In the following document I have provided

a) Comments on the pdf versions of the NTP Draft Brief on BPA,

b) A copy of the text of the Puberty section from the Brief with my comments in CAPS,

c) Graphs and statistical analyses of the data on the effects of BPA on puberty in the mouse as measured by either the ages at vaginal opening and first estrus.

Given the limitations of these three studies with mice, the lack of effect of BPA at “low doses” on the age at vaginal opening in several studies using mice, and the lack of acceleration of puberty by “low doses” of BPA in the rat the CERHR BPA Expert Panel concluded that these data provided “minimal” evidence of concern for the effects of BPA on puberty in humans. I hope that these comments are helpful and constructive.

Sincerely,

Leon Earl Gray Jr, PhD

Former member of the CERHR BPA Expert Panel

**Can Bisphenol A Affect Human Development or Reproduction?**

*Possibly.* Although there is no direct evidence that exposure of people to bisphenol A adversely affects reproduction or development, studies with laboratory rodents show that exposure to high dose levels of bisphenol A during pregnancy and/or lactation can reduce survival, birth weight, and growth of offspring early in life, and delay the onset of puberty in males and females. These effects were seen at the same dose levels that also produced some weight loss in pregnant animals ("dams"). The administered dose levels associated with delayed puberty (≥ 50 mg/kg bw/day), growth reductions (≥ 300 mg/kg bw/day), or survival (≥ 500 mg/kg bw/day) are far in excess of the highest estimated daily intakes of bisphenol A in children (< 0.0147 mg/kg bw/day), adults (< 0.0015 mg/kg bw/day), or workers (0.100 mg/kg bw/day) (Table 1). These "high" dose effects of bisphenol A are not considered scientifically controversial and provide *clear evidence* of adverse effects on development in laboratory animals.

In addition to effects on survival and growth seen at high dose levels of bisphenol A, a variety of effects related to neural and behavior alterations, precancerous lesions in the prostate and mammary glands, altered prostate gland and urinary tract development, and early onset of puberty in females have been reported in laboratory rodents exposed during development to much lower doses of bisphenol A (≥ 0.0024 mg/kg bw/day) that are more similar to human exposures. In contrast to the "high" dose developmental effects of bisphenol A, there is scientific controversy over the interpretation of the "low" dose findings. When considered together, the results of "low" dose studies of bisphenol A provide *limited evidence* for adverse effects on development in laboratory animals (see **Figures 2a & 2b**).

Recognizing the lack of data on the effects of bisphenol A in humans and despite the limitations in the evidence for "low" dose effects in laboratory animals discussed in more detail below, the possibility that bisphenol A may alter human development cannot be dismissed (see **Figure 3**).

**Supporting Evidence**

The NTP finds that there is clear evidence of adverse developmental effects at "high" doses of bisphenol A in the form of fetal death, decreased litter size, or decreased number of live pups per litter in rats (≥ 500 mg/kg bw/day) (*28, 29*) and mice (≥ 875 mg/kg bw/day) (*30-32*), reduced growth in rats (≥ 300 mg/kg bw/day) (*28, 29*) and mice (≥ 600 mg/kg bw/day) (*30, 31, 33*), and delayed puberty in male mice (600 mg/kg bw/day) (*33*), male rats (≥ 50 mg/kg bw/day) (*29, 34*) and female rats (≥50 mg/kg bw/day) (*29, 35*).

In addition to these "high" dose effects on survival and growth, the NTP recognizes that there are studies that provide evidence for a variety of effects at much lower dose levels of bisphenol A related to neural and behavioral alterations in rats and mice (≥ 0.010 mg/kg bw/day) (*36-42*), preneoplastic lesions in the prostate and mammary gland in rats (0.010 mg/kg bw/day and 0.0025 mg/kg bw/day, respectively) (*43-45*), altered prostate and urinary tract development in mice (0.010 mg/kg bw/day) (*46*), and early onset of puberty in female mice (0.0024 and 0.200 mg/kg bw/day) (*40, 47*).



# Summary of Comments on NTP Brief on Bisphenol A

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why is the NTP using this method for determining human BPA exposures as opposed to the data from urine samples? There is no discussion of this decision or discussion of the fact that they provide very different pictures of what human exposures to BPA are. I believe that many experts like Dekants et al (2008) do not rely on this approach. His article should be included and I have attached the last page of their paper to the supplemental information I am sending. They conclude that even the "low dose" studies are not using environmentally relevant daily doses of BPA.

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not signficant...no effect on age at first estrus or age at vo.

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no early puberty at 2.4

account for the inconsistencies. In other cases, particularly for findings based on studies with very specific experimental questions, variations in experimental design are large enough to conclude that the reproducibility of the finding is essentially unknown. A number of these effects have not been addressed in traditional toxicity studies carried out to assess the toxicity of bisphenol A. Typically the safety studies do not probe for potential organ effects with the sam degree of specificity or detail as those studies with specific experimental questions. The NTP evaluated the biological plausibility of findings with unknown reproducibility in light of supporting data at the mechanistic, cellular, or tissue level.

Another issue is that the "low" dose studies generally have not tested higher dose levels of bisphenol A, i.e., > 1 mg/kg. Testing over a wide range of dose levels is necessary to adequately characterize the dose-response relationship. Typically, effects are easier to interpret when the dose-response curve is monotonic and the incidence, severity, or magnitude of response increases as the dose level increases. Effects that have biphasic, or non-monotonic dose response curves, are well documented in toxicology, endocrinology and other scientific disciplines (*56, 57*), but can be more difficult to interpret, which often limits their impact in risk assessments or other health evaluations. Testing higher dose levels may also identify additional effects that aid in interpreting the "low" dose finding with respect to potential health risk.

- Do the *in vivo* effects represent adverse health findings in laboratory animals and/or humans?

A general limitation in the "low" dose literature for bisphenol A is that many studies have addressed very specific experimental questions and not necessarily established a clear linkage between the "low" dose finding and a subsequent adverse health impact. For example, when an effect is observed in fetal, neonatal, or pubertal animals, investigations may not have been conducted to determine if the effect persists or manifest as a clear health effect later in life. Establishing a linkage to an adverse health impact is important because many of the "low" dose findings can be described as subtle, which can make them difficult to utilize for risk assessment purposes. An additional factor in considering the adversity of a finding is determining if the experimental model is adequate for predicting potential human health outcomes.

- How should studies that use a non-oral route of administration be interpreted?

Because the majority of exposure to bisphenol A occurs through the diet (*1*), laboratory animal studies that use the oral route of administration are considered the most useful to assess potential effects in humans. However, a large number of the laboratory animal studies of bisphenol A have used a subcutaneous route of administration to deliver the chemical, either by injection or mini-pumps that are implanted under the skin. The consideration of these studies in health evaluations of bisphenol A has proven controversial (*2, 58*). There is scientific consensus that doses of bisphenol A administered orally and subcutaneously cannot be directly compared in adult laboratory animals because the rate of metabolism of bisphenol A differs following oral and non-oral administration. There is also consensus that fetal and neonatal rats do not metabolize bisphenol A as efficiently as adult rats at a given dose because the enzyme systems that are responsible for the metabolism of bisphenol A are not fully mature during fetal or neonatal life.

alter DNA methylation (an epigenetic mechanism to alter phenotype) following exposure during development and that this effect may be offset by dietary exposure to methyl donors or the phytoestrogen genistein (*55*).



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most of these are in vitro or in studies using limited endpoints. In contrast, multigenerational studies with EE, E2, methoxychlor, nonlyphenol and other xenoestrogens have not found reproducible nonmonotonic dose responses. See Ema, Biegel, Howdeshell, etc studies on estrogens in rats and mice. Where are they data to support this statement, not a review article that claims they exist. Most regulatory agencies have found that the data do not support this hypothesis (see EFSA 2007 or Wilhite et al 2008).
this remains an unproven hypothesis for multigenerational studies, not a fact.

However, there is scientific debate on whether the reduced metabolic capability of neonatal rats is sufficient to adequately metabolize low doses of bisphenol A.

In adult rats and monkeys, bisphenol A is metabolized to its biologically inactive form, or glucuronidated, more quickly when administered orally than by a non-oral route, e.g., subcutaneously, intraperitoneally, or intravenously (*59-61*). This is because bisphenol A administered orally first passes from the intestine to the liver where it undergoes extensive conjugation primarily with glucuronic acid before reaching the systemic circulation ("first pass metabolism"). Because non-oral administration bypasses the liver, and therefore first pass metabolism, these routes of dosing in adult rats and monkeys result in higher circulating concentrations of biologically active, free bisphenol A compared to oral administration. Although not tested directly in adult laboratory mice, the impact of first pass metabolism is predicted to be similar. Thus, a subcutaneous dose is expected to have a greater biological effect than the same dose delivered by mouth in adult laboratory animals, including in the offspring of dams treated with bisphenol A during pregnancy.

Studies that administer bisphenol A through non-oral routes are most useful for human health evaluations when information on the fate, e.g., half-life, and concentration of free bisphenol A in the blood or other tissue is also available. For example, if the peak and average daily concentrations of free bisphenol A in blood were measured following non-oral administration, these values could then be compared to levels of free bisphenol measured in rodent studies where bisphenol A is administered orally or to levels measured in humans. However, none of the reproductive and developmental toxicity studies that treated animals by non-oral routes of administration determined the circulating levels of free bisphenol A or its metabolites. As a result, studies that treat laboratory animals using non-oral routes of administration have often been considered of no or of limited relevance for estimating potential risk to humans (*2, 19, 48*).

As discussed previously (see "Are People Exposed to Bisphenol A?"), fetal and neonatal rats do not metabolize bisphenol A as efficiently as the adult and, as a result, have higher circulating concentrations of free bisphenol A for some period of time compared to adults receiving the same dose (*12-14*). The peak concentrations of free bisphenol A in the blood of 4-day old male and female rat pups orally dosed with 10 mg/kg are 2613 and 162-times higher than the peak blood levels measured in male and female adult rats treated with the same mg/kg dose (*12*). A measure of how long it takes the body to eliminate free bisphenol A, referred to as "half-life," was also slower at this dose in neonatal rats: > 6.7 hours in male or female pups compared to well under an 1 hour in adult animals (*12*). Thus, for a given administered dose, blood levels of bisphenol A are higher in neonatal rats than in adults, and remain so longer following exposure. However, neonatal rats do have the ability to metabolize bisphenol A as indicated by the presence of bisphenol A glucuronide in the blood and the inability to detect the free form within the measurement sensitivity of the assay by 12 to 24-hours after treatment in females and males respectively (*12*).

Neonatal rats appear to be able to more efficiently metabolize bisphenol A when given at lower dose levels than at higher dose levels. Although Domoradzki *et al.* (*12*) also treated neonatal and adult animals with a lower dose level of bisphenol A, 1 mg/kg, making a direct comparisons based on age at exposure was not possible at that dose because free bisphenol A was too low to



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why include this number? This dose is irrelevant to human exposures and misrepresents what is happening at environmentally relevant dose levels.

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this also is not an environmentally relevant dose level.

be quantified in the blood of adults. However, in 4-day old male and female rats treated with 1 mg/kg of bisphenol A, 98 – 100% of administered bisphenol A was detected as bisphenol A-glucuronide[6] compared to 71 – 82% at 10 mg/kg, i.e., a smaller proportion of administered bisphenol A is glucuronidated at 10 mg/kg compared to 1 mg/kg. This would be expected when the limited capacity of young animals to metabolize bisphenol A is overwhelmed by dose levels of the compound. These data suggest more efficient metabolism by neonatal rats at 1 mg/kg compared to 10 mg/kg and imply that the age at exposure differences described above may be less profound in the "low" dose range (≤ 5 mg/kg bw/day).

Taken together these data indicate that, compared to adults at a given dose, neonatal rats (and presumably mice) metabolize bisphenol A more slowly and suggest that differences in circulating levels of free bisphenol A arising from oral and subcutaneous routes of administration as a result of "first-pass metabolism" are reduced in fetal or infant animals compared to adults. This prediction is supported by a recent study that did not detect differences in the blood concentration of free bisphenol A as a function of route of administration (oral versus subcutaneous injection) in 3-day old female mice following treatment with either 0.035 or 0.395 mg/kg of bisphenol A (*58*).

While more research in this area is warranted, data from studies where bisphenol A was given by subcutaneous injection were considered as useful in the NTP evaluation as oral administration when treatment occurred during infancy when the capacity to metabolize bisphenol A is low. Studies in adult animals, including pregnant dams, that administered bisphenol A by subcutaneous injection or by a subcutaneous mini-pump were considered informative for identifying biological effects of bisphenol A but not for quantitatively comparing exposures in laboratory animals and humans.

- What is the impact of limitations in experimental design and how should studies with these limitations be interpreted?

The impact on study interpretation due to limitations in experimental design has been a significant point of discussion for bisphenol A, especially for the issues of (1) small sample size, (2) a lack of experimental or statistical control for litter effects, and (3) failure to use a positive control (*2, 62*).

In general, studies with larger sample sizes will have more power to detect an effect due to bisphenol A exposure than studies with small sample sizes. For this reason, "negative" results from small sample size studies are viewed with caution. On the other hand, "negative" results from studies with larger sample sizes are usually considered more credible (*63*). However, there is no single sample size that can be identified as appropriate for all endpoints. The ability to detect an effect is affected by the background incidence, e.g., tumor or malformation rates in control animals, variability of a particular endpoint, and the magnitude of the effect. A sample size of at least six may be reasonable for many endpoints with low or moderate degrees of variability, such as body weight, but could be insufficient to detect statistically significant differences in endpoints with a higher degree of variability such as hormone level or sperm

[6] Based on percentage of plasma area under the curve (AUC) for radioactivity that was bisphenol A glucuronide.



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this is not proven for low doses of BPA.

count, or that occur infrequently such as malformations or tumor formation. These factors can make consistent detection of relatively small changes especially difficult on endpoints that have a high degree of inherent variability.

Lack of statistical or experimental control for litter effects was perhaps the single most common technical shortcoming noted in the developmental toxicity studies evaluated by the CERHR Expert Panel for Bisphenol A (*2*). Adequate control for litter effects when littermates are used in an experiment is considered essential in developmental toxicology. In 2000, the NTP co-sponsored a workshop with the U.S. Environmental Protection Agency referred to as the "Low Dose Endocrine Disruptors Peer Review." As part of the peer review, a group of statisticians reanalyzed a number of "low" dose studies (*63*). Based on studies that used littermates, they determined that litter or dam effects were generally present such that pups within a litter were found to respond more similarly than pups from different litters. The overall conclusion on this issue was that "[f]ailure to adjust for litter effects (e.g., to regard littermates as independent observations and thus the individual pup as the experimental unit) can greatly exaggerate the statistical significance of experimental findings." Studies that did not adequately control for litter effects were given less weight in the NTP evaluation and were generally only used as supportive material.

The NTP concurs with the opinion of several scientific panels that positive control groups can be very useful to evaluate the sensitivity and performance of a given experimental model (*2, 52, 63*). However, the NTP does not consider use of a positive control to be a required study design component particularly in animal model systems that are well characterized regarding the background incidence of "effects" and their variability. For bisphenol A studies, potent estrogens, such as diethylstilbestrol, ethinyl estradiol, 17β-estradiol, and estradiol benzoate, are the most commonly used positive control chemicals given bisphenol A's historical classification as a weak estrogen. Failure to obtain predicted responses with these chemicals is generally interpreted as a "failed" experiment, perhaps reflecting the selection of a relatively insensitive animal or experimental model or insufficient chemical challenge. Studies where no responses are observed in the positive control group have generally contributed less weight to evaluations of bisphenol A (*2, 52*). The significance of a "failed" positive control for bisphenol A varies from endpoint to endpoint and reflected more negatively on a study in the NTP evaluation when the predicted effect on reproductive tissue or function was not observed at dose levels that should be sufficiently high to produce an effect.

Although potent estrogens are used as positive controls for bisphenol A, an increasing number of molecular or cell-based ("*in vitro*") studies suggest that interpreting the toxicological effects of bisphenol A solely within the context of their consistency with a classic estrogenic mechanism of action, or even as a selective estrogen receptor modulator (SERM),[7] is overly simplistic. In addition to binding to the nuclear estrogen receptors ERα and ERβ, bisphenol A interacts with a variety of other cellular targets [reviewed in (*2, 64*)] including binding to a non-classical membrane-bound form of the estrogen receptor (ncmER) (*65-67*), a recently identified orphan nuclear receptor called estrogen-related receptor gamma ERR-γ (*68-72*), a seven-transmembrane estrogen receptor called GPR30 (*73*), and the aryl hydrocarbon receptor (AhR) (*74, 75*).

[7] A selective estrogen receptor modulator, or SERM, is a compound that binds nuclear estrogen receptors and acts as an estrogen agonist in some tissues and as an estrogen antagonist in other tissues.



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This workshop also identified some studies that were so poorly designed that they would not reanalyze the data and other studies where the "effects" of BPA could not be reproduced. One study did not run concurrent controls. These studies/data also were excluded from the CERHR BPA Expert Panel review. They should not be discussed in the NTP brief either. Delete them. This gives the appearance that the NTP document is based in part on poor quality studies or studies that used inappropriate statistical analyses. These data and not interpretable and should not be used as "supportive" information.

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what criteria did the NTP employee to decide what reported "effects" they could interpret? How did they discriminate "false positives" resulting from faulty analyses from effects considered supportive? It is reliance on these studies that leads to so much confusion in the BPA literature and explains why effects cannot be replicated. In fact, many of them were not effects at all and the subsequent lack of effect in a large robust study actually is replicating what was found in the original study: No effect!

Several *in vitro* studies show that bisphenol A can act as an androgen receptor antagonist (*74, 76-82*) and is reportedly mitogenic in a human prostate carcinoma cell line through interactions with a mutant tumor-derived form of the androgen receptor (*83*). Bisphenol A also interacts with thyroid hormone receptors (TRs) and, based on *in vitro* studies, is reported to either inhibit TR-mediated transcription (*84*), inhibit the actions of triiodothyronine (T3) or its binding to TRs (*85, 86*); or stimulate cell proliferation in a thyroid hormone responsive cell line (*87*). One *in vivo* study suggests that bisphenol A acts as a selective TRβ antagonist (*88*). Bisphenol A may also inhibit activity of aromatase, the enzyme that converts testosterone to estradiol (*74, 89*).

The toxicological consequences of the non-nuclear estrogen receptor interactions identified so far are unclear. In some instances, the physiologic role of the receptor is unknown or not well characterized, i.e., ERR-γ, GPR30, which makes interpreting the consistency of the data impossible with respect to the implicated mechanism based on the cellular or molecular studies and the observed *in vivo* toxicology. However, even when the physiological effects are generally understood, e.g., AhR or AR binding, aromatase function, scientists can only speculate as to the possible *in vivo* impacts when multiple receptor or other cellular interactions are considered together. Nevertheless, the identification of a growing number of cellular targets for bisphenol A may help explain toxicological effects that are not considered estrogenic or predicted simply based on the lower potency of bisphenol A compared to estradiol. Effects mediated through the ncmER are of interest because of its role in regulating pancreatic hormone release and because bisphenol A has been shown to activate this receptor *in vitro* at a concentration of 1 nM, which is similar to the active concentration of the potent estrogen diethylstilbestrol (*65, 67*).

***Human Studies***

Only a very small number of studies have looked at associations between bisphenol A exposure and disorders of reproduction or developmental effects in humans [(*10, 90, 91*), studies prior to mid-2007 reviewed in (*2, 3*)]. The human studies have looked at the relationship between urine or blood concentrations of total or free bisphenol A and a variety of health measures including levels of certain hormones that help regulate reproduction (*24, 92*), markers of DNA damage (*93*), miscarriage (*94*), chromosomal defects in fetuses (*95*), fertility and obesity in women (*90, 96, 97*), effects on the tissue that lines the uterus ("endometrium") (*90, 98*), polycystic ovary syndrome (*92, 97*), and birth outcomes and length of gestation (*10, 91*).

In these studies, there are reports of associations between higher urine or blood concentrations of bisphenol A and lower levels of follicle-stimulating hormone in occupationally exposed men (*24*), higher levels of testosterone in men and women (*92, 97*), polycystic ovary syndrome (*92, 97*), recurrent miscarriage (*94*), and chromosomal defects in fetuses (*95*). In addition, one study reported that patients with endometrial cancer and complex endometrial hyperplasia had lower blood levels of bisphenol A than healthy women and women with simple endometrial hyperplasia (*98*). Bisphenol A was not associated with decreased birth weight or several other measures of birth outcome in two recent studies (*10, 91*). Drawing firm conclusions about potential reproductive or developmental effects of bisphenol A in humans from these studies is difficult because of factors such as small sample size, cross-sectional design, lack of large variations in exposure, or lack of adjustment for potential confounders. However, the NTP



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but there are no antiandrogenic effects in vivo in any short term or long term study. If you are going to discuss these in vitro mechanisms you need to include the attempts to demonstrate them in vivo and whether they were positive or negative for the proposed mechanism of action.

While bisphenol A has not been shown to cause cellular changes or cancer of the mammary gland in female rats and mice exposed as adults (*163*), two recent studies suggest that exposure of rats to bisphenol A during gestation may lead to the development of lesions in adulthood, ductal hyperplasia and carcinoma *in situ*, that may potentially progress to tumors, i.e., "preneoplastic" lesions (*44, 45*). In the study by Murray *et al.* (*45*) rats were treated with 0.0025 – 1 mg/kg bw/day bisphenol A during pregnancy by subcutaneous mini-pump. Significant increases in the incidence of hyperplastic ducts were reported in all dose groups of female offspring on post-natal day 50 and only in the lowest dose group of 0.0025 mg/kg bw/day on post-natal day 95 (sample sizes range from 4 – 6). A more severe lesion, carcinoma *in situ,* was present in female offspring in the 0.25 and 1 mg/kg bw/day groups on postnatal day 50 (25% incidence for both treatment groups) and postnatal day 95 (33% incidence for both treatment groups). These findings are supported by a study by Durando *et al.* (*44*)[10] where pregnant rats were treated with 0.025 mg/kg bw/day, again using a subcutaneous mini-pump. In this study, the percent of hyperplastic ducts was significantly increased in the female offspring at both postnatal days 110 and 180 (~2 – 5-fold). A non-significant increase in the incidence of ductal carcinoma *in situ* was noted following adult treatment with a subcarcinogenic dose of *N*-nitroso-*N*-methylurea, a chemical used in cancer research to assess susceptability to carcinogens (2/15 compared to 0/10 in control animals).

These findings are generally consistent with other reports of changes in mammary gland growth and development following perinatal exposure to bisphenol A that are related to an altered rate of maturation, e.g., advanced fat pad maturation, delayed lumen formation, enhanced duct growth, adoption of a pregnancy-like state, enhanced responsiveness to secondary estrogenic exposures, and potentially increased susceptibility to carcinogenesis, e.g., increased number or density of terminal end buds and ducts (*44, 45, 164-170*). Overall, these findings have been interpreted as indicating that developmental exposure to bisphenol A causes differential effects on maturation of epithelial and stromal elements in the breast tissue that may lead to a predisposition to disease onset later in life.

With the exception of an oral dosing study conducted by Moral *et al.* (*170*) that reported an increased number of mammary gland terminal ducts in the female offspring of rats treated during gestation with 0.250 mg/kg/day, the cellular and tissue-level effects on the mammary gland occurred following subcutaneous treatment via mini-pump with bisphenol A at doses of 0.000025 to 10 mg/kg/day (*44, 45, 164, 166-169*). The findings most closely linked to an "adverse" outcome, ductal hyperplasia and carcinoma *in situ*, were reported at 0.0025 – 1 mg/kg/day (*44, 45*).

[10] The study by Durando *et al.* (*44*) implied that 99.9% DMSO was used in the mini-pump ["Pumps are designed to deliver 25 BPA (Sigma-Aldrich de Argentina S.A., Buenos Aires, Argentina) or only DMSO (99.9% molecular biology grade, Sigma-Aldrich de Argentina S.A.)"]. The manufacturer of the mini-pump does not recommend use of DMSO concentrations greater than 50% because it can degrade the pump reservoir material and potentially result in tissue inflammation and edema. For this reason, the CERHR Expert Panel on Bisphenol A considered this study critically flawed (*2*). The NTP concurs that use of a high concentration of DMSO is a technical short-coming, but is not convinced that this factor could account for the observed results. The NTP also considered the possibility that potential pump degradation could result in variations in administered dose, but concluded that the study was still useful to consider in the context of other findings.



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this sentence should be deleted. It is not statistically significant. The NTP cannot claim that is or may be an effect of BPA. It is not even close to statistical significance. The p value for such an effect is 0.5. This means you would see an effect like this 50% of the time due to chance. Hardly an "effect" that should be included in a scientific NTP document. to do so is misleading as suggests that this is related to BPA treatment.

Certain aspects of mammary gland cancer differ between rats and humans, e.g., metastases are uncommon in rodents, but the lesions identified in these two recent studies, ductal hyperplasia and carcinoma *in situ*, are generally recognized as intermediary steps in chemical-induced mammary gland cancer in the rat and as pre-neoplastic lesions in the human (*171-174*). The appearance of ductal hyperplasia and carcinoma *in situ* are similar enough between rats and humans that these findings in the rat are considered relevant to humans (*172*). In humans, a greater than mild degree of ductal hyperplasia and ductal carcinoma *in situ* are associated with increased relative risk of developing invasive breast carcinoma. It is important to note that the development of these lesions does not guarantee the formation of tumors or cancer in rats or humans and they are most appropriately interpreted as risk factors. If similar changes occur in women, the increased relative risks for developing invasive breast cancer range from 1.5 to 5-fold for moderate and atypical ductal hyperplasia and 8.0 to 10.0-fold for ductal carcinoma *in situ* (*175*). The relative risk is based on a comparison to women of the same age in the general population. For example, a 50-year old woman has a 1 in 39 chance of developing invasive breast cancer in the next 10 years. If a 50-year woman has atypical ductal hyperplasia, a form ductal hyperplasia associated with a moderate level of increased relative risk (4 to 5-fold), then her chance of developing invasive breast cancer in the next 10 years increases to approximately 1 in 10 to 1 in 8.

The current literature is not sufficient to establish the reproducibility of the ductal lesion findings by multiple independent investigators. Bisphenol A was not shown to induce neoplastic or non-neoplastic lesions in the mammary gland of female rats (~74 and 135 mg/kg bw/day) or mice (650 and 1300 mg/kg bw/day) in two-year dietary cancer bioassays where exposure was initiated in young adult animals (5-weeks of age) (*163*). However, these studies did not include perinatal exposure and the NTP recognizes that adult-only exposure may not be sufficient to detect chemical carcinogens in hormonally-responsive tissues such as the mammary gland (*174*). Most of the toxicology studies of bisphenol A that included assessment of females following developmental exposure either (1) did not report examination of the mammary gland (*29, 35, 111, 176, 177*), or (2) collected mammary gland tissue but did not prepare the tissue in a manner that would readily reveal these changes, i.e., whole mounts (*33, 99*). The limited assessment of the mammary gland in these studies is critical because it is not clear that, if present, intraductal epithelial proliferations would have been detected during the routine histopathologic examinations. While more severe lesions, such as the presence of a mammary mass, would be detected during routine necropsy, the studies by Ema *et al.*, (*99*) and Tyl *et al*., (*33*) were primarily designed to detect effects on reproduction and development and not tumor incidence. Animals were not followed-up for a sufficiently long period of time to necessarily expect to observe tumors in control animals or differences in tumor incidence between treatment groups. In both of these studies, mammary gland tissues in the parental (F0) and F1 generations of females were only examined after weaning of their pups, and the animals would have been well under one year of age at the time of tissue collection.

The NTP concurs with recent reviews (*2, 178*) that additional data are needed to more completely understand the possible long-term consequences of disrupting mammary gland development in animals by bisphenol A exposure and its significance for human health. Namely, long-term follow-up studies with sufficient statistical power should be conducted to evaluate if the ductal hyperplasia and carcinoma *in situ* progress to mammary gland tumors, preferably



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So, after all this discussion about the limitations of these studies, did they or did they not find any lesions in the mammary tissue? this should be included since they are the only studies that have looked at the tissues at all.

the interpretation of this study when considering its relevance to human bisphenol A exposure. However, as discussed in more detail below, rodents are normally resistent to developing prostate cancer and the use of hormone treatment, chemical treatment, or other alternative animal model to obtain a more sensitive rodent model is considered an acceptable and recommended strategy in prostate cancer research (*174*).

The findings of Ho *et al.* (*43*) are consistent with a recent report of increased expression of cytokeratin 10 (CK10), a cell-marker associated with squamous differentiation, in adult male offspring of pregnant mice orally treated with 0.020 mg/kg bw/day bisphenol A during gestation (*181*). Chronic exposure to high doses of potent estrogens, such as diethylstilbestrol, leads to squamous metaplasia of the prostate, a tissue change characterized by a multilayering of prostatic basal epithelial cells. Squamous metaplasia is associated with benign prostatic hyperplasia or long-term estrogen treatment in patients with benign or malignant prostatic disease. The induction of CK10 expression in basal epithelial cells is an early indicator of changes leading to estrogen-induced squamous metaplasia. While the long-term health consequences of such an alteration are unclear, prostatic basal epithelial cells are implicated in the initiation and early progression of prostate cancer due to their function in maintaining ductal integrity and regulating the differentiation of luminal epithelial cell differentiation (*182*). It is important to note that prostates in the Ogural *et al.* study appeared morphologically the same as control animals based on the staining technique normally used in pathology (hematoxylin and eosin, or H&E). A stain specific for squamous keratin was required to detect the change. Thus, it is unclear whether similar changes in basal epithelial cell phenotype were present in other studies that evaluated the prostate using only an H&E stain.

The NTP concurs with the CERHR Expert Panel on Bisphenol A and another recent evaluation (*2, 178*) that additional studies are needed to understand the effects of bisphenol A on the development of the prostate gland and urinary tract. Studies should attempt to confirm these findings and include longer periods of follow-up to understand the significance of the structural and cellular effects observed in fetuses and to clarify the relevance of prostate intraepithelial neoplastic lesions resulting from bisphenol A exposure to the development of prostate cancer in these animals. Future research to clarify the role of bisphenol A in the development of prostate cancer presents a scientific challenge. Unlike humans where prostate cancer is common, it is the most common non-skin cancer in American men (*183*), rodents rarely develop prostate cancer. Of the almost 4,550 rats and mice used as controls in NTP 2-year inhalation or feed studies conducted during the last decade, only 1 cancerous tumor and 17 benign tumors ("adenoma") of the prostate gland were detected (*183*). No substances, including bisphenol A (*163*), have been identified as causing prostate tumors in NTP studies (*174*). The NTP has long recognized the limits of the traditional rodent cancer bioassay for detecting chemical-induced prostate tumors and organized a workshop in May 2006 to address this issue (*174*). Suggested strategies to improve the sensitivity of rodent models for detecting prostate cancer included using alternative models, e.g., genetically modified, and/or initiating exposure in perinatal life. In addition, NTP workshop participants suggested a more detailed histopathologic evaluation of the prostate because the assessment of human carcinogenic potential may be better determined based on chemical-induced preneoplastic changes rather than tumor incidence.



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Author: reviewer
Subject: Note
Date: 4/21/2008 8:18:48 AM
several of the multigenerational rat and mouse studies examined the prostate and other tissues for histopathological lesions. Include them and what they found as they are the only long term data available. New studies should also be included that have been published in the last few months. Perinatal administration of estrogens like EE does produce histopathological lesions in the prostate of the rat and other reproductive tissues as well. EE also reduces sperm counts. BPA does not produce these lesions. This should be included. If one is to assume that BPA is acting as and estrogen and producing effects on the prostate by sc injection that are relevant to oral exposures at equivalent doses why are effects not being detected later in life?

Author: reviewer
Subject: Note
Date: 4/21/2008 8:19:45 AM
They also need to use a relevant route of exposure.

During its evaluation of bisphenol A exposure and prostate development, the NTP also considered a number of studies in rats or mice that have detected increased prostate weight at low doses (*107, 184*) or failed to detect this effect (*29, 33, 35, 99, 108, 113, 179, 185-190*). Prostate weight effects have taken on a special significance in the controversy surrounding bisphenol A because elevated prostate weight was the first "low" dose finding reported in laboratory animals (*107*) and prompted numerous follow-up studies. Attempts to understand the basis for discordant findings has generated considerable scientific discussion and debate including their review at the NTP-EPA Low-Dose Peer Review workshop mentioned earlier (*62*). In brief, the NTP believes that the overall conclusions of the Bisphenol A Subpanel of the NTP Low-Dose Peer Review remain valid with respect to "low" dose effects on prostate weight, i.e., increased prostate weight cannot be considered a general or reproducible finding.

More importantly, it is not clear that prostate weight should continue to be considered a critical endpoint in risk evaluations of bisphenol A given the relative crudeness of this measure. Changes in organ weight may be useful to identify potential target tissues, but become less important when additional data relating to structural, cellular, or functional integrity are available. Prostate enlargment does not correlate with the development of prostate histopathology or cancer in rodents, and the evaluation of prostate weight without corresponding assessment of histopathologic changes is not considered useful for determining carcinogenic potential (*191*).

In addition, changes in prostate weight are not necessarily observed in the same bisphenol A studies that report prostatic cellular or tissue-level changes. For example, no effects on prostatic lobe weight were observed in studies that reported (1) increased incidence and susceptibility to develop prostate intraepithelial neoplastic lesion (*43*), (2) changes in the prostatic periductal stroma and decreases in androgen-receptor positive stromal cells and epithelial cells positive for prostatic acid phosphatase (PAS), an enzyme produced by the prostate that can be found in higher amounts in men with prostate cancer (*192*), and (3) increased expression of CK10 in adult mice exposed as fetuses to 0.020 mg/kg bw/day via treatment of the dam or during adulthood to high doses of bisphenol A (2 – 200 mg pellets implanted under the skin for 3-weeks) (*181*).

- Puberty

NTP concurs with the CERHR Expert Panel on Bisphenol A that limited data are available at low doses to suggest an effect of accelerating the onset of puberty in female mice. Early onset of puberty has been observed in offspring of CF-1 mice orally treated with 0.0024 mg/kg/day during gestation (*47*) or C57BL/6 mice orally dosed with 0.2 mg/kg/day during gestation and lactation (*40*). These findings are supported by another study that noted an early onset of puberty in female ICR/Jcl mice whose mothers were treated with 0.02 mg/kg bw/day bisphenol A during gestation by subcutaneous injection (*176*). Two studies reporting effects on mammary gland growth and differentiation in female offpsring of CD-1 mice treated with bisphenol A during pregnancy through a subcutaneous mini-pump are consistent with an impact of bisphenol A on timing of puberty [(*164, 167*), reviewed in (*193*)]. In humans, early onset of puberty in girls is associated with elevated risk of developing breast cancer, early bone age maturation, and psychosocial impacts that include influencing age at first sexual intercourse and increasing risk for certain adolescent risk behaviors (*194-196*). Depending on the magnitude of the finding, early onset of puberty in laboratory animals can be considered an "adverse" effect in



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Author: reviewer
Subject: Note
Date: 4/21/2008 8:23:33 AM
what is meant by this? regulatory agencies have used prostate weight in risk assessments as the critical effect. Are you saying they were wrong to do this? Since permanent changes in prostate weight can occur in the absence of histopathological alterations, risk assessors should use both kinds of data. Delete this statement.

Author: reviewer
Subject: Note
Date: 4/21/2008 8:27:49 AM
this statement is inaccurate. what measure of early puberty was significantly altered in this study, which is referred to over and over again in this document. There also are questions about the effect on puberty cited for female ICR mice. see attached document. If the NTP is going to rely so heavily on these studies then they need to resolve the apparent discrepancies between the reported effects and the data. I suggest they query the authors for the data and reanalyze them unless they are will to accept my reanalysis or explain why the analysis I provided is in error.

In the following section I have copied the text from the NTP Draft Brief on Puberty and added editorial comments in CAPS

Puberty

NTP concurs with the CERHR Expert Panel on Bisphenol A that limited data are available at low doses to suggest an effect of accelerating the onset of puberty in female mice.

INCORRECT STATEMENT
Early onset of puberty has been observed in offspring of CF-1 mice orally treated with 0.0024 mg/kg/day during gestation (*47*)

or C57BL/6 mice orally dosed with 0.2 mg/kg/day during gestation and lactation (*40*).

NOT SO, LOOK AT THE DATA IN THE FIGURE AN THE ENCLOSED DATA ANALYSIS.
These findings are supported by another study that noted an early onset of puberty in female ICR/Jcl mice whose mothers were treated with 0.02 mg/kg bw/day bisphenol A during gestation by subcutaneous injection (*176*).

The magnitude of the acceleration in puberty reported in the mouse studies ranges from 1 to 4.5 days (*40, 47, 176*).

Other studies have reported no effects on the timing of puberty in female mice [CF-1(*185*) or CD-1 (*33, 165*)] whose dams were treated with "low" doses of bisphenol A delivered orally or by subcutaneous mini-pump during gestation or during gestation and lactation.

It is unclear if the inability of these studies to reproduce the advanced onset of puberty finding was due to variations in mouse strain and stock, timing of exposure, diet, or other facets of experimental design. The most consistent difference between the "positive" and "negative" studies lies in the approach used to measure onset of puberty.

THIS IS NOT ALWAYS THE CASE. THE MOST ACCURATE INDICATOR OF PUBERTY IN RODENTS IS THE ONSET ESTROUS CYCLICITY. WHILE THIS NORMALLY OCCURS WITH THE FIRST ESTRUS IT MAY NOT BE THE CASE WITH EXPOSURE TO ESTROGENS.
Age at first estrus is the most accurate indicator of puberty in rodents. This occurs at the same time as vaginal opening in rats. However, in mice, vaginal opening does not correlate well with puberty and the first day of detecting cornified cells in a vaginal smear, a sign of first estrus, is used to indicate the onset of puberty (*197*).

The studies by Ashby *et al*., Markey, *et al.*, and Tyl *et al.*, (*33, 165, 185*) that did not detect an effect of bisphenol A relied on age at vaginal opening in mice rather than the use of vaginal smears to assess onset of puberty.

INACCURATE STATEMENT. HOWDESHELL DID NOT DETECT A STATISTICALLY SIGNIFICANT ACCELERATION IN EITHER THE AGE AT FIRST ESTRUS OF VAGINAL OPENING. THEY FOUND A REDUCTION IN THE NUMBER OF DAYS BETWEEN VO AND FIRST ESTRUS, BUT THIS IS NOT AN INDEX OF PUBERTY. FURTHERMORE, THE HONMA ET AL STUDY THAT REPORTED AN ACCELERATION IN VO AND FIRST ESTRUS, EFFECTS OF UNCERTAIN STATISTICAL SIGNIFANCE, DID NOT SEE A CHANGE IN THE NUMBER OF DAYS BETWEEN VO AND FIRST ESTRUS.
The study by Howdeshell *et al.*, (*47*) reported a ~ 2.5 day acceleration of puberty in female offspring of mice orally treated with 0.0024 mg/kg bw/day during pregnancy based on a measure that is not standard in toxicology (the interval between vaginal opening and first estrus).

Using the more standard interval of days from birth to first estrus, Ryan *et al*. (*40*) found ~ 4.5 day acceleration in puberty in the female offspring of dams treated during gestation and lactation with an oral dose of 0.2 mg/kg bw/day, but no effect at 0.02 mg/kg bw/day.
USING A VERY MARGINAL SAMPLE SIZE FO 4-5 PER BPA GROUP.

The study by Honma *et al*. (*176*) reported a ~1 day earlier onset of puberty in the offspring of mice treated with 0.02 mg/kg bw/day by subcutaneous injection during pregnancy.
WHICH IS OF UNCERTAIN STATISTICAL SIGNFICANCE.

YOU DESCRIBE THE ABOVE DATA AS “COMPELLING”.
The data in female rats are less compelling for a possible “low” dose effect on puberty. A finding of accelerated puberty has been reported in Wistar rats (*44*), but most of the “low” dose literature does not support an effect (*29, 35, 45, 99, 113, 198, 199*).

The effects of bisphenol A on puberty in rats at “high” doses are generally inconsistent with the “low” dose effects reported in the mouse studies by Howdeshell *et al.* (*47*), Ryan *et al.* (*40*), and Honma *et al.* (*176*).

Only one study has reported an effect on puberty in the predicted direction, i.e., acceleration following subcutaneous treatment on postnatal days 0 to 9 (*111*).
IF YOU ARE GOING TO DISCUSS THIS PAPER YOU NEED TO INDICATE THAT IS SC NEONATAL INJECTIONS (PND 0-9) OF VERY HIGH DOSES OF BPA (AS HIGH AS 656 MG/KG/D) WHICH IS VERY DIFFERENT THAN THE PREVIOUSLY DISCUSSED LOW DOSE STUDIES (IN TERMS OF DOSE, ROUTE AND TIMING OF EXPOSURE).

Other studies reported no effect (*108-110*) or a delay in puberty at ≥ 50 mg/kg bw/day (*29, 35*). Four of these studies used a positive control group (*35, 108, 110, 111*). In these studies, responses to potent estrogens based on age at vaginal opening ranged from no effect (*108*), to statistically significant small or moderate acceleration [1.7 days (*35*); 2.4 days (*111*); 3.6 days (*110*)].

An area of uncertainty in the assessment of puberty is reconciling the general absence of an

effect at "low" doses in rats with the mouse studies that found early onset of puberty in females when puberty was assessed by age at first estrous.
THIS IS MAY NOT BE HARD TO RECONCILE AT ALL. THE FEW STUDIES WITH MICE REPORTING ACCELERATIONS IN PUBERTY ARE NOT STRONG STUDIES AND SUFFER FROM LIMITATIONS SO IT IS NOT SURPRISING THAT EFFECTS ARE NOT SEEN IN RATS: THEY MAY NOT BE REPEATABLE IN MICE EITHER.

The differences in outcomes cannot be attributed to use of single insensitive strain or stock as a variety of rat models were used in the "negative" studies: Sprague-Dawley, Wistar, Wistar-Furth rats, Wistar-derived Alderley Park, CD, and Donryu. Moreover, three of the "negative" puberty studies reported other "low" dose effects (*45, 113, 198*).
THIS DISCUSION IMPLIES THAT THERE ARE STRAIN DIFFERENCES IN RESPONSE TO BPA OR OTHER ESTROGENS. THIS ISSUE WAS THOROUGHLY REVIEWED BY THE EXPERT PANEL, AND IS DISCUSSED BY HOWDESHELL ET AL (2008) AND FOUND NOT TO BE THE CASE FOR ALL ENDPOINTS: STRAIN SPECIFICTY TO ESTROGENS IS TARGET TISSUE DEPENDENT. SEE EXPERT PANEL REPORT DISCUSSION, TABLE 54 IN THE REPORT AND DISCUSSION BY HOWDESHELL ET AL (2008).

Based on an evaluation of two negative studies that included "low" dose treatment groups and that used a positive control compound (*35, 113*), there is some support for a conclusion that vaginal opening may not be a sensitive indicator of estrogenic response in all strains of rat or experimental designs. The study by Tinwell *et al.* (*35*) reported a relatively small acceleration in puberty, 1.7 days, in Wistar-derived Alderley Park rats treated with what is considered a high dose level of ethinyl estradiol (0.2/0.1 mg/kg bw/day orally to dams during pregnancy). In contrast, the study by Kubo *et al.* (*113*) reported a more profound acceleration in puberty of 5.9 days in female offspring of Wistar rats exposed to diethylstilbestrol (0.050 mg/L in drinking water) during pregnancy and lactation (*113*).

Another observation made from the rat studies that used a positive control group is that larger impacts on puberty onset (> 3 days) were more likely to be observed in studies that exposed animals during gestation and lactation or lactation (*110, 111, 113*) compared to gestation only (*35*); although, the Kwon *et al*. study (*108*) does not fit this profile (no effect on puberty following oral treatment with 3.2 – 320 mg/kg/day during gestation and lactation).
PUBERTY CAN BE ACCELERATED BY POTENT ESTROGENS DURING LACTATION BY AS MUCH AS 15 DAYS. THE DISCUSSION SHOULD INCLUDE EXPOSURES DURING THE PUBERTAL STAGE OF LIFE WHEN ESTROGEN OR XENOESTROGEN CAN ACCELERATE VO AND FIRST ESTRUS IN THE RAT BY AS MUCH AS 10 DAYS. THIS EFFECT HAS BEEN DEMONSTRATED FOR EE, E2, METHOXYCHLOR, GENISTEIN, TAMOXIFEN (A SERM), BUT BPA WAS WITHOUT EFFECT: IT DID NOT ACCELERATE PUBERTY IN THE RAT OR ALTER ESTROUS CYCLES.

THE PUBERTAL RAT DATA, DATA ANALYSES AND THE FINAL REPORT ON

THE LACK OF EFFECTS OF BPA ARE NOW AVAILABLE AT THE EPA EDSP WEBSITE. THIS SHOULD BE INCLUDED IN THE REFERENCES AND DISCUSSED HEREIN.

In summary, additional research is needed to assess the robustness of altered puberty at dose levels in the very low µg/kg bw/day range in mice, i.e. 0.0024 mg/kg bw/day.

Research directed towards understanding the apparent differences in response between rats and mice on this measure would also be valuable. This issue has implications not just for the evaluation of bisphenol A, but also for characterizing possible effects on puberty for other weakly estrogenic compounds. YOU SHOULD DELETE THIS LAST SENTENCE. THE PUBERTAL EFFECTS OF XENOESTROGEN EXPOSURES HAVE BEEN WELL CHARACTERIZED FOR MANY OF THEM ALREADY. IN THIS ASSAY BPA SHOWS NOT ESTROGENICITY, IN CONTRAST. IT DOES NOT ACCELERATE VO OR INDUCE CORNIFIED ESTROUS SMEARS.

•

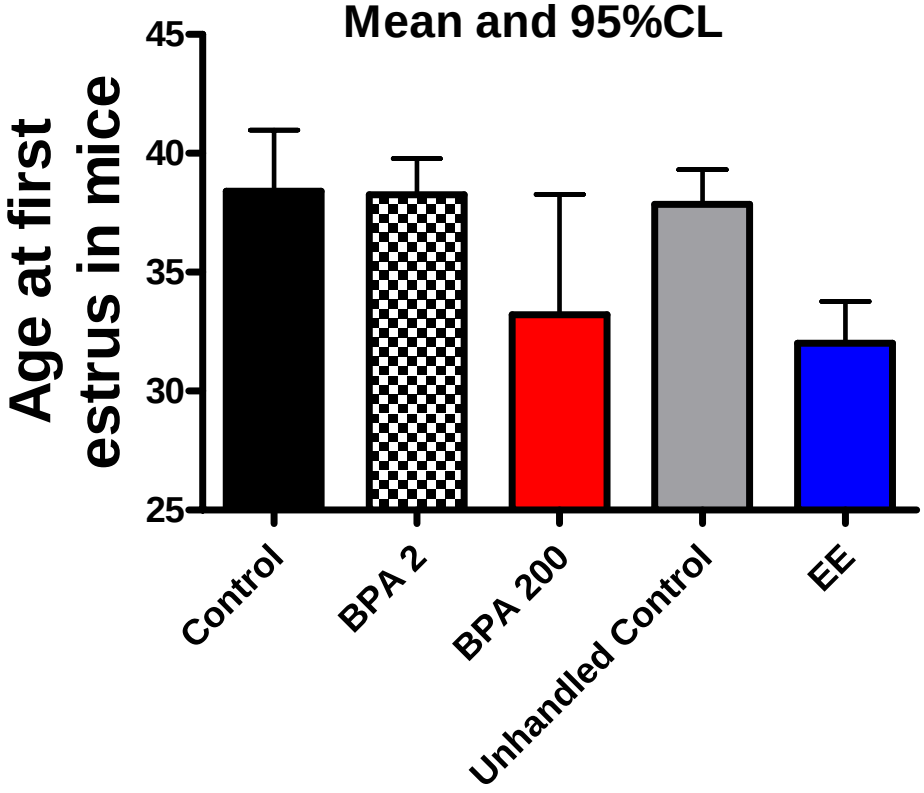
Other Effects Considered

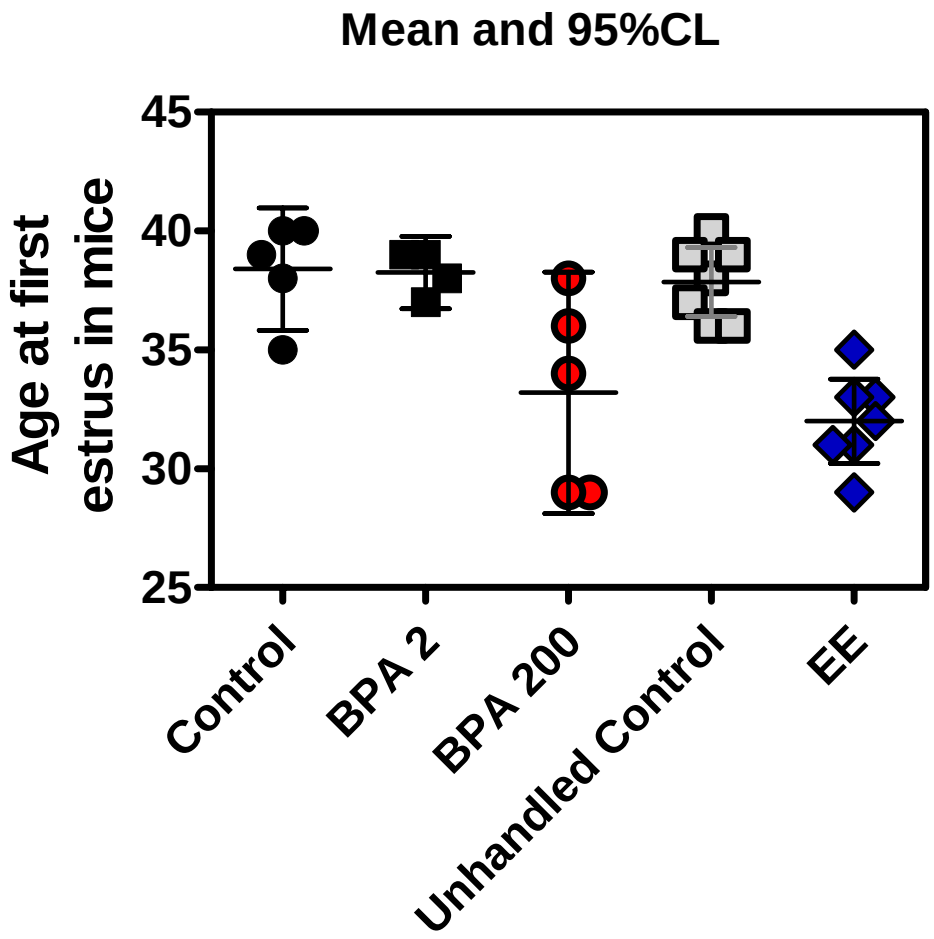
A variety of other effects in laboratory animals have been linked to "low" dose bisphenol A exposure during development, including decreased sperm quantity or quality, obesity, disruption of meiosis, changes in reproductive hormone levels, or cellular effects in reproductive tissues. These effects had less impact in shaping NTP's conclusions on potential risks to humans from bisphenol A exposure than the developmental effects observed at "high" doses on survival and growth and the "low" dose effects on brain and behavior, mammary gland, prostate gland, and onset of puberty in females described above.

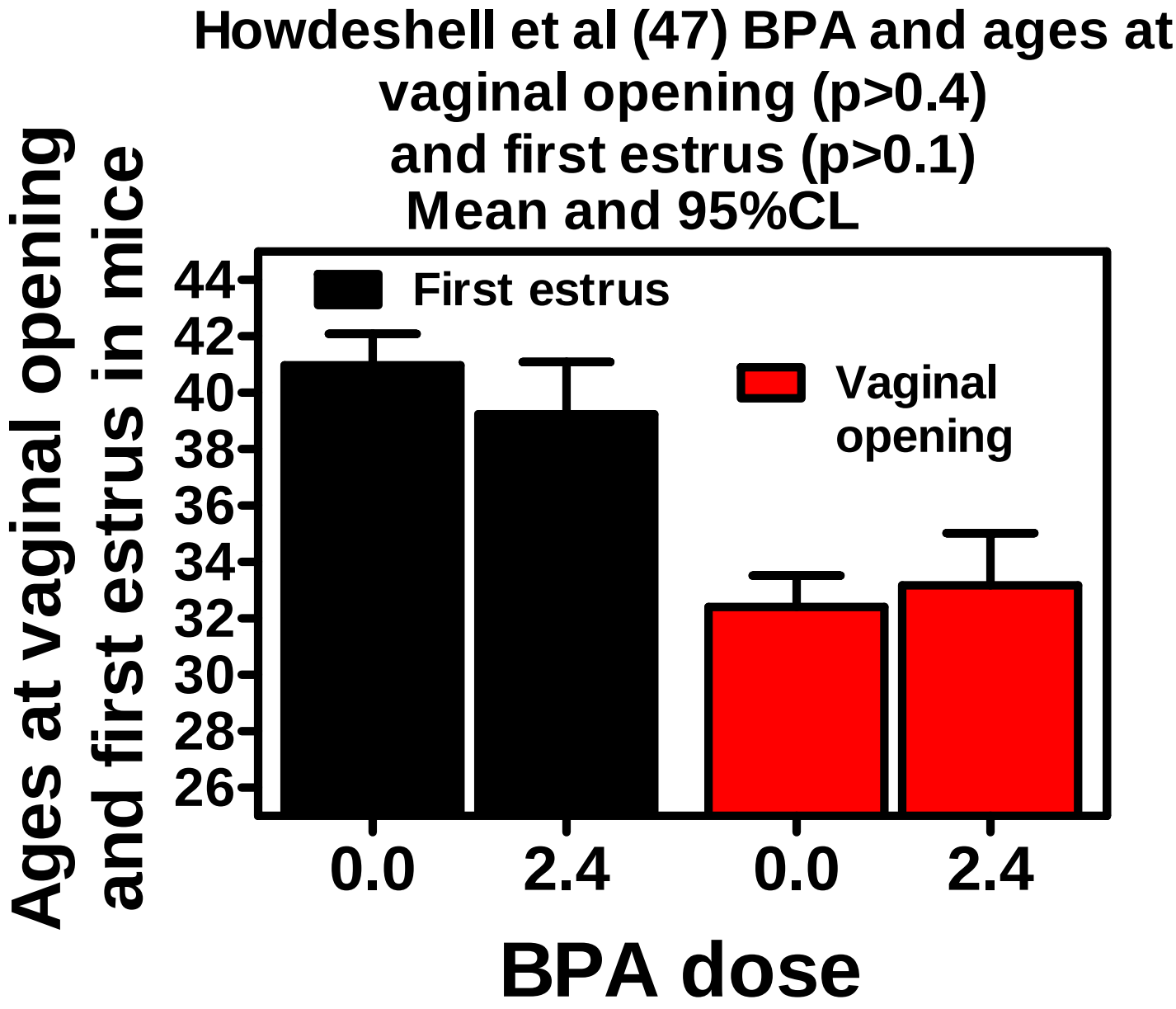
In some cases, the relationship between a specific cellular- or tissue-level finding and a potential health effect in the whole organism is unclear. This is because there is often uncertainty about the functional impact of a cellular or mechanistic finding, such as the altered level of a receptor protein or change in enzyme activity. For example, the potential health impact that may result from uterine changes characterized by altered ERα and ERβ expression and from an increase in the number and appearance of uterine epithelial cells is unclear (*200*).

In the following section I have copied provided my graphs of the data from the three papers reporting effects of BPA on puberty in female mice followed by my statistical analyses. These analyses have not been reviewed by the original authors and they may have significant comments or clarification. In particular, the analyses of the data from Honma et al. (2002) are not based upon raw data, but rather, information derived from the figure, figure legend and methods in the paper.

Ryan et al (40) BPA and age at first estrus data




Howdeshell et al (47) BPA and ages at vaginal opening (p>0.4) and first estrus (p>0.1)
Mean and 95%CL
Ages at vaginal opening and first estrus in mice
44
42
40
38
36
34
32
30
28
26
First estrus
Vaginal opening
0.0
2.4
0.0
2.4
BPA dose

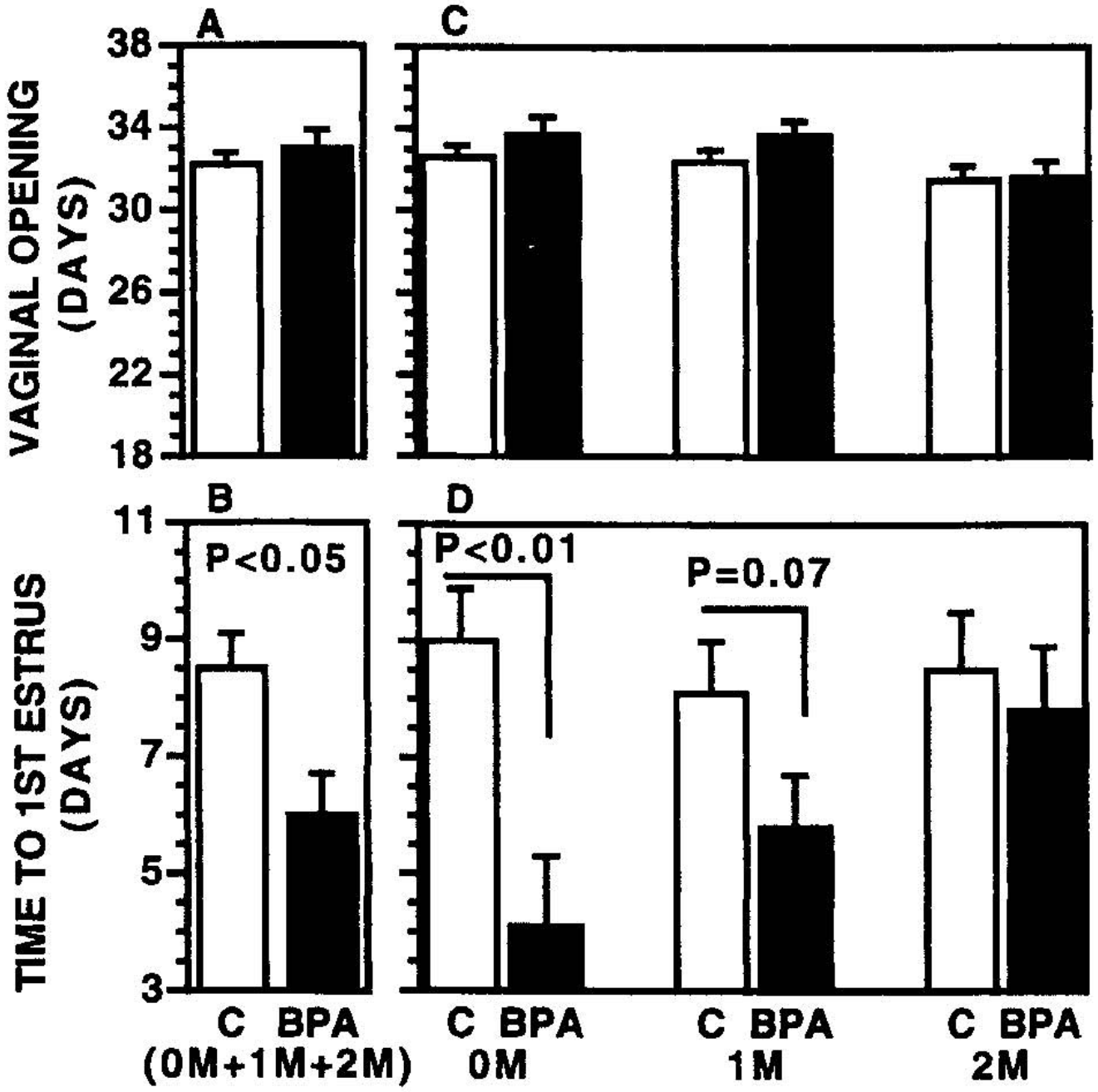
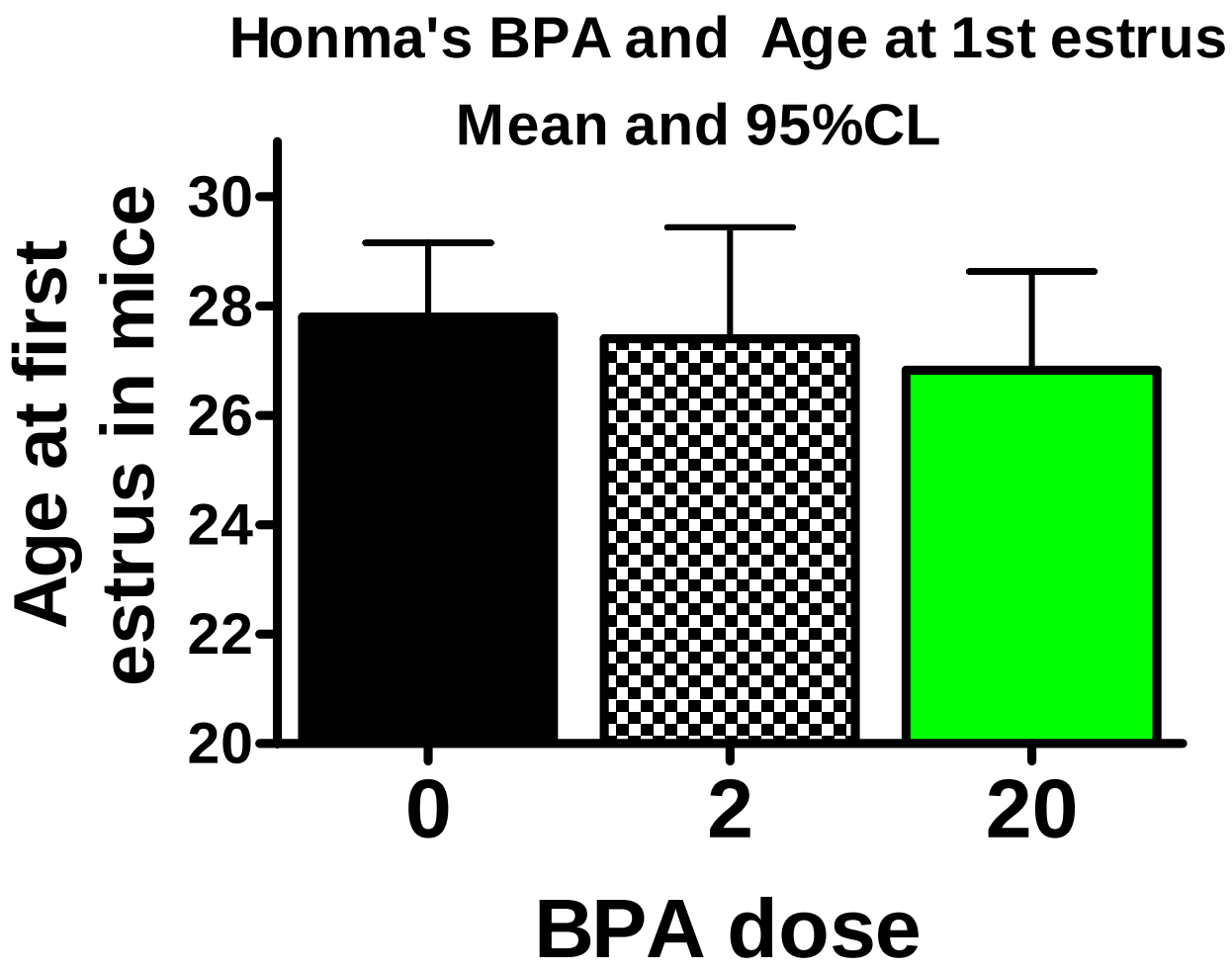



FIG. 3. Mean (±SEM) age at vaginal opening and interval between vaginal opening and first vaginal estrus for all females combined (A, B) and as a function of intrauterine position (C, D). All data were adjusted for litter to control for maternal effects. Data were also corrected by analysis of covariance for body weight at weaning. Vaginal opening and interval data were calculated on 19 0M, 20 1M and 19 2M control females (open bars) and 19 0M, 21 1M and 11 2M bisphenol A-treated females (closed bars).


Honma's BPA and Age at 1st estrus
Mean and 95%CL
Age at first estrus in mice
30
28
26
24
22
20
0
2
20
BPA dose

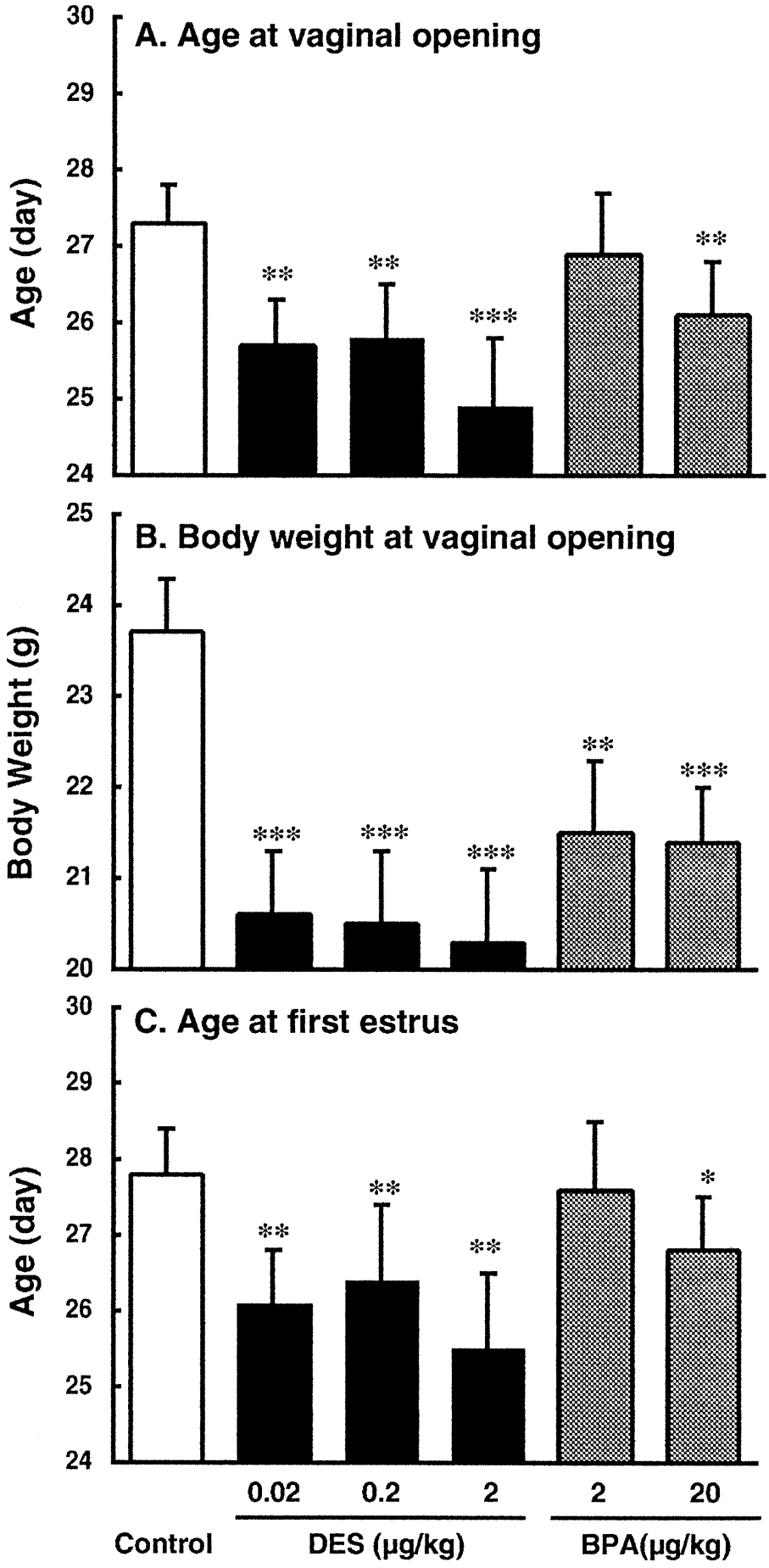



Fig. 1. Days of age at vaginal opening (A), body weight at vaginal opening (B) and age at first estrus (C). Values are mean ± sem. *, **, and *** indicate significantly different from the control at $P \leq 0.05$, 0.01, and 0.001 (Student's *t*-test), $n = 10$ for each dose group.

**Pubertal data from Howdeshell et al. 1999. Raw data are not included here, but the SAS input statements and authors' method of statistical analysis are listed. These were reanalyzed using data provided by the author on May 12 2008, using**

**a) the nested PROC GLM provided and other methods including**

**b) PROC GLM using the individual pups as the unit of analysis without regard to intrauterine position since it did not have a significant effect on pubertal landmarks in this study in the mouse. This analysis does not account for litter effects.**

**c) PROC MIXED accounting for litter effects, using all females from both control and BPA groups.**

**d) PROC GLM on litter means, accounting for litter effects, using all females from both control and BPA groups.**

**Puberty, as measured by the Age at First Estrus and the Age at Vaginal Opening was not significantly affected by in utero BPA treatment in the mouse using the results of any of the statistical analyses.**

SAS File provided by the author. The individual animal data have been deleted from the file.

DATA USED IN THE MANUSCRIPT:

IUP = intrauterine position; OM=no adjacent male fetuses, 1M= 1adjacent male fetus, 2M = 2 adjacent male fetuses.

WW = wean weight; this file includes the wean weights for only the females used in the puberty study.

VO = vaginal opening.

VOA = (VO + 1); this is the measure that we reported for vaginal opening in the paper.

E1 = interval from vaginal opening to 1st estrus confirmed by Hotchkiss/Vandenbergh.

AGE1E = (VOA + E1); this is the age at 1st estrus.

DATA NOT USED IN THE MANUSCRIPT:

E2, E3 and E4 were subsequent cycles that were not reviewed by Hotchkiss and were not reported in the paper

```
DATA ONE;
INPUT OBS  ID$ TRT$ LIT IUP$  WW  VO E1 E2 E3 E4 VOA AGE1E L1 L2  L3 LAV3;
* The SAS System
*INPUT ID TRT$ LIT SEX$ IUP$ BWB BWW LIVE$ BWA;
*IF SEX='F';
*ANIMALS REMOVED FROM PREVIOUS ANALYSES WERE DEAD L-43 M 2M 0.92 D AND L-45 F 0M 0.9 D;
CARDS;
```



INDIVIDUAL MOUSE DATA FOR 109 FEMALES WERE HERE.

```
PROC SORT;BY TRT IUP;PROC PRINT;
PROC GLM;CLASSES TRT IUP LIT;
MODEL WW VOA AGE1E E1 LAV3 L1 L2 L3 =  WW TRT|IUP LIT(TRT IUP);
TEST H=TRT|IUP E=LIT(TRT IUP);LSMEANS TRT|IUP/P S E=LIT(TRT IUP);
TITLE '1st day estrus Vandenbergh - WW and Litter Controlled';
RUN;
```

**Individual animal and litter means of pubertal data from Howdeshell et al. 1999.**

**Analyzed using data provided by the author on May 12 2008.**

| | | Control | BPA |
|---|---|---|---|
| **Individual Animal means and SE (n of mice)** | **AGE AT VO** | **32.5 ±0.36 (58)** | **31.6 ±0.31 (51)** |
| | **AGE AT FIRST ESTRUS** | **40.5 ±0.58** | **39.4 0.48±** |
| | **DELAY FROM VO TO ESTRUS** | **7.9 ±0.52** | **6.8 ±0.48** |

| | | Control | BPA | Control 0 M | BPA 0 M | Control 1 M | BPA 1 M | Control 2 M | BPA 2 M |
|---|---|---|---|---|---|---|---|---|---|
| **Litter means And SE (n of litters)** | **AGE AT VO** | **32.3 ±0.44 (19)** | **32.8 ±0.46 (15)** | **32.7 ±0.83 (12)** | **32.8 ±0.56 (11)** | **32.4 ±0.63 (15)** | **33.2 ±0.65 (14)** | **32.2 ±0.62 (14)** | **31.5 ±0.26 (9)** |
| | **AGE AT FIRST ESTRUS** | **40.3 ±0.62** | **39.4 ±0.52** | **41.3 ±1.12** | **38.8 ±0.70** | **40.6 ±0.77** | **40.0 ±0.89** | **39.3 ±0.98** | **39.4 ±1.01** |
| | **DELAY FROM VO TO ESTRUS** | **8.0 ±0.44** | **6.6 ±0.50** | **8.5 ±0.73** | **6.1 ±0.82** | **8.2 ±0.91** | **6.8 ±0.89** | **7.1 ±0.83** | **7.9 ±1.04** |

Statistical analysis A) LSMEANS OUTPUT FROM A NESTED ANALYSIS ON PROC GLM SHOWING CONTRASTS AMONG CONTROL AND BPA MICE USING LITTER MEAN VALUES WITHOUT (A) AND WITH (B) CORRECTION FOR WEANING WEIGHT AS A COVARIATE. INTRAUTERINE POSITION (IUP) INTERACTIONS OF IUP WITH BPA TREATMENT WERE NOT SIGNIFICANT. (USING LITTER(TREATMENT IUP) AS THE ERROR TERM). THE AGES AT VAGINAL OPENING AND FIRST ESTRUS ARE NOT AFFECTED BY BPA TREATMENT.

(A)

| TRT | WW LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 9.7540351 | 0.0942 |
| L | 10.6007778 | |

| TRT | VOA LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 32.3307018 | 0.4335 |
| L | 32.8355556 | |

| TRT | AGE1E LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 40.3394737 | 0.2927 |
| L | 39.4411111 | |

| TRT | MVOA LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 32.1508098 | 0.1560 |
| L | 33.0634186 | |

(B)

| TRT | MAGE1E LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 40.1125568 | 0.6483 |
| L | 39.7285391 | |

| TRT | ME1 LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 7.96174700 | 0.0761 |
| L | 6.66512046 | |

| TRT | E1 LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 8.00877193 | 0.0436 |
| L | 6.60555556 | |

Statistical analysis B) LSMEANS OUTPUT FROM PROC GLM SHOWING CONTRASTS AMONG CONTROL AND BPA MICE USING INDIVIDUAL VALUES WITHOUT CORRECTION FOR WEANING WEIGHT AS A COVARIATE.



The GLM Procedure
Least Squares Means
Standard Errors and Probabilities Calculated Using the Type III MS for LIT(TRT*IUP) as an Error Term

| TRT | WW LSMEAN | Standard Error | H0:LSMEAN=0 Pr > \|t\| | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|---|---|
| | 9.7307143 | 0.2526250 | <.0001 | 0.0415 |
| CL | 10.5177008 | 0.2823230 | <.0001 | |

| TRT | VOA LSMEAN | Standard Error | H0:LSMEAN=0 Pr > \|t\| | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|---|---|
| | 32.4686508 | 0.3789427 | <.0001 | 0.9728 |
| CL | 32.4880952 | 0.4234903 | <.0001 | |

| TRT | AGE1E LSMEAN | Standard Error | H0:LSMEAN=0 Pr > \|t\| | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|---|---|
| | 40.4293651 | 0.5328463 | <.0001 | 0.2030 |
| CL | 39.4023569 | 0.5954863 | <.0001 | |

| TRT | E1 LSMEAN | Standard Error | H0:LSMEAN=0 Pr > \|t\| | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|---|---|
| | 7.96071429 | 0.48447075 | <.0001 | 0.1543 |
| CL | 6.91426166 | 0.54142393 | <.0001 | |

STATISTICAL ANALYSIS C) USING PROC MIXED ON SAS USING ALL THE FEMALES SINCE THERE IS NO IUP EFFECT OR INTERACTION OF IUP WITH TREATMENT.
BPA DID NOT SIGNIFICANTLY AFFECT ANY OF THESE THREE ENDPOINTS

proc mixed; class LIT TRT;model  ENDPOINTS=TRT/solution;random intercept/subject=LIT;
AND
proc mixed; class LIT TRT;model  ENDPOINTS=TRT WW/solution;random intercept/subject=LIT;

1. AGE AT VAGINAL OPENING

| Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|
| TRT | 1 | 75 | 0.30 | 0.5858 |

AND

| Effect | DF | DF | F Value | Pr > F |
|---|---|---|---|---|
| TRT | 1 | 74 | 1.63 | 0.2054 |
| WW | 1 | 74 | 9.65 | 0.0027 |

2. AGE AT FIRST ESTRUS

| Effect | DF | DF | F Value | Pr > F |
|---|---|---|---|---|
| TRT | 1 | 75 | 1.62 | 0.2072 |

AND

| Effect | DF | DF | F Value | Pr > F |
|---|---|---|---|---|
| TRT | 1 | 74 | 0.70 | 0.4043 |
| WW | 1 | 74 | 3.80 | 0.0551 |

3. DIFFERENCE BETWEEN VAGINAL OPENING AND FIRST ESTRUS

| Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|
| TRT | 1 | 75 | 2.72 | 0.1036 |

AND

| Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|
| TRT | 1 | 74 | 2.65 | 0.1077 |
| WW | 1 | 74 | 0.03 | 0.8573 |

STATISTICAL ANALYSIS d) USING LITTER MEANS ANALYSIS USING PROC GLM WITH ALL FEMALES FROM THE 34 LITTERS (19 CONTROL AND 15 BPA). WITHOUT WEANING WEIGHT AS A COVARIATE. THE AGES AT FIRST ESTRUS (PUBERTY) AND VAGINAL OPENING ARE NOT STATISTICALLY SIGNIFICANT.

1. AGE AT VAGINAL OPENING

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 1 | 2.1364720 | 2.1364720 | 0.63 | 0.4335 |
| Error | 32 | 108.6384055 | 3.3949502 | | |
| Corrected Total | 33 | 110.7748775 | | | |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| TRT | 1 | 2.13647199 | 2.13647199 | 0.63 | 0.4335 |

2. AGE AT FIRST ESTRUS

| Source | DF | Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 1 | 6.7650225 | 6.7650225 | 1.14 | 0.2927 |
| Error | 32 | 189.1239318 | 5.9101229 | | |
| Corrected Total | 33 | 195.8889542 | | | |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| TRT | 1 | 6.76502247 | 6.76502247 | 1.14 | 0.2927 |

3. DIFFERENCE BETWEEN AGES AT VAGINAL OPENING AND FIRST ESTRUS

| Source | DF | Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 1 | 16.5049887 | 16.5049887 | 4.41 | 0.0436 |
| Error | 32 | 119.6822417 | 3.7400701 | | |
| Corrected Total | 33 | 136.1872304 | | | |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| TRT | 1 | 16.50498868 | 16.50498868 | 4.41 | 0.0436 |

| TRT | MVOA LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 32.3307018 | 0.4335 |
| L | 32.8355556 | |

| TRT | MAGE1E LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 40.3394737 | 0.2927 |
| L | 39.4411111 | |

| TRT | ME1 LSMEAN | H0:LSMean1=LSMean2 Pr > \|t\| |
|---|---|---|
| C | 8.00877193 | 0.0436 |
| L | 6.60555556 | |

STATISTICAL ANALYSIS USING LITTER MEANS ANALYSIS USING PROC GLM WITH ALL FEMALES FROM THE 34 LITTERS (19 CONTROL AND 15 BPA).
WITH WEANING WEIGHT AS A COVARIATE. THERE ARE NO SIGNIICANT EFFECTS OF BPA

1. AGE AT VAGINAL OPENING Source

| | DF | Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 2 | 17.1309003 | 8.5654501 | 2.84 | 0.0740 |
| Error | 31 | 93.6439772 | 3.0207735 | | |
| Corrected Total | 33 | 110.7748775 | | | |
| MWW | 1 | 14.99442829 | 14.99442829 | 4.96 | 0.0333 |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| TRT | 1 | 6.38757801 | 6.38757801 | 2.11 | 0.1560 |
| MWW | 1 | 14.99442829 | 14.99442829 | 4.96 | 0.0333 |

2. AGE AT FIRST ESTRUS

| | DF | Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 2 | 30.6233544 | 15.3116772 | 2.87 | 0.0717 |
| Error | 31 | 165.2655998 | 5.3311484 | | |
| Corrected Total | 33 | 195.8889542 | | | |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| TRT | 1 | 1.13101786 | 1.13101786 | 0.21 | 0.6483 |
| MWW | 1 | 23.85833197 | 23.85833197 | 4.48 | 0.0425 |

3. DIFFERENCE BETWEEN AGES AT VAGINAL OPENING AND FIRST ESTRUS

| Source | DF | Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Model | 2 | 17.5296087 | 8.7648044 | 2.29 | 0.1182 |
| Error | 31 | 118.6576217 | 3.8276652 | | |
| Corrected Total | 33 | 136.1872304 | | | |

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| TRT | 1 | 12.89426877 | 12.89426877 | 3.37 | 0.0761 |
| MWW | 1 | 1.02462006 | 1.02462006 | 0.27 | 0.6086 |

| TRT | MVOA LSMEAN | Pr > \|t\| |
|---|---|---|
| C | 32.1508098 | 0.1560 |
| L | 33.0634186 | |

| TRT | MAGE1E LSMEAN | H0:LSMean1= LSMean2 Pr > \|t\| |
|---|---|---|
| C | 40.1125568 | 0.6483 |
| L | 39.7285391 | |

| TRT | ME1 LSMEAN | H0:LSMean1= LSMean2 Pr > \|t\| |
|---|---|---|
| C | 7.96174700 | 0.0761 |
| L | 6.66512046 | |

In the following section I have copied the final page from an exposure article by Dekant et al. (2008) on human BPA exposure levels and their relationship to dosage levels used in many “low dose” studies.

arising from temporal factors within a day (e.g., time of sampling after food consumption and last urination) and across days (e.g., variable diets from day to day). Accordingly, mean values from studies reporting bisphenol A concentrations in spot urine samples with a larger number of participants correlate well with those using cumulative excretion over 24 h.

The biomonitoring data demonstrate that the average concentrations of bisphenol A in urine samples from the general population are low (at best a few μg/L) and confirm that bisphenol A is mainly present as glucuronide in human urine. The available data from Japan and the US, which contain samples from a significant number of individuals and report concentrations of bisphenol A in pooled 24 h urine samples of 1–3 μg/L thus serve as a basis for assessing daily exposures to bisphenol A in adults. Based on a total urine volume of 1.2–1.6 liters (Siegenthaler 1987; ICRP 2003) excreted over 24 h, a median daily intake of bisphenol A of 3.75–5 μg/day for adults can be concluded with maximum values up to 6–8 μg/day not including spot urine samples with high concentrations. This translates to average daily doses for 60 kg adults from 0.06 μg/kg bw to a reasonable worst case of 0.1–0.13 μg/kg bw in adults. Measured urinary concentrations of bisphenol A were recently used in Japan to define bisphenol A exposure of the population giving estimates for the daily intakes (95% confidence intervals) as 0.037–0.064 μg/kg bw/day for males and 0.043–0.075 μg/kg bw/day for females in the 95th percentile high-exposure populations (Miyamoto and Kotake, 2006). In the average exposure concentration, daily doses of bisphenol A for males were 0.028 to 0.049 μg/kg bw/day and for females 0.034 to 0.059 μg/kg bw/day.

Unfortunately, no data on the urinary excretion of bisphenol A in infants are available to determine if the higher exposures calculated from food consumption and measured bisphenol A concentrations in migration studies are consistent with actual exposures measured by biomonitoring (EFSA, 2006).

The daily exposure of humans to bisphenol A established by biomonitoring is thus well below the daily exposure as delineated from estimates of exposure based on food consumption and migration in adults, but in the same range as recent exposure assessments using food concentrations of bisphenol A and consumption patterns, e.g. 4.7 μg bisphenol A/day or 0.078 μg/kg bw/day for a 60 kg adult (Thomson and Grounds, 2005) or 0.001 μg/kg bw/day (Miyakawa et al., 2004). A low intake of bisphenol A for young children is also supported by a detailed exposure assessment of bisphenol A using measured concentrations in air, dust, and food. Delineated daily doses were between 0.052 and 0.074 μg/kg bw/day in preschool children (Wilson et al., 2007). The average daily doses of bisphenol A in adults delineated by biomonitoring and supported by the exposure assessment based on concentrations of bisphenol A in the diet are more than 500-fold below the TDI set by EFSA and the US EPA reference dose (both 50 μg/kg bw/day) suggesting that the exposure to bisphenol A does not result in a health risk to the general population. In addition, the bisphenol A exposures of the general population are also well below the daily doses of bisphenol A that sometimes have been reported to cause responses of unknown toxicological relevance in highly sensitive animal systems (20 μg/kg bw/day) (Timms et al., 2005) giving Margins-of-Exposure of 200 or more. For a comparison with human intake of other weakly estrogenic compounds, bisphenol A intake is at least 30 fold lower than that of phytoestrogens, which are more potent estrogens as compared to bisphenol A (Moors et al., 2007, Safe, 2004, Valentin-Blasini et al., 2005).

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