples (Table E16).

Table E16. Distribution of the urinary concentration of BPA (μg/L)[a] in hospitalized premature infants (Adapted from Table 1 (Calafat et al., 2009)).

Species	# of Infants	No.< LOD	Geometric Mean (SD)	Median	Range		NHANES 2003–2004	
					Minimum	Maximum	Median	95th percentile
Total	41	0	30.3 (5.2)	28.6	1.6	946	3.7	16.0
Free	37	3	1.8 (3.2)	1.7	< LOD (0.4)	17.3	N.A.	N.A.

[a] The total concentrations are the sum of the free plus conjugated species of each phenol.
LOD limit of detection
N.A. not analyzed

Free BPA was found, but most was in a conjugated form (e.g. glucuronide, sulfate). The authors excluded the possibility that the measured BPA concentrations resulted primarily from contamination. The authors conclude conjugated species were the primary urinary metabolites of BPA, suggesting the premature infants have some capacity to metabolize BPA. The quantitative capacity of the young to metabolize BPA remains unanswered. Glucuronidation is less mature than adults at birth, and is likely even more so in utero. Data from Wistar rats discussed below suggests newborns have less capacity compared with adults to metabolize BPA (Matsumoto et al., 2002).

Physiological pharmacokinetic model was used to explore the age dependence of BPA pharmacokinetics. Both BPA and glucuronidated BPA were modeled (Edginton and Ritter, 2009). Using information gathered from toxicokinetic studies in adults, Edginton et al. built the PBPK model (Figure E1). The researchers then scaled the model to children < 2 years of age based on the age dependence of physiologic parameters. Edginton et al. assumed that 100% of the elimination of BPA was attributable to metabolism to its glucuronidated metabolite, BPA-Glu. At all ages, 100% of the applied BPA dose was modeled as absorbed to the portal vein, although there was an increase in the efficiency of first-pass metabolism with increasing age. Bioavailability in newborns, 3-month-, 6-month-, 1.5-year olds, and adults was 88%, 48%, 32%, 23%, and 18%, respectively. In all simulations, BPA and BPA-Glu steady state was reached within the first 48 h. Figure E2 presents average plasma concentrations at steady state and urinary concentrations for children and an adult male after administration of 1 μg BPA/kg/day. Edginton et al. estimated the average steady-state BPA plasma concentration in newborns to be

11 times greater than that in adults when given the same weight-normalized dose. Because of the rapid development of the glucuronidation process, this ratio dropped to 2 by 3 months of age. Simulation of typical feeding exposures, showed a 5-fold greater steady-state BPA plasma concentration in 3- and 6-month olds compared with adults, reflecting both a reduced capacity for BPA metabolism and a greater weight-normalized BPA exposure (Edginton and Ritter, 2009).

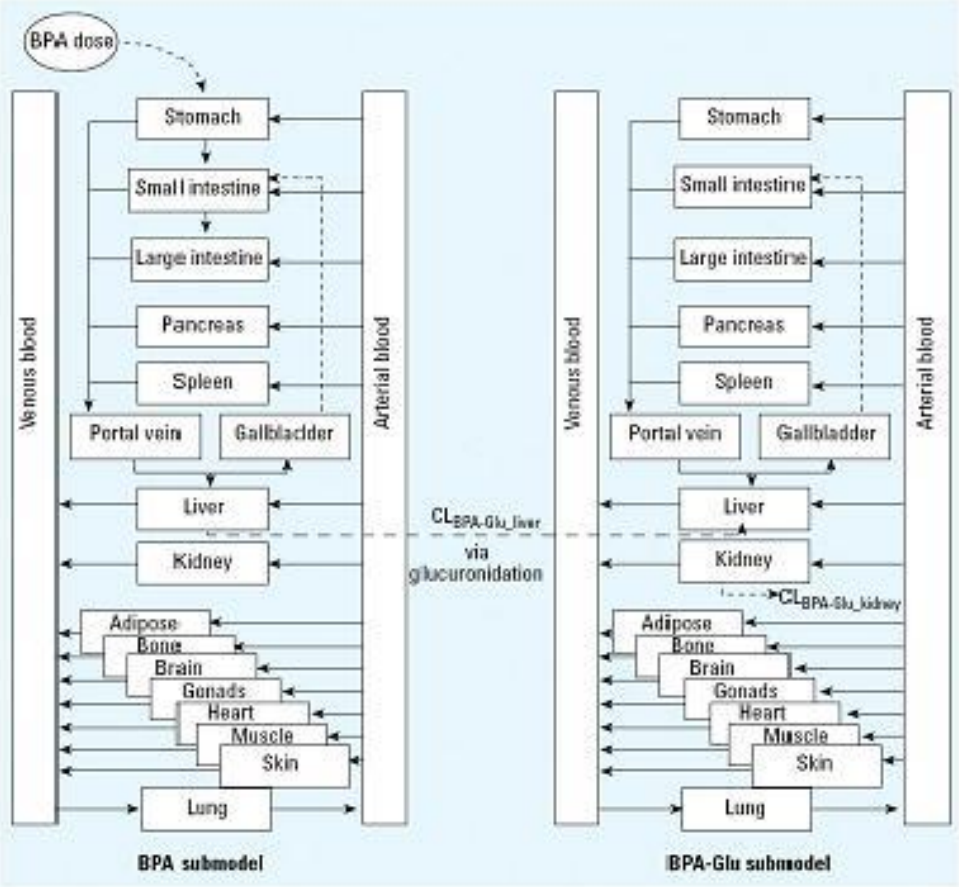

Figure E1. Schematic of the PBPK model structure consisting of BPA and BPA-Glu submodels. Input of BPA was to the stomach, thus simulating oral administration. Input of BPA-Glu was the hepatic metabolism of BPA to BPA-Glu in the liver (Edginton and Ritter, 2009).

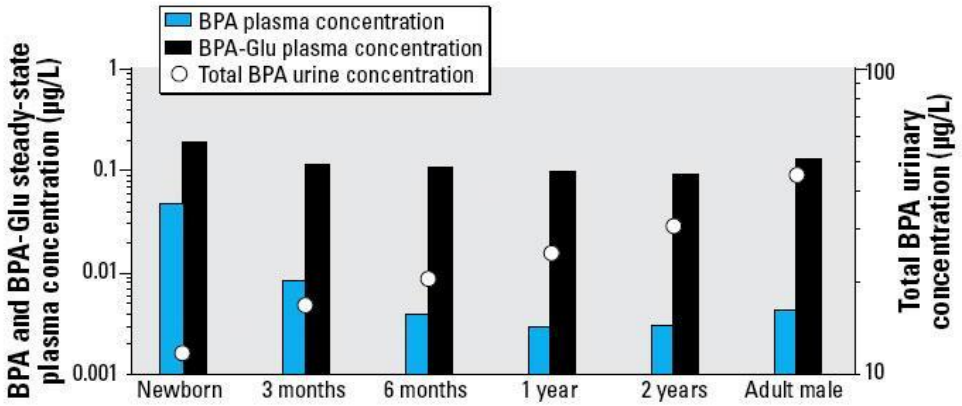

Figure E2. Estimated BPA and BPA-Glu average steady-state plasma concentrations in children and an adult after a 1 µg BPA/kg/day oral administration. Total BPA=BPA+BPA-Glu urinary concentration (Edginton and Ritter, 2009).

E.2.6. Age and pregnancy dependent pharmacokinetics (laboratory animal studies)

The ontogeny of glucuronyl transferases (GT) differs with age. The GT activity in fetal/neonatal rat liver varies by the type of substrate (Lucier, 1981), which may make fetal and neonatal animals more susceptible to BPA toxicity if there is insufficient GT activity relative to the BPA dose administered. Radio-labeled BPA was administered via gavage at 1 or 10 mg/kg to Sprague-Dawley rats on PND 4, 7, and 21, or to 11-week old adult rats (10 mg/kg dose only) (Domoradzki et al., 2004). Female neonates of all ages dosed with 10 mg/kg had higher plasma concentrations of BPA at earlier times post-dosing than adult female rats (Domoradzki et al., 2004). Male pups showed a similar pattern. Bisphenol A glucuronide and BPA concentrations in the plasma were greater in neonates than in adults, except at 24 hours post-dosing, suggesting an immaturity in the development of hepatic excretory function in neonatal rats (Domoradzki et al., 2004). Domoradzki and colleagues observed age-related differences in the plasma metabolite profiles as well as in the pharmacokinetics of BPA and BPA-glucuronide.

Domoradzki et al. examined the metabolism and pharmacokinetics of BPA in pregnant Sprague-Dawley rats at three gestational stages. Female rats were 11–14 weeks of age at dosing. Radio-labeled BPA (^{14}C-BPA) was administered orally at 10 mg BPA/kg to nongravid rats and to other groups on GD 6, 14, and 17 (Domoradzki et al., 2003). Radioactivity derived from ^{14}C-BPA was quantified in maternal blood, selected tissues, and the embryo or fetus (Domoradzki et al., 2003). Despite physiological changes associated with pregnancy, the tissue distribution, metabolism rates and routes of excretion of BPA and the plasma concentration-time profiles of BPA-glucuronide do not appear to be altered at any stage of gestation as compared to nonpregnant rats (Domoradzki et al., 2003). Similarly, a toxicokinetic study by Moors et al., demonstrated in DA/Han rats after a single i.v. administration of 10 mg BPA/kg the plasma concentration-time curve for BPA aglycone (a BPA metabolite) in pregnant DA/Han rats resembles that of nonpregnant DA/Han rats (Moors et al., 2006).

In pregnant mice, BPA is rapidly transferred to placenta and fetus and the BPA concentration declines slower in fetuses than in the dams (Kawamoto et al., 2005). A single dose of radio-

labeled BPA (^{14}C-BPA) at 1, 10 or 100 mg/5 ml/kg was given orally to pregnant ICR mice (8 wks old) on GD 15. Tissue and blood were collected. After a single dose of 10 mg ^{14}C-BPA/kg, Cmax in maternal blood was 3.36 μg BPA eq/ml and was obtained at 15 min (Tmax) after dosing. The radioactivity decreased rapidly after Tmax to increase again at 6 h to 0.48 μg BPA eq/ml. The peak radiolabel in tissues from dams was found at 20 min in liver, kidney, and stomach and at 6 h in other organs such uterus, ovary and placenta. In fetuses, the radioactivity was found soon after dosing (20 min) and in many tissues continue increasing up to 24 h after treatment. At 24 h, there was a similar radioactive distribution in reproductive tissues and brain between fetuses and dams. However, the distribution of radiolabel in digestive organs was one order of magnitude lower in fetuses than in dams at that time. The glucuronide conjugated substrate in maternal blood was the major metabolite of BPA (70% of radioactivity) at 20 min. In maternal liver, the major component was BPA (about 60% of radioactivity) at 6 h and the major metabolite was BPA-glucuronide (24% at 20 min and 9.7% at 6 h). In whole fetuses, the majority of the radioactivity was in the form of BPA at 20 min (63%). At 1 h and later, the major metabolite was BPA-glucuronide.

Zalko et al. investigated the metabolic fate of a low dose of BPA s.c. injected into pregnant CD1 mice using a tritium-labeled molecule (^{3}H- labeled BPA) (Zalko et al., 2003). Eleven pregnant CD1 mice were s.c. injected with 25 μg ^{3}H-BPA/kg on GD 17 and sacrificed 0.5, 2, or 24 h after administration. Additional studies were performed to explore the fate of BPA and to identify metabolites when a single oral dose of 25 μg ^{3}H-BPA/kg was administered to CD1 mice (n=3). Bisphenol A was extensively metabolized by CD1 mice. In the reproductive tract, residual levels ranged from 2.2 to 4.8 ng/g, respectively, for ovaries and amniotic fluids. Fetuses accounted for a total of 4% (3.7 ng/g) of the administered radioactivity, regardless of litter size. Fetal radioactivity was associated with unchanged BPA, BPA-glucuronide and a disaccharide conjugate. Identified metabolite structures in urine included the glucuronyl acid conjugate of BPA, several double conjugates, and conjugated methoxylated compounds demonstrating the formation of potentially reactive intermediates. The major compound in most samples, (maternal plasma, placenta, fetus, amniotic fluid, maternal liver) was BPA-glucuronide.

In a perfusion study, male (300–400g), non-pregnant female (240–280 g) and 20–21 day pregnant Sprague-Dawley rats (270–340 g; GD 20–21) were perfused with BPA by the portal vein. In total either 1.5 or 7.5 μM BPA was infused into the liver of each rat. Pregnant Sprague Dawley rats had lower total excretion of BPA-glucuronide compared with nonpregnant rats ($P<0.05$) (Inoue et al., 2004). During a 1-h perfusion, total excretion of the glucuronide from the liver of the male, non-pregnant female, and pregnant rats was 889.5 ± 69.6, 1256 ± 54.8, and 1038.8 ± 33.3 nmoles, respectively. Bilious excretion of the resulting glucuronide in pregnant rats was half of that in nonpregnant rats during a 1-h perfusion (Inoue et al., 2004). The non-pregnant rat liver absorbed more than 90% of all BPA substrate. From that, 84% and 91% (for low- and high-dose, respectively) was glucuronidated and excreted mainly into the bile. The pregnant rat glucuronidated about 69% of the substrate and the distribution of excretion was 55% by the bile duct and 45% by the vein (Inoue et al., 2004). Similarly, Matsumoto et al. reported rat hepatic microsomal UDP-glucuronosyltransferase activities of mother rats toward BPA was reduced by about half during pregnancy (Matsumoto et al., 2002). In addition, UDP-glucuronosyltransferase activities towards BPA were not detected in fetal rat liver (Matsumoto et

al., 2002). At three weeks of age, the activities had reached the same level as that of adult rats (Figure E3) (Matsumoto et al., 2002).

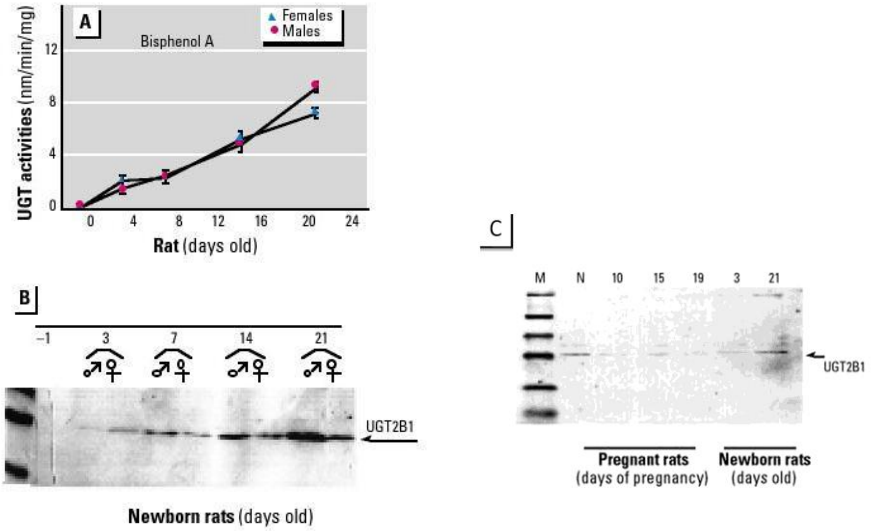

Figure E3. (A) Developmental increase in fetal and neonatal rat liver microsomal UDP-glucuronosyltransferase activities toward BPA. Assayed by HPLC. Results are means ± SE (error bars). (B) Western blotting of the liver microsomes from fetal and neonatal rats was performed using anti-UGT2B1. (C) Western blotting analysis of the liver microsomal proteins prepared from the pregnant and newborn rats was performed using anti-UGT2B1. Arrowhead show the UGT2B1bands. (Adapted from Figures 1 and 4 of (Matsumoto et al., 2002)).

Orally administered BPA has been reported to easily cross the placenta and enter the fetus (Miyakoda et al., 1999), but BPA-glucuronide does not easily pass through the placenta (Miyakoda et al., 2000). After administration of an oral dose of 10 mg BPA/kg to pregnant female rats, BPA-glucuronide in the fetus was not detected (Miyakoda et al., 2000). Similarly, BPA easily passes into the testis. One hour after administration of an oral dose of 10 mg BPA/kg to mature male rats, approximately 90% of the BPA was present as BPA-glucuronide in both blood plasma and testes. Intact BPA steadily decreased in blood plasma and increased slightly in testis 8 h after administration. In contrast to the gradual decrease of BPA-glucuronide observed in testis, the level of BPA-glucuronide in blood plasma measured as a percentage of the Cmax detected following oral BPA administration first decreased to 55% at 3 h and then increased to 100% at 8 h (Miyakoda et al., 2000).

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APPENDIX 1

Summaries of relevant studies of Bisphenol A not reviewed in the NTP-CERHR Monograph (NTP-CERHR, 2008) or EU Documents (EU 2003, 2008)

Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

October 2009

The studies are divided into three sections: developmental toxicity (Section 1), female reproductive toxicity (Section 2) and male reproductive toxicity (Section 3). Within each section, human and animal studies are listed separately and are ordered alphabetical by the first author.

Section 1: Developmental Toxicity Studies

Human Studies

*Padmanabhan, V., K. Siefert, et al. (2008). "Maternal bisphenol-A levels at delivery: a looming problem?" J Perinatol **28**(4): 258-63.*

The stated objective of this study was to determine if BPA is found in the circulation of pregnant women in the U.S. population. However, this study also examined the association between maternal plasma BPA concentrations and gestational length and birth weight. Blood samples from 40 mothers were collected at the time of delivery at the University of Michigan Hospital, between August 2006 and November 2006. The authors did not specify how the mothers were selected to participate. The freely available form of BPA in plasma was measured using HPLC-MS/MS with a LOD of 0.5 ng/ml. Maternal BPA concentrations (mean 5.9 ± 0.94 ng/ml, range 0.5–22.3 ng/ml) did not differ by maternal age, body mass index (BMI) or sex of the offspring. No differences were seen in length of gestation or birth weight relative to BPA concentrations $\leq$ 5 or > 5 ng/ml. Limitations identified in this study include the measures of BPA being obtained at birth and not earlier in gestation; the small sample size; the lack of control for potential confounders, such as race or socioeconomic status; and the lack of a measure of the glucuronide conjugates of BPA. Although the study did find levels of free BPA in maternal circulation, no measurements were made of BPA metabolites, BPA-glucuronide and BPA-sulfate. Finally, an important limitation is that with the small sample size of only 40 mothers the study lacked the power to detect significant effects on birth weight or length of gestation.

*Wolff, M. S., S. M. Engel, et al. (2008). "Prenatal phenol and phthalate exposures and birth outcomes." Environ Health Perspect **116**(8): 1092-7.*

In this prospective cohort study of prenatal exposures to phenols and phthalates and birth outcomes, pregnant women were enrolled at Mount Sinai Hospital in New York City from March 1998 to March 2002. This ethnically diverse cohort included 404 primiparous women who had their first prenatal visit before 27 weeks gestation. A prenatal questionnaire was administered to the women during the third trimester to obtain information on pesticide and other environmental exposures, sociodemographics information, maternal health and lifestyle habits. Maternal blood samples were taken and a urine sample was collected mostly during the third trimester. Birth outcome information was acquired from the hospital database. Urine samples were analyzed for 5 phenols and 10 phthalate urinary metabolites. BPA was analyzed using HPLC-MS (limit of detection = 0.4 μg/L). Sufficient specimen amounts were available for phenol analysis in 367 women. Of the phenols, BPA, 2,5-dichlorophenol, (2,5-DCP), and 2,4-dichlorophenol (2,4-DCP) were positively correlated with maternal prepregnancy BMI if the biomarker was expressed as micrograms per liter, but not if expressed as micrograms per gram

creatinine. None of the phenols were significantly associated with any birth outcomes in regression models, adjusted for covariates. Birth outcomes examined in this study included birth weight, birth length, head circumference and gestational age. Interaction terms revealed possible sex-specific effects in four models of birth weight or birth length for three phenols (2,5-DCP, triclosan and benzophenone-3). No effects were observed with BPA; however, as noted by the authors, urinary concentrations of BPA were much lower than those of 2,5-DCP, triclosan, and benzophenone-3. As shown below, BPA levels in these women were considerably lower than the levels reported in women from the National Health And Nutrition Examination Survey (NHANES), while levels of triclosan and 2,5-DCP were higher. At the upper end of the distributions, levels of triclosan, benzophenone-3 and 2,5-DCP, but not BPA, were much higher in these women than the levels reported for women in NHANES. The exposure assessment for BPA relied on a one time measure during the third trimester. Since the half-life of BPA is short, this may not be a reliable indicator of exposure throughout or during critical periods of pregnancy. A concern for the study in general is the number of multiple comparisons conducted in the analyses (72), which could have led to spurious significant associations.

Table A1-1. Urinary levels of BPA and other phenols in women as reported by NHANES and by Wolff et al., (2008).

	Geometric Mean (95% CI) (µg/L)	*50th percentile*	*75th percentile*	*95th percentile (NHANES) Maximum (Wolff et al.)*
BPA				
NHANES	2.4 (2.1 – 2.8)	2.4	5.0	20.1
Wolff et al.		1.3	2.3	35.2
Triclosan				
NHANES	10.6 (9.3 – 12.1)	7.4	33.2	430
Wolff et al.		11	42	1,790
Benzophenone-3				
NHANES	30.7 (23.7–39.8)	26	137	1,790
Wolff et al.		7.5	31	92,700
2,5-DCP				
NHANES	*	1.41	24.6	1,320
Wolff et al.		53	135	13,300

* Not calculated. Proportion of results below limit of detection was too high to provide a valid result.
NHANES values - 2,5-DCP values were from the third report, survey years '01-'02 (CDC, 2005),
- BPA (Calafat et al., 2008c), triclosan (Calafat et al., 2008b), and BP3 (Calafat et al., 2008a) were from survey years '03-'04

Animals Studies

Gioiosa et al. (2007). Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice.

Gioiosa et al. (2007) studied the effects of developmental BPA administration on sex-differentiated behavior. Pregnant CD-1 mice, were administered 10 µg/kg-d BPA in tocopherol-

stripped corn oil by oral instillation (n=16). Vehicle control mice (n=15) received 0.1 mL corn oil, and a comparison estrogen group (n=12) received 20 µg methoxychlor (MXC)/kg-d. Mice were housed in polycarbonate cages under a 12-h light/dark cycle with light onset at 1100 h. They were fed commercial mouse feed 4RF2221 from Mucedola srl. From GD11 (plug detection = GD0) to PND8 (day of birth=PND1) dams were dosed at 1700 h each day. Dams were housed 3/cage before dosing and individually after dosing was initiated. Within 12 h of birth litters were culled to 10 (5±1 males and females). At 25 days of age, offspring were weaned into groups of like-sexed littermates. Different offspring participated in adolescent (<PND30) and adult (PND70) testing. One male and one female from each litter were selected for adolescent testing and a second set of one male and one female per litter were selected adult testing. This provided for a litter-based statistical analysis. Each endpoint was analyzed by 2-say ANOVA (treatment, sex) with Tukey post hoc tests.

Behavioral tests of exploration and anxiety which reflected sex-differentiation were administered to prior to puberty (novelty preference task) or in adulthood (open field test, elevated plus maze). Females were tested in the diestrous phase of the estrous cycle. Testing was conducted between 1600 and 2000 h. Novelty preference and elevated plus maze tests were scored by observers blind to the experimental group of the subjects, while open field test was scored by computer image analysis. The measures reflected the mouse's spontaneous entry into new, unfamiliar and anxiety-provoking areas (brightly lit area, open/elevated area, center area) of the apparatus. Measures taken during the tests were evaluated for sex differences within each treatment group (vehicle, BPA, MXC). Additionally mice of each sex in the treated groups were compared to vehicle mice of the same sex. Only females who were in the diestrous stage of the estrous cycle were included in the analysis.

Females in the vehicle control group demonstrated more activity and less anxiety than males as reflected in several measures:

- For the Novelty Preference Test: time spent in novel compartment, risk assessment behavior (forward elongation of the head and neck and scanning of the area ahead),
- For the Open Field Test: time spent in the open field, time spent in the center of the open field, time spent in the bright zone of the open field
- For the Elevated Plus Maze: higher frequency of entrance into the open arm; less time in the closed arm, more time in the center of the maze (open area).

There was no effect of sex on these measures in the BPA-treated group. Additionally female BPA treated mice differed from vehicle-control mice on several of these measures (time spent in the novel compartment, self-grooming, while male BPA-treated mice differed from male controls on other measures (latency to enter the novel compartment, rearing in the novel compartment, number of returns to the home cage, time in the center of the elevated plus maze). MXC-treated mice failed to show sex differences on any of the measures taken in the study. Neither sex nor treatment effects were identified for a few of the measures (latency to enter the open field, time spent far from the home-cage in the open field, time spent in the open arms of the elevated plus maze). The study was supported by the University of Parma and the Italian Ministry of University and Scientific and Technological Research.

Ohshima et al. (2007). Transmaternal exposure to bisphenol A modulates the development of oral tolerance.

Ohshima et al. (2007) studied the effects of developmental BPA exposure on immune tolerance in mice based on the known role of estrogens in expanding populations of CD4+CD25+ regulatory T cells, which are crucial to maintenance of tolerance. Failure to maintain tolerance can result in allergic reactions, such as food allergies in infants. The mouse model was a transgenic mouse expressing a T-cell receptor sensitive to a specific ovalbumin antigen. They would be expected to show a discrete and well-defined immunological response to injection of ovalbumin antigen. Pregnant mice were fed diets containing 0, 0.1 or 1.0 ppm BPA. The resulting dose was estimated at 0, 10 and 100 µg/kg-d based on 10% body weight food intake and continued throughout lactation. Serum samples at the time of weaning detected 1.41 ± 0.66 ng BPA/mL in the dams and 3.97 ± 2.40 ng BPA/mL in the offspring. One male offspring per litter was used in a given experiment, resulting in litter-based statistics. Experiment s and findings included:

- *Splenic T lymphocytes in offspring at 21 days of age*. Spleens of weanling mice contained fewer cells with the T cell receptors sensitive to ovalbumin. In vitro stimulation of splenocytes led to aTh2 cytokine profile.
- *Effect on development of tolerance to ovalbumin antigen*. Lymphocyte proliferation in response to ovalbumin injection was greater in BPA-treated offspring. Cytokine production by lymphocytes in response to ovalbumin was also greater than in controls. This demonstrates less tolerance
- *Effect on numbers of CD4+CD25+ T cells and CD4+foxP3 T cells.* Fox P3 is a transcription factor that regulates T cell development. These two cell types increased in controls, but not in the BPA-treated offspring, in response to ovalbumin. Reduction in the number of regulatory cells leads to a greater immune response.
- Effect on ovalbumin specific immunoglobulin production. Ovalbumin specific antibodies were greater in BPA-treated than control offspring. This effect occurred at the low dose, but not at the high dose, of BPA.

These results support an altered ability of T-cells from BPA-treated mice to modulate response to antigen. The study also reported serum levels of BPA at weaning in the dams (1.41 ± 0.66 ng/mL) and offspring (3.971 ± 2.40 ng/mL). BPA was measured by ELISA with a detection limit of 0.2 ng/mL. Group sizes for the serum BPA analysis were not stated. This work was supported by the Japan Chemical Industry Association, Pollution-Related Health Damage Compensation and Prevention Association, and 21st Century COE program.

Yang et al. (2008). Proteomic biomarkers for prenatal bisphenol A-exposure in mouse immune organs.

Yang et al. (2008) investigated biomarkers for developmental BPA effects on immune tissues through gene expression studies in the spleen and thymus. Pregnant ICR mice were given 0, 15 or 300 mg BPA/mL drinking water from GD 7 to PND 21. The resulting dose was estimated at 0, 9 and 171 mg/kg-d. Offspring were sacrificed either at weaning (PND 21) or as adults (PND 49). Protein was extracted from two immune system organs, spleen and thymus for proteomic

analysis (identification of all proteins in the sample by protein gels or western plot analysis. RNA was also isolated for analysis of mRNA expression. There were 5 pregnant mice in each treatment group. Both male and female offspring were studied. Litter representation in the various assays was not discussed. In the proteomics analysis, four proteins were upregulated and three proteins were down regulated in BPA-treated offspring compared to controls. Western plot confirmed this result for three of the upregulated proteins of potential interest due to their possible relation to endocrine-related disease. Of these three proteins, mRNA expression was increased in spleen and thymus for only one, *apo-A1*. This protein was described as having a potential anti-inflammatory role. The authors suggest that the three upregulated proteins could serve as biomarkers for BPA developmental immunotoxicity. This work was supported by the Korean Research Center for Women's Diseases.

Yan et al. (2008). Exposure to Bisphenol A prenatally or in adulthood promotes TH2 cytokine production associated with reduction of CD4+CD25+ regulatory T cells.

Yan et al. (2008) examined the response to a bacterial pathogen after exposure to BPA either in adulthood or during development. For the developmental exposure, pregnant mice of two strains differing in their sensitivity to infection by the pathogen, Leishmania major, were exposed to BPA in drinking water at concentrations of 0, 1, 10, or 100 nM. Mice were housed in poly-methylpentene cages fed commercial diet (FR-2, Funabishi Farms) and drank water from glass bottles. Bedding was not described. The dose from this exposure can be estimated as 0, 0.03, 0.3, and 3 μg/kg-d.

Measure were as follows:

- Swelling in the foot pad, the site where the pathogen was injected
- Production of cytokines when spleen cells from infected mice were challenged in vitro with a pathogen antigen.
- Counts of the relative number of CD4+CD25+ regulatory T-cells.

C57Bl6/J mice did not become infected with L. major and their immune system measures were not affected by prenatal BPA exposure. Effects of BPA were seen in adult male offspring of BALBc mice. Three-four mice per treatment group were included in each assay. Litter representation in the test groups was not discussed.

BPA-treatment effects were as follows:

- BPA treatment increased swelling of the foot pad.
- BPA treatment increased cytokine production of splenocytes,
- BPA treatment decreased the number of CD4+CD25+ regulatory T cells.
- Many of these effects occurred at the highest BPA dose, but some were also seen at the second highest dose.

Section 2: Female Reproductive Toxicity Studies

Human Studies

Itoh et al. (2007). Urinary Bisphenol-A concentration in infertile Japanese women and its association with endometriosis: A cross-sectional study.

In this cross-sectional study, urinary concentrations of BPA were measured in infertile women to investigate a possible association with endometriosis. Subjects were recruited from 166 women who had complained of infertility and were consulting with a university hospital in Tokyo, Japan. A total of 148 women out of 166 agreed to participate, with the final sample size of 140 women, who had a laparoscopic examination, providing a urine sample (136 of which were first morning samples). A structured interview was conducted with the participants which collected information on demographics, personal and family medical, reproductive and menstrual histories, oral contraceptive use, food and alcohol consumption frequencies, and smoking history. Concentrations of BPA were measured in urine following deconjugation using HPLC isotope dilution tandem MS. The LODs were 0.30–0.55 µg/L.

BPA was detected in 93% of urine samples (median = 0.80, 25^{th}–75^{th} percentile = 0.45–1.3 µg/g creatinine). No association was reported between BPA concentrations and stage of endometriosis. No statistical difference was found between urinary BPA concentrations (median, 25^{th}–75^{th} percentile creatinine-adjusted) for women grouped as stage 0–1 (0.74, 0.45–1.21) and those grouped as stage II–IV (0.93, 0.5 –1.48).

The strengths of this study include:

1) the good participation rate (84%)
2) the use of HPLC-MS to analyze urinary BPA concentrations
3) the appropriate use of the rank sum test since the distribution of BPA was non-normal.

Potential limitations of this study include:

1) cross-sectional study design
2) inclusion of nine women with no complaint of infertility
3) urine samples, stored for about five years at -80^{o}C, were thawed and refrozen several times during that period
4) no control for potential confounders in multivariate analysis.

With regard to the issue of potential confounders, although the authors reported that endometriosis stage was associated with menstrual cycle length, regularity of menstrual cycle, and history of dyspareunia, no adjustment was made for such variables in the analyses. In addition, some potentially important covariates may have been assessed from the questionnaire (such as family history, menstruation that started before age 12); however, no mention was included about other important risk factors such as abnormal structure of the uterus, cervix, or vagina. An important limitation of the statistical analysis is the grouping of women with

endometriosis classified as stage 0 with those classified as stage 1. This could effectively have combined women with no diagnosis of endometriosis with women classified as having early stage endometriosis, thus resulting in misclassification of disease status. From the information provided in the article it is not possible to determine how many of the 81 women grouped as stage 0–1 were actually classified as stage 0.

Wolff, M. S., J. A. Britton, et al. (2008). Environmental exposures and puberty in inner-city girls.

In this study, Wolff et al. investigated pubertal status in association with environmental exposures to hormonally active substances in a multi-ethnic group of 9-year old girls. A total of 192 girls were recruited from Mount Sinai Hospital and a nearby pediatric private practice in New York City during 1996–1997. The participation rate was 89%. Dietary intake of phytoestrogens was measured using a food-frequency questionnaire (Harvard Youth/Adolescent Questionnaire). Three phytoestrogens (daidzein, enterolactone, and genistein) and bisphenol A were measured in urine. Additional chemicals were measured including DDE, PCBs, and lead. Pubertal stages, including breast stage (B1–B4, B1 = no development, B2+ = any development) and pubic hair stage (H1 and H2), were assessed by pediatricians. The authors reported that median creatinine corrected urinary concentrations of the three phytoestrogens were similar to those reported in children in the 1999 – 2000 NHANES. Although the authors did not make the same comparison for BPA levels or provide overall mean values, the following comparisons suggest that levels of BPA were lower in this population than in children measured in NHANES (Calafat et al., 2008b) (NHANES - geometric mean (µg/g creatinine) = 4.3; Wolff et al., geometric mean (µg/g creatinine) for breast stage 1 = 0.24, breast stage 2+ = 0.11.

Breast development was present in 53% of girls and pubic development in 31%. Urinary phytoestrogen concentration was highest for enterolactone, then daidzein and genistein. Girls with breast stage 1 had significantly higher mean urinary levels of BPA, genistein, and enterolactone compared with girls at breast stage 2 or higher (BPA values; geometric mean (geometric standard deviation) expressed µg/g creatinine, for breast stage 1 = 0.24 (10.3) compared breast stage 2 or higher = 0.11 (12.9); $p < 0.05$). No significant differences were observed in hair stage for any of the urinary phytoestrogens levels. Dietary phytoestrogen intake was not significantly associated with either breast or hair stage. In the multivariate analysis, BPA was not significantly associated with either breast stage (prevalence ratio = 0.92, 95% CI 0.92–1.01) or hair stage (prevalence ratio = 0.98, 95% CI 0.89–1.08). Regression models for BPA were adjusted for height and race being Black. Additional covariates included urinary creatinine, and maternal education. As the outcome measure pubertal development was not rare, modified Poisson regression was used to estimate prevalence ratios. Delayed breast development was observed among girls with below median BMI and the highest exposure of urinary daidzein and genistein compared to girls with lower exposures. The authors also reported that BPA appeared to be unrelated to risk, with or without consideration of BMI.

Limitations of this study, as identified by the authors, include the relatively small sample size, the cross-sectional study design, and possibly inadequate adjustment for socioeconomic status and residual confounding among the urinary biomarkers, BMI and creatinine. In addition,

phytoestrogen intake at age 9 may not be relevant to pubertal stage at the same age. Previous research suggests that diet and exposures early in life influence onset of puberty. Of note, concentrations of BPA were low in this sample of girls. BPA concentrations may not have reached a level of biologic significance, as was previously suggested by Wolff in relation to birth outcomes (Wolff et al., 2008). In addition, as mentioned above, BPA concentrations in this population, sampled in 1996–1997, seem to be considerably lower than levels measured in girls 6–11 years old in the NHANES 2003–2004 sample. It may be that these two samples are not comparable or it may be that BPA levels are increasing over time in this population.

Animal Studies

Berger et al. (2008). Impact of acute bisphenol-A exposure upon intrauterine implantation of fertilized ova and urinary levels of progesterone and 17β-estradiol.

The goal of this study was to assess the impact of acute and repeated s.c. BPA administration on intrauterine implantation of fertilized ova, and urinary levels of 17β-estradiol and progesterone in inseminated female mice. Sexually naïve female CF-1 mice aged 3–6 months, with an average weight of 35 g at the beginning of experimental procedures, were used in this study. Before beginning the experiment, an anogenital distance index (AGDI) was generated for each female. Females were then immediately isolated in the collection apparatus for a 1-week adaptation period and left undisturbed until mating. Females were randomly paired with a 5–12 month old CF-1 male. Inspection for a vaginal sperm plug occurred 3 times daily during the dark phase of the light cycle. The day of sperm plug detection was considered day 0 of pregnancy. Pregnancy outcome was measured on GD 6 at 3–6 hours after commencement of the dark phase of the light cycle. Females were sacrificed by CO_2 asphyxiation, uteri were excised and the number of implantation sites counted.

In experiment #1, females received doses of 0, 0.0005, 0.0045, 0.05, 0.125, 1.125, 3.375, 6.75, and 10.125 mg BPA/animal/d dissolved in peanut oil (approximately 0, 0.01, 0.1, 1.5, 3.5, 30, 100, 200 or 300 mg BPA/kg) on days 1–4 of gestation via s.c injection. In experiment #2, females received BPA dissolved in peanut oil. A single dose of 6.75 or 10.125 mg BPA/animal/d on days 0, 1 or 2 of gestation was administered via s.c injection. Urine collections occurred on days 2–5 of pregnancy, with the exception of the day 0 single administration group where collections occurred on days 1–5. Creatinine, 17β-estradiol, and progesterone concentrations were assessed by enzyme immunoassays. (The concentration of urine samples were adjusted for creatinine to compensate for variations in fluid intake and output.)

In experiment #1, one-way ANOVA was conducted comparing the number of implantation sites to condition. In experiment #2, planned orthogonal t-tests were conducted comparing each dose with the control (0 mg BPA) dose for the respective day. In experiment #1, daily doses of 6.75 and 10.125 mg/animal significantly reduced the number of implantation sites ($p<0.01$). A clear decrease was observed in both the 6.75 and 10.125 mg/d groups; in the former dose no animals showed any implantation sites, while only one animal did at the 10.125 mg dose. A significant negative correlation was found between the creatinine-adjusted progesterone level across groups and AGDI on day 2 and 4 of pregnancy. No significant correlations were found between

estradiol levels and AGDI. In experiment #2, a single dose of 10.125 mg reduced the number of implantation sites when given on day 0 or day 1, and 6.75 mg on day 1 also produced fewer implantation sites, but there was no such effect on any dose when administered on day 2.

Among animals treated with a single dose on day 0 of gestation, creatinine levels showed a significant effect of day, but no other significant effects. No significant differences in creatinine-adjusted urinary estradiol levels in animals treated on day 0 were found. Among animals treated on day 1, urinary creatinine levels showed a significant effect of day ($p<0.0001$), and a significant effect of day was also seen in the creatinine-adjusted estradiol levels ($p=0.0002$). Among animals treated on day 2, urinary creatinine levels did not show a significant effect of group but did show a significant effect of day ($p=0.0106$). No significant impact of BPA administration on mean adjusted progesterone or estradiol levels was found. The authors concluded that these data show a lower threshold for BPA-induced pregnancy disruption, which is due to actions of BPA on implantation sites and show that higher doses can influence systemic progesterone levels.

Bredhult et al. (2007). Effects of some endocrine disruptors on the proliferation and viability of human endometrial endothelial cells in vitro.

The purpose of this study was to examine the proliferation and viability of human endometrial endothelial cells (HEEC) *in vitro* after exposure to a variety of chemicals, including BPA. Endometrial biopsy samples were obtained from hysterectomy specimens, which came from women of fertile age who had regular menstrual cycles. The HEEC were exposed to 0.01 μM (low), 1 μM (medium), and 100 μM (high) BPA for 24 hours at 37^{o}C in an atmosphere of 5% CO_2 in humidified air. Cell proliferation was assessed using immunocytochemistry for proliferating cell nuclear antigen (PCNA) expression and a 5-bromo-2'deoxyuridine (BrdU) assay. Cell viability was studied by vital staining with propidium iodide and Hoescht 33258. Mean values for PCNA expression, the BrdU assay, and vital staining were assessed by a univariate ANOVA.

None of the BPA treatments altered the number of PCNA-positive cells compared to the dimethylsulfoxide (DMSO) control. The HEEC proliferation after treatment with 0.01, 1, or 100 μM BPA was lower compared to the DMSO control ($p<0.001$, <0.001, and <0.001, respectively). Treatment with 100 μM BPA also resulted in decreased proliferation compared to 0.01 and 1 μM BPA ($p<0.001$, and <0.001, respectively). Bisphenol A in a concentration of 100 μM decreased cell viability and increased necrosis compared to the DMSO control. The authors concluded that BPA could have effects *in vivo* as well as *in vitro*, and influence processes involving, for example, fertile age, endometrial angiogenesis.

Fukumori et al. (2001). Low-dose effects of Bisphenol A on the uterine and vaginal ultrastructure of suckling female mice.

The objective of this study was to examine the estrogenic effects of BPA on the uterus and vagina of suckling female mice at the ultrastructural level by electron microscopy. Suckling

female mice were treated with 0 (DMSO-control), 0.8, 4, 20, or 500 μg BPA/kg-d by s.c. injection. 100 μg 17β-estradiol/kg-d was used was a positive control. Mice were treated 5 d/week from the PND 1–21, and sacrificed on PND 22. The uterus and vagina were immediately fixed in 2.5% glutaraldehyde. In the uterus, luminal epithelial cells of the endometrium in 4, 20 and 500 μg BPA/kg-d groups showed taller cell height than those of the controls. In the vaginal epithelium of the 4, 20 and 500 μg BPA/kg-d groups, increased cell layers (5–7 layers in BPA vs. 3–4 layers in the control), enlargement of the nuclei, and widening of the intercellular space among the stratified cells were observed in comparison with the controls. The authors suggest that the luminal uterine cells and vaginal epithelium responded to 4, 20, and 500 μg BPA/kg-d, and that electron microscopic observation may be useful to detect these subtle changes.

Howdeshell et al. (2003). Bisphenol A is released from used polycarbonate animal cages into water at room temperature.

Bisphenol A reportedly hydrolyzes and leaches from food packaging, dental sealants, polycarbonate plastics and many other products under high heat and alkaline conditions. Howdeshell et al. examined whether new and used polycarbonate animal cages passively release bioactive levels of BPA into water at room temperature ($23 \pm 2^{o}C$) and neutral pH (a neutral solvent and high-pressure liquid chromatography (HPLC)-grade water, pH 7). Purified water was incubated at room temperature in new polycarbonate and polysulfone cages and used polycarbonate cages, as well as control (glass and used polypropylene) containers for one week. The resulting water samples were characterized with gas chromatography/mass spectrometry (GC/MS) and tested for estrogenic activity using an MCF-7 human breast cancer cell proliferation assay. MCF-7 cells were treated with reconstituted water samples in media on days 3–6 of cell culture, and on day 7 the cell culture wells were assayed for cell proliferation. The *in vivo* estrogenic bioactivity of the used polycarbonate cages was tested by measuring the uterine wet weight of prepubertal female mice housed in the cages. Three CD-1 females per litter that were 19 days of age were housed in used polycarbonate cages, and polypropylene cages, respectively. This experiment was conducted in three replicates of approximately six litters per cage type, for a total of 57 animals per cage type. After 1 week, the body weight of the female mice was recorded. Females were euthanized with CO_2, uteri were removed and wet weights recorded. The uterine wet weight data were log-transformed to achieve homogeneity of variance and then analyzed by analysis of covariance (ANCOVA). Uterine weight data were adjusted for body weight by ANCOVA.

Significant estrogenic activity from used polycarbonate animal cages was found. Two polycarbonate animal cages leached 110 and 51 μg BPA/L water. Water from the negative control glass dishes did not contain detectable BPA. A small amount of BPA (0.3 μg/L) migrated out of the new polypropylene cages, and the used polycarbonate cages released much higher concentrations of BPA than did the new polycarbonate cages. All water samples from the used polycarbonate cages stimulated MCF-7 cell proliferation. Bisphenol A exposure as a result of being housed in used polycarbonate cages produced a 16% increase in uterine weight in prepubertal female mice compared with females housed in used polypropylene cages, although the difference was not statistically significant ($p=0.31$). There was no significant difference in uterine wet weight based on housing in used polycarbonate versus used polypropylene cages.

The authors conclude that laboratory animals housed in polycarbonate and polysulfone cages are exposed to BPA via leaching, with exposure reaching the highest levels in old cages.

Kawamoto et al. (2005). Disposition of Bisphenol A in pregnant mice and fetuses after a single and repeated oral administration.

In this study, the authors investigated the distribution pattern of ^{14}C-BPA-derived radioactivity in fetal tissues and analyzed BPA metabolites in whole fetuses following administration of 10 mg/kg ^{14}C-BPA to pregnant mice. Pregnant ICR mice were orally given a single dose of 1, 10 or 100 mg^{14}C-BPA /5ml/kg bw on GD 15 and placed into plastic cages until necropsy. One or two mice were sacrificed at 20 minutes, 1, 3, 6 or 24 hours following administration of ^{14}C-BPA. In another experiment, three pregnant mice were orally given a dose of 10 mg ^{14}C-BPA/5ml/kg bw once a day for 3 days starting on GD 15, and placed into plastic cages until necropsy. All mice treated for 3 d were necropsied 6 hours after the last dose. In dams, the concentrations of radioactivity in many tissues, as well as blood concentration, achieved peak concentrations twice, at 20 minutes and 6 hours post-dosing. The concentration in whole fetuses as well as many tissues in the fetus continued to increase up to 24 hours post-dosing. The concentration in whole fetuses at 24 hours was almost half that in maternal blood. The radioactivity was rapidly transferred through the placenta and distributed to all fetal organs including reproductive organs and brains at a similar level. The concentration declined slowly compared with dams. Radioactivity level in most of the fetal tissues on repeated administration was higher than single administration. The authors concluded that their results added the following new information on the pharmacokinetics of BPA:

1. The transfer of ^{14}C-BPA-derived radioactivity through placenta to a fetus was distributed to all fetal organs at a similar level. Fetal reproductive organs and brains were exposed to similar levels of BPA and metabolites as were other organs.
2. There was no clear effect of fetal position in the uterus on the total ^{14}C-BPA-derived radioactivity concentration in the fetus.
3. The pharmacokinetics of BPA is linear below an oral dose of 10 mg/kg in mice and could be linearly extrapolated to lower doses.
4. When repeated doses were administered, the ^{14}C-BPA-derived radioactivity levels in most of the fetal tissues were higher than those resulting from a single dose.

Lenie et al. (2008). Continuous exposure to bisphenol A during in vitro follicular development induces meiotic abnormalities.

The purpose of this study was to analyze the effects of chronic BPA exposure (3 nM to 30 μM) on follicle-enclosed growth and maturation of mouse oocytes *in vitro*. Female F1 hybrid (C57BL/6j x CBA/Ca) mice were bred. Early preantral follicles (Type 3b-4 in the Pedersen classification) with a diameter between 100 and 130 μm containing an immature oocyte centrally located within the follicle and an intact basal membrane surrounded by some theca cells were selected for follicle culture. Follicles from 32 mice were used and randomly allocated to experimental groups. Treatments included the following: control, DMSO, 3 nM, 30 nM, 300 nM,

3 μM and 30 μM. Cells were cultured for 12 d at 37°C, 5% CO_2 in air at 100% humidity in the dark after which an ovulatory stimulus was administered to induce oocyte resumption of meiosis and ovulation in vitro. Spindles and chromosomes of oocytes were stained by α-tubulin immunofluorescence and ethidium homodimer-2, respectively. Follicles grown under BPA concentrations from 3 nM to 3 μM were generally morphologically normal. Only follicles exposed to 30 μM BPA during follicular development showed a slightly reduced granulosa cell proliferation and a lower total estrogen production, but they still developed and formed antral-like cavities.

Eighteen percent of oocytes were unable to resume meiosis after stimulation of oocyte maturation, and 37% arrested after germinal vesicle breakdown (GVBD) compared with the solvent control. Only 45% of the oocytes from the highest concentration of BPA extruded a first polar body compared with controls and other lower concentrations of BPA ($p<0.05$). Unalignment of chromosomes was more pronounced at the lowest concentrations of BPA (3–300 nM) compared with the higher concentrations (3 and 30 μM). Oocytes that were able to progress beyond meiosis I frequently arrested at an abnormal telophase I. Additionally, many oocytes exposed to low chronic BPA that matured to meiosis II chromosomes failed to congress at the spindle equator. The study authors concluded the follicle bioassay employed in the present study was instrumental to reveal potentially adverse effects of chronic low dose exposure to BPA on mammalian oocytes at the level of whole follicle exposures, while steroidogenesis was not affected.

Muhlhauser et al. (2009). Bisphenol A effects on the growing mouse oocyte are influenced by diet.

The Hunt research group has noted that variations in the results of BPA studies conducted at different times appears to correlate with changes in mill dates of animal feed. Thus, the purpose of this study was to evaluate the effect of diet on the results of BPA studies of the periovulatory oocyte. C57Bl/6J mice were placed at least one week prior to mating on one of two rodent diets:

1. TestDiet (American Institute of Nutrition) AIN-93G
2. Harlan Teklad Sterilizable Rodent Diet 8656, a soy based diet.

Female offspring from matings were used to assess the influence of diet on BPA effects on the oocytes. Twenty-one day old females were treated with BPA in a corn oil carrier. Daily oral doses of 20, 40, 100, 200, or 500 μg/kg bw were given for 7 d preceding oocyte collection. Germinal vesicle stage oocytes were collected from the ovaries of 28 d old females, with the exception of oocytes from adults (6–11 weeks) on the soy-based diet. Oocytes were cultured for 16–17 hours, then those exhibiting a polar body were fixed in formaldehyde, washed with phosphate buffered saline, and blocked with a phosphate buffered saline containing normal goat serum. Immunolabeling of oocytes followed.

Significant diet-related variation in both the frequency of abnormalities in oocytes from untreated females and in the response to BPA were observed. Specifically, 2% of eggs from control females on the casein diet were identified as showing abnormal meiosis-II, but the abnormality rate increased to nearly 8% for the soy diet ($p=0.002$). The casein diet produced an apparent linear dose response, with significant increases in spindle/chromosome alignment at the

200 μg/kg exposure level (p=0.03). In studies comparing oocytes from juvenile and sexually mature females maintained on the standard diet, the authors have found few, if any meiotic differences. Six out of 85 (7.1%) metaphase II arrested oocytes exhibited severe aberrations in chromosome alignment and/or spindle formation. This was in agreement with the 6.2% baseline abnormality rate observed in oocytes from juvenile females, and authors suggested that the aberrations observed in their studies of females on this diet are not a reflection of the immature female model used. The authors also suggested that variation in the conclusions of recent BPA studies reflect differences in the diets used, as well as other methodological differences. Meiotic differences are a feature of all BPA studies on oocytes to date; thus, the authors concluded that low levels of BPA adversely affect the meiotic process.

Newbold et al. (2009). Prenatal exposure to Bisphenol A at environmentally-relevant doses adversely affects the murine female reproductive tract later in life.

The aim of this study was to investigate whether prenatal BPA at low environmentally-relevant doses causes long-term adverse effects in aged female reproductive tissues. Timed pregnant CD-1 mice were treated by s.c injections on GD 9–16 with 0.1, 1, 10, 100, or 1000 μg BPA/kg-d. Pregnant mice delivered their pups on GD 19. At PND 21, offspring were weaned and held without further treatment. Pups were 16–18 months of age when reproductive tissues were evaluated. Mice were euthanized with CO_2. Reproductive tract tissues plus ovaries/oviducts were removed and prepared for histological evaluation. Cystic ovaries were common in all groups but only the 1 μg BPA/kg-d group showed a statistical significant difference compared with controls ($p<0.05$). Progressive proliferative lesion of the oviduct was increased following BPA, similar to that described following DES exposure. Progressive proliferative lesion was seen in all groups of the BPA treated mice, but not controls. In some BPA animals, atypical hyperplasia and stromal polyps of the uterus, sarcoma of the uterine cervix, and mammary adenocarcinoma were observed. Females from the 0.1 μg BPA/kg-d group showed the highest tumor incidence at 36% of treated mice (p=0.01). The authors suggest BPA causes long-term adverse reproductive and carcinogenic effects if exposure occurs during critical periods of differentiation.

Smith et al. (2007). Xenoestrogen exposure imprints expression of genes (Hoxa10) required for normal uterine development.

Smith et al. tested the ability of BPA to alter expression of the HOXA10, a gene necessary for uterine development. Hoxa10 (mouse)/HOXA10 (human) in particular is the target of endocrine disruption by DES both in mice and in human reproductive tract cell lines. Bisphenol A affects HOXA10 expression through the HOXA10 estrogen response element (ERE) and indirectly though the autoregulatory element (ARE). The authors hypothesized that the purported endocrine disruptor BPA would impact the expression of HOXA10. Human Ishikawa cells, a well-differentiated endometrial adenocarcinoma cell line, were cultured *in vitro* with concentrations of BPA, which ranged from 0.1 nM to 25 mM for 24 hours. Semiquantitative and quantitative polymerase chain reaction (PCR) were conducted to assess gene expression. To test whether *in utero* BPA exposure resulted in a lasting alteration of uterine HOXA10 expression,

pregnant CD-1 mice were injected i.p. with 0.5, 1.0, 5.0, 50 or 200 mg/kg BPA on GD 9–16. Female offspring were euthanized 2 or 6 weeks after birth, and reproductive tracts were resected and examined using immunohistochemical techniques. An increase in HOXA10 gene expression was seen *in vitro* with increasing concentrations of BPA treatment. Treatment with 200 mg BPA/kg resulted in death of all pregnant females. After administration of 0.5 mg/kg to 1.0 mg/kg, a dose-dependent increase was seen in stromal cell Hoxa10 expression in 2- and 6-week old mice exposed *in utero*. A statistically significant increase in Hoxa10 expression was seen at 2-weeks in the 1.0 and 5.0 mg BPA/kg groups, and at 6-weeks in the 0.5, 1.0, and 5.0 mg BPA/kg groups ($p<0.01$). No gross abnormalities of the reproductive tract were observed in any of the female offspring.

Vandenberg et al. (2008). Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice.

Previous work by these authors focused on the effects of perinatal exposure to BPA on mammary gland development from GD 8–PND 2. *In utero* exposure to BPA caused a decreased invasion of the stromal compartment, an increased number of highly proliferative structures known as terminal end buds and an enhanced sensitivity to estradiol. The effects of prolonged exposure to BPA through lactation have not yet been determined. Therefore, the authors hypothesized in this study that exposure during gestation and the lactation period during which pups are reliant solely on milk for their nutrition (through PND 16) is likely to lead to phenotypes that are not predicted by gestational exposure alone.

Sexually mature CD-1 mice were exposed to environmentally relevant doses of BPA during gestation and lactation (GD 8–PND 16). On the evening of pregnancy day 8, dams were weighed and s.c. implanted with Alzet osmotic pumps designed to deliver 50% DMSO or BPA in 50% DMSO. The pumps continually released 0.25 μl/hour until day 16 of lactation. Exposure groups included: 0 (control), 2.5 or 25 μg BPA/kg bw-d. Dams were allowed to deliver naturally and litters were culled to 8 pups per dam on PND 1. Litters were weaned on PND 22–24. At 3, 9 and 12–15 months of age, female offspring were killed and mammary glands collected to assess any structural changes using whole mounts, and immunohistochemical methods. Analysis of variance followed by Bonferroni posthoc tests were used to assess differences between treatment groups for each paradigm. A Chi-square test was performed to compare the incidence of beaded ducts in the mammary gland epithelium of control and BPA-exposed animals.

Bisphenol A exposed females demonstrated altered mammary phenotypes including the appearance of alveolar buds. At 3 months of age, significant differences were noted for the volume fraction of alveolar buds for the 0.25 μg BPA/kg bw-d group compared with all other treatment groups ($p<0.05$). A decrease in the volume fraction of ducts, and an increase in total epithelial structures was also noted for the 0.25 μg BPA/kg bw-d group although the differences were not statistically significant. At 9-months of age, the volume fraction of alveolar buds was significantly increased in the 2.5 μg BPA/kg bw-d group compared with control females ($p<0.05$), and a significant decrease in the volume fraction of ducts in the 0.25 μg BPA/kg bw-d group compared with controls ($p<0.05$). Intraductal hyperplasias were also observed exclusively

in BPA-exposed females. Intraductal hyperplasias were described by the authors as "beaded ducts" with epithelial cells present inside the ductal lumen and increased proliferation indexes compared to normal ducts. Proliferating cells are generally not apparent in normal adult virgin mammary duct epithelium. "Beaded ducts" were observed in only 1 animal at 3 months of age, but in multiple animals at 9 months and 12–15 months of age. The authors conclude the results of this study provide further evidence that perinatal BPA exposure can alter the morphology of the rodent mammary gland in adulthood.

Section 3: Male Reproductive Toxicity Studies

Human Studies

Bennetts et al. (2008). Impact of estrogenic compounds on DNA integrity in human spermatozoa: Evidence for cross-linking and redox cycling activities.

This in vitro study investigated the ability of various estrogenic compounds including natural, synthetic, and environmental estrogens to create oxidative stress and DNA damage in human spermatozoa. Semen samples were obtained from students, the fertility of whom was unknown. High doses of estrogen compounds were used (range 30–5000 μM) to assess their direct effects on spermatozoa. DNA integrity was assessed using the Comet and TUNEL assays and lesion frequencies were quantified by quantitative polymerase chain reaction using targets within the mitochondrial and nuclear genomes. DNA adducts were characterized and redox activity was monitored. Although there was evidence that certain estrogenic compounds, such as catechol estrogens and quercetin, stimulated redox activity and produced DNA damage, other estrogens, such as BPA and 17β-estradiol, showed no effects on these parameters. The compounds that exhibited high redox activity induced a complete loss of sperm motility. However, BPA did not show an effect on any outcome measure.

Cha et al. (2008). Influence of occupational exposure to bisphenol A on the sex hormones of male epoxy resin painters.

This occupational study of workers at a shipyard reported higher concentrations of urinary BPA in epoxy resin painters (geometric mean ± geometric standard deviation: 2.61 ± 1.08 μg/g creatinine; N = 25) than in non-painters (1.38 ± 0.5 μg/g creatinine; N = 25). Plasma testosterone, LH and FSH concentrations of all subjects were within the reference values. However, painters had significantly lower testosterone concentrations (3.51 ± 0.74 ng/mL) than non-painters (5.18 ± 1.04 ng/mL) ($p = 0.000$); significantly higher LH concentrations (5.34 ±1.68 IU/L) than non-painters (3.16 ± 1.40 IU/L) ($p = 0.000$); and significantly higher FSH concentrations (7.68 ± 2.54 IU/L) than non-painters (5.53 ± 2.11 IU/L) ($p = 0.002$). Urinary BPA concentrations were positively correlated with LH concentrations ($r = 0.482$; $p = 0.05$). Multiple regression analysis performed for each of the hormones included urinary BPA concentrations, work duration and exposure index as variables, while controlling for age, smoking and drinking habits. Urinary BPA concentration was a significant variable only in the model for LH ($\beta = 0.487$; $p = 0.04$). Both work duration and exposure index were significant variables for testosterone, while work duration was the only significant variable for FSH. Urinary BPA concentrations in the exposed and control groups in this study were similar to those reported by Hanaoka et al. (2002), (median; exposed group = 2.14 μg/g creatinine; control group = 1.05 μg/g creatinine). Levels in the exposed group were similar to those reported for the NHANES 2003–2004 sample (geometric mean: 2.6 μg/g creatinine), which is representative of the U.S. population (Calafat et al., 2008c).

Some of the limitations of this study include the following:

1) the LOD for the HPLC method of analysis was not reported;
2) no mention was made of the whether the data were normally distributed, or log-transformed if non-normal;
3) although information was reportedly collected on the number of years workers had been employed and the amount of BPA contained in the epoxy resin (10–30%), the regression variables work duration and exposure index were not clearly defined.

Dechaud et al. (1999). Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG).

This study examined the ability of BPA to bind hSHBG in plasma. Plasma was obtained from normal, healthy men and women; however, no other details about the subjects were reported. Ammonium sulfate precipitation assay was used to determine the ability of BPA to displace testosterone and estradiol from hSHBG. A solid phase binding assay was used to determine the binding affinity constants of BPA. The study reported that BPA, along with some other xenoestrogens, bind hSBHG with a reversible and competitive binding activity for testosterone and 17 β-estradiol. BPA was one of the substances identified as a potent hSHBG-ligand. As the authors state, the data suggest that hSHBG binding may transport some contaminant xenoestrogens into the plasma and modulate their bioavailability to cell tissues.

Mulligan et al. (1998). Competitive binding of xenobiotic oestrogens to rat alpha-fetoprotein and to sex steriod binding proteins in human and rainbow trout (oncorhynchus mykiss) plasma.

In this in vitro study the authors examined the competitive binding ability of BPA, along with other "environmental estrogens", to sex steroid binding proteins in humans using plasma from three pregnant women. These parameters were also examined in rainbow trout and rats. The effect of exogenous compounds on the binding of estradiol ($[^3H]E_2$) and dihydrotestosterone ($[^3H]$DHT) to steroid binding proteins was examined using a filter assay technique. The relative binding affinity of BPA in human plasma was very weak, being <0.01%. The results suggested that BPA is not likely to produce biological effects by displacing endogenous steroids from plasma steroid binding proteins unless it is present in very high concentrations.

Inoue et al. (2002). Application of liquid chromatography-mass spectrometry to the quantification of bisphenol A in human semen.

In this study two analytical methods were compared for quantifying BPA in biological samples: liquid chromatography-mass spectrometry with electrospray ionisation technique using a deuterium-labeled surrogate standard (LC-MS) and a competitive BPA enzyme-linked immunosorbent assay (ELISA). Human semen samples from 41 healthy males were used. Using ELISA, measures of BPA in semen ranged from no detection to 12.0 ng/ml, with an

average of 5.1 ng/ml. No BPA was detected using LC-MS. The LC-MS was determined to be a more accurate and more sensitive method (limit of detection = 0.5 ng/ml) than the ELISA, which may give erroneously high values possibly due to the non-specific binding to the antibody.

Animal Studies

Ashby and Lefevre (2000). The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, estrogens and metabolic modulators.

The authors investigated the effects of a number of potential endocrine disruptors (including BPA, dibutyl phthalate, fenitrothion, flutamide, mexoychlor, etc.) on the weights of the testis, epididymis, seminal vesicles, and prostate in rats of 22 or 36 days of age. In the experiments with BPA, AP (Wistar) rats, 22 or 35 days of age, 8–10 animals per group were treated with BPA for 14 or 20 days in the following dosing regimens:

- 200 mg/kg-day in 22-day old rats for 14 days (PND 22–36)
- 100 mg/kg-day in 35-day old rats for 14 days (PND 35 to 50)
- 100, 150 or 200 mg/kg-day in 35-day old rats for 20 days (PND 35–55).

BPA was prepared in hydroxypropyl methoxycellulose (HPMC) as the vehicle (5 ml/kg). The body weight, the weights of the kidney, liver, testis, epididymis, prostate, and seminal vesicles, and the day of preputial separation (PPS) were evaluated and statistically analyzed. The authors found that body weights of the animals underwent a near linear increase during development from PND 22–50, but the weights of all organs measured had non-linear growth, indicating the complex relationship between tissue weights and animal body weights during this developmental period. All the male reproductive organs examined (testis, epididymis, seminal vesicles, and prostate) had rapid growth, especially the seminal vesicles and epididymis, suggesting that this period may represent a sensitive time window for male reproductive organs to the action of endocrine modulating chemicals. The day of PPS was clearly dependent on the initial body weight of the animals on PND 22. However, chemicals (e.g., fenitrothion, methoxychlor) that produced significant reduction in body weight gain did not delay PPS. DES caused delayed PPS only when the animals were treated from PND 22–36 or PND 55, but not in animals that received the treatment from PND 35. These data suggest that the day of PPS is affected by the body weight, the chemical, the beginning time and the duration of the treatment. With regard to the BPA, the authors found BPA had no effect on the weights of testis, epididymis, seminal vesicles, or prostate in any treated groups. There was no effect on the day of PPS. Histopathological evaluation of the organs was not included in the study.

Deng et al. (2004). Experimental studies on male reproductive toxicity of bisphenol A in vitro and vivo.

This study was conducted at a university research laboratory and reported in Chinese. The authors investigated the male reproductive toxicity of BPA in SD rats using both in vivo and in

vitro approaches. In the in vivo experiments, 9-week old male SD rats (17 rats in the control and high-dose groups, 8 in the low-dose group) were fed diets that contained 0, 1or 5 g BPA /kg for 14 days. The authors estimated that the animals received 0, 50, or 250 mg/kg-d of BPA. The BPA treatment caused a slight decrease in testis weight (statistically significant at 250 mg/kg-d), and an obvious reduction in the number of "round germ cells" per Sertoli cell (statistically significant at both doses). The authors also reported histopathological changes (e.g., degeneration of the germ cells, distortion or sloughing-off of the seminiferous epithelium, reduced or altered histoimmunostaining for vimentin or the cytoskeletal structure of the Sertoli cell). However, the figures for the histopathology of the testis presented in the paper showed poor qualification of histological processing and thus made interpretation of the histopathological data difficult. There was no significant difference in the blood levels of E_2 or T between the control and BPA-treated groups. In the *in vitro* experiments, the Sertoli cells were isolated from male SD rats 28–30 days of age, cultured and treated with BPA at concentrations of 0, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} mol/l (0, 0.1, 1, 10 100 µM, respectively) for 72 hrs. Bisphenol A treatment produced degenerative changes in the cultured Sertoli cells and altered immunostaining for vimentin.

Hess-Wilson et al. (2007). Unique bisphenol a transcriptome in prostate cancer: Novel effects on ER beta expression that correspond to androgen receptor mutation status.

The authors used LNCaP cells to investigate the molecular actions of BPA in prostate cancer cells. LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells that carry the AR-T877A mutation in the AR. This mutation was found in up to 12.5% of hormone-refractory prostate cancers in humans and it permits usage of E_2, progesterone, and specific antiandrogens as AR ligands, possibly leading to the failure of anti-AR activity therapy. Bisphenol A at 1 nM for 24-hr treatment caused significantly increased proliferation of LNCaP cells to a degree that was similar to the stimulating effect of 0.1 nM DHT, indicating that BPA acted as an AR ligand in T877A-carrying prostate cancer cells. Microarray analysis revealed that the expression of 283 genes was significantly altered, and the gene profiles after exposure to BPA and DHT are divergent, though the two AR ligands in this cell line had similar proliferative effect. Both microarray and real-time PCR analyses also found that BPA down-regulate the expression of ERβ in this cell line, but not in other cell lines, such as 22Rv1 (androgen-independent prostate cancer cells carrying AR-H874Y mutation) or LAPC4 cells (androgen-dependent prostate cancer cells having the wild-type AR). One function of ERβ is to antagonize AR function and AR-dependent proliferation. The authors concluded that BPA-induced down-regulation of ERβ may represent a novel mechanism by which BPA likely regulates cellular proliferation in prostate cancer cells.

Howdeshell et al. (2008). Gestational and Lactational Exposure to Ethinyl Estradiol, but not Bisphenol A, Decreases Androgen-Dependent Reproductive Organ Weights and Epididymal Sperm Abundance in the Male Long Evans Hooded Rat.

In this study, the authors examined the effects of ethinyl estradiol (EE, an oral contraceptive) and BPA on the development of the male reproductive system in LE rats in two blocks of

experiments. In the first block conducted in 2005, 13–29 dams per group were treated by oral gavage (corn oil as vehicle) with EE (0, 0.05, 0.5, 5 or 50 µg/kg-d) or BPA (0, 2, 20 or 200 µg/kg-d) from GD 7 to PND 18. In the second block conducted in 2006, 6–14 dams per group were gavaged with EE (0, 0.05, 0.15, 0.5, 1.5, 5, 15 or 50 µg/kg-d) or BPA (20 or 200 µg/kg-d) for the same period as in the first block. Compared to the first block, the second block included three more intermediate doses of EE (0.15, 1.5, and 15 µg/kg-d) and eliminated the low-dose of BPA (2 µg/kg-d). One (out of 14) untreated dams in the first block and 5 (out of 10) untreated dams in the second block were combined with those treated with vehicle only (corn oil) in the statistical analysis. The authors stated that there were no significant differences between the vehicle-only and untreated controls, and thus they were pooled together as the control.

Male offspring from the first block were necropsied in two batches: Block 1a at PND 150, and Block 1b at PND 229. All the male offspring were necropsied on PND 150, but the epididymal sperm in half of the animals (Block 2a) were counted electronically with a Coulter Counter Mutisizer II device, whereas the other half (Block 2b) were counted with a Multisizer III device. The unit for the epididymal sperm count was not clearly reported in the paper, but stated in the text as "the number of mature sperm per epididymis." According to the description of methods, the epididymis was divided into cauda and corpus + caput and the sperm from each part were counted separately. The authors cited an earlier publication by Gray et al. (1999) as giving the details of the method. However, that paper has no description of the sperm counting method. The sperm counting unit in the paper by Gray et al. (1999) was the number of cauda epididymal sperm, not the whole epididymis, as described in the current study report. In addition to the issue of the unit, the authors used two methods to count sperm numbers in this study. The number of sperm per epididymis in the control group from Block 2b was apparently greater (134.3 ± 3.9) than that in Block 1a, 1b or 2a controls (112.8 ± 6.5, 122.2 ± 54.4, 118.0 ± 5.9, respectively, a difference of about 10%). Because the group size, the age of the animals at necropsy, and the method of sperm counting among the blocks were different, the authors normalized the data into "percent of control values using the respective control values for each block" for final statistical analysis. These technical issues should be considered when interpreting the data, especially sperm count.

The authors reported that developmental exposure to EE reduced the weights of androgen-dependent male reproductive organs at doses of ≥ 5 µg/kg-d and significantly decreased the epididymal sperm count at 50 µg/kg-d. Routine histopathological evaluation of the testis from Block 1a males revealed no significant increase in the incidence of testicular degeneration (0/16, 2/20, 1/29, 4/26 in the 0.05, 0.5, 5 and 50 µg/kg-d groups, respectively, vs. 1/31 in the control group). Hyperplasia of the ventral prostate was also observed in the groups treated with ≥ 5µg/kg-d EE. Re-examination of the data as presented by the authors (also summarized below in Table A-1) indicates that the impacts of EE on testis weight and sperm count were remarkably greater in Block1a animals than those in Block 2 or 2bb animals, indicating the experiments in Block 2 were not as effective as those in Block 1a in detecting the effects of EE on the testis. This observation is important in interpreting the findings on BPA, since EE was used as a positive control, and there were differences in the testicular effects of BPA between the two blocks of experiments.

Table A1-2. Testicular effects of EE as observed in the study by Howdeshell et al. (2008).

EE doses (µg/kg-d)	0	0.05	0.5	5	50
Testis weight (mg)					
Block 1a	4011.63 ± 105.54	3832.79 ± 111.24	3810.88 ± 113.86	3488.26 ± 125.90	3249.36 ± 141.87
Block 1b	3934.75 ± 95.47	No data	No data	3839.141 ± 56.10	3885.75 ± 510.25
Block 2	3776.58 ± 135.44	3758.73 ± 183.79	3573.09 ± 106.06	3739.79 ± 146.52	3317.40 (1 rat)
Total	3894.47 ± 71.31	3795.76 ± 103.02	3699.91 ± 81.93	3691.37 ± 85.18	3364.54 ± 162.67
Total as % of control	100.3 ± 1.8	97.8 ± 2.7	95.1 ± 1.9	95.0 ± 2.3*	85.1 ± 4.3*
Epididymal sperm count (unit not reported)					
Block 1a	112.8 ± 6.5	112.5 ± 5.1	107.3 ± 4.5	97.6 ± 3.4	75.7 ± 9.1
Block 2a	118.0 ± 5.9	No data	No data	108.9 ± 6.4	95.0
Block 2b	134.3 ± 3.9	115.0 ± 23.2	127.0 ± 8.7	130.5 ± 5.3	No data
Total	120.1 ± 3.3	113.7 ± 11.3	110.1 ± 4.98	113.5 ± 3.76	84.1±9.8
Total as % of control	101.1 ± 2.5	102.3 ± 4.7	93.7 ± 3.6	95.2 ± 2.1	74.1± 7.8*
Histopathology					
Block 1a	1/31 (3.2%)	0/16 (0%)	2/20 (10.0%)	1/29 (3.4%)	4/26 (15.4%)

Notes: Block2 at 50 g/kg-d only had one male from one litter for Block 2a. Only the doses that were included in both blocks were included for comparison. *: $P < 0.05$.

In the animals treated with BPA, there was no effect on maternal body weight gain or the weights of non-reproductive organs. There was no effect on AGD (PND 2) or nipple retention in adulthood. Detailed data on testis weight, epididymal sperm count and testis histopathology are summarized in the table below.

Based on the data summarized in the table, it is apparent that there was no obvious difference in testis weight between the control and BPA-treated groups. For the data on epididymal sperm count and testis histopathology, several observations should be noted:

1. There was an apparent (about 8–12%), but not statistically significant, reduction in epididymal sperm count in Block 1a. The unit for sperm count was not reported in the methods or presented in the table of the publication, but was most likely the number (x 10^6) of sperm per epididymis.
2. Increased incidence of testicular degeneration in Block 1a, usually indicated by presence of dying germ cells, was supportive of reduced sperm count.
3. The authors did not explain the reasons for the apparent inconsistent results between Block 1a and Block 2. Treatment with EE reduced testis weights and sperm count at doses of 5 and 50 µg/kg-d, though the reduction was not statistically significant. There was no apparent reduction in these two endpoints in Block 2 or 2b, especially sperm count in Block 2b (using the new method of counting). These observations suggest that EE was not as effective as it was in Block 1a. Considering these factors, it may not be appropriate to rely on the data from Block 2 to reject those from Block 1a experiments.

Table A1-3. Testicular effects of BPA observed in the study by Howdeshell et al. (2008).

BPA doses (µg/kg-d)	0	2	20	200
Testis weight (mg)				
Block 1a	4011.63 ± 105.54	3946.86±84.27	3820.43±258.18	3651.81±87.45
Block 1b	3934.75 ± 95.47	No data	No data	No data
Block 2	3776.58 ± 135.44	No data	3951.08±204.50	3714.33±97.15
Total	3894.47 ± 71.31	3946.86±84.27	3946.86±84.27	3880.73±162.30
Total as % of control	100.3 ± 1.8	98.9±2.1	99.9±4.3	95.0±1.8
Epididymal sperm count (unit not reported)				
Block 1a	112.8 ± 6.5	104.0 ± 2.6	104.0 ± 10.1	98.7 ± 3.1
Block 2a	118.0 ± 5.9	No data	No data	114.5 ± 4.0
Block 2b	134.3 ± 3.9	No data	130.7 ± 6.9	138.0 ± 2.9
Total	120.1 ± 3.3	104.0 ± 2.6	116.3 ± 7.2	113.4 ± 4.7
Total as % of control	101.12.5	94.6 ± 2.4	95.4 ± 5.3	95.7 ± 2.2
Histopathology				
Block 1a	1/31 (3.2%)	0/18 (0%)	3/18 (16.7%)	7/20 (35%)**
Block 2	2/35 (5.7%)	No data	0/16 (0%)	1/34 (3%)

**: $p<0.005$.

Lee et al. (2004). Analysis of differentially regulated proteins in TM4 cells treated with bisphenol A.

The authors studied the effect of BPA on cultured TM4 cells, a mouse Sertoli cell line. Treatment with BPA at concentrations of 50, 100, 150, 200 or 250 µM of BPA reduced the viability of TM4 cells to about 90, 85, 78, 55, and 30% of the control, respectively. Two-dimensional electrophoresis (2-DE) revealed that expression levels of 11 proteins were significantly changed. Among these proteins were heat shock protein 27, Bcl2-associated athanogene 2, G protein pathway suppressor 1 and placental calcium binding protein. The authors concluded that BPA strongly affected TM4 cell viability, morphology, and expression of certain proteins that are associated with cellular viability and function.

Liu et al. (2006). Effect of bisphenol A on apoptosis of male mice reproductive cells.

This short communication paper in Chinese reported the findings from a study in mice (Kunming strain of outbred Swiss mouse) on the testicular effects of high doses of BPA. Groups of adult male mice (7 per group, weight 18–20 g) were treated by i.p. injection with 0, 250, 500, or 1000 µmol/kg-d of BPA in corn oil for 5 days. The doses are equivalent to 0, 57, 114 or 228 mg/kg-d. The animals were necropsied on day 7 or 17 from the beginning of the treatment. The treatment did not induce any significant change in testis weight, but caused significantly increased levels of nitric oxide (NO) and nitric oxide synthase (NOS) in testicular tissues from the animals in the mid- and high-dose groups. Flow cytometry analysis of the testicular cells (mainly consisting of germ cells) found that BPA treatment at all doses significantly increased the proportions of cells

in the G2/M and S phases on day 7. At the high dose, there was a significant increase in the proportion of apoptotic cells. By day 14 of the treatment, significant changes in the cell cycle were limited to the high-dose group, suggesting the effect on the cell cycle was reversible. The authors concluded that BPA-induced alteration in the cell cycle and apoptosis in germ cells were related to the increased level of lipid peroxidation.

Ogura et al. (2007). Bisphenol A induces permanent squamous change in mouse prostatic epithelium.

The authors studied the effects of BPA and diethylstilbestrol (DES) on differentiation of the prostate epithelium in mice, using both *in vivo* (adult or fetal exposure) and *in vitro* approaches. In the experiments in adult mice, male BALB/c mice (8–9 weeks of age, 7–9 mice per group) were treated for 3 weeks with s.c. implants of 0, 0.2, 2, 20 or 200 mg BPA pellets or 2 mg DES. The control group was treated with 20 mg of cholesterol. In addition, one group of mice was castrated at the beginning of the BPA or DES treatment and used as a castration control. All animals were necropsied at the end of treatment (12 weeks of age). Castration and DES treatment caused significant decreases in the weights of anterior prostate (AP), dorsaolateral prostate (DLP) and seminal vesicles (SV). The weight of ventral prostate was reduced in the castration and DES groups, but the reduction was only statistically significantly in the castration group. Immunostaining for cytokeratin 10 (CK10), a biomarker for estrogen-induced squamous metaplasia in the prostatic epithelium, revealed that DES, but not castration, caused squamous metaplasia in the AP, but not in the DLP or VP. Treatment with BPA had no effect on the weights of the three lobes of the prostate, but reduced SV weight significantly at 20 and 200 mg. There was no apparent morphological alterations in hemotoxylin-eosin (HE)-stained paraffin sections (routine histopathology) in the ducts of all prostatic lobes of BPA-treated mice. However, at doses ≥ 2 mg, BPA induced expression of CK10 in the basal epithelial cells of all lobes (AP>VP>DLP in the staining density) in a dose-dependent manner. This observation suggests that s.c. treatment with BPA in adult mice caused prostatic squamous metaplasia, an estrogen-induced abnormal morphological alteration that has been observed in a number of species including humans, mice, rats, dogs, sheep, goats, and bulls.

In the experiment on gestational exposure, three pregnant Balb/c mice per group were treated by oral gavage with 0.2 µg/kg-d of DES or 20 µg/kg-d of BPA in tocopherol-stripped corn oil (TSCO) from GD 13–18. The litters were culled to ≤ 4 males per litter on PND 6 and weaned on PND 42. All the animals were terminated at 12 weeks of age. The prostate was dissected into three lobes and subject to routine histopathology and immunostaining for CK 10 and 14. Treatment with DES or BPA caused no noticeable morphological changes in the routine histopathological evaluation, but produced squamous metaplasia in the basal epithelial cells as observed in the experiment with adult mice (treated with DES or BPA by s.c. implants).
In the *in vitro* experiment, the authors isolated prostatic ducts from individual prostate lobes of Balb/c mice 8–9 weeks of age and cultured them on Millicell CM filters in serum-free medium. The cultured prostatic ducts were treated with DES (1 nM) or BPA (1 or 1000 nM) for 6 days. Immunostaining for CK 10 in cultured ducts found that BPA at 1000 nM caused squamous metaplasia that was similar to the observation *in vivo* or to the cultured ducts treated with 1 nM of DES. At 1 nM (which is below the average level of BPA detected in the serum samples of

human populations), BPA-induced squamous metaplasia was noticeable, but not as striking as that induced by 1000 nM of BPA.

Okada and Kai (2008). Effects of estradiol-17beta and bisphenol A administered chronically to mice throughout pregnancy and lactation on the male pups' reproductive system.

This study investigated the reproductive effects of estradiol-17beta (E_2) and BPA in mice following the perinatal administration via s.c. implant. Three days before mating, female mice (5–9 pregnant mice) were implanted with silicone tubes filled with E_2 (10 ng, 500 ng, 1 µg or 10 µg) or BPA (0.1 or 5 mg) in sesame oil. The litters were culled to 8 pups per litter on the day of birth and all the pups were terminated for necropsy at postnatal week (PNW) 4. Citing a previous paper by Okada et al. (2005), the authors stated that the silicone tube used in the study released E_2 or BPA at a rate of 1.2% of the total amount per day. Based on this release rate, the authors estimated that the treatment was equivalent to 0.12, 6, 12 or 120 ng/d of E2, or 1.2 or 60µg/d of BPA.

Treatment with 1 or 10 µg of E_2 significantly reduced the birth rate and no pup was produced for subsequent analysis. E_2 at 10 and 500 ng had no effect on body weight or relative weight of the testis, epididymis or seminal vesicles with coagulating glands, but caused significant reduction in the proportion of seminiferous tubules (STs) with elongated spermatids. Blood levels of T at PNW 4 were significantly increased at 500 ng, but not at 10 ng.

BPA treatment had no effect on reproductive outcome, body weights of pups, or the relative weights of the testis, epididymis, or seminal vesicles with coagulating glands. Compared to the control group, the proportion of STs with elongated spermatids was significantly reduced in the 5-mg BPA group, but not in the 0.1 mg group. On the other hand, blood levels of T at PNW 4 were significantly increased at 0.1 mg, but not at 5 mg.

Richter et al. (2007). Estradiol and bisphenol A stimulate androgen receptor and estrogen receptor gene expression in fetal mouse prostate mesenchyme cells.

In this study, the authors isolated mesenchymal cells from the prostatic region of the urogenital sinus (UGS) at the bladder neck of GD 17 fetal male CD-1 mice. The cells were cultured in estrogen-free medium containing 5% charcoal-stripped fetal bovine serum (FBS) and 690 pM DHT. The cultured cells were treated with E_2, BPA or tamoxifen for 4 days. A wide range of concentrations of BPA, from 0.0001 to 100,000 nM, was tested for effects on the content of DNA and RNA (as indicators for growth) and expression of genes for the androgen receptor (AR) and estrogen receptor α (ESR 1), respectively.

The authors found that AR and ESR1 mRNA expression was increased in response to E_2, with thresholds of 0.001 and 0.037 nM, respectively. BPA at concentrations of 1,000 and 10,000 nM, but not at any other concentrations, significantly increased the DNA content, indicating that BPA stimulated the growth of the cultured cells. At concentrations of $\geq$ 1 nM, BPA significantly increased the expression of AR and ESR1. Co-treatment with tamoxifen (100 nM) significantly

attenuated increased AR or ESR1 expression induced by BPA at 10 nM, but not at 1,000 nM, suggesting that tamoxinfen at 100 nM was not able to block the effects of BPA at high concentrations. The authors concluded that their findings suggest that prenatal exposure to estrogens and xenoestrogens at physiological concentrations could result in an increase in the numbers of ARs or ERs in the developing prostate mesenchyme, which subsequently increases in growth in response to estrogens.

Saunders et al. (1997). Fetal and perinatal influence of xenoestrogens on testis gene expression.

The authors treated neonatal Wistar male rats (8–16 per group) by s.c. injection with DES (10 µg), BPA (0.5 mg), or octylphenol (2 mg) in corn oil on PND 2, 4, 6, 8 and 12. On PND 18, all the animals were necropsied. Treatment with BPA did not affect the testis weight, the diameter of STs, or the density of immunostaining for ERα or FSH β in the pituitary. No other endpoints were evaluated in this study.

Takamiya et al. (2007). Effect of bisphenol A on human chorionic gonadotrophin-stimulated gene expression of cultured mouse Leydig tumour cells.

The authors studied the effect of BPA on gene expression in cultured mLTC-1 cells, a mouse Leydig tumor cell line, using the semi-quantitative RT-PCR analysis. The cultured cells were treated with BPA at concentrations of 10^{-11}–10^{-4} M for 1 or 3 hr in the absence or presence of hCG (0.1–100 µg/l). Bisphenol A treatment for 1 hr had no apparent effect on cell viability. In the absence of human chorionic gonadotrophin (hCG) stimulation, BPA exposure for 3 hr significantly increased expression of the AR gene at concentrations of 0.01 and 1 µM, but not at 0.1, 10, 100 µM. Within the same range of concentrations, BPA increased the expression of StAR at 10 and 100 µM, but not at lower concentrations. Bisphenol A had no effect on the expression of Cyp17α1. Similar effects on the expression of these three genes were observed in cultured cells stimulated with 100 µg/l of hCG, indicating that BPA had no effect on the action of hCG in the cultured cells. Global gene expression analysis by Affymetrix microarray revealed that BPA caused alterations in the expression of a number of genes that are involved in steroidogenesis.

Wetherill et al. (2005). Xenoestrogen action in prostate cancer: pleiotropic effects dependent on androgen receptor status.

Based on their previous findings on the BPA-induced androgen-independent proliferation of prostate cancer cells via activation of a tumor-derived AR mutant (T877A), the authors examined the underlying molecular mechanisms for BPA-induced activation of AR mutants in prostatic tumor cell lines. The authors found that BPA at concentrations of $\geq 10^{-8}$ M (0.01 µM) potentiated DHT-induced activation of AR in a slightly reversed dose-dependent manner (lower concentrations were slightly stronger than are the higher concentrations) in LNCaP cells. At 10^{-5} M (10µM), BPA inhibited proliferation of AR-positive cells (LNCaP and LARC-4 cell lines). An in vitro radio-ligand binding assay revealed that BPA altered DHT binding to AR-T877A, likely through non-competitive inhibition. By contrast, AR-negative prostate cancer cells (PC-3

or DU-145 cell lines) failed to show growth inhibition after exposure to a high dose of BPA. The authors postulated that BPA can serve as a potential "hormone sensitizer" of the mutant ARs present in advanced prostate adenocarcinomas, thereby possibly contributing toward therapeutic relapse in advanced prostate cancer patients.

APPENDIX 2

Male Reproductive Toxicity of Bisphenol A in Laboratory Animals

Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

October 2009

APPENDIX 2 OUTLINE

APPENDIX 2

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  - Section 1.1.2.1 Prenatal exposure: fertility or reproductive outcome in rats
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SECTION 2.3 STUDIES IN OTHER ADULT ANIMALS
Section 2.3.1 Rabbit
Section 2.3.2 Dog

SECTION 3 STUDIES *IN VITRO*

SECTION 3.1 SERTOLI CELLS
SECTION 3.2 LEYDIG CELLS
SECTION 3.3 PROSTATE CELLS

SECTION 4 REFERENCES

Because of the extent and complexity of the data on male reproductive toxicity of BPA, this appendix supplements the discussion of male reproductive toxicity in Section D of the main document. More detailed information on the main findings from all the relevant studies of male reproductive toxicity is presented to help the readers to understand the findings from those studies in an integrative approach. The information in this appendix is organized first by the developmental windows of exposure and then by endpoints indicative of male reproductive effects.

There are a large number of studies on the potential effects of BPA on the development and/or function of the male reproductive system in laboratory animals. The majority of these studies have been reviewed and summarized in comprehensive review documents such as the NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A (CERHR, 2008) and the European Union Risk Assessment Report 4,4'-Isopropylidenediphenol (Bisphenol A) (EU, 2003) and its update (EU, 2008).

The majority of the studies were conducted in mice and rats. Development of the male reproductive system in rodents begins around gestation day (GD) 12 (Magre and Jost, 1991), through the neonatal (first week) and infantile (second week) period, until postnatal week (PNW) 7, when sexual maturation of the whole male reproductive system is basically complete (Flickinger, 1971; Sun and Flickinger, 1979; Nazian and Mahesh, 1980; Vergouwen et al., 1993; Marty et al., 2003; Sharpe et al., 2003a). The male reproductive system consists of organs mainly of three embryonic origins: undifferentiated gonads (for the testis), Wolffian ducts (for the internal genital organs, such as seminal vesicles, epididymides, and external genitalia), and the urogenital sinus (for the prostate). Development of these organs is under the active and balanced control of numerous hormones, such as androgens (including testosterone (T) and dihydrotestosterone (DHT)), estrogens, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), in a time-, dose-, duration-, and even frequency-dependent manner. The exact biological effects of a hormone on the development and function of a particular organ depends on many factors. For example, the level of 17β-estradiol (E_2) in free (active) form, the level and function of its receptors estrogen α (ERα) and ERβ, and the ratio of these two receptors in the testis or prostate determines the exact effects of estrogen on these two organs at different stages of development. In addition, the functions of androgens (T or DHT) can also influence the effects of estrogens significantly (O'Donnell et al., 2001; Akingbemi, 2005; Morani et al., 2008; Prins and Korach, 2008). Therefore, evaluation of evidence on the potential effects of an endocrine-modulating chemical requires careful consideration of all the potential compounding factors, including the pharmacokinetic characteristics at the level and time of exposure.

It is also important to recognize that every animal model or laboratory experiment in toxicological studies has its own scientific merits as well as limitations. This document presents the major findings from all relevant studies, but notes significant limitations or study design features that may impact the interpretation of the results. For studies that were designed to replicate or verify the findings from other researchers using the same or similar animal models or study designs, comparisons will be presented.

Integrative evaluations of the major findings from all the relevant studies are presented below in three subsections. Section 1 includes studies that treated the animals during one or multiple

periods of development, namely the prenatal, perinatal, neonatal, or pubertal period. Traditional one-, two-, or three-generation reproductive studies are also included in this subsection. Section 2 focuses on the studies that used adult animals (≥ 8 weeks of age). Section 3 presents a brief overview of *in vitro* studies.

Section 1 Effects on development of the male reproductive system

Major findings from studies under this subsection are evaluated by treatment period (prenatal, neonatal, perinatal, pubertal, and two- or three-generation reproductive studies) and then by species (mice and rats) if there are multiple studies in each species. Following a summary table under each exposure period and the animal species, the major findings are evaluated by major endpoints observed in the studies. These endpoints include:

- **Effects on Fertility or Reproductive Outcome** include a number of parameters are commonly used to measure fertility or reproductive outcome. These parameters include mating index, fertility index (pregnancy rate), number of litters per pair, number of live pups per litter at birth, still birth index (percent of dead pups among the total pups at birth), post-implantation loss per litter, etc.
- **Testicular effects** include data on testicular weights, histopathology, sperm parameters, and mechanistic data such as molecular, cellular or biochemical observations.
- **Effects on the Epididymis or Seminal Vesicles** include effects on organ weights and histopathology. Data on epididymal sperm parameters are usually included in testicular effects, unless indicated in the text.
- **Effects on the Prostate** include organ weights, histopathology, molecular and biochemical observations. The rodent prostate is comprised of three distinct lobes: anterior, dorsolateral, and ventral prostate. The weight (absolute or relative to body weight) of the whole or ventral prostate is one of the commonly used endpoints indicative of prostate effects.
- **Effects on Sexual Maturation** include data on anogenital distance (AGD) and the age of preputial separation (PPS) or testicular descent, nipple retention in the male, etc.
- **Hormonal Effects** include data on blood or tissue levels and production of hormones, including T, FSH, LH, estradiol (E_2), or prolactin (PRL).

Section 1.1 Prenatal Exposure

A total of 21 prenatal studies were found through a comprehensive literature search. Key study design features and major findings from these studies are briefly summarized in Table A2-1 (mice) and A3-3 (rats). In addition, the literature search also identified 16 abstract-only study reports. Thirteen of them are studies in rats, and three in mice. The findings from these studies are not included in this summary report.

Table A2-1. Studies in mice on the effects of prenatal exposure to BPA on the male reproductive system.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Nagel et al., 1997 NTP-A	CF-1 mice, 5-7 mice per group. One male pup from each litter for examination at 6 months of age.	Oral instillation, Vehicle: tocopherol-stripped corn oil (TSCO).	Control: one with no dosing, and another one with TSCO. BPA: 2.0 or 20.0 µg/kg-day from GD 11 to 17. Necropsied at 6 months of age.	Increased prostate weights (absolute and relative) at both doses. No histopathological data.
vom Saal et al., 1998 NTP-A	CF-1 mice, 5-7 mice per group. One male pup from each litter for examination at 6 months of age.	Oral instillation, Vehicle: TSCO.	Control: one with no dosing, and another one with TSCO. BPA: 2.0 or 20.0 µg/kg-day from GD 11 to 17. Necropsied at 6 months of age.	Reduced efficiency of sperm production at 20 µg/kg-day. Reduced weights of seminal vesicles and epididymis at 2 µg/kg-day, but not at 20 µg/kg-day.
Cagen et al., 1999a NTP-A	CF-1 mice, 28 pregnant mice per group for analysis of male reproductive toxicity data. Pups were culled to 4 males per litter at weaning and examined on PND 90±2. Some litters were not included in the analysis.	Oral instillation, Vehicle: TSCO.	Ctrl: one with no dosing, and another one with TSCO. BPA: 0.2, 2.0, 20, or 200 µg/kg-day from GD 11 to 17. Necropsied on PND 90±2.	Increased body weights of male pups at 20 or 200 µg/kg-day. No effect on weights of testis, epididymis, seminal vesicles, or prostate. No effect on sperm production. No histopathological changes.
Ashby et al., 1999 NTP-A	CF-1 mice, 5-7 pregnant mice per group for analysis of the male reproductive toxicity data. No culling. 3 males per litter randomly selected on PND 112 for final necropsy at 6 months of age.	Oral instillation, Vehicle: TSCO.	Control: one with no dosing, and another one with TSCO. BPA: 2.0 or 20.0 µg/kg-day from GD 11-17.	No effect on weights of testis, epididymis or seminal vesicles. No effect on testicular sperm counts. No histopathological data.
Gupta, 2000 NTP-A	CD-1 mice, 15 pregnant mice/group. Litters culled to 8 per litter at birth. 1-3 pups from each litter pooled for group comparison.	Oral (possibly gavage). Vehicle: corn oil containing 10% ethanol.	Control: vehicle only. BPA: 50 ng/g (50 µg/kg) from GD 16 to 18. Examined on PND 3, 15, 21, or 60.	Increased AGD and prostate weights in male pups, with/without adjusting to body weight. Relative epididymal weights decreased. Histopathological enlargement of the prostate on PND 15. No evaluation of testis.

Table A2-1. Studies in mice on the effects of prenatal exposure to BPA on the male reproductive system (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Ogura et al., 2007 NTP-B	BALB/c mice, 3 mice per group. Litters culled to ≤ 4 male pups.	Oral (possibly gavage). Vehicle: TSCO.	Control: TSCO only BPA: 20 μg/kg-day from GD13 to18. Evaluated at PNW 12	Positive staining for cytokeratin 10 (CK-10) in basal epithelial cells of the prostate from BPA-treated male pups at PNW 12, indicative of abnormal proliferation and differentiation of basal epithelial cells.
Nagao et al., 2002 NTP-A	C57BL/6N mice, 10 mice per group. All fetuses removed by cesarean section on GD 18, fostered by untreated mice, culled to 3 males only per litter on PND 4, examined at PNW 12.	Oral gavage. Vehicle: 0.5% carboxymethyl cellulose.	Control: vehicle only. BPA: 2, 20, or 200 μg/kg-day, GD 11-17. Examined at PNW 12.	No effect on weights of the testis, epididymis, or seminal vesicles (SV). No effect on epididymal sperm count. No histopathological changes in testis. No data on the prostate. More discussions on this study in the text.
Iida et al. 2002 NTP-A	ddY mice, 3 dams per group, 4-5 male offspring from each group for histopathology on PND 60. 3 pups from the10-mg/kg-BPA group were examined on PND 120.	Oral gavage. Vehicle: corn oil.	Control: vehicle only. BPA: 1, 10, or 100 mg/kg-d from GD 10 to 17. Male pups examined on PND 60.	The number of seminiferous tubules with abnormal morphology was increased in all BPA-treated groups on PND 60 and in the 10-mg/kg BPA group on PND 120.
Kawai et al., 2003 NTP-A	CD-1 mice, 7-9 mice per group. All male offspring were pooled by group and randomly selected (8-16) for examination at PNW 8, 12, and 16.	Oral gavage. Vehicle: corn oil.	Control: vehicle only. BPA: 2 or 20 ng/kg-day from GD 11 to 17. Examined at PNW 8, 12, and 16,	2 ng/kg-day: reduced relative testis weights at PNW 8 and 12, but not at PNW 16. 20 ng/kg-day: reduced relative testis weights at PNW 12. No consistent effect on the blood levels of T.
Timms et al., 2005 NTP-A	CF-1 mice, 4-6 mice per group. Male fetuses positioned between a female and a male fetus in the uterus were used for morphometric evaluation on GD 19. One male fetus per litter was selected.	Oral instillation Vehicle: TSCO.	Control: one with no dosing, and another one with TSCO. BPA: 20.0 μg/kg-day from GD 11 to 17. Examined on GD 19.	Increased the number and volume of prostate ducts in the dorsal and dorsolateral lobes. Increased percentage of cells with positive PCNA staining in the dorsal lobe. Abnormal morphology in the portion of the urethra associated with the neck of the bladder.

Section 1.1.1 Prenatal exposure: studies in mice

Among the ten prenatal studies conducted in mice (Table A2-1), five used CF-1 mice and the remainder used other strains including CD-1, BALB/c, C57BL/6N, or ddY. All the studies used the oral route of exposure.

Section 1.1.1.1 Prenatal exposure: fertility or reproductive outcome in mice

There are no prenatal studies that evaluate male-mediated reproductive outcome of male mice exposed to BPA prenatally. Studies that assessed the fertility of F_1 or F_2 generations after perinatal exposure or through multiple generations, which include both pre- and post-natal exposure, are included in the corresponding subsections.

Section 1.1.1.2 Prenatal exposure: testicular effects in mice

Testis weights, morphology, or sperm parameters were investigated in seven studies. Reduction in relative testis weight was observed in a study in CD-1 mice at PNW 8 and 12, following prenatal exposure to BPA at 2 ng/kg-day, (Kawai et al., 2003). At a higher dose (20 ng/kg-day), BPA caused an increase (not statistically significant) in relative testis weights at PNW 8, and a reduction at PNW 12 (statistically significant). BPA did not cause statistically significant alterations in relative testis weights at PNW 16. No other studies in mice that included data on testicular weights reported changes in testis weights.

Iida et al. (2002) reported an increased number of seminiferous tubules with abnormal histopathological changes on PND 60 in mice treated orally with 1, 10, or 100 mg/kg-d of BPA from GD 10 to 17. No other prenatal studies in mice included similar observations. Nagao et al. (2002) included histopathological evaluation of the testis at PNW 12, but the authors reported no histopathological changes in the BPA-treated male pups.

vom Saal et al. (1998) reported that BPA at 20 µg/kg-day reduced the efficiency of sperm production (daily sperm production/g testicular tissue; a reduction of approximately 16% compared to the control). However, the authors found no difference in testicular weights or daily sperm production per testis between the BPA-treated and the control groups. Using the same strain of mice (CF-1) and study design, Cagen et al. (1999a) and Ashby et al. (1999), respectively, did not find any significant effect of BPA on sperm production. However, neither of these two studies observed any effect of DES (a positive control) on the testis or the sperm production. Comparison of the major findings among the four studies that used the same study design is presented in Table A2-2. In addition to the studies in CF-1 mice, Nagao et al. (2002) reported no change in epididymal sperm count in C57BL/6N mice. However, it should be noted that the fetuses were removed by cesarean section on GD 18 and fostered by untreated dams until weaning. The perinatal period in rodents is critical for the development of the male reproductive system. Development of certain male internal genital organs (e.g., epididymis, seminal vesicles) begins on GD 16–19 (Sun and Flickinger, 1979, 1982; Staack et al., 2003). The surgical procedure of cesarean section is uncommon in studies designed to evaluate development of the male reproductive system. Interpretation of relevant findings, especially of those on the

development of internal organs (e.g., seminal vesicles, see summaries below on this organ) should be made with caution.

Section 1.1.1.3 Prenatal exposure: effects on the epididymis or seminal vesicles in mice

Five studies evaluated the effects of prenatal exposure to BPA on development of the seminal vesicles or epididymis. vom Saal et al. (1998) found slight (about 10–11%), but statistically significant reduction in the weights of the seminal vesicle or epididymis in CF-1 mice treated with 2 µg/kg-day BPA (but not at 20 µg/kg-day) between GD 11 to 17. Gupta (2000) also reported reduced relative weights of epididymis in CD-1 mice following oral treatment with 50 µg/kg-day from GD 16–18, the most critical period of time for the development of these organs. However, using the same stain of mice as that used by vom Saal (1998), Cagen et al. (1999a) and Ashby et al. (1999) found no effect on the weights of the epididymis or seminal vesicles. In addition, Nagao et al. (2002) reported no effect on the weights of these two organs in C57BL/6N mice, but the cesarean section surgery at the time most critical for the development of these two organs makes interpretation of these findings difficult.

Section 1.1.1.4 Prenatal exposure: effects on the prostate in mice

Six studies investigated the effect of prenatal BPA exposure on the development of the prostate in mice. Increase in prostate weights was reported in CF-1 mice (Nagel et al., 1997) and CD-1 mice (Gupta, 2000a). Histopathological or histochemical changes indicative of abnormal proliferation and/or differentiation of the prostate epithelial cells were reported in CF-1 mice (Timms et al., 2005) and in BALB/c mice (Ogura et al., 2007). However, Cagen et al. (1999a) and Ashby et al. (1999) did not find any statistically significant increase in prostate weights in CF-1 mice, using the same protocol as that used by Nagel et al. (1997). Table A2-2 presents the comparison of four studies that used the same strain of animal and the same study design.

Table A2-2. Comparison of four prenatal studies in CF-1 mice.

	Nagel et al.,1997; vom Saal et al., 1998			**Ashby et al., 1999**			**Cagen et al., 1999a**		
BPA doses (µg/kg-day)	0	2	20	0	2	20	0	2	20
Body weights (g)	37.9	34.6	36.7	43.5	47.9	45.9	34.68	35.70	36.99
Testis, left (mg)	ND	ND	ND	118	129	133	116	117	116
Testis, right (mg)	ND	ND	ND	125	137	139	123	123	123
Testis, total (mg)	229	216	232	243	266	272	239	240	239
DSP(10^6/day)	5.26	5.25	4.65	3.5	3.9	4.1	3.5	3.3	3.4
DSP/g testis	46.6	45.0	**37.6**	30.1	30.6	31.0	29.5	27	28
Epididymis (mg)	94.3	83.3	87.2	92	97	103	94	95	96
Seminal vesicle (mg)	48.9	43.1	49.5	57.3	64	62	107	105	113
Prostate (mg)	41	**53**	**55**	48.4	53.2	50.3	39	39	41

Note: ND: no data. DSP: Daily Sperm Production, expressed as millions of sperm produced per day. All values are mean value from the tables or estimated values from the figures presented in the original publications as cited. The values for the control groups are from the vehicle-only control groups. Number in bold indicate statistically significant.

There are several differences among the studies in CF-1 included in the table above. For example, even though all the studies used the same strain of mice, the body weights of mice used in the study by Ashby et al., are noticeably higher than those by Nagel et al. (1997), vom Saal et al. (1998) or Cagen et al. (1999a). The average weight of seminal vesicles in the control group of the study by Cagen et al. (1999a) was about twice that in the studies by vom Saal et al. (1998) or Ashby et al. (1999), respectively. The differences between these studies indicate that the animals used in these studies, even though exactly the same strain, are different, possibly due to differences in genetic background or nutrients in the feed. It should be noted that the control values presented in A2- 2 are from the vehicle-only control, not from the naïve control (no dosing control) or the combination of the two control groups. The body weights of the dams and pups from the naïve control in the study by Ashby et al. (1999) were smaller than those of the vehicle-only control. The authors also found that prostate weight was associated with the body weights. Therefore, it may not be appropriate to use the naïve control for comparison analysis.

The values of daily sperm production (DSP) or DSP/g testis (efficiency of sperm production) from the study by vom Saal et al. (1998) are much higher than those from the studies by Ashby et al. (1999) or Cagen et al., (1999a). The reduction in sperm production reported by vom Saal et al. (1999) did not reach the level of statistical significance until the testicular weights were incorporated into the calculation for the efficiency of sperm production. Therefore, if BPA indeed causes reduction in sperm production, the degree of this effect under the experimental conditions used by the authors would likely be small. Similarly, slight, but statistically significant (Nagel et al., 1997), or insignificant (Ashby et al., 1999), increases in prostate weights was observed, while Cagen et al. (1999a) observed no apparent difference between the treated

and the control group. Slight differences in a biological effect can be impacted by factors beyond the chemical treatment, highlighting the importance of factors beyond the treatment. On the other hand, using histopathological or histochemical approaches, Timms et al. (2005) and Ogura et al. (2007) observed BPA-produced significant changes in the prostate that are consistent with increased weights of the prostate. Several reports have shown that careful histopathological evaluation of the testis is a more sensitive means to detect the testicular toxicity of chemicals (e.g., Mangelsdorf et al., 2003). However, it is not known whether histopathological evaluation of the prostate or prostate weight is a more sensitive indicator of toxicity in this organ.

Section 1.1.1.5 Prenatal exposure: effects on sexual maturation in mice

The study by Gupta et al. (2000) found increased AGD on PND 3 or 21 in male pups from dams exposed to 50 ng/kg-day BPA from GD 16 to 18. The authors also reported that 0.1 g/kg-day of DES caused increased AGD, while 2,000 g/kg of DES produced a significant reduction in the AGD. None of the other studies evaluated this endpoint.

Section 1.1.1.6 Prenatal exposure: hormonal effects in mice

Kawai et al. (2003) observed no obvious effect on the blood levels of testosterone among the male pups of CD-1 mice treated prenatally with 2 or 20 ng/kg-day BPA from GD 11 to 17. None of the other prenatal studies in mice measured hormonal levels in the male pups.

Section 1.1.2 Prenatal exposure: studies in rats

Among the eleven prenatal studies conducted in rats (Table A2-3), seven used SD rats, and the remaining five used Wistar or Wistar-derived rats. Seven studies used the oral route of exposure and four treated the animals by s.c. injection. The studies by Tinwell et al. (2002) were designed to replicate the findings by Talsness et al. (2000).

Table A2- 3. Prenatal studies on the male reproductive system in rats.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Tinwell et al., 2002 NTP-A	SD rats, 7 per group. Culled to 8 pups per litter on PND 5, weaned on PND 23, terminated on PND 90-91.	Oral, gavage. Vehicle: arachis oil.	Control: AO only. BPA: 20, 100, or 50,000 µg/kg-day from GD 6 to 21.	No effect on pregnancy of the dams or pup body weights. No effect on AGD (PND 1), PPS, or weights of testis or seminal vesicles or sperm parameters. No histopathological changes in testis. Prostate weights appeared to be slightly higher than those of the controls, but not statistically significant.
Tinwell et al., 2002 NTP-A	AP (Wistar) rats, 7 per group. Culled to 8 pups per litter on PND 5, weaned on PND 23, terminated on PND 90-91.	Oral, gavage. Vehicle: arachis oil .	Control: AO only. BPA: 20, 100, or 50,000 µg/kg-day from GD 6 to 21.	No effect on pregnancy of the dams or body weights of pups. No effect on AGD, PPS or testis weight. Sperm production (total sperm count, DSP, DSP/ g testis) lower in BPA-treated groups, but only statistically significant at 50 mg/kg-d. Prostate weights appeared to be slightly lower in the lower and higher doses, and higher in the mid-dose, but not statistically significant.
Talsness et al., 2000 NTP-A	SD rats, 18-20 per group. No culling. Necropsied PND 70 or 170.	Oral, gavage. Vehicle: 2% Mondamin (corn starch).	Control: vehicle only. BPA: 0.1 or 50 mg/kg-d from GD 6 to 21.	Alterations in AGD, PPS, organ weights, sperm production and hormonal levels. See Table 4.
Thuillier et al., 2003. NTP-A	SD rats, no data on the number of dams per group. All male pups examined at birth or on PND 3.	Oral gavage, Vehicle: DMSO in corn oil.	Control: vehicle only. BPA: 0.1 – 200 mg/kg-d from GD 14 to birth.	BPA dose-dependently increased the expression of platelet-derived growth factor (PDGF) receptors α and β in testis.
Wang et al., 2004. NTP-A	SD rats, no data on the number of dams per group. All male pups examined at birth, on PND 3, or 21.	Oral gavage, Vehicle: DMSO in corn oil.	Control: vehicle only. BPA: 0.1 – 200 mg/kg-d from GD 14 to birth.	BPA dose-dependently altered the expression of estrogen receptor-associated proteins (e.g., Hsp 90) in the gonocytes at birth or on PND 3, but not in germ cells on PND 21.
Tanaka et al., 2006 NTP-A	SD rats, no data on the number of dams per group. 12-45 male fetuses were examined on GD 22 following cesarean section, and 11-20 pups at 2 hrs after birth.	Oral, drinking water. Vehicle: drinking water.	Control: no BPA in drinking water. BPA: 0.2 – 20 µg/ml in drinking water from GD 1 to GD 22.	Dose-dependent increase in serum levels of BPA in the fetuses. No apparent effect on serum levels of T in fetuses removed by cesarean section on GD 22. Dose-related reduction in the serum levels of T in neonates at 2 hrs after birth, indicative of suppression of the normal T surge right after parturition. No data on organ weights or histopathology.

Table A2- 3. Prenatal studies on the male reproductive system in rats (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Wistuba et al., 2003 NTP-A	SD rats, 5-15 per group. Pups from one (50 mg/kg-d BPA) to 4 litters per dam (0.1 mg/kg-d BPA) were used for analysis.	Oral gavage. Vehicle: 2% Mondamin (cornstarch).	Control; vehicle only. BPA: 0.1 or 50 mg/kg-d from GD 6 to 21. Male pups were examined at ≥ 90 days of age.	No significant inter-litter, but high inter-individual (within the same litter; 20-27%) variations in the number of Sertoli cells per testis. No effect on testis weight or morphology. Increased number of Sertoli cells per testis, but no effect on number of Sertoli cells per g testis.
Ramos et al., 2001	Wistar rats, 4 dams per group. Culled to 8 pups per group. Examined on PND 30.	s.c. implant of osmotic pumps. Vehicle: DMSO.	Control: DMSO only. BPA: 25 or 250 µg/kg-day, from GD 8 to GD 23 (birth).	Stated no effect on the AGD, with no data reported. Reduced expression of androgen receptors (AR). Reduced expression of prostatic acid phosphatase (PAP, indicative of prostate function) in the epithelial cells. Altered phenotype of periductal stromal cells.
Ramos et al., 2003 NTP-A	Wistar rat, 7-9 dams per group. Culled to 8 pups per group, weaned on PND 22. 6-8 pups from different litters per group were examined on PND 15, 30, or 120.	s.c. implant of osmotic pumps. Vehicle: DMSO.	Ctrl: DMSO only. BPA: 25 or 250 µg/kg-day, from GD 8 to GD 23 (birth).	Stated no effect on the AGD, with no data reported. No effect on weights of the ventral prostate. Altered proliferative activity, decreased expression of androgen receptors, and abnormal morphological changes in periductal stromal cells on PND 30, but not PND 120. Increased expression of ERβ in the anterior hypothalamic structures of the brain on PND 30 and 120. Slight, but statistically significant increase in blood levels of T and PRl on PND 15 and 30, but not on PND 120.
Naciff et al., 2005 NTP-A.	SD rats, 8 dams per group. 4 male fetuses from different litters per group for necropsy GD 20.	s.c. injection. Vehicle: DMSO.	Control: DMSO only. BPA: 0.002 to 400 mg/kg-d from GD 11 to 20.	Nipple retention in males at 400 mg/kg-d. No obvious histopathological changes in testis or epididymis. BPA-induced gene expression profile in testis and epididymis: consistent with that by ethinyl estradiol or genistein. BPA at all doses tested induced significant changes in the expression of certain genes that are critical for testicular develop or function.

Table A2- 3. Prenatal studies on the male reproductive system in rats (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Saito et al., 2003 NTP-A	Wistar, 2-4 dams per group. A total of 11 and 22 male pups from the control and BPA groups, respectively, were examined at PNW 13.	s.c. injection. Vehicle: corn oil.	Control: corn oil only. BPA: 50 µg/dam from GD 12 to 19. Examined at PNW 13.	No effect on absolute testis weights. Reduced T levels in blood.

Section 1.1.2.1 Prenatal exposure: fertility or reproductive outcome in rats

There are no prenatal studies that evaluate male-mediated reproductive outcome of male rats exposed to BPA prenatally. Studies that assessed the fertility of F_1 or F_2 generations after perinatal exposure or through multiple generations, which include both pre- and post-natal exposure, are included in the corresponding subsections.

Section 1.1.2.2 Prenatal exposure: testicular effects in rats

Talsness et al. (2000) found that prenatal exposure to BPA at 0.1 or 50 mg/kg-d produced certain alterations in the endpoints indicative of abnormal alterations in testicular development or function in SD rats. However, Tinwell et al. (2002) found no such alterations, using similar study designs. In addition, Tinwell et al. (2002) used both SD and AP (Wistar-derived) rats. A comparison of the key study design features and major findings from these three studies are presented in Table A2-4 below.

Table A2- 4. Comparison of the study by Talsness et al. (2000) to those by Tinwell et al. (2002). (ND: No data).

	Talsness et al. (2000)		**Tinwell et al. (2002)**	
Strain	SD		SD	AP
No. of litters	18-20		7	7
Culling	No		Yes (8 per litter)	Yes (8 per litter)
Statistical units	Pups		Litters and pups	Litters and pups
Dosing method	Oral gavage		Oral gavage	Oral gavage
Vehicle	2% Mondamin		Arachis oil	Arachis oil
Doses (mg/kg-d)	0.1 or 50		0.02, 0.1 or 50	0.02, 0.1 or 50
Dosing period	GD 6-21		GD 6-21	GD 6-21
Terminal ages (PND)	70	170	90	90
Testis weights (absolute)				
Left	ND	ND	No effect	No effect
Right	ND	ND	No effect	No effect
Paired	Reduced at 0.1, but not 50 mg/kg-d	No effect	ND	ND
Testicular histopathology	Minimal changes	Minimal changes	No effect	No effect
Sperm counts (10^6/testis)	Reduced at 50, but not 0.1 mg/kg-d.	Reduced at 0.1, but not 50 mg/kg-d.	No effect	No effect
Sperm counts (10^6/ g testis)	ND	ND	No effect	No effect
DSP (10^6/testis)				
Control (pup-based)	43 ± 8	47 ± 7	26.6 ± 4.0	25.5 ± 4.4
0.1 mg/kg-d BPA	41 ± 8	38 ± 11**	26.0 ± 3.2	25.2 ± 5.1
50 mg/kg-d BPA	37 ± 8**	48 ± 8	26.9 ± 3.4	22.3 ± 3.6
DSP (10^6/g testis)	ND	ND	No effect	No effect
Epididymal weights (mg)				
Left	ND	ND	No effect	No effect
Right	ND	ND	No effect	No effect
Paired	Reduced at 0.1, increased at 50 mg/kg-d	No effect	ND	ND
Seminal vesicles weights	Reduced at 0.1, but no effect at 50 mg/kg-d	No effect	No effect	No effect
Prostate weights (mg)	Increased at 50, not 0.1 mg/kg-d	No effect	No effect	No effect
Blood testosterone (ng/ml)	Reduced at 50, but not at 0.1 mg/kg-d	No effect	ND	ND
Blood LH (ng/ml)	No effect	No effect	ND	ND
AGD (mm)	Reduced on PND 3, 15, 21 at 50 mg/kg-d		No effect on PND 1	No effect on PND 1
Preputial separation (PND)	Delayed at 0.1, but no effect at 50 mg/kg-d		No effect	No effect

As shown in Table A2-4, Tinwell et al. (2002) found no testicular effect in SD or AP rats on PND 90 (adulthood). On the other hand, Talsness et al. (2000) observed noticeable, but not dramatic, testicular effects (indicated by changes in testicular weights, histopathology, sperm parameters, and blood levels of testosterone) on PND 70 (considered by the authors as pubertal age). In adulthood (PND 170), no obvious effect was observed.

Although the studies reported by Tinwell et al. (2002) were designed to replicate the study by Talsness et al. (2000), there are some obvious differences between these studies. For example, Tinwell et al. (2000) did not examine the animals on PND 70. DSP was 47 ± 7 (pup-based mean ± SD) in the control animals on PND 170 in SD rats in the study by Talsness et al. (2000). Tinwell et al. (2002) reported a DSP value of 25.5 ± 4.4 (pup-based mean ± SD) or 25.6 ± 2.6 (litter-based mean ± SD) in the same strain of rats on PND 90, which is approximately 50% lower than that reported by Talsness et al. (2000). There are also similar differences in the values for AGD and prostate weights in the control animals among these three studies (data not summarized). Compared to the differences between the control and treated groups reported by Talsness et al., the inter-experimental or inter-study variations in the same endpoints from the control groups (thus the background values) appear to be much higher. Without a full understanding of what caused the large differences in the background values in the endpoints used in the studies, it may not be appropriate to reject the findings from one study based on different findings from another. In addition, the pup-based average DSP (10^6 per testis or per g of testis) in AP rats treated prenatally with 50 mg/kg-d in the study by Tinwell et al. (2002) was lower than that of controls, but the difference was not statistically significant. However, using the litter as the statistical unit, the authors found that the reduction in DSP in the 50-mg/kg-d group was significantly lower than that of the control group (22.5 ± 2.0 in BPA group vs. 25.6 ± 2.6 in controls for DSP per testis or 14.3±1.0 in the BPA group vs 15.9 ± 1.0 in controls for DSP per g of testis, $P<0.05$). Therefore, Tinwell et al. (2002), in fact, observed a small but statistically significant reduction in DSP in AP rats, but not in SD rats.

Prenatal treatment with BPA by s.c. injection of the dam induced no effect on testicular weights in adult male offspring (Saito et al., 2003; Wistuba et al., 2003). Wistuba et al. (2003) observed increased number of Sertoli cells per gram testicular tissue, indicative of stimulatory effects of BPA on proliferation of Sertoli cells during the fetal period.

However, there was no effect on the number of Sertoli cells per testis at ≥90 days of age, suggesting that even if BPA stimulated Sertoli cell proliferation during the fetal period under the experimental condition, it is likely minimal, and thus difficult to confirm.

At the molecular level, Thuillier et al. (2003) and Wang et al. (2004) observed changes in the expression of certain genes (e.g., platelet-derived growth factor receptors or PDGF receptors, ER-associated proteins) in the testis of neonatal rats, following oral exposure of dams to BPA at 0.1–200 mg/kg-d from GD 14 to birth. The BPA-induced changes in the expression of these genes were dose-dependent. Following s.c. injection of BPA from GD 11 to 20, BPA induced a gene expression profile that is similar to the genomic effect of EE (Naciff et al., 2005). Although there are numerous studies on the functions of the genes reported in the studies by Thuillier et al. (2003) or Naciff et al. (2003), there is no study to determine if the BPA-induced changes in the

expression of those genes ultimately result in functional or histopathological changes in the testis.

Section 1.1.2.3 Prenatal exposure: effects on the epididymis or seminal vesicles in rats

BPA reduced weights of the epididymis and seminal vesicles on PND 70 in SD rats, following prenatal oral exposure to 0.1 mg/kg-d BPA, but not at 50 mg/kg-d of BPA, or at either dose on PND 170 (Talsness et al., 2000b). No reduction in the weights of the epididymis or seminal vesicles was observed by Tinwell et al. (2002) in SD or AP rats on PND 90 (Table A2-5).

Naciff et al. (2005) reported changes in the expression of certain genes in the epididymis of SD rats on GD 20, following s.c. injection of 0.002 to 400 mg/kg-d of BPA from GD 11 to 20. The pattern of changes in gene expression induced by BPA was similar to that induced by ethinyl estradiol. It should be noted that the epididymis at this age (GD 0) should be considered as pre-epididymis and pre-vas deferens, since the epididymis in rats begins to differentiate from the Wolfian duct on GD 20–21 and does not complete the development until approximately PND 44 (Sun and Flickinger, 1979, 1982).

Section 1.1.2.4 Prenatal exposure: effects on the prostate in rats

Prenatal oral exposure to 50 mg/kg-d BPA caused a slight, but statistically significant increase in prostate weight (0.28 ± 0.08 vs. 0.22 ± 0.07 in the controls, units not reported) in SD rats on PND 70, but not at 0.1 mg/kg-dmg/kg-d, or at either dose on PND 170 (Talsness et al., 2000b). No alteration in weight of the prostate was observed by Tinwell et al. (2002) in SD or AP rats on PND 90. In the study by Talsness et al. (2000), the absolute weight of the prostate in control animals was 0.22 ± 0.07 on PND 70 and 0.47 ± 0.10 on PND 170, but the authors did not report the weight units, nor did they specify whether it was the weight of the whole prostate or any single lobe of the prostate. The ventral prostate from the control SD rats in the study by Tinwell et al. (2002) weighed 339.9 ± 54.6 mg on PND 90. The limitations (e.g., lack of details for the method used in evaluation of the prostate) in the study report by Talsness et al. (2000) and the differences in the study design and background values (in the control groups) between the study by Talsness et al. (2000) and that by Tinwell et al. (2002) make it difficult to evaluate the findings from these studies.

At the molecular level, prenatal treatment with BPA at 25 or 250 µg/kg-day via s.c. implantation of osmotic pumps caused altered proliferative activity, decreased expression of androgen receptors, and abnormal morphological changes in the periductal stromal cells of the prostate in Wistar rats (Ramos et al., 2001; Ramos et al., 2003). Theoretically, these changes support an increase in prostate weight, but the authors did not report any data on prostate weight.

Section 1.1.2.5 Prenatal exposure: effects on the sexual maturation in rats

Talsness et al. (2000) reported that oral treatment with 50 mg/kg-d of BPA caused a reduction in AGD of male pups on PND 3, 15, and 21 (3.52 ± 0.44, 5.54 ± 1.10, and 9.91 ± 2.33 mm, compared to 4.09 ± 0.50, 8.47 ± 0.94, and 11.33 ± 2.04 mm in controls, respectively). At 0.1 mg/kg-d, reduced AGD was observed on PND 15 and 21, but not on PND 3. After adjusting to

body weights, AGD in BPA-treated groups was still significantly lower than that in the time-matched controls. In contrast, Tinwell et al. (2002) observed no effect on absolute AGD of SD or AP rats on PND 1. The authors did not report data on body weight-adjusted AGD.

Section 1.1.2.6 Prenatal exposure: hormonal effects in rats

Reduced blood levels of T in SD rats (on PND 70 following prenatal oral exposure at 50 mg/kg-d, but not at 0.1 mg/kg-d; Talsness et al., 2000) or s.c. injection (at PNW 13 at 50 µg/dam; Saito et al., 2003) have been reported. Tanaka et al. (2006) found that prenatal exposure to BPA via drinking water at concentrations of 0.2–20 µg/ml reduced serum levels of T in the neonatal male pups at two hours after birth, suggesting that BPA suppressed the normal T surge right after birth.

Section 1.2 Neonatal Exposure

There is no clear definition for the neonatal period in mice or rats, though it is generally refers to the first week after birth (Ojeda et al., 1980; Orth et al., 2000; Sharpe et al., 2003a). During this and the subsequent infantile period (the second week after birth), numerous cell types in the testis and the accessory organs of the male reproductive system undergo rapid changes in proliferation and differentiation. Therefore, studies in rodents that treated the animals, either prenatally, neonatally, or perinatally, have been frequently identified as "developmental" studies in the literature. Many events that occur during the neonatal period in rodents are largely completed before birth (prenatal period) in human males, though maturation of the male reproductive system in men continues until about 15–20 years of age (Marty et al., 2003; Sharpe et al., 2003a). Thus, many findings from studies in neonatal rodents are relevant to the fetal period in human.

A total of 11 studies that treated animals neonatally (from birth to PND 14) were included in the current integrative evaluation. All but two studies (Aikawa et al., 2004; Toyama and Yuasa, 2004) used rats of various strains. Key study design features and major findings from the studies in rats are briefly summarized in Table A2-5. Four abstract-only study reports that were identified in the literature search are not included in this integrative evaluation.

Section 1.2.1 Neonatal exposure: studies in mice

The studies in mice by Aikawa et al. (2004) and Toyama and Yuasa (2004), respectively, focused on the potential effects of BPA on testicular development. Aikawa et al. (2004) found that s.c. injection of BPA at 0.5 or 50 µg/mouse per day increased the percentage of epididymal sperm with abnormal morphology. Reduced sperm motility was also observed with BPA treatment at 50 µg/mouse per day. The effects of BPA on sperm motility or morphology were attenuated by co-treatment with vitamin A, and enhanced if the treated mice were nursed by vitamin A-deficient dams. The authors reported no histopathological changes in the testis.

In the study by Toyama and Yuasa (2004), the authors treated newborn mice and rats by s.c. injection of BPA at doses ranging from 0.1 to 10 µg/animal in mice or 1 to 100 µg/animal in rats. In mice, treatment with BPA at doses of 1–10 µg/animal from PND 1 to 11 resulted in formation of multinucleated giant cells, exfoliation of step 8 spermatids, and deformation of spermatids in the testis at ≥ 21 days of age. When the treated mice were mated to untreated females at PNW 12, there was no effect on fertility. However, only one mouse per dosing group was used to measure fertility. These results were also similar to those observed in BPA-treated rats (summarized in Table A2-5) by the same researchers.

BPA had no effect on the immunostaining pattern for ERα in the efferent duct or epididymis (Aikawa et al., 2004). Beyond this finding, there is no study on the potential effects of BPA in the epididymis, seminal vesicles, prostate, sexual maturation or hormonal levels in neonatal mice.

Section 1.2.2 Neonatal exposure: studies in rats

All studies but one (Aloisi et al., 2002) in neonatal rats treated the newborn male pups by s.c. injection of BPA in oil (corn oil, olive oil, unspecified oil, or in ethanol diluted with corn oil). Aloisi et al. (2002) treated the dams by oral instillation or suckling with BPA in peanut oil either from mating to PND 2 (gestational exposure) or from PND 3 to PND 21 (lactational exposure). The findings from gestational exposure are summarized in Table A2-6 (perinatal exposure). The authors found no changes in the blood levels of E_2 or T in male pups nursed by SD rats exposed to 40 µg/kg-day of BPA during the lactational period. There were no other data from the experiments using neonatal exposure in this study. Therefore, the findings from the neonatal exposure experiment are not included in the summary. Major findings from all other studies are summarized in Table A2-5.

Section 1.2.2.1 Neonatal exposure: effects on fertility or reproductive outcome in rats

There are three studies that evaluated male-mediated fertility or reproductive outcome in adult animals treated with BPA neonatally by s.c. injection. None of them found any effect on fertility or reproductive outcome (Nagao et al., 1999; Toyama and Yuasa, 2004; Kato et al., 2006).

Section 1.2.2.2 Neonatal exposure: testicular effects in rats

Toyama and Yuasa (2004) reported that neonatal treatment with BPA at doses of 1–100 µg/animal caused the same histopathological changes as those observed in ICR mice in the same series of experiments.

The studies reported by Sanders et al. (1997), Fisher et al. (1999), Atanassova et al. (2000), and Sharpe et al. (2003b), respectively, used same treatment protocol and seemed to be conducted in the same laboratory, the Center for Reproductive Biology, Edinburgh, UK. None of them found the histopathological changes in the testis reported by Toyama and Yuasa (2004). No effect on

testis weights, the number of Leydig cells, or the morphology of the testis was observed by Saunders et al. (1997), Fisher et al. (1999), or Sharpe et al. (2003b). Similarly, no effect on testis weight, histopathology, sperm parameters or fertility was observed in SD rats following neonatal treatment with BPA (Nagao et al., 1999; Kato et al., 2006). However, Atannassova et al. (2000) observed several histopathological changes that may suggest accelerated development of the testis (advanced formation of the lumen of seminiferous tubules, advanced differentiation of Sertoli cells and germ cells, reduced level of germ cell apoptosis) on PND 18, but not on PND 25 or beyond.

Table A2-5. Neonatal studies on the male reproductive effects of BPA.

References	Species/Strains	Exposure Route	Doses and Duration	Major Findings
Aikawa et al., 2004 NTP-A	SHN mice, newborn, 10-20 mice per group.	s.c. injection. Vehicle: sesame oil.	Control: vehicle only. BPA: 0.5 or 50 µg/mouse for 5 days. Groups co-treated with retinol acetate (vitamin A) or nursed by vitamin A-deficient dams included. Necropsy at PNW 10.	No data on body weights or testis weights. No histopathological changes in the testis. Reduced sperm motility (50 µg/mouse) and increased percentage of sperm with abnormal morphology at both doses. Vitamin A attenuated the sperm effects. Nursing by vitamin A-deficient dams enhanced the effects. No effect on the immune-staining pattern for ERα in the epithelia of the efferent ducts or epididymis.
Toyama and Yuasa, 2004 NTP-A	ICR mice, newborn, 3-5 mice per dose per observation time point.	s.c. injection Vehicle: olive oil.	Control: vehicle only. BPA: 0.1, 1, 5, or 10 µg/mouse on PND 1, 3, 5, 7, 9, and 11. Necropsied weekly at PNW 2-10. One mouse per group for fertility tests at PNW 12.	No histopathological changes in testis until PND 21. Multinucleated giant cells, exfoliation of step 8 spermatids, deformed spermatids or spermatozoa from mice treated with BPA at 1-10 µg/mouse. No effect on fertility in one male from each group examined at PNW 12.
Toyama and Yuasa, 2004 NTP-A	Wistar rats, newborn, 3-5 rats per dose per observation time point. One rat per group for fertility tests.	s.c. injection Vehicle: olive oil.	Control: vehicle only. BPA: 1, 10, 100, or 600 µg/rat on PND1, 3, 5, 7, 9, and 11. Necropsied weekly at PNW 2-10. Fertility tests at PNW 15.	All rats in the 600 µg/rat of BPA group died by PND 20. Histopathological changes in testis were similar to those in mice (above). No effect on fertility at PNW 15.
Saunders et al., 1997	Wistar rats, newborn, 8 (BPA group) or 16 (control group) were used.	s.c. injection. Vehicle: oil (type not reported).	Control: vehicle only. BPA: 0.5 mg/rat per day on PND 2,4,6,8, and 12. Terminated on PND 18.	No effect on testicular weights, diameters of round sections of seminiferous tubules, or immune-staining for inhibin α in the testis or FSHβ and ERα in the pituitary.
Fisher et al., 1999 NTP-A	Wistar rats, newborn, 3-5 pups per group per time point.	s.c. injection, Vehicle: corn oil.	Control: vehicle only. BPA 20 mg/animal (about 37 mg/kg-d, from PND 2 to 12. Necropsied on PND 18 - 75.	No effect on testicular weights, morphology of the rete testis or efferent ducts, or immunostaining for water channel aquaporin 1 (AQP-1).
Atanassova et al., 2000 NTP-A	Wistar rats, newborn, 7-14 pups per group per time point.	s.c. injection. Vehicle: corn oil.	Control: vehicle only. BPA: 0.5 mg/rat per day from PND 2 to 12. Examined on PND 18, 25, or 90-100.	PND 18: advanced lumen formation, increased nuclear volume of germ cells, testis weight, and serum levels of FSH, reduction in the apoptotic rate of germ cells, indicative of accelerated differentiation of Sertoli cells or germ cells and testicular development. No obvious effect on PND 25 or 90-100.
Sharpe et al., 2003b NTP-A	Wistar rats, newborn, 3-6 pups per group per time point.	s.c. injection. Vehicle: corn oil.	Control: vehicle only. BPA: 0.5 mg/rat per day from PND 2 to 12. Examined on PND 18, 25, 35 or 90.	Increased plasma levels of T on PND 18, but not on PND 25, 35, or 90. No effect on testis weights or the number of Leydig cells.

Table A2-5. Neonatal studies on the male reproductive effects of BPA (continued).

References	Species/Strains	Exposure Route	Doses and Duration	Major Findings
Nagao et al., 1999 NTP-A	SD rats, newborn, 30-31 pups per group. 5 pups per group for necropsy on PND 21. 25 rats per group for fertility evaluation at PNW 12, and 10 rats per group for sexual behavior analysis at PNW 14.	s.c. injection vehicle: corn oil.	Control: vehicle only BPA: 300 mg/kg-d from PND 1 to 5. Necropsy on PND 21 (histopathology), fertility test at on PNW 12, sexual behavior evaluation and histopathology at PNW 14.	No effect on PPS, testis decent, or weights of testis, epididymis, and brain (PNW 14). No histopathological changes in testis on PND 21 or at PNW 14. No effect on reproductive outcome (copulation index, fertility index, or number of live embryos per litter). No effect on sexual behavior.
Kato et al., 2006 NTP-A	SD rats, newborn, 8 pups per group per observation time.	s.c. injection. Vehicle: mixture of ethanol and corn oil.	Control: vehicle only. BPA: 0.024, 0.12, 0.6, 3, or 1,000 µg/animal (2, 11, 56, 277, 97,000 µg/kg-day from PND 0 to 9. Necropsies on PND 10 (testis weight, histopathology, gene expression, and serum T), PND 35 (+weights of the epididymis, seminal vesicles, and prostate), and PND 150 (+ sperm and fertility).	No effect on PPS and weights of testis, epididymis, seminal vesicles, or prostate (PND 10, 35, or 150). No histopathological changes in testis on PND 10, 35, or 150. No effect on sperm parameters (counts, motility, morphology). No effect on reproductive outcome (copulation rate, fertility rate, implantation rate, or number of live festuses per litter). No effect on serum testosterone levels on PND 10, 35, or 150. No effect on expression of genes for T production or receptors for estrogen, androgen, or progesterone.
Ho et al., 2006 NTP-A	SD rats, newborn, 20-30 neonatal pups per group.	s.c. injection. Vehicle: TSCO.	Control: TSCO only. BPA: 0.1 µg/pup (10 µg/kg) on PND 1, 3, and 5. Half of the animals received E2 +T s.c. implant for 16 wks from PND 90.	Examined the prostate only. No effect with BPA on prostate weights. Neonatal BPA and adult E+T: increases in the incidence and severity of prostate neoplastic lesions; increased proliferation and reduced apoptosis of the epithelial cells; abnormal epigenetic alterations in genes such as phosphodiesterase type 4 variant 4.
Khurana et al., 2000 NTP-A	F344 rats, newborn, 8-10 pups per group.	s.c. injection. Vehicle: TSCO.	Control: vehicle only. BPA: 100 or 500 µg/pup per day from PND 1 to 5. Blood levels of PRL measured on PND 15, 20, 25. Necropsy on PND 30.	Increased levels of PRL on PND 25 and 30, but not on PND 15 or 20, when PRL was also low in control animals. Increased expression of ERα and ERβ in the anterior pituitary on PND 30. No effect on expression of ERβ in the prostate.

Section 1.2.2.3 Neonatal exposure: effects on epididymis or seminal vesicles in rats

Nagao et al. (1999) found no effect on the weights of epididymis in SD rats at PNW 14 following neonatal s.c. injection of BPA at 300 mg/kg-d. Similarly, Kato found no effect on the weights of the epididymis or seminal vesicles in SD rats. No other evidence is available on the potential effects of BPA on the epididymis or seminal vesicles after neonatal exposure.

Section 1.2.2.4. Neonatal exposure: effects on the prostate in rats

Neither Kato et al. (2006) or Ho et al. (2006) found BPA-produced alterations in prostate weights following neonatal treatment. However, using a "two-hit" animal model, Ho et al. found that neonatal BPA exposure at10 µg/kg-day followed by adult exposure to estradiol and testosterone significantly increased the incidence and severity of prostate neoplastic lesions when the pups developed into adulthood. The authors also observed increased proliferation and reduced apoptosis of epithelial cells of the prostate and reported that abnormal epigenetic alterations in genes such as phosphodiesterase type 4 variant 4 were involved in the BPA-induced increase in susceptibility of the prostate to cancer.

Section 1.2.2.5. Neonatal exposure: effects on sexual maturation in rats

Neonatal exposure to BPA had no effect on the day of testicular decent or PPS in SD rats (Nagao et al., 1999; Kato et al., 2006). In addition, Nagao et al. (1999) found no effect of BPA on sexual behavior in adult male SD rats treated neonatally with BPA. No other evidence is available on the effect of BPA on sexual maturation.

Section 1.2.2.6. Neonatal exposure: hormonal effects in rats

Oral exposure to BPA via nursing dams during lactation (PND 3–21) had no effect on blood levels of E_2 or T in SD rats at PNW 22 (Aloisi et al., 2003).

Neonatal exposure to BPA by s.c. injection had no effect on the blood level of T on PND 10, 35, or 150 in SD rats (Kato et al., 2006) or on PND 25, 35, or 90 in Wistar rats (Sharpe et al., 2003b). However, a transient increase on PND 18 in SD rats has been observed by Sharpe et al. (2003b). This transient increase appears to be consistent with increased blood levels of FSH and accelerated testicular development at the same age in the same animal model (Atanassova et al., 2000). There was on no alteration in the blood levels of FSH in rats at 25 days or 90 days of age (Atanassova et al., 2000).

Hyperprolactinemia (high blood levels of PRL) has been observed by Khurana et al. (2000) in F344 rats on PND 25 and 30, but not on PND 15 or 20, following s.c. injection of 100 or 500 µg/pup per day from PND 1 to 5. Levels of PRL in control male pups were also relatively higher on PND 25 and 30 than that on PND 15 or 20, indicative of pubertal development in the pituitary and hypothalamus. The authors postulated a number of reasons for BPA-induced hyperprolactinemia, such as xenoestrogen-induced reprogramming effects in one or more components of the prolactin regulatory system.

Section 1.3 Perinatal Exposure

Most of the perinatal studies identified in the literature were conducted in rats (10 of a total of 12 studies) and used oral route of exposure (11 studies; one study in mice used s.c. implants). The full reports for two one-generation reproductive toxicity studies sponsored by General Electric (GE, 1976 and 1978, respectively) are not available to OEHHA, but major findings from these two studies were included in the NTP-CERHR Monograph (CERHR, 2008) and in the EU document on BPA (EU, 2003). There were three NTP-sponsored studies in CD-1 mice. A feed study used the standard design of Reproductive Assessment through Continuous Breeding (RACB; (NTP, 1985; Morrissey et al., 1989)). The RACB study is usually conducted in mice and includes four components or tasks. Details of the study design can be found in the NTP report (1985) or in the paper by Chapin and Sloane (1997). Briefly, Task 1 is a 14-day dose-finding study in adult mice that evaluates the general toxicity of a test chemical, using endpoints such as clinical signs, mortality, body weights and food consumption. Task 2 is the continuous breeding phase that evaluates the reproductive performance of the treated animals. Both sexes are treated (for a total of 15 weeks, one week before mating and 14 weeks of mating) and usually 4–5 litters are generated through continuous mating for 14 days. If significant adverse effects on reproductive performance are observed in Task 2, a cross-over mating trial (Task 3) will be performed to determine the affected sex. In Task 3, animals from the high-dose group are mated to the control animal of the opposite sex for one week (treated male X control female and treated female X control male). A control group (control male X control female) is also included for comparison. Task 4 uses the offspring (F_1) from the last litter of Task 2 to evaluate the effects of treatment in the parent generation (F_0) on the reproductive system of F1 animals. Animals in Task 4 receive treatment through lactation (via F_0 nursing dams) until adulthood (PND 74 ± 10 days). F_1 animals are also mated to generate one litter of offspring (F_2) for developmental toxicity assessment. In addition to the feed study, NTP also sponsored an abbreviated one-generation study (Tyl et al., 2002a), and a s.c. implant study that only included Tasks 1 and 2 (NTP, 1984, 1985; Morrissey et al., 1989). The implant study is included in this section, since it did not include Task 3 or 4 (hence no F_2 generation) and is equivalent to a perinatal study with extended exposure. The feed study is included in the two- or three-generation studies in the next section, since the F_2 generation was included in the observation. The abbreviated one-generation study was designed to confirm the effects on the reproduce outcome of dietary exposure to high doses of BPA as observed in the feed study (Tyl et al., 2002b). There was no assessment of male-specific reproductive toxicity in this study. Therefore, it is considered as part of the feed study and included in the summary of the two-generation studies. Table A2-6 presents the summaries of the design and major findings relevant to the male reproductive effects from all the perinatal studies.

Section 1.3.1 Perinatal exposure effects on fertility or reproductive outcome

The majority of perinatal studies included at least some data on the reproductive outcome of the females following gestational exposure to BPA. The NTP-sponsored s.c. implant study in CD-1 mice and the traditional one-generation studies in rats sponsored by GE (1976; 1978) treated both males and females in the parental generation. None of these studies found any obvious

changes in reproductive outcome following treatment with BPA by s.c. injection (1.6–10.5 mg/mouse) or via feed (5 to 650 mg/kg-d). None of the perinatal studies listed in Table A2-6 evaluated the fertility of male animals in the second generation (exposed to BPA during the perinatal period).

Section 1.3.2 Perinatal exposure: testicular effects

Except for the study in ICR mice by Kabuto et al. (2004), none of the other studies that evaluated testis weights found significant change in this endpoint (GE, 1976a, b, 1978; NTP, 1984; Cagen et al., 1999b; Kwon et al., 2000; Kobayashi et al., 2002; Ichihara et al., 2003; Akingbemi et al., 2004; Howdeshell et al., 2008; Okada and Kai, 2008). Kabuto et al. (2004) found that perinatal treatment with BPA at concentrations of 5 or 10 µg/ml in drinking water resulted in reduced testis weights (73.4 ± 10.0 mg or 76.6 ± 10.3 mg, respectively, vs. 89.3 ± 12.6mg in the control, $P<0.05$). The decrease in the low-dose group (about 19%) is slightly higher than that in the high-dose group (about 13%). The authors did not report the estimated doses or any data on water consumption. In the study by Howdshell et al. (2008), apparent but statistically insignificant decrease in testis weight was observed in one block (Block 1a) of rats treated with 20 or 200 µg/kg-d.

There was no effect on testicular or epididymal sperm counts in rats in the study by Cagen et al. (1999b). Howdeshell et a. (2008) stated that their study found no significant effects on sperm production; however, close examination of the reported data indicate that BPA at 20 and 200 µg/kg-d caused an apparent, but statistically insignificant decrease in epididymal sperm count in the experiments (Block 1a).

Traditional histopathological evaluation of the testis was conducted in several studies in mice (NTP, 1984) or rats (GE, 1976a, b, 1978; Cagen et al., 1999b; Kwon et al., 2000; Howdeshell et al., 2008). Except for one set of experiments (Block 1a) in the study of Howdeshell et al. (2008), none of these studies found obvious histopathological changes in the testis. In the study by Howdeshell et al. (2008), the authors found increased incidence of histopathological changes in the testis of animals treated with 20 µg/kg-d (16.7%, compared to 3.2% in the control, but not statistically significant) or 200 µg/kg-d (35%, statistically significant). Reduced (but statistically insignificant) testis weight and epididymal sperm counts were also observed in these animals. However, similar findings were not found in another series of experiments (Block 2) conducted by the same authors. In this study, the authors also evaluated the male reproductive effects of perinatal exposure to EE. Notably, the effects of EE on testicular weight and epididymal sperm count were more obvious in the first series of experiments (Block 1a) than those in the second series of experiments (Block 2). The authors pooled experimental data from both series (Block 1 and 2) for statistical analysis and concluded that perinatal treatment with BPA caused no male reproductive effects in the offspring (based on statistical analysis of the data pooled from both series of experiments). However, examination of the data from the first block separately from the second block indicates that BPA may cause subtle but noticeable effects on the testis.

Using a morphometric method to count germ cells in the seminiferous epithelium, Okada and Kai (2008) found that the percentage of seminiferous tubules having elongated spermatids in male pups at 28 days of age was significantly decreased. The dams of the pups were treated with 5 mg BPA/mouse by s.c. implant from three days before mating, through gestation, until termination on PND 28. The authors found no effect of the BPA treatment on testis weight or histopathological changes. Increased levels of oxidative stress in testicular tissues in mice or reduced rate of T production by Leydig cells in rats have also been reported by Kabuto et al. (2004) or Akingbemi et al. (2004), respectively.

Section 1.3.3 Perinatal exposure: effects on epididymis or seminal vesicles

Perinatal exposure to BPA caused no significant changes in weight of the epididymis in male mice (NTP, 1984; Okada and Kai, 2008) or in adult rats (Cagen et al., 1999b; Kwon et al., 2000; Ichihara et al., 2003; Howdeshell et al., 2008).

Akingbemi et al. (2004) observed reduced weights of seminal vesicles in association with reduced testicular production of T in rats. However, no effect in seminal vesicles was observed in mice by Okada and Kai (2008) or in rats by Cagen et al. (1999b), Kwon et al. (2000), Ichihara et al. (2003), and Howdeshell et al. (2008).

Table A2-6. Perinatal studies on the male reproductive effects of BPA.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
NTP, 1984; Morrissey, 1989 NTP-A	CD-1 mice. Standard RACB study design by the NTP. Task 1 and 2 studies only. 40 (control) and 20 (treated) mice per sex per group.	s.c. implants. Vehicle: corn oil.	Control: corn-oil-only implants. BPA: implants containing 0, 25, 50 and 100 mg of BPA (approximately 11.65, 20.05, and 38.60 mg/mouse) from 7 days premating, through 98 days of mating, and 21 days post-mating. Necropsy for the control and high dose groups.	Approximately 40% of BPA released from the implants, but some implants expelled during the treatment. No effect on fertility index or number of litters per pair. Slight but significantly increased number of live pups per litter at 50 mg/mouse (10.66 vs. 9.93 in the control), but not at 25 or 100 mg/mouse. No effect on weights of the testis, epididymis, prostate or seminal vesicles in the high dose group. No histopathological changes in the testis. No assessment of sperm parameters.
Kabuto et al., 2004 NTP-A	ICR mice, 6 female mice per group.	Oral, drinking water. Vehicle: 1% ethanol in drinking water.	Control: vehicle only. BPA: 5 or 10 µg/ml in drinking water, from 1 wk before mating, through mating and gestation, until necropsy on PND 28.	BPA detected in tissues of brain, kidney, liver and testis of mice treated with BPA, but not in controls. Testis: weights reduced at both dose levels. Increased levels of thiobarbituric acid-reactive substance (TBARS, indicator of peroxidation) and increased levels of glutathione peroxidase (GPx). No effect on the levels of superoxide dismutase (SOD), catalase, glutathione (GSH), or L-ascorbic acid.
Okada and Kai, 2008	ICR mice, 5-7 female mice per group.	s.c. implants. Vehicle: sesame oil.	Control: vehicle only. BPA: 50 µl of 2 or 100 mg/ml (0.1 or 5 mg/mouse), from 3 days before mating, through mating and gestation, until necropsy on PND 28.	No effect on relative weights of the testis, epididymis, or seminal vesicles. Decreased percentage of seminiferous tubules having elongated spermatids at 5 mg/mouse. Increased serum levels of T at 0.1, but not at 5 mg/mouse.
GE, 1976 NTP-A	CD Rats [likely Crl:CD (SD) rats]. 10 rats per sex per group for the F0 generation. F0 males and females were mated around 100 days of age. 15 pups/sex/group for the F1 generation.	Oral, feed.	Control: regular diets. BPA: 1000, 3000, or 9000 ppm in deits (≈ 70, 200, or 650 mg/kg-d in males) for 17 weeks (no data on the beginning time of exposure). F_1 pups were treated until 13 wks of age.	No effect on reproductive outcome of the F_0 males. No effect on testis weights or histopathology. No effect on prostate weights or histopathology.

Table A2-6. Perinatal studies on the male reproductive effects of BPA (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
GE, 1978 NTP-A	CD Rats [likely Crl:CD (SD) rats]. 10 rats per sex per group for the F0 generation. F_0 males and females were mated around 100 days of age. 15 pups/sex/group for the F1 generation.	Oral, feed.	Control: regular diets. BPA: 100, 250, 500, 750, or 1000 ppm in deits (≈ 5, 15, 30, 50, or 60 mg/kg-d in males) for 18 weeks (no data on the beginning time of exposure). F_1 pups were treated until 13 wks of age.	No effect on reproductive outcome of the F_0 males. No effect on testis weights or histopathology. No effect on prostate weights or histopathology.
Cagen et al., 1999b NTP-A	Han-Wistar rats, 28 female rats per group. Two non-treatment control groups were included and pooled for analysis. Up to 4 male pups per litter were selected for necropsy on PND 90.	Oral, drinking water. Vehicle: water only.	Control: regular drinking water. BPA: 0.01 – 10 ppm in drinking water (estimated to be 0.001-0.004, 0.008-0.038, 0.1-0.391, or 0.775-4.022 mg/kg-d), from 2 wks before mating, through mating and gestation, until PND 22 (weaning). Male pups (up to 4 per litter) necropsied on PND 90.	BPA concentration in water confirmed by chemical analysis (method not reported). No effect on weights of the testis, epididymis, prostate, or seminal vesicles. No effect on epididymal sperm concentration (10^6 per g epididymal tissue), daily sperm production (10^6/day), or efficiency of sperm production (10^6 per g testicular tissue per day). No histopathological changes in testis.
Kwon et al., 2000 NTP-A	SD rats, 8 dams per group. No culling.	Oral (possibly by gavage). Vehicle: corn oil.	Control: vehicle only. BPA: 3.2, 32, or 320 mg/kg-d, from GD 11 to PND 20. Pups necropsied on PND 180.	No effect on weights of the testis, epididymis, ventral or dorso/lateral prostate, or seminal vesicles. No histopathological changes in ventral prostate. No histopathological evaluation of testis.
Kobayashi et al. 2002 NTP-A	SD rats, 6 dams per group. Litters culled to 10 pups per litter on PND 7. 4-9 male pups per time point (1 male pup at PNW 1 in the 40-mg/kg-d BPA group).	Oral, gavage, Vehicle: corn oil.	Control: vehicle only. BPA: 4, 40, or 400 mg/kg-d from GD 6 to PND 20. Pups examined at PNW 1, 3, and 9 .	All dams in the 400 mg/kg-d group died during gestation. No effect on testis weights at PNW 9. No effect on AGD (absolute, adjusted to body weights, or cube root of body weights) at PNW 1,3, or 9.

Table A2-6. Perinatal studies on the male reproductive effects of BPA (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Yoshino et al., 2002 NTP-A	F344 rats, 19-22 and 11-12 dams/group in the 1st and 2nd experiments, respectively. Culled to 8 pups per litter on PND 4.	Oral gavage. Vehicle: 0.5% CMC-Na.	Control: vehicle only. BPA: 7.5 or 120 mg/kg-d from GD 0 to PND 21. 7.5, 120 mg/kg-d. 5 males pups each time-point necropsied on PND 23, 28, and 91. Testis evaluated on PND 91 only.	No effect on relative testis weight on PND 91. No effect on ventral prostate weight on PND 23, 28 or 91. Reduced number of sperm per testis at 120 mg/kg-d in the 1st experiment, but not observed in the 2nd experiment. No histopathological changes in testis or prostate.
Aloisi et al., 2002 NTP-A	SD, 13 dams for control, 7 for the BPA group. Male pups produced from each group were used.	oral instillation. Vehicle: peanut oil.	Control: vehicle only. BPA: 40 µg/kg-day, from the day of mating to PND 2. Pups then fostered by dams from the control. Blood hormonal levels assessed at PNW 22.	No effect on blood levels of E_2 or T.
Ichihara et al., 2003 NTP-A	F344 rats. 8 -15 dams per group. Pups culled on PND 4 to 8/litter. 12 or 21 F1 males per group received s.c. injection of corn oil or DMAB (carcinogen, 50 mg/kg-d) at PNW 5.	Oral, gavage. Vehicle: 0.5% CMC-Na (sodium carboxymethyl cellulose	Control; vehicle only. BPA: 0.05, 7.5, 30 or 120 mg/kg-d from GD 0 to PND 21. Male pups received s.c. injection of DMBA at PNW 5. All animals necropsied at PNW 65.	No effect on relative weights of the testis, epididymis, or prostate in male offspring at PNW 65. BPA caused no increase in incidence of preneoplastic or neoplastic lesions in prostate or seminal vesicles. BPA had no apparent effect on DMAB-induced preneoplastic or neoplastic lesions in prostate or seminal vesicles. [DMAB = 3,2'-dimethyl-4-aminobiphenyl. A carcinogen.]
Watanabe et al., 2003 NTP-A	IGS (SD) rats. No data on the number of dams per group. Pups culled to 10 per dam. 4-6 male pups per group necropsied.	Oral, gavage. Vehicle: corn oil.	Control; vehicle only. BPA: 4, 40, and 400 mg/kg-d, from GD 6 and PND 20. Male pups necropsied at PNW 9 and 36.	All but one dam survived in the 400-mg/kg-d group. Stated that no effect on the development of the male reproductive system at 4 or 40 mg/kg-d group, but no data reported. Increased blood levels of T in the 4- and 4-mg/kg-d groups at PNW 9. No effect on levels of T in testicular tissues or on blood levels of other hormones measured (LH, FSH, E_2).
Akingbemi et al., 2004 NTP-A	LE rats, 7 dams per group. 12-14 male pups per group for necropsy.	Oral, gavage Vehicle: corn oil.	Control: vehicle only. BPA: 2.4 µg/kg-day from GD 12 to PND 21. Necropsied on PND 90.	No effect on testis weight, serum levels of LH or T. Reduced rate of T production by Leydig cells and reduced levels of T in testicular interstitial fluids. Reduced weights of the seminal vesicles, but no effect on prostate weights.

Table A2-6. Perinatal studies on the male reproductive effects of BPA (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Howdeshell et al., 2008	LE rats, 13-29 dams per group for Block 1 experiment and 6-14 dams per group for Block 2 experiment. No culling of the offspring.	Oral, gavage. Vehicle: corn oil.	Control: corn oil. BPA: 2, 20, or 200 ug/kg-day in Block 1 and 20 or 200 ug/kg-day in Block 2 experiment, from GD 7 to PND 18. Male pups necropsied on PND 150 or later.	No effect on AGD and nipple retention. Apparent, but not statistically significant reduction in testis weight, epididymal sperm count in Block 1a animals at 20 and 200 µg/kg-d. Increased incidence of histopathological changes in the testis in Block 1a animals at 20 (not siginificant) and 200 µg/kg-d (siginificant). No similar effects in Block 1b or 2 animals.

Section 1.3.4 Perinatal exposure: effects on the prostate

The NTP study in CD-1 mice found no effect of BPA on prostate weight (NTP, 1984). Following oral administration during the perinatal period, BPA did not cause obvious alterations in weight or histopathology of the prostate in rats (GE, 1976a, b, 1978; Kwon et al., 2000; Ichihara et al., 2003; Akingbemi et al., 2004; Howdeshell et al., 2008). The doses of BPA used in these studies ranged from 1–4 µg/kg-day (Cagen et al., 1999b) or 2.4 µg/kg-day (Akingbemi et al., 2004) to 650 mg/kg-d (GE, 1976b). The strains of rats included SD, F344, and LE. In addition, perinatal exposure to BPA had no effect on DMAB-induced neoplasia in the prostate. While all the studies in neonatal rats listed in Table A2-6 treated the animals by s.c. injection, all the studies in rats in Table A2-7 (perinatal studies) treated the animals by oral administration.

Section 1.3.5 Perinatal exposure: effects on sexual maturation

Two studies that evaluated the effect of BPA on AGD (Kobayashi et al., 2002 and Howdeshell et al., 2008, respectively) found no significant alterations in AGD in SD and LE rats, respectively. Howdeshell et al. (2008) also found that perinatal exposure to BPA caused no increased incidence of nipple retention in LE rats.

Section 1.3.6 Perinatal exposure: hormonal effects

Okada and Kai (2008) observed increased level of T in 28-day old male ICR mice exposed to 0.1 mg/dam of BPA via s.c. implant from one week before mating, through mating and gestation, until necropsy on PND 28. The increased level of T was not observed in the group treated with 5 mg/dam of BPA.

In rats, none of the studies that measured blood levels of T (Aloisi et al., 2003; Watanabe et al., 2003; Akingbemi et al., 2004; Howdeshell et al., 2008) found any significant effect of BPA on this parameter. However, Akingbemi et al. (2004) observed a significantly reduced rate of T production by Leydig cells from 90-day-old male rats exposed to 2.4 µg/kg-day of BPA from GD 12 to PND 21, in association with reduced levels of T in testicular interstitial fluids and reduced weights of seminal vesicles. None of the other studies measured T level in testicular fluids, an indicator that has been used to measure the status of T production by the Leydig cell accurately (Sharpe and Cooper, 1983; Adams et al., 1998).

Section 1.4 Pubertal Exposure

Like the neonatal period, there is no clear definition for the pubertal period in mice or rats. The pubertal period in this document refers to the period from PNW 2 to PNW 7. During this period, the testis develops from the beginning of spermatogenesis to completion of the first cycle of spermatogenesis (Vergouwen et al., 1993; Marty et al., 2003; Sharpe et al., 2003a). This period also marks the rapid growth and differentiation of the epididymis, seminal vesicles, and prostate (Flickinger, 1971; Sun and Flickinger, 1979; Nazian and Mahesh, 1980; Ashby and Lefevre, 2000). All of these rapidly-developing events critical for the final establishment of the permanent male reproductive system in the adult are under the active control of sex hormones, such as T or E (O'Donnell et al., 2001; Akingbemi, 2005). Therefore, this period of development presents another sensitive time window of the male reproductive system to chemical insults.

There are 14 studies in rats and four studies in mice of pubertal age (about 3–7 weeks old at the beginning of treatment). Major findings from these 11 studies are summarized in Table A2-7 (mice) and A2-8 (rats). Findings reported in two abstracts (one in rats and one in mice) are not included in this review.

Section 1.4.1 Pubertal exposure: studies in mice

All of the four the studies were conducted in 3–5 week-old C57BL/6 mice, using oral administration.

Section 1.4.1.1 Pubertal exposure: effects on fertility or reproductive outcome in mice

There was no evaluation of reproductive outcome in the treated mice in any study.

Section 1.4.1.2 Pubertal exposure: testicular effects in mice

There was no change in testis weight or traditional histopathology in the treated mice in any study. No effect on sperm count or production was observed in the studies by Nagao et al. (2002) or Takahashi and Oishi (2003), respectively. However, treatment with 50μg/ml of BPA in drinking water for eight weeks caused an increased number of multinucleated giant germ cells (Takao et al., 2003) in mice age three weeks at the beginning of treatment. Takao et al. (2003) also observed abnormal alterations in the expression of ERα and ERβ in the germ cells.

Section 1.4.1.3 Pubertal exposure: effects on epididymis or seminal vesicles in mice

No effect on weighs of the epididymis or seminal vesicles was found in the study by Nagao et al. (2002) or Takahashi and Oishi (2003).

Section 1.4.1.4 Pubertal exposure: effects on the prostate in mice

Takahashi and Oishi (2003) did not find any significant change in prostate weight. None of the other studies evaluated the prostate.

Section 1.4.1.5 Pubertal exposure: effects on sexual maturation in mice

None of the studies included observations to evaluate sexual maturation.

Section 1.4.1.6 Pubertal exposure: hormonal effects in mice

Takao et al. (1999) found reduced serum level of T in mice after exposure to 50µg/ml of BPA in drinking water for eight weeks. The authors did not include this measurement in their second study reported in 2003 (Takao et al., 2003). No other studies included measurement of sex hormone levels in the blood.

Table A2-7. Male reproductive effects of pubertal exposure to BPA in mice.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Takao et al., 1999 NTP-A	C57BL/6 mice, 5-wk-old, 7 mice per group.	Drinking water. Vehicle: 0.005% ethanol in water.	Control: vehicle only. BPA: 0.5 or 50 µg/ml in drinking water for 4 or 8 wks.	No effect on testis weights. Reduce serum level of free T. Increased number of multinucleated giant germ cells in the 50-µg/ml BPA group at 8-wk, but not at 4-wk. No effect on testis weight, histopathology, or free T level in the low-dose group.
Takao et al., 2003 NTP-A	C57BL/6 mice, 3-wk-old, 7 mice per group.	Drinking water. Vehicle: 0.005% ethanol in water.	Control: vehicle only. BPA: 0.5 or 50 µg/ml in drinking water for 8 wks.	No effect on testis weights at either does. At 50 µg/ml, reduced number of germ cells positive for ERβ protein. Reduce ERβ mRNA in the testis. Increased number of germ cells positive for ERα protein. Increased ERα mRNA in the testis. No effect on ER proteins or mRNA at 0.5 µg/ml.
Nagao et al., 2002 NTP-A	C57BL/6N male mice, 3-wk-old, 30 mice per group.	Oral gavage. Vehicle: 0.5% carboxymethyl cellulose.	Control: vehicle only. BPA: 2, 20, or 200 µg/kg-day for 3 weeks. Necropsy at PNW 6.	No effect on weights of the testis, epididymis, or seminal vesicles or number of sperm in the caudal epididymis. No histopathological change.
Takahashi and Oishi, 2003 NTP-A	C57BL/6CrSlc and Crj:CD-1 (ICR) mice, 4-wk-old, 8 mice per group	Feed.	Control: normal diets. BPA: 0.25% in diets for 2 months.	No effect on weights of testis, epididymis, seminal vesicles, or prostate. No effect on blood level of T. No histopathological change in testis. No effect on sperm production.

Table A2-8. Male reproductive effects of pubertal exposure in rats.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Ashby and Lefevre, 2000	AP rats, 22 or 35 days old, 8-10 animals per group	Oral, gavage Vehicle: hydroxypropyl methoxycellulose (HPMC).	Control: vehicle only. BPA: 200 mg/kg-d in 22-day-old rats for 14 days (PND 22-36). In 35-day-old rats, 100 mg/kg-d for 14 days (PND 35 to 50); 100, 150, or 200 mg/kg-d X 20 days (PND35 -55).	No effect on weights of testis, epididymis, seminal vesicles, or prostate in any treated groups. No effect on day of preputial separation.
Kim et al., 2002 NTP-A	SD rats, castrated at 5 or 6 wks of age, treated with BPA 7 days after castration (6 or 7 wks of age). 10 rats per group. [Hershberger assay]	Oral gavage. Vehicle: 2.5% ethanol in corn oil.	Control: vehicle only. BPA: 10, 100, or 1000 mg/kg-d for 7 days in rats castrated at 5 wks of age, or 50, 100, 250, and 500 mg/kg-d for 7 days in rats castrated at 6 wks of age in the absence or presence of testosterone injection.	In rats castrated at 5 wks of age: no effect on weights of the ventral prostate, seminal vesicles or LABC (levator ampins bulbocavernosus muscles). In rats castrated at 6 wks of age: no effect on weights of ventral prostate, seminal vesicles or LABC. In rats castrated at 6 wks of age in the presence of T stimulation: no effect on T-stimulated growth of ventral prostate, seminal vesicles or LABC.
Yamasaki et al., 2002a NTP-A	SD rats, 7-wk-old, 10 rats per group	Oral gavage. Vehicle: olive oil.	Control: vehicle only. BPA: 40, 200 mg/kg-d, or 1000 mg/kg-d (changed to 600 mg/kg-d after the 1st wk of treatment) for 28 days.	Decreased weights of ventral and dorsolateral prostate and seminal vesicles at 600 mg/kg-d. Decreased weight of ventral prostate at 200 mg/kg-d. No effect on sperm parameters or blood levels of sex hormones. No histopathological change in testis, epididymis, prostate, or seminal vesicles.
Chitra et al., 2003a NTP-A	Wistar rats, 45-day-old, 6 rats per group.	Oral gavage. Vehicle: olive oil.	Control: vehicle only. BPA: 0.2, 2, or 20 µg/kg-day for 45 days.	Reduced weights of testis and epididymis. Increased weight of ventral prostate. Reduced epididymal sperm counts and motility. Increased levels of oxidative stress in the sperm.
Chitra et al., 2003b NTP-A	Wistar rats, 45-day-old, 4 rats per group.	Oral gavage. Vehicle: olive oil.	Control: vehicle only. BPA: 0.2, 2, or 20 µg/kg-day for 60 days, with or without co-treatment with vitamin C (40 mg/day).	Reduced epididymal sperm counts and motility. No effect on sperm viability. Increased levels of oxidative stress in the sperm. Degeneration of epithelia in the caput, corpus, and cauda epididymis. Co-treatment with vitamin C attenuated all effects.
Tan et al., 2003 NTP-A	SD rats, 23-day-old, 12 rats per group.	Oral gavage. Vehicle: 10% Tween-80 in water.	Control: vehicle only. BPA: 100 mg/kg-d for 30 days.	No effect on weights of testis, epididymis, or seminal vesicles, but adverse effects in liver, kidney and thyroid.

Table A2-8. Male reproductive effects of pubertal exposure in rats (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Takahashi and Oishi, 2001 NTP-A	F344 rats, 4-wk-old,	Feed.	Control: normal diets. BPA: 0.25, 0.5, or 1.0% in diets for 44 days (author's estimates: 235, 466, or 950 mg/kg-d).	No effect on weights of testis, epididymis or ventral prostate. Reduced weights of seminal vesicles and dorsal-lateral prostate at the high dose. Reduced weights of preputial glands at all doses. Histopathological changes in seminiferous epithelia at≥ 0.5%. No effect on blood T level.
Takahashi and Oishi, 2003 NTP-A	Wistar and Holtzman (SD) rats, 4-wk-old, 8 rats per group	Feed.	Control: normal diets. BPA: 0.25% in diets for 2 months.	No effect on weights of testis, epididymis, seminal vesicles, or prostate. [No data on the weights of seminal vesicles or prostate in SD rats.] No effect on blood T level. No histo-pathological changes in testis. No effect on sperm production.
Akingbemi et al., 2004 NTP-A	LE rats, 21-day-old, 8-10 rats per group.	Oral, gavage Vehicle: corn oil.	Control: vehicle only. BPA: 2.4, 10 µg/kg-day (low doses) , 100 or 200 mg/kg-d (high doses) for 15 days.	2.4 µg/kg-day: reduced blood level of LH and T. Reduce testicular production of T under LH stimulation. Reduced LHβ mRNA, increased ERβ mRNA, but no effect on ERα mRNA. 10µg/kg-day: Reduce testicular production of T under LH stimulation. 100 or 200 mg/kg-d: no effect.
Akingbemi et al., 2004 NTP-A	LE rats, 21-day-old, 10-12 rats per group.	Oral, gavage Vehicle: corn oil.	Control: vehicle only. BPA: 2.4 µg/kg-day for 90 days.	No effect on testis weights or blood level of T, but increased blood level of LH. No effect on prostate weights, but significantly reduced weights of seminal vesicles.
Stoker et al., 1999 NTP-A	Wistar rats, 22-day-old, 6 animals per group for the PND 29 examination. Number of rats per group for the PND 120 terminal examination was not reported.	s.c. injection. Vehicle: sesame oil. Volume: not reported.	Control: vehicle only. Positive control: Estradiol (s.c. implants) BPA: 50 mg/kg-d from PND 22 to 29 for the PND 29 necropsy or from PND22 to PND 32 for the PND 120 termination.	PND 29: increased blood level of PRL. PND 120: Increased weights of the lateral prostate, but not of the ventral prostate. Increased incidence of inflammation in the prostate.
Saito et al., 2003a NTP-A	Wistar rats, 3 wks old, 8-9 rats per group.	s.c. injection. Vehicle: corn oil. Volume: not reported.	Control: vehicle only. BPA: 5 µg/day or 5 mg/day for 8 wks. Examined at age of 13 wks (2 wks after the last injection).	No effect on weights of testis, epididymis, prostate, or preputial glands. BPA at 5 µg/day, but not at 5 mg/day, reduced blood levels of T.

Table A2-8. Male reproductive effects of pubertal exposure in rats (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Takahashi and Oishi, 2003	Wistar rats, 4-wk-old, 5 rats per group	s.c. injection. Vehicle: propylene glycol.	Control: vehicle only. BPA: 200 mg/kg-d, 4 days a week, for one month.	Reduced weights of testis, epididymis, seminal vesicles, ventral prostate, dorsal and lateral prostate, and preputial gland. Histopathological changes indicative of degeneration in the seminiferous epithelium. Reduced sperm production. No apparent effect on blood level of T.
Takahashi and Oishi, 2003	Wistar rats, 4-wk-old, 5-6 rats per group	Intraperitoneal injection. Vehicle: propylene glycol.	Control: vehicle only. BPA: 2 or 20 mg/kg-d, 4 days a week, for one month.	Reduced ventral prostate weight and blood levels of T at 20 mg/kg-d. No effect on weights of testis, epididymis, seminal vesicles, dorsal and lateral prostate, and preputial gland. No histopathological changes or effect on sperm production.

Section 1.4.2 Pubertal exposure: studies in Rats

Among the 14 studies in rats, ten used oral treatment and four treated the animals by s.c. or i.p. injection. The oral studies used different strains of rats, including AP, F344, LE, SD, and Wistar rats. All of the injection studies were conducted on Wistar rats.

Section 1.4.2.1 Pubertal exposure: effects on fertility or reproductive outcome in rats

There was no evaluation of the reproductive outcome in the treated rats exposed to BPA during the pubertal period of development.

Section 1.4.2.2 Pubertal exposure: testicular effects in rats

Chitra et al. (2003a) found decreased testis weights in Wistar rats treated by oral gavage with 0.2–20 µg/kg-day of BPA for 45 days from age 45 days onwards. The authors also found significant reduction in epididymal sperm count and motility, with increased levels of oxidative stress in the sperm (Chitra et al., 2003a; Chitra et al., 2003b). Co-treatment of BPA with vitamin C significantly attenuated the BPA-induced effects in sperm (Chitra et al., 2003b). However, none of other oral studies in rats found significant change in testis weights, testicular histopathology, or sperm parameters (Ashby and Lefevre, 2000; Takahashi and Oishi, 2001, 2003; Tan et al., 2003; Akingbemi et al., 2004).

Treatment by s.c. injection with 5 or 5,000 µg/kg-day of BPA for 13 weeks did not cause significant change in testis weight of Wistar rats age three weeks at the beginning of treatment (Saito et al., 2003). BPA treatment at 200 mg/kg-d by s.c. injection for one month significantly reduced testis weight in 4-wk-old Wistar rats (Takahashi and Oishi, 2003). Reduced sperm production and degenerative changes in germ cells were also observed in the BPA-treated rats. Comparing the toxicity of BPA between s.c. and i.p injection, Takahashi and Oishi (2003) found that 200 mg/kg-d of BPA was lethal by i.p. injection. At lower doses (2 or 20 mg/kg-d), BPA had no effect on testis weights, histopathology or sperm production.

Section 1.4.2.3 Pubertal exposure: effects on epididymis or seminal vesicles in rats

A significant decrease in epididymal weight and degenerative changes in the epithelium of the epididymis were observed in Wistar rats treated with 0.2–20 µg/kg-day of BPA for 45 days from age 45 days onwards (Chitra et al., 2003a; Chitra et al., 2003b). Similar effects on the epididymal weight was not observed in other studies in rats (Ashby and Lefevre, 2000; Takahashi and Oishi, 2001; Tan et al., 2003). None of these studies evaluated the histopathology of the epididymis. Seminal vesicles were evaluated in the comparison study by Takahashi and Oishi (2003). Similar to their findings on testis weight (see above), the authors found reduced weights of epididymis and seminal vesicles in rats treated by s.c. injection of 200 mg/kg-d of BPA for one month, but no effects after i.p. injection of 2 or 20 mg/kg-d of BPA.

The findings on the effect of oral exposure to BPA on seminal vesicles in rats of pubertal age are mixed. Table A2-9 provides an overall outline of the findings from the relevant studies that

evaluated seminal vesicles or prostate. It appears that BPA caused a decrease in prostate weight at very high doses in SD (600 mg/kg-d; (Yamasaki et al., 2002a)) or F344 (950 mg/kg-d; (Takahashi and Oishi, 2001)) rats. In LE rats, exposure to 2.4 µg/kg-day for 90 days beginning at 21 days of age caused a significant decrease in the weight of the seminal vesicles, consistent with the reduced level of T production in the testis (Akingbemi et al., 2004).

Table A2-9. Effects of pubertal exposure to BPA on seminal vesicles and prostate.

References	Animals	Treatment	Seminal Vesicles	Prostate
Ashby and Lefevre, 2000	AP rats, 22 or 35 days old.	Gavage. 100 – 200 mg/kg-d for 14-20 days.	No effect on weight.	No effect on weight.
Kim et al., 2002	SD rats, castrated at 5 or 6 wks of age.	gavage. 10 – 1000 mg/kg-d, with or without T stimulation.	No effect on weight, with or without T stimulation. [Hershberger assay]	No effect on weight, with or without T stimulation.
Tan et al., 2003	SD rats, 23-day-old.	Gavage. 100 mg/kg-d for 30 days.	No effect on weight.	No evaluation.
Takahashi and Oishi, 2003	Wistar rats, 4-wk-old.	Feed. : 0.25% in diets for 2 months.	No effect on weight.	No effect on weight.
Takahashi and Oishi, 2001	F344 rats, 4-wk-old.	Feed. 0.25, 0.5, or 1.0% in diets for 44 days (235, 466, or 950 mg/kg-d).	Reduced weight at 950 mg/kg-d.	No effect on weight of the ventral prostate, but reduced weight of the dorsal-lateral prostate at high dose.
Yamasaki et al., 2002a	SD rats, 7-wk-old.	Gavage. 40-600 mg/kg-d for 28 days.	Reduced weight at 600 mg/kg-d. No histopathological changes.	Reduced weight at 200 and 600 mg/kg-d. No histopathological change.
Akingbemi et al., 2004	LE rats, 21-day-old.	Oral, gavage. 2.4 µg/kg-day for 90 days.	Reduced weight.	No evaluation.

Section 1.4.2.4 Pubertal exposure: effects on prostate in rats

The reported effects of pubertal exposure to BPA via oral administration on the prostate are outlined in Table A2-9 (above). It appears that BPA at very high doses (e.g., 200 or 600 mg/kg-d) may reduce prostate weight in rats, following exposure of four weeks or longer (Takahashi and Oishi, 2001; Yamasaki et al., 2002a), but not at lower doses or shorter periods of exposure (Ashby and Lefevre, 2000; Takahashi and Oishi, 2003).

Brief treatment (5 or 10 days) by s.c. injection with 50 mg/day of BPA in 22-day old rats caused increased weight of the lateral prostate, but not the ventral prostate, at 120 days of age (Stoker et al., 1999). On the other hand, reduced weight of the ventral and lateral prostate has been observed in rats of approximately seven weeks of age, following one-month s.c. injection of 200 mg/kg-d or i.p. injection of 20 mg/kg-d of BPA (Takahashi and Oishi, 2003). The different effects of BPA on prostate weights in the same strain of rats treated by the same route of

exposure highlight the importance of the evaluation time, as well as of the physiological differences between different lobes of the prostate.

Section 1.4.2.5 Pubertal exposure: effects on sexual maturation in rats

Ashby and Lefevre (2000) observed no effect of BPA at doses of 100–200 mg/kg-d on the day of preputial separation in AP rats. The authors evaluated the relationship of PPS day to body weights of the animals in the control and BPA-treated groups. No other studies evaluated PPS or other hallmarks for sexual maturation.

Section 1.4.2.6 Pubertal exposure: hormonal effects in rats

Several studies measured the blood level of T in rats exposed to BPA via oral treatment or by s.c. or i.p injection. Oral administration of BPA caused no change in the blood level of T in AP rats (Ashby and Lefevre, 2000), in SD rats (Yamasaki et al., 2002a) or in F344, Holzman, or Wistar rats (Takahashi and Oishi, 2001, 2003). In LE rats, daily oral gavage of 2.4 µg/kg-day of BPA for 90 days beginning on PND 21 caused no significant change in the blood level of T (Akingbemi et al., 2004). However, treatment with the same dose of BPA for 15 days significantly reduced the blood levels of T and LH, decreased the LH-stimulated production of T by Leydig cells, and altered the expression of LHβ and ERβ mRNA in the pituitary gland in BPA-treated rats of about 36 days of age (Akingbemi et al., 2004). These effects were not observed in rats treated with 100 or 200 mg/kg-d of BPA, following the identical dosing protocol.

A reduced blood level of T was observed in rats exposed to BPA by s.c. or i.p injection (Saito et al., 2003; Takahashi and Oishi, 2003). In addition, an increased blood level of PRL was observed in Wistar rats of 29 days of age following s.c. injection of 50 mg/kg-d of BPA from PND 22 to 29.

Section 1.5 Two- or Three-generation Reproductive Studies

There are four two- or three-generation reproductive toxicity studies of BPA in rodents: two in SD rats (Ema et al., 2001; Tyl et al., 2002b) and two in CD-1 mice (Peknicová et al., 2002; Tyl et al., 2008b). These four studies were reviewed by the NTP-CERHR (2008). The original full reports (Tyl et al., 2000; Tyl et al., 2007) of these studies (Tyl et al., 2002b; Tyl et al., 2008b) were also available to OEHHA for review.

Major findings that are relevant to female reproductive or developmental toxicity are summarized in Section B (developmental toxicity) or C (female reproductive toxicity), respectively. This section summaries the findings that are relevant to the male reproductive toxicity. Table A2-10 presents the major findings that are relevant to the male reproductive toxicity of BPA from these studies.

The studies by Ema et al. (2001) and Tyl et al. (2002b; 2008b), respectively, followed standard designs for two- or three-generation reproductive toxicity studies of environmental chemicals. The study by Peknicova et al. (2002) focused on the acrosome integrity of sperm from male

offspring exposed to BPA via a two-generation study design. The paper lacks details of the study design (e.g., unknown number of treated dams, no information on the exposure periods, etc.). Therefore, this study is not included in this review.

Section 1.5.1 Effects on fertility or reproductive outcome in multi-generation studies

The NTP-sponsored RACB study in mice found that BPA at 0.25–1.0% in diets (approximately 437–1750 mg/kg-d) caused reduction in the number of litters per pair, live pups per litter, and increased birth weight at all doses with significant differences at 0.5% and 1.0% (NTP, 1985; Morrissey et al., 1989). The reduced number of live pups per litter was most noticeable in the 4^{th} and 5^{th} litter from the continuous breeding phase (Task 2). The reduced number of live pups per litter was also confirmed in the abbreviated one-generation study that only included the top two doses (0.5% and 1.0%; (Tyl et al., 2002a)). When males in the 1.0% group were mated with females from the control group (Task 3), the mean number of live pups per litter was significantly lower than that in the litters from the controls. This finding suggests that the poorer reproductive outcome observed in Task 2 (treated males mated with treated females) was partly attributable to the males.

The majority of the findings from the two- or three-generation studies are already reviewed and summarized in the previous sections on the developmental or female reproductive toxicity of BPA. However, since both the male and female animals were treated in these studies, and the findings from RACB studies indicate that the male-mediated effects are, at least in part, responsible for the reduced number of live pups, the fertility data from the two- or three-generation reproductive toxicity studies are relevant to the male reproductive effects.

The potential effects of BPA on reproductive outcome were evaluated in a recent two-generation study in CD-1 mice (Tyl et al., 2002b). There was no apparent reduction in the average number of live pups per litter at birth. However, the live birth index (percent of live pups among total pups at birth) had a declining trend with BPA treatment at the 0.18 to 3500 ppm dose range in the F_1 generation. The still birth index (percent of dead pups among total pups at birth) in all BPA-treated F_1 groups appeared to be increased (Table A2-11). Meanwhile, the post-implantation loss per litter was noticeably reduced in the F_1 generation following BPA treatment. Although none of these changes in the BPA groups reached statistical significance and were absent in the F_1 generation (producing F_2 pups), the biological significance should not be totally ignored.

Table A2-10. The male reproductive toxicity of BPA in two- or multi-generation reproductive studies in mice or rats.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
NTP, 1985; Morrissey et al., 1989 NTP-A	CD-1 mice. Standard RACB study design by the NTP. Both males and females were treated in Task 2. Treated males mated to untreated females in Task 3 (cross-over mating trial). 40 (control) or 20 (BPA) mice per sex per group in Task 2.	Feed.	Control: regular diets. BPA: 0.25, 0.5, or 1.0 % in diets (437, 875, 1750 mg/kg-d) from 8 wks premating in F0 mice to PND 21 in F_2.	F_0: reduced litters per pair, live pups per litter, and increased birth weight at all doses, but only significant at 0.5% and 1.0%. Necropsy at 1.0%: reduced weight of the seminal vesicles. Reduced sperm motility. No data on histopathology. No necropsy at 0.25%, 0.5%. Cross-over mating: reduced mean number of live pups per litter, increased offspring birth weight, but no effect on other reproductive outcome. F_1: no effect on reproductive outcome. Reduced testis weight at 0.5% and 1.0%. Reduced weights of epididymis and seminal vesicles at all doses. Reduced sperm count at 0.5% and 1.0% (not significant). Reduced sperm motility at all doses, but only statistically significant at 0.5%. No effect on sperm morphology. Increased prostate weight at all doses, but not significant. No assessment of F_2 pups.
Tyl et al.,2007; 2008b	CD-1 mice. Standard two-generation reproductive toxicity study design. 28 mice per sex per group in F0 generation. Litters were culled to 10 pups per litter on PND 4.	Feed.	Control: normal diets. BPA: 0.018, 0.18, 1.8, 30, 300, 3500 ppm (≈ 0.003, 0.03, 0.3, 5, 50, 600 mg/kg-d).	3500 ppm: reduced sperm count in F_0 males. Delayed PPS. Reduced AGD in F1 males on PND 21, but not on PND 0 or in F_2 males on PND 0 or PND 21. Reduced testis weights, and increased incidences of undescended testes and hypoplasia of the seminiferous tubules. 300 ppm: Reduced AGD in F1 males on PND 21, but not on PND 0 or in F_2 males on PND 0 or PND 21. 30 ppm or lower doses: no effect on the male reproductive system.
Ema et al., 2001 NTP-A	SD rats. Standard design for two-generation reproductive toxicity study. 20-25 rats per sex per group. Litters culled to 8 pups per litter on PND 4.	Oral, gavage. Vehicle: distilled water.	Control: water only. BPA: 0.2, 2, 20, or 200 µg/kg-day (or 0.0002, 0.002, 0.02, 0.2 mg/kg-d).	No obvious effects on the following endpoints: F_0: copulation index, fertility index, weights and histopathology of reproductive organs, blood levels of T, thyroxine (T4), triiodothyronine (T_3), E_2, PRL, LH, FSH, and thyroid stimulating hormone (TSH), and sperm parameters. F_1: all endpoints examined in F_0 males and two endpoints on sexual maturation: PPS, AGD (mm per cube root of body weight) from PND 0 to adulthood. F_2: PPS, AGD, weights and histopathology of the reproductive organs,
Tyl et al., 2000; 2002b NTP-A	SD rats. Design for three-generation reproductive toxicity study. 30 F_0 animals per sex per group for at least 20 litters per group in F_1 generation. F_1 litters culled to 10 pups per litter on PND 4. One litter per generation.	Feed.	Control: no-BPA normal diet. BPA: 0.015, 0.3, 4.5, 75, 750, or 7500 ppm (0.001, 0.02, 0.3, 5, 50, or 500 mg/kg-d).	750 and 7500 ppm: reduced body weights, reduced organ weights including testis, epididymis, prostate, and seminal vesicles, delayed PPS adjusted to body weights in F_2 and/or F_3 males. 75 ppm or lower doses: no effect on the following endpoints: F0: mating index, fertility index, weights and histopathology of non-testis reproductive organs, sperm parameters. F_1: all endpoints examined in F_0 males, PPS, retained nipples. F_2: all endpoints examined in F_1 males and AGD at birth. F_3: all endpoints examined in F_2 males except for reproductive outcome.

Table A2-11. Reproductive outcome of CD-1 mice in the two-generation study by Tyl et al. (2007; 2008b)

Concentration in feed (ppm)	**0**	**0.018**	**0.18**	**1.8**	**30**	**300**	**3500**
Estimated dose (mg/kg-d)	0	0.003	0.03	0.3	5	50	600
No. of live pups/litter							
F_1	12.5 ± 0.3	12.0 ± 0.4	11.7± 0.7	11.9 ± 0.7	12.7 ± 0.5	11.0 ± 0.8	11.1 ± 0.7
F_2	12.4 ± 0.4	12.8 ± 0.3	12.6 ± 0.3	12.4 ± 0.5	12.4 ± 0.5	12.8 ± 0.6	11.5 ± 0.6
Still Birth Index (%)							
F_1	0.4 ± 0.4	**0.9 ± 0.7**	**6.2 ± 3.9**	**6.0 ± 3.9**	**2.9 ± 2.6**	**9.5 ± 5.7**	**9.1 ± 4.5**
F_2	1.6 ± 0.7	0.9 ± 0.7	1.6 ± 0.8	1.4 ± 0.8	0.9 ± 0.6	2.2 ± 1.4	0.0 ± 0.0
Live Birth Index (%)							
F_1	99.6 ± 0.4	99.1 ± 0.7	**93.8 ± 3.9**	**94.0 ± 3.9**	**97.1 ± 2.6**	**90.5 ± 5.7**	**90.9 ± 4.5**
F_2	98.4 ± 0.7	99.1 ± 0.7	98.4 ± 0.8	98.6 ± 0.8	99.1 ± 0.6	97.8 ± 1.4	100.0 ± 0.0
Post-implantation loss/litter (%)							
F_1	11.7 ± 3.6	**2.9 ± 1.0**	**8.5 ± 3.0**	**8.4 ± 3.2**	**6.8 ± 3.6**	**17.9 ± 6.5**	**5.6 ± 1.5**
F_2	6.0 ± 1.5	6.2 ± 3.8	4.2 ± 1.2	5.3 ± 1.6	9.7 ± 3.8	15.3 ± 5.5	9.4 ± 3.3

Note: Numbers in bold indicate apparent reduction. However, none of them was statistically significant.

Table A2-12. Reproductive outcome of SD rats in the three-generation study by Tyl et al. (2000; 2002b)

Concentration in feed (ppm)	**0**	**0.015**	**0.3**	**4.5**	**75**	**750**	**7500**
Estimated dose ranges (mg/kg-d)	0	0.0007–0.003	0.015–0.062	0.22–0.73	4.1–15.4	37.6–167.2	434–1823
Number of implant sites/dam							
F_1	14.23 ± 0.62	15.04 ± 0.51	14.93 ± 0.49	13.93 ± 0.61	14.74 ± 0.64	14.04 ± 0.48	**11.89 ± 0.52 ****
F_2	15.86 ± 0.44	16.33 ± 0.46	15.13 ± 0.64	14.85 ± 0.79	15.33 ± 0.39	16.00 ± 0.38	**11.93 ± 0.43 *****
F_3	15.25 ± 0.33	15.03 ± 0.38	14.03 ± 0.53	14.19 ± 0.73	15.11 ± 0.39	14.44 ± 0.33	**12.44 ± 0.29 *****
Post-implantation loss/litter (%)							
F_1	3.45 ± 1.23	**6.96 ± 2.67**	**7.02 ± 1.70**	**5.66 ± 1.48**	**13.81 ± 4.21**	**9.96 ± 3.03**	**11.33 ± 3.64**
F_2	9.35 ± 1.83	9.11 ± 1.51	7.59 ± 1.97	6.44 ± 1.70	7.04 ± 1.44	7.37 ± 1.98	**11.08 ± 2.21**
F_3	5.02 ± 1.14	7.17 ± 1.60	6.59 ± 1.57	**10.88 ± 3.92**	**9.26 ± 1.77**	**6.87 ± 1.35**	**12.30 ± 2.17**
No. of total pups/litter							
F_1	14.4 ± 0.6	14.9 ± 0.7	14.3 ± 0.5	**13.5 ± 0.6**	**14.0 ± 0.5**	**13.1 ± 0.6**	**11.8 ± 0.4 ****
F_2	14.9 ± 0.6	15.1 ± 0.5	14.5 ± 0.7	14.7 ± 0.7	14.5 ± 0.5	15.0 ± 0.5	**11.1 ± 0.5** ***
F_3	14.9 ± 0.4	14.3 ± 0.4	**13.3 ± 0.5 ***	**13.8 ± 0.6**	**14.1 ± 0.4**	**13.8 ± 0.4**	**11.2 ± 0.4 *****
No. of live pups per litter							
F_1	14.3 ± 0.6	14.7 ± 0.7	14.1 ± 0.5	**13.3 ± 0.6**	**13.7 ± 0.5**	**12.9± 0.6**	**11.5 ± 0.4 ****
F_2	14.6 ± 0.6	14.9 ± 0.4	14.3 ± 0.7	14.7 ± 0.7	14.3 ± 0.4	14.9 ± 0.5	**10.8 ± 0.5 *****
F_3	14.8 ± 0.4	14.1 ± 0.4	**13.2 ± 0.5 ***	**13.6 ± 0.6**	**13.9 ± 0.4**	**13.7 ± 0.4**	**10.9 ± 0.4 *****

* P<0.05; ** P<0.01; *** P<0.001; statistically significant difference as compared to control values; data presented as mean ± SEM.
numbers in bold indicate apparent difference from the control group, with or without statistical significance.

There is no RACB study in rats available. There was no effect on reproductive outcome of F_0 or F_1 generations in the two-generation study reported by Ema et al. (2001). In the three-generation study reported by Tyl et al. (2008b), a significant reduction in the average number of implant sites per dam and the total number of live pups per litter was observed in all three generations of pups in the 7500 ppm group (Table A2-12). At lower doses, a reduced total number of pups per litter was noticeable in the 4.5–750 ppm groups in the F_1 generation and in the 0.3–750ppm groups in the F_3 generation, but the changes were not statistically significant. Post-implantation loss per litter was increased in the F_0 generation (producing F_1 pups) in all the treated groups, but the increase was only significant at 7500 ppm. The reducing trend in the number of live pups per litter seems to be in line with the observation in CD-1 mice, but the increasing trend of reduced post-implantation loss per litter was in contrast to that in CD-1 mice (Tyl et al., 2007; Tyl et al., 2008b).

Section 1.5.2 Testicular effects in multi-generation reproductive studies

Reduced sperm motility was observed in F_0 CD-1 mice exposed to 1.0% BPA in diet (NTP, 1985). In the F_1 generation, BPA at 0.5% and 1.0% in diet (437 and 875 mg/kg-d, respectively) reduced testis weights. Epididymal sperm count was decreased at 0.5% and 1.0%, but the reduction was not statistically significant. Compared to controls, sperm motility was lower at all doses. It was statistically significant at the 0.5% dose. Similarly, reduced testis weights, increased incidence of hypoplasia in the seminiferous tubules, and reduced sperm count were observed in mice treated with 3500 ppm in diet (approximately 600 mg/kg-d during gestation; (Tyl et al., 2007; Tyl et al., 2008b)). In rats, a slight decrease in testis weight was observed in F_3 male rats at all doses; the reduction was statistically significant in the 0.015, 0.3, 750, 7500 ppm groups, but not in the 4.5 and 75 ppm groups (Tyl et al., 2000; Tyl et al., 2002b). Testis weights in F_1 and F_2 males were also lower in all BPA-treated groups, but only the reductions in F_2 males at 0.3 and 750 ppm were statistically significant. Consistent with the reduced testis weights in F_3 males, daily sperm production (DSP) per testis at all doses were slightly reduced (by about 9–19%) in F_3 males. The reduction was only statistically significant at 7500 ppm (Tyl et al., 2000). There was a similar declining trend in the efficiency of DSP per g testis, but it was not as obvious as that in the DSP. Histopathological evaluation detected no obvious abnormal changes in the testes of SD rats treated with BPA at doses ranging from 0.2 µg/kg-day (Ema et al., 2001) to 500 mg/kg-d (Tyl et al., 2000; Tyl et al., 2002b).

Section 1.5.3 Effects on epididymis or seminal vesicles in multi-generation reproductive studies

Continuous exposure to BPA caused apparent reduction in weights of the epididymis and seminal vesicles in CD-1 mice at doses of 0.25%–1.0% in diets, but only the reduction at 0.25% in F_1 males was statistically significant (NTP, 1985). There was no effect on the epididymis or seminal vesicles at concentrations up to 3500 ppm in diets, equivalent to approximately 600 mg/kg-d, in the two-generation study (Tyl et al., 2002b).

Ema et al. (2001) found that BPA at doses from 0.2 to 200 µg/kg-day caused no change in the weight or histopathology of the epididymis or seminal vesicles in SD rats. Reduced weight of the epididymis and seminal vesicles was observed in SD rats of all four generations exposed to 7500 ppm of BPA in diets, equivalent to 500 mg/kg-d, but not at lower doses (Tyl et al., 2002b).

Section 1.5.4 Effects on the prostate in multi-generation reproductive studies

Dietary exposure to BPA did not produce obvious changes in prostate weight or histopathology in CD-1 mice in either the RACB study by the NTP (1985) or the two-generation reproduction study by Tyl et al. (2008). The prostate weights in F1 males exposed to 0.5% and 1.0% BPA in diets were higher than those of the controls (46 ± 5 mg and 48 ± 6 mg, respectively, vs. 42 ± 5 mg in the control), but the difference was not statistically significant.

BPA at doses of 0.2 to 200 µg/kg-day had no observed effect on the prostate of SD rats (Ema et al., 2001). Dietary exposure to 0.018–3500 ppm of BPA caused no change in the weight or histopathology in adult CD-1 mice (Tyl et al., 2007; Tyl et al., 2008b). In the three-generation study in SD rats, the authors found significantly decreased weight of the prostate in all four generations of male rats in the 7500-ppm (500 mg/kg-d) group, but not in the groups at lower doses. It should be noted that the weight of the prostate decreased gradually from the F_0 generation to the F_3 generation in all the groups, including the control group (see Table 2 in the original publication).

Section 1.5.5 Effects on sexual maturation in males in multi-generation reproductive studies

No effect on PPS or AGD was observed in the study in SD rats by Ema et al. (2001). Tyl et al. (2002b) reported delayed PPS in the F_1 and F_3 generations at 7500 ppm, and in the F_2 generation at 750 and 750 ppm (50 and 500 mg/kg-d, respectively). After adjusting the day of PPS to the body weights either at the day of PPS acquisition or PND 14, the authors concluded that the delayed PPS was due to smaller body weights on PND 14 in the high dose groups. The rationale for using the body weight of PND 14 was not reported in the publication (Tyl et al., 2002b) or the original full report (Tyl et al., 2000). No effect on AGD was found in this study. Delayed PPS and reduced AGD were also found in CD-1 mice at 3500 ppm (Tyl et al., 2008b), but not at lower doses.

Section 1.5.6 Hormonal effects in males in multi-generation reproductive studies

No apparent effect on blood levels of hormones was found in any of the three multigenerational studies.

Section 2 Studies in Adult Animals

The majority of the studies in adult animals (eight weeks or older in mice or rats when the treatment began) were conducted in mice or rats. There is also a study in beagle dogs (GE, 1976a) and a study in New Zealand white rabbits (Moon et al., 2001).

Section 2.1 Studies in Adult Mice

Seven studies investigated the male reproductive toxicity of BPA in adult mice. Five different strains of mice (C57BL/6N, ICR, Kuming, and Balb/c) and three different routes of administration (oral, s.c injection or implant, and i.p. injection) were used in these studies. Table A2-13 presents the major findings from these studies, followed by an endpoint-by-endpoint review.

Section 2.1.1 Effects on fertility or reproductive outcome in adult male mice

The NTP-sponsored RACB study in ICR mice included cross-over mating trials in the F_1 males from the last litter (litter 5) in the high-dose group (1.0% of BPA in diet from 8 weeks premating in the F_0 mice to PNW 11 in the F_1 males; (Morrissey et al., 1989); see discussions on page 43 under Section 1.5.1.). Mating of these F_1 males to females from the control group produced litters that had significantly reduced mean numbers of live pups per litter, but there were no significant effects on any other indices of reproductive outcome (including mating index, fertility index, mean number of live male or female pups per litter, pups birth weights, etc.).

Al-Hiyasat et al. (2002) evaluated reproductive outcome in male Swiss mice exposed to 5–100 µg/kg-day of BPA by oral gavage for 30 days (the unit of the doses in the original report in 2002 was corrected from ng/kg-day to µg/kg-day in the erratum in 2003). The authors found that BPA treatment caused significant increases in the percentage of females with resorptions and in the ratio of the total number of resorptions per group to the total number of implantation sites per group at all the doses tested. Toyama et al. (2004) injected ICR mice s.c. for six days with 20 or 200 µg/kg-day of BPA. Two months after treatment, fertility was evaluated and no effect was found, but the author only used one mouse per group in the fertility trial. No other studies included evaluation of fertility.

Table A2-13. Male reproductive effects of BPA in adult mice.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Nagao et al., 2002 NTP-A	C57BL/6N male mice, 10-wk-old, 20 mice per group (15 per group for organ weight analysis)	Oral gavage. Vehicle: 0.5% carboxymethyl cellulose.	Ctrl: vehicle only. BPA: 2, 20, or 200 µg/kg-day for 6 days, examined in week 17 (6 wks after last dosing).	No effect on weights of the testis, epididymis, or seminal vesicles (SV) or number of sperm in the caudal epididymis. No obvious histopathological changes.
Al-Hiyasat et al., 2002 (2003 for the erratum) NTP-A	Swiss mice, 10 male mice per group.	Oral gavage Vehicle: 0.1% ethanol in drinking water.	Control: vehicle only. BPA: 5, 25, or 100µg/kg-day for 30 days. Fertility was assessed by mating to untreated females (1 male to 2 females) for 10 days after the BPA treatment. Necrosied after the mating trial.	5 µg/kg-day: increased resorptions. No effect on absolute testis weight. Reduced weight of the seminal vesicles. Reduced total sperm number per epididymis, but not sperm number per mg of the epididymis. At 25 and 100 µg/kg-day, all effects observed at 5µg/kg-day dose occurred; in addition, there was reduced pregnancy rate, reduced sperm production in the testis and reduced sperm number per mg of the epididymis.
Toyama et al., 2004 NTP-A	ICR mice, 3 (control group) or 6 (BPA group) mice per group.	s.c. injection. Vehicle: DMSO in olive oil.	Control: vehicle only. BPA: 20 or 200 µg/kg-day for 6 days. 2 mice from the control group and 5 from BPA group necropsied on Day 7. Fertility test 2 months after dosing.	Deformed spermatids and multinucleated giant germ cells under light microscopy, and abnormal changes under electron microscopy on Day 7. No histopathological changes or effects on fertility 2 months after the treatment, but only one mouse per group.
Anahara et al., 2006 NTP-A	ICR mice, 5-7 mice per group.	s.c. injection. Vehicle: corn oil.	Control: vehicle only. BPA: 2.4 µg/kg-day for 5 days.	Reduced protein levels of cortactin in the testis, indicative of alterations in the structure of the apical ectoplasmic specialization in the seminiferous epithelium.
Liu et al., 2006	Kunming mice (Outbred Swiss mice), 7 mice per group per time point.	i.p. injection. Vehicle: corn oil.	Control: vehicle only. BPA: 250, 500, or 1000 µmol/kg-day (57, 114, or 228 mg/kg-d) for 5 days. Necropised on Day 7 or 14 from the beginning of the treatment.	No effect on relative weight of the testis. Increased levels of nitric oxide (NO) and nitric oxide synthase (NOS) in the testicular tissues at all doses on Day 7 and in the middle- and high-dose groups on Day 14. Altered cell cycles of the testicular cells and increased percentage of apoptotic cells at the high-dose on Day 7.
Ogura et al., 2007 NTP-B	BALB/c male mice of 9-week-old, 7-9 mice per group.	s.c. implants.	Ctrl: pellets containing cholesterol only. BPA: 0.2 – 200 mg/animal for 3 wks.	At 20 and 200 mg/animal, reduced weight of seminal vesicles. No effect on prostate weight or histopathology. Positive staining for cytokeratin 10 (CK-10) in basal epithelial cells of the prostate from BPA-treated mice in a dose-dependent manner, indicative of abnormal proliferation and differentiation of basal epithelial cells.

Section 2.1.2 Testicular effects in adult mice

Treatment with BPA by oral gavage (Al-Hiyasat et al., 2002; Nagao et al., 2002)or i.p. injection (Liu et al., 2006) did not result in a reduction in testis weight. Nagao et al. (2002) also observed no histopathological changes in testis or reduction in epididymal sperm count in mice evaluated six weeks after six days of oral treatment with 2–200 µg/kg-day of BPA. Similarly, Toyama et al. (2004) found no histopathological changes in testis of the BPA-treated mice necropsied two months after six days of s.c. injection with 20 or 200 µg/kg-day of BPA. However, in studies that evaluated the testis soon after the treatment, reduced sperm count or production and histopathological changes were observed. Al-Hiyasat et al. (2002) reported reduced sperm count or production in Swiss mice. Toyama et al. (2004) found deformed spermatids and multinucleated germ cells at the light microscopic level and abnormal changes under the electron microscopic level in the seminiferous epithelium. BPA-induced alteration in the expression of cortactin, indicative of structural changes, increased levels of oxidative stress, or increased number of apoptotic cells in the seminiferous epithelia has also been reported (Anahara et al., 2006; Liu et al., 2006).

Section 2.1.3 Effects on epididymis or seminal vesicles in adult mice

No effect on epididymis weight was observed by Nagao et al. (2002) or Al-Hiyasat et al. (2002). No other studies evaluated the epididymis.

Nagao et al. (2002) found no change in the weight of the seminal vesicles in mice six weeks after six days of oral treatment with 2–200 µg/kg-day of BPA. However, reduced weight of the seminal vesicles was observed in Swiss mice treated with 5–100 µg/kg-day of BPA for 30 days (Al-Hiyasat et al., 2002) or in Balb/c mice exposed to 20 or 200 mg/mouse of BPA via s.c. implants for three weeks (Ogura et al., 2007).

Section 2.1.4 Effects on the prostate in adult mice

Ogura et al. (2007) treated the adult Balb/c mice with 0.2–200 mg BPA/mouse for three weeks by s.c. implant. While no effect on prostate weight or general histopathology was observed, BPA treatment caused increased positive immunostaining for cytokeratin 10 in the basal epithelial cells in a dose-dependent manner. This observation suggests that BPA exposure in adult Balb/c mice can cause abnormal changes in the proliferation and differentiation of epithelial cells in the prostate. No other studies in adult mice evaluated the prostate.

Section 2.1.5 Hormonal effects in adult mice

There are no data on the potential effects of BPA on blood levels or testicular production of T in mice treated in adulthood.

Section 2.2 Studies in adult rats

A total of six studies evaluated the effects of BPA in adult rats exposed to BPA during adulthood. Three studies used SD rats and oral administration. Two used Wistar rats with s.c. injection, and one used Brl han:Wist jcl rats (Wistar-derived) exposed by oral gavage. Major findings from these studies are summarized in Table A2-14.

Table A2-14. Male reproductive effects in adult rats.

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Sakaue et al., 2001 NTP-A	SD rats, 5 rats per group per evaluation time point in the 1st experiment. 8 rats per group per evaluation time point in the 2nd experiment.	Oral gavage. Vehicle: 6.5% ethanol in corn oil.	Control: vehicle only and untreated (2nd experiment). BPA: 0.02, 0.2, 2, 20 or 200 mg/kg-d for 6 days. Necropsy on Day 8 or Day 36 from the beginning of treatment. Lower doses (2, 20, 200 ng, 2-2000 µg per kg-day) in the 2nd experiment on sperm production analysis.	No effect on testis weights. Reduced daily sperm production (DSP; number of sperm per testis) or the efficiency of sperm production (DSP per gram testis) at ≥20 µg/kg-day. Reduction in sperm production was confirmed in the 2nd experiment. Altered patterns of protein profile in two-dimensional gel electrophoresis of testicular proteins.
Ashby et al., 2003 NTP-A	SD rats, 10 rats per group. Aimed to replicate the findings by Sakaue et al. (2001).	Oral gavage. Vehicle: 6.5% ethanol in corn oil.	Five experiments. Four included BPA treatment at doses of 0.02, 2, or 200 mg/kg-d for 6 days between PND 91 and 97. Necropsy at 18 weeks of age.	No effect on weights of the testis, epididymis, seminal vesicles, or prostate. No effect on sperm production.
Deng et al., 2004	SD rats, 17, 8, or 17 rats in the control, 1g/kg-BPA, or 5 g/kg-BPA group), respectively.	Feed. Vehicle: 10% BPA in ethanol and then to diets.	Control: diets without BPA. BPA: 1 or 5 g/kg in diets (estimated by the authors to be approximately 50 or 250 mg/kg-d) for 2 wks.	Reduced testis weight at 250 mg/kg-d. Reduced number of round spermatids and spermatocytes per Sertoli cell at both doses. Histopathological changes in the seminiferoous epithelium and altered immunostaining for vimentin. No effect on blood levels of T or E2.
Yamasaki et al., 2003 NTP-A	Brl Han:WIST Jcl rats (Wistar). Castrated at 7 wks of age. 56-day-old at the beginning of treatment. 6 rats per group.	Oral gavage. Vehicle: olive oil.	Control: vehicle only, with or without s.c injection of 0.2 mg/kg-d of testosterone propionate. BPA: 50, 200, or 500 mg/kg-d for 10 days. [Hershberger assay]	No effect on weights of the ventral prostate, seminal vesicles, LABC, glans penis, or Cowper's gland, in the presence or absence of T stimulation.

Table A2-14. Male reproductive effects in adult rats (continued).

References	Species/Strains	Exposure Routes	Doses and Duration	Major Findings
Tohei et al., 2001 NTP-A	Wistar-Imamichi rats, 5-6 rats per group.	s.c. injection. Vehicle: sesame oil.	Control: vehicle only. BPA: 0.1 (hCG stimulation experiment only) or 1 mg/kg-d (all experiments) for 2 wks.	Increased blood levels of LH and PRL, and reduced blood levels of T and inhibin. Suppression of hCG-stimulated production of progesterone and T. No effect on LHRH-stimulated production of LH in the pituitary. No effect on male sexual behavior.
Herath et al., 2004 NTP-A	Wistar-Imamichi rats, 50-day-old, 10-11 rats per group.	s.c. injection. Vehicle: DMSO.	Control: vehicle only. BPA: 3 mg/kg-d for 5 wks. At the end of 2-wk treatment, blood samples were collected before and after injection of LHRH (250 ng).	Week 2: reduced blood level of LH. No effect on blood levels of T. Increased levels of progesterone. No obvious effect on LHRH stimulation. At Week 5, no effect on weights of testis or seminal vesicles. Increased weight of the prostate. Reduced blood level of T. Reduced epididymal sperm count. No effect on blood levels of LH or epididymal sperm motility.
Toyama et al., 2004 NTP-A	Wistar rats, 3 (control group) or 6 (BPA group) rats per group. [Same design as the experiments in ICR mice]	s.c. injection. Vehicle: DMSO in olive oil.	Control: vehicle only. BPA: 20 or 200 µg/kg-day for 6 days. 2 rats from the control group and 5 from each BPA group necropsied on Day 7. Remaining rats for fertility test 2 months after dosing.	Deformed spermatids and multinucleated giant germ cells under light microscopy, and abnormal changes under electron microscopy on Day 7. No histopathological changes or effects on fertility 2 months after treatment, but only one rat per group.

Section 2.2.1 Effects on fertility or reproductive outcome in adult rats

Toyama et al. (2004) evaluated the fertility of Wistar rats at two months after six days of s.c. injection with 20 or 200 μg/kg-day of BPA and found no effect, but only one rat per group was used in the fertility trial. No other studies included evaluation of fertility.

Section 2.2.2 Testicular effects in adult rats

All three studies in SD rats evaluated testicular effects of BPA following oral administration. In the feed study by Deng et al. (2004), reduced testis weight was observed following exposure at 250 mg/kg-d for two weeks, but not at 50 mg/kg-d. The authors also reported a reduced number of germ cells and degenerative changes in the seminiferous epithelium following exposure at 50 and 250 mg/kg-d.

The study by Ashby et al. (2003) was designed to replicate the study by Sakaue et al. (2001). Both studies treated SD rats at 13 weeks of age for six days by oral gavage with BPA in corn oil containing 6.5% ethanol. Sakaue et al. (2001) evaluated the testis at PNW 14 and 18, but did not report the PNW 14 data from the BPA-treated groups. The authors found no effect on testis weight or histopathology, but sperm number per testis or per gram of testis (efficiency of sperm production) were significantly reduced at doses $\geq$ 20 μg/kg-day. The reduction in sperm counts was confirmed in the second experiment that included doses as low as 2 ng/kg-d. In the study by Ashby et al. (2003), there was no effect on testis weight or sperm production, although the authors designed their experiments carefully to mimic the study design used by Sakaue et al. (2001). Ashby et al. (2003) analyzed historical control values for sperm count from a number of studies and tried to find answers to the inconsistence in the findings between their study and that by Sakaue et al. (2001). One of the potential factors raised by Ashby et al. was the possible differences in the genetic make-ups of the animals between the two studies. This possibility remains to be resolved, but the apparent difference in body weights of rats at 18 weeks of age in the control group between the two studies may indicate that the rats used in the two studies have some genetic and/or nutritional differences. The average body weight (556 g) of SD rats used in the study by Ashby et al. was approximately 10% higher than that of the animals used in the study by Sakaue et al. (2001; approximately 500 g, based on the Figure 2 in the paper).

None of the studies in Wistar rats evaluated testis or sperm parameters.

Section 2.2.3 Effects on the epididymis or seminal vesicles in adult rats

No effect on the weight of the epididymis and seminal vesicles was observed in SD rats by Ashby et al. (2003). Using Brl Han:WIST Jcl (Wistar) rats in a Hershberger assay, Yamasaki et al. (2003) did not find any effect on the weight of the seminal vesicles in castrated rats in the absence or presence of T stimulation. No other studies evaluated the epididymis or seminal vesicles.

Section 2.2.4 Effects on the prostate in adult rats

No effect on prostate weight was observed in the studies by Ashby et al. (2003) and Yamasaki et al. (2003), respectively. No other studies in adult rats investigated effects of BPA on the prostate.

Section 2.2.5 Hormonal effects in adult rats

Deng et al. (2004) found no effect on blood levels of T or E_2 in SD rats treated with 50 or 250 mg/kg-d of BPA in diets for two weeks. However, treatment with 1.0 mg/kg-d of BPA by s.c. injection for two weeks caused increased blood levels of LH and PTL, reduced levels of T and inhibin, suppressed production of T and progesterone under hCG stimulation in Wistar-Imamichi rats (Tohei et al., 2001). Blood levels of sex hormones were not measured in other studies in adult rats.

Section 2.3 Studies in Other Adult Animals

Section 2.3.1 Rabbit

Moon et al. (2001) studied the effect of BPA on erectile function in New Zealand rabbits. The authors treated the animals (15 animals per group) with 150 mg/kg of BPA by i.p. injection every other day for 12 days and evaluated erectile function four and eight weeks after treatment. They found that contraction and relaxation of the cavernosal tissue strips were significantly suppressed in the BPA-treated animals. Histopathological changes in the penis were also observed.

Section 2.3.2 Dog

A 90-day dietary study in beagle dogs of unknown age (weighing 6.6–13.4 kg) found no apparent effects on testis weight or histopathology (GE, 1976a). The study focused on the general toxicity of BPA and treated the dogs with BPA in diet at concentrations of 1000, 3000, and 9000 ppm.

Section 3 Studies *in vitro*

Compared to the large volume of *in vivo* studies on male reproductive effects of BPA, there are only a limited number of *in vitro* studies, although studies with *in vitro* models can provide valuable mechanistic data. The major features of and the findings from several *in vitro* studies are summarized in Table A2-15 below.

Section 3.1 Sertoli cells

Table A2-15 lists the major findings from four studies using Sertoli cell culture models. BPA at concentrations of 100 µM or higher caused cell death and damaged functions of cultured Sertoli cells isolated from immature rats (Monsees et al., 2000; Iida et al., 2003). Similar treatment resulted in significant changes in the expression of certain genes or proteins in Sertoli cell lines (Tabuchi et al., 2002; Tabuchi and Kondo, 2003; Lee et al., 2004). These findings indicate that exposure to BPA can directly damage Sertoli cells.

Table A2-15. Effects of BPA on cultured Sertoli cells.

References	Model	Treatment	Major findings
Monsees et al., 2000. NTP-A	SC from SD rats 18-21 days of age	10-50 µM for 24 hrs.	No effect on viability. Increased production of lactate and inhibin.
Iida et al., 2003. NTP-A	SC from Wistar rats 18 days of age	50-300 µM for 48 hrs.	At ≥ 150 µM, reduced viability and production of transferin, increased apoptosis.
Tabuchi et al., 2002; Tabuchi and Kondo, 2003. NTP-A	TTE3 cell line (mouse)	50-400 µM for 24 hrs.	cDNA microarray analysis. Increased expression of 31 genes. No reduction in any gene analyzed. Chop-10, a gene induced by DNA damage or ER stress, is the most sensitive to BPA.
Lee et al., 2004. Not in A	TM4 cell line (mouse)	50-250 µM for 48 hrs.	Reduced viability at ≥ 100 µM. Altered expression of numerous proteins.

Section 3.2 Leydig cells

Table A2-16 summarizes the major findings from five *in vitro* studies on the Leydig cells, using either primary cells or cell lines. Murono et al. (2001) found that BPA at 1–1000 nM did not affect conversion of cholesterol to T or binding of hCG to the LH receptor in cultured Leydig cells from SD rats at 55–65 days of age. In contrast, exposure to 0.01 nM, but not any higher concentrations (up to 1000 nM) reduced hCG-stimulated T production in Leydig cells isolated from 90-day-old LE rats (Akingbemi et al., 2004). Expression of the mRNAs for $P450_{17\alpha}$ and aromatase, key enzymes of steroidogenesis, was also significantly reduced. Using mouse Leydig tumor cells, Song et al. (2002), Nikula et al. (1999), and Takamiya et al. (2007), respectively, also found that BPA at concentrations of ≥ 10 nM affected T production and/or expression of several key steroidogenic enzymes. The findings from these *in vitro* studies indicate that BPA can impair steroidogenesis by altering the expression or function of key components of steroidogenesis, but that it does not affect the binding of LH or hCG to the LH receptor in Leydig cells.

Table A2-16. Effects of BPA on cultured Leydig cells.

References	Model	Treatment	Major findings
Murono et al., 2001 NTP-A	LC from SD rats 55-65 days of age	1-1000 nM for 24 hrs.	No effect on viability. No effect on conversion of cholesterol to T or binding of hCG to LH receptors.
Akingbemi et al., 2004 NTP-A	LC from LE rats 90 days of age	0.01-1000 nM for 18 hrs.	Reduced T production under hCG stimulation at 0.01 nm, but not at higher doses. Reduced expression of $P450_{17\alpha}$ and aromatase mRNA.
Song et al., 2002 NTP-A	K28 cell line (mouse Leydig tumor cells)	1-10000 nM for 30 min or 1000 nM for 30 min	Increased expression of Nur77 (orphan nuclear receptor). Increased activity of mitogen-activated protein kinase at ≥0.01 μM.
Nikula et al., 1999 NTP-A	mLTC-1 cell line (mouse Leydig tumor cells)	0.1 – 100 μM for 48 hrs.	Reduced cAMP and progesterone production under hCG stimulation. No effect on hCG binding to LH receptor or 8-Br-cAMP-stimulated production of progesterone at ≥ 0.1μM.
Takamiya et al., 2007 Not in A	mLTC-1 cell line (mouse Leydig tumor cells)	0.01 pM – 100 μM for 1 or 3 hrs.	Reduced expression of StAR, increased expression of Cyp17α and AR expression, at ≥ 1 μM.

Section 3.3 Prostate cells

There are six studies using *in vitro* models to investigate the effects of BPA on the prostate tissues or epithelial cells (Table A2-17). BPA at nanomolar concentrations has been shown to cause increased proliferation of prostate tumor cells (Wetherill et al., 2002; Wetherill et al., 2005; Hess-Wilson et al., 2007) or primary cells isolated from fetal CD-1 mice (Gupta, 2000b; Richter et al., 2007). BPA-induced proliferation in prostate cells appears to result from activation or increased expression of AR. It should be noted that low concentrations of BPA were more potent in inducing cell proliferation or AR activation than higher concentrations. This finding may support, at least in part, the lack of clear dose-response relationships in certain testicular or prostate effects observed in some studies *in vivo*.

Similar to the effects observed in adult mice treated with BPA (0.2–200 mg/animal for 12 weeks by s.c. implantation), abnormal differentiation (squamous metaplasia) and positive immunostaining for CK10 were observed in basal epithelial cells in cultured mouse prostatic ducts (Ogura et al., 2007). These findings indicate that BPA can cause abnormal differentiation of prostatic epithelial cells directly.

Table A2-17. The effects of BPA on cultured prostate cells.

References	Model	Treatment	Major findings
Wetherill et al., 2002 NTP-A	LNCaP cell line (androgen-sensitive human prostate adenocarcinoma cells expressing the mutant AR-T877A)	0.1 – 100 nM for 24- 192 hrs.	Mitogenic effects. Increased proliferation at 1 (maximum) and 10 nM, but not at 100 nM. At 1 nM, increased expression of cyclin D1 and A. AR nuclear translocation resulting in T-independent activation of AR and increased expression of prostate-specific antigen.
Wetherill et al., 2005 Not in A	LNCaP cell line	0.1 – 10 nM for 48 hrs in the absence or presence of DHT stimulation.	Potentiated DHT-induced activation of AR in a reversed dose-dependent manner (0.01 nM>1 nM>10 nM). Inhibited DHT binding to AR via non-competitive competition. High concentrations reduce proliferation of cancer cells with functional AR.
Hess-Wilson et al., 2007 Not in A	LNCaP, 22Rv1 (androgen-insensitive), LAPC4 (expressing wildtype AR) cell lines	1 nM for 24 hrs.	Altered profiles of gene expression in LNCaP cells. Reduced expression of ERβ in LNCaP, but not in other cell lines.
Gupta, 2000 NTP-A	Urogenital sinus (UGS) from GD-17 fetal CD-1 mice	5 or 50 pg/ml (0.022 or 0.22 nM) for 6 days.	50 pg/ml: increased volume, increased number of branches, and increased T binding. No effect at 5 pg/ml.
Richter et al., 2007 Not in A	Prostate mesenchyme cells from GD-17 fetal CD-1 mice	10^{-5} – 10^{5} nM for up to 96 hrs.	Slight but significant increase in total DNA at 1,000 and 10,000 nM, but not at lower or higher concentrations. Slight decrease in total RNA at 10-5 – 10-2 nM, and increase in total RNA at 103 nM. At ≥ 1nM, increased expression of mRNAs for AR and ERα, respectively. Tamoxifen attenuated increased expression of AR or ERα mRNA induced by 10-nM, but not 100-nM BPA.
Ogura et al., 2007 Not in A	Prostatic ducts from Balb/c mice 8-9 weeks of age	1 or 1000 nM for 6 days.	Squamous metaplasia of the epithelial cells with positive staining for cytokeratin 10, indicating abnormal differentiation. Effects at 1000 nM more obvious than at 1nM.

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