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

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

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:** ovariectomized 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 approximately 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).

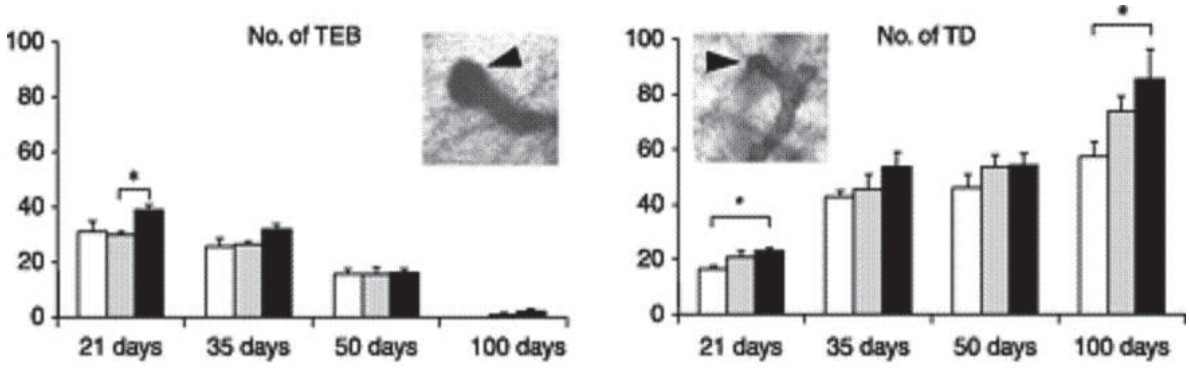

Figure F1. Morphological analysis of the mammary gland. Total number (mean ± SEM) of TEB and TD in the mammary glands from control (white bars) and BPA-exposed (grey bars: low dose [25 µg BPA/kg bw], black bars: high dose [250 µg BPA/kg bw]) rats at different ages of development. * significantly different ($P<0.05$) (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 ♀ (pregnant)	*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 (pregnant) 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.2.9. 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.

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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

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°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 ± 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.